Freezing subsequently alters mating behavior in *D. melanogaster*.

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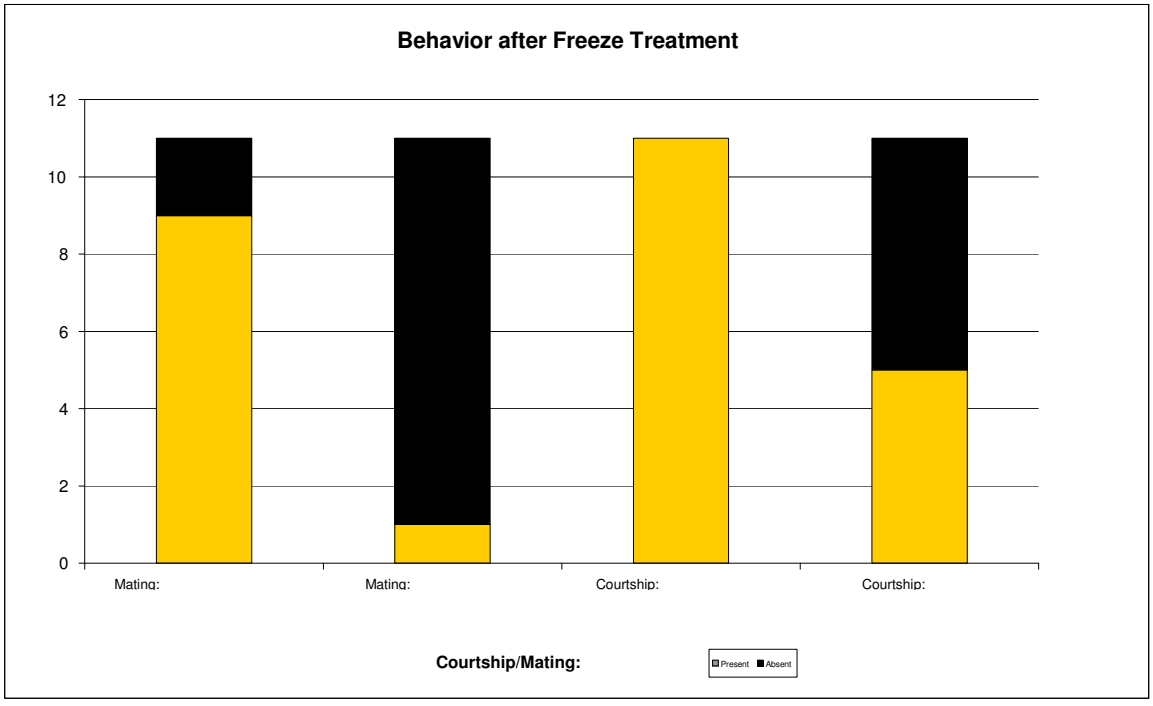
In 2000, we determined that the fertility, evidenced by flies who contributed to one or more offspring, of male *D. melanogaster* subsequently ceased after freezing, whereas female fertility decreased from 91.7% to 12.9%. Since male fertility depends upon complex, aggressive courtship (Partridge *et al*., 1987), we hypothesized that the freezing of male flies caused subsequent changes in courtship and reproductive behavior that resulted in decreased fertility after thawing.

Virgin males and females were collected and maintained in stock vials until they reached 6-7 days of maturity, whereupon half of the males were frozen in glass vials in a freezer with a mean temperature of –21.9 ± 1.8 °C. After 5 minutes, the frozen flies were placed in room temperature plastic vials with food and water and left to recuperate for 24 hours. Fruit fly-sized droplets of water froze after 5 minutes exposure to this temperature. After recuperation, one male fly was chosen at random from those that survived and was placed in a clear plastic cuvet. A control male that had been collected at the same time as the treated male was then chosen at random and placed in a separate cuvet. A virgin female chosen at random was added to each cuvet and the behavior of the flies as recorded with a video cassette recorder as they interacted during the next six hours.



In eleven trials, nine control males successfully mated, whereas only one treated male successfully mated. This difference is significant with $\alpha = 0.05$ using the Fisher Exact Probability Test. In every trial, the control male courted his female, whereas courtship was only observed in frozen males in five instances. Courtship was defined as visible pursuit of the female. This difference, too, is significant with $\alpha = 0.05$ using the Fisher Exact Probability Test. These results strongly support the assertion that freezing subsequently alters virgin male mating behavior in *D. melanogaster*.

Acknowledgments: Lawrence Reuter, Winona State University Biology Department.

Reference: Partridge, Linda, Arthur Ewing, and Amanda Chandler 1987, Animal Behavior 35: 555-562.

Comparison between two sampling methods for Drosophilidae (Diptera) using banana baits.

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Introduction

Studies of species composition, population dynamics and community structure were realized using the genus *Drosophila.* For these ends, various sampling and collection methods have been described (Spencer, 1949; Lanke and Telfer, 1952; Wheeler, 1955; Levitan, 1962; Carson and Heed, 1983; Tidon and Sene, 1988). These methods can be divided into two basic types (Carson and Heed, 1983):

(a) collection of breeding sites such as fermented plant material, fungus and others;
(b) collection of individuals flying over substrates or baits of fermented fruit.

Community studies make greater use of the second collection type, as it is possible to capture adult individuals, facilitating their identification. Bait consists of fruits with high potential for attraction, such as banana, orange, pineapple and others, or a combination of these.

The method of fly collection can also vary. Entomological nets, aspirators or traps are commonly used.

Variations in bait composition as well as fly capture methods can lead to different results regarding the community composition sampled, as was observed by Sene *et al.* (1981) and Valente *et al.* (1981).

Materials and Methods

Four sites on Santa Catarina Island, in the south of Brazil, were chosen for the collection of flies during the month of August 2001.

Two methods were used simultaneously to collect drosophilids for a period between three and six days. The first method (**M1**) consisted of a plastic box, placed at ground level, containing five kilos of banana seeded with *Saccharomyces cerevisae*. After the determined period, individuals flying over the bait were collected using an entomological net. The second method (**M2**) made use of 50 traps constructed according to Tidon and Sene (1988), with five kilos of fermented banana (bait) distributed equally among traps. Traps were placed 1.5 meters from ground level.

To compare the results obtained, we used the Jaccard (**C**) index (Magurran, 1988) and the χ^2 test of homogeneity.

Table 1. Similarity index (C) and statistical significance (χ^2) comparing the two sampling methods in each collection site. A – Morro da Lagoa da Conceição; B – Morro da Cruz; C – Campus da Universidade Federal de Santa Catarina; D – 14ª Brigada Motorizada da Infantaria.

Place	C	χ^2	Gl	P%
A	0.47	2,188.80	42	0
B	0.43	1,919.81	51	0
C	0.47	3,593.99	48	0
D	0.64	2,120.73	42	0

Table 2. Diversity index (H'), species number (S) and total flies sampled (N) in each collection site with each sampling method.

Place	Method 1 (M1)			Method 2 (M2)		
	H'	S	N	H'	S	N
A	0.40	25	4,889	1.56	38	6,558
B	1.67	25	2,856	1.74	51	17,577
C	1.49	26	3,810	1.28	45	47,894
D	1.88	29	3,589	1.52	40	43,300

Table 3. Absolute abundance (AA) and relative abundance (AR) for the principal species sampled with each sampling method used (* AR < 0.01).

Place	Species	Method 1 (M1)		Method 2 (M2)	
		AA	AR	AA	AR
A	*Drosophila sg. willistoni*	4,463	0.91	3,322	0.51
	D. capricorni	144	0.03	469	0.07
	D. polymorpha	83	0.02	1,396	0.21
	D. simulans	3	*	268	0.04
	Zaprionus indianus	3	*	176	0.03
	Others	193	0.04	927	0.14
	TOTAL	4,889	1.00	6,558	1.00
B	*D. sg. willistoni*	1,171	0.41	2,507	0.14
	D. simulans	612	0.21	4,498	0.26
	Z. indianus	338	0.12	6,567	0.37
	D. polymorpha	153	0.05	717	0.04
	Others	582	0.21	3,288	0.19
	TOTAL	2,856	1.00	17,577	1.00
C	*Z. indianus*	2,146	0.56	20,290	0.42
	D. malerkotliana	542	0.14	16,731	0.35
	D. simulans	334	0.09	7,660	0.16
	D. polymorpha	262	0.07	376	0.01
	Others	526	0.14	2,837	0.06
	TOTAL	3,810	1.00	47,894	1.00
D	*D. malerkotliana*	931	0.26	10,769	0.25
	Z. indianus	760	0.21	17,189	0.40
	D. simulans	731	0.20	9,928	0.23
	D. sg. willistoni	481	0.13	1,555	0.04
	Others	686	0.20	3,859	0.08
	TOTAL	3,589	1.00	43,300	1.00

The characterization of the communities studied was conducted using the species diversity index (**H'**) of Shannon and Weaver adapted by Hutcheson (1970), and the number of species (**S**) and the total number of individuals collected (**N**) at each site.

Results and Discussion

As shown in Table 1, the values of **C** do not exceed 0.50, with the exception of site **D**. De Toni (unpublished results), using the same index, obtained higher values than those encountered in this study, when she analyzed drosophilid communities collected from five collection sites in coastal islands in Santa Catarina.

High χ^2 values indicate, in all sites, a difference between results obtained using the two methods, strengthened by the results found by calculating **C**. Similar results were obtained by Sene *et al.*(1981).

In Table 2, **H'**, in site **A** using the **M1**, was low probably due to a large relative abundance (**AR**) of individuals from the subgroup *willistoni*, which composed more than 90% of the sample, in comparison with approximately 50% **AR**

when **M2** was used (Table 3). For sites **B** and **D**, the subgroup *willistoni* continued to be encountered at higher **AR** when **M1** was used.

The species *Zaprionus indianus* was found in higher **AR** using **M2** at sites **A**, **B**, and **D** (Table 3). This is likely to be related to the saltatory flight behavior observed in this species. Such behavior makes capturing individuals difficult when using entomological nets.

Also note that the values of **H'** are more homogeneous when **M2** was used.

The values of **S** and **N** are always high in collections using **M2**.

These differences could be caused by two reasons:

- the way the flies were captured – in **M1**, the entomological net was used at a single point both in space and time and, in **M2**, the traps were spread over a larger area and individuals were stored during the entire collection period;
- the fermentation process of the bait – in **M1** all the bait is contained within one recipient, whereas in **M2** it is divided in 50 parts.

The first reason reflects the ability to attract and store individuals from each method, which in the case of **M2** was greater for both, and also reflects the flight behavior of flies, that may have influenced collections using **M1**.

When collections were finally made, it was noted that the bait in **M1** was always very humid, sometimes to the point where the box was full of water. In contrast, the bait placed in the traps in **M2** were generally dry on the surface at the end of the collection period. Taking into account that when the bait was placed in its respective recipient it was of the same quality, the processes of drying and fermentation were distinct, producing a different composition of yeast and bacteria. Dobzhansky *et al.* (1956) observed that different lines of yeast attract different species of drosophilids with greater or lesser intensities, which could be the case in this work.

On the basis of the results obtained, we conclude that **M2** was more efficient, presenting higher values of both **S** and **N**, and more homogeneous values of **H'** and is, therefore, more faithful to reality in each of the communities studied.

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Mutability of unstable sex-linked alleles of *Drosophila melanogaster* and their interaction with mutations of the genes of the repair system.

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Almost all of the known unstable alleles in *Drosophila melanogaster* are associated with insertions/excisions of mobile elements or recombination between two copies of mobile elements leading to deletions, duplications or inversions of chromosomal segments flanked by these transposons. All these processes occur through the generation of double-strand breaks, and the general repair system of the host is believed to be involved in the process of insertional mutagenesis. It is therefore important to study the effect of particular genetic components of the repair system (mutagen-sensitive mutations) on the mutability of insertional alleles, as the mobile elements can be considered as "internal mutagens" of the genome.

A class of genes in *Drosophila melanogaster*, the so-called mutagen-sensitive mutations (*mus*), are known to disturb the normal process of repair. These mutations are specific to different stages and pathways of repair differing in their specific activity – repair of single- and double-strand breaks, removal of DNA interstrand crosslinks, and so forth.

In the present work, we studied the frequencies of mutation of the series of sex-linked unstable alleles (*white*, *yellow*, and *singed*) in the genomic environment deficient for different factors of general repair system (in females homozygous for *mus206*, *mus207*, *mus302*, and *mus308* alleles).

The unstable alleles were isolated in different years from different natural populations of *Drosophila melanogaster* of the former USSR. These alleles remain unstable after many generations of maintenance as laboratory stocks.

A molecular analysis of some of the *singed* and *yellow* alleles revealed the presence of the *hobo* transposon in the unstable loci. Mutations/interallelic transitions are associated with chromosomal rearrangements near the unstable loci, presumably, mediated by *hobo* (Gracheva *et al.,* 1998; O'Hare *et al.,* 1998).

We synthesized the tester lines of *Drosophila* homozygous for different *mus* mutations and carrying attached X-chromosomes in females (see the scheme in Figure 1). These lines enabled us to place the male X-chromosome carrying an unstable allele in the female genomic environment deficient for different components of the repair system and evaluate the mutability of unstable alleles on the repair-deficient background. The basic mutability level of studied alleles was determined in control crosses of males of unstable lines to females of laboratory stock C(I)DX, *ywf*/Y, which were used for establishing the tester lines.

#1 (unstable allele in repair-deficient environment)

1 male um_i/Y × females C(1)DX, *ywf* / Y; mus_j /mus_j

#2 (control – unstable allele in normal environment)

1 male um_i/Y × females C(1)DX, *ywf* / Y,

where um_i (unstable mutation) = $w^{883\text{-}6}$, $sn^{m859\text{-}2}$, sn^{s99}, $y^{+743\text{-}66}$, $y^{1\text{-}85}$, $y^{2\text{-}717}$, $y^{2\text{-}771}$ or $y^{2\text{-}836}$; and mus_j = *mus207*, *mus302* or *mus308*.

Figure 1. Schemes of crosses.

The results of experiments are given in Table 1. The mutability of all studied alleles in the genomic environment of C(I)DX, *ywf* /Y females (control laboratory stock) is within the range of 8.4×10^{-4} - 2.5×10^{-2}.

Table 1. Mutation frequencies of white, yellow and singed unstable alleles in repair-deficient genomic environment.

Line	Direction of mutation	Control: Number of progeny studied	Control: Exceptional progeny: Number	Control: Exceptional progeny: Mutation frequency	Control: Families studied: Total	Control: Families studied: With mutations, per cent and (number)
w^{883-6}	$w \rightarrow w^+$	9113	18	2.0×10^{-3}	120	10.8 (13)
snm^{859-2}	$snm \rightarrow sns$	5332	133	2.5×10^{-2}	120	80.0 (96)
sn^{s99}	$sn \rightarrow sn+$	9722	36	3.8×10^{-3}	120	25.8 (31)
$y^{+743-66}$	$y^+ \rightarrow y^2$	7845	48	6.1×10^{-3}	120	31.7 (38)
y^{1-85}	$y^1 \rightarrow y^2$	8976	31	3.4×10^{-3}	120	24.2 (29)
y^{2-717}	$y^2 \rightarrow y^+$	8721	44	5.1×10^{-3}	120	28.3 (34)
y^{2-771}	$y^2 \rightarrow y^+$	9441	19	2.0×10^{-3}	120	12.5 (15)
y^{2-836}	$y^2 \rightarrow y^+$	10847	9	8.4×10^{-4}	120	5.8 (7)

Line	Direction of mutation	mus308: Number of progeny studied	mus308: Exceptional progeny: Number	mus308: Exceptional progeny: Mutation frequency	mus308: Families studied: Total	mus308: Families studied: With mutations, per cent and (number)
w^{883-6}	$w \rightarrow w^+$	9875	11	1.1×10^{-3}	120	7.5 (9)
snm^{859-2}	$snm \rightarrow sns$	9850	39	4.0×10^{-3}	120	22.5 (27)
sn^{s99}	$sn \rightarrow sn+$	11774	11	9.7×10^{-4}	120	7.5 (9)
$y^{+743-66}$	$y^+ \rightarrow y^2$	8590	11	1.3×10^{-3}	120	6.7 (8)
y^{1-85}	$y^1 \rightarrow y^2$	11865	9	7.7×10^{-4}	120	6.7 (8)
y^{2-717}	$y^2 \rightarrow y^+$	10742	9	8.4×10^{-4}	120	7.5 (9)
y^{2-771}	$y^2 \rightarrow y^+$	11794	6	5.1×10^{-4}	120	5.0 (6)
y^{2-836}	$y^2 \rightarrow y^+$	13870	3	2.3×10^{-4}	120	2.5 (3)

Line	Direction of mutation	mus302: Number of progeny studied	mus302: Exceptional progeny: Number	mus302: Exceptional progeny: Mutation frequency	mus302: Families studied: Total	mus302: Families studied: With mutations, per cent and (number)
w^{883-6}	$w \rightarrow w^+$	9977	9	9.0×10^{-4}	120	5.8 (7)
snm^{859-2}	$snm \rightarrow sns$	9651	49	5.1×10^{-3}	120	25.8 (31)
sn^{s99}	$sn \rightarrow sn+$	10271	9	9.2×10^{-4}	120	7.5 (9)
$y^{+743-66}$	$y^+ \rightarrow y^2$	8660	19	2.2×10^{-3}	120	13.3 (16)
y^{1-85}	$y^1 \rightarrow y^2$	12100	11	9.4×10^{-4}	120	7.5 (9)
y^{2-717}	$y^2 \rightarrow y^+$	8799	11	1.3×10^{-3}	120	8.3 (10)
y^{2-771}	$y^2 \rightarrow y^+$	10930	7	6.1×10^{-4}	120	5.0 (6)
y^{2-836}	$y^2 \rightarrow y^+$	10391	3	3.0×10^{-4}	120	2.5 (3)

Line	Direction of mutation	mus207				
		Number of progeny studied	Exceptional progeny		Families studied	
			Number	Mutation frequency	Total	With mutations, per cent and (number)
w^{883-6}	$w \rightarrow w^+$	6979	30	4.3×10^{-3}	120	20.8 (25)
snm^{859-2}	$snm \rightarrow sns$	5200	265	5.1×10^{-2}	120	85.8 (103)
sn^{s99}	$sn \rightarrow sn+$	7122	43	6.0×10^{-3}	120	31.7 (38)
$y^{+743-66}$	$y^+ \rightarrow y^2$	6885	56	8.2×10^{-3}	120	33.3 (40)
y^{1-85}	$y^1 \rightarrow y^2$	7349	45	6.1×10^{-3}	120	32.5 (39)
y^{2-717}	$y^2 \rightarrow y^+$	6992	61	8.7×10^{-3}	120	34.2 (41)
y^{2-771}	$y^2 \rightarrow y^+$	7284	36	5.0×10^{-3}	120	25.0 (30)
y^{2-836}	$y^2 \rightarrow y^+$	8120	28	3.5×10^{-3}	120	21.7 (26)

The genomic environment of C(I)DX, *ywf* /Y; *mus308* tester line reduces the mutation frequency of all studied alleles by 2-10-fold and the number of mutation events – by 3 (number of families in which mutation event occurred is 6.6% in the experiment as compared to 18.3% in control crosses). The same change of mutability of the unstable alleles occurs in the genomic environment of C(I)DX, *ywf* /Y*; mus302* tester line (mutation frequency is reduced by 2-5 in all studied alleles).

Conversely, the genomic environment of the other tester line, C(I)DX, *ywf* /Y*; mus207*, increases the mutability of studied alleles by 2-5 times (with the exception of the $yellow^{+743-66}$ allele, which demonstrates an insufficient increase of mutability, increase factor 1.3).

No effect of *mus206* mutation on mutability of unstable alleles was detected.

These data support the notion that the processes of general genomic repair are coupled with the process of locus-specific mutagenesis. Apparently, insertions-excisions of mobile elements initiate the cascade of repair events under the control of different components of the repair system.

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Ecology of two *Scaptodrosophila* flower breeding species.

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Scaptodrosophila hibisci (Cook *et al*., 1977) and *S. aclinata* (McEvey and Barker, 2001) are endemic Australian species that breed in the flowers of a number of *Hibiscus* species belonging to the section *Furcaria*. *Scaptodrosophila hibisci* has been bred from the flowers of five species in eastern Australia, and *S. aclinata* from 11 species in the Northern Territory (Table 1).

For non-*Furcaria Hibiscus* species, *H. tiliaceus* is common within the distributions of both *Scaptodrosophila* species, and *H. panduriformis* is present within the distribution of *S. aclinata*. In

Table 1. Months during which flowering has been recorded (herbarium records and field observations) for various *Hibiscus* species in Australia. In southern locations, *H. heterophyllus* and *H. splendens* flowering periods are about two months shorter

Hibiscus species	Flowering period
S. hibisci host species	
H. divaricatus	March – November
H. diversifolius	January – December
H. heterophyllus	May – March
H. meraukensis	January – December
H. splendens	January – December
S. aclinata host species	
H. aneuthe	May-June[a]
H. arnhemensis	January – October
H. byrnesii	September – May
H. fallax	February-October[a]
H. menzeliae	October – June
H. meraukensis	January – December
H. petherickii	April-May[a]
H. riceae	February-October[a]
H. symonii	January – July
H. zonatus	January – October

[a] Flowering periods may be longer as few records are available.

addition, the introduced species *H. sabdariffa* (also sect. *Furcaria*) is widespread within the distribution of *S. aclinata*. These three species are often in close proximity to section *Furcaria* species with *S. hibisci* or *S. aclinata* present, but no adult *Scaptodrosophila* have been seen in hundreds of flowers of each of these species. Thus an apparently strict host-plant association of both *S. hibisci* and *S. aclinata* with endemic Australian *Hibiscus* species of the section *Furcaria* has evolved. However, as both *Scaptodrosophila* species use a range of different *Hibiscus* species (only *H. meraukensis* common to both), it seems likely that *Furcaria* specialization pre-dated the speciation of *S. hibisci* and *S. aclinata.*

In the laboratory using glasshouse grown flowers, we have bred *S. hibisci* on *Hibiscus esculentus* (≡ *Abelmoschus esculentus*, Pfeil *et al.,* 2002) and *H. cannabinus* (which is sect. *Furcaria*), both of which are introduced species that do not occur as natural populations in Australia. Detailed quantitative data were not kept, but progeny numbers per flower were low as compared with those from Australian section *Furcaria* species. However, Okada and Carson (1982) found *S. hibisci* breeding in village garden plantings of *H. esculentus* at Wau, Papua New Guinea.

Tsacas *et al.* (1988) described six species of the *aterrima* group of *Scaptodrosophila* in Africa, and noted that some Australian species (including *S. hibisci*) have affinities with this group. Although Tsacas *et al.* (1988) state that all these species breed in flowers of various species of Convolvulaceae, Cucurbitaceae and Malvaceae, the evidence is that adults have been collected directly from their flowers in the field. Only three of the species are recorded as having been bred from flowers – *S. aterrima* from *Ipomoea involucrata* and *S. nicolae* and *S. rufuloventer* from *Hibiscus tileaceus*. In earlier records, *aterrima*-like species were found as adults in *H. asper*, *H. surratensis* and *H. rostellatus*, all of which also are section *Furcaria*, while two unidentified sibling species of *S. aterrima* were recorded breeding in flowers of native *H. esculentus* and other unidentified *Hibiscus* (Lachaise and Tsacas, 1983). Lachaise and Tsacas (1983) argue that it is unlikely that African flower-breeding *Scaptodrosophila* display host specificity for *Hibiscus*, as Burla (1954 – cited by Lachaise and Tsacas 1983) reported continuous breeding site transfer from *Hibiscus* to *Ipomoea* flowers and vice versa, and Couturier *et al.* (1986) showed that species of the *aterrima* complex also breed in *Gossypium* and *Ipomoea*-like Tubiflorales. However, as species of the *aterrima* complex are not well characterized, some may be specific to *Hibiscus* species and others not.

Lachaise and Tsacas (1983) also argue that the flowering period of *Hibiscus* species is restricted during the year, and that a strictly *Hibiscus*-dependent fly population could not survive year round. Although most *Hibiscus* species in Australia do not flower continuously throughout the year (Table 1), the flowering season is not as restricted as inferred by Lachaise and Tsacas (1983), and possible alternative flowering species have not been identified. *Ipomoea* spp. (*I. pes-caprae* (native)

and *I. purpurea*, *I. indica* and *I. cairica* (introduced)) are common in a number of locations together with *Hibiscus* species, but we have never seen flies in those flowers. This is not to say that the flies do not use alternative resources, but if so, such resources have not been identified. If alternate resources are not utilized, the question remains as to how fly populations are maintained during any non-flowering season. Our limited observations suggest adult aestivation and/or an extended larval or pupal period. Two population cages were maintained in a glasshouse and given up to four fresh flowers daily throughout the flowering period. Population sizes during this time averaged about 500. After the last available flower was added, daily inspection of the cages continued. Adult fly numbers gradually decreased, but at least one fly was seen every day until 43 and 50 days after the last flower addition. Over the following 30 days, one or two flies were seen occasionally. At the same time as these cage observations were made, a natural *H. heterophyllus* population was checked about weekly, and sporadic flowering (1-2 flowers) was sometimes observed, but these flowers could not be checked for the presence of flies (out of reach). These two observations indicate the possibility for continuous maintenance of *S. hibisci* populations without the need to postulate an alternative breeding host.

Table 2. Oviposition pattern during the day – measured as numbers of adults emerging from flowers collected at intervals at the one site

Time of collection	No. of flowers	Total no. flies emerged	Av. No. flies/flower (sd)
1100	20	26	1.30 (1.78)
1200	20	40	2.00 (2.08)
1300	20	181	9.05 (5.38)
1400	20	178	8.90 (5.99)
1500	22	70	3.18 (2.34)
1600	20	133	6.65 (5.58)
1700	24	139	5.79 (5.68)
Next day – 0800	20	82	4.10 (2.99)

Hibiscus flowers are ephemeral, opening in the morning, closing late in the day and falling from the plant that evening or occasionally the next day. Flies appear on the flower even before it opens, and can be found in the flowers throughout the day. When the flower closes and falls, many flies may remain in it, but by next morning when new flowers are opening, no or very few flies remain in the fallen flowers. Graber (1957 – cited by Lachaise and Tsacas, 1983) suggested that females may await the fall of the flower before ovipositing. In fact, oviposition commences some time after the flower opens and continues throughout the day (Table 2). As noted by Cook *et al.* (1977), the fresh flowers provide courting and feeding sites for the adults, and the fallen decaying flowers the resource for larval development. Late stage larvae leave the decaying flowers and pupate in the leaf litter or soil.

Acknowledgments: This research was part of a larger collaborative program on the reproductive biology and evolution of *S. hibisci* funded by a grant from the NSF (INT-9512767) to W.T. Starmer and L.L. Wolf. Scientists from Australia (J.S.F. Barker), Canada (M-A. Lachance, J. Bowles) and the USA (M. Polak, W.T. Starmer, L.L. Wolf) took part in field observations.

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Distribution of viability of chromosomes 2 from Uman natural population of *Drosophila melanogaster* in heterozygote with *Df(2)62* overlapping the region of *l(2)gl* locus.

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In the 1960-1970s, in a series of works by M.D. Golubovsky and collaborators dedicated to a study of lethal mutations on chromosome 2 in natural populations of *Drosophila melanogaster* of the former USSR, the frequency of lethal mutations and their spatial and temporal dynamics were studied. In many populations, not only the concentration but also the kinds of occurring lethal mutations were studied. In particular, the alleles of *lethal (2) giant larvae - l(2)gl*, were shown to be permanently present in populations in high concentration (Golubovsky, 1978; Sokolova and Golubovsky, 1979).

The males used in the present research were collected in natural population Uman (Ukraine) in July, 1979.

Drosophila stocks: we used the *Df(2)62* line obtained by X-ray irradiation of line #62 (L.A.Kulakov, *Drosophila* stocks of the Laboratory of Genetics of Populations, Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences). *Df(2)62* is a deficiency overlapping *net* and *l(2)gl* loci.

Scheme of Crosses:

1 male n_1/ n_2 × females *Bl* L^2*/SM1, al Cy* sp^2

1 male n_i */Bl* L^2 × females *Df(2)62/SM1, al Cy* sp^2

(where i = 1, 2)

genotypes:

n_i */ SM1, al Cy* sp^2 ····*Bl* L^2*/ SM1, al Cy* sp^2 *Bl* L^2*/ Df(2)62* n_i */Df(2)62*

phenotypes and expected proportion of classes:

1 [*Cy*] : 1 [*Cy Bl L*] : 1 [*Bl L*] : 1 [+]

Viability (percentage) was calculated by the formula:

$$V_i^{Df(2)62} = 3\frac{\sum[+]_i}{\sum\{[Cy]+[Cy\,Bl\,L]+[Bl\,L]\}_i} x\,100\%$$

(where i – is the number of individual culture)

Table 1. Concentration of l(2)gl and net mutations in Uman population (1979).

Number of chromosomes studied	Frequency of lethal and semilethal chromosomes in region Df(2)62	Frequency of chromosomes carrying net mutation
766	0.0065	0.0104

The overall frequency of lethal and semilethal chromosomes from the natural population (according to the Table 1 data) made 0.0065 for *l(2)gl* and 0.0104 for *net* mutation, which confirmed the previous data reporting the high frequency of lethal and visible mutations in the *net - l(2)gl* region of chromosomes 2 isolated from the wild.

The data on the viability of heterozygotes n_i */Df(2)62* presented in the histogram demonstrates a characteristic bimodal character of distribution (Figure 1). The first mode corresponds to the lethal mutations. The high frequency of *l(2)gl* lethal is, possibly, associated with the selective advantage of heterozygotes in which this lethal is present in haplo-state. According to the histogram, the lethal effect of mutations in *l(2)gl* locus in populations is compensated by an increased viability of normal variants for this locus.

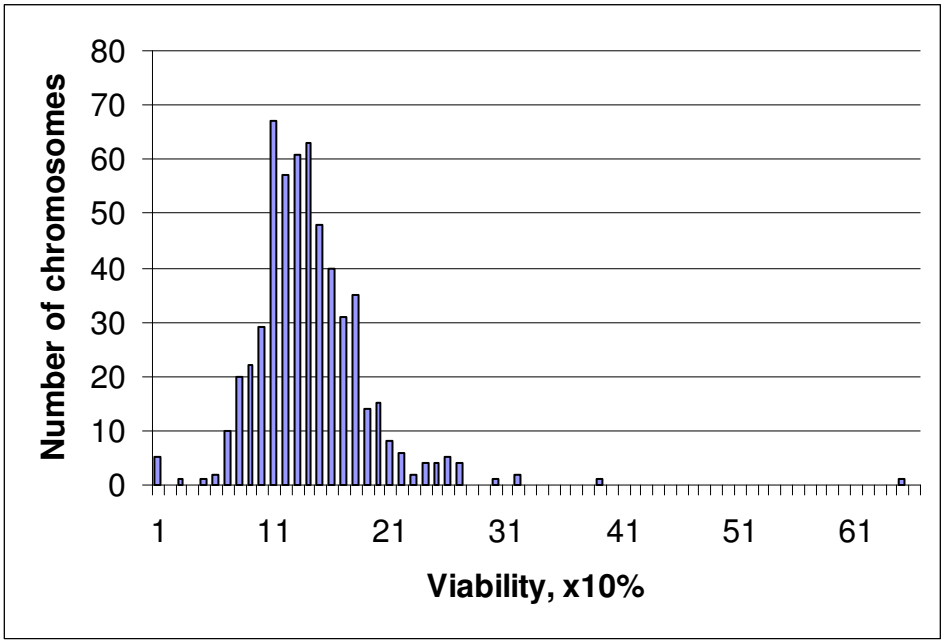

Figure 1. The viability of heterozygotes n_i */Df(2)62*.

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Comparison of female post-copulatory behavior in six species of *Drosophila.*

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Abstract

Female remating is an important component of *Drosophila* mating systems as it determines the pattern of sexual selection and sexual conflict. During the course of present study, female remating behavior of six species of *Drosophila*: *D. ananassae, D. nasuta, D. eugracilis, D. melanogaster, D. simulans, D. pseudoananassae* was observed and compared. Periodic confinement design (2-h daily observation) was used for female remating experiments. The frequency of female remating ranges from 16% (*D. eugracilis*) to 82% (*D. melanogaster*) in different species of *Drosophila*, and the differences among different species are statistically significant. Remating latency also varies from 5.51 days (*D. melanogaster*) to 9.85 days (*D. pseudoananassae*) in different species,

and variation among different species is statistically significant. Duration of copulation in first (virgin mating) and second matings (remating) was observed and compared in each of the six species. Among all the species tested, females of *D. ananassae, D. nasuta, D. eugracilis*, and *D. pseudoananassae* show significantly shorter duration of copulation in second mating as compared to first mating while *D. simulans* females show shorter duration of copulation in second mating compared to first mating but the difference is statistically not significant. However, *D. melanogaster* females show significantly longer duration of copulation in second mating as compared to first mating. Based on these findings, it may be suggested that different species of *Drosophila* may vary in the incidence of remating and duration of copulation due to differences in their reproductive biology and adaptation.

Introduction

Female remating is an important component of *Drosophila* mating systems because after mating females store the large number of sperm in the paired spherical spermathecae and a single elongate tubular seminal receptacles (Pitnick *et al.,* 1999), and utilize them to fertilize eggs as they are laid. Once a virgin female *Drosophila* has mated, she is usually unwilling to accept another male for some time because after mating, behavioral and physiological changes occur, including decrease in attractiveness to males (Wolfner, 1997), decreased receptivity to further mating (Fuyama, 1995), increasing of oogenesis, ovulation and oviposition rates (Wolfner, 1997), storage and utilization of sperm (Gromko *et al.*, 1984), and decreased life span (Chapman *et al.,* 1995). Female remating is common in many species of *Drosophila* under both natural and laboratory conditions (Richmond and Ehrman, 1974; Markow, 1985; Barbadilla *et al*., 1991; Joly *et al.,* 1991; Swartz and Boake, 1992; Etges and Heed, 1992; McRobert *et al.*, 1997; Harshman and Clark, 1998; Snook, 1998; Singh and Singh, 1999; Bundgaard and Barker, 2000; Iliadi *et al.*, 2001; see Singh *et al*., 2002). It has also been proved from the DNA marker technology that across a wide array of species, females frequently mate with more than one male (Eberhard, 1996; Imhoff *et al.,* 1998). The phenomenon of female remating has received considerable attention because it is associated with pattern of sperm usage and sexual selection (Parker, 1970). Parker (1970) used the term "sperm competition" and defined sperm competition as the "competition within a single female between the sperm from two or more males for the fertilization of the ova". Sperm competition offers a unique opportunity to study adaptations shaped by the interacting forces of natural, sexual and antagonistic selection (Rice, 1996). The existence of sperm competition in *Drosophila* has been inferred from the proportion of progeny produced by the second male in double mating experiments (Clark *et al.,* 1995; Singh and Singh, 2001). This approach has been used to quantify genetic variation underlying sperm competition, to elucidate its genetic basis, to identify the dependence of different male competition ability on the genotype of the females with which they mate, and to discern the potential role of sperm competition in species isolation (Price *et al.,* 1999).

The main goal of the present study was to compare the mating and post-mating behavior among six species of *Drosophila*. These species are: *D. ananassae*, *D. melanogaster*, *D. simulans*, *D. nasuta*, *D. eugracilis,* and *D. pseudoananassae*. Among them only *D. ananassae*, *D. melanogaster*, and *D. simulans* were previously tested for female remating and others such as *D. nasuta*, *D. eugracilis,* and *D. pseudoananassae* have never been tested for female remating behavior. In view of this, we conducted experiments to test the female remating in these six species of *Drosophila* with particular reference to the frequency of mating and remating, remating latency, and duration of copulation in first and second matings and the results are reported in the present paper.

Material and Methods

(a) Flies and culture conditions

To study female remating behavior, flies of six species of *Drosophila* were used. These species are *D. ananassae*, *D. nasuta, D. eugracilis, D. melanogaster, D. simulans,* and *D. pseudoananassae*. The data on the female remating behavior of *D. ananassae* given in the present study is based on the data reported by Singh and Singh (1999). The *D. nasuta* stock was obtained from the *Drosophila* Stock Centre, Mysore, India. The stocks of *D. eugracilis* (isofemale line)*, D. melanogaster* and *D. simulans* (mass culture) used in the present study are being maintained in our laboratory. The *D. pseudoananassae* stock (14024-0421.0) used in this study was obtained from the National *Drosophila* Species Resource Center, Bowling Green, OH, USA. All the stocks are being maintained under uncrowded conditions on a simple culture medium containing agar-agar, dried yeast, maize powder, brown sugar (crude sugar), nipagin, propionic acid, in the laboratory in a 12:12 light dark cycle at 24 ± 1ºC. From each species virgin flies (males and females) were collected on the day of hatching, anaesthetized with ether to facilitate sorting of the sexes, and stored in food vials. Flies were aged for seven days in food vials for sexual maturity. The males and females were then paired according to the protocols described below without being anaesthetized.

(b) Female remating

In each species, to obtain once-mated females, a single seven days old virgin female was placed individually in a fresh food vial (3" length × 1" diameter) with a single seven days old virgin male and the pair was observed for 60 min. When mating occurred, courtship time and duration of copulation were recorded for each mated pair. Observation was continued until 50 females had mated, usually within 60 min. Females failing to mate during a 60 min observation period were discarded. Following completion of copulation, males were removed by aspiration, usually within 30 min. The next morning, 50 once-mated females were individually paired with virgin males in fresh food vials and were observed continuously for 2 h. After 2 h observation, the males were discarded from the vials and the same procedure was repeated on twelve consecutive mornings with fresh males (Singh and Singh, 1999). When remating occurred, the duration of copulation was noted for each pair in each species. The number of remated females per day was also noted in each species from 1-12 days after first mating. Remating days (the number of days spent after the first mating until the female accepts to copulate again within 12 days) were also noted for each remated female in each species. Females that remated on any one of the 12 testing days were no longer given the opportunity to remate. In this way 50 once-mated females were observed in species for remating frequency, remating days and the duration of copulation in first and second matings (remating).

Results

Table 1 presents the mean courtship time (min), number of copulations and frequency of copulation in virgin females in different species of *Drosophila*. The mean courtship time varies from 8.22 min (*D. ananassae*) to 17.63 min (*D. pseudoananassae*) (Figure 1). The analysis of variance for courtship time shows significant variation among different species (Table 1). Frequency of copulation in virgin females varies from 54.35% (*D. pseudoananassae*) to 96.15% (*D. melanogaster*). The χ^2-test on the number of copulations in virgin females shows significant variation among different species (Table 1). Table 2 shows the number of remated females, remating frequency and remating latency (min) in different species of *Drosophila*. The female remating frequency varies from 16% (*D. eugracilis*) to 82% (*D. melanogaster*). The χ^2-test for remating frequency shows significant variation

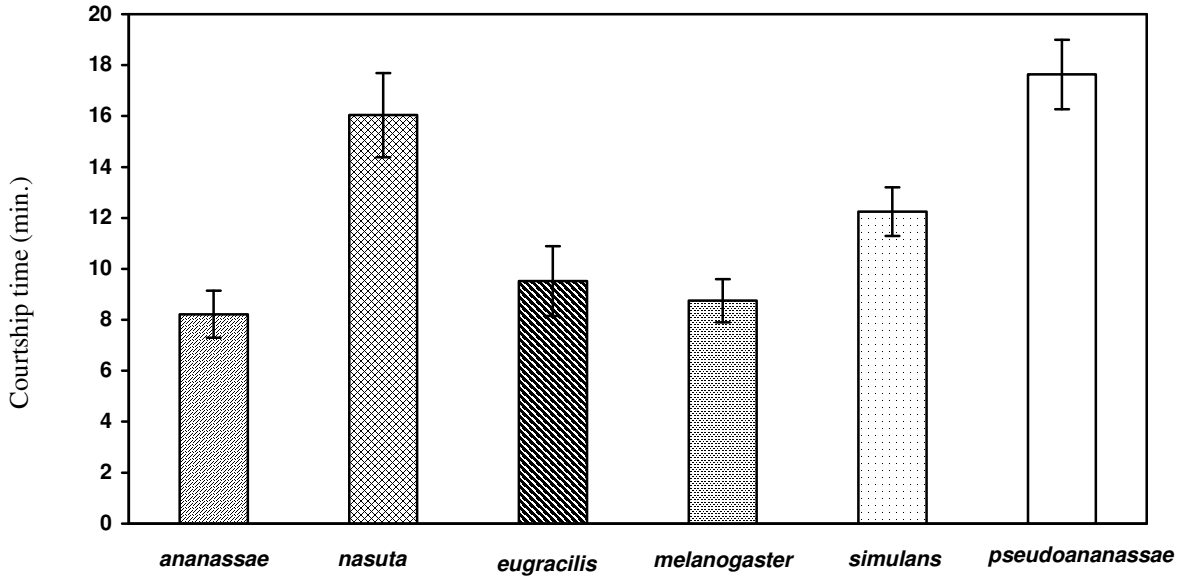

Figure 1. Courtship time (in minutes) in different species of *Drosophila*.

among different species (Table 2). Figure 2 shows the cumulative percentage of remated females in different species of *Drosophila*. Female remating was observed from 1 to 12 days after first mating. The mean remating latency (in days) varies from 5.51 (*D. melanogaster*) to 9.85 (*D. pseudoananassae*). The analysis of variance (ANOVA) for mean number of remating latency (days) shows a significant variation among different species (Table 2). Table 3 presents the comparison of the duration of copulation between first (DC I) and second matings (remating-DC II) in different species of *Drosophila* (Figure 3). Among all the species tested, *D. ananassae*, *D. nasuta, D. eugracilis* and *D. pseudoananassae* show significantly shorter duration of copulation in second mating as compared to first mating. The *D. simulans* females also show shorter duration of copulation in second mating but the difference was not significant statistically (Table 3). However, females of *D. melanogaster* show significantly longer duration of copulation in second mating as compared to first mating. The duration of copulation for first mating varies from 4.22 min (*D. ananassae*) to 20.06 (*D. nasuta*) in different species and the analyses of variance for DC I shows significant variation among different species (Table 3). The duration of copulation for second mating also varies from 3.38 min (*D. ananassae*) to 18.03 (*D. melanogaster*) in different species and the analyses of variance for DC II shows significant variation among different species (Table 3).

Discussion

During the course of the present study, six species of *Drosophila* were tested for female mating and remating frequency, remating latency (days) and the duration of copulation in first and second matings (remating). It is evident from the results that there are inter-specific differences in mating frequency, remating frequency, remating latency and duration of copulation in first and second matings. Further, when the duration of copulation in first and second mating is compared there are inter-specific differences. Out of six species tested *D. melanogaster* stand out distinct from rest of species as it shows longer duration of copulation in second mating as compared to first

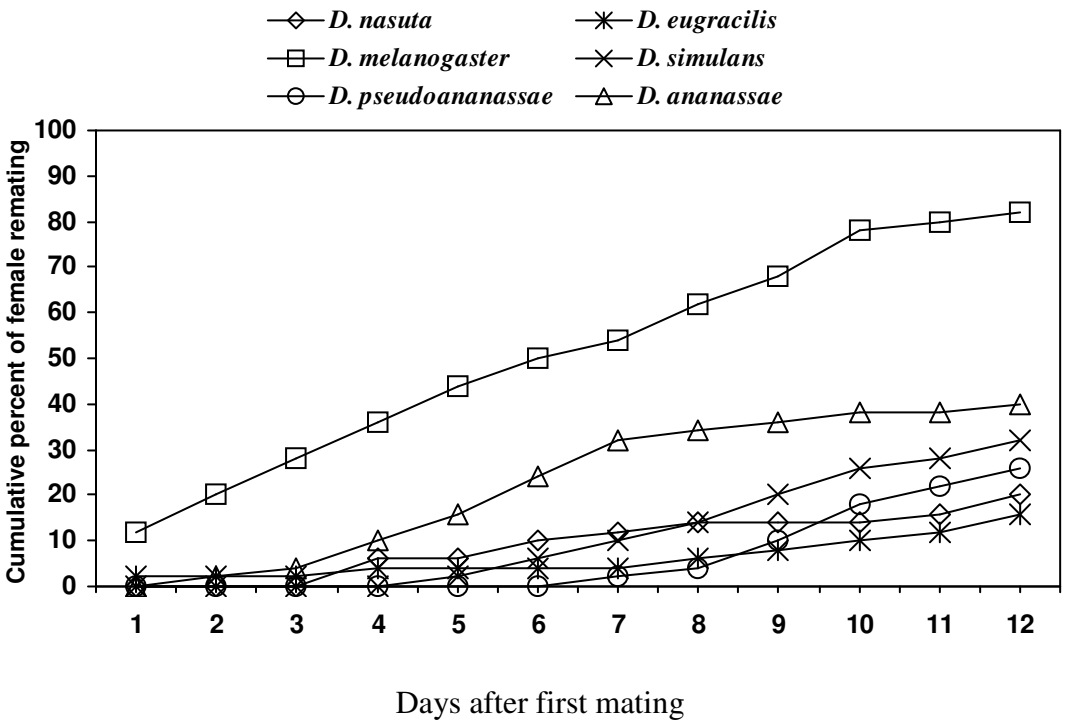

Figure 2. Cumulative percentage of remating in females in different species of *Drosophila*. Female remating was observed from 1 to 12 days (every day 2 hrs) after the first mating.

matings. It also shows higher mating and remating frequency but lower remating latency when compared with other species tested during the present study. Out of six species tested, five belong to *melanogaster* species group and one species, *i.e*., *D. nasuta* belongs to the *immigrans* species group. *D. melanogaster*, a cosmopolitan and domestic species is most wide spread geographically in distribution. It is also genetically most variable as compared to other species.

In *Drosophila,* successful mating depends upon male activity and female receptivity because usually the female is the discriminating partner in the mating act (Bastock, 1956). Courtship time (mating speed), the time from the beginning of the courtship to copulation is a good estimate of sexual activity in males and sexual receptivity in females (Speith and Ringo, 1983). The courtship behavior of *Drosophila* enables conspecific to distinguish non-conspecific and enables males to distinguish fe-males, including the physiological readiness of the female to copulate (Speith and Ringo, 1983). Mating activity is correla-ted with fitness in many species of *Drosophila* (Fulker, 1966; Singh and Singh, 1999). The differences observed in the present study regarding the

Table 1. Mean (± S. E.) courtship Time (min.), number of copulations and frequency copulations in virgin females in different species of *Drosophila.*

Species	Courtship time (min.)	Number of copulations	Proportion (%)
D. ananassae	8.22 ± 0.93	50/80	62.50
D. nasuta	16.22 ± 1.65	50/78	64.10
D. eugracilis	9.52 ± 1.37	50/65	76.92
D. melanogaster	8.75 ± 0.85	50/52	96.15
D. simulans	12.25 ± 0.96	50/85	58.82
D. pseudoananassae	17.63 ± 1.37	50/92	54.35
$F_{(5, 294)} = 11.68$, $P < 0.001$		$\chi^2 = 8.49$, $P < 0.001$	

Table 2. Number of remated females, remating frequency and mean remating latency (days) in different species of *Drosophila.*

Species	Remating	Frequency (%)	Remating latency (mean ± S. E.)
D. ananassae	20/50	40	6.15 ± 0.46
D. nasuta	10/50	20	7.40 ± 1.02
D. eugracilis	8/50	16	8.37 ± 1.40
D. melanogaster	41/50	82	5.51 ± 0.52
D. simulans	16/50	32	8.69 ± 0.53
D. pseudoananassae	13/50	26	9.85 ± 0.40
χ^2 = 22.90, *P* <0.001		$F_{(5,102)}$ = 7.48, *P* <0.001	

courtship time and mating are due to differences in the courtship patterns in these species which leads to fast mating in some species (*D. melanogaster*) and slow in other species.

In female remating studies the 2h periodic confinement technique has been used by Newport and Gromko (1984), Letsinger and Gromko (1985), in *D. melanogaster* and by McRobert *et al.* (1997) in *D. biarmipes* and *D. melanogaster*. In *D. melanogaster*, it was found that about 80% of the females remate when remating was observed for 12 days (Pyle and Gromko, 1978). McRobert *et al.* (1997) studied remating in females of *D. melanogaster* and *D. biarmipes* during 2-h periodic confinement for 14 days and compared the post-copulatory behaviour of *D. biarmipes* and *D. melanogaster* females. Females of both species were shown to undergo a series of behavioural changes following mating, including significant reduction in both sexual attractiveness and receptivity. However, while both attractiveness and receptivity return to "virgin like" levels within a few days in *D. melanogaster*, *D. biarmipes* females, which regained their sexual attractiveness within a few days, remained unreceptive to copulation for at least two weeks. They also tested remating frequency in both species and found that about 26% of *D. biarmipes* females mated at least twice and that the mean remating latency was 10.8 days. In contrast, *D. melanogaster*, 87% of the females remated and remating latency was 6.5 days.

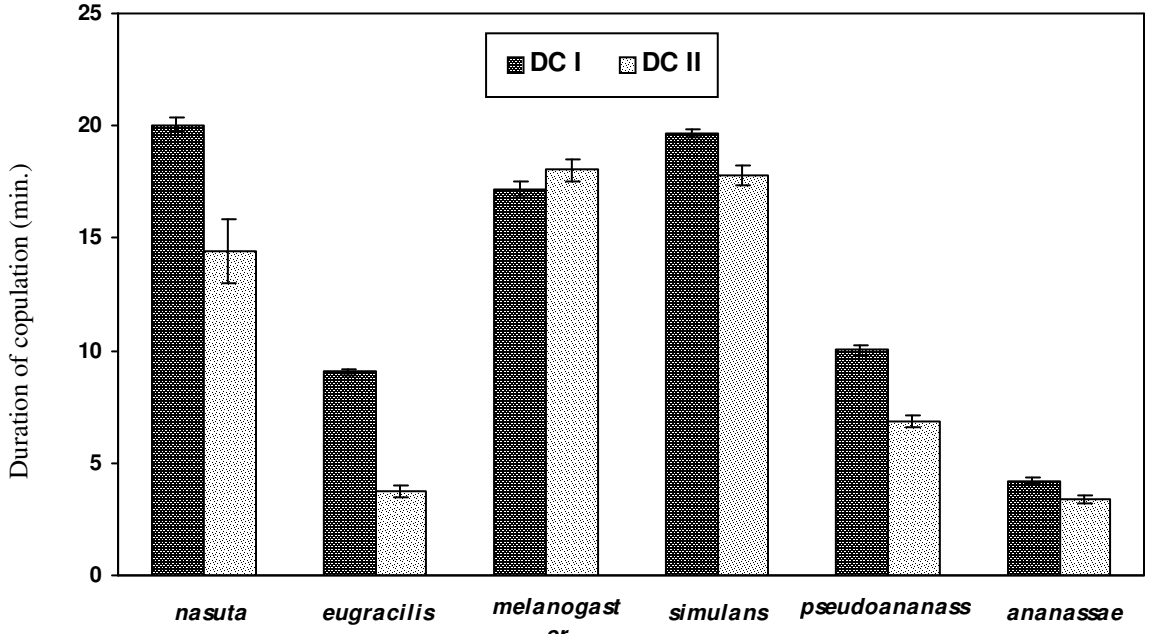

Figure 3. Mean (± S.E.) duration of copulation (min.) in first (DC I) and second mating (DC II-remating) in different species of *Drosophila*.

In *D. ananassae*, Singh and Singh (1999) used 2-h periodic confinement technique and remating was observed for 12 days. They found average female remating frequency 39.4% and the mean remating latency was 7.17 days. In the present study six species of *Drosophila* were used for female remating, using 2-h periodic confinement technique and remating was observed for 12 days. The differences observed here in the present study for remating frequency and remating latency in different species may be due to differences in the amount of sperm and seminal fluid transferred by males during mating and also due to the reproductive biology of the females in these species. In *Drosophila melanogaster*, a longer delay of female remating is due to the transfer of large amount of sperm (Letsinger and Gromko, 1985). However, Service and Vossbrink (1996) found that a longer delay in remating was associated with the slower use of stored sperm. Remating is also delayed in *lozenge* mutant females of *D. melanogaster*, possibly because this mutation results in spontaneous ovulation that serve to confuse the normal signal causing refractoriness to mating (Fuyama, 1995). Snook (1998) suggested that oviposition is more important than sperm in delaying female receptivity to remating and that sperm hetero-genotypes may play a role in affecting female remating because some species are having monomorphic and some have heteromorphic sperm types. Van-vianen and Bijlsma (1993) showed that female remating frequency is affected by their first male, and suggested that this could be due to differences in the amount or quality of seminal fluid transferred during mating. Newport and Gromko (1984) have shown that females with less sperm in the first mating are more likely to remate.

Table 3. Comparison of the duration of copulation (min.) between first (DC I) and second matings (DC II- remating) in different species of *Drosophila.*

Species	DC I	DC II	*t*	*df*	*P*
D. ananassae	4.22 ± 0.15	3.38 ± 0.15	7.43	19	<0.001
D. nasuta	20.06 ± 0.35	14.44 ± 1.43	2.46	9	<0.05
D. eugracilis	9.05 ± 0.15	3.71 ± 0.25	9.17	7	<0.001
D. melanogaster	17.15 ± 0.36	18.03 ± 0.48	2.41	40	<0.02
D. simulans	19.66 ± 0.21	17.78 ± 0.42	1.48	15	>0.05
D. pseudoananassae	10.01 ± 0.19	6.83 ± 0.28	6.08	12	<0.001
	$F_{(5,294)}$ = 700.70	$F_{(5,102)}$ =145.90			
	P <0.001	*P* <0.001			

Data are presented as means ± standard error of mean.

Joly *et al.* (1991) and Joly and Lachaise (1993) studied female remating and compared the duration of copulation in first and second matings in two geographic strains of *D. teissieri*, which originated from two geographic localities in Africa [(i) Mount Silinda, Zimbabwe, established in 1970; (ii) Tai forest, Ivory coast, established in 1981]. Joly and her collaborators used periodic confinement technique (8h) and scored remating after 24, 48 and 72h daily. Joly and Lachaise (1993) found a significant strain variation in female remating frequency. In one stock (Mount Silinda) all females remated, while only about 16% females of the other strain (Tai) remated. In both these strains the duration of copulation was longer during second mating (Joly *et al.,* 1991). Interestingly, the two strains of *D. teissieri* differ in sperm morphology. The Silinda males have very large sperm and females of this strain remate with a high frequency. Based on the findings in *D. teissieri,* Joly *et al*. (1991) proposed that the stable coexistence of two fertile sperm morphs (short and long) in one ejaculate is a mixed strategy, which has evolved via sperm competition possibly in response to female facultative polyandry. Bressec *et al.* (1991) found high recurrence polyandry in *D. littoralis* and *D. latifasciaeformis* (up to 3 mates within 8 h) and low recurrence polyandry in *D. affinis* (females have only two rematings every 7 days). Furthermore, they also measured the duration of copulation in first as well as in subsequent matings. Interestingly, their results clearly indicate that there is no significant difference in the duration of copulation when compared between different matings in *D. littoralis* and *D.*

latifasciaeformis. However, there is significant decrease in the duration of copulation from first to second mating in *D. affinis*, which is characterized by low recurrence polyandry.

Snook (1998) observed female remating in *D. pseudoobscura, D. persimilis,* and *D. affinis* using the periodic design and found > 80% remating in *D. pseudoobscura,* > 80% in *D. persimilis* and > 90% in *D. affinis*. She also compared the duration of copulation between first and second matings in each species and found that invariably all species show shorter duration of copulation in second mating. Singh and Singh (1999) reported female remating in ten strains of *D. ananassae* and compared the duration of copulation between first and second matings and found that invariably all the strains show significantly shorter duration of copulation in second mating. Recently, Bundgaard and Barker (2000) also found shorter duration of copulation in second mating as compared to first mating in *D. buzzatii* females.

There is considerable variation in copulation duration among *Drosophila* species (Grant, 1983), but causal factors influencing variation in copulation duration have been described for some species. These factors are complex and depend on the form of sperm precedence, female mating status and oviposition patterns, size of males, and age of males (Krebs, 1991; Snook, 1998; Koref-Santibanez, 2001). In general, however, longer copulation leads to a higher reproductive success for males. Among the six species examined here, males that mated with nonvirgin females and thus, experienced sperm competition, copulated for an unexpected shorter duration than males that mated with virgin females (*D. ananassae*, *D. nasuta, D. eugracilis, D. pseudoananassae* and *D. simulans*). However, in *D. melanogaster* males mated with non-virgin females, experienced sperm competition, copulated for longer duration than males that mated with virgin females. These results provide no evidence that males respond predictably to sperm competition risks through ejaculate in different species of *Drosophila* examined here, except *D. melanogaster* characterized by longer duration of copulation in second mating. Copulation duration and ejaculate characteristics of the male may be influenced by the discerned risk of sperm competition (Snook, 1998). As copulation duration increases, paternity inclined to increase. Males of certain species prolong copulation with previously mated females, allowing males to increase paternity by (i) delivering more sperm, (ii) mate guarding, or (iii) volumetrically removing more of a rival male's sperm from the female sperm storage organs (Parker, 1970). Males may alter the number and type of sperm transferred to females in response to female mating status, presence of rival males and female age. These results indicate that males increase their reproductive success by transferring larger number of fertilized sperm to compete with prior male's sperm and suggest that, because younger females are likely to have higher future fecundity, males transfer more non-fertilizing sperm to delay female remating behavior and prolong the use of their sperm (see Snook, 1998). Longer copulation is an adaptation to males which could reduce the risk of sperm competition with future ejaculates with the help of mating plug, which prevents the female from remating before oviposition (Gilchrist and Partridge, 2000). According to Eberhard (1996) copulation duration may be a component of post-mating courtship, *i.e*., females may require some initial period of mating to evaluate the male before they permit the transfer of ejaculate.

Finally, it has been reported that copulation (the seminal fluid transferred during mating) reduces the life span of *D. melanogaster* females (Chapman *et al.,* 1995). If this phenomenon is common in other *Drosophila* species, the females of *D. ananassae, D. nasuta, D. eugracilis, D. simulans* and *D. pseudoananassae* live longer due to their low remating frequency. However, recently Bundgaard and Barker (2000) reported that this is not true for *D. buzzatii* because this species is known for fast remating among *Drosophila* species. Thus, it may be suggested that different *Drosophila* species may vary in the incidence of remating and duration of copulation due to differences in their reproductive biology and adaptation.

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Tetraploid male mosaics induced by pressure.

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Within animal species, Fankhauser (1955) showed that cell volume is typically proportional to ploidy, whereas body size is not. His rules make sense because (1) cytoplasmic mRNA and protein reflect the number of active gene copies (Osborn *et al.*, 2003), while (2) organ sizes are dictated by the diffusion ranges of morphogens and the timing of hormones (Martín-Castellanos and Edgar, 2002; Stern, 2003; Vincent and Dubois, 2002).

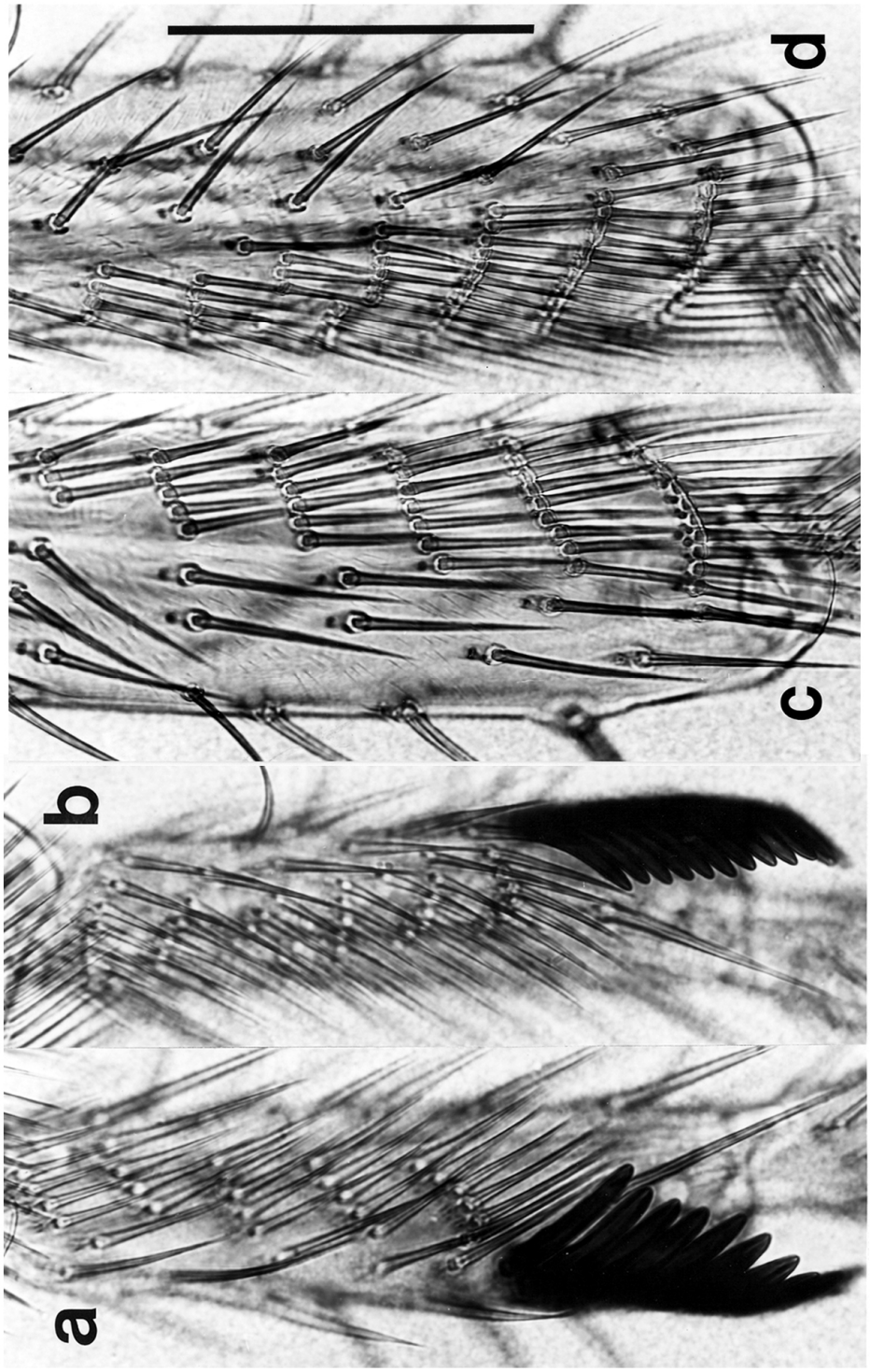

Figure 1. Left vs. right forelegs of a putative 2n/4n mosaic male (#e183). **a**, **b**. Right basitarsus with thick sex comb teeth (**a**) vs. left basitarsus with normal teeth (**b**). Bristles of the transverse rows are also thicker (and more widely spaced) in panel **a**. **c**, **d**. Right (**c**) vs. left (**d**) tibia of the same legs, showing a similar discrepancy in bristle thickness and spacing. Scale bar in **d** is 100 microns. All pictures are at the same magnification.

Wild-type *D. melanogaster* are diploid (2n). Triploid (3n) flies arise as offspring of adults that are fed colcemid (a tubulin-binding drug) during the larval period (Held, 1982), and they obey Fankhauser's rules (Held, 1979). Attempts to produce tetraploid (4n) flies by this method have failed, however, as have attempts using compound autosomes (Novitski, 1984; L. Held, unpublished). In each case, a serious obstacle is sterility.

Rare cases of 4n females have occurred as offspring of 3n females crossed with 2n males (Bridges, 1925; Morgan, 1925), but no 4n males have ever been found (Ashburner, 1989). The lack of 4n males calls into question the viability (or gender) of cells that carry 2Xs, 2Ys, and 4 sets of autosomes. My research was undertaken, in part, to investigate this issue at a tissue level.

In the present study, pressure was used to disable the spindle as a means of doubling the chromosomes somatically (Dasgupta, 1962), thus avoiding the problem of sterility. Eggs were collected on agar plates (ethanol-acetic acid) for 60 mins. at 25˚C, aged for 22 mins. at 21˚C, then submerged and exposed to 5000 pounds per square inch for 10 mins. (≈ 1 mitotic cycle). Pressure was applied by a hydraulic press (Carver) connected to a custom-made bomb chamber via a pressure transducer (Aminco). Other pressures and times were also tried but were less effective.

Age at the treatment midpoint thus ranged from 27 mins. (~cycle 3) to 87 mins. (~cycle 10) after egg laying, when the embryo has ~8 to 750 syncytial nuclei dividing in waves (Foe *et al.*, 1993). After treatment, pressure was reduced gradually (-500 psi/sec.). Control batches were submerged but not pressurized. Treated embryos were transferred to food bottles for the duration of development. Survival of pressurized embryos to the adult stage was only 8.5% (N = 200), compared with 90.5% (N = 200) for submerged controls.

Wings are easier to screen for ploidy than legs due to their flatness and uniform hairs, each of which is made by one cell. Wings were examined at 150× magnification for sparse hairs indicative of large cells. Patches of sparse hairs were found in 17/185 experimental males (9%) on one (15 cases) or both (2 cases) wings and in 12/222 experimental females (5%) on one (11 cases) or both (1 case) wings. Patches ranged in size from ~10% of one surface to virtually the entire wing (7 male and 3 female wings). No such patches were found on 108 control wings.

The legs of these 17 males were mounted between cover slips (in Faure's solution) and examined at 200× for abnormal sex combs. Three abnormal combs were found on 3 different adults. In two cases the comb had one or two teeth (bristles) that were obviously thicker.

The third case (right foreleg of male #e183) was striking. All 11 teeth in this comb were thick (Figure 1a), whereas the left comb (12 teeth) was normal (Figure 1b). The rest of the right foreleg had thick bristles as well, and they were widely spaced (Figure 1c)—indicating 4n ploidy. Indeed, the entire right 2nd and 3rd legs appeared 4n, as did the right wing (not shown). Evidently, this male came from an embryo, most of whose nuclei on the right side were dividing when pressurized.

The existence of combs with 4n-size teeth implies that 2Xs, 2Ys, and 4 sets of autosomes specifies maleness. This inference is consistent with the orthodox model of sex determination, where gender depends on the X:autosome ratio (Cline and Meyer, 1996). The sample size here is too small, however, to draw any conclusion about penetrance.

Because bristle spacing is proportional to cell diameter (Held, 1979; Stern, 2003), Fankhauser's rules imply that bristle number should decrease on polyploid legs—a correlation already documented in 3n flies (Held, 1979.). This prediction is also met in the 4n legs here. For example, rows 1-8 on the 2nd-leg basitarsi of male #e183 had the following numbers of bristles, where each ordered pair gives left (2n) vs. right (4n) data as "(L, R)": row 1 (11, 10), row 2 (10, 7), row 3 (7, 4), row 4 (5, 4), row 5 (6, 6), row 6 (7, 6), row 7 (10, 8), row 8 (13, 11).

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Sperm management in the sperm heteromorphic species, *Drosophila teissieri*.

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Apart from the species of the *D. obscura* group, in which all examined species showed a sperm heteromorphism (Joly and Lachaise, 1994; Snook, 1997), *D. teissieri* is another drosophilid species showing such a phenomenon (Joly *et al*., 1991; Lachaise and Joly, 1991). The heteromorphism in *D. teissieri* differs from the one observed in the *D. obscura* group species because the two sperm length morphs are not discrete classes but two major peaks in a wide and continuous distribution. Furthermore sperm length in *D. teissieri* is always longer than in the *D. obscura* group species (*i.e*., from 0.139 mm for *D. obscura* to 0.925 mm for *D. azteca* for the long sperm morph within the *D. obscura* group species, and 1.606 mm for the long sperm morph in *D. teissieri*; Joly *et al.,* 1989). If it now seems clear that only long sperm are fertilization competent in the *D. obscura* group species (Bressac and Hauschteck-Jungen, 1996; Snook, 1997; Snook and Karr, 1998), this question is still open for *D. teissieri*. To a better understanding of the reproductive strategy of this species, we performed a quantitative analysis of sperm production, transfer and storage at different times after mating.

Sperm production in *D. teissieri* males was determined by dissecting the two seminal vesicles of one-week-old non-mated males. The sperm mass was spread and the number of sperm determined by visual observation under a fluorescence microscope, after ethanol fixation and DAPI staining method for nucleus (Bressac and Hauschteck-Jungen, 1996). The number of sperm transferred during copulation was recorded dissecting the female genital tract just after the end of copulation of one-week-old pair of flies. In order to determine the number of stored sperm, the females were isolated just after the copulation and kept in vial with 10 ml of standard food for one, five or eight days. At the end of this period, the females were dissected and the number of sperm present in the storage organs, *i.e*. the ventral receptacle and the two spermathecae, was counted after DAPI staining.

Table 1. Sperm management in *Drosophila teissieri.* Means (M) and Standard Errors (SE) of sperm are given for males (it is considered that the two male seminal vesicles contain the same number of sperm), in uterus and storage organs in females just after mating (Transferred sperm), and in the storage organs in females after an egg laying period of 1, 5 and 8 days (Stored sperm D1, D5 and D8, respectively). N is the number of flies dissected.

Storage organ			M	SE	N	Min-Max
One seminal vesicle			2136.0	118.3	25	1262-3496
Transferred sperm			1644.0	90.1	20	887-2714
Stored sperm	D1	Receptacle	139.1	14.5	20	134-528
		2 Spermathecae	161.4	20.0	20	27-271
		Total	300.5	26.9	20	38-346
	D5	Receptacle	56.2	10.8	20	0-163
		2 Spermathecae	70.8	9.8	20	0-131
		Total	127.0	16.9	20	3-281
	D8	Receptacle	45.7	9.3	17	2-114
		2 Spermathecae	57.0	11.9	17	0-128
		Total	102.7	18.8	17	4-255

Results are shown in Table 1. Assuming each seminal vesicle in a male approximately contained the same number of sperm, about 4300 sperm are stored in an eight days old non-mated male. Almost 40% of available sperm in a male were transferred to the female during a single mating. The greater part, 90%, was present in the uterus, while 10% (160 sperm) were already found in the storage organs (75% of this stored sperm was in the ventral receptacle and 25% in the two spermathecae). Thus, a part of the sperm transferred during copulation was already stored as early as the end of mating. This unusual result could be linked to a long copulation duration, *i.e.* 48.45 ± 1.36 minutes (mean ± standard error calculated from 40 couples).

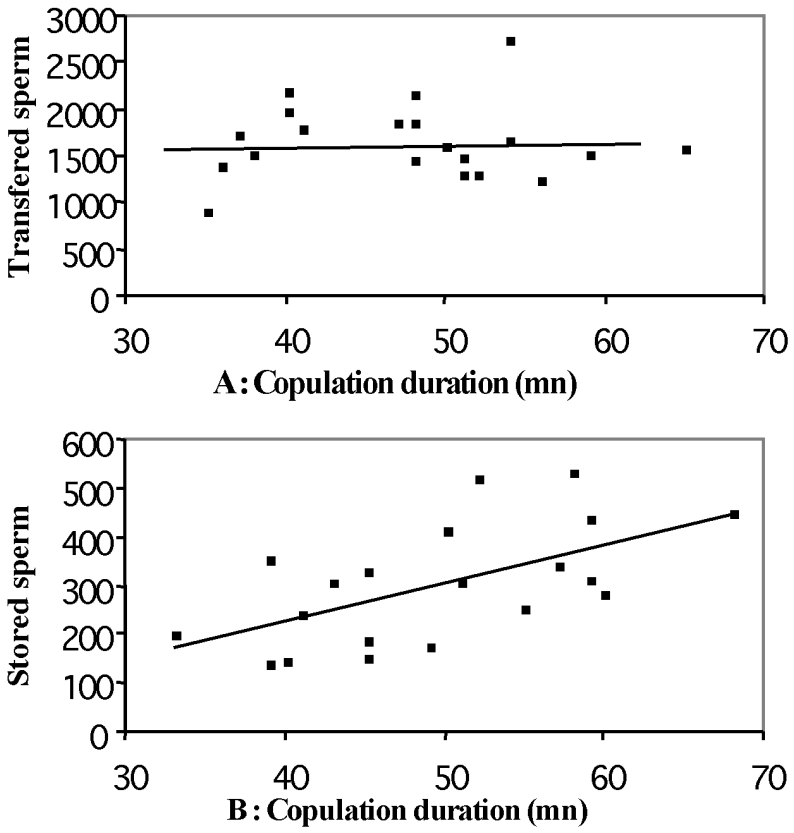

Figure 1. Number of sperm transferred by the male (A) and stored in the female (B) plotted against copulation duration. Correlations are indicated by the lines (see the statistics within the text).

Both types of sperm-storage organs in female's *D. teissieri* (*i.e.*, the two spermathecae and the ventral receptacle) are used, which is not always the case in the Drosophilidae (see Pitnick *et al.*, 1999, for a review). Less than 20% of the transferred sperm are still present in the female's storage organs 24 hours after mating. Therefore 80% were lost or used to produce the first offspring (11.7 ±

1.4 adult offspring was produced during the first day of egg-laying). It is interesting to note that the number of sperm stored in both types of storage organs is decreasing at the same pace. Then, the proportion of sperm stored in the two spermathecae and the ventral receptacle was the same after one, five or eight days of egg-laying ($\chi^2 = 4.018$, ddl = 1, $P < 0.05$), which suggests that the sperm are used without any preference from one of the storage organs. This result contrasts with observations carried out on *D. subobscura* females which preferentially use the sperm from the spermathecae (Bressac and Hauschteck-Jungen, 1996).

Figure 1 shows that the number of sperm stored in the females was clearly correlated with the copulation duration ($r = 0.597$, $P = 0.005$) in contrast to the number of sperm transferred (Pearson's correlation: $r = 0.039$, $P = 0.872$). Then increasing the copulation duration leads to an increase of the potential number of offspring for the males.

In our study, a mean of 51 offspring was produced per female between the first and the eighth day of egg laying. During the same period, nearly 200 sperm disappear in the storage organs. The high number of sperm wasted after storage in *D. teissieri* contrasts with results obtained in *D. melanogaster* (Gromko *et al*., 1984; Miller and Pitnick, 2003) and in *D. subobscura* (Bressac and Hauschteck-Jungen, 1996) in which nearly 50% and 83% of the stored sperm are used to fertilized eggs.

This study confirms large differences in the sperm management between sperm-heteromorphic species, namely *D. teissieri*, and those species of the *D. obscura* group for which only long sperm are fertilization competent. The reproductive strategy leading to various sperm morphs in *D. teisseiri* probably differs from the one found in the *D. obscura* group, and the question about the fertilization competence for the short sperm in this species remains open.

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Toxic effect of Fluoxetine - an antidepressant drug on the rate of development and viability of *Drosophila melanogaster.*

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Depression is a medical condition often people experience. People suffering from depression feel hopeless. They may have feelings of worthlessness and experience loss of interest in day to day activities such as work, hobbies, or sex. Fluoxetine {N-methyl-3-phenyl-3- [(alpha, alpha, alpha-trifluoro-p-tolyl) oxy] propylamine hydrochloride}, an antidepressant drug belonging to the class selective serotonine reuptake inhibitors (SSRI), is administered to get rid of depression (www.mentalhealth.com). Fluoxetine affects certain chemicals in the brain and release depression or

mood disturbances. However it may cause eating disorders or obsessive-compulsive disorders, and bulimia - binge eating and purging (www.discountrxpills.com).

The effect of fluoxetine other than this has not been studied either in man or any other animal. *Drosophila* is a sub-mammalian test system used in genotoxic studies (Vogel and Sobels, 1976). Since the effects of fluoxetine has not been studied earlier, its effect on rate of development and viability of *Drosophila malanogaster* is studied here.

Table 1. Mean developmental time of *Drosophila melanogaster* in control and different concentrations of Fluoxetine (in days).

Concentration (%)	For group	For males	For females
Control	12.41±0.05	12.52±0.08	12.29±0.07
0.02	14.43±0.11*	14.43±0.15	14.43±0.16
0.04	18.51±0.15*	18.34±0.21	18.70±0.22
0.06	19.34±0.16*	18.89±0.22	19.77±0.22
0.08	20.95±0.17*	21.01±0.26	20.90±0.22

*Control versus treatment significant at 5% level by t-test.

Table 2. Effect of Fluoxetine on viability of *Drosophila melanogaster* (No. of males and females emerged out of 1000 eggs laid).

Concentration (%)	Males	Females	Total	Viability (%)
Control	469	457	926	92.60
0.02	366	368	734	73.40*
0.04	316	289	605	60.50*
0.06	254	266	520	52.00*
0.08	162	199	361	36.10*

*Control versus treatment significant at 5% level by paired t-test.

Oregon-K strain of *Drosophila melanogaster* was used for the present studies. The flies were reared on a standard food medium consisting of 100 g wheat cream, 100 g jaggery (sugar), and 10 g agar, cooked well in 1000 ml water to which 7.5 ml propionic acid (antimouldant) was added. The chemical treated media was prepared by separately mixing 0.02, 0.04, 0.06 and 0.08% of fluoxetine for the above. Media without fluoxetine served as control. Larval feeding method was used to administer the chemical. To study the effect of fluoxetine on rate of development and viability, eggs of the same age were collected by modified procedure of Delcour (1969). Equal number of eggs (25/vial), were added to each vial containing fluoxetine, as well as control. One thousand eggs were allowed to develop in the respective concentrations of fluoxetine and control at uniform temperature of 24 ± 1°C. After emergence the flies were counted and sexed every day. Mean developmental time, viability and sex-ratio were calculated. The data were analyzed using the SPSS software ver. 10.0.

The results revealed significant variation ($P < 0.05$) in the developmental time at different concentrations of fluoxetine. Eclosion of flies in control started on 9^{th} day, while in treated eclosion commenced on 10^{th}, 13^{th}, 13^{th}, and 14^{th} day in 0.02, 0.04, 0.06, and 0.08% concentrations, respectively. Similarly eclosion ended on 19^{th} day in control, 25^{th}, 27^{th}, 29^{th}, and 30^{th} day in 0.02, 0.04, 0.06, and 0.08%, respectively. Mean developmental time for control was 12.41 ± 0.05 days, while for highest concentration (0.08%), it was 20.95 ± 0.17 days. Thus the mean developmental time at different concentrations of fluoxetine has been extended in proportion with the concentration used.

Table 1 also reveals that none of the concentrations had any discernible effect on the mean developmental time of either sexes ($P < 0.05$). Therefore males and females develop at the same rate and exploit the chemical environment at the same speed starting from the egg to the adult stage.

Table 2 shows that the viability has been reduced from 92.60% in control to 36.10% in 0.08% concentration. The extent of lethality is directly proportional to the concentration. It is also observed that fluoxetine induced significant changes in viability even in the lowest concentration (0.02%). Also the male and female larvae exhibit the same viability in different concentrations of fluoxetine.

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Distribution of telomeric transposable elements *TART* and *HeT-A* in *Drosophila melanogaster* and *D. simulans.*

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Incomplete replication at the end of the DNA molecule makes chromosome ends become shorter and shorter in successive cell generations, leading to programmed cell death. Chromosome termini thus need to be protected from degradation and fusion. This protection is provided by short tandem repeats that cap chromosomes in most eukaryotes. However, in some *Drosophila* species it involves transposable elements (TEs) (Pardue and DeBaryshe, 1999). Two LINE-like retrotransposable elements, *HeT-A* and *TART*, are involved in this phenomenon and are thought to be restricted to telomeric and chromocentric regions, no hybridisation ever having been detected in euchromatic regions of any strain. Transposition of these TEs to the telomeres opposes the loss of DNA of 50-100 bp per fly generation (Biessmann and Mason, 1988; Levis *et al.*, 1993). *Het-A* has been detected by *in situ* hybridisation at all salivary gland polytene chromosome tips in many strains of *Drosophila melanogaster*, with quantitative variation in labelling strength, due to copy number variation (Rubin, 1978; Young *et al.*, 1983; Valgeirsdottir *et al.*, 1990; Traverse and Pardue, 1989). In comparison, *TART* element is less abundant and not present at all tips, which may be due to a lower transposition rate for this element (Levis *et al.*, 1993). This lower abundance of *TART* makes it unlikely to be the supplier of reverse transcriptase for *HeT-A*, the latter element not encoding this enzyme, needed for transposition (Biessmann *et al.*, 1992), although the presence of both these elements across the *Drosophila* genus, suggests that they collaborate (Casacuberta and Pardue, 2003). By *in situ* hybridisation to polytene chromosomes and by Southern blots, we analysed the insertions of *HeT-A* and *TART* on individuals from populations of *D. melanogaster* and *D. simulans*. We showed that *HeT-A* and *TART* protect chromosome termini equally frequently. The presence, in all *D. simulans* populations, of a labelling with the *TART* probe in the 42C region of the 2R chromosome arm indicates that a sequence or a part of a sequence homologous to *TART* exists on the euchromatin away from telomeres. We observed *D. simulans* genomes that are devoid of either *TART* or *HeT-A* telomeric insertions, and no labelling was detected on chromosome 4. The absence of full-lenght copy of either *TART* or *HeT-A* on telomeres in certain populations, combined with the total absence of labelling in chromosome 4, raises the question of how these elements are maintained in these populations.

Materials and Methods

We worked with fly samples collected from several geographically distinct, natural populations. The 10 *D. melanogaster* populations considered were from Arabia, Argentina (Virasoro), Bolivia, China (Canton), Congo (Brazzaville), France (St Cyprien), Portugal (Chicharo), Réunion Island, Senegal, and USA (Seattle). The 10 populations of *D. simulans* were from Australia (Canberra, Can River), Kenya (Kwale, Makindu), Madagascar, New Caledonia (Amieu), Polynesia (Noumea, Papeete), Portugal (Madeira), and Russia (Moscow). These populations were maintained in the laboratory as isofemale lines or small mass cultures. It has been shown that these lines were highly homozygous (Vieira *et al.*, 2000).

We used as *TART* probes: 1) the internal 2.2 kb *Sac*I fragment (region 434-2683 of the element *TART A441* (accession number: U02279)) in the vector pIC19H provided by Lewis *et al.* (1993). This probe was denoted *TART*- 3', and 2) a 1.9 kb fragment obtained by PCR amplification of the region 5090 to 6987, which comprises part of the 3'UTR of the element *TART* A441 (accession number: U02279). This probe was denoted *TART*- 5'. We used as *HeT-A* probes: 1) a 2.7 kb fragment obtained by PCR amplification of the region 640 to 3393, which includes the coding sequence of the element *Het-A* 9D4 (accession number: X68130) (Valgeirsdottir *et al.,* 1990; Biessmann *et al.,* 1992). This probe was denoted *HeT-A*- 3', and 2) a 1.3 kb fragment obtained by PCR amplification of the region 4867 to 6129, which comprise the 3'UTR of the element *Het-A* 23 Zn-1 (accession number: U06920) (Danilevskaya *et al.,* 1994). This probe was denoted *HeT-A*- 5'.

Polytene chromosomes from salivary glands of third instar female larvae were prepared and treated with nick-translated, biotinylated DNA probes, as previously described (Biémont, 1994). Insertion sites were visualised as brown bands resulting from a dye-coupled reaction with a peroxidase substrate and diaminobenzidine (DAB). For each TE, slides from different populations and from the two species *D. melanogaster* and *D. simulans* were hybridised simultaneously so as to remove any problem associated with labelling intensity. Ten larvae for one isofemale line per population were analysed for *TART* and *HeT-A* insertions.

To be sure that the absence of labelling at the chromosome termini was not due only to a lower TE copy number not detected by the usual *in situ* hybridisation technique, we amplified the DAB labelling with tiramide following the NEN Life Science protocol. This amplification gave strongly-labelled signals. We, however, always detected the same number of labelled termini as with the classical conditions of the *in situ* technique. This strongly suggests that the absence of a labelling at a termini was really due to the absence of an insertion of the element or at least to a very short sequence not detectable by our technique and our probes.

Total genomic DNA was extracted from 20 females adult flies of the populations Makindu, Kwalé, and Can River for *Drosophila simulans*, and the population Canton 8 for *Drosophila melanogaster*, by a standard phenol-chloroform method after proteinase K digestion. DNA was digested with the enzymes *Bam*HI, *Eco*RI, *Eco*RII, and *Hind*III, which cut within or outside the sequence of *TART* and *HeT-A*. Agarose gel electrophoresis, transfer of DNA to membranes, pre-hybridization, and hybridization procedures were carried out at low stringency in 20% formamide at 42°C following standart procedures. Nylon Hybond N^+ membranes (Amersham) were washed at 42°C in 1 × SSC and 0.1% SDS. The 3' and 5' probes were random prime labeled with a Megaprime kit (Amersham), and the same blot was hybridized successively with the 4 probes defined above. The X-ray films were exposed at -80°C overnight or up to 4 days, with intensifying screens. The autoradiographs were scanned and the patterns assessed with a DNA molecular weight ladder.

Because the data of presence/absence of an element at telomere were not binomially distributed, we used a Wilcoxon rank sum test (Bauer, 1972; Hollander and Wolfe, 1973) with the

alternative hypothesis that for the two species over all populations the genomic number of insertions differed for the two elements *TART* and *HeT-A*. To analyse the distribution of the two elements between chromosomes within each species, we used the Friedman rank sum test with different treatments and blocs that corresponded respectively to the chromosome arms and the populations. These analyses were done with the R package (The R Development Core Team version 1.3.1, August 2001).

Results and Discussion

Tables 1 and 2 show the number of polytene chromosome termini labelled by *TART* (Levis *et al.,* 1993) and *HeT-A* (Biessmann *et al.,* 1990) 3'probes in *D. melanogaster* and *D. simulans* populations. In both species and for both TEs, not all chromosome ends were labelled in a given population. Most labelled chromosome termini were labelled in all 10 larvae analysed. The few termini that were labelled in only a subset of larvae could result from residual labelling heterogeneity, recent transposition of the TEs in some termini not yet fixed in this state by the homozygosity of the line, or heterogeneity in loss of tip sequences between individuals (Biessmann and Mason 1988). Globally in *D. melanogaster*, *TART* labelled fewer chromosomal ends than *HeT-A* (see Table 1; the probabilities of chromosome ends to be labelled were 0.49 for *TART* and 0.64 for *HeT-A*; $p-value \approx 0.01$). No chromosomal effect was observed to explain the higher observed value for HeT-A ($p-value \approx 0.1$). Both *TART* and *HeT-A* labelled the chromocenter but not in all genomes (data not shown).

The picture is quite different in *D. simulans* genomes (Table 2). The two elements *TART* and *HeT-A* did not differ for their mean probabilities of ends being labelled (probabilities: 0.30 for *TART* and 0.35 for *HeT-A*; $p-value \approx 0.54$). The probability of end labelling differed between chromosome arms, with the 3L and 3R ends being less often labelled and no labelling being seen on chromosome 4 for *TART* (probabilities: 0.45 for 2L, 0.33 for 2R, 0.24 for 3L, 0.25 for 3R, 0.54 for X; $p-value \approx 0.04$), with, in addition, an elevated number of insertions on 2L for *HeT-A* which reinforce the chromosomal effect (probabilities: 0.77 for 2L, 0.58 for 2R, 0.12 for 3L, 0.29 for 3R, 0.37 for X; $p-value < 10^{-3}$). Surprisingly, a labelling with the *TART* probe was observed in the euchromatic region 42C of the 2R chromosome arm in all *D. simulans* larvae, while no labeling was observed in the centromeric regions.

HeT-A and *TART* thus have more similar behaviours for presence/absence on telomeres than was previously thought (Walter *et al.,* 1995; Pardue *et al.,* 1997), with very similar probabilities of being inserted at the tips of chromosomes. However, our *in situ* technique cannot eliminate the hypothesis of more *HeT-A* copies in comparison with *TART* in the terminal TE arrays, which are known to differ between chromosome tips and change with time (Walter *et al.*, 1995; Mason and Biessmann, 1995; Fortunati and Junakovic, 1999). Since *HeT-A* lacks a reverse transcriptase (RT) gene and cannot mediate its own transposition, *TART* offers a possible source of RT for *HeT-A*, in addition to the host organism or other transposable elements. Although this idea of *TART* as the supplier of RT for *HeT-A* was disfavoured by the first reported observation that *TART* was less abundant than *HeT-A* (Levis *et al.,* 1993), our present work makes this hypothesis more reliable.

Because *TART* and *HeT-A* can transpose in any sequence at the end of a chromosome, they do not require a specific DNA sequence on the chromosome tips to target their insertion (Biessmann *et al.,* 1992; Danilevskaya *et al.,* 1992). Thus, the targeting may be mediated by interaction between the TEs and end-specific proteins (Biessmann *et al.,* 1990; Ahmad and Golic, 1999). The difference in number of labelled chromosome tips in *D. simulans*, for both elements, and the absence of detectable insertion on chromosome 4, argue in favour of variation in the efficacy of interaction between the

Table 1. Number of polytene chromosome termini labeled with *TART* and *HeT-A* in larvae from *D. melanogaster* populations (10 larva analysed per population).

	Populations										
Chromosomes	Arabie	Bolivie	Brazza	Canton	Chicharo	Reunion	Senegal	St Cyprien	Seattle	Virasoro	mean[#]
TART element											
X	10	10	0	0	0	0	10	10	10	0	0.50
2L	10	0	8	7	10	0	0	10	4	10	0.59
2R	0	10	7	4	7	0	0	10	10	0	0.48
3L	0	6	10	8	0	10	3	2	2	0	0.41
3R	2	8	0	0	10	0	10	0	8	10	0.48
4	0	0	10	8	10	10	0	1	3	8	0.50
HeT-A element											
X	0	10	1	0	1	2	10	10	0	10	0.44
2L	10	10	3	8	10	3	10	10	8	2	0.74
2R	1	3	10	10	10	0	0	10	10	1	0.55
3L	10	2	0	0	10	10	7	6	3	9	0.57
3R	1	6	3	8	10	9	5	0	10	10	0.62
4	10	10	10	10	2	10	10	10	10	10	0.92

[#] Mean number of labeled termini per chromosome arm

Table 2. Number of polytene chromosome termini labelled with *TART* and *HeT-A* in larvae from *D. simulans* populations (10 larvae analysed per population).

	Populations										
Chromosomes	Amieu	Canberra	Can River	Kwale	Madagascar	Madere	Makindu	Moscou	Noumea	Papeete	mean[#]
TART element											
X	0	10	10	7	0	0	0	10	7	10	0.54
2L	0	0	10	10	0	10	0	10	0	5	0.45
2R	1	0	10	3	9	0	0	0	9	1	0.33
3L	0	10	8	1	0	0	0	0	0	5	0.24
3R	4	10	0	1	0	0	0	0	10	0	0.25
4	0	0	0	0	0	0	0	0	0	0	0.00
HeT-A element											
X	0	1	10	0	1	10	0	2	4	9	0.37
2L	10	10	9	0	10	10	10	10	0	8	0.77
2R	0	8	10	0	10	10	10	0	1	9	0.58
3L	0	2	7	0	0	1	0	0	0	2	0.12
3R	9	2	3	0	1	1	2	1	0	10	0.29
4	0	0	0	0	0	0	0	0	0	0	0.00

[#] Mean number of labeled termini per chromosome arm
TART; w: weak labeling. Values in brackets are numbers of larvae analysed per species

TEs and the putative telomeric proteins. But the absence of labelling of the chromosome 4 end in *D. simulans* is puzzling. We could suspect the *in situ* technique, if the number and size of TEs was below the level the technique allowed us to detect, perhaps because of a specific configuration of the

chromosome 4 end. But why were the ends of chromosome 4 labelled in many larvae of *D. melanogaster*? It may be that the elements inserted in the chromosome 4 end in *D. simulans* had deletions in regions which include the sequence of the probe used. Although we cannot reject the possibility of a lower polyploidisation of the chromosome 4 end (Valgeirsdottir *et al.,* 1990; Leibovich *et al.,* 1990), which, however, appeared normal and not underreplicated, it may be that chromosome 4 is using another mechanism of telomere protection. The absence of *TART* or *HeT-A* at all chromosome ends in the populations Makindu (*TART*) and Kwale (*HeT-A*) of *D. simulans*, suggests that this mechanism could exist in addition to *TART* and *Het-A* protection at the other chromosomal termini of this species. A role of the usual telomerase-generated repeats that protect chromosome termini from degradation and fusion in various organisms is unlikely because such repeats have not been found in *D. melanogaster* (Pardue, 1994). This remains, however, to be demonstrated in *D. simulans*. Mechanisms such as those involving recombination in yeast (Nakamura *et al.,* 1998; Kass-Eisler and Greider, 2000), homologous recombination between DNA satellites, as reported in species of the virilis group (Biessmann *et al.,* 2000), and supposed to occur in lower dipteran insects (Roth *et al.,* 1997), may be at work. Gene conversion and recombination have even been proposed as an alternative mechanism to transposition in *Drosophila melanogaster* (Mikhailovsky *et al.,* 1999; Kahn *et al.,* 2000) and the mosquitos (Walter *et al.,* 2001). This could thus suggest coexistence of different mechanisms of telomere protection pathways in the same organism. Chance alone could of course account for the absence or an undetectable amount of short sequences of *TART* or *HeT-A* on all telomeres of larvae from Makindu and Kwale.

To check whether the absence of *TART* or *HeT-A* in the populations Makindu and Kwale, respectively, could be due to the presence of deleted or truncated elements not detected by our 3' probes, we did *in situ* hybridization on the chromosomes of these two populations with the 5' probes from these two elements. We did not detect more telomeric insertions with the 5' than with the 3' probes (data not shown), suggesting that if deleted elements existed in the telomeres, they should be very short so as not to be detected by the in *situ* technique. In addition, we did Southern blots on DNA from adult flies, that we probed with *TART*-3', *TART*-5', *and HeT-A*-3', *HeT-A*-5'. As seen for the *TART* element, only one to three bands were detected with both the 3' and 5' probes in Makindu and Kwale populations. This suggests that these two populations had very low *TART* insertions in their genome, the bands observed in the filters coming eventually from the centromeric or 42C regions. Cann River had more band than Makindu and Kwale, in agreement with the *in situ* data, but less than the Canton population of *Drosophila melanogaster.* The element *HeT-A* presented a different picture. Both Makindu and Kwale had many bands on the filters, although less than Cann River and Canton. Various deleted *HeT-A* insertions may thus subsist in the genome of populations, even in the Kwale population of *D. simulans* in which no insertion was detected in the telomeres and chromocenter. These insertions of deleted elements might thus be located in the chromocenter, even in the centromeric region of the Y chromosome (Agudo *et al.,* 1999), but got undetected by the *in situ* technique. Globally there were less bands detected with the 5' probe than with the 3' probe for both TEs in the Canton population of *Drosophila melanogaster*, which agrees with the fact that non-LTR retrotransposons are usually 5' deleted. The same tendency was observed for *HeT-A* in *D. simulans* but not for *TART*, for which more bands were observed with the 5' probe, especially in the Cann River populations. This suggests that the *TART* sequences in at least some populations of *D. simulans* differed from that of *Drosophila melanogaster* for their expected enzymatic restriction map (Danilevskaya *et al.,* 1998).

The absence of detectable labelling of the chromocenter and the very few bands detected by Southern blots for the two Makindu and Kwalé populations of *D. simulans* raise thus the question: from where do new transpositions of complete elements come? The 42C region could bear *TART* elements able to promote transposition to the chromosome termini in that species, although we have

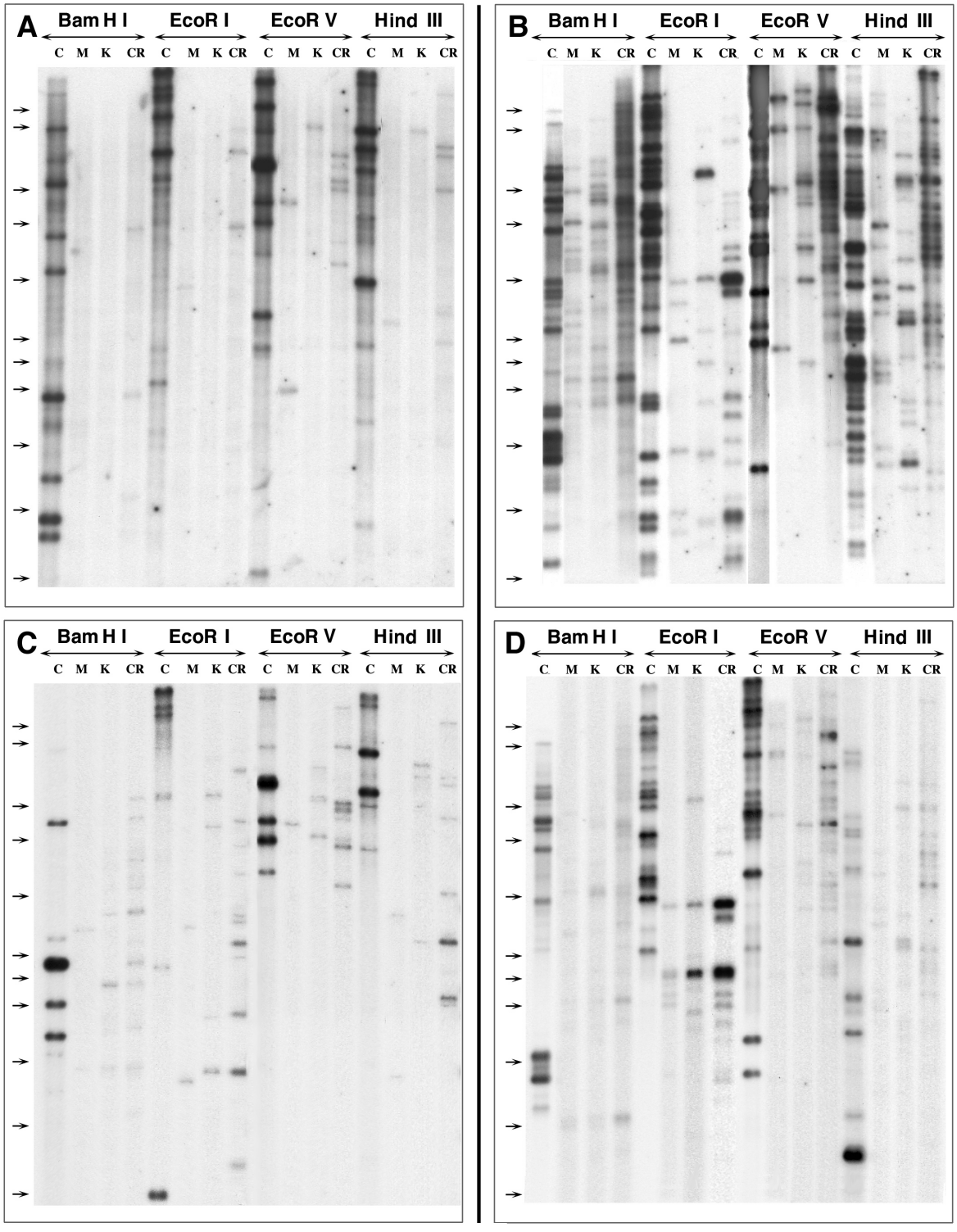

Figure 1. Southern blots of genomic DNA from adult flies of populations of *Drosophila melanogaster* (C: Canton) and *D. simulans* (M: Makindu, Kenya; K: Kwale, Kenya; CR: Cann River, Australia), digested with *Bam*H1, *Eco*RI, *Eco*RII or *Hin*dIII, transferred to filters, and probed with *TART* –3' (A), *HeT-A*-3' (B), *TART* –5' (C), and *HeT-A*-5' (D). The arrows indicate the band of the DNA molecular weight ladder.

no evidence for the presence of a complete *TART* element into this region. We have, however, to suppose that *HeT-A* transposition depends on centromeric sequences undetected by the *in situ* hybridisation technique as suggested by the bands observed in the Southern blots or on new sequences derived from internalisation of telomeres or centromeres by chromosome rearrangements (Losada *et al.,* 1999). As alternative hypotheses, it may be that the populations devoid of apparent telomeric sequences will not acquire new TE copies with time, or that the polytene chromosomes of the larvae are eroded more quickly than in other populations. The *TART* and *HeT-A*-devoid populations could thus suffer physiological decline and shorter longevity because of progressively eroded chromosome ends (Ahmad and Golic, 1999, but see Blackburn, 2000, for a discussion), or could suffer changes in gene expression if dependence of telomere position effect on telomere length is effective on *Drosophila* as it is in yeast or human cells (Kyrion *et al.,* 1993; Baur *et al.,* 2001). This dead end could of course be avoided if recombination of subtelomeric sequences, as seen in the mosquito *Anopheles gambia* (Roth *et al.,* 1997) and in yeast (Lundblad and Blackburn, 1993; McEachern and Blackburn, 1996) and in immortalized human cells lacking telomerase expression (Bryan *et al.,* 1997). is still operating.

Other TEs than *TART* and *Het-A* could participate in telomere protection. This is a likely possibility because during previous analyses of distribution of many TEs in *Drosophila melanogaster* and *Drosophila simulans* (Vieira *et al.,* 1999), we observed sporadic insertions of *412*, *roo/B104*, *copia*, *HMS Beagle*, *stalker* and other TEs at telomeres. The element *stalker* was even detected only on telomeres in certain populations (unpublished data). The presence of these various TEs at telomeres might of course be coincidental, which agrees with the idea that *TART* and *HeT-A* may play a role at telomere because of their preferential insertions, rather than because of a specific telomere function. This conclusion is strengthened by the absence of a negative correlation between the presence/absence at telomeres of both *TART* and *HeT-A* ($r = 0.18$, $P < 0.05$ for *Drosophila melanogaster*; $r = 0.07$, $P < 0.05$ for *Drosophila simulans*), as expected if natural selection was reducing the class of individuals with neither element. The presence of other TEs than *TART* and *HeT-A* could thus explain the absence of labelling of chromosome 4 ends if other TEs have preferential insertions to these ends. But it remains to explain why *TART* and *HeT-A* do not insert to this chromosome in *D. simulans* while they do in *Drosophila melanogaster*.

The existence in an euchromatic site in *D. simulans* of a sequence homologous to *TART* is puzzling. The complete sequence of this insertion, the host sequence adjacent to it, and the chromatin conformation of the 42C region, which is known to bear various TEs in *D. melanogaster* (Biémont and Gautier, 1989), will be of great interest for understanding the complex biology of telomeres and the special case of *D. simulans*.

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Proliferative genes and copy number control.

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Abstract

Mutant proliferative genes are responsible for cell overgrowth in *Drosophila* tumors. They can induce genome instabilities by asymmetric replication of homologous chromosomes. In bacteria, one of the loci, *efendi*-regulator, gains control over the replication origin of plasmid DNA, reduces its copy number and multimerizes the plasmid. A possible biological mechanism of proliferative genes functions is discussed.

Introduction

The *Drosophila* line Malignant Brain Tumor (MBT) has been subject to genetic analysis. This has shown the existence of proliferative genes involved in tumor formation (Riede, 1997). MBT carries additional mutations in tumor suppressor genes and oncogenes. Mutant oncogenes and tumor suppressor genes can destabilize the differentiation pattern in different tissues. Due to incomplete differentiation of the cells, overgrowth occurs and a tumor appears. However, neoplasms depend on

at least one additional proliferative gene defect. One strong proliferative gene defect alone is sufficient for tumor formation (Riede, 2002).

Mutant proliferative genes break the restriction of the cell cycle. They induce somatic pairing gaps of polytene chromosomes (Riede, 1997). The intimate contact between homologous chromosomes is initiated during S-phase and leads to an apparent haploid set of polytene chromosomes. Mutant proliferative genes additionally induce polytenization in brain cells, allowing replication. Thus, the genes are expected to break the restriction of the cell cycle by interference with part of the chromosome, allowing onset of replication.

Like in human tumor cells, chromosome aberrations are frequent in *Drosophila* tumors and correlate with the malignancy of the cells (Ryo *et al.,* 1984). The cause of this phenomenon is unknown. Chromosome aberration is a result of inadequate genome stability. It could be due to incomplete replication of the genome or to overreplication of certain regions or to asymmetric DNA division into daughter cells.

Here I present evidence that defect copy number control at the origins of replication might be involved in the process or tumor formation by mutant proliferative genes.

Results

Proliferative genes induce genome plasticity by replication errors: The chromosomes are not always symmetrically replicated and telomeres might miss (Riede, 1998). The proliferateve gene mutation *bellatrix* frequently shows somatic pairing gaps of the end of chromosomes, unreplicated telomeres and asymmetric amplification of homologous chromosomes (Figure 1). Asymmetric amplification is the result of asymmetric replication initiation. Thus, the gene is expected to interfere in a direct or indirect fashion at origins of replication.

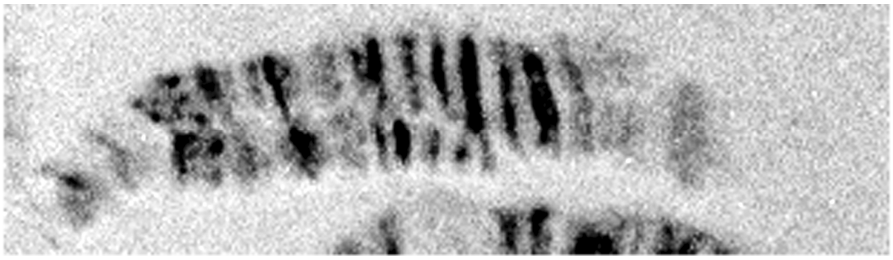

Figure 1. Asymmetric replication of homologous chromosomes at a somatic pairing gap induced by *bellatrix*^{P} in region 62: The homologous chromosomes at the unpaired sites are asymmetrically replicated, one chromosome appears without telomere replication.

The proliferative gene *efendi* is localized in 97F, within a region of about 8 kb (Gateff *et al.,* 1993; Wismar *et al.,* 1995). Cloning of that region in cosmid or plasmid libraries failed, only genomic lambda clones were stable. Subcloning of the region revealed, that one *Bam*HI-*Sal*I fragment of 4.1 kb induced lethality in bacteria. The lethality induction shows temperature dependence and is complete at 37^{o}C, whereas some growth is possible at 22^{o}C. The fragment is transcribed intensely in *Drosophila* into overlapping poly-A transcripts of 7.0, 2.1, 1.85, 1.35, 1.2, 1.0, 0.8 and 0.65kb in telomeric to centromeric direction and into one poly-A-transcript of 0.65 kb (*efendi*-regulator) in the opposite direction. The lethality induction is dependent on the direction of transcription from the bacterial promoter: most lethal is the transcription of *efendi*-regulator. The lethality induction is dependent on the copy number of the plasmid DNA: plasmids at low copy number are stable with the fragment, even at 37^{o}C. Despite great efforts, none of the transcripts could be cloned from cDNA libraries.

Incomplete lethality of the fragment at 22^{o}C opens the way for analysis of the character of the interaction. Freshly transformed bacteria were grown at the permissive temperature of 22^{o}C. The

plasmid starts to form multimers and the copy number decreases. After two days, monomers are absent and most of the plasmid DNA appears as highly polymeric. The plasmids still contain their original DNA. Digestion with restriction enzymes show the same size fragments as the original plasmid.

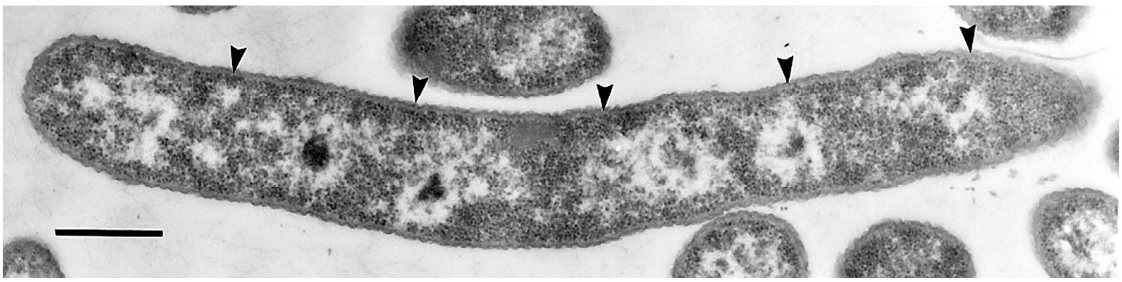

Figure 2. Hyperlong bacterium. At the sites of the arrows, the cell should have been divided. The bar represents 1 µm.

Analyzing the bacteria by electron micoscopy reveals that they form hyperlong fibers, the cell division signal is suppressed (Figure 2). The genomic DNA, usually visible as white mass within the cytoplasm, and within one or two areas of one cell, is spread in multimeric spots, indicating that coordinate replication - cell division fails.

Shifting the bacteria to 37°C induces multimerization of the plasmids within an hour. After that period, the genomic DNA starts to degrade and the bacteria digest their cytoplasmic proteins.

The DNA originating from *Drosophila* shows an effect not only onto the DNA replication in *Drosophila* but as well onto similar processes in bacteria. This ubiquity raises the question whether the human genome contains DNA homologous to those *Drosophila* sequences. Therefore the fragment of *Drosophila* was hybridized to human genomic DNA. *efendi*-regulator hybridizes to about 40 copies in human DNA. The centromeric neighborhood of the fragment is not present in human DNA, whereas the telomeric neighborhood shows as well repetitive occurrence in human DNA.

Discussion

Mutant proliferative genes induce somatic pairing gaps, allow replication and interfere with the DNA repair system (Riede, 2000). Three major biochemical defects of human cancer cells involve recombination, replication, and repair, all of the items are touched by the *Drosophila* proliferative genes. Here I present evidence, that the biochemical mechanism involves DNA copy number control.

The *Drosophila* fragment interferes in bacteria with the plasmid origin, reducing the copy number and inhibiting separation of the two copies after one replication round - allowing multimerization. In addition, the replication - cell division cycle of the bacterium is affected. *efendi*-regulator interferes with the bacterial genomic DNA - which is degraded under lethal conditions. Under sublethal conditions, cell division is suppressed. The resulting hyperlong bacteria reveal a similar phenotype than bacteria deficient in the recombination system (Handa and Kobayashi, 2003; Amundsen and Smith, 2003).

Bacterial DNA copy number is regulated by molecular systems localized at the origin of replication, usually coupling a set of transcrips in one direction with a regulative transcript in anti-

sense. The concentration of the anti-sense transcript regulates the onset of replication at the origin (Scott, 1984; Messer, 1987). Reguation of replication or origins in higher organisms are unknown.

The *Drosophila* region discussed here is transcribed intensely into a set of transcripts in one direction and into *efendi*-regulator in anti-sense. This indicates, that this region has the prerequisite for a similar biochemistry for copy number control.

A tumor cell circumvents the restrictions of the cell cycle. Mutations in proliferative genes allow replication and lead to hyperplasic growth of tissue. A proliferative gene mutation is thought to be the primary initiative event in tumor formation. The defect leads to overgrowth of cells. Wild type proliferative genes therefore inhibit cell overgrowth. *efendi*-regulator inhibits cell division of bacteria and inhibits replication of the plasmid in reducing its copy number. Thus, *efendi*-regulator reacts as an overgrowth blocking gene by interfering with the origin of replication. *efendi* and all of the proliferative genes might belong to the class of genes, strictly measuring and regulating the quantitative DNA level of each chromosomal region. They might be guardians of the origins.

Materials and Methods

Chromosomes were prepared as described from larvae grown at 18^{o}C (Riede, 1997).

Colony svcreening, nucleic acid isolation, protein analysis, and blotting were performed according to standard molecular biology protocols. Southern transfer hybridization occured at high stingency with random primed isolated fragments, Northern transfers were hybridized with single stranded RNA probes. As vectors served pBluescriptIIKS (Stratagene), lambdaEMBL4 (Promega), cos Pneo (Steller and Pirotta, 1985), pHSG575 and pHSG575/6 (Takeshita *et al.,* 1987) in HB101 (Boyer and Roulland-Dussoix 1969). The 4.1 *Bam*HI-*Sal*I fragment with *efendi*-regulator is localized in *Drosophila* Oregon in 97F, about 2 kb downstream of l(3)mbt (Wismar *et al*., 1995) and part of a 8.5kb*Sal*I fragment. For electron microscopy, bacteria were fixed with glutaraldehyde (2.5%) on ice for 45 minutes, embedded in agarose, refixed wiith OsO_4 (1%) and incubated in uranylacetate for 30 minutes. The samples were dehydrated in ethanol and embedded in epon for thin sections.

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Larval behavior in unfamiliar environment of two populations of *Drosophila pavani* that live naturally in very different breeding sites in the wild.

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In *Drosophila*, larval foraging and pupation behavior depend, between other factors, upon larval patterns of movement (Sokolowski, 1980; Godoy - Herrera and Silva - Cuadra, 1997). We investigated how larvae of two populations of *Drosophila pavani* that, in the wild, live in very different breeding sites move and use space and food provided by unfamiliar habitat. This type of investigation could of importance to understand: i) the behavioral bases of dispersal and distributional patterns of *Drosophila* larvae in the wild and ii) genetic differentiation between populations and species of ecologically important behavioral traits of *Drosophila* larvae.

One of the populations of *D. pavani* used (the Chillán strain) was formed with parents that had emerged from rotten apples (the Red Delicious variety). Another stock (the Til – Til strain) was originated from parents that had emerged from fermented cactus tissue (*Echinopsis chilensis*) (Manríquez and Benado, 1994). *D. pavani* belongs to the *mesophragmatica* species group of *Drosophila* (Brncic, 1957). The stocks were bred at 18 ºC because the flies that live in the cactus tissue die at 24 ºC. In contrast, the Chillán strain can grow well at 18 and 24 ºC.

One hundred and twenty petri dishes were each filled up to a depth of 2 cm with 3% agar. Once cooled, the agar surface was covered with a film of 44% of baker yeast paste. Larvae were individually deposited on the centre of the corresponding petri dish. Because of locomotion and turning are two behavioral elements that configure a *Drosophila* larval pattern of movement (Godoy – Herrera *et al*., 1997), we drew the trail made by each of the larvae on the agar - yeast surface of the petri dishes. Locomotion was estimated by measuring the length of each trail (Sokolowski, 1980). Turning behavior was recorded by counting number of directional changes in each trail. Each of the larvae was observed in a new petri dish. The observation period was 2 min. Larvae recorded were of 96 and 192 hours of development. At 96 hours (late second instar) feeding rate is maximal (Godoy - Herrera *et al*., 1994). At 192 hours of development, *D. pavani* larvae leave the nutritive medium crawling and wandering on glass wall of the breeding bottles searching for pupation sites. Additionally, latency (the time elapsed since a larvae is placed into the petri dish to the onset of the first occurrence of behavior) was also measured. Number of larvae tested per species and age was 30.

At 96 hours of development, larval locomotion of the Chillán strain was 18.60 ± 1.01 cm in 2 min. In this same time period, Til – Til larvae of the same age moved 19.27 ± 1.46 cm (t = 0.27, NS, df = 28). However, at 192 hours of age, Chillán larvae decreased locomotion (8.37 ± 1.07 cm), while larvae of the Til – Til strain increased this behavior (32.43 ± 1.91 cm) (t = 7.50, $P < 0.05$, df = 28). Mean number of turns made by 96 hours old – larvae were: i) the Chillán strain, 2.30 ± 0.30, ii) the Til – Til strain, 4.15 ± 0.64 (t = 1.97, $P < 0.05$, df = 28). At 192 hours of larval development, the mean number of turns was: i) the Chillán strain, 2.00 ± 0.50, ii) the Til – Til strain, 5.82 ± 0.73 (t = 2.91, $P < 0.05$, df = 28). Thus, depending on their developmental time, larvae of the two populations of *D. pavani* show important differences in ecologically relevant behavioral traits. Latency duration (seconds) also depend upon larval development and the strain. For example, at 96 hours of larval development the results were: i) the Chillán strain, 1.97 ± 0.19 sec, ii) the Til – Til strain, 11.77 ± 1.00 sec (t = 8.18, $P < 0.05$, df = 28). At 192 hours old, mean duration of latency (seconds) was: i) the Chillán strain, 42.97 ± 4.53 sec, ii) the Til – Til strain, 4.83 ± 0.55 sec (t = 7.41, $P< 0.05$, df = 28).

Our data indicate that under the same environmental conditions, larvae of two ages of two populations of *D. pavani* that, in the wild, live in very different breeding sites show important differences in their patterns of movement. The larvae crawl to explore feeding sites identifying microenvironments in which there are preadults of the same or the other *Drosophila* species. On the other hand, *Drosophila* breeding sites change their ecological conditions in a short time (Atkinsons and Shorrocks, 1984). The rapidity of occurrence of these changes depends, between other factors, on: i) type of breeding site (for example, fruit or tissue), ii) accumulation of toxic substances as ethanol, acetic acid, and larval waste substances, etc., and ii) physical characteristics of microenvironments surrounding the breeding sites (illumination conditions, humidity, etc.). The changes in larval patterns of movement reported here could be in line with the ecological features of the natural breeding sites of *D. pavani*. These factors could act as natural selection pressures participating in the evolution and development of larval behaviors of *Drosophila* species that live in decaying breeding sites.

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Mating activity in three populations of Chilean *Drosophila.*

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Drosophila pavani, an endemic species of the *mesophragmatica* group is found mainly in Chile from Lat 28º S to Lat 40º S and in some parts on the Eastern slope of the Andes Mountains in Argentina. Besides, it was recently reported from Ecuador. *D. pavani* has been captured on fermented banana baits and also on rotting fruits in orchards. Some flies were found on a rotten columnar cactus (*Echinopsis chilensis*) that contains alkaloids and triterpens (Barker, 1994). Descendants of these latter flies were found to differ from other *D. pavani* strains in a translocation between chromosomes 2 and 3 (Brncic and Manriquez, 1994). They can only be bred at 19ºC and hybridize with great difficulty with other populations.

Previous studies (Koref-Santibañez and del Solar, 1961) have shown that courtship in stocks of *D. pavani* of different geographic origin is very similar, although males prefer to court females of their own population, although this does not result in more homogamic matings. With *D. gaucha* (a sibling species found in Brazil and Argentina), *D. pavani* prefer to court and mate within their own species. However, they do court and mate with *D. gaucha* and form sterile hybrids.

The present study aims to investigate whether the different ecological conditions in which the Til Til population (bred from cactus) lives, may be associated with precopulatory sexual isolation. Courtship and mating patterns were studied in descendants of these flies and in stocks descended from flies caught in an orchard (Chillan) and on banana baits (La Florida).

Mating activity was studied by registering courtship in about 30 individual couples of each population. Males and females were isolated within 24 hours of hatching and kept separately for 10 days. Observations were made in empty 2.5 × 1 cm glass vials, at a constant temperature of 23ºC under a 100W light bulb for a period of 60 minutes. All activity was recorded with a tape recorder and elements of courtship were later analyzed quantitatively. Duration of copula was also timed, and couples which did not mate were placed in clean food vials. After 3-4 days female spermathecae were examined for insemination.

For male choice experiments, one male was placed with one female of its own strain together with one female of another population. Courtship was examined and recorded in the same manner described above.

Results and Discussion

The behavioral elements of courtship are those common to most species of the genus *Drosophila* (Spieth and Ringo, 1983; Greenspan and Ferveur, 2000). However, qualitative and quantitative differences were found among the three populations.

The ritual performed by the couples from Chillan (CH) and La Florida (LF) correspond to that described for *D. pavani* by Koref-Santibañez (1963). However, the couples from Til Til (TT) behave differently. Most courtship activity occurs on the cottonwool stopper of the vial or along the upper third of the vial. (CH and LF court along the lower third or bottom of the vial.) Circling of TT males around females is slow with abdominal movements and little scissoring. Males from the other two populations circle rapidly.

Table 1. Mean number (N°) and mean percentage (%) of courtship elements performed by males and females of three populations assigned to *Drosophila pavani*. N: Number of males and females that courted.

Males	Population and numbers that courted (N)						Females	Population and numbers that courted (N)					
	Til Til (TT) N=33		Chillán (CH) N=27		La Florida (LF) N=25			Til Til (TT) N=33		Chillán (CH) N=27		La Florida (LF) N=25	
Behavior	Nº	%	Nº	%	Nº	%	Behavior	Nº	%	Nº	%	Nº	%
Orient	71	10.84	48	7.89	56	6.55	Stand	48	9.98	34	16.67	39	10.86
Circle[1]	23	3.51	126	20.72	122	14.27	Ignore	29	6.03	26	12.74	24	6.68
Tap	191	29.16	95	15.63	176	20.58	Kick	31	6.44	37	18.14	66	18.38
Vibrate	215	32.82	115	18.91	173	20.23	Run	53	11.02	24	11.76	42	11.70
Scissor[2]	33	5.04	65	10.69	70	8.19	Abd.mov	41	8.52	18	8.82	6	1.67
Follow[3]	85	12.98	19	3.13	67	7.84	Tap[4]	106	22.04	12	5.88	46	12.81
Abd. mov.	56	8.55	78	12.83	89	10.41	Scissor	81	16.84	25	12.25	78	21.73
Lick	40	6.11	49	8.06	89	10.41	Open wg	73	15.18	22	10.78	40	11.14
Mount	9	1.37	13	2.14	13	1.52	Close wg	19	3.95	6	2.94	18	5.01
Total	655		608		855		Total	481		204		359	

Student t-test: [1]TT-CH t=2.54 P_{56} 0.01; TT-LF t=2.05: P_{50} 0.005; [2]TT-CH: t=-2.02, P_{56}<0.05; [3]TT-CH: t=2.28 P_{50} <0.05, CH-LF t=2.32 P_{50} <0.05; [4]TT-CH: t= 3.92 P_{56}<0.005, TT-LF t= 2.03 P_{50} <0.05

Wing vibration in TT is slower, discontinuous and repeated at short intervals, while in the others it is rapid and more prolonged.

Once the TT male mounts the female, she initially moves, and then remains in the upper part of the vial. On terminating the copula, she actively kicks off the male. CH and LF mounted females move around the sides of the vial during most of the copula, but do not go upwards. CH and LF males climb off actively.

The quantitative overview of the courtship elements is shown in Table 1. The mean number of courtship elements performed by each of the three populations is shown. There are few quantitative differences among them, although the qualitative characteristics mentioned above are

Table 2. Number of couples of three Chilean populations of *Drosophila* observed individually for 60 minutes. a.- that courted; b.- that mated within the observation period and c.- females inseminated after 3-4 days.

Population	Nº couples obs	a Courting couples		b Mating couples		c Inseminated females	
		Nº	%	Nº	%	Nº	%
Til Til	36	33	91.6	9	25	27	75.0
Chillán	30	27	90	13	43.3	17	56.7
La Florida	30	25	83	12	40	14	60.0

sustained. Thus TT males circle less than do either CH or LF, they scissor less than CH and follow the females more frequently than the other two. TT females establish contact with the males more frequently than do CH and LF females.

Table 2 shows that only a fraction of the couples examined courted (a), and those that did, not always copulated within the observation period (b). However, after 4 days all females were inseminated (c).

Copula lasted a mean of 20 ± 0.69 minutes in TT, 69 ± 3.3 min in CH, and 64.5 ± 2.76 min in LF.

In the male choice experiments (Table 3), CH and LF males court and mate equally with either CH or FL female and ignore TT of the opposite sex. The courtship patterns follows that described above. On the other hand, TT males only court and mate with females of their own population. Both TT males and females ignore the individual of the opposite sex of the CH and FL populations. Here also, their activity is restricted to the upper part of the tube.

These results indicate that the TT population behaves very differently from the other two populations of *D. pavani*. They respond to different types of sensory stimulation. Thus, although the type and number of courtship elements is similar in all three, the overall courtship pattern of the TT population is different from that of CH and TT.

Table 3. Number of courtships and matings by CH, LF and TT males with females of their own (homogamic) or another (heterogamic) population in male choice experiments. (Some CH and LF males courted both females).

Male	N	Courtship Female		χ^2	Copula Female		χ^2
		Homogam	Heterogam		Homogam	Heterogam	
CH	100	54	50	0.15	41	37	0.21
LF	100	60	50	0.91	37	26	1.92
TT	100	88	0		73	0	-

Thus, for example, courtship song determined by the type of wing vibrations that emit sound pulses, is probably very different in TT, as has been described for many species (*i.e*. species of the *virilis* group by Hoikkala *et al.,* 1982).

Precopulatory courtship differences have also been described in many sibling species, *i.e*., *D. heteroneura* and *D. silvestris* (Boake *et al.,* 2000) or different forms of the cactophyllic *D. mojavensis* from Arizona (Zourus and d'Entremont, 1988).

Another significant trait in which TT differs from CH and LF is the duration of copula, that amounts to almost 1/2 -1/3 of that in the other two populations.

As courtship is an important component of precopulatory sexual isolation, and constitutes one of the first steps in the origin of new species (Coyne and Orr. 1989), all these facts indicate that the precopulatory sexual isolation of TT from CH and LF most probably corresponds to a process of speciation that must be studied further.

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Molecular phylogeny of the *mesophragmatica* species group inferred from Cytochrome Oxidase II sequence.

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Introduction

The *mesophragmatica* group includes eight Andean species of *Drosophila* subgenus, distributed from Colombia to Chile and Argentina. The group was proposed by Brncic and Koref (1957) on the basis of morphological and chromosomal characters. Throckmorton (1975) proposed that *mesophragmatica* was originated from the *repleta* - *virilis* radiation during the New World Miocene. The phylogenetic relationships among *mesophragmatica* species were previously analyzed by Nair *et al.* (1971) and Brncic *et al.* (1971). Here, we analyzed the phylogenetic relationship among the species in the group using mitochondrial cytochrome oxidase II (COII) complete DNA sequence. In this analysis we include the sequence of sixty one other species belonging to several groups of *Drosophila* subgenus, obtained from Genbank. The monophyly of the group and the agreement between our results and those reported by Nair *et al.* and Brncic *et al*. (*op cit*) are discussed.

Materials and Methods

Samples: the specimens were obtained from the stocks of the Laboratory of Genetic Analysis of Behavior and Evolutionary Biology of *Drosophila*, Faculty of Medicine, University of Chile. The species used were: *D. pavani* (Chillán, Chile), *D. gaucha* (San Luis, Argentina), *D. viracochi* (Bogotá, Colombia), *D. brncici* (Bogotá, Colombia), *D. mesophragmatica* (La Paz, Bolivia), and *D. gasici* (Arica, Chile). Sixty one sequences from species of *Drosophila* subgenus were obtained from Genbank (see Table 1).

DNA extraction: flies were ground individually in tubes containing STE (400 µl), SDS 20% (20 µl) and PK (2 µl). Tubes were incubated at 45°C overnight. Chloroform - Phenol DNA extraction method and ethanol precipitation was used.

PCR amplification: fragments containing the COII complete sequence (688 bp) were amplified in *mesophragmatica* group species using the next set of primers:

FOXI2: 5´ AATATGGCAGATTAGTGCAA 3´ (Forward)
ROXI2: 5´ CCAGTACTTGCTTTCAGTCA 3´ (Reverse)
FOXI2b: 5´ TTGCTCTCCCTTCTTTACGA 3´ (Forward, internal primer)

Master mix and conditions for the PCR reactions were the standard for mitochondrial DNA with 55°C for annealing. PCR products were cleaned from agarose gel using Montage DNA Gel Extraction Kit (Millipore) and sequenced by automated sequence method.

Data analysis: Maximum Likelihood trees were obtained using *PAUP* 4.0b10 for Windows (Swofford, 2003). The evolutionary molecular model was selected comparing the likelihood of fifty-six models using MODELTEST 3.06 for Windows (Posada and Crandall, 1998). Bootstraps values (500 replicates) were obtained for parsimony analysis using heuristic search algorithm.

Table 1. List of species and Genbank Accession used in this study.

species	Genbank Accession	species	Genbank Accession	species	Genbank Accession
***melanica* group**		***pallidipennis* group**		***quinaria* group**	
micromelanica	AF478430	*pallidipennis*	AY162982	*unispina*	AF519344
dreyfusi group		***calloptera* group**		*curvispina*	AF519320
camargoi	AF478421	*calloptera*	AF478419	*falleni*	AF147117
***nannoptera* group**		*atrata*	AY162972	*kuntzei*	AF519326
nannoptera	AF478425	*schildi*	AY162973	*transversa*	AF519342
***bromeliae* group**		***cardini* group**		*occidentalis*	AF519333
bromeliae	AF478418	*cardini*	AY162974	*munda*	AF519330
***repleta* group**		*cardinoides*	AY162975	*quinaria*	AF478428
hamatofila	AF146176	*acutilabella*	AF519317	*nigromaculata*	AF519332
eohydei	AF145889	***tripunctata group***		***guttifera* group**	
hydei	AF145888	*tripunctata*	AF519343	*guttifera*	AF147119
nigrohydei	AF145890	*cuaso*	AY162984	***testacea* group**	
bifurca	AF145891	*metzii*	AY162992	*testacea*	AF519341
richardsoni	AF146175	*paraguayensis*	AY162987	*neotestacea*	AF519331
stalkeri	AF146174	*mediosignata*	AY162985	*orientacea*	AF519334
venezolana	AF146166	*mediopunctata*	AY162989	*putrida*	AF478431
starmeri	AF146165	*medioimpressa*	AY162994	***planitibia* group**	
martensis	AF146168	***macroptera group***		*obscuripes*	AY006443
serido	AF146173	*macroptera*	AF519329	*hanaulae*	AY006427
borborema	AF146171	***histrio* group**		*neoperkinsi*	AY006436
buzzatii	AF146169	*histrio*	AF519322	*planitibia*	AY006438
***immigrans* group**		***funebris* group**		*heteroneura*	AY006434
immigrans	AY162993	*funebris*	AF478422	*hemipeza*	AY006429
ruberrima	DRU243196	***guarani* group**		*substenoptera*	AY006440
***polychaeta* group**		*ornatifrons*	AY162978	*neopicta*	AY006439
polychaeta	AF478427			*picticornis*	AY006430

Results and Discussion

The model of molecular evolution selected by Hierarchical Likelihood Ratio Tests (hLRTs) was TRG+I+G (Gamma distribution shape parameter = 0.4404).

The maximum likelihood tree (see Figure 1) show that *mesophragmatica* group is a well defined monophyletic group (bootstrap = 64). In this analysis, *mesophragmatica* group is phylogenetically related with the *repleta* group, in agreement with the results obtained by Durando *et*

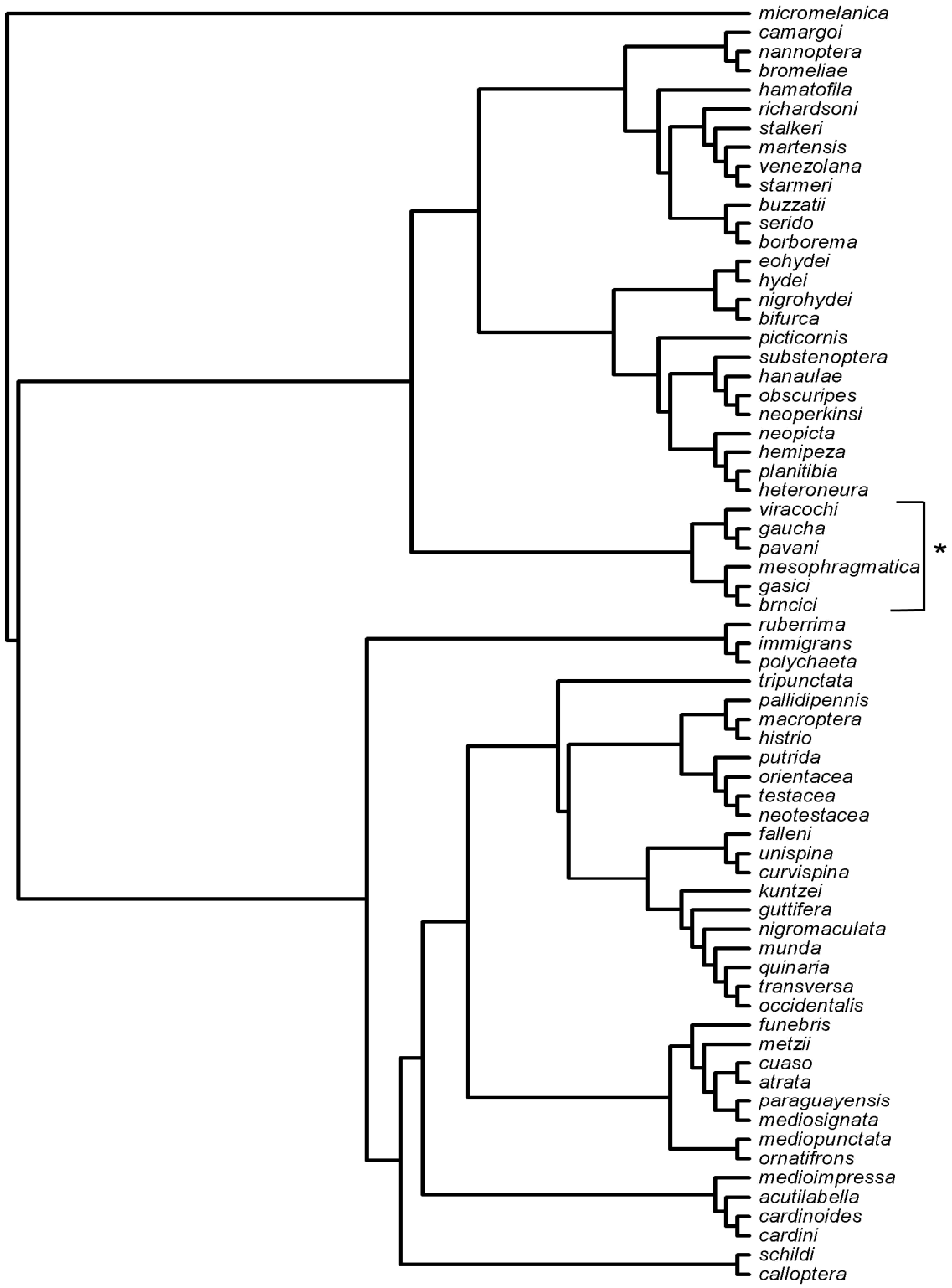

Figure 1. Maximum likelihood tree using the GTR+I+G model of molecular evolution. Heuristic search; Branch-swapping algorithm: TBR (tree-bisection-reconnection). * = *mesophragmatica* group.

al. (2000). In that article several species of the *repleta* and *virilis* groups but only two species of *mesophragmatica* group were included (*D. pavani* and *D. gaucha*). Our results are also in agreement with Throckmorton´s hypothesis of the *Virilis - Repleta* origin. *Virilis* group sequences for COII are not available in Genbank; therefore, this group was not included in the present study.

The *mesophragmatica* group topology obtained is very similar to those obtained by Nair *et al.* (1971) and Brncic *et al.* (1971). In that work phylogenetic relationships were inferred using isozyme distance matrix and polytene inversions analysis, respectively. In the present tree the group is formed by two well supported clades: the *pavani-gaucha-viracochi* clade (named the **pavani clade;** bootstrap **=** 54), and the *brncici-gasici-mesophragmatica* clade (named the **mesophragmatica clade;** bootstrap **=** 100). The *pavani - gaucha* clade (bootstrap = 100) could be the result of recent speciation. These sibling species are distributed very closely and overlapped in San Luis, Argentina (Koref-Santibanez, 2001).

Acknowledgments: Funded by FONDECYT 1020130.

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Stability of olfactory and locomotory response in third instar larvae.

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Abstract

The larvae of *Drosophila melanogaster* have a rich repertoire of behavioral complexity paralleling imago, albeit having a simpler nervous system. Despite the ease in handling, they have not been frequently employed in neurogenetic studies like isolation of learning and memory mutants. This is due to lack of information on the stability and robustness of many behavioral responses. In this study, the stability of olfactory and locomotory responses of third instar larvae were assessed. If larvae are maintained on thin layer of Ringers - agar (2%), they show stable olfactory and locomotory response for more than 8 hours after separation from growth medium.

Introduction

Stable and robust behavioral response of an animal is an essential characteristic for it to be used as a model for neurogenetic studies. *Drosophila* larvae have a much simpler nervous system than imago and have a translucent body wall. Many behavioral assays of larvae take less time than that of imago and are easier to conduct. Thus, larvae promise to be a suitable system for isolation of behavioral mutants and mapping of behavior. Till now, larvae have not been the favored model system for neurogenetic studies, like isolation of learning and memory mutants. In fact, no

conditioning mutant has been isolated using olfactory conditioning in larvae, and very few olfactory response mutants reported from larval screens (Cobb, 1996). Larval studies have lagged behind due to doubts about the capability of larvae to perform several behavioral tasks and the lack of information on the stability of larval responses. In recent years, many studies have demonstrated olfactory (Bala *et al*., 1998; Cobb and Domain, 2000; Wuttke and Tompkins, 2000) and olfactory learning capabilities (Dukas, 1999; Scherer *et al*., 2003) of larvae. This study attempts to address stability of olfactory response of third instar *Drosophila melanogaster* larvae.

Experimental Procedures

Synchronous cultures of larvae were reared on standard cornmeal yeast medium at 24°C and maintained on a 12 h day/night cycle. Standard procedures were followed in handling cultures (Ashburner and Thompson, 1978; Roberts, 1988). Early third instar larvae were separated from cornmeal medium by floating on 30% Poly ethylene glycol solution, as described in this issue of DIS (technique article by Khurana). After separation the larvae were maintained on a thin layer of *Drosophila* Ringers - Agar (2%).

The olfactory response of larvae was measured using the olfactory assay described by Heimbeck *et al*. (1999). The numbers of larvae in odor and control zone were counted from the images obtained by Intel image capture. The response index was defined as:

(larvae in odor zone – larvae in control zone)/ (total larvae in odor and control zones).

Liquid paraffin was used as a diluent for all the odorants tested.

The larval locomotion was measured in absence of any olfactory cue, as described by Roberts (1988), with some minor modifications. The size of the etchings was reduced to 0.5 cm by 0.5 cm and the test was run for 2 minutes, in a 15 cm plate. All the experiments were later confirmed with automated tracking (tracking software kindly provided by Veeresh Neginhal). All the experiments were preformed under uniform overhead white light to avoid the effect of stray light cue. All the fine reagents used were procured from Sigma Aldrich. Errors were calculated as standard deviation of mean of means. Student's t test was used to calculate significance of results.

Results and Discussion

The size of the larvae increases during different instars and so does its speed. Hence mixed instars of larvae cannot be used for most purposes. All the experiments mentioned here employed only third instar larvae.

To counter the effects of separation of larvae from the food medium, in the earlier studies (Aceves-Pina and Quinn, 1979), larvae were placed along with food. For conditioning, locomotory and olfactory experiments such procedure will introduce confounding variables in the experiments. Contact with cornmeal medium makes larvae sticky and affects the locomotion. The odor of the cornmeal medium can guide odor driven response. Food can act as reward and errors in the conditioning experiments, thus introducing other errors.

Keeping larvae out of cornmeal medium for several hours also has its problems. Frequently the Ringers solution turns white, after approximately two hours of keeping third instar larvae in it. This is probably due to condensation of salt on larval cuticle and resulting changes in osmolarity. Larvae become fragile and unresponsive if kept for longer time period. It has been a general practice to conduct the larval olfactory assay within an hour after separating them from cornmeal medium.

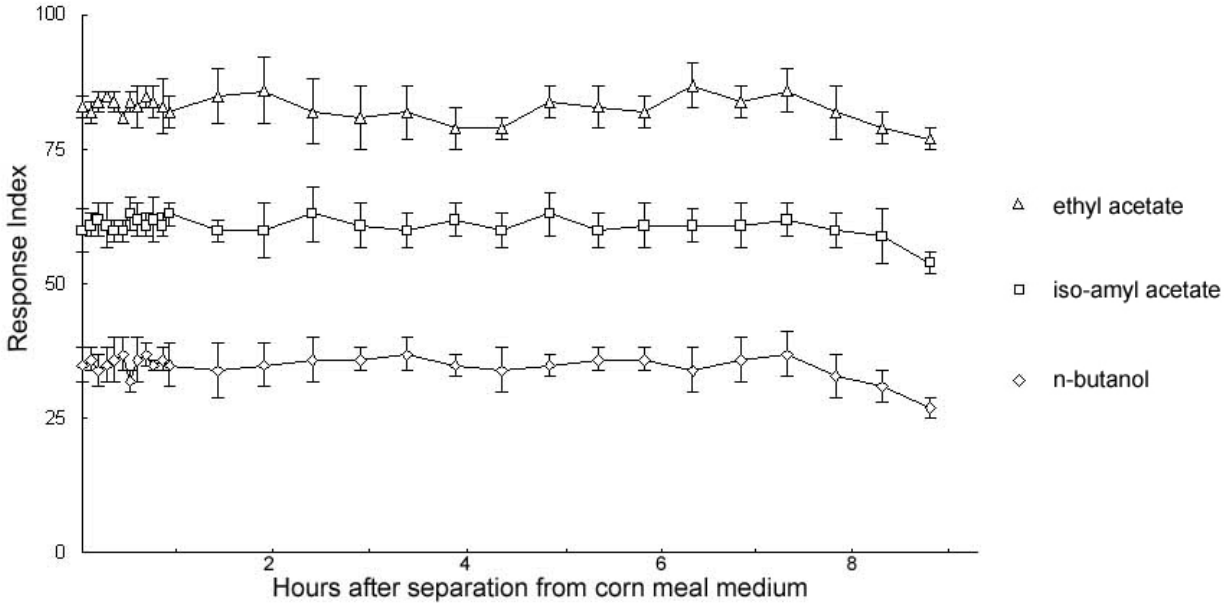

Figure 1. Responses of third instar larvae to 10^{-2} ethyl acetate (open triangles), 10^{-3} iso-amyl acetate (open squares) and 10^{-4} n-butanol (open diamonds) after different periods of separation from cornmeal medium. N = 8 independent experiments for each data point.

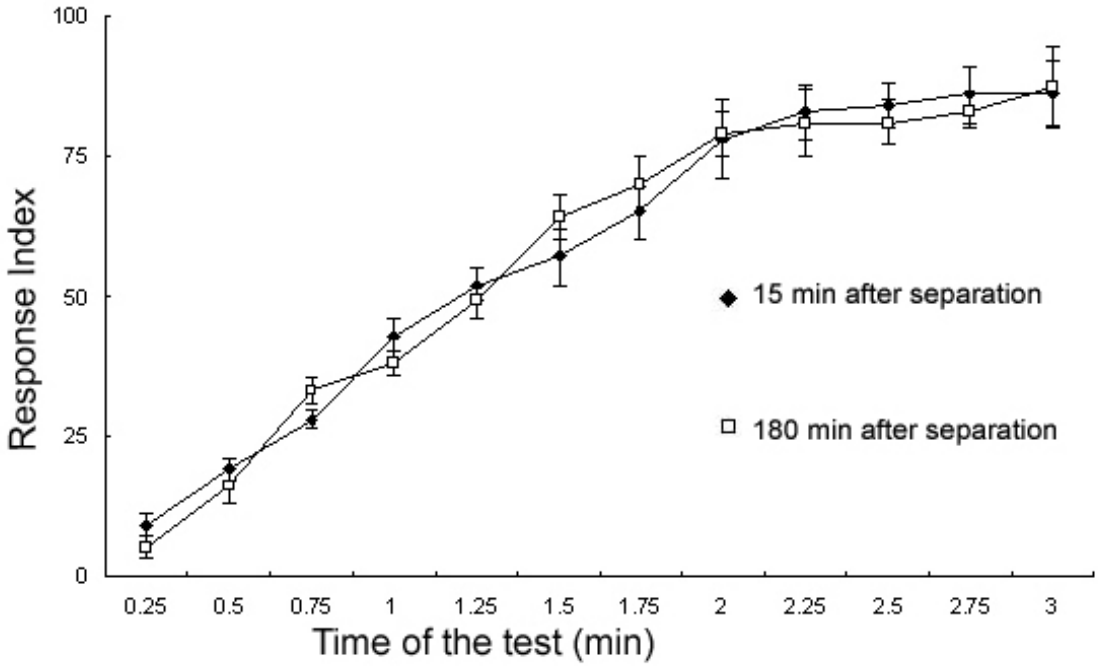

Figure 2. Rate of larval entry after 15 minutes (closed diamonds) and 3 hours of separation (open squares) to 10^{-3} ethyl acetate. N = 8 independent experiments for each data point.

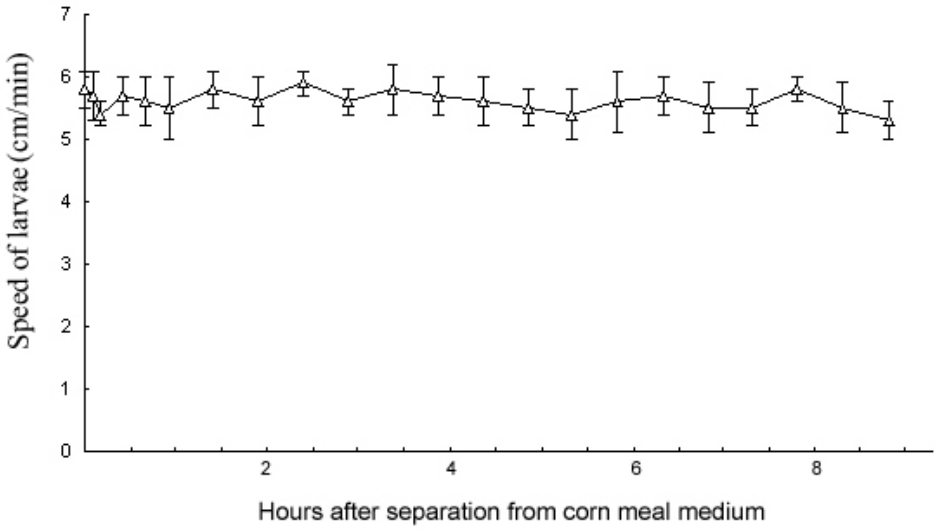

Figure 3. Speed of larvae after different periods of separation from cornmeal medium. N = 12 independent experiments for each data point.

During these studies it was observed that if larvae were maintained on a thin layer of Ringers –agar (2%), they stayed healthy and the medium did not turn white even after keeping larvae for more than 12 hours in it. Thus the response of the third instar larvae for all the studies presented here was measured after waiting on a thin layer of Ringers agar instead of Ringers solution.

Larval olfactory responses were measured for ethyl acetate, iso-amyl acetate, n-butanol, and ethyl valerate for every ten-fold dilution in the range of 10^{-7} to 10^{-1}. Olfactory measurements were taken after every 5 minutes in the first hour and after every 30 minutes during 1 hour to 9 hour. The responses of the larvae to all the odorants were found to be stable till 8 hours. Figure 1 shows some typical responses. A slight reduction in response was observed only in the 9^{th} hour of starvation. Thus till 8 hours, which is practically a whole day of measurement, the olfactory response of larvae can be observed without any significant reduction.

To verify the stability of response, rate of entry was measured. Figure 2 gives one such comparison of rate of entry of larvae in response to 10^{-3} ethyl acetate after 15 minutes and 180 minutes after separation. No significant difference in the rate of entry was observed, for any test.

The speed of larval locomotion increases with increasing age and it tends to slow down before prepupa stage. Thus, there is a possibility that the apparent stability of olfactory response is due to increased locomotion and decreased olfactory response, compensating each other. To test this possibility, larval locomotion was assayed after different periods of waiting in Ringers-agar (2%). Measurement of locomotory response of larvae till 9 hours showed a stable response (Figure 3). Hence, after separation from cornmeal medium, there is no change in speed and olfactory response of third instar larvae, if maintained on thin layer of Ringers-agar.

Thus the third instar larvae were found to be a robust system for olfactory and olfactory conditioning experiments running for a whole day.

Acknowledgements: Without the help of Professor Obaid Siddiqi this work would not have been possible. Thanks to Veeresh Neginhal, for allowing using of his larval tracking software.

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Isolation of amorphic alleles of the *lesswright* gene by P element-mediated male recombination in *Drosophila melanogaster.*

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Abstract

We isolated two molecular null alleles of the *lesswright* (*lwr*) gene via P element-mediated male recombination. We used a PZ insertion line, in which the PZ element was inserted 3' (distal) to the *lwr* gene, and attempted to isolate deletions proximal to the element. We isolated 13 recombinant chromosomes, and two of them were associated with a deletion. One such chromosome has a deletion from the PZ element toward the *lwr* gene, and the deletion removes the entire *lwr* gene. The other chromosome lost the entire PZ element except for an eight bp long segment at the end of the element, and its deletion extended to remove the entire *lwr* gene. Therefore, these two new alleles are molecular nulls. The existing *lwr* alleles were also classified using one of the new null alleles; the lwr^{4-3} and lwr^{5} alleles were hypomorphic and the lwr^{14} allele was a null allele. Lethal phases of the null alleles are around the early third instar larval stage.

A ubiquitin-conjugating enzyme functions in the second step in ubiquitination, which designates proteins for the 26S proteasome-mediated degradation. There are many enzymes that belong to this group and they are collectively called E2 enzymes. There are 26 E2 enzymes identified based on their sequence similarities in *Drosophila melanogaster*. Interestingly, enzymes in the Ubc9 subfamily conjugate small ubiquitin-like modifiers (SUMO) instead of ubiquitin. Thus, they are often called SUMO-conjugase. SUMO-conjugation (SUMOylation) regulates subcellular localizations and stability of target proteins and it is involved in development and tumorigenesis (Melchior, 2000; Muller *et al.,* 2001; Yeh *et al.,* 2000). Therefore, it is important to understand how SUMOylation functions in different biological processes.

Mutations of the *Drosophila* Ubc9 gene, *lesswright* (*lwr*), exhibit pleiotropic phenotypes Epps and Tanda, 1998; Apionishev *et al.,* 2001). The previous studies of the *lwr* mutants indicated that *lwr* plays a role in nuclear transport and disjunction of homologues in female meiosis. In order to gain more insights into the functions of *lwr*, we attempted to isolate null alleles of the gene because only hypomorphic alleles have been isolated. Taking advantage of the presence of P element insertion distal (3') to the *lwr* gene, we induced P element-mediated male recombination in the hope of

isolating deletions that remove at least a part of the *lwr* coding region. We also genetically classified the existing *lwr* alleles using a newly isolated null allele of the *lwr* gene.

Materials and Methods

Fly culture: Flies were raised in Jazz Mix-based medium at 25°C in uncrowded conditions. Jazz Mix was purchased from Fisher Scientific.

Mutagenesis: In order to obtain a null allele of *lwr*, we attempted to isolate deletions created by P element-mediated male recombination (see a review at http://www.wisc.edu/genetics/CATG/engels/Pelements/index.html). The $lwr^{semi710}$ allele was used as the P element source. The *PZ* element responsible for this allele, $P\{PZ\}lwr^{semi710}$, is inserted in the 3' regulatory region of the *lwr* gene, which is distal to the gene (Figure 1). The ex^1 ds^1 S^* ast^* chromosome was used to identify chromosomes resulting from male recombination between the *ex* and the *S* loci. Note that the *lwr* gene is between the *ex* and the *ds* loci. The mating scheme is depicted in Figure 2. In the F_2 generation, we set up more than 300 cultures with a few ex^1 females and a single $P\{PZ\}lwr^{semi710}/\ ex^1$ ds^1 *S* ast*;* $P\{\Delta 2\text{-}3\}$, $Sb^1/\ ry^{506}$ male. From them, 269 males produced F_3 progeny. In the F_3 generation, we searched for males with rough-surfaced eyes (*S*) with wild type wings (ex^+). Such males were crossed with *y* w*; CyO/ L* Pin** females to establish balanced stocks.

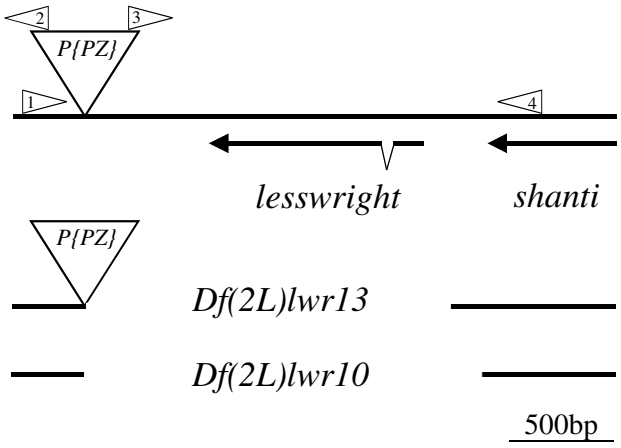

Figure 1. Schematic drawing of a genomic segment around the *lwr* gene. The horizontal bar at the top represents the genomic segment of wild type, and lines with arrows indicate the locations of the *lwr* and the *shanti* genes. An inverted open triangle marked with *P{PZ}* indicates an approximate location of the $P\{PZ\}lwr^{semi710}$ element. Open arrowheads with numbers show the location of primers used in this study: primer 1, lwr35P1; primer 2, pry4; primer 3, plac1; primer 4, lwrF1. The telomere is to the left. Two lines at the bottom are schematic presentations of the deficiencies *Df(2L)lwr10* and *Df(2L)lwr13*. The solid lines indicate the segments present in these deficiency chromosomes.

Sequencing of new lwr alleles: To determine the molecular structures of the new *lwr* alleles, lwr^{10} and lwr^{13}, genomic DNA was purified from these mutant stocks and the genomic region of *lwr* was amplified by PCR. To characterize the lwr^{13} allele, we used the plac1 primer located at the 5' end of the *PZ* element (Berkeley Drosophila Genome Project, http://www.fruitfly.org/) and the lwrF1 primer, which is located 582bp upstream of the 5' most end of *lwr* cDNA clones (Figure 1). We used a primer distal to the *PZ* insert (lwr35P1) and lwrF1 to characterize the lwr^{10} allele (Figure 1). The pry4 primer, which hybridizes to the 3' end of PZ element was also used to verify the presence or the absence of this element in these new *lwr* alleles. The sequences of the primers specific to the *lwr* gene are the following: lwrF1; 5'-CCATCTACCGCAGTCCATAGCTC, and lwr35P1; 5'-GTGCTGCCAGATTAGCG. The fragments shorter than the expected size were cloned into the pCR2.1 vector (TOPO-TA cloning kit, Invitrogen) and sequenced at the Plant-Microbe Genomics Facility at Ohio State

University.

Allele Classification: We used the following stocks for allele classification: *y* w*; lwr$^{4-3}$ FRT40A/ CyO, y$^+$, y* w*; lwr^5 b^1 cn^1 bw^1/ CyO, y$^+$, y* w*; Df(2L)dUbc9-14/ CyO, y$^+$, y* w*; lwr^{13} ds^1 S* ast*/ CyO, y$^+$,* and *y* w*; lwr^{13} ds^1 b^1 cn^1 bw^1/ CyO, y$^+$*. The *y* w*; lwr^{13} ds^1 b^1 cn^1 bw^1/ CyO, y$^+$* was used for assay of the *lwr^{13}* homozygotes. Larval viability was estimated by counting the number of *y* mutants and *y$^+$* larvae at the fifth day of development. A *yellow$^+$* transgene on the *CyO* balancer allows us to determine genotypes of larvae. Larval instar was determined by counting the number of teeth on the mouth hooks (Ashburner, 1989).

Results and Discussion

Isolation and molecular characterization of new lwr *alleles:* In order to obtain a null allele of the *lwr* gene, we induced deletions via P element-mediated male recombination. PZ insertion located distal to the *lwr* gene was used for this purpose. The experiment was designed to identify recombination proximal to this PZ element (Figures 1 and 2). We set up 269 F_2 cultures with a single male and isolated 13 independent chromosomes with the *ds^1, S*,* and *ast** alleles, but not the *ex^1* allele. Two of them failed to complement the *lwr^5* and *lwr^{14}* alleles, indicating that these two chromosomes carry new *lwr* alleles. Thus, we named them *lwr^{10}* and *lwr^{13}*.

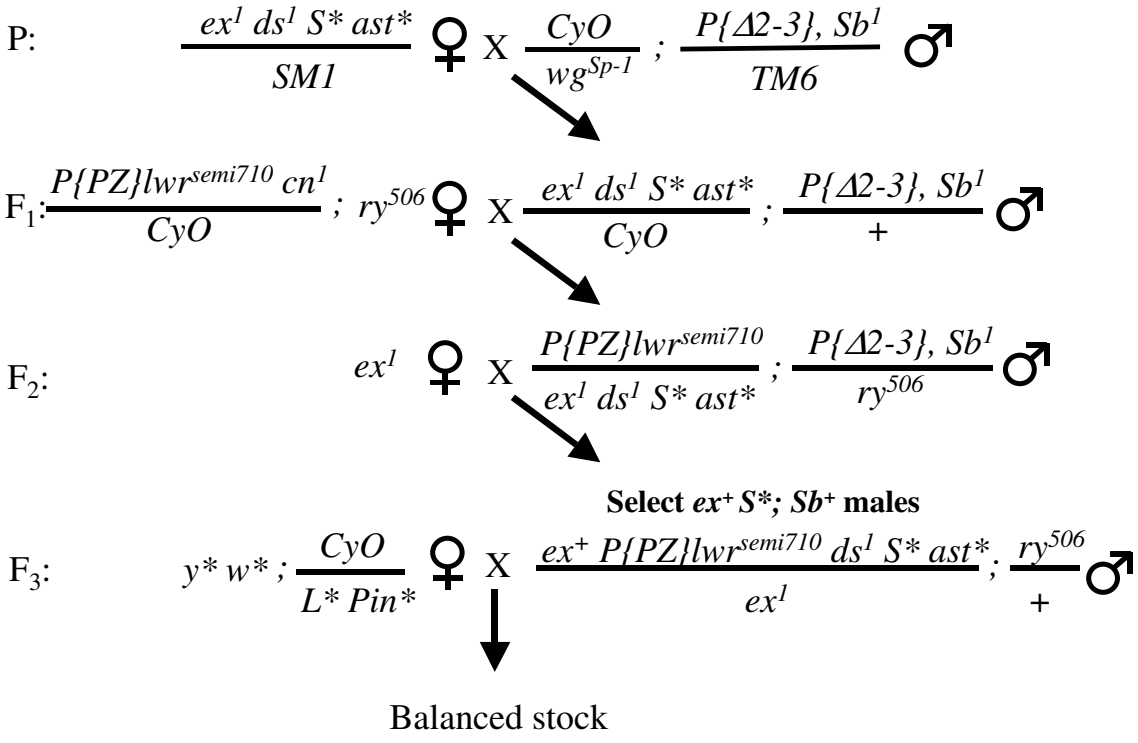

Figure 2. Mating scheme to identify recombinant chromosomes via P element-mediated male recombination. Asterisks indicate mutations with uncertain allele numbers. All mutations and stocks are described in FlyBase (http://flybase.bio.indiana.edu). P{Δ2-3} is a P element transposase source at 99B (Robertson *et al.,* 1988).

Molecular characterization of the new *lwr* alleles was done by Polymerase Chain Reaction (PCR) and sequencing of PCR products. A PCR fragment was obtained from the lwr^{13} allele using a set of primers, plac1 and lwrF1 (primers 3 and 4 in Figure 1, respectively), and the sequence of this fragment revealed a deletion of a 1,603bp-segment from the P element insertion site (Figure 1). We named this the deficiency *Df(2L)lwr13*. The proximal breakpoint of this deletion is 18bp upstream of the 5' end of the *lwr* gene and 299bp downstream of the poly(A) addition site of *shanti* (Apionishev *et al.,* 2001),which suggests that this deficiency is most likely to affect *lwr* function only. The molecular configuration of this deletion suggests that it was probably produced by the hybrid element insertion (HEI) model (Preston *et al.,* 1996).

On the other hand, the molecular analysis of the lwr^{10} allele showed that the PZ element was also lost during P element-mediated male recombination. We obtained no PCR product with any primer combination including P element primers, pry4 or plac1 (primers 2 and 3 in Figure 1, respectively). However, we obtained a PCR fragment using lwr35P1 and lwrF1 primers (primers 1 and 4 in Figure 1, respectively). The sequence of this fragment indicated a deletion of a 1,885bp genomic segment and the presence of an 8bp-long piece of the 3' end of the P element. This deletion was named the deficiency *Df(2L)lwr10*. This deletion extends to the 3'end of the next gene, *shanti*, and the distance between its proximal breakpoint and the poly(A) addition site of *shanti* is only 17bp in length. Therefore, it is possible that this deficiency affects *shanti* function to some extent. The molecular configuration of this deficiency suggests that this may be a byproduct of double-strand break repair following P-element excision (Engels *et al.,* 1990; Gloor *et al.,* 1991). Taken together, we consider both new *lwr* alleles as molecular null.

Classification of the lwr alleles: We wanted to genetically classify the existing *lwr* alleles since we have isolated molecular null alleles. We classified the existing *lwr* alleles, lwr^{4-3}, lwr^{5}, and lwr^{14} using the lwr^{13} allele. All the alleles used are zygotic recessive lethal. The majority (more than 90%) of the transheterozygous embryos of lwr^{4-3}, lwr^{5}, and lwr^{14} with lwr^{13} as well as lwr^{13} homozygotes hatched, indicating that the zygotic loss of *lwr* function does not affect embryonic development too much. However, some portion of the dead embryos exhibited cuticle defects that were previously reported (Epps and Tanda, 1998). In order to determine a lethal stage of these allelic combinations, we first compared the number of transheterozygous larvae with those of heterozygous control animals in culture at the fifth day of development (Table 1). This period roughly corresponds to the late third instar larval stage. The larval viability ranges from 50.0% (lwr^{13}/ lwr^{14} heteroallelic combination) to 64.6% (lwr^{3}/ lwr^{13} heteroallelic combination). We also determined the larval stages of *lwr* mutant animals in culture based on the number of teeth on the mouth hooks (Ashburner, 1989). Table 1 shows a rough positive correlation between larval viability and the developmental stage of the mutant larvae. The lwr^{13}/ lwr^{14} transheterozygous larvae stopped developing most prematurely like the lwr^{13} homozygote. The lwr^{5}/ lwr^{13} larvae developed to the late third instar larval stage although their development was delayed one day or so. Few mutant larvae advanced to the prepupal stage. Taken together, we conclude that the lwr^{14} allele is null, and that the lwr^{4-3} and lwr^{5} alleles are hypomorphic. The lwr^{4-3} allele seems stronger than the lwr^{5} allele.

Table 1. Viability and developmental retardation of the *lwr* alleles.

Genotype	Larval Viability[a]	Larval Stage[b]
lwr^{4-3} X lwr^{13}	59.2%	Middle Third Instar
lwr^{5} X lwr^{13}	64.6%	Late Third Instar
lwr^{13} X lwr^{13}	55.6%	Early Third Instar
lwr^{14} X lwr^{13}	50.0%	Second Instar

[a] The number of heterozygotes in culture was used for calculation.
[b] The latest stage to which the *lwr* mutant animals developed.

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Failure to suppress *polo* sterility but an hypothesis for why males are so very sterile (plus somatic reversion of st^1).

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All extant alleles of *polo* are female sterile (no surprise, the female-sterile phene was used to identify most of them), as homozygotes if viable, as transheteroallelic combinations of weaker allele/stronger if the stronger allele is lethal; however, weaker combinations can be male fertile although stronger combinations are completely male sterile. I therefore reasoned that it might be possible to identify dominant suppressors of *polo* male sterility in a genotype that is just sterile as the rare male who is fertile. Moreover, since in a *polo* background only the sons who inherit such a suppressor will be fertile, the suppressor can be anywhere in the genome, being kept in stock by virtue of conferring fertility; it can be located and properly balanced at leisure.

The test cross is: mutagenized $polo^x$/*st* $polo^1$ *ca (extraneous lethal)* males crossed back to *st* $polo^1$ *ca (extraneous lethal)/Balancer* females; should any of the $polo^x/polo^1$ males be fertile, cross their $polo^x/polo^1$ sons again back to $polo^1$ stock females. Since $polo^1$ is homozygous male fertile, the extraneous lethal its chromosome carries guarantees that all of the non-Balancer sons are $polo^x/polo^1$ and sterile unless they have also inherited the suppressor. $polo^1$ was chosen as the tester allele because it is homozygous male fertile; its lesion in *polo* (Tavares, Glover, and Sunkel, 1996) reduces Polo activity (since $polo^1$/*strong polo* males are sterile) but does not abolish it. Consequently, fertility could be restored to $polo^x/polo^1$ sons by mutations that a) increase levels of Polo protein per se (since two doses of $polo^1$ confers fertility), or b) reduce the requirement for Polo protein during spermatogenesis, or c) anything I haven't thought of.

Two different $polo^x$ alleles were chosen: *"*$polo^{6"}$, which is viable but male sterile/deficiency for *polo,* and $polo^8$, which is lethal both homozygous and over the deficiency. However, *"*$polo^{6"}/polo^1$ males are already fertile and indeed this chromosome behaves as though it carries $polo^1$ -- there seems to have been historical stock confusion. Consequently, only mutagenized