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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

polo^{8}/polo^{1} males were tested for fertility; however, females of both genotypes were also tested -- since I had them after all and suppressing female sterility would also be interesting.

th st "polo^{6}" ca/TM6B and *ru h th st polo^{8} red sr e/TM6C, Sb* males were given 5000 rads of X rays (Torrex 150 X-ray machine) and mated to *st polo^{1} ca/TM6B* females; F_1 *"polo^{6}"/polo^{1}* females, *polo^{8}/polo^{1}* females, and *polo^{8}/polo^{1}* males were mated back to *polo^{1}* stock flies of the opposite sex in batches of 5. It should be noted that both these combinations of *polo* alleles have good viability, as indeed does *polo^{1}/deficiency;* the reduction in viability originally noted for *polo^{1}* homozygotes (Herrmann, Amorim and Sunkel, 1998) must therefore reflect extraneous sub-vital mutations present on the original chromosome.

For *polo^{8}/polo^{1},* 3805 females and 3393 males were sterile; the mutagenesis was effective because in addition one presumptive new allele of *Dichaete* was recovered on a *polo^{8}* chromosome(= *D^{12})* and one of *Auricular* (= *Au^{2})* on *TM6C, Sb;* and one *scarlet* on *TM6C, Sb (Minute* and too female-sterile to be kept), two *clarets* on *TM6C, Sb* kept, one not tested, two sterile plus lots of other dull phenotypes not tested. For *"polo^{6}"/polo^{1}*, 2618 females were sterile and one new allele of *scarlet* was recovered on *TM6B*. There do not seem to be many genes capable of mutating to dominant suppressors of *polo* sterility -- anywhere in the genome, since the flies tested were F_1s and therefore carried a mutagenized full haploid complement.

One *polo^{8}/polo^{1}* male test was fertile but it does not seem to have been a suppression event; first lots of *ca Tb Hu* (= *polo^{1}/TM6B)* progeny eclosed, I thought "non-virgin" of course, but eventually one sole *e Tb Hu* female (= *polo^{8}/TM6B)* appeared. Attempts to rescue any suppressor from either genotype failed but the putative *polo^{8}* progeny did carry a strong *polo* allele with the expected markers for *polo^{8}*. Since *polo^{1}* is male-fertile homozygous, I think this fertile test was the result of a germ-line event that generated a *polo^{1}* homozygous clone (either a somatic crossover or somatic nondisjunction). That such a large clone would succeed in producing *polo^{1}*-bearing functional sperm is not surprising but the inclusion of the sole *polo^{8}*-bearing functional sperm is, and provides an hypothesis for why *polo^{8}/polo^{1}* males are completely sterile in the first place despite producing moderate numbers of apparently normal onion-stage spermatids. Although the processes of individualization and coiling can sort the normal spermatids in a half normal/half abnormal bundle *(SD/+* males are fertile even though no *SD^{+}* sperm are produced; Peacock, Tokuyasu and Hardy, 1972), there may well be an upper limit on this, both within each individual bundle and throughout the testis; one normal spermatid in the midst of lots of abnormal ones may not make it to being a mature sperm either (and *polo^{8}/polo^{1}* testes contain no motile mature sperm although lots of elongated immature bundles, and even apparently individualized, coiled bundles, are present). Alternatively, females may reject an ejaculate containing only (say) six sperm. This may well provide an experimental bypass for separating specific gene effects from testis-environmental effects: can your mutant of interest make a functional sperm in a testis with a sperm-producing clone even though it can't on its own?

Early on I noticed that the occasional *st^{1}/st^{1}* fly had *st^{+}//st* mosaic eyes, so I scored for this; 28 of the *polo^{8}/polo^{1}* females were such mosaics, 12 of the *polo^{8}/polo^{1}* males, and 11 of the *"polo^{6}"/polo^{1}* females, for an overall frequency of 51/7299 = 0.7%. I have subsequently spotted such mosaics in a variety of other genotypes so they are unlikely to be caused either by *polo* or by X-ray mutagenesis. The *st^{1}* mutation is known to result from insertion of a *412* transposable element

(ten Have, Green and Howells, 1995); it seems to be slightly unstable somatically.

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Effect of intra- and interspecific competition on pupation height in *Drosophila*.

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D. nasuta belongs to nasuta subgroup of immigrans species group and *D. rajasekari* belongs to suzukii subgroup of melanogaster species group. These two species are sympatric in distribution (Krishna and Hegde, 2003). The isofemale lines of these two species were established from females collected in semi domestic localities of Mysore city (Karnataka, India). After 10 generation of inbreeding the flies were isolated and aged for 5 days. 50 males and 50 females of the same species were introduced together into a culture bottle. This formed the pure culture exhibiting only interspecific competition. In addition to pure culture, a mixed culture was also prepared by mixing 25 pairs of male and females of *D. nasuta* and 25 pairs of *D. rajasekari* were allowed to develop. Five replicates of such culture were maintained. When pupae formed, the parental flies were transferred to fresh bottles and pupal heights were measured. The pupae of two species in mixed culture could be differentiated with their size and nature of spiracles. When adults emerged they were added to bottles containing parents. This procedure was followed until the elimination of one of the two species in the mixed culture. The pupal height was measured in all generations of pure and mixed cultures. The pupal height at the interval of four generations was used for computation.

D. nasuta with their higher productivity eliminate *D. rajasekari* in a span of 14 generations. The overall pupation height has been assessed during intra- and interspecific competitive condition. The mean pupation height in the pure culture of *D. rajasekari* (6.10 ± 0.49 to 6.47 ± 0.49cm) was higher than *D. nasuta* (5.01 ± 0.42 to 6.21 ± 0.47). The larvae of *D. rajasekari* can crawl to a greater height than *D. nasuta*. In pure culture the pupal height of *D. nasuta* gradually increased over generations. The intraspecific competition, therefore, has led to the increase in pupal height in this species. In the interspecific competition, the pupal height of *D. rajasekari* decreased significantly, indicating that the interspecific competition has a serious impact on the pupation height. *D. nasuta* slowly replaces *D. rajasekari* in interspecific competition. Though in pure culture the species

Table 1. Showing pupation height during intra and inter specific competition.

	D. rajashekari		*D. nasuta*	
Generation	Pure culture	Mixed culture	Pure culture	Mixed culture
1	6.47±0.49	5.71±0.49	5.01±0.42	6.18±0.45
4	6.10±0.48	6.15±0.49	5.86±0.45	5.81±0.48
8	6.37±0.48	5.57±0.45	6.12±0.45	5.40±0.48
12	6.14±3.46	3.20±0.52	6.21±0.47	5.26±0.45

Values are Means ± SE (cms).

showed increase in pupation height, in mixed culture the height decreased. Thus *D. nasuta* is a better competitor than *D. rajasekari.*

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A revised cytological location of *stambh A* and of the breakpoints of Df(2R)44CE of *Drosophila melanogaster.*

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The mutation *stambh A* (*stmA*) (2-56.8cM) in *Drosophila melanogaster* was discovered through its phenotype of reversible temperature sensitive (ts) paralysis (Shyngle and Sharma, 1985). Adults and larvae homozygous for $stmA^{1}$ and $stmA^{2}$ paralyse within 2-4 minutes at 38ºC temperature and recover to normal behaviour when brought back to 23-24ºC . Mutations isolated later included several unconditional embryonic lethal alleles $stmA^{7}$, $stmA^{12}$, $stmA^{18\text{-}2}$ and several multiphasic lethals (Chandrashekaran and Sarla, 1993; Kumar *et al.,* 2001). *stmA* was mapped to the 42A16-42B2 interval (Chandrashekaran and Sarla, 1993) based upon the following observations:

1. Flies of the genotype $stmA^{1}$ / $stmA^{1}$; Tp2;3 P32, containing two copies of $stmA^{1}$ and a wild type copy of the 2nd chromosome region 41A to 44D4-8 (Figure 1, Table 1), transposed on chromosome 3 showed wild type behaviour. On this basis *stmA* was roughly mapped to the 41A-44D4-8 interval.
2. Deletions spanning 41A through 41C and 42A16 through 43 F8.9 (Figure 1, Table 1) were wild type over *stmA*. The $stmA^{12}$ bearing chromosome was associated with a visible cytological deficiency 42A8-42B2, which could not be separated by recombination from the *stmA* embryonic lethal phenotype at the time of the study.

On the basis of (1) and (2) *stmA* was placed between 42A8-42A16 (Figure 1). Deletions spanning 43 F8.9 and 44 D were not tested because of the deficiency associated with $stmA^{12}$.

Table1. Aberrations spanning 41A–42B2 used in mapping *stmA* (From Chandrashekaran and Sarla, 1993).

Rearrangement	Breakpoints	Whether *stm A+*
Tp 2;3 P 32	41A-44D4-8	Yes
Df (2R) rl ^{10}	41A	Yes
Df (2R) V30	41B-41C	Yes
Df (2R) D5 ^{L} *TE 146-SZ 4*	42A16.19-43A1	Yes
Df (2R) pk 78s	42C1.7-43F8.9	Yes
Df (2R) stm A ^{12}	42A8-42B2	No

Recently we were able to separate, by meiotic recombination, the $stmA^{12}$ allele from the deficiency 42A8-42B2, without altering the ts paralytic phenotype of $stmA^{12}$ / $stmA^{1}$ flies or the recessive lethality of $stmA^{12}$ / $stmA^{12}$homozygotes. It became obvious that the 42A8-42B2 map position of *stambh A* was incorrect and it became essential to re-determine, the cytological position of *stmA*.

We have tested five deficiencies (Figure 1, Table 2) all with at least one breakpoint distal to 43F8.9 to map *stmA*.

Df(2R)H3C1 and *Df(2R) H3E1* both uncover the ts paralytic phenotype and lethality of the

various *stmA* alleles (Table 3). *stmA* therefore maps to the 44D1-4 to 44D3-8 interval defined by the proximal breakpoint 44D1-4 of *Df(2R) H3E1* and the distal breakpoint 44D3-8 of *Df(2R) H3C1*. The deficiency, *Df(2R) 44C-E* whose proximal breakpoint is 44C1-2 and distal breakpoint is 44E1-4, according to Flybase (www.flybase.bio.indiana.edu) ought to be deficient for the relevant 44D1-4 to 44D3-8 region. *Df(2R)44CE* was therefore expected to uncover the mutant phenotype of *stmA*. Contrary to expectations, *Df(2R)44CE* complemented *stmA* (Table 3). This unexpected observation suggested that *Df (2R)H3E1* was not deficient for all or some part of the 44D1 to 8 region.

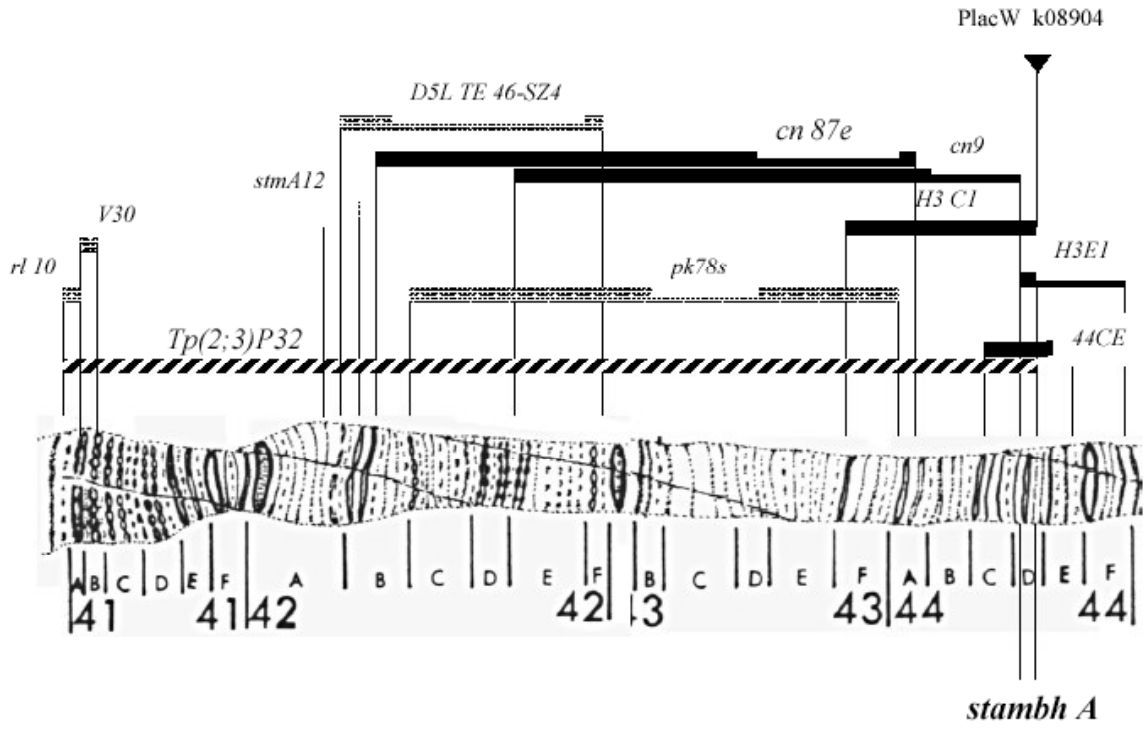

Figure 1. Chromosomal aberrations used to map *stambh A*. Dotted bars: deficiencies used in 1993 . Solid Black bars: deficiencies used in the present study. Stippled bar: Transposition (1993). Solid triangle: PlacW element at 44D4.5.

Table 2. List of deficiencies with breakpoints distal to 43F8.9 used to map *stambh A*. * as published by Flybase.

S. No	Deficiency (2R)	Bloomington Stock Numbers	Breakpoints		Whether *stmA*$^{+}$
			Proximal	Distal	
1	cn 87e	224	42B4.C1	43F.44A1	Yes
2	cn 9	3368	42E	44C	Yes
3	**H3 C1**	**198**	**43F**	**44D3.8**	**No**
4	44CE	3643	44C1.2*	44E2.4*	Yes
5	**H3E1**	**201**	**44D1.4**	**44F12**	**No**

The breakpoints of Deficiency (2R) 44CE: We examined polytene chromosomes of *Df (2R) 44C-E* /+ larvae and found that it is not entirely deficient for the 44C1-2 to 44E2-4 region. The following observations were made about the cytology of *Df(2R)44CE*: (1) It consists of a proximal deficiency (P) of a deeply staining doublet; (2) a large distal (D) deficiency; and (3) that P and D are

separated by a single band (S) (Figure 2).

Table 3. Viability and temperature sensitive paralytic response of *stmA* alleles over deficiencies H3C1,H3E1 and 44CE. TsP: TS paralytic homozygous viable allele; L:Recessive Lethal allele; P: Paralytic; NP:Non-Paralytic.

stmA Allele	Viable ts Paralytic / Lethal	H3 C1 43F to 44D3-8		H3E1 44D1-4 to 44F12		44CE 44C1.2-44E2.4	
		% viability	Paralytic response	% viability	Paralytic response	% viability	Paralytic response
stmA^{1}	Ts P	112	P	104	P	81	NP
stmA^{2}	Ts P	66	P	101	P	52	NP
stmA^{7}	L	29	P	27	P	222	NP
stmA^{12}	L	0	-	34	P	80	NP
stmA^{PEL1}	L	0	-	0	-	0	NP
stmA ^{PEL7}	L	0	-	0	-	29	NP
stmA ^{PEL8}	L	0	-	0	-	18	NP
stmA ^{PEL11}	L	0	-	0	-	ND	ND
stmA ^{PEL4}	L	0	-	0	-	42	NP
stmA ^{PEL3}	L	0	-	0	-	29	NP

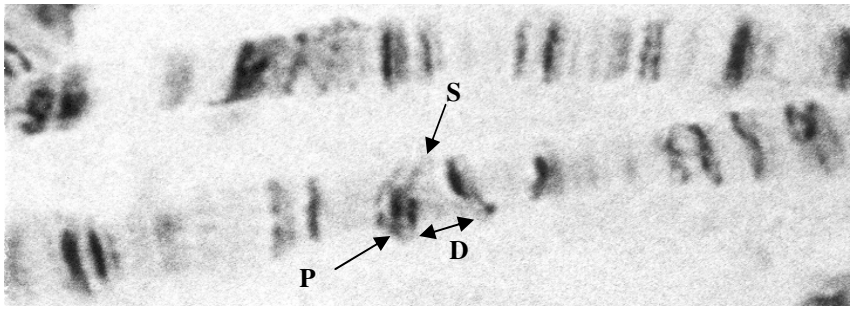

Figure 2. A lacto aceto-orcein stained polytene chromosome preparation of Df (2R)44CE/+ P: Proximal deficiency 44 C1.2; S: 44D1.2 ; D: 44 D3-44E2.4.

The presence of band 'S' supports the genetic complementation of *Df(2R) 44C-E* over *stmA*. Complementation data also suggest that `S' should comprise of bands in the 44D1 to 8 interval. It is difficult to resolve the identity of `S' unequivocally at the current level of microscopy. For the present, `S' has been defined as 44D1-2 based upon the argument that the doublet band 44D1-2 is the only band common to *Df (2R) H3C1* and *Df (2R)H3E1*. The proximal deficiency `P' is defined as a deficiency for the doublet 44C1-2. The breakpoints of the distal deficiency (D) were defined as 44D3 (proximal) and 44E1.4 (distal). On the basis of this interpretation, we have mapped *stambh A* to the doublet 44D1.2, based on the observation that 44D1.2 is the only band common to the overlapping *Df (2R) H3C1* and *Df (2R)H3E1* and is present in *Df(2R)44CE. Df(2R)44CE* is also reported to complement l(2)k05118 a multiple PlacW insert line that has one insertion at 44D1-2 (www.fruitfly.org, visited on 21.6.2001).

We have come across published reports that the proximal breakpoint in *Df(2R) 44C-E* might be 44C4-5, and not 44C1-2 Df(2R)44CE (Table 4). Among seven papers surveyed, five list the proximal breakpoint of *Df(2R)44CE* as 44 C4.5 and not 44 C1.2 as listed in Flybase. We interpret the conflicting reports to the twin deficiencies associated with *Df(2R)44CE.*

The unexpected complementation pattern of *Df(2R)44CE* over *stmA* and its cytology point to

the need for its breakpoints be determined accurately, for it to be of greater utility to the *Drosophila* research community.

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The extension of the geographic range of *Drosophila malerkotliana* on the American continent.

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Numerous species, either plants or animals, may become successful invaders, and biological invasions are a major and recurrent problem for the protection and conservation of biodiversity in ecological communities (Vermeij, 1996; Kolar and Lodge, 2001). Besides this practical purpose, the study of such phenomena provides an opportunity to access many general questions in evolutionary biology, including demographic bottle necks and climatic adaptations.

Among more than 3000 described species the Drosophilidae family harbors a small number of widespread species which have reached a cosmopolitan status thanks to artificial modern-man transportation. Since an earlier publication (David and Tsacas, 1981) which listed about 20 such species, other cases of recent invasions have been documented, such as the European *D. subobscura* in South and North America (Brncic *et al.*, 1981; Huey *et al.*, 2000) and the very recent case of *Zaprionus indianus* in Brazil (Vilela, 1999; Tidon *et al.*, 2003).

Here we focus on the case of *D. malerkotliana*, which has appeared on South American continent several decades ago, and which now seems to extend its range in North America. Due to a continuity of favorable habitats from South to North America, we expect that the extension of *D. malerkotliana* will be limited only by climatic factors.

D. malerkotliana belongs to a cluster of 4 related species, within the *D. ananassae* subgroup of the *D. melanogaster* group in the *Sophophora* subgenus. This cluster, the *D. bipectinata* complex, comprises, besides *D. bipectinata, D. parabipectinata, D. malerkotliana* and *D. pseudoananassae* (Bock, 1971a). These species are closely related and, in most combinations, make hybrids, that is fertile F1 females but sterile males (Bock, 1971b). *D. malerkotliana* has the broadest distribution, and indeed is subdivided into two subspecies. The type subspecies is found in India (Malerkotla is the name of an Indian city), Thailand, and Malaysia; it is easily recognized by the fact that the male's last abdomen segments (5 and 6) are black. The *pallens* subspecies is defined by the fact that males have an abdomen entirely yellow (as in the other species of the complex as well as in all females). It has a more oriental distribution, being found in Borneo and Philippines (Bock, 1971a). Differences in the

shape of the Y chromosome have also been described between the subspecies (Kaneshiro and Wheeler, 1970).

The species in the *D. bipectinata* complex are generalists, breeding in a diversity of sweet decaying resources, and often have a domestic status like *D. melanogaster*. This is a favorable situation for becoming an invader, and indeed the type subspecies of *D. malerkotliana* is known to be cosmopolitan (David and Tsacas, 1981). Ancestral populations are presumably found in India, where the species is abundant everywhere, from North to South. From there, the species has been introduced in the Afrotropical region, and it is generally abundant at low elevations on the mainland (Tsacas *et al.*, 1981). It has been introduced to the Seychelles during the 20th century, where it is very abundant (Louis and David, 1986). Worth mentioning is the fact that *D. malerkotliana* is absent from Réunion island and Mauritius (in the Indian Ocean) where another species of the complex (*D. parabipectinata*) has been introduced (David and Tsacas, 1975; David *et al.*, 1989a).

D. malerkotliana is now very abundant in South America, and especially in Brazil. It is a dominant species in domestic Drosophilid communities (Val and Sene, 1980; Sene *et al.*, 1980) and has a strong invading capacity. In the Amazon region, its abundance may reach 70% in disturbed forest habitats (Martins, 1989, 2001). It is also found, but with a much lesser frequency, in the undisturbed, native forest. *D. malerkotliana* thus appears as a good biological indicator of habitat disturbance in the Amazon forest (Martins, 2001).

The date of appearance of *D. malerkotliana* in Brazil is quite well documented (Val and Sene, 1980). Apparently it was absent in the 1950s and 1960s while it became quite abundant in the 1970s. The origin of the propagule which invaded Brazil so quickly is not known. It seems likely to be Africa, since *D. malerkotliana* is frequent there. An introduction from south East Asia is unlikely, since this part of the world harbors the *pallens* subspecies. *D. malerkotliana* has extended its range to most South American countries, provided the climate is sufficiently warm. For example it is abundant in coastal places of Equator (Rafael *et al.,* 2000) and Panamá (Sevenster, 1992; Sevenster and Van Alphen, 1996; Shorrocks and Sevenster, 1995). Going South, it has been collected on Santa Catarina island (SC, Brazil 27°42S) (Toni and Hofmann, 1995) but never in Porto Alegre (RS, Brazil, 30°03S) (V.L.S. Valente, personal communication), nor in Uruguay (Goni *et al.*, 1998). Going North of Equator there is a continuity of favorable habitats and warm climatic conditions, up to the South of USA. Indeed, the presence of *D. malerkotliana* in Mexico and Cuba was mentioned in the late ‘80s (Chassagnard *et al.*, 1989). The sudden appearance of the species in French Caribbeans (Martinique and Guadeloupe) is also documented. In 1980 we investigated these communities (David and Capy, 1983) and no *D. malerkotliana* was found (only *D. melanogaster* and *D. ananassae* were representatives of *Sophophora*). Ten years later, *D. malerkotliana* was very abundant in both islands, especially at sea level (J. David, unpublished observation). The origin of the Caribbean *D. malerkotliana* is not known, but might be Mexico or Cuba. Worth mentioning is the fact that cosmopolitan, domestic species are certainly transported by man, but such introductions are not so common, nor efficient. For example, *D simulans* is very abundant in North American mainland and in Cuba. It is however completely absent (last observation in 1998) from Martinique and Guadeloupe, while it is very common in St. Martin island, about 150 km north of Guadeloupe (see Morin *et al.*, 1999).

When a new species is introduced into different islands, the sizes of the propagules are not known, but could be inferred by investigating the diversity of genetic markers. We may mention a likely case for *D. melanogaster*. All populations in the world so far investigated are polymorphic for the two widespread alleles at the *Adh* locus, although the frequencies are extremely variable across latitudinal clines (David *et al.*, 1989b). We know only one place in the world, Martinique Island, where the *Adh* locus seems monomorphic. In the Caribbean region, the Fast allele is rare, at a frequency less than 5%. Any fly sample of more than 50 individuals always reveals some

heterozygotes, but not in Martinique. From that island, we have analyzed more than 500 wild collected flies, and never found a Fast allele. This observation suggests that the extant population was founded by a very small propagule.

From an ecophysiological point of view, *D malerkotliana* is typically a tropical species, whose distribution is limited by cold temperature in winter time. The cold sensitivity of *D. ananassae* is well documented, since it cannot grow below 16°C (Morin *et al.*, 1997). *D. malerkotliana* is not so well known, but some unpublished data suggest the lower developmental limit to be 15°C (J.R. David), and that fertility is reduced below 20°C (M.B. Martins). So in North Mexico, we expect that proliferation of *D. malerkotliana* will favor the colonization of northern places, while winter low temperature will destroy the summer populations. We do not know where the limit is, but recent observations suggest that *D. malerkotliana* is now established in Southern USA. The species has been regularly collected in the Tallahassee (FL) area at a latitude of 30°25N (Birdsley, 2003). In September 2002, *D. malerkotliana* was collected (J.R. David) farther north, in the suburbs of Athens, GA, at a latitude of 33°57N. In this community, two species were dominant, *D. simulans* and *D. affinis*. *D. malerkotliana* should be searched for in all USA Southern states were its extension will be limited only by low winter temperatures. From an ecological point of view, the species might be a convenient indicator of climatic warming in the next decades. From an evolutionary point of view, we know that *D. malerkotliana* is proliferating under a diversity of climates from the stable warm environment of the Equator to subtropical places and even warm temperate climates. Several *Drosophila* species are known to exhibit latitudinal clines in response to such climatic variation, and indeed such clines may become established in less than 20 years, as such has been the case for *D. subobscura* (Huey *et al.*, 2000). Natural populations of *D. malerkotliana* might thus become a good model for analyzing thermal adaptations, for comparing ancestral and derived populations and for investigating the possible effects of recurrent demographic bottle necks.

Another cosmopolitan species, *Zaprionus indianus*, is likely to reach a similar distribution in the forthcoming years. This African species was intoduced to Brazil in 1998 and spread very rapidly (Tidon *et al.*, 2003). It is now abundant in most places up to the Equator and was collected in Igarapé Açu (Pará, Brazil) in December 2000 and in Belem in December 2002 (J.R. David and M. Martins, unpublished). Since this species has apparently a very strong dispersal and colonizing ability, we expect it will invade Mexico and Southern USA in a few years, and it should be searched there in the same way as *D. malerkotliana*.

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Colonization of Northeast Region of Brazil by the drosophilid flies *Drosophila malerkotliana* and *Zaprionus indianus*, a new potential insect pest for Brazilian fruitculture.

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The drosophilid fauna from Northeast region of Brazil is poorly studied. According to Val *et al.* (1981) and Ehrman and Powell (1982), the main species in the region were *Drosophila nebulosa*, *D. willistoni*, *D. paulistorum,* and *D. tropicalis* (*willistoni* group) and *D. simulans*, *D. melanogaster* and *D. kikkawai* (*melanogaster* group). This list was enlarged to include the colonizing species *D. malerkotliana* (Sene *et al.*, 1980; Val and Sene, 1980). Since 1996 we have made several sampling collections of drosophilids at different localities in the States of Pernambuco (PE), Bahia (BA), and Paraíba (PB), in the Northeast region of Brazil. These localities encompass different degrees of urbanization, which is high at UFPE *Campus* (08^o 03' 01" S, 34^o 56' 54" W) in Recife City (PE), and Olinda (08^o 03' 45" S, 34^o 48' 45" W, PE), medium at Bezerros (PE, 08^o 11' 15" S, 35^o 48' 45" W), Moreno (PE, 08^o 06' 24" S, 35^o 10' 32" W), and in Santa Rita (PB, 07^o 03' 45" S, 34^o 56' 15" W), and is low at Sobradinho (BA, 09^o 29' 13" S, 40^o 49' 49" W), Vila Velha (07^o 41' 15" S, 34^o 48' 45" W, Itamaracá, PE), Estação Ecológica de Tapacurá (08^o 03' 45" S, 35^o 03' 45" W, São Lourenço da Mata, PE), Estação Itapirema (07^o 33' 45" S, 35^o 03' 45" W, Goiana, PE), Parque Dois Irmãos (08^o 00' 05" S, 34^o 57' 23" W), a preserved secondary forest inside the Recife City and in the Refúgio Ecológico Charles Darwin (07^o 48' 57" S, 34^o 41' 52" W), which is also a secondary forest, very well protected and not urbanized at all, which is part of the Atlantic Forest, near Igarassu (PE). While Sobradinho is in the Sertão (semiarid interior), all the other locations are at the litoranean stripe of Brazilian Northeast.

The diversity of species was higher at low and medium urbanization localities, as can be drawn from Table 1. The *willistoni* group of species was represented only by few individuals of *D. nebulosa*, which means that this group is now extremely reduced in the Northeast of Brazil both in number of species and individuals, unravelling an extensive modification in the drosophilid fauna of the region. For the *melanogaster* group the situation is also completely different from that reported in

1980 (Sene *et al.*, 1980; Val and Sene, 1980; Val *et al.,* 1981), represented by *D. melanogaster* and *D. simulans*, since there is now a remarkable dominance of the colonizing species *D. malerkotliana*, which could have displaced the other species from their niches, including that of the *willistoni* group. The replacement of *D. willistoni* by *D. malerkotliana* was reported earlier by Morais *et al.* (1995) in the Amazonian region (North Brazil). The invasion of Northeast region by *D. malerkotliana* is, thus, probably the cause of the annotated drop in frequency of individuals of both the *willistoni* and *melanogaster* group of species. Also from the *melanogaster* group we have registered the presence of few individuals of *D. kikkawai*, exclusively in the Refúgio Ecológico Charles Darwin, a preserved park not urbanized. An unexpected finding, however, was the significant number of individuals of *D. maculifrons* (*guarani* group) in Parque Dois Irmãos and Refúgio Ecológico Charles Darwin, both with low urbanization. The *guarani* group is generally believed to occur mainly in cold regions (Sene *et al.*, 1980), being never registered before in Northeast region of Brazil, characterized by elevated temperatures during all the year and a low and irregular pluviometry.

Moreover, these were not the only changes in the drosophilid fauna of this region. Our register of collections shown in Table 1 also documents the invasion of Northeast of Brazil by another drosophilid, *Zaprionus indianus.* This fly was first reported in Brazil by Vilela (1999), who found it colonizing fermenting fruits of persimmon trees (*Diospyros kaki*) in São Paulo State. Unexpectedly, this fly adopted a pest behaviour in Brazil, whose larvae colonized orange, peach and the *Ficus carica* fruits, causing a severe lost of exportation profits (Vilela *et al.*, 1999; Vilela *et al.*, 2001). This fly was also found in the Amazonian region (Tidon *et al.*, 2003), where it has dispersed rapidly, resulting in a marked alteration in drosophilid fauna. Although not found in our collecting from 1996 to 1999, *Z. indianus* is now widespread in the Northeast region, suggesting a very new colonization. In fact, in March 2000 this species was firstly found in the Basin of São Francisco River (Sobradinho, BA), colonizing fruits of *Spondias tuberosa* (umbuzeiro), a native non-cultivated tree. In following collections in this same year we have found it in the litoranean stripe of Pernambuco, colonizing fruits of *Jambosa vulgaris* (jambo) in the city of Recife (PE), *Spondias purpurea* (siriguela) in Bezerros (PE), and *Genipa americana* (genipapo) in Moreno (PE) and Santa Rita (PB). The populations grew rapidly, and by the end of 2000, *Z. indianus* already represented 12.4% of individuals frequency, being the third main drosophilid species (Table 1). It was suggested by Vilela (1999) and Vilela *et al.* (2001) that *Z. indianus* could have reached the São Paulo State through infested fruits imported from Europe, or, alternatively, this species can have escaped from laboratory cultures in USA and widespread to Central America and then throughout Amazonia to Brazil in South America. Whatever the source, from Amazonia or São Paulo, it is clear from our data that *Z. indianus* has first colonized the interior of Bahia State and then has reached the litoranean stripe of Northeast of Brazil. As in the case of *D. malerkotliana*, *Z. indianus* also seems to be a powerful species which can displace other drosophilid species. The main concern about this fly is its behaviour as a potential fruitculture pest in Brazil, undocumented earlier in other countries. Vilela (1999) and Vilela *et al.* (2001) reasoned that the species seems to be restricted to cultivated fruits, which could facilitate the biological control. However, as we demonstrate here, *Z. indianus* is now, at least in Northeast region of Brazil, very well adapted to several native resources, which can make the species more difficult to control and can become a pest for the fruitculture developed at the Basin of São Francisco River.

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Morphometric changes of the *Drosophila saltans* salivary glands prior to and during apoptosis.

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Drosophila salivary glands develop from two ventral ectodermal plates with about 100 cells each, in the region of the presumptive posterior head. They differentiate without further cell division and increase in size by increasing the volume of individual cells (Andrew *et al*., 2000). *Drosophila* salivary glands are eliminated in development, in general during the pupal phase. This elimination, which is named apoptosis or programmed cell death, is preceded by morphological and physiological modifications resulting from the activation of an intrinsic, genetically controlled "suicide" program

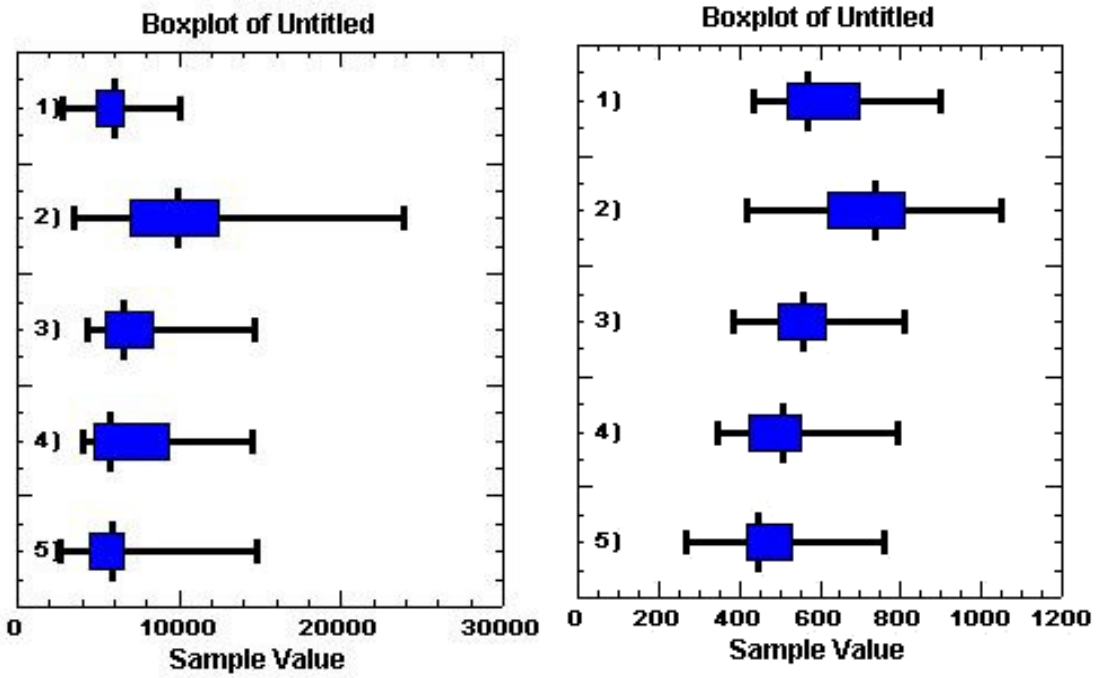

Figure 1. A, B. Boxplots for area (A) and perimeter (B) values of *Drosophila saltans* salivary glands during development. 1= early third instar larvae; 2 = late third instar larvae; 3 = puparium; 4 = prepupae; 5 = pupae.

(Steller, 1995). There is already fairly good knowledge about the regulatory pathways controlling salivary gland formation (Andrew *et al*., 2000). Also several steps involving specific gene activation for apoptosis are already known (White *et al.*, 1994; Kumar and Doumanis, 2000; Farkas and Mechler, 2000).

In the present study, variations in the area and perimeter of salivary glands were analyzed in *Drosophila saltans* development, from early third instar larvae to the pupal phase preceding disintegration of the glands due to apoptosis. This study was carried out in parallel with the study of modifications involving chromosomes and nucleoli in the salivary gland cells (data being published elsewhere). Area and perimeter measurements were taken in early and late third instar larvae, puparium (everted spiracles), prepupae (white pupae) and pupae (yellow pupae, before the eyes turn red). They were aged about 248, 250, 251, 278 and 305 hours from the egg, respectively. The strain used was the 180.40, from San Jose, Costa Rica, maintained in the stocks of the Biology Department, IBILCE-UNESP. The salivary glands were dissected in Demerec solution (Demerec and Kaufman, 1945), carefully cleaned of the fat tissue present around them, and stained with lacto-acetic orcein. The slides were made permanent with crystal varnish. Measurements of area and perimeter were taken using the computer program Global Lab Image SP0550 (Data Translation, 1995; Marlboro, MA, U.S.A.). From thirty to thirty-three complete salivary glands from each stage were measured.

Table 1. Statistics for area and perimeter values of *D. saltans* salivary glands, in five developmental stages. N= number of salivary glands examined; eL3 = early third instar larvae, lL3 = late third instar larvae, pup = puparium, pp = prepupae, p = pupae.

		Area in μ^2		Perimeter in μ	
stage	*Nº*	Mean	St.Dev.	Mean	St.Dev.
eL3	30	6034	1645.20	602	119
lL3	32	10348	5171.79	728	155.59
pup	31	7462	2693.48	565	97.59
pp	33	7003	3023.88	506	100.62
p	30	5815	2138.74	474	102.09

Table 2. Student's t test for comparison of the area values (above the diagonal) and perimeter values (below the diagonal) of *D. saltans* salivary glands, in different developmental stages. eL3 = early third instar larvae, lL3 = late third instar larvae, pup = puparium, pp = prepupae, p = pupae. Asteriscs indicate significant differences: * = p < 0.05; ** = < 0.001.

	eL3	lL3	pup	pp	p
eL3	xxxxxxxxx	**			
l L3		xxxxxxxxx	*	*	**
pup		**	xxxxxxxxx		*
pp		**	*	xxxxxxxxx	
p		**	**		xxxxxxxx

Data for area and perimeter are shown in Table 1 and Figure 1. The Student's t test results for comparison of area and perimeter values of salivary glands from different stages are set out in Table 2. The area and perimeter of salivary glands from the late third instar larvae were significantly greater than those in the early third instar and significantly greater than the subsequent developmental stages (puparium, prepupae, and pupae). The area and perimeter of the salivary glands from the puparium stage were also significantly greater than those from pupae. The perimeter in pupae was also significantly smaller than in prepupae. Although not significant statistically, pupae also showed a smaller mean area than prepupae. In pupae older than those examined in the present study, the salivary glands are smaller and take the appearance of a rounded body. At this time they are very soft and disintegrate when touched with the dissection needles.

The fact that the salivary glands attain their greatest size in the late third instar lavae is due to a feature that has been understood for a long time. In that stage, the giant chromosomes from the salivary glands cells exhibit the highest degree of polyteny (Berendes, 1965). This means that there are multiple rounds of DNA replication without cell division. In *Drosophila*, at this developmental

time, about 1024 strands are present in the salivary gland polytene chromosomes. In consequence nucleus and cell size increase. The high degree of polyteny is related to the high degree of synthesis that characterizes the salivary gland cells in this stage. In addition, the nucleolar size and the number and size of puffs (chromosome regions enlarged due to the unraveling of the chromosome threads for RNA synthesis) are the greatest and the cytoplasm shows a high amount of synthesized material. After this stage, the involvement of the salivary glands in the fly development apparently ceases. The significant decrease of size, here described, occurs along with changes that denote a decrease in nuclear activity. From the late third instar larve to the time of the gland disappearance, occurring later in the pupal stage, the polytene chromosomes and the nucleolus exhibit morphological changes that culminate with the disintegration of both (Oliveira and Bicudo, unpublished data). Since the size increase of the salivary glands is due to an increase in the volume of individual cells, the decrease is also due to a reduction in the volume of the same cells, in consequence of the mentioned changes. Besides, in the process of apoptosis, cytoplasmic components are released into the lumen of the glands, also contributing to the size decrease. The reduction of salivary gland size progresses till the occurrence of histolysis. As shown by the present data, in the last phase in which the salivary glands could be manipulated for measurement, their mean area was about half of that found in the late third instar larvae.

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Novel isoforms of *Drosophila* annexins produced by alternative splicing.[1]

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[1]Nucleotide sequence data presented in this paper have been deposited in GenBank under accession numbers AY007377 (*AnxB9a*) and AF261718 (*AnxB9b*) or can be found under GadFly gene number CG9968 (*AnxB11*).

Keywords: annexin B9, annexin B11, RT-PCR, cDNA, *Drosophila melanogaster*

Abstract: We report the complete sequence analysis of the *AnxB9* gene and a novel, related gene, *annexin B11* (*AnxB11*), from *Drosophila*. Analysis of *AnxB9* cDNAs revealed two closely related isoforms expressed from this gene that arise from alternative splicing. We identified the complete open reading frame for the previously reported transcript, *AnxB9a*, in addition to a novel isoform, *AnxB9b*. The novel *AnxB11* gene also encodes two annexin isoforms, *AnxB11-short* and *AnxB11-long*, that arise from alternative exon usage. All four annexin isoforms described are expressed at all *Drosophila* life stages, suggesting broad cellular roles. Our analyses also suggest a

dissociation between conservation in the functional organization of *Drosophila* annexin genes and conservation of the amino acid sequence of their proteins.

The annexins are a large family of proteins found in diverse eukaryotes including fungi, plants, *Caenorhabditis elegans*, *Drosophila*, fish, birds, and mammals, but not yeast or prokaryotes (Benz and Hofmann, 1997). These proteins typically contain four repeated domains (Sudhof *et al.*, 1988) and bind phospholipids in a calcium-dependent manner (Sudhof *et al.*, 1984). Many aspects of annexin structure and function have been intensively studied including their interactions with lipids and membranes, binding to other proteins, and ion channel activity (Benz and Hofmann, 1997). Possible cellular roles as regulators of membrane fusion or cell surface receptors have been suggested. However, no clear biological function has been shown for any member of the annexin family (Benz and Hofmann, 1997).

Two annexins from *Drosophila* were previously identified using antibodies directed against a consensus annexin repeat found in mammalian annexins (Gerke, 1989). These antibodies recognized 30 and 34 kD *Drosophila* proteins that interacted with phospholipids in a calcium-dependent manner, suggesting that these proteins were annexins. cDNAs for two annexins from *Drosophila* were cloned using a probe derived from conserved regions of vertebrate annexins (Johnston *et al.*, 1990). These annexins were designated *annexin IX* and *annexin X* (hereafter called *AnxB9* and *AnxB10,* respectively, in accord with the revised nomenclature for the annexin family (Morgan *et al.*, 1999)). Sequence analyses and *in situ* hybridizations to chromosomes showed that these two annexins are encoded by two distinct genes. The reported *AnxB10* cDNA contained the complete open reading frame (ORF). However, the published *AnxB9* cDNA appeared to be truncated, resulting in the predicted ANXB9 protein having only three of the four annexin repeat domains found in other annexins (Johnston *et al.*, 1990).

We identified a cDNA clone (V1.39) encoding a novel isoform of *AnxB9* as a false positive in a yeast two-hybrid screen. Pair-wise alignments (Smith *et al.*, 1996) of the nucleotide sequence of clone V1.39 with GenBank accession number M34068.1, the previously reported cDNA for *AnxB9* (Johnston *et al.*, 1990), revealed that the first 840 residues of V1.39 have 99.8% identity with M34068.1. These two sequences diverge at the 3' end, suggesting that there might be multiple *AnxB9* isoforms.

A Blast search (Altschul *et al.*, 1997) of the expressed sequence tag (EST) database from the Berkeley *Drosophila* Genome Project (BDGP, http://www.fruitfly.org/) revealed that V1.39 has greater than 97% nucleotide identity with the 21 ESTs in Clot number 2657:3 (BDGP, unpublished). Twenty of these are 5' ESTs and only one, GH08594.3, is a 3' EST. Clone LD09947, the EST that extended the furthest in the 5' direction, was obtained from BDGP via Research Genetics (Huntsville, AL) and sequenced from the 5' and 3' ends. The 5' end of LD09947 is 97% identical to M34068.1 in regions of overlap but contains additional 5' bases encompassing the 5' end of the ORF, confirming that the original *AnxB9* cDNA sequence was truncated (Johnston *et al.*, 1990). The 3' end of LD09947 was 98% identical to V1.39 and GH08594.3. However, all three of these clones diverge from the previously published *AnxB9* cDNA (M34068.1; (Johnston *et al.*, 1990)) at the same nucleotide, indicating that they represent a novel isoform of *AnxB9*.

Together, these analyses show that two distinct transcripts are produced from the *AnxB9* locus. We designate one isoform as *AnxB9a* (GenBank accession number AY007377; represented by the previously published truncated sequence, M34068.1 (Johnston *et al.*, 1990), and several BDGP ESTs that complete the ORF) (Figure 1A) and the novel isoform as *AnxB9b* (GenBank accession number AF261718; represented by clones LD09947, GH08594.3, and V1.39) (Figure 1B). Based on these analyses we predicted that the *AnxB9a* and *AnxB9b* transcripts are identical except for alternatively spliced fifth exons.

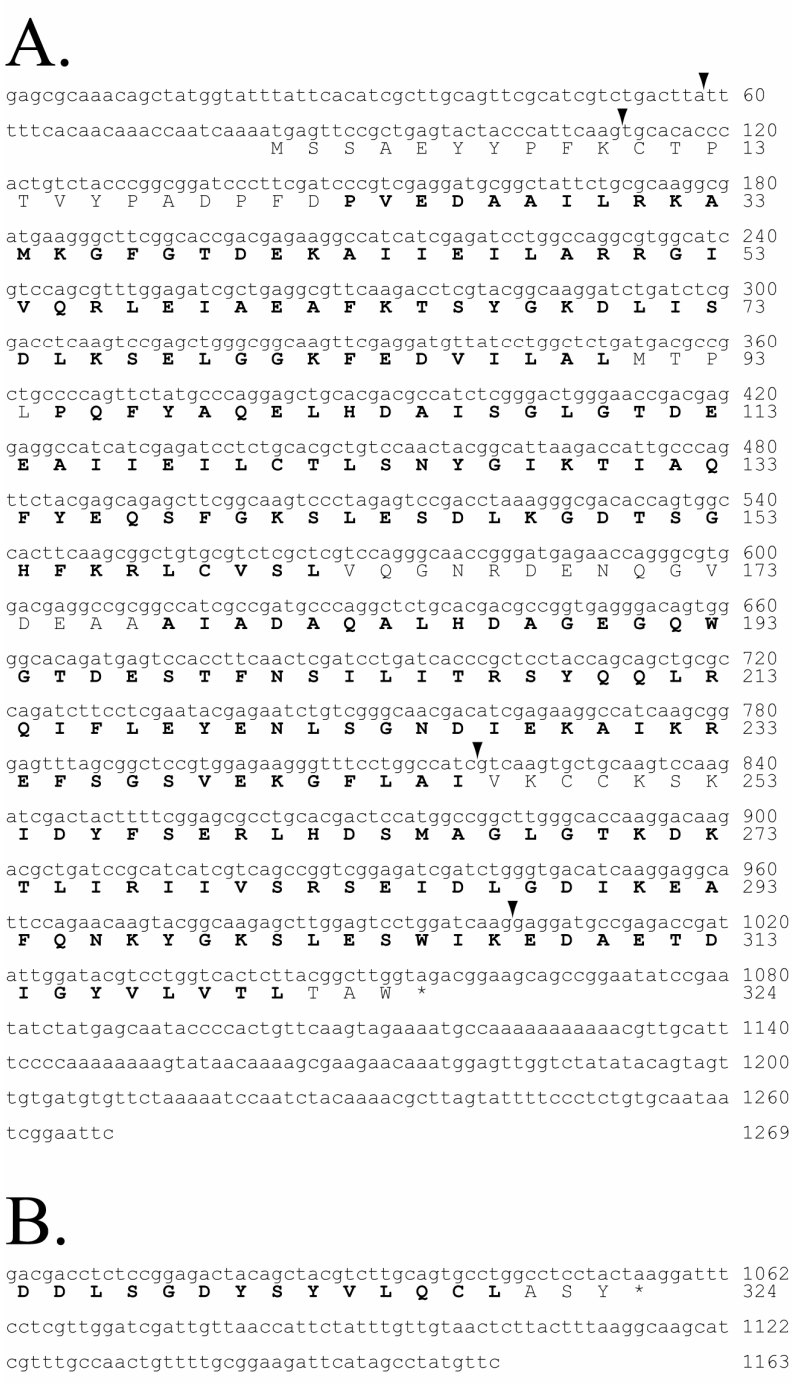

Figure 1. *AnxB9* cDNAs and proteins. (A) Nucleotide sequence of *AnxB9a* with translation (GenBank accession number AY007377). Arrowheads indicate exon junctions. (B) Nucleotide sequence of the fifth exon from *AnxB9b* with translation (GenBank accession number AF261718). The first four exons of *AnxB9a* are identical to those from *AnxB9b*. The nucleotide sequence numbering begins with base 1003 (from *AnxB9a*) where *AnxB9b* diverges. The annexin repeats, identified using Pfam (Bateman *et al.*, 1999) via the Institute for Chemical Research, Kyoto University, Japan (http://www.genome.ad.jp/), are shown in bold.

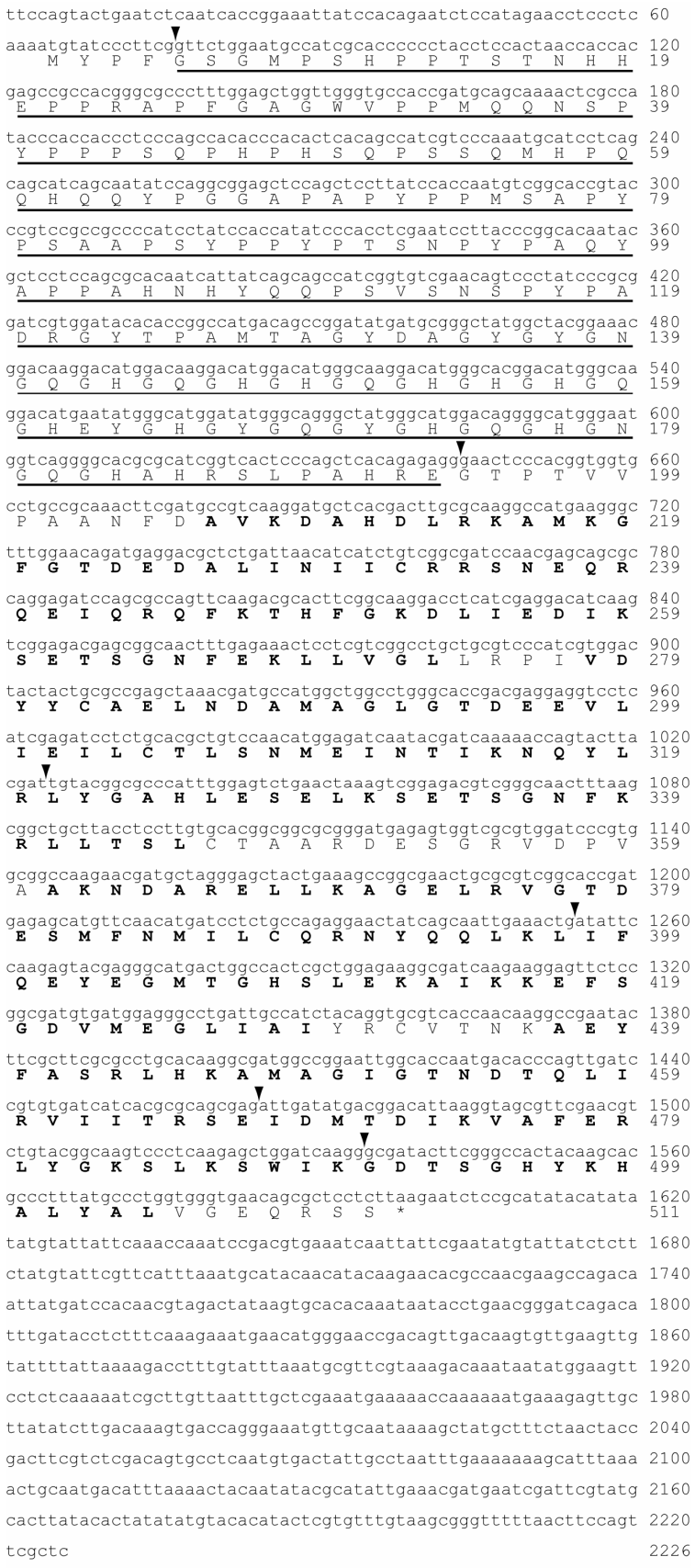

Figure 2. *AnxB11* cDNAs and proteins. Nucleotide sequence of *AnxB11-long* with translation (GadFly gene CG9968, GenBank accession AJ276963). Arrowheads indicate exon junctions. ANXB11-*short* and ANXB11-long are identical except for the underlined sequence which is specific to the long isoform. The annexin repeats, identified using Pfam (Bateman *et al.*, 1999) via the Institute for Chemical Research, Kyoto University, Japan (http://www.genome.ad.jp/), are shown in bold.

The structure of the *AnxB9* locus was investigated by sequence analysis. Genomic sequence (GenBank accession number AC019736; Adams and Venter, unpublished) for the *AnxB9* locus was obtained by searching the BDGP database with the *AnxB9b* and *AnxB9a* sequences. In each case the analysis revealed five blocks of identity representing five exons from which each transcript is derived. The first four exons are common to both transcripts, whereas the fifth exon is alternatively spliced (Figure 3A). Each intron begins with GT and ends with AG, following the GT-AG rule (Breathnach *et al.*, 1978; Stephens and Schneider, 1992).

Analyses of all ESTs from *AnxB9* cDNA clones (BDGP EST clot #2657:3) support this splicing pattern. Nineteen ESTs are consistent with splice sites one and two, three ESTs are consistent with splice three, and two ESTs are consistent with splice site 4b. Two ESTs, LD03123 and GH13750, are derived from alternative splicing within the 5' untranslated region such that the 3' end of the first exon is extended by 25 bases (5'-GTACGTTGAAAATCGAATATCATTG-3') relative to the sequence shown in figure 1A. The nucleotides in this extended exon are identical to those in the genomic sequence and are followed by GT residues, conforming to the GT-AG rule (Breathnach *et al.*, 1978; Stephens and Schneider, 1992). Thus, all available sequence information is fully consistent with the splicing patterns shown in Figure 3A.

While all available 5' *AnxB9* ESTs share the same 5' residues, these data do not exclude the possibility that *AnxB9a* and *AnxB9b* are derived from different first exons. We used reverse transcription polymerase chain reaction (RT-PCR) analysis to verify the presence of *AnxB9a* and *AnxB9b* transcripts containing the common first exon and the alternatively spliced fifth exons. First-strand cDNAs were generated via reverse-transcription using total RNA isolated with Trizol (Life Technologies, Inc., Rockville, MD) as template. cDNAs were amplified by PCR using primer B9.1F (5'-CGCTTGCAGTTCGCATCG-3') which anneals to the first exon in combination with primers B9a.R (5'-TGAACAGTGGGGTATTGC-3') and B9b.R (B9b.R, 5'-ACCGCGATTTTACAGTGC-3'), which are specific for exon 5a or 5b, respectively. Primer pairs B9.1F/B9a.R and B9.1F/B9b.R amplified the predicted 1076 and 1161 base pair products corresponding to portions of *AnxB9a* and *AnxB9b*, respectively (data not shown). Additionally, alternative splicing of the first exon was confirmed by RT-PCR studies using a forward primer specific for the extended region of exon one and reverse primers specific for either the *AnxB9a* or *AnxB9b* isoform (data not shown). These data, in conjunction with the available 5' EST sequence data presented above, show that the *AnxB9a* and *AnxB9b* transcripts are derived from the same first exon and confirm the pattern of exon utilization depicted in Figure 3A.

Blast searches of the BDGP sequence database identified a novel annexin gene in *Drosophila*, *AnxB11* (Genome annotation database of *Drosophila* (GadFly) gene CG9968 (Adams *et al.*, 2000)). This gene is predicted by GadFly to have two transcripts resulting from alternative splicing. One transcript is derived from six exons, whereas the other is derived from the same six plus an additional exon in the second position. We designate these two transcripts *AnxB11-short* and *AnxB11-long*, respectively. The additional exon within *AnxB11-long* is composed of 567 nucleotides and encodes 189 amino acids (Figure 2). Alignment of the nucleotide sequences of the two *AnxB11* cDNAs to the *AnxB11* genomic region (BACR48A09, GenBank accession AC016020) revealed six blocks of homology for *AnxB11-short* and seven blocks for *AnxB11-long* (Figure 3B), consistent with the prediction of exon usage from GadFly (Adams *et al.*, 2000). Additionally, all of the introns follow the GT-AG rule (Breathnach *et al.*, 1978; Stephens and Schneider, 1992).

Eight ESTs from the BDGP database were found that corresponded to the *AnxB11* locus. One EST was consistent with splice site one in *AnxB11-long* and three were consistent with splice site one in *AnxB11-short*. Additionally, four were consistent with splice site three, two with sites four and five, and three with site six in both isoforms (numbering relative to splice sites in *AnxB11-long*) (Figure 3B). RT-PCR analyses determined that both *AnxB11* isoforms are expressed (Figure 4, see

below). Together, these data confirm that the *AnxB11* gene produces two alternatively spliced transcripts.

Expression of *AnxB9a* and *AnxB9b* was analyzed using RT-PCR. First-strand cDNAs were generated via reverse-transcription using as template total RNA isolated from embryos, each larval instar, and adult body parts. A primer that anneals to the common third exon (B9.2F, 5'-ACCAGTGGCCACTTCAAG-3') was used with primers B9a.R or B9b.R to specifically amplify *AnxB9a* and *AnxB9b* cDNAs, respectively (Figure 3A). The identities of amplified fragments were verified by direct sequencing using a nested primer (B9.3F, 5'-TCAGCCGGTCGGAGATCG-3') that anneals to the common fourth exon (Figure 3A). Expression of both *AnxB9a* and *AnxB9b* was found in embryos, all larval instars, and both heads and bodies of adult flies (Figure 4A and 4B). These data directly show that the two distinct transcripts of *AnxB9*, encoding different isoforms of the ANXB9 protein, are present at all life stages in *Drosophila*, consistent with previous results showing two transcripts on northern blots that hybridize with an *AnxB9* probe (Johnston *et al.*, 1990).

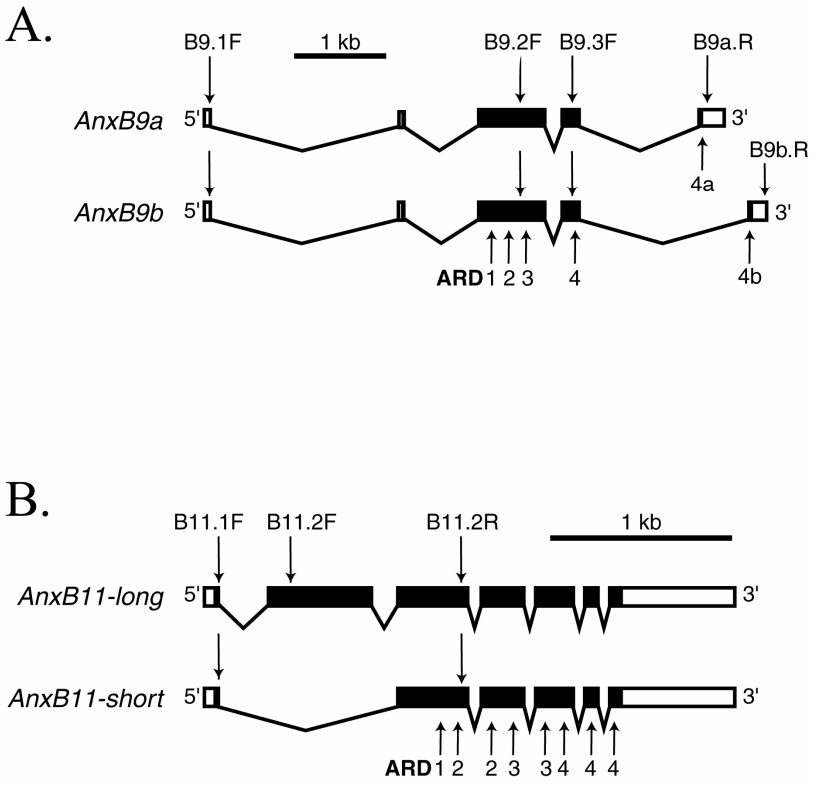

Figure 3. Genomic structures of *AnxB9 and AnxB11*. Exons are shown as boxes with the protein coding regions shaded. The positions of annexin repeat domains (ARD) 1-4 relative to exons are indicated by upward arrows. (A) Structure of *AnxB9*. Nucleotide sequences from *AnxB9* cDNAs and sequences from the BDGP database were used to determine the genomic structure of the *AnxB9* locus. The locations of primers (B9.1F, B9.2F, B9a.R, B9b.R, B9.3F) used in RT-PCR and sequencing are indicated. (B) Structure of *AnxB11*. Nucleotide sequences from *AnxB11* cDNAs (GadFly gene CG9968, GenBank accession AJ276963) and genomic sequences (BDGP BACR48A09, GenBank accession AC016020) were used to determine the genomic structure of the *AnxB11* locus. The locations of primers (B11.1F, B11.2F, B11.2R) used in RT-PCR are indicated.

Expression of *AnxB11-short* and *AnxB11-long* was also analyzed by RT-PCR. Amplification with a common reverse primer (B11.2R, 5- ATCTCCATGTTGGACAGC -3') in combination with a common forward primer (B11.1F, 5'-CCCTCAAAATGTATCCCTTCGG-3') generated a strong band corresponding to *AnxB11-short* (Figure 4C) and a much weaker band that was consistent with *AnxB11-long* expression (data not shown). Expression of *AnxB11-long* was confirmed by RT-PCR using an *AnxB11-long* specific forward primer (B11.2F, 5'-AAACTCGCCATACCCACC-3') in combination with the same common reverse primer (B11.2R) (Figure 4D). These studies revealed that *AnxB11-short* and *AnxB11-long* are both expressed in all life stages analyzed in addition to heads and bodies of adults. Together, our results on expression of the *AnxB9* and *AnxB11* genes suggest that these two genes might have basic cellular roles at all life stages.

Our studies, in combination with previous experiments (Johnston *et al.*, 1990; Benevolenskaya *et al.*, 1995), identify three annexin genes in *Drosophila*: *AnxB9*, *AnxB10*, and *AnxB11*. Collectively, these three genes encode five protein isoforms (Johnston *et al.*, 1990; Benevolenskaya *et al.*, 1995) (Figures 1 and 2). All five isoforms contain the characteristic four annexin repeat domains (Figures 1 and 2) (Sudhof *et al.*, 1988) and are 25-50 percent identical to other known annexins (data not shown).

The predicted ANXB9a and ANXB9b proteins are both 324 amino acids in length and differ only in the carboxy-terminal 17 amino acids which are encoded by alternatively spliced fifth exons. These 17 carboxy-terminal residue are identical at six positions and are similar at 11 positions (Figure 1). ANXB11-short and ANXB11-long are 322 and 511 amino acids in length, respectively. They are identical except that ANXB11-long has an additional 189 amino acid residues derived from *AnxB11-long* specific exon two that are inserted after the fourth amino acid residue (Figure 2). This alternative splicing makes ANXB11-long similar in overall structure to vertebrate annexins VII and XI which have long amino termini followed by the four annexin repeats (Smith and Moss, 1994). In contrast, ANXB11-short (Figure 2) along with ANXB9a, ANXB9b (Figure 1) and ANXB10 (Benevolenskaya *et al.*, 1995) are similar to all other annexins containing short amino termini preceding the four repeat domains (Smith and Moss, 1994). Blast searches of protein and nucleotide databases via NCBI and BDGP revealed that this additional region of ANXB11-long does not have significant homology to any other known protein, leaving its functional significance obscure. However, its presence suggests that ANXB11-long might have a unique function among annexins.

Comparisons of the full length amino acid sequences revealed that the ANXB9 proteins and ANXB11-short are somewhat more related to each other than they are to ANXB10 (54-55% amino acid identity versus 44-47%). Interestingly, *AnxB10* is expressed in embryos and in adults, but not at other stages (Johnston *et al.*, 1990), whereas *AnxB9* and *AnxB11* are expressed at all life stages in *Drosophila* (Figure 4). The conservation in primary amino acid sequence between the ANXB9 isoforms and ANXB11-short, in combination with parallels in their temporal patterns of expression (Figure 4), is consistent with a conserved function for this group of proteins.

Interestingly, though, the functional organizations of the *AnxB9* and *AnxB10* genes are strikingly similar to each other and markedly different from that of *AnxB11*. The two most prominent similarities between *AnxB9* and *AnxB10* is that the first three annexin repeat domains in both genes are encoded by the second protein-coding exon and the fourth annexin repeat is encoded by the third and fourth protein-coding exons (Benevolenskaya *et al.*, 1995) (Figures 1 and 3A). In contrast, the first annexin repeat in *AnxB11-short* is encoded by the second exon, the second repeat by exons two and three, the third repeat by exons three and four, and the fourth repeat by exons four, five and six (Figures 2 and 3B). Thus, the conservation in primary amino acid structure between the ANXB9 isoforms and ANXB11-short does not correlate with conservation of functional organization of their genomic loci.

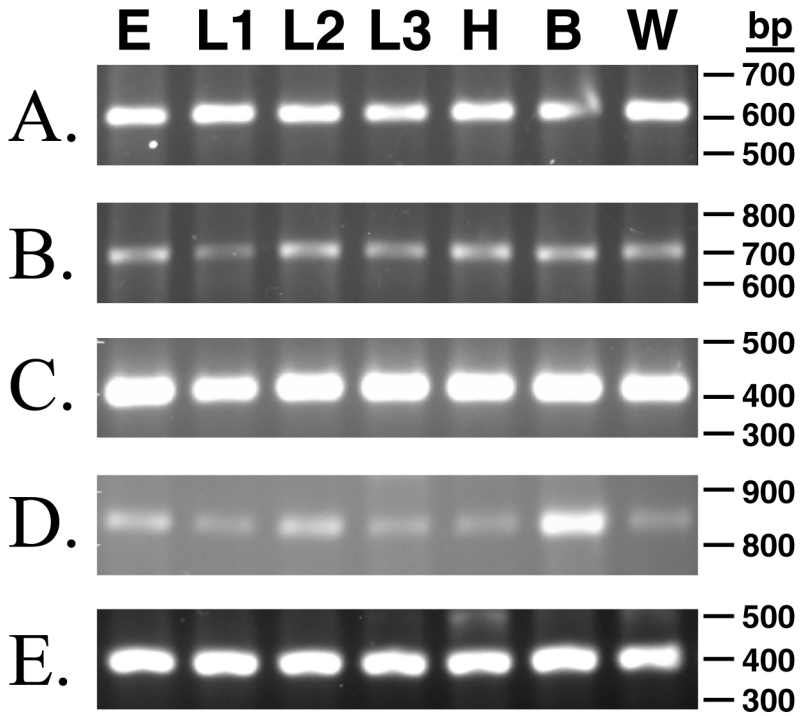

Figure 4. Expression of *AnxB9* and *AnxB11* isoforms**.** Gels showing RT-PCR products amplified with 30-35 cycles from RNA isolated from embryos (E), first instar larvae (L1), second instar larvae (L2), third instar larvae (L3), adult heads (H), adult bodies (B) and whole adults (W). (A) Amplification using primers B9.2F and B9a.R, specific for *AnxB9a*. (B) Amplification using primers B9.2F and B9b.R, specific for *AnxB9b*. (C) Amplification using primers B11.1F and B11.2R which amplify both *AnxB11-short* and *AnxB11-long* (long not shown). (D) Amplification using primers B11.2F and B11.2R which specifically amplifies *AnxB11-long*. (E) RT-PCR analysis of expression from the ribosomal protein gene *rp49* (O'Connell and Rosbash, 1984) was the internal control. No DNA fragments were amplified in reactions with RNase added prior to reverse transcription (data not shown). Amplification products were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized with ultraviolet light. Molecular weight markers (in base pairs) are indicated.

In accord with a model of annexin gene evolution involving intron loss (Smith and Moss, 1994; Benevolenskaya *et al.*, 1995; Morgan and Fernandez, 1997), we propose that a single ancestral gene similar in organization to the *AnxB11-short* transcription unit gave rise to a common precursor of *AnxB9* and *AnxB10* via gene duplication and subsequent elimination of the second, third and fourth introns. This common precursor in turn would have undergone gene duplication to produce *AnxB9* and *AnxB10*, whereas the presumptive ancestral gene would have evolved into *AnxB11*. Thus, our analyses suggest that the conservation in functional organization of annexin genes and the conservation of the amino acid sequences of their gene products are influenced by distinct evolutionary forces.

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The influence of the *p53* mutation on the tumor frequency in *wts*/+ heterozygotes.

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Recently a homologue of mammalian *p53*, the key gene in the tumor suppression and apoptosis control, was identified in *Drosophila* (Brodsky *et al.*, 2000; Ollmann, M. *et al.,* 2000). The influence of *Drosophila p53* mutations on the radiation-induced apoptosis had been thoroughly characterized (Brodsky *et al.*, 2000; Lee *et al.*, 2003). In the present article we describe the *p53* dominant negative mutation effects on the frequency, both spontaneous and mutagen-induced, of somatic recombinant tumor clone formation in heterozygotes for the tumor suppressor gene *warts* (*wts*).

Three crossings were performed:
(I): ♀ *y w; wts^{X1}/TM6B, Hu Tb* × ♂ *w sn^3* to obtain control *wts*/+ heterozygotes without the *p53* mutation; (II): ♀ *y w; P{w^+ GMR-$p53^{259H.GUS}$} wts^{X1}/TM6B, Hu Tb* × ♂ *w sn^3* to obtain *GMR-$p53^{259H.GUS}$ wts^{X1}* / + + heterozygotes carrying a dominant negative *p53* mutation in a transgenic construct; (III): ♀ *y w; P{w^+ GMR-$p53^{259H.GUS}$} wts^{X1}/TM6B, Hu Tb* × ♂ *w; act-GAL4/TM6B, Hu Tb* to produce + *GMR-$p53^{259H.GUS}$ wts^{X1}/act-GAL4* + + heterozygotes with the *p53* mutation under Glass Multiple Reporter (GMR) promoter activated by *GAL4*. For the characteristics of the strains see (Brodsky *et al.*, 2000; Xu *et al.*, 1995)

The 1st instar F_1 larvae from the crosses were treated either with distilled water (control group) or with a mutagenic substance, oxoplatin (2 mg/ml aqueous solution). In the F_1, *wts^{X1}*/+ (crossing I), *P{w^+ $p53^{259H.GUS}$} wts^{X1}*/ + + (crossing II) and + *P{w^+ $p53^{259H.GUS}$} wts^{X1}/ act-GAL4* + + (crossing III) flies selected by the absence of *Hu* and *Tb* markers were examined for *wts* tumorous

clones. Clone frequency (p) was calculated as (Number_of_clones / Number_of_flies) × 100%. The significance of differences between the series was calculated in Student's t-test with Fisher's correction $\varphi = 2\arcsin\sqrt{p}$.

Table 1. The influence of *GMR-p53*$^{259H.GUS}$ on the tumor clone frequency in *wts*/+ heterozygotes.

Flies genotype	Substance, concentration	flies	*wts* clones	P_{wts}, %
wts^{X1}/+	Oxoplatin, 2 mg/ml	581	144	24.78!
	Distillated water	1383	11	0.80
GMR-p53$^{259H.GUS}$ *wts*^{X1} / + +	Oxoplatin, 2 mg/ml	1064	826	77.63! **
	Distillated water	686	16	2.33*
+ *GMR-p53*$^{259H.GUS}$ *wts*^{X1}/*act-GAL4* + +	Oxoplatin, 2 mg/ml	228	220	96.49! **
	Distillated water	765	66	8.63**

! – the *wts* tumor frequency is significantly higher than the control one, *P < 0.001*; ** – the *wts* tumor frequency is significantly higher than the frequency in the corresponding variant with less active or absent *p53* mutation; *P < 0.001; * - the same, P<0.01*.

The results are summarized in the Table 1. We have made the following conclusions.

1). The *p53*$^{259H.GUS}$ dominant mutation increases both spontaneous and induced tumor frequency in *wts*/+ heterozygotes (3.13 times for the induced mosaicism and 2.91 times for the spontaneous mosaicism). Probably, the *p53* dominant mutation leads to the increase in the number of surviving cells with DNA breaks that are the substrate for the somatic recombination producing tumorous clones homozygous for *wts* in *wts*/+ individuals.

2). The *P{w*$^{+}$ *GMR-p53*$^{259H.GUS}$*}* construct is sufficient to increase the frequency of tumor clones even without *GAL4*. It is possible that the GMR promoter provides some minimum rate of the mutant *p53* transcription in absence of *GAL4*.

3). The activation of *p53*$^{259H.GUS}$ transcription in *GAL4* presence results in the increase in the frequency of *wts* clones. This may suggest that in absence of *GAL4* in *GMR-p53*$^{259H.GUS}$ *wts*^{X1} / + + flies the production of the mutant form of the p53 protein is insufficient to eliminate the wild type product completely.

4). The relative tumor frequency increase observed upon *GMR-p53*$^{259H.GUS}$ transcription activation by *GAL4* is higher in case of the spontaneous mosaicism (3.70 times) than in case of the induced mosaicism (1.24 times).

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Overexpression of *phosphatidylinositol 3-OH kinase (PI3K)* in dopaminergic neurons dramatically reduces life span and climbing ability in *Drosophila melanogaster*.

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Abstract

Parkinson's disease (PD) is a prevalent neurodegenerative disease marked by the selective loss of dopaminergic neurons that is accompanied by resting tremors and other symptoms. The study of organismal models of PD, including the well-studied α-synucleinopathic model, in *Drosophila melanogaster* has led to a greater understanding of the biological basis of the disease. In an attempt to establish additional *Drosophila* models of PD via the manipulation of cell survival signaling, the UAS/GAL4 system was used to overexpress two forms of *phosphatidylinositol 3-OH kinase* (*PI3K*) in the dopaminergic neurons of flies. The directed expression of *PI3K* in this manner dramatically reduces life span and climbing ability while an inhibitory form, a dominant negative version of *PI3K*, reduces life span in a far less dramatic way. These novel models should provide the basis for a series of investigations into the role of cell survival signaling in Parkinson's disease.

Introduction

Parkinson's disease (PD) is a common, age-related neurodegenerative disease characterized by muscle rigidity, resting tremors, and postural instability (Spacey and Wood, 1999; Lansbury and Brice, 2002). Post-mortem analysis of patients reveal that PD appears to be due to the selective loss of dopaminergic neurons in the *substantia nigra* region of the brain. The underlying cause of this distinctive loss of neurons may be classified as either sporadic or familial in origin. Although the underlying mechanism is not well understood, defects in several genes as well as a number of environmental toxins have been linked to the cause of this neuronal loss. As it is difficult to research the pathogenesis of PD in living patients, a number of animal models (Dawson, 2000; Hashimoto *et al.*, 2003), including a well established *Drosophila* model (Feany and Bender, 2000), have been developed to investigate aspects of PD.

A promising series of investigations into the biological basis of PD have been initiated through the generation of a PD model by the conditional expression of human *α-synuclein* in transgenic *Drosophila* (Feany and Bender, 2000). The expression of *α-synuclein*, in both a pan-neural and dopaminergic neuron-specific manner, produce an age-dependent loss of dopaminergic neurons. The neuronal loss is accompanied with the premature loss of climbing ability and the formation of cytoplasmic inclusions in the dopaminergic neurons. In addition, expression of *α-synuclein* in the developing eye results in an age-dependent degeneration of the retina. In further experiments, the dopamine precursor levodopa, dopamine receptor agonists, and the anticholinergic agent atropine act to counter the age-dependent loss of climbing ability (Pendleton *et al.*, 2002). Expression of the molecular chaperone gene *hsp70* with *α-synuclein* prevents dopaminergic neuronal

degeneration (Auluck *et al*., 2002). The expression of *parkin* can suppress the loss of dopaminergic neurons (Yang *et al*., 2003), the premature loss of climbing ability and the age-dependent degeneration of the retina (Haywood and Staveley, in preparation) induced by α-synuclein in *Drosophila*. In addition, another model has recently been established with the description of mutants in the *parkin* gene (Greene *et al.*, 2003). The *Drosophila* models of PD are proving to be very effective tools in the investigation of the biological basis of this disease.

Dopaminergic neurons may die as a result of apoptosis in PD (for review see Lev *et al*., 2003). This process may be caused by the accumulation of endogenous toxic proteins or environmental toxins. Exploration of the role of cell survival signaling in the selective loss of dopaminergic neurons in *Drosophila* may provide further insight into the basis of PD. The insulin receptor/ PI3 kinase/ akt anti-apoptotic signaling pathway is highly conserved between mammals and *Drosophila* (Fernandez *et al*., 1995, Leevers *et al*., 1996; Staveley *et al*., 1998; Datta *et al*., 1999; Oldham *et al*., 2000). To initiate this signal, insulin or insulin-like growth factors bind to receptor tyrosine kinases at the cell membrane and activate the protein phosphatidylinositol 3-OH kinase (PI3K) via phosphorylation (Vanhaesebroeck *et al*., 2000). In turn, PI3K phosphorylates inositol lipids on the inner membrane of the cell, which leads to the co-localization of akt and phosphoinosotide-dependent kinase 1 (PDK-1) and, as a result, the activation of akt. An anti-apoptotic or cell survival signal results from activated akt. Consequently, manipulation of the InR/PI3K/akt pathway in the dopaminergic neurons of *Drosophila melanogaster* may produce selective apoptotic death of those cells and produce flies with symptoms similar to other models of PD. As PI3K is an essential component of this pathway, it is a good candidate for manipulating cell survival signaling.

The UAS/GAL4 ectopic expression system (Brand and Perrimon, 1993) was used to overexpress wild type and mutant forms of PI3K in the dopaminergic neurons. Climbing and longevity assays were performed and the results demonstrate that overexpression of PI3K dramatically reduces climbing ability and viability of the flies from the time of eclosion. Overexpression of an inhibitory PI3K also reduces the length of life span when compared to controls but does not prematurely reduce the climbing ability of the flies.

Materials and Methods

Fly stocks and culture: The *Ddc-GAL4*$^{4.3D}$ and *Ddc-GAL4*$^{4.36}$ transgenic lines (Li *et al.*, 2000) were obtained from Dr. Jay Hirsh at the Department of Biology, University of Virginia. The *UAS-PI3K-dp110* and *UAS-PI3K-dp110*^{D954A} flies were obtained from Dr. Sally Leevers at the Ludwig Institute for Cancer Research and the Department of Biochemistry and Molecular Biology, University College, London. The w^{1118} strain was provided by Dr. Howard D. Lipshitz of the Hospital for Sick Children and the University of Toronto. All flies were cultured on standard cornmeal/yeast/agar medium at 25°C.

Transgene Expression: The UAS/GAL4 ectopic expression system (Brand and Perrimon, 1993) was used to express wild type and mutant forms *of phosphatidylinositol 3-OH kinase* (*PI3K*; Leevers *et al*., 1996) in the dopaminergic neurons using *Ddc-GAL4* transgenes (Li *et al*., 2000). The progeny of crosses of the *Ddc-GAL4* lines to transgenic *UAS-PI3K-dp110* flies will express the catalytic subunit of PI3K (dp110) in the dopaminergic neurons. The same *Ddc-GAL4* driver lines were crossed to *UAS-PI3K-dp110*^{D954A} to induce the expression of an inhibitory form of this subunit of PI3K. The controls were produced by crossing w^{1118} to the *Ddc-GAL4* transgenics.

Aging assay: Adult male flies were collected within 24 hours of eclosion and scored for viability every two to three days to determine the adult life span characteristics as previously

described (Staveley *et al*., 1990). Flies were maintained under non-crowded conditions of approximately 5 to 15 individuals upon standard cornmeal/yeast/agar medium at 25°C.

Climbing assay: The climbing ability of male flies of the same age were assayed every four days to determine their locomotor abilities throughout their life span as previously described (Feany and Bender, 2000). To be precise, the proportion of a cohort of ten (or fewer) flies to climb a distance of 8 centimetres within a period of 18 seconds was determined. In total, twenty trials were carried out at each time point. From this data, the average number of flies that successfully completed the climb at each time point was calculated.

Data Analysis: Data from the aging and climbing assays were compiled and graphed using Microsoft Excel.

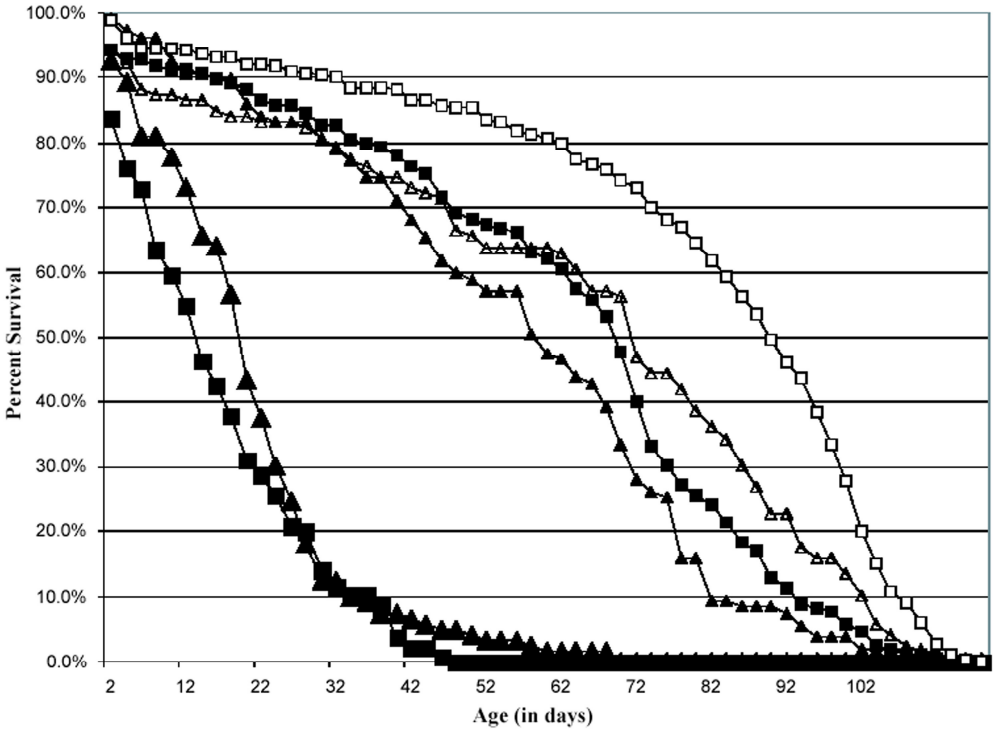

Figure 1. Survival of flies expressing wild type (*PI3K*) and dominant negative *PI3K* (*PI3K*^{DN}) in the dopaminergic neurons. Adult males that express the wild type version of *PI3K* in the dopaminergic neurons *Ddc-GAL4*$^{4.36}$*/UAS-PI3K-dp110* (large solid triangles) and *Ddc-GAL4*$^{4.3D}$*/UAS-PI3K-dp110* (large solid squares) have a greatly reduced life span when compared to controls. Expression of the dominant negative *PI3K* transgene under the same circumstances, *Ddc-GAL4*$^{4.36}$*/UAS-PI3K-dp110*^{D954A} (small solid triangles) and *Ddc-GAL4*$^{4.3D}$*/UAS-PI3K-dp110*^{D954A} (small solid squares), leads to a slightly reduced life span, when compared to the *GAL4*-expressing controls, *Ddc-GAL4*$^{4.36}$/+: (small open triangles) and *Ddc-GAL4*$^{4.3D}$/+: (small open squares). The number of individuals aged was as follows: *Ddc-GAL4*$^{4.36}$*/UAS-PI3K-dp110*, n = 122; *Ddc-GAL4*$^{4.3D}$*/UAS-PI3K-dp110*, n = 129; *Ddc-GAL4*$^{4.36}$*/UAS-PI3K-dp110*^{D954A}, n = 107; *Ddc-GAL4*$^{4.3D}$*/UAS-PI3K-dp110*^{D954A}, n = 195; *Ddc-GAL4*$^{4.36}$/+, n = 119; *Ddc-GAL4*$^{4.3D}$/+, n = 280.

Results and Discussion

Transgenic flies expressing one of the two forms of *PI3K* in the dopaminergic neurons were tested for viability with an aging assay (Figure 1). Overexpression of *PI3K-dp110* with both of the *Ddc-GAL4* transgenes greatly decreased the life span of the flies. The median age of survival (50%) for flies expressing *PI3K-dp110* was between 18 and 20 days when expressed by *Ddc-GAL4*$^{4.3D}$ and between 12 and 14 days when expressed by *Ddc-GAL4*$^{4.36}$. Expression of the dominant negative form of *PI3K* (*PI3K-dp110*^{D954A}) produced a small decrease in survival. The median age of survival (50%) was between 58 and 60 days under the control of the *Ddc-GAL4*$^{4.36}$ driver and between 68 and 70 days with the *Ddc-GAL4*$^{4.3D}$ transgene. The *GAL4* heterozygotes, *Ddc-GAL4*$^{4.3D}$ and *Ddc-GAL4*$^{4.36}$, were tested and the results show a median age of survival between 70 and 72 days for the former and between 82 and 84 for the latter. The expression of *PI3K-dp110*^{D954A} resulted in a decrease in median survival of approximately 14 days when compared to the *Ddc-GAL4* heterozygote controls while the expression of *PI3K-dp110* resulted in a major decrease in life span by between 50 and 70 days.

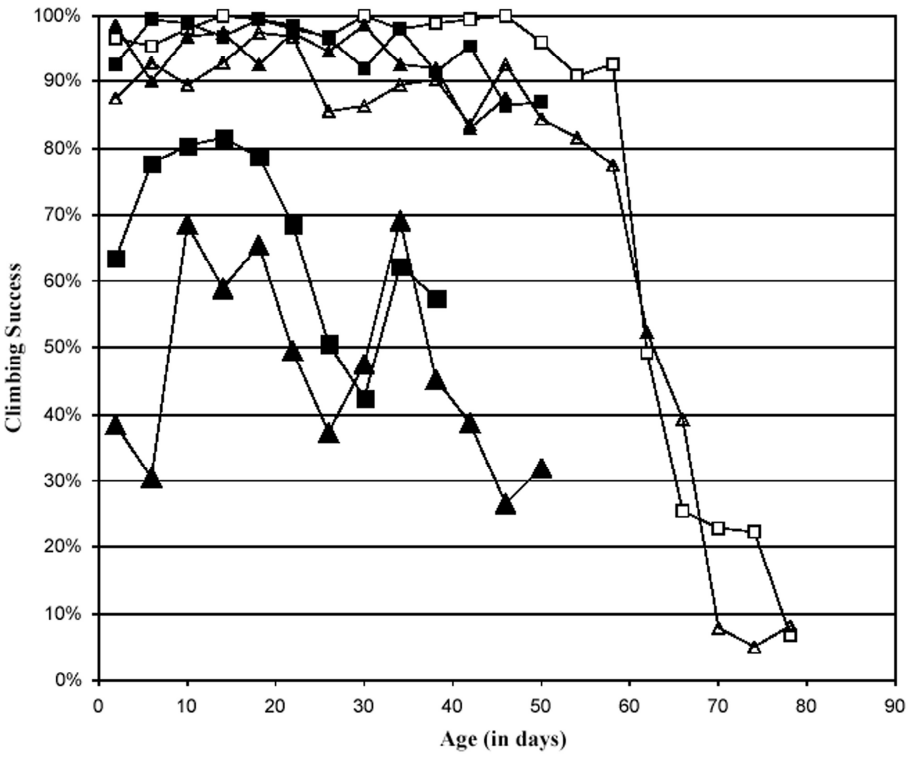

Figure 2. The measurement of climbing ability of flies expressing wild type (*PI3K*) and dominant negative *PI3K* (*PI3K*^{DN}) in the dopaminergic neurons. Adult males that express the wild type version of *PI3K* in the dopaminergic neurons *Ddc-GAL4*$^{4.36}$*/UAS-PI3K-dp110* (large solid triangles) and *Ddc-GAL4*$^{4.3D}$*/UAS-PI3K-dp110* (large solid squares) have a poor ability to climb when compared to controls. Expression of the dominant negative *PI3K* transgene under the same circumstances, *Ddc-GAL4*$^{4.36}$*/UAS-PI3K-dp110*^{D954A} (small solid triangles) and *Ddc-GAL4*$^{4.3D}$*/UAS-PI3K-dp110*^{D954A} (small solid squares), maintain the ability to climb in a manner similar to the controls, *Ddc-GAL4*$^{4.36}$/+ (small open triangles) and *Ddc-GAL4*$^{4.3D}$/+ (small open squares). The climbing experiments were discontinued when death reduced the number significantly.

To monitor the effects upon locomotion, the climbing ability of these transgenic flies were tested (Figure 2). Flies that express the wild type version *PI3K-dp110* under the control of *Ddc-GAL4* climb poorly while those expressing *PI3K-dp110*^{D954A} appear to climb as well as the controls throughout the duration of the experiment.

In addition to the defects in climbing ability and the greatly reduced life span, flies overexpressing *PI3K-dp110* exhibit a blistered wing phenotype shortly after emerging from the pupae cases (data not shown). Within a day or so, most adult *Ddc-GAL4/UAS-PI3K-dp110* flies have shriveled wings. This defect may be indirectly caused by neuronal loss.

Overexpression of *PI3K* in the dopaminergic neuron during development may lead to selective apoptotic death of these neurons. Contrary to the common role of *PI3K* in supporting cell survival, overexpression of *PI3K* has been shown to cause apoptosis. In cultured rat embryo fibroblasts, prolonged activation of *PI3K* in the absence of other stimuli (serum) results in apoptosis (Klippel *et al.,* 1998). Prolonged overexpression of *PI3K* increases in the proportion of cells in G2/M and induces apoptosis in *Drosophila* (Vanhaesebroeck *et al*., 2000). This may be due to deregulation of the cell cycle or the induction of an apoptotic feedback program by the hyperactivation of many signaling pathways. The selective loss of the dopaminergic neurons via a cell death mechanism could be responsible for the observed poor climbing ability and reduced life span of adult *Ddc-GAL4/UAS-PI3K-dp110* flies.

Although active PI3K acts to prevent apoptosis of cells, larvae survive for twenty days without PI3K (Weinkove *et al.,* 1999). In contrast, the inhibitory form of *PI3K* has been shown to cause cell death when expressed in embryos (Scanga *et al.,* 2000). In our experiments, the expression level of *PI3K-dp110*^{D954A} may have been sufficient to induce neuronal loss only in late life. The small decrease in life span may have resulted from this late loss in neurons in the *Ddc-GAL4/UAS-PI3K- dp110*^{D954A} flies.

In conclusion, this experiment analyzed the viability and climbing ability of flies expressing two forms of *PI3K* in an attempt to model characteristics of Parkinson's disease. Unexpectedly, the ectopic expression of *PI3K* showed dramatically reduced life span coupled with poor climbing ability. Unlike Parkinson disease patients, the locomotor dysfunction begins early, rather than arising in a gradual manner, which may be due to the larval expression of *PI3K*, and subsequent loss of dopaminergic neurons at that stage. The dominant negative version of *PI3K* reduced life span by a modest amount but did not seem to influence the ability of these flies to climb. In summary, our experiments show that the overexpression of *PI3K* in dopaminergic neurons can produce defects that may recapitulate some aspects of Parkinson's disease in *Drosophila melanogaster*.

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Drosophila malerkotliana and *D. ananassae* in Florida.

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Drosophila malerkotliana is probably native to Asia. It has spread through Africa and the New World tropics where it was first collected in the latter part of the twentieth century (Chassagnard *et al*., 1989, and references therein). Here I report the first collections of this species in North America. I compare its distribution to that of its relative *D. ananassae*, long considered to be the only representative of the *ananassae* subgroup in North America (*e.g*., Patterson and Wagner, 1943).

I identified the specimens as *D. malerkotliana* by genital and sex comb characters. As members of the *bipectinata* complex in the *ananassae* subgroup (Bock, 1971), *malerkotliana* males have a bifid aedeagus. Males in the related *ananassae* complex have a single, fused aedeagus. Sex combs in *malerkotliana* consist of short, transverse rows of bristles (Bock and Wheeler, 1972). There are one or two rows on the metatarsus, the proximal row having 0-2 teeth, the distal row having about 3. There are two rows on the second tarsal segment, the proximal row having about 1 tooth, the distal row having about 2. This formula distinguishes *malerkotliana* from the very similar *D. pseudoananassae* (Bock, 1971). In September 2003 I performed crosses between recent Florida stocks and a *D. malerkotliana* stock collected May 2002 in Chiapas, Mexico, and distributed by the Tucson Stock Center. All crosses produced F1 and F2 flies.

I collected the first specimens of *malerkotliana* at Tallahassee, Florida, 22 Sep 2001. My weekly year-around collections on fruit compost in central Tallahassee show it to be present only between August and December, some years becoming very abundant in October (see http://bio.fsu.edu/~birdsley). It may be re-colonizing north Florida from the south each summer. In fall 2000 I made less regular collections at this same Tallahassee site but did not identify any *malerkotliana*. I think it is likely that *malerkotliana* was not present in 2000, though inexperience may have caused me to confuse them with *D. simulans* or *D. willistoni*.

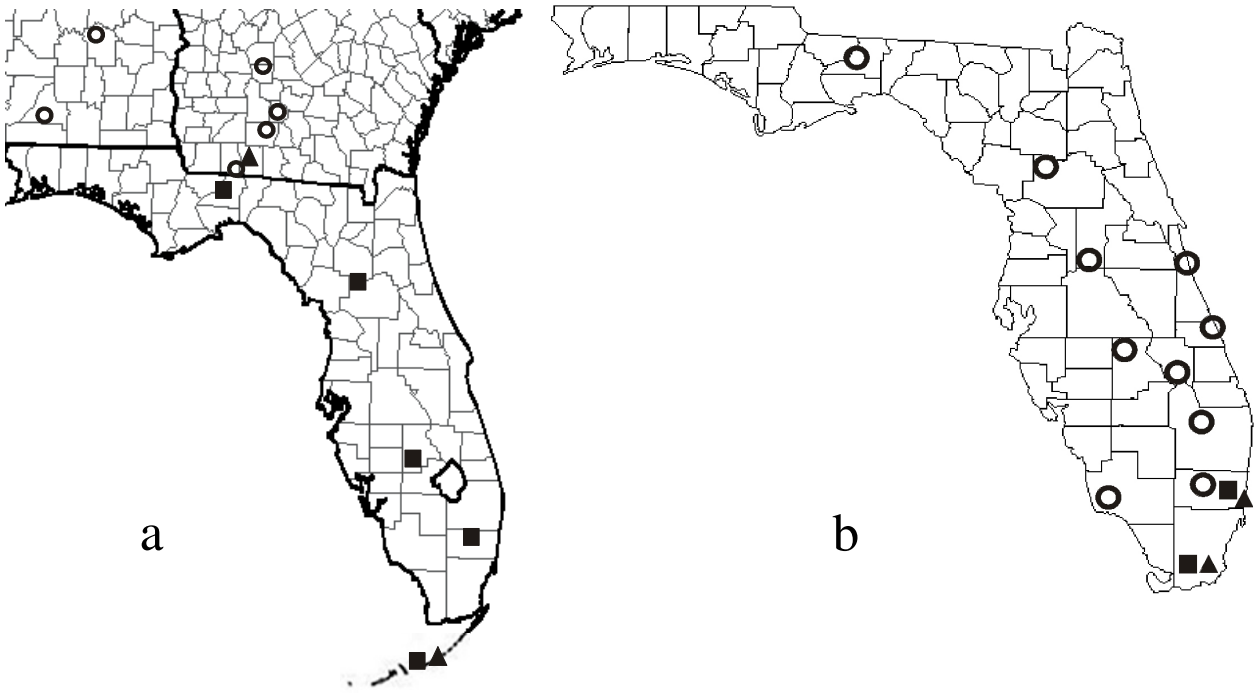

Figure 1. Distribution of *D. malerkotliana* (■), *D. ananassae* (▲), or neither species (O) in collections made (a) in Aug-Oct 2002 and Sep 2003 and (b) in Feb-Mar 2002 and Mar 2003.

I have collected *malerkotliana* throughout peninsular Florida. In far south Florida, I have also collected small numbers of what appears to be a sexually monomorphic form of *ananassae*. The abdomen of males of this species is not black-tipped, but has thin, dark bands like that of the female. The aedeagus is single. Sex combs consist of four or five metatarsal rows, three or four rows on the second tarsal segment, and 1 tooth on the distal end of the third tarsal segment. Figures 1a and 1b show the distributions of both species in Florida and neighboring states in fall and spring, respectively. I generally collected samples of hundreds of flies on rotting fruits. Note the apparent winter refuge in far south Florida and the spread north by fall. The Georgia collections were made 6 Sep 2003 when I collected a single male *ananassae* at a large produce distributor in Thomasville, Georgia. I had collected a single female *ananassae* at the same site on 28 Jun 2003, suggesting frequent immigration from the south via produce shipments. The *malerkotliana* population may spread north into Georgia after 6 Sep, but it is less likely that it spreads into Alabama. I made the collection shown in Conecuh County, Alabama, on 19-20 Oct 2002, long after *malerkotliana* had become abundant in Tallahassee.

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A summary of the polytene chromosome bands not seen by EM.

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In the literature, the number of bands among dipteran polytene chromosomes ranges from 1500 to 5000 (Zhimulev, 1996). According to Zhimulev, this variation is probably due to subjective or objective factors such as the way preparations are made. The most extensively studied dipteran, *Drosophila melanogaster*, has been attributed the highest number of bands. The Bridges' drawn maps for *D. melanogaster* polytene chromosomes (C.B. Bridges, 1938; P.N. Bridges, 1941a,b, 1942; Bridges and Bridges, 1939) reprinted in Lindsley and Zimm (1992) and in Sorsa (1988) show more than 5000 bands when the so called double bands are counted as two bands each (Bridges, 1942), but less than 3800 bands when the doublets are counted as one band each (Zhimulev, 1996). The latter number may be a good approximation of reality.

The size of the single bands on the Bridges' maps ranges from very small and faint to large and dark. The smallest bands are difficult to see on a chromosome preparation even using an electron microscope (EM). Our electron micrograph maps based on thin sections of the long salivary gland chromosomes of *D. melanogaster* were published piecemeal in 1979-1996 and finally completed with the micrographs on the fourth or the dot chromosome (Saura *et al*., 2002). Our original goal was to identify as many of Bridges' bands as possible. Of course, the interpretation of the banding given on our micrograph maps is subjective and the amount of evidence needed to demonstrate the existence of many of the faint bands is open to debate (Semeshin *et al*., 1985).

Table 1 lists the Bridges' bands that were regarded as missing in our electron micrographs. One reason many of the faintest bands have not been detected through EM is that a micrograph shows only a very thin section, 80-100 nm, of a chromosome. One might assume that successive longitudinal thin sections through the same chromosome would show some of the now missing Bridges' bands, even though Lefevre (1976) suggested that Bridges drew certain bands only to even up otherwise excessively long interbands.

Because our EM mapping was done during a long period of time, our ways to interpret banding pattern may have changed at least slightly, if we compare the first papers (Saura and Sorsa, 1979a-d) and the later ones (*e.g*., Saura *et al*., 1993, 1994, 1996). In papers published later we have applied a more stringent interpretation in comparison with the first papers but not as stringent as the rules of Semeshin *et al.* (1985). They propose that the banding pattern of a region should be reproducible on serial sections of at least two or three chromosomes. In addition, single electron dense structures less than 1/20 of the chromosome diameter should not be regarded as bands. The list of bands in Table 1 contains now some 320 (-350) bands. The list might be much longer, if the banding on the micrographs (*e.g*., Saura *et al*., 1997) were interpreted by some other person or strictly by the above rule of Semeshin *et al*. (1985), since the amount of evidence needed to demonstrate the existence of many other of the Bridges' faint bands might be considered to be too meager.

Acknowledgments: Veikko Sorsa and Tapio. I. Heino have commented upon the MS. This work has been supported by a grant from the Jenny and Antti Wihuri Foundation.

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Table 1. The bands of the Bridges' maps not been seen on the electron micrographs. The bands in parentheses (in particular in 2R) may exist but they are weakly visible in the micrographs in comparison with other bands. Chromocentral parts (20B-F, 40B-F, 41A-E, 80B-F) were mostly omitted from detailed mapping.

Chromosome arm and region	Bridges' bands not seen in micrographs. Numbers denoting a chromosome division are in **bold**	Reference (see also Saura *et al*., 1997)
X1-2	-	Sorsa and Saura (1980a)
X3-5	**4**A6, E3; **5**A10 (or A11), B5, C4	Sorsa and Saura (1980b)
X6-10	**10**(B12, B17)	Sorsa *et al*. (1983)
X11-20A	**11**B12, D7, D11, E5, E7; **12**A3, C3; **13**A4, (C8); **14**C3; **15**A6, A9, A12, B3, C3, C6, D3, F6; **16**A3, A7, B3, C9; **18**A7, B8	Saura *et al*. (1993)
2L21-22	-	Saura and Sorsa (1979a)
2L23-26	**24**B3, C4, C6, F5, F8; **26**A7	Saura (1980)
2L27-29	**27**E8, F6; **28**A3, B4, C6, C9, E6 (or E5)	Saura and Sorsa (1979d)
2L30-31	**30**B7; **31**A3	Saura and Sorsa (1979b)
2L32-36(-40)	**32**E3; **33**A4, A8, B4, B7, C6, D2, D5, E? F?; **34**A11, B3, B4, B8, B12, C7, E3, E6; **35**E3, E4, E5-6, F10; **36**B3, C3, C8	Saura (1983)
2L37-40A	**37**(F3); **38**C4; **39**C3, C4	Saura and Sorsa (1979c)
2R41F-50	**41**F7; **42**A13, A15, A17, B5, (C2), (C3), D3; **43**E18; **44**A8, D7, (E3); **45**B5, B9, C9, D3, D6, (D7), D10, (E4), F3, F6; **46**B9, B13, C3, C7, C10, C12, (D5), D6 (or D8), E5, E7, E9, F3 (or F4), (F8), (F11); **47**A4 or A5, A6, A10, A15, (A16), (B6), B10, B14, C5, C6, (D3, D7, E3), E6, (F3), F5, F6, F10, F17; **48**A6, (B4), C7, (D7), (E8, E9), F4, F9, F10; **49**A6, (B4), (C4), D4, D5, F12; **50**(A6), A11, (A15), B4, (C5), D3, (D6), (E9)	Saura (1986)
2R51-60	**51**B3, C4, C7, D3, E8, E9, F5; **52**A5, A13-14, D6, E11, F4, F6, F8, F11; **53**C5, C8, E3, E9, F7; **54**C11, D6, E5; **55**F13; **56**D9, D10, D12, D15, E3, E6, F5; **57**B8, C4; **58**E5; **59**B3; **60**(A7, E7, E10, E12), F4	Saura *et al*. (1991)
3L61-63	**-**	Sorsa *et al*. (1984)
3L64-70	**64**B3, E3, E4, E10; **65**A13, F9; **66**A4, A16, D13; **68**C5; **69**C3, C11; **70**D6, E8	Saura *et al*. (1989)
3L71-73	**-**	Sorsa *et al*. 1987)
3L74-80A	**74**C4; **75**F8; **76**A7; **77**C5, D5, E5, F5; **78**B4	Saura *et al*. (1988)
3R81-90	**83**C9, D3, F3; **84**A6, F16; **85**A4, A10, C6, D10, D13, D20, D23, D26, F11, F14, **86**A3, A8, C9, C14, D3, D10, E3, E9, F5; **87**B3, B12, C4, D6, D8, D10, D13, E3, E7, F4, F6, F9, F11; **88**A3, A11, B4, B9, C5, C11, E5, E10, E13; **89**A10, A13, B5, B8; **90**A4, A7, D6, E7, F5, F8	Saura *et al*. (1994)
3R91-100	**91**A7; **92**A9, A12, B5, B10, C3, D3, D5, D6, D9, E5, E7, E15, F9, F12; **93**A3, A6, C6, D5, D10, E3, E10, F3, F8; **94**A5, A13, B7, B11, C6, D6, D9, E2, E5; **95**A3, A9, B4, C3, C6, C9, C14, E4, E8, F13; **96**A12, A16, A21, A25, B3, B12, B16, B18, D3, D6, E7, E9, E12, F7, F13; **97**A10, C2, C5, D10, D13, D15, E4, E8, F4, F7, F9; **98**A5, A8, A13, B4, D3, D6, F10; **99**B3, B6, C3	Saura *et al*. (1996)
4:101-102	**-**	Saura *et al*. (2002)

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A further study on the expansion of *Drosophila simulans* in Japan.

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Drosophila simulans, a sibling species closely related to *D. melanogaster*, is distributed throughout subtropical and temperate zones of the Northern and Southern Hemispheres in association with human habitation and agriculture. This species was apparently absent from Japan prior to its discovery in 1936 in the Bonin Islands, 1000 km south of Tokyo (Komai, l937; Okada, l956). Watanabe and Kawanishi (l976) first reported *D. simulans* individuals in 1975 in many places in Fukuoka (Kitakyushu), Ehime (Shikoku), Shizuoka (Pacific coast of Middle mainland). Watanabe and Kawanishi (l978) reported the expansion of *D. simulans* in l976 in the Kyushu district and the Kanto-Tokai district along the Pacific coast of Middle mainland, but not in the intervening areas. However, no *D. simulans* was found in the southwestern islands aside from a few individuals, probably temporarily introduced. In field surveys of domestic and semi-domestic species of *Drosophila* from l977 to l985, Watada *et al.* (l986) confirmed the abundance of *D. simulans* in the Kyushu and Kanto-Tokai districts. Newly established populations of *D. simulans* were also found in the western mainland, in the Kinki and Chugoku districts. Some isolated populations were observed in Sapporo and Akita, the northern parts of Japan, and some of the southwestern islands.

In an attempt to know how a newly immigrant species adapts to new environments and colonizes, we examined temporal changes in the distribution of *D. simulans*. Wild *Drosophila* flies were collected at 32 sites in Japan from 1993 to 2003. Here we report the *D. simulans* distribution in Japan for this period.

Flies were collected, within seven days after trap setting, using banana-bait traps put near human habitation. At vineyards, flies were collected by sweeping a net over pomace heaps. The collection seasons were restricted to the most preferable for *D. simulans* and *D. melanogaster* in each site: from summer to autumn in the mainland and from winter to spring in the southwestern islands. Collected flies were classified under a dissection microscope. The presence of a dark longitudinal stripe at the tip of egg-guide of *D. simulans* females is a reliable measure to distinguish them from those of *D. melanogaster* (Okada, l956). Males were distinguished by their genitalia.

A total of 32 collection sites throughout Japan were chosen for the present study (Figure 1). Collection data, combining males and females, are summarized in Table 1. *D. simulans* was found at all sites except in Toyotomi (site 1), the most northern part of Japan, and some islands in the

Table 1. Number of *D. melanogaster* and *D. simulans* collected.

Locality	Date	*D. melanogaster*	*D. simulans*
Hokkaido			
1. Toyotomi	Aug. 2000	26	0
Northern mainland			
2. Inakadate,	Aug. 2000	243	45
3. Tsuruta	Aug. 2000	78	33
4. Ohazama	Sep. 2000	155	1
5. Higashine	Sep. 2000	262	1
Middle mainland			
6. Obuse	Sep. 2000	22	399
7. Katsunuma	Oct. 1997	1,055	430
	Oct. 1999	484	126
8. Mishima	Oct. 1999	177	283
9. Fukui	Jul. 1999	60	4
10. Minogamo	Sep. 2001	96	101
Western mailand			
11. Aito	Sep. 2001	105	7
12. Kyoto	Oct. 2001	30	41
13. Kawachinagano	Oct. 1993	12	162
14. Tozugawa	Jul. 2000	71	68
15. Nishinomiya	Sep. 2001	42	36
16. Kobe	Aug. 1993	15	282
17. Tottori	Oct. 2001	61	174
18. Akasaka	Sep. 2001	93	409
19. Tokuyama	Sep. 2001	44	192
Shikoku			
20. Oe	Sep. 2001	28	425
21. Nankoku	Sep. 2001	85	138
Kyushu			
22. Shonai	Sep. 2001	6	399
23. Tanushimaru	Sep. 2001	27	95
24. Seihi	Sep. 2001	15	44
25. Kobayashi	Sep. 2001	7	128
South-west Islands			
26. Naga	Nov.1996	71	73
27. Kumejima	Apr. 2002	141	454
28. Miyakojima	Dec. 1999	61	8
29. Ishigakijima	Mar. 1998	20	0
30. Iriomotejima	Nov. 2001	896	1
	Apr. 2003	985	0
31. Harerumajima	Nov. 1998	174	0
32. Yonagunijima	Dec. 1998	199	0

Data from sites 26, 28, 29, 31, and 32 are from Watada and Itoh (1999).

southwestern islands (sites 29-32). The abundance of *D. simulans* in Kanto-Tokai (sites 7, 8, and 10), Chugoku (sites 17-19), Kinki (sites 12-16), Shikoku (site 20 and 21), and Kyushu (sites 22-25) districts was confirmed. Although isolated colonies were found in sites 2 and 3, *D. simulans* was very rare in the Tohoku district (sites 4 and 5). In the southwestern islands, new colonies of *D. simulans* were found in Nago (site 26), Kumejima (site 27) and Miyakojima (site 28). The distribution of *D. simulans* is more extensive than in the 1980s (Watada *et al*., l986), suggesting that the expansion of *D. simulans* has continued in Japan during the last 20 years. The speed of spreading, however, might be slowing down.

D. melanogaster were significantly few in some areas, for instance, in the Kyushu district (site 22 and 25) where the invasion of *D. simulans* was first reported (Watanabe and Kawanishi, l978). In such sites, *D. simulans* was almost predominant to *D. melanogaster*. Therefore, it is likely that the reduction of *D. melanogaster* population size is caused by the expansion of *D. simulans*. Similar cases of competitive exclusion of *D. melanogaster* were reported in Colombia and Egypt (Hoenigsberg, l968; Tantawy *et al.,* l970). *D. melanogaster* may be at the doorstep of eradication in some areas.

It has generally been thought that *D. simulans* and *D. melanogaster* have overlapping niches in nature, since they are very close sibling species and their distributions are largely overlapping in association with human habitation or activity. Nonetheless, some physiological and behavioral differences suggest that there may be microhabitat separation between the two species. *D. simulans* is less adapted to both cold and hot temperature stresses, and both high and low humidity than *D. melanogaster* (Parsons, l979, l983; but Kojima and Kimura, 2003). Some wild *Drosophila*, including

D. melanogaster, come indoors, but *D. simulans* does not (Dobzhansky, l965; Okada, 1971) because of its stronger light preference (Parsons, l975; Kawanishi and Watanabe, l978). What factors determine the success of *D. simulans* colonization in an area is the question to be solved.

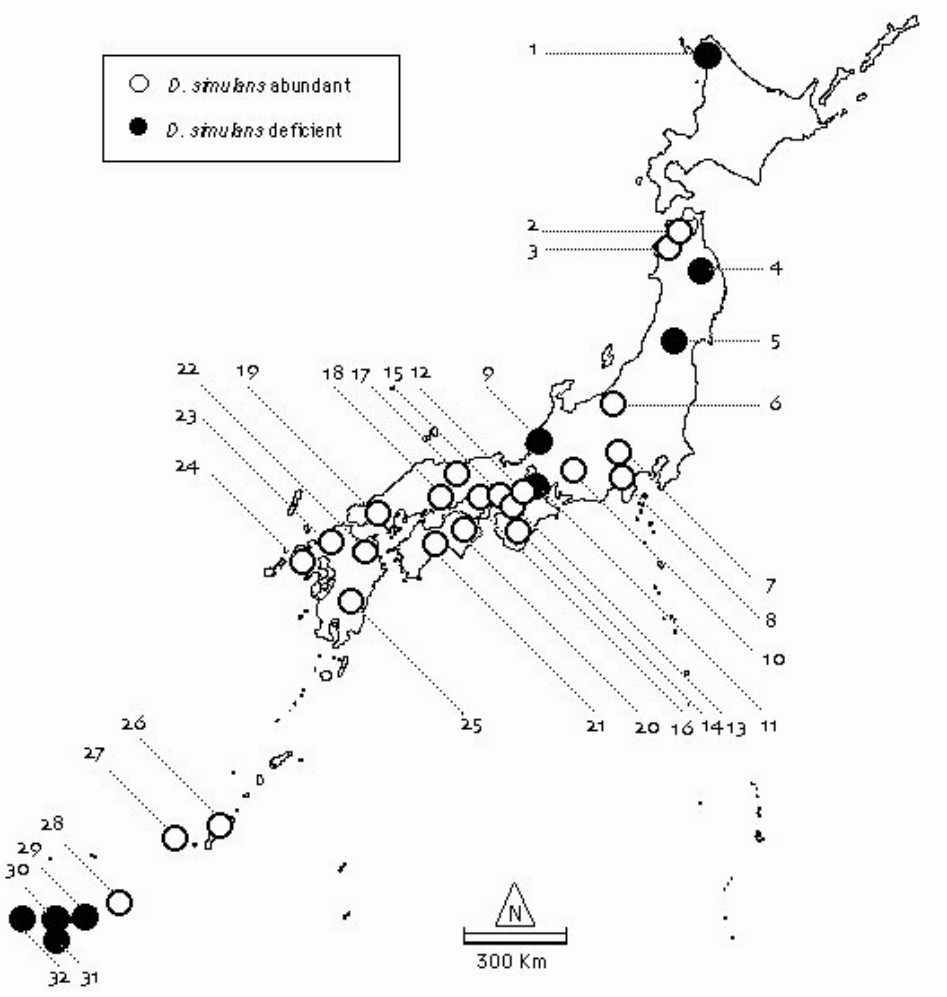

Figure 1. Geographical distribution of *Drosophila simulans* in Japan. White circles are *D. simulans*-abundant sites (the number of *D. simulans* was more than 10% of the total number of *D. simulans* and *D. melanogaster*) and black ones are *D. simulans*-deficient sites (the number of *D. simulans* was less than 10%). The number of each site is the same as in Table 1.

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Sterile female of *lozenge* of *Drosophila melanogaster* has activated eggs in ovary.

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Many alleles of the *lozenge* (*lz*) of *Drosophila melanogaster*, on the X chromosome (1-27.7), show a wide range of phenotype, namely, reduced eye size, abnormal parovaria, tarsal claws reduction, female sterility and so on (Lindsley and Zimm, 1992). The extent of abnormality is different by alleles. The *lozenge* gene controls eye development as a transcription factor (Daga *et al*., 1996). But its roles of other organs were not known.

We observed ovaries of homozygous flies for several alleles of *lz* to know the effect of *lz* gene on female fertility. The strains of *lz* were given from Bloomington *Drosophila* Stock Center, BL65 (lz^{34}), 66 (lz^{36}), 114 (lz^{y4}), 3613 (lz^{37}), 4069 (lz^{BS+46}), 4495 (lz^{48f}), and Tokyo Metropolitan University, lz^{85}. Flies were fed and maintained in nutritious condition at room temperature. Five- or six-day-old females of *lz* / *lz* or six- and eight-day-old Canton-S females were dissected in *Drosophila* ringer solution (Ephrussi and Beadle, 1936). We found activated eggs in ovaries of homozygous female for *lz*. We immersed these eggs into ten times ringer solution in order to check permeability of the membrane of eggs. Mature oocyte of *D. melanogaster* (Hastumi, *et al*., 2001) is more rehydrate than stage 14 oocyte described by King (1970) and looked more similar to activated. When they were immersed into ten times ringer solution, mature oocytes shrunk because of nature of semipermeable membranes, while the vitelline membrane of activated eggs were not permeable and shape of activated eggs did not change. By using ten times ringer solution we tried to confirm that the egg in the ovary of the *lz* female was activated. Table 1 shows the results. Canton-S females, control strain of wild type, and female homozygous for female fertile *lz* allele, lz^{77a7} / lz^{77a7} and lz^{85} / lz^{85}, had no activated eggs in their ovaries. Activated eggs were found in ovaries of females homozygous for sterile *lz* alleles. We immersed ovaries of sterile *lz* females into 50% commercial bleach which destroys unactivated oocytes. A few eggs were not destroyed after ovaries were soaked

Table 1. Number of females with activated eggs in ovary and mean number of activated eggs per female.

Genotype	Fertility	N*	NAE**	Mean***
lz^{34}/lz^{34}	Sterile	20	1	1.00
lz^{36}/lz_{36}	Sterile	20	2	2.00
lz^{y4}/lz^{y4}	Sterile	20	1	1.00
lz^{37}/lz^{37}	Sterile	20	2	1.00
lz^{Bs+46}/lz^{Bs+46}	Sterile	20	4	1.75
lz^{48f}/lz^{48f}	Sterile	20	5	1.80
lz^{77a7}/lz^{77a7}	Fertile	20	0	0
lz^{85}/lz^{85}	Fertile	20	0	0
CantonS 6-day-old	Fertile	20	0	0
CantonS 8-day-old	Fertile	20	0	0

N*: Number of females dissected. NAE**: Number of females with activated eggs in ovary. Mean***: Mean number of activated eggs per female.

in bleach. These results suggested that abnormal egg activation in ovary is related to sterility of *lz* / *lz* females. It was reported that storage of stage 14 oocytes is characteristic in females homozygous for lz^{34k} and King (1970) discussed that in this mutant the musculatures of the uteri and genital segments are unable to generate the peristaltic contractions required for oviposition. We confirmed eggs laid on medium by female homozygous for *lz* allele except one, lz^{BS+46} allele. Our results suggest sterile *lz* female can lay eggs, but activated eggs in ovary disturb ovulation frequently. It was suggested that the *lz* gene product controls the repression of egg activation in ovaries.

Mechanisms of egg activation at fertilization are not understood well. Our observation suggests that there are not only the mechanisms of egg activation at fertilization but also the mechanisms that disturb egg activation before fertilization when eggs are staying in the ovary and that both mechanisms are important for egg fertility.

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Cooperation of *net* and EGFR signalling in the wing vein pattern development in *Drosophila*.

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A great number of genes are involved in the development of wing veins pattern. Expression of those genes in the embryonic imaginal disc is specific both for the cells competent to the development into veins as well as for their neighbors developing into the wing cell regions. In *Drosophila melanogaster*, *net* (*net,* 2-0.0) is one of the major players in the development of wing veins pattern.

net mutations cause the development of vein nets and additional longitudinal segments in all wing cells except for the first behind cells (Figure 1B). Mutations differing in their phenotypical expression from the known *net*-alleles have been found in the natural populations of *D. melanogaster*. The phenotype of $net^{extra\text{-}analis}$ allele isolated from Belokurikha population in 2000 shows a single additional vein in the 3rd back wing cell near the center of a longitudinal anal vein (Figure 1C). Another non-typical net^{Ch86}allele from Chernobyl-1986 population causes the development of additional vein segments in the marginal, submarginal, 2nd and 3rd back wing cells. In addition, up to 10% of flies in net^{Ch86} stock have an additional vein segment in the 1st back wing cell, which is not typical for the previously described *net* mutations (Figure 1D). net^{ST91} mutation was discovered in Tashkent-2001 population of *D. simulans*, the closest relative of *D. melanogaster* species. Vein patterns in males and females of net^{ST91} stock is disturbed leading to the formation of a net and several additional vein segments in different wing sectors including the 1st back cell (Figure 1E). *net-extra-analis* is a recessive mutation while, net^{Ch86} and net^{ST91} are semi-dominant mutations as compared to the normal wild-type allele.

$net^{extra\text{-}analis}$ and net^{Ch86} alleles are characterized by a less strong disturbance of vein pattern as compared to the standard mutant allele $net^{2\text{-}45}$ and are dominant to that allele. net^{Ch86} allele is also

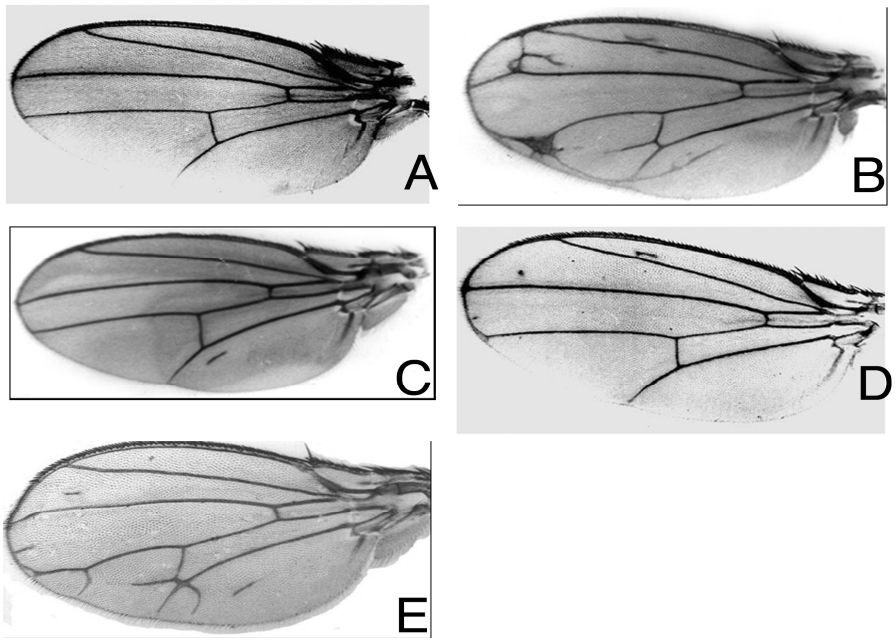

Figure 1. Phenotypical expression of different *net*-alleles in homozygote *D. melanogaster*: a - net^{+}; b - net^{2-45}; c- *net-extra-analis*; d - net^{Ch86}; *D. simulans*; e - net^{ST91}.

dominant in net^{Ch86} (*D. melanogaster)* / net^{ST91} *(D. simulans)* interspecies hybrids leading to the development of mutant veins pattern typical for net^{Ch86} homozygotes. However, in heterozygotes for different *net* alleles and *Df(2)* net^{62} deletion – namely, in net^{Ch86} / *Df(2)* net^{62}, net^{2-45}/ *Df(2)* net^{62} and net^{ST91} / *Df(2)*net^{62} flies – the strongly disturbed veins pattern in the form of net and vein segments develops in the wings, including an additional vein in the 1st back wing cell.

EGFR signalling pathway plays a major role in the wing veins pattern development in *Drosophila*. Veins pattern develops in response to the local EGFR signals. The extracellular domain of the TGF-α-family transmembrane protein encoded by *spitz* (*spi*, 2-54.0) gene can, possibly, work as the EGFR ligand. Activation of the ligand is regulated by the membrane proteins. Thus, Star controls the intracellular migration of Spitz, while Rhomboid – its proteolysis and activation (Lee *et al.,* 2001; Gallio *et al.,* 2002; Klambt, 2002). The two isoforms of Pnt transcription factors are encoded by *pointed* (*pnt*, 3-79.0) gene. Likewise the other tyrosine kinase receptors, EGFR activates Ras/MAP-kinase phosphorylation cascade. The kinases change the activity of Pnt P2 transcription factor (Kuo *et al.,* 1996).

net gene encodes a polypeptide containing bHLH-domain, and it is classified as the transcription repression factor. EGFR and Net act as antagonists in the wing cells specialization. The role of Net protein in wing veins pattern development is to control the development of the wing cells other the veins cells by means of the EGFR pathway genes repression, mainly, *rhomboid* (*rho,* 3-0.2) gene. Mutations leading to the lack of *net* function cause the expanded *rho* expression in all wing cells except for C sector (1st back cell between L3 and L4 veins), disturb the correct specification of the wing disc cells, and initiate the development of additional non-typical veins. In turn, *net* transcription in presumptive veins cells is suppressed by the Rho-dependent EGFR cascade (Brentrup *et al.,* 2000). Rho and Net expression is, therefore, observed in the different regions of the wing disc, namely, in its primordial veins and sectors (Figure 2).

The phenotypical differences of our natural *net*-alleles can be, possibly, explained by different molecular defects of *net* gene and differential expression of Net protein. Natural alleles $net^{extra-analis}$, net^{Ch86}, and net^{ST91} can, possibly, differentially control the competence of the cells to blocking of EGRF pathway signal through the *rho* repression in different inter-veining sectors of imaginal wing disc.

There are the other genes working in the imaginal wing disc of *D. melanogaster* and allowing the development of sectors, blocking the development of veins and, possibly, repressing *rho*, such as

blistered (*bs*, 2-107.3), *daughterless* (*da*, 2-39.3), *knot* (*kn*, 2-72.3*)*, *plexus* (Fristrom *et al.,* 1994; Brentrup *et al.,* 2000). Phenotypical expression of the different mutants can depend on the activity of those genes in different wing sectors as well.

The role of EGFR pathway in ontogenesis considerably depends on its interactions with the other signalling systems, for example, the Notch cascade. The coordinated interaction of signalling systems lays the foundation for the coordinated regulation of genome functioning in live organisms. Thus, the cooperation of *net* and EGFR in the wing veins pattern development in *Drosophila* is an important link in a complicated chain of events.

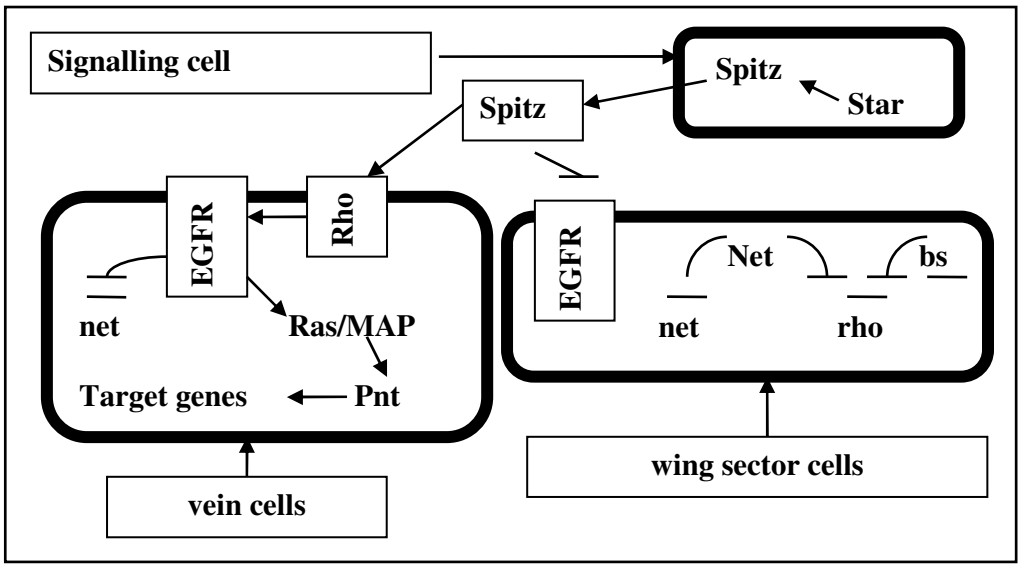

Figure 2. Antagonism between Net and EGFR pathway proteins.

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Movement patterns on two substrates of Chilean third instar larvae of *D. melanogaster*, *D. hydei* and *D. pavani* collected in the same orchard.

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Animal patterns of movement are related with dispersal and distribution of these organisms in space. They are also associated with genetic flow between animal populations. The patterns of

movement also determine what parts of the habitat are accessible to animals (Irschick and Garland, 2001). More specifically, the patterns of movement are involved in, for example, habitat selection, searching for conspecifics, feeding strategies, and avoidance of predators and/or parasites. Thus, the way that an animal moves in its environment affects its fitness. In the case of *Drosophila* species that breed naturally in the same fruits in the wild, it is of importance to relate larval patterns of movement with dispersal and searching for food and pupation sites. This type of investigation could help us to understand the role of larval behavior in the ecology of *Drosophila*, particularly with respect to those species that coexist in the same breeding sites sharing resources as food and space. Here we studied patterns of movement of third instar larvae of *Drosophila melanogaster*, *Drosophila hydei* and *Drosophila pavani* while searching for pupation sites. The goal is to investigate the role of physical features of substrates on that larval behavior to understand how do *Drosophila* larvae used their patterns of movement when select pupation sites. The strains used were founded with parents collected in an orchard in Chillán (410 km South from Santiago).

Two types of substrates were used to perform the experiments: i) agar (humid, smooth surface substrate), and ii) sand (dry, rough surface substrate). A set of 120 petri dishes were each filled up to a deep of 2 cm with 3% agar. Once cooled, a half agar of the petri dishes was removed along one side leaving a dry surface. Then this part was filled with sand forming a 2 cm thick layer. Each larvae tested was deposited on the center of the corresponding petri dish on the limit between the two substrates. The trail made by each of the larvae on the substrates was drawn for a total period of 2 min or until they dug disappearing into the sand. A Wild M5 camera lucida was used to drawn the trails. Each one of the larvae tested was observed in a "virgin" petri dish. Number of third instar larvae tested per strain and species was 30.

Locomotion and turns are the principal behavioral elements which define a pattern of movement in *Drosophila* larvae (Sokolowsky, 1980). The locomotion was estimated by measuring the length of the trail made on the agar and sand (Sokolowski *op.cit.*). Turning behavior was recorded by counting number of changes in direction in the trails. To have an additional estimation of turning behavior, we also measured size of turns by tracing tangent lines where the trails showed changes in direction. The angle size formed by two adjacent tangents cutting in a point was another estimation of larval turning behavior.

Third instar larvae of the *Drosophila* species tested crawl and wander on the substrates exploring environment searching for pupation sites. The larvae spent most of the observation time on the agar: i) *D. melanogaster*, 58.33%, ii) *D. hydei*, 70.83%, and iii) *D. pavani*, 67.92%. These differences are statistically important (the 2 × 2 G-test of independence produced values that exceeded the critical value, $\chi^2 = 3.84$, $df = 1$). Larvae that crawl and wander on agar may touch the sand in the limit between the two substrates. Some of them carry on crawling on agar, while other may cross towards the sand. The mean number of these type of contacts was about one per larva in the three species. The number of larvae of each species that crossed towards the sand was: i) *D. melanogaster* 21 out of 30 (70.00%), ii) *D. hydei*, 10 out of 30 (33.33%), and iii) *D. pavani*, 12 out of 30 (40.00%). Once on the sand, the larvae move and start to dig into the substrate. One hundred percent of the *melanogaster* larvae pupated underneath the sand, about 70 % of *hydei* larvae also dug into the substrate, while no more of 10% of *pavani* larvae formed puparia underneath the sand. Thus, substantial differences in digging and preferences for pupation substrate were found between the three species collected in the same orchard.

Table 1 shows the locomotion and turning behavior of third instar larvae of *D. melanogaster*, *D. hydei* and *D. pavani* (the Chillán populations). On agar, *D. hydei* larvae show higher locomotion, while those of *D. pavani* the lowest. The differences in larval locomotion between the three species are statistically important (*t*.test: i) *D. melanogaster* versus *D.hydei*, $t = 5.15$, $df = 58$, $P < 0.05$, ii) *D.*

melanogaster versus *D. pavani*, $t = 5.05$, $df = 58$, $P < 0.05$, iii) *D. hydei* versus *D. melanogaster*, $t = 6.78$, $df = 58$, $P < 0.05$). On the sand, third instar larvae of the three species substantially decrease their locomotion (Table 1). In contrast with agar, on the sand, *D. melanogaster* larvae show higher locomotion, while *D hydei* the lowest. On the other hand, *D. pavani* larvae exhibit a locomotion intermediate respect to those of the other two species (Table 1). These interspecific differences in larval locomotion are statistically important (*t*-test).

Table 1. Locomotion and turning behavior of third instar larvae of *D. melanogaster*, *D. hydei* and *D. pavani*. The behaviors were recorded while larvae crawling on agar and dry sand. The observation time was two minutes. Locomotion was estimated measuring length of the trail made on each of the substrates. Number and size of changes in direction were also recorded.

Species	Locomotion (cm)		Number of turns		Size of turns (angles)	
	agar	sand	agar	sand	agar	sand
D. *melanogaster*	9.70 ± 1.25	0.73 ± 0.19	6.30 ± 4.07	0.73 ± 0.18	125.00 ± 2.04	92.98 ± 43.90
D. hydei	18.30 ± 1.94	0.22 ± 0.05	9.80 ± 0.93	0.17 ± 0.10	137.50 ± 2.31	117.10 ± 1.73
D. pavani	6.30 ± 1.30	0.44 ± 0.15	2.80 ± 0.36	0.20 ± 0.17	103.40 ± 4.70	157.17 ± 1.73

Table 1 also shows that, on agar, third instar larvae of the three species differ in turning behavior. Thus, *D. hydei* larvae exhibit the higher number of turns which are also wider than those turns made by larvae of *D. melanogaster* and *D. pavani*. The *t* values for number of turns on agar were: i) *D. melanogaster* versus *D. hydei*, $t = 8.98$, $df = 483$, $P < 0.05$, ii) *D. melanogaster* versus *D. pavani*, $t = 14.76$, $df = 272$, $P < 0.05$, iii) *D. hydei* versus *D. pavani*, $t = 26.78$, $df = 478$, $P < 0.05$). Similar results were obtained for size of the turns made by the larvae of the three species. Interestingly, by comparison with turning behavior exhibited on agar, on the sand, third instar larvae of *D. melanogaster* and *D. hydei* decrease amplitude of the turns. By contrast, in that same substrate, *D. pavani* larvae made turns wider than on the agar (Table 1). Interspecific differences in size of turns made on the sand are also statistically important (t-test).

The present results suggest how third instar larvae of *D. melanogaster*, *D. hydei* and *D. pavani* use their patterns of movement when they search for and select sites where form puparia. Our findings should also help to understand the behavioral bases of aggregation patterns and density of pupae found in microhabitats that surround overripe fruits fallen on the ground. These fruits are used as breeding sites by several Chilean *Drosophila* species. Thus, in Chile, *Drosophila* females oviposit on fruits as cherries (*Prunus avium*), plums (*Prunus domestica*) and mulberries (*Morus alba*) (Brncic, 1987). At the end of larval period, most of *D. melanogaster, D. hydei* and *D. pavani* third instar larvae leave their breeding sites forming, each species, pupae aggregations away from those of the other species (Medina and Godoy-Herrera, 2003). Some of these preadults also dig and pupate underneath substrates that surrounding their natural breeding sites.

Agar and sand substrates differ in their structure varying in their resistance to larval movement. As a consequence, larvae of the three species here studied travel at different rates and over different distances depending on that type of substrate they are moving. For example, agar, which shows a humid and smooth surface, seems to favor larval movements. By contrast, on a dry substrate that has rough surface as occur with the sand, larval movement decrease. However, it is interesting to note that, on the sand, *D. pavani* larvae increase the size of their changes in direction suggesting that for this local *Drosophila* species this substrate could be ecologically less restrictive for dispersal activities than for *D. melanogaster* and *D. hydei* third instar larvae. The larval patterns of movement here studied could be a part of the process by which *Drosophila* larvae evaluate the

relative quality of the microhabitas surrounding their breeding sites in the wild, before selecting and form puparia in one of them.

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References: Brncic, D., 1987, Medio Ambiente 8(2): 3-9; Irschick, D.J., and T. Jr. Garland 2001, Ann. Rev. Ecol. Syst. 32: 367-396; Medina, M.C., and R. Godoy-Herrera 2003, Behav. Ecol. (submitted); Sokolowski, M.B., 1980, Behav. Genet. 10: 291-302.

Guide to Authors

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