Variation of the male specific wing pigment in the natural populations of *Drosophila rajasekari.*

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In *Drosophila* abdominal, wing and thoracic pigmentations are sexually dimorphic characters and are often variable both in laboratory and natural populations (Robertson *et al*., 1977; Gibert *et al.*, 1996). Fuyame (1979) has shown that the wing pigmentation in *D. suzukii* acts as a visual stimulus in courtship, while according to Gibert *et al*. (1996) body colouration of *Drosophila* has adaptive significance in natural populations. Unlike abdominal and thoracic pigmentations, not much is known about wing pigments, although *D. suzukii, D. prostipennis, D. pulchrella, D. elegance and D. silvestris* (picture winged group) possess species specific apical wing pigments. Males of *D. rajasekari* a synonym of *D. biarmipes* belonging to the *suzukii* subgroup of the *melanogaster* species group also possess such apical wing pigment, which extends from the apical margin of the wing to the 3rd longitudinal vein. In the present study variation of male specific apical wing pigment in geographical populations of *D. rajasekari* is investigated.

Drosophila flies were collected from five different places of Karnataka namely, Bangalore, Mysore, Belagola, Nagamangala, and Shimoga. Flies from these areas were collected by placing mixed fermented fruits in the form of small lumps in 5 different spots in each of the above wild localities. After 24 hours flies were collected by net-sweeping method and transferred to bottles containing wheat cream agar medium. In the laboratory, collected flies were isolated, categorized, sexed, and the females thus obtained were placed in individual vials to build up isofemale lines. The males obtained from isofemale lines were used to identify the species of their mothers. The number of males and females of *D. rajasekari* were recorded (Table 1). *D. rajasekari* males collected from the wild localities in the above five geographical areas and fifty laboratory bred males of the same species were used for measuring the area of apical wing pigment. Both right and left wings of each individual was removed at the costal region of the wing base and mounted horizontally on a glass slide. The total area of the wing pigment was measured using an ocular micrometer calibrated to a stage micrometer using microscope with a magnification of 10×. The irregular area of pigment was determined by geometric angle of trapezium for the wing pigment measurement. The values were calculated using formula, Area = ½ ht, where 'h' is the height and 't' is the sum of parallel sides. The values were summed to obtain a single measure of area, which was then converted in to mm^2. The data were subjected to analysis of variance followed by DMRT.

In the present study, the polymorphism of wing pigmentation was observed among different geographic populations. The mean pigment area of the right wing of different populations of *D. rajasekari* varied from 0.2501 ± 0.0054 to 0.2760 ± 0.0055 while the area of left wing varied with the mean value from 0.2497 ± 0.0051 to 0.2686 ± 0.0039. The differences between the populations were significant by ANOVA. It was observed that the intensity of coloration of wild males also varied compared to laboratory cultured males. Table 1 also shows that in nature all the males possess the wing pigments while in laboratory some males have no wing pigment. Among the populations studied, Mysore and Shimoga are situated away from Bangalore. The mean wing pigment area in these two populations is lesser than others. Thus it is evident that the variation is in correspondence with geographic distance.

Table 1. Total area of wing pigment in different natural populations of *D.rajasekari.*

Population	Total males collected		Total females collected	Mean area of wing pigment ($mm^2 \pm SE$)	
	Males with pigment	Males without pigment		Right wing	Left wing
Bangalore	80	0	49	0.2760 ± 0.0053^{c}	0.2686 ± 0.0039^{b}
Mysore	60	0	45	0.2501 ± 0.0054^{a}	0.2497 ± 0.0051^{a}
Belagola	72	0	30	0.2601 ± 0.005^{abc}	0.2678 ± 0.0073^{b}
Nagamangala	58	0	32	0.2702 ± 0.0055^{bc}	0.2638 ± 0.0046^{ab}
Shimoga	75	0	40	0.2564 ± 0.0055^{ab}	0.2509 ± 0.0055^{a}
Laboratory	45	5	-	0.2678 ± 0.0049^{bc}	0.2664 ± 0.0057^{ab}

F-values by ANOVA between different populations, Right wing =3.171,left wing =2.392, df = 5, 294. P<0.05
a,b,c; The populations with the same alphabet as superscript are not significantly different at 5% level according to DMRT.

The pigment traits among insects are often polygenic. True *et al.* (2002) suggested that in *D. melanogaster* several genes are known to be necessary for cuticular melanization. He has also demonstrated that yellow and ebony genes are complementary for black melanin pattern in *D. biarmipes* wings. Gompel *et al*. (2005) have shown that the regulatory region of the yellow gene which binds unknown transcription factors is responsible for wing pigment expression. The significant difference in wing pigment in the geographical populations may be due to difference in environmental conditions of different geographic areas. Thus like other morphometric traits wing pigment also exhibits polymorphism in natural populations.

Acknowledgment: The facilities provided by Chairman of the Department of Zoology, University of Mysore are gratefully acknowledged.

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Stability of sex ratio at mutagenesis in *Drosophila melanogaster.*

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The sex ratio (**SR**) is the ratio of the number of females to that of males. In *D. melanogaster*, when parents of one sex are irradiated, F_1 females and males obtain through heterosomes, unlike autosomes, different numbers of induced dominant lethal mutations (**DLM**), due to which different survivals of sexes and a shift of ST are expected. When recording the crosses, we shall designate the irradiated chromosomes with bold letters and write the female first, *i.e.*, on the left. When irradiating

P_1 males, *i.e.*, in the cross XX × **XY**, the **X** spermia that form females bring more radiation-induced damage to the zygote than the **Y** spermia that form males do, because the Y chromosome of *D. melanogaster* is completely heterochromatic, so that even its complete loss does not influence the viability of the zygote. Therefore it is expected that in the F_1 the survival of females will be lower than that of males, and the SR will be lower than in the control (without irradiation): $r_1 < r_0$. See Figure 1 (crossing 1).

When P_1 females are irradiated, the cross **XX** × XY produces daughters **X**X and sons **XY.** The number of DLM obtained through the irradiated **X** chromosome is the same in females and in males, but recessive lethals (RLM) induced in it turn out to be in hemizygote in males and behave like DLM. That is why males die more than females and the SR is expected to be higher than in the control: $r_2 > r_0$. See Figure 1 (crossing 2).

In this way, theoretically irradiation of males must be lower, and irradiation of females, on the contrary, increase the SR in their F_1 progeny: $r_1 < r_0 < r_2$ (Figure 1).

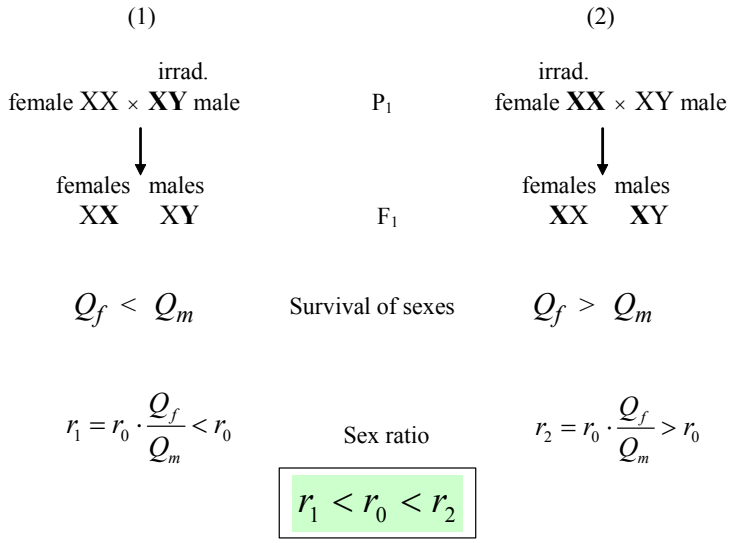

Figure1. Transmission of irradiated sex chromosomes to F_1 females and males when P_1 males (crossing 1) and females (crossing 2) were irradiated and the expected sex ratio in their F_1 progeny (r_1 and r_2, respectively) versus the control without irradiation (r_0). The irradiated chromosomes are designated by bold letters. Q_f and Q_m are survival of F_1 females and males, respectively.

Hower, in fact the SR does not change in the expected direction even at considerable radiation doses, and its shift does not correlate with the dose, is not constant with respect to the sign and is very small with respect to the module. In all the more than 10 experiments, the expected SR shift was practically absent despite the gamma-ray doses of up to 15000 R and the effect of EMS which was equivalent to the dose of about 19500 R. Examples for comparison of SR under irradiation and in the control without irradiation are presented in Table 1. For demonstration we limited ourselves to only two experiments carried out on the highest irradiation doses in which the stability of SR was observed in a very clear-cut and significant form. Formulae for the expected SR r_E were taken from our recent work (Ivanov, 2003) where it was demonstrated how they were deduced.

The considerable deviation of data from the theory seemed to be a curiosity and a defect of the experiment, but it was confirmed and could even be accounted for in the experiments with the strain XXY × XY (attached X chromosomes). Its remarkable peculiarity consists in the fact that X spermia produce males, and Y spermia produce females. That is why irradiation of males (cross XXY × **XY**) more eliminates sons than daughters, and the SR is expected to be higher than in the control: $r_1 > r_0$. See Figure 2 (crossing 1).

Table 1. Sex ratio in the F_1 progeny of wild type strain Canton-S (+) in irradiation of P_1 parents of one sex and without irradiation (control).

Conditions of experiment	Sex ratio r		n	χ^2 as the criterion of concordance ($H: r_i = r_E$)	χ^2 as the criterion of homogeneity ($H: r_i = r_0$)
Irradiation of P_1 Males $D = 10000$ P	Expected r_E*	0.718 ± 0.018	-	74.1	-
	Factual r_1	1.127 ± 0.060	1438	-	1.234
	Control r_0	1.055 ± 0.026	6402		
Irradiation of P_1 Females $D = 15000$ P	Expected r_E**	1.390 ± 0.031	-	108.6	-
	Factual r_2	1.027 ± 0.030	4722	-	0.0016
	Control r_0	1.030 ± 0.023	7930		

* $r_E = r_1(D) = 0.997 \exp(-0.0000382D) \cdot r_0$

** $r_E = r_2(D) = 0.997 \exp(0.0000202D) \cdot r_0$

Because of large values of the concordance criterion χ^2 at $df = 1$, the zero hypothesis $H: r_i = r_E$ ($i = 1; 2$) in the two experiments is rejected as incredible in favor of its alternative $\overline{H}: r_i \neq r_E$ at the level of doubtfulness $\alpha = 0.001$.

Because of small values of the homogeneity criterion χ^2 at $df = 1$, the zero hypothesis $H: r_i = r_0$ in the two experiments is not rejected.

When females are irradiated (cross **XX**Y × XY), the chromosomal compound **XX** with all its lethal damage is transmitted to daughters, and the harmless **Y** chromosome is so to sons, preferential death must happen to females, and a decrease of SR in F_1 is expected: $r_2 < r_0$. See Figure 2 (crossing 2).

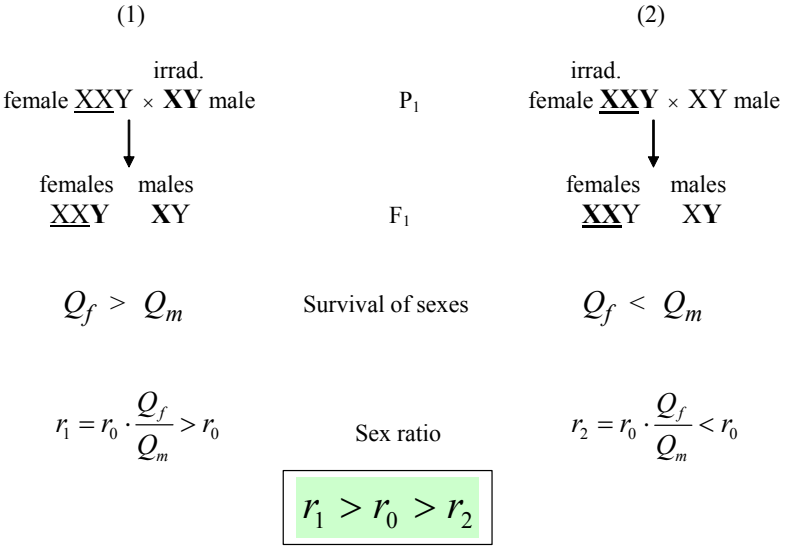

Figure 2. Transmission of irradiated sex chromosomes to F_1 females and males in the strain XXY × XY when P_1 males (crossing 1) and females (crossing 2) were irradiated and the expected sex ratio in their F_1 progeny (r_1 and r_2, respectively) versus the control without irradiation (r_0). The irradiated chromosomes are designated by bold letters. Q_f and Q_m are survival of F_1 females and males, respectively.

In this way, in the strain XXY × XY it is expected that irradiation of males will increase and irradiation of females decrease the SR in F_1: $r_1 > r_0 > r_2$ (Figure 2). However, here too SR shifts were not large, and although they were larger than in the normal strain, they were quite different than it follows from the theory. The phenomenon of SR conservation about the control level in spite of the effect of strong disturbing factors (in our case, mutagenesis) was called SR homeostasis. The factors that make SR closer to the normal level, despite the deviating influences of mutagenesis, astonish one by the fact that SR and even the frequency of each gender are abstract quantities so that it is impossible to imagine how they can be the moving cause for regulatory factors unless one ascribes a certain intelligence. How on earth is SR regulated and what are the causes of its homeostasis?

Let us consider the cross (1) in Figure 2 where the irradiation did not produce any effect, so that SR $r_1 \approx r_0$ was observed. Here, an increased mortality of **XY** males as compared with XXY females is beyond any doubt, because in the cross M5 × **XY,** according to measurements by Muller-5 method, the mutability (frequency of RLM incidence) in **X** spermia at the used irradiation doses is high, and it cannot decrease due to replacement of M5 females by XXY females. This means that in F_1 the mortality of males is really higher than that of females. For conservation of the same proportion of males as that in the control ($r_1 \approx r_0$), one has to hypothesize an excessive mortality in the females, additional to that caused by irradiation, *i.e.*, death caused not by radiation-induced genetic factors, but by regulatory ones - death for the sake of conservation of SR norm. It seems that something similar to apoptosis at the organismal level happens, when it is not tissue cells that eliminate themselves, but individuals, zygotes that had female sex possibly still at one cell stage before the beginning of cleavage.

In all the cases when in F_1 prevalence of zygotes of certain sex is expected, their partial and adequate self-elimination occurs, and it is impossible to give a different interpretation of SR homeostasis. If one admits a sex redetermination in a part of zygotes of the excess sex, then the impossibility thereof becomes clear from the fact that the sex is determined by the heterosomal contents of the zygote, which cannot be changed during the ontogenesis. Environmental sex determination without a change of heterosome composition would have been confirmed by the presence of XY females and XX males, which is not the case.

This interpretation of SR homeostasis has a teleological character, for it resorts to a final goal (*causa finalis*) as the cause of the phenomenon. Allocentric death of the excess sex seems to occur in order to conserve the SR at the level of r_0 which is normal for the given conditions. We fail to find an efficient cause (*causa efficiens*) sufficient for SR homeostasis and it seems that it does not exist. That is why the SR stability seems to be considered as a fundamental law of nature and accepted as a basic datum without any explanation, similarly to the law of universal gravitation, interaction of magnetic or electric charges, etc. Let us explain the situation by means of the well-known example of gravitation. The Kepler's laws of planet movement are not basic laws of nature, for they are deduced from gravitation as consequences. It is for them the efficient cause and has no efficient cause of its own, but only the final cause, and for this reason is considered to be a basic law of nature. Believers in mechanistic conception of the world try in vain to interpret the gravitation not as a basic law, but to find efficient causes for it. They believe that the understanding of the universe can be reduced to a few, and ideally one, basic principle; however, the experience of natural sciences, especially quantum mechanics, demonstrates that there are a lot of basic principles (Peierls, 1957). That is why a complete clarity is unattainable. Truth is always pregnant with vagueness, and a complete clarity points unerringly to departure from the truth either due to ignoring some inconvenient facts, or due to idealization whose extreme degree is vulgarization. That is why the absence of efficient causes in SR homeostasis is nothing special and must not be very astonishing.

Homeostasis is a rather conventional name for the phenomenon described. If the SR deviated from the normal value had already been realized in the population and some of its consequences had been able to influence it in the opposite direction stabilizing its level near the normal, such a regulation of SR would have meant the existence of a mechanism that had to be called homeostat, and its regulation would have been called homeostasis of SR. But in reality, still prior to realization there is a virtual SR of whose existence we judge by r_0 and which is achieved due to an adequate death of zygotes of the excess sex as if they had known. According to Bohr, the truth and the clarity are mutually supplementing, so that proximity to the in what numbers they need die for normalization of SR. It seems that here no mechanism can exist similarly to its absence in gravitation and in other basic laws of nature.

A definition of homeostasis can be given through homeostat. Homeostasis is the limitation of variability of some parameter of the system by preset limits by means of a regulatory mechanism - homeostat. If one uses this definition, then homeostasis is ensured by the existence of the homeostat, and if the latter is absent, like in SR in the case discussed here, then stability is preset without any mechanism, directly by the law of nature, and then this stability should not be referred to as homeostasis, like the basic laws of preservation of matter, energy and impulse are not referred to. It is just for this reason that in the title of the present paper we preferred to use the term *stability* and not *homeostasis*. However, before renouncing completely the term *homeostasis* in application to SR stability, it seems expedient to wait for further clarification of the situation. Although there is little hope, yet it is not ruled out that there exists nevertheless a homeostat for SR.

SR homeostasis serves as an indication of an important role of SR in the regulation of numbers of a species, *i.e*. in the ecosystem. It is remarkable that SR in *D. melanogaster* close to unit is strongly deviated from the optimal one, responding to the maximum of Darwinian fitness. One male can fertilize many females, but a female usually does not mate more than 1 - 2 times in her life. Therefore, advantageous would be a SR larger than unit by several times, *i.e*., a great excess of females over males. But in fact, there is a strong excess of males over their theoretically necessary number, which is a squander from the point of view of adaptation, and this phenomenon cannot be considered otherwise than an allocentric property of the species. When the population is numerous, SR decreases, and when it is scarce, SR increases (Grechany and Pogodaeva, 1996), which indicates its regulatory role, since reproduction in a population is an increasing function of SR.

The cognitive significance of the SR homeostasis consists also in the fact that in it a clearly demonstrated phenomenon is discovered which is determined not by genetic, but by external, environmental, ecological factors and occurs despite the genetic determination of SR. Here, we have another confirmation of the fact that in the study of the phenomenon of life the priority role belongs to ecology and not to genetics as anyone usually thinks at present. Life is based on biotic circulation, and it does not depend on the genetic structure of individuals and species, but is created by the properties of the planet as a whole. Species are transient, and the circulation is continuously renewed and supported by the conservation of CO_2 (Ronov, 1978) and O_2 (Bgatov, 1982) concentration in the atmosphere due to effusion vulcanism, the CO_2 ensuring the photosynthesis, or production, and O_2 ensuring respiration, or reduction of organic matter in the circulation.

Conclusions

When parents of one sex are irradiated, the sex ratio (SR) in their immediate progeny does not change, or changes considerably less than it is expected according to the sex difference by the number of lethal mutations obtained through heterosomes. The SR stability phenomenon is referred to as SR homeostasis.

However, stability of SR is a necessary but insufficient condition of SR homeostasis. For homeostasis, still a homeostatic mechanism (homeostat) is necessary. But if the latter is not available, then the stability of SR is not a homeostasis in the general sense, but a primary, fundamental law of nature.

SR homeostasis is achieved here by the fact that the sex that suffers less from the lethals induced by the irradiation of the parents, *i.e*., that available in excess, is self-eliminated and dies to the level at which SR is restored that is normal for the given conditions without irradiation.

The final cause, or goal, of the additional mortality of the excess sex is maintenance of SR at the normal level. Immediate, or efficient, causes are not known and require investigation. They do not exist, as it seems probable, in general, and then the SR homeostasis belongs to fundamental laws of nature.

SR changes in the fruit fly in nature demonstrate that SR serves as one of factors of regulation of the population size; that is why an important role of SR homeostasis in the ecosystem seems undoubted, although its concrete significance is still to be clarified. SR determination by external factors occurs despite the action of genetic factors and independently of them.

Acknowledgment: The author is sincerely grateful to Dr. V.G. Kolpakov for his help in translating the paper into English, and Dr. D.A. Afonnikov for his consistent help in preparation of the manuscript for publication.

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Flies collected in orchards.

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For the fly collection in fruit plantations in five counties of the State of São Paulo, Brazil (Figure 1), a standard wooden box (30 × 40 × 4 cm) was used. Its inner part was coated with a plastic sheet, over which fruits of the plantation were placed, in a way to fill all its interior. During 20 minutes, the flies were captured with an entomological net. Seventeen collections were carried out (Table 1 and Figure 2), with 16, 225 captured flies and 23 identified species. The only exception to this procedure took place in Valinhos, where the collections were carried out directly in the fig's infructescence, fixed in the plant and in the disposable material placed in deposits in the ground (holes) with approximately the same surface of the bait box.

The most frequent species were: *Zaprionus indianus*, *Drosophila simulans*, *D. latifasciaeformis*, *D. paranaensis*, "willistoni" group, and *D. melanogaster* (Table 1). This last species, although being the sixth most common one, presented individuals in just two locations, Olimpia and Jaboticabal, where the baits were close to houses (50 m), confirming that this species is not common in orchards, but common in synantropic locations (Bélo, 2000; Bélo and Gallo, 1977; Mourão, 1966; Pavan, 1959). The species frequencies and relationships (Table 1) show that the collections were harmed during the dry season (April to September). In winter (July to September),

in the avocado plantation in the FCAV-UNESP Campus, in Jaboticabal, and on 08/17/01, in the orange plantation in Olimpia, on 07/19/01, in the guava plantation in Monte Alto, the number of captured flies was small, due to the conditions of low moisture and temperature, associated with the presence of winds in August. In the rain season, in Olimpia (12/22/01), 2,535 flies were captured.

Table 1. Species and number of captured flies in the orchards.

Species	Jaboticabal		Olimpia		Jaboticabal		Monte Alto		Valinhos		Brotas			Total
	Avocado		Orange		Mango		Guava		Fig	Guava	Peach	Guava	Plum	
	06/14/00	08/09/00	08/17/00	12/22/01	10/03/00 02/14/01	11/22/01	04/17/01	07/19/01	04/24/01	08/14/01	12/12/01			
Anastrepha sp.	-	1	-	-	2	-	-	-	-	-	-	-	2	5
Ceratitis capitata	-	-	-	-	-	1	-	-	-	-	-	-	-	1
Crysomya putoria	-	-	-	-	2	-	-	-	-	-	-	-	-	2
Hippelates sp.	-	1	-	-	-	-	-	-	-	-	-	-	10	11
D. cardini	4	9	-	5	21	2	10	-	-	-	-	10	24	85
D. maculifrons	17	-	-	-	30	3	-	-	-	-	-	-	-	50
D. immigrans	17	25	-	5	70	13	4	-	-	-	6	-	-	140
D. latifasciaeformis	25	3	4	46	402	144	144	-	495	-	70	13	64	1410
D. mediostriata	20	-	-	-	19	2	-	-	-	-	6	2	1	50
D. melanogaster	-	-	22	24	194	-	-	-	-	-	-	-	-	240
D. hydei	-	-	-	-	-	1	-	-	-	-	-	-	-	1
D. mirassolensis	1	-	-	-	-	-	-	-	-	-	-	-	-	1
D. nebulosa	-	-	-	25	48	9	2	6	1	-	1	5	1	98
D. paranaensis	2	10	3	696	210	91	4	-	3	2	-	4	23	1048
D. polymorpha	-	-	-	-	7	-	10	1	-	-	22	1	12	53
D. simulans	6	18	70	70	3584	1442	7	116	13	589	102	58	130	6205
D. sturtevanti	-	-	-	4	104	3	-	-	10	-	3	-	-	124
Drosophila sp.	-	-	-	1	-	-	-	-	-	-	-	-	1	2
Drosophilidae 1	-	4	-	-	-	1	2	1	-	-	-	-	6	14
Drosophilidae 2	-	-	-	-	-	9	-	-	-	1	-	-	2	12
Grupo willistoni	4	-	-	12	153	51	3	-	7	-	7	59	-	296
Megaselia scalaris	-	2	-	-	-	-	-	1	-	-	-	-	-	3
Zaprionus indianus	2	4	1	1647	1853	724	260	29	414	208	89	128	1015	6374
Total	98	77	100	2535	6699	2496	446	154	943	800	306	280	1291	16225

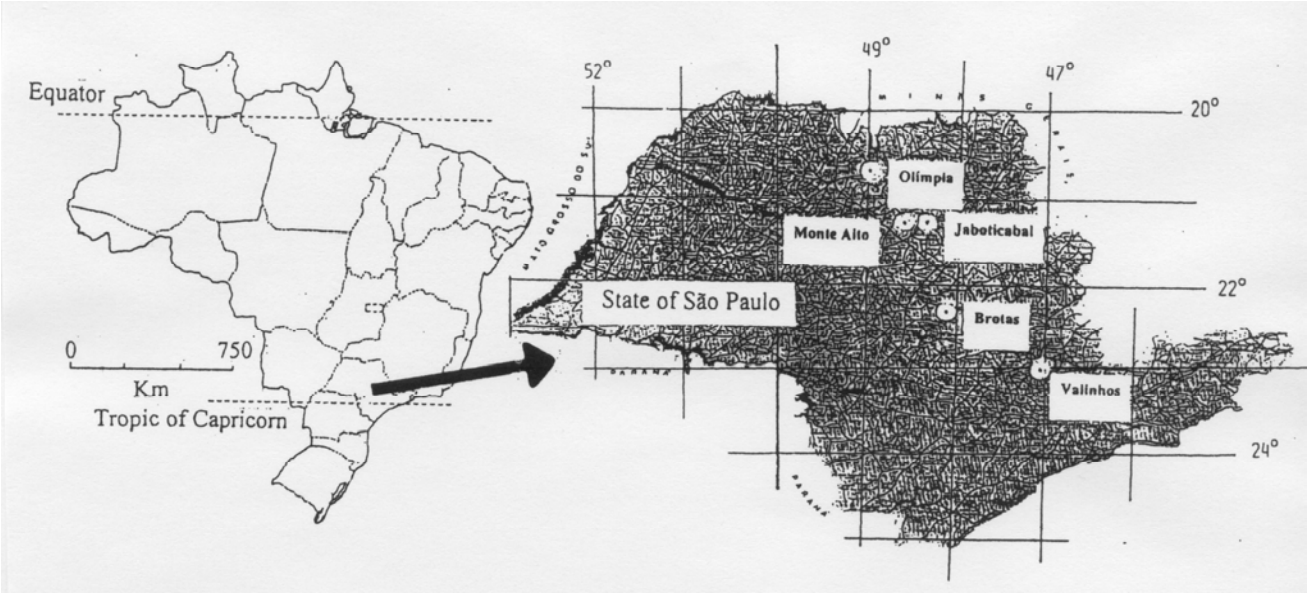

Figure 1. Map of Brazil, map of the State of São Paulo, in amplified projection, showing the locations where the collections were carried out.

Among the total amount of captured flies, *Z. indianus* and *D. simulans* were represented with the values of 39% and 38%, respectively. *D. simulans* predominated in the mango plantation with 53% and 58%, in the guava plantation in Monte Alto (07/19/01), with 75%, and in the guava plantation in Valinhos, with 74%. Meanwhile, *Z. indianus* was the most abundant in the orange

plantation in Olimpia (12/22/01) with 65%, in the guava plantation in Monte Alto (04/17/01), with 58%, in the fig plantation with 44% and in the guava and plum plantations in Brotas, respectively, with 46% and 79%. The county of Santa Isabel, where *Z. indianus* was captured by Vilela (1999) for the first time in South America, is 78 km from Valinhos. In this last county, in the fig plantation, 414 individuals of the species were captured; 267 were captured in the infructescence fixed in the plant; of these flies, 234 were females. Only 29 were captured in disposed ripe fruit, placed in a hole in the ground and 118 were obtained from a ripe infructescence deposit, in the plantation surroundings.

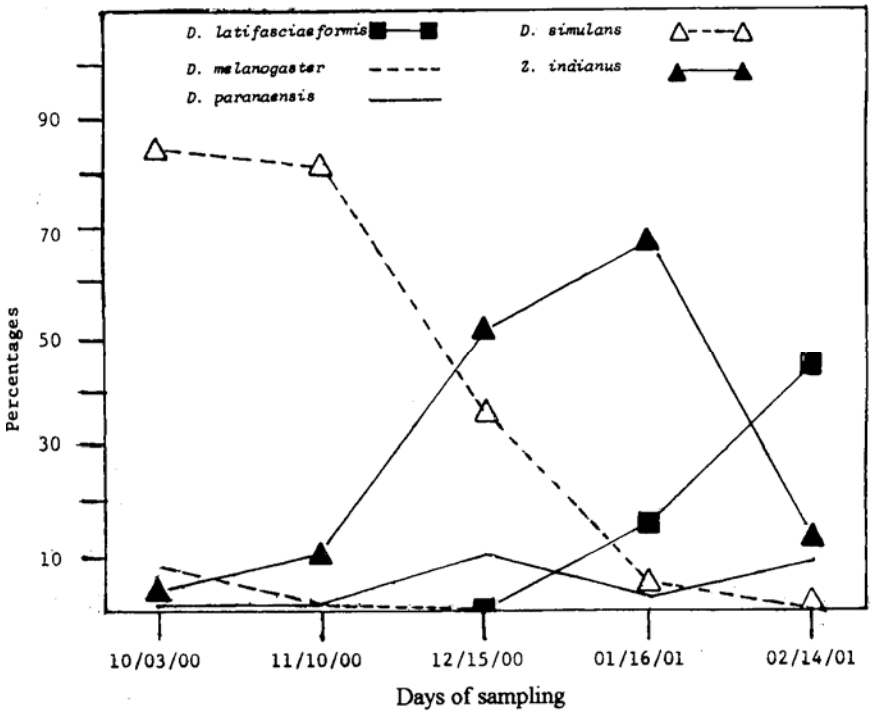

Figure 2. Number of captured flies during the fructification of *Mangifera Indica*.

Lachaise *et al*. (1982) have been showing, in Africa, the succession of individuals which are exploiters of the species of native figs (*Ficus sur*, *F. ovata*, *F. elasticoides*, etc.). Coincidentally, the data pointed at the exploitation of *Ficus carica* by *Z. indianus* in the period of floral ripening, females being 90% of the flies from this species that have visited the synconium, indicating that they probably laid their eggs in that place, where the imagoes must have emerged when the infructescences were still fixed in the plant. *Drosophila latifasciaeformis* was an abundant species in the mango, guava (Monte Alto) and fig plantation, not presenting representatives in the guava plantations of two locations, Valinhos and Monte Alto. In the collections carried out in infructescences fixed in the plants, only 12 individuals of the species were captured in infructescences deposited in holes in the plantation, where it was the most common species, 483 individuals were collected, and D. simulans was the second most common in those holes. These pieces of information seem to be suggesting a possibility of succession in the exploitation of these resources.

Of all the flies, *D. cardini* (17 females and 4 males) and *D. paranaensis* (153 females and 57 males) were the only species (in the mango plantation) which showed numbers of females where were significantly bigger than the number of males. The last species was the fourth most common in the collections, not presenting representatives in the guava cultures in Monte Alto (07/19/01), and in the peach culture in Brotas. In the orange plantation (12/22/01), it was the second most common species. In Brotas, although the cultures were just 2 km from each other, the plum plantation attracted a significantly greater number of flies (1,291) compared to the guava (280) and peach (306) cultures.

The mango plantation in Jaboticabal serves as a germoplasma bank for experiments which aim at improving the culture, presenting several varieties and hybrids, where approximately 300 leafy trees were planted in a condensed way in a three-hectare area, forming a vegetal covering on the soil. Usually, the produced fruits are not sold and, after ripening, naturally fall to the soil, serving as a remarkable substratum for the *Drosophila*. The colonization of these areas by the fly species was registered from its beginning (August 2000) up to its end (February 2001). The totals of these collections are shown in Table 1. The results, obtained in October 2000 (Figure 2), are related to a few trees, whose fruits precociously ripened if compared to the others, being the case that many of them fell down due to the wind effects and ripened on the ground. On that date, the captured flies must have come from nearby areas, since previously in these areas there were no apparent resources to be exploited by the flies. During the period from 11/10/00 to 01/16/01, there was a progressive increase in the ripening, with the increment of the fruit which had fallen to the ground. On 12/12/00, all the trees presented ripe fruits and in the end, 02/14/01, there were rare fruits in the trees, and those which had fallen to the ground were in an advanced stage of decomposition.

The most common species, in the beginning of the invasion (Figure 2), were: *D. simulans* (1,841 flies) and *D. melanogaster* (167), which progressively had their populations decreasing to the end of the collection period, whereas *Z. indianus* presented a marked increase in the collections on 12/15/00 (533) and 01/16/01 (1,025), starting from an initial low frequency (83 flies). After the collection of 01/16/01, all the fly populations must have suffered the influence of the putrefaction of the fruits, which became unsuitable for the majority of the species, except for the *D. latifasciaeformis* that was not captured in the initial period, just presenting representatives in the last two collections, respectively, with 243 and 153 flies. The fruit putrefaction must have been the cause of the low number of captured flies in the last collection (341).

Acknowledgments: The authors are grateful to FAPESP for the fellowship to D.J.P.

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Non-additive combined effect of multiple mutations in tumor suppressor genes on the frequency of hyperplastic mosaic clones in *Drosophila* imagoes.

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Tumor progression is a chain of mutations in a somatic clone leading to increase of its malignancy. As far back as ten years ago, modeling of tumor formation in adult flies was complicated, because it was impossible to induce a hyperplastic outgrowth visible in an adult fly, in spite of the fact of existence of numerous tumor suppressors, which mutations cause lethal neoplastic transformation in larval imaginal discs. Findings made during the last decade in the *Drosophila* genome and the progress achieved in somatic mosaic techniques revealed the tumor suppressor genes perspective for induction of mosaic clones visible in imagoes. Among them are the tumor suppressors *discs overgrown* (*dco)*, *warts* (*wts*), and *p53.* The *dco* gene is involved in cell communication, control

of imaginal disc growth and regulation of the circadian rhythm. Noteworthy, mutations in *dco* reduce the number of gap junctions on cell membranes (Jursnich *et al*., 1990). Its allele dco^3 is responsible for hyperplastic transformation of imaginal discs in dco^3 homozygous larvae, and formation of hyperplastic mosaic clones in heterozygous imagoes (Zilian *et al*., 1999). The *wts* gene is a part of cell cycle control machinery. It is responsible for maintaining two cell cycle checkpoints in *Drosophila*. Its mutation leads to rapid cell division allowing the mosaic clones in heterozygous individuals to survive the clonal competition and reveal themselves as imaginal hyperplastic clones. The *p53* gene is a member of the wide interspecies *p63/p53/p73* gene family mediating the mutagenic stress response, DNA repair, control of cell death *via* apoptosis. *Drosophila p53* is known to be involved in mutagen-induced apoptotic response rather than into developmental apoptosis (Brodsky *et al*., 2000; Ollmann *et al.*, 2000). All the known *p53* mutations in *Drosophila* are homozygous viable and do not lead to any tumor phenotype.

Since the experiments demonstrating synergetic effects of the *Ras* mutation with the cell polarity disrupting mutations, such as *lethal(2)giant larvae, discs large2, scribble*, onto clone metastatic behavior in larvae, modeling the corresponding progression step, were presented (Pagliarini *et al*., 2003), it's tempting to study the combined effects of mutations in tumor suppressor genes on imaginal somatic clones.

We studied the influence of combination of dco^3, *wts* and $p53^{259H.GUS}$ (dominant-negative *Drosophila p53* transgene marked with $white^{+mC}$) mutations on the frequency of (dco^3), (*wts*) and (dco^3 *wts*) clones on wild type and $p53^{259H.GUS}$ background. For this purpose, flies of the following genotype classes were generated:
(I) *w/+/Y;* dco^3*/ +*; (II) *y w/+ +/Y; wts/+*; (III) *y w/+ w/Y;* $p53^{259H.GUS}$ *wts / + act-GAL4* ;
(IV) *w/+/Y;* dco^3*/* $p53^{259H.GUS}$; (V) *w/+/Y;* dco^3 *wts / + +*; (VI) w; $p53^{259H.GUS}$ /TM6B, +.
(Here, notation "*w/+/Y*" means "*w/+ or w/Y* ")
To generate these flies, the following crosses were performed:
(a) ♀ *w;* dco^3 */ TM6B, Hu Tb e* × ♂ *w;* $p53^{259H.GUS}$ */+* to obtain classes IV and VI
(b) ♀ *w;* dco^3 */ TM6B, Hu Tb e* × ♂ D-32 (wild type) to obtain the class I
(c) ♀ *w; wts* dco^3*/TM6B* × ♂ D-32 to obtain the class V
(d) ♀ *y w;* $p53^{259H.GUS}$ *wts/TM6B* × ♂ *w; act-GAL4 / TM6B* for the class III
(e) ♀ *y w; wts/TM6B* × ♂ *w; +/+* for the class II.

The 1st instar F_1 larvae from the crosses (a – e) were treated either with a mutagenic substance, oxoplatin (2 mg/ml aqueous solution), or with distilled water (control group). After eclosion males and females of the above-mentioned genotype classes I – VI were screened for tumors under a stereomicroscope. Individuals of all other genotypes were rejected.

Clone frequency (p) was calculated as (Number_of_clones / Number_of_flies) × 100%. Significance of differences between the series was calculated in Student's t-test with Fisher's correction $\varphi = 2\arcsin\sqrt{p}$. The results are summarized in Table 1. We have made the following conclusions.

1. Dominant-negative p53 background is sufficient to significantly increase clone frequency for both *wts* and *dco*, especially in mutagenized series (3.3 times for *wts* and 1.8 times for *dco*, correspondingly).
2. Effect of combination of both *dco* and *wts* mutations in a mosaic clone[1] is comparable to the influence of *p53* dominant-negative background onto either (*wts*) or (*dco*) clone frequency (in induced variant it is ~ 6 times higher then (*dco*) clone frequency, ~ 2.7 times higher than (*wts*) clone frequency and ~ 1.8 times higher than their sum. Thus, the clone frequency increase in

result of combination of *dco*^{3} and *wts* mutations is non-additive. This effect remains pronounced in spontaneous mosaicism series also.

3. The effect of a single mutation on the clone frequency increases in the row: *p53* < *dco* < *wts*. In case of combination of two mutations, the clone frequency is higher in variants with *wts* mutation than in the same variant without *wts*.

Table 1. Frequency of mosaic clones with single mutation or a combination of mutations.

Genotype		Treatment							
		Spontaneous mosaicism (distillated H_2O)				Induced mosaicism (2 mg/ml oxoplatin)			
Principal fly genotype	Clone	flies	tumor clones	clone frequency (%)		flies	tumor clones	clone frequency (%)	
dco^3/+	dco^3	1003	14	1.40		1134	139	**12.26**	
wts/+	wts	1414	28	1.98	/P> 0.05/	962	271	**28.17**	/P< 0.001/
$p53^{259H.GUS}$/TM6B,+	$p53^{259H.GUS}$	424	0		0.00	282	0		0.00
+ $p53^{259H.GUS}$ wts / act-GAL4 + +	$p53^{259H.GUS}$ wts	1158	102	8.81	[P< 0.001] {P< 0.001}	377	351	**93.10**	[P< 0.001] {P< 0.001}
wts dco^3/+ +	wts dco^3	262	21	8.02	/P< 0.001/ [P< 0.001] [/P< 0.01]/] {P< 0.05}	360	269	**74.72**	/P< 0.001/ [P< 0.001] [/P<0.001/] {P< 0.001}
dco^3 + / $p53^{259H.GUS}$ +	$p53^{259H.GUS}$ dco^3	535	20	3.74	/P< 0.01/	343	77	**22.45**	/P< 0.001/

Legend: Within the same treatment variant
/ / – the frequency is higher than the (*dco*) clone frequency;
[ ] – the frequency is higher than the (*wts*) clone frequency
[/ /] – the frequency is higher than the sum of (*dco*) and (*wts*) clone frequency
{ } – the frequency is higher than (dco^3) clone frequency on a *p53* background.
Corresponding P-values are given in parentheses.
Frequencies given in **bold** are significantly higher than the corresponding ones in H_2O-treated series

Acknowledgments: We are grateful to Dr. Peter Bryant and Dr. M. Brodsky for the *wts, p53* and *dco Drosophila* mutants. This work was sponsored in part by the Russian Foundation for Basic Research grants # 05-04-49859a and # 04-04-49468a.

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Stress effects on spatial pattern of *Drosophila* wing sensilla in stress-sensitive and resistant genotypes.

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Abstract

Longitudinal placement of the campaniform sensilla along the third vein (L3) on the *Drosophila* wing provides an excellent quantifiable trait for studying developmental variances caused by genetic and environmental differences. Fluctuating asymmetry is an indicator of developmental stress within individuals and populations (Palmer, 1996). The defective HSP-90 protein stress-sensitive strain (*Hsp83* mutant strain #5696, Bloomington Stock Center) and stress-resistant Methuselah (Lin *et al.*, 1998) were compared to the genetically normal Canton S strain in several treatments. Methuselah had previously been shown to have a 35% longer lifespan and an increased resistance to several stresses (Lin *et al.*, 1998). Treatments of hypergravity and vibration were administered to the experimental strains at several stages of development. The placement of each of the campaniform sensilla were computed by converting the longitudinal position, measured by microscope graticule, into percentages of total wing distance measured from the anterior crossvein. The variances and mean asymmetries for paired wings were calculated and compared across treatments.

Introduction

Environmental and genetic factors interact during every biological process. Deviations from symmetrical development can indicate the strength of homeostatic coordination of developmental events and suggest the key mechanisms that influence them. The campaniform sensilla function as proprioceptors allowing constant monitoring of wing torsion and angle during flight (Figure 1). Multiple sensilla are found at various locations on each wing with good symmetry between left and right wings. Sensilla placement is strongly correlated with vein development (Jan and Jan, 1994), and the overall pattern of support veins and sense organs has fitness value to a flying insect. Thus, the system may be a good model for studying homeostatic mechanisms in the face of environmental stress. The stresses we chose to use are among those that would be experienced when an organism is exposed to the novel combination of gravitational, vibration, and radiation conditions in space.

Comparisons of Canton S wild type (non-mutant) and a balanced heterozygous *Hsp83* strain (*w[*]*;*Hsp83[e6A]* / TM6B, *Tb[1]*; Bloomington Stock Center #5696), which is mutant for the gene producing the HSP-90 protein, were used to test if quantifiable differences exist between deficient strains and functional strains. The HSP-90 protein is classified as a heat shock chaperone protein and is specifically linked with signal transduction pathways within the cell (Gething, 1997). Chaperone proteins have many functions including assistance in protein folding and transportation throughout the cell. The chaperone helper macromolecule ensures greater efficiency within a cell and in essence creates a buffering capacity. The HSP-90 family of proteins is suspected to assist in structural changes of kinase and steroid receptors in the endoplasmic reticulum of eukaryotes. A deficient HSP-90 metabolic pathway marks a decreased production of the stress protein. *Hsp83* heterozygotes have displayed a greater variance due to stress in prior studies (Rutherford and Lindquist, 1998), but

Milton *et al.* (2003) found that the Hsp buffering was limited to specific genetic variation and Hsp inhibition did not necessarily affect all phenotypic traits. Our experiments test this relationship in a spatial patterning trait.

Fluctuating asymmetry was also measured in the stress-resistant strain, Methuselah (*mth*, Lin *et al.*, 1998). The *mth* abnormality has been traced to a P-element insertion at 61C1 (Brody, 2005). The *mth* gene sequence encodes a peptide strand homologous to several G-protein-coupled seven trans-membrane domains receptors. *Drosophila* may use signal transduction pathways to modulate stress response and life span.

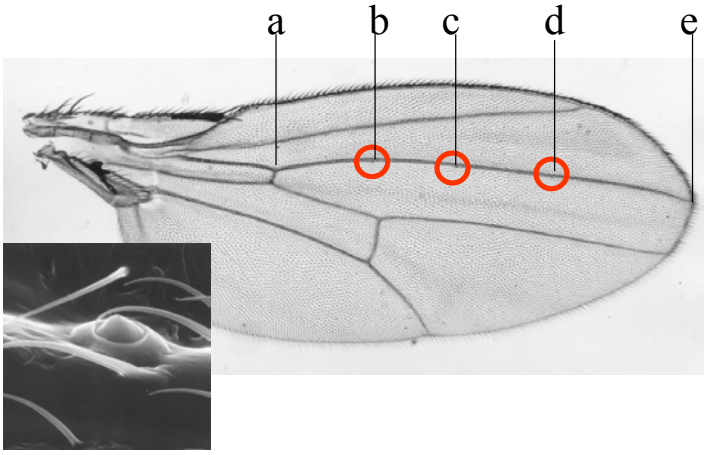

Figure 1. The placement of three campaniform sensilla (b, c, and d) on the wild type *Drosophila melanogaster* L3 wing vein from the anterior crossvein (a) to the distal wing margin (e). Measurements were taken of the distances between a and b, b and c, c and d, and d and e and used to assess sensilla distribution. The inset is a scanning electron micrograph image of a campaniform sensilla.

Materials and Methods

All *Drosophila* stocks were maintained on a culture medium of cornmeal, molasses, agar, and yeast kept at 23 ± 1°C in half-pint bottles. Crosses, mate pairing, and treated samples were placed in 8 dram vials with the same food. During treatments, flies were placed in smaller 2 ml centrifuge tubes containing 0.5 ml of medium to provide humidity. This medium consisted of 13% agar, 43.5% glucose, and 43.5% dry yeast and had a thicker consistency to prevent the flies from sinking and sticking to the food during increased gravitational stresses.

The first experiment tested the background variability of the *Hsp83* strain compared to the wild type Canton S. The trial was used to test the hypothesis that strains with a functionally homozygous *Hsp83* gene (Canton S) were more symmetrical than the strain with only one functional copy of the *Hsp83* gene (mutant strain 5696).

The second set of experiments were carried out at the NASA Ames Research Center (ARC), Moffett Field, California. Vibration table stresses were programmed to model the frequencies experienced on a Space Shuttle mid-deck during take-off (random vibrations ranged from 20 to 2000 Hz and could be divided into separate treatments of low range (20-150 Hz), mid-range (150-1000Hz), and high range (1000-2000 Hz) vibration frequencies; see profile in Thompson *et al*., 2001). The 1 Foot Diameter Centrifuge (CGBR, 2004) is a modified Beckman centrifuge was used to simulate increased gravitational stress for treatments of 1.4 to 180 times Earth Gravity. All fly preparations and genetic breeding were done prior to arrival at the NASA laboratories; they were shipped to California in 8 dram vials by FedEx. At Ames Research Center, four to six female virgins were placed in 2 ml centrifuge tubes with food for treatments. Eggs were also collected for separate treatment. Eggs were collected on yeasted plates in a population cage. Plates were exposed to the three treatment and the control conditions. The treated eggs were then removed from the media by

washing the yeast and eggs through a cell strainer and placing them in fresh food tubes. Four treatment groups were 5 minutes of vibration, 5 g hypergravity for four hours, 5 g followed within an hour by 5 minutes of vibration, and an untreated control. In a separate set of hypergravity treatments, females from Canton S, *Hsp83*-deficient, and *mth* were exposed to a range of gravitational treatments in 1G increments for four hours each using a centrifuge at the University of Oklahoma (see Potthoff and Thompson, 2002).

After treatments, the ARC samples were sent by FedEx back to the University of Oklahoma and females were singly mated and transferred to new food tubes to yield three successive 3-day broods. Wings were microdissected and mounted from the progeny. Mean placement of the campaniform sensilla on the third longitudinal vein as a percentage of the wing length, asymmetric mean, variance of the placement as a percentage of the length, and asymmetric variance were all studied with the aid of graphical representation. Fluctuating asymmetry was measured as $| L - R | / (0.5 \times (L + R))$, which standardizes the difference between trait expression on the two sides of a symmetrical trait.

An analysis of variance (ANOVA) was used to observe possible differences in fluctuating asymmetry in different treatments and broods for each of the three campaniform sensilla. In addition, *t*-tests were calculated for each of the three sense organs to compare average placement as a percentage of the wing in eggs to that of the broods.

Results

There was consistently larger variation in the *Hsp83* deficient strain (#5696) compared to the wild type Canton S (CS; Table 1). The *F-ratio* showed that both male and female comparisons of FA on all three campaniform sensilla in #5696 are more variable (ratio of FA in 5696/CS are all greater than 1: females, 1.24, 1.29, and 3.01*; males, 1.54, 1.06, and 5.80*** for the three sensilla, respectively; * $P < 0.05$; *** $P < 0.001$). Although consistent, the variation was significant in only two of the six ratios, *i.e.*, the third sensilla in females and males.

Table 1. Variance, mean, and standard deviation in contrasting genotypes (5696 = *Hsp83* deficient; CS = Canton S wild type) (mean ± SD).

Strain	Sex	n	1st Sensilla	2nd Sensilla	3rd Sensilla
CS	F	22	2.44 ± 2.30	2.62 ± 2.77	1.60 ± 1.65
CS	M	22	2.61 ± 1.75	2.47 ± 2.22	1.59 ± 1.42
5696	F	23	3.11 ± 2.56	3.44 ± 3.14	2.65 ± 2.87
5696	M	21	2.42 ± 2.17	3.54 ± 2.29	3.63 ± 3.42

Table 2. F ratios comparing variation in fluctuating asymmetry of *Hsp83*-deficient (5696), normal (Canton S), and Methuselah (*mth*) in treated versus untreated groups. Degrees of freedom = 49 for all compared groups. All values calculated by taking the treated variance divided by the untreated variance of each individual strain.

Strain	1st Sensilla	2nd Sensilla	3rd Sensilla
CS	1.32	1.46	1.80
5696	0.81	1.00	1.36
mth	1.34	1.15	1.30

For vibration and hypergravity treatments of Canton S, there were no significant trends or statistical differences among treatments or broods derived from treated females in FA (Williams, 2005, for data), indicating that the treatments did not reduce developmental stability in spatial pattern of sensilla. But, graphs of average campaniform sensilla placement as a percentage of the wing length suggested a more proximal placement of the sense organs in the treated eggs than in any

of the three broods (Figure 2). The *t*-tests confirmed significant variation ($P < 0.001$) for each of the three sense organs.

In the hypergravity exposures using the Oklahoma centrifuge, FA was compared in treated versus untreated *Hsp83* deficient, *mth*, and Canton S strains (Table 2). The treated individuals showed an elevated level of variance in asymmetry in 7 of the 9 *F-ratios* calculated. Although none were found significant, there is a noticeable trend that treated individuals develop greater asymmetries than untreated individuals. Not all sensilla showed the same level of sensitivity; the second sensilla was typically less variable than the other two.

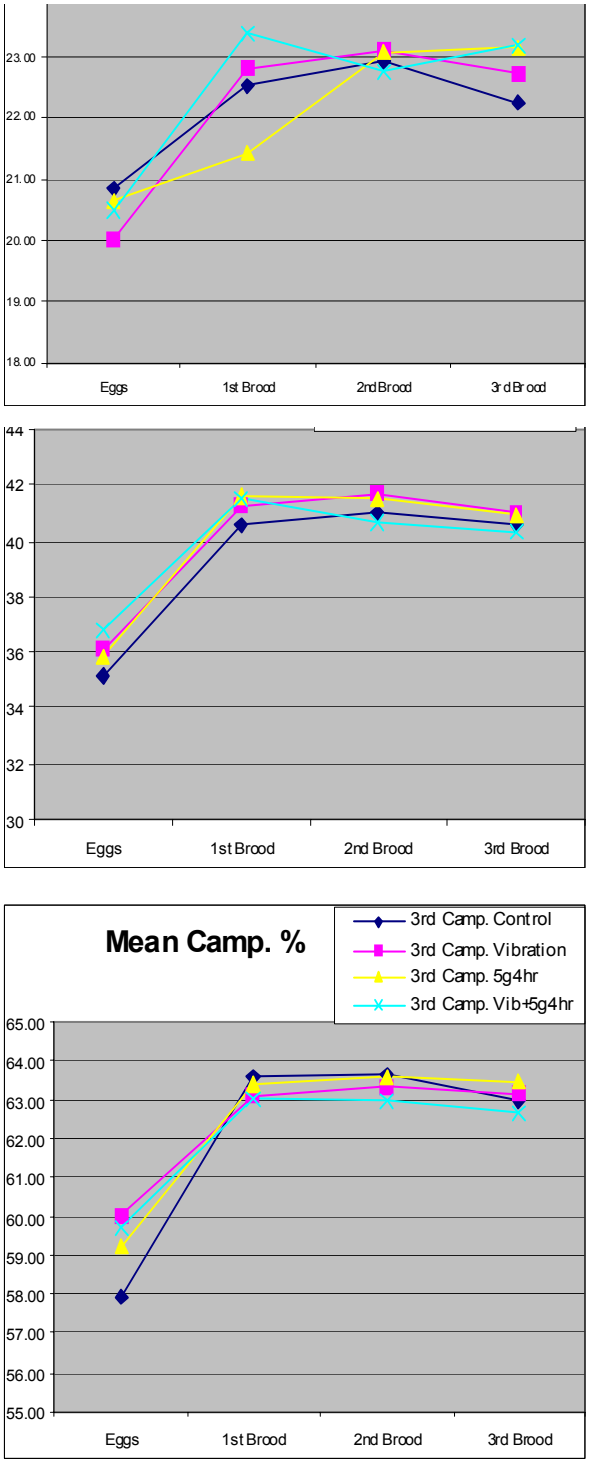

Figure 2. Average campaniform placement each of the three sense organs. The first, second, and third sensory organs are represented in the top, middle, and bottom graphs, respectively.

Discussion

We found a slightly increased variation among HSP-90 defective (*Hsp83* #5696) individuals relative to the Canton S strain. This would be predicted if the HSP-90 protein contributes to developmental stability in *Drosophila melanogaster*. The females of both strains were also consistently more variable than their male counterparts. But the variation among groups of flies would be expected to be reduced by of the expected overlapping roles of different mechanisms contributing to overall developmental stability. Redundancy of molecular mechanisms is well documented in many different systems.

Vibration and hypergravity exposures of Canton S females showed no consistent effect on FA or trait variation. But, interestingly, the flies that developed from treated eggs had a significantly more proximal mean campaniform placement on their wings than did those from broods from treated mothers. We suspect that the water wash used to collect eggs is the most likely factor and tests of this hypothesis are planned.

Similarly, the trend of increased FA in *Hsp83* deficient and *mth* flies derived from treated mothers, relative to that in Canton S, suggests that stresses like hypergravity can lead to greater phenotypic variation and within-individual asymmetry in genotypes with altered stress responses. This was predicted for the *Hsp83* strain, but the response of *mth* was unexpected. It is reported to be stress-

resistant for variables like nutrient reduction and environmental conditions. But the ability to survive such conditions might, in turn, leave the strain more vulnerable to superficial developmental effects on less fitness-related traits like spatial patterning of sense organs. This hypothesis is also being tested further.

Acknowledgments: We thank Seymour Benzer for providing a culture of Methuselah and Clayton Hallman and other students in the laboratory for helpful discussions. We also thank Sharmila Bhattacharya, Max Sanchez, Chris Chen, and other colleagues at Ames Research Center. Experimental treatments and analyses were supported by NASA grants NAG 2-1427 and NCC 2-1355.

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Targeted mutagenesis of *Drosophila atm* and *mre11* genes.

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Results

Targeted mutagenesis of the Drosophila *atm/tefu locus*

The *Drosophila CG6535* gene has been identified to encode the fly ortholog of mammalian ATM (Bi *et al.,* 2004). This gene was named *telomere fusion* (*tefu*) based on its mutant phenotype (Queiroz-Machado *et al.*, 2001). We use an 8 kb genomic fragment internal to the ATM coding region as homology for ends-in gene targeting (Rong and Golic, 2000). In addition, we introduced two point mutations into this fragment (Figure 1A). At a position that corresponds to codon 195 of ATM, we inserted a linker DNA that carried stop codons in all six reading frames. At codon 1187, we introduced a +1 frame shift mutation that would lead to a STOP 31 codons downstream. As shown in Figure 1A, a tandem duplication of the *tefu* locus was expected after ends-in targeting. The copy on the left could produce a truncated ATM protein that is missing the last 1241 amino acid residues, which includes the conserved C-terminal kinase domain. The right hand copy lacks the *tefu* promoter as well as the first 93 codons. It is not unreasonable to suggest that such a disrupted locus would result in a null mutation of *tefu*. By a similar approach, we were able to generate a null allele for the *pugilist* gene (Rong and Golic, 2001).

Using a targeting scheme previously described (Rong and Golic, 2001), we screened 330 female germlines and recovered five independent events that harbored the w^+ marker gene on chromosome *3*. Molecular and genetic analyses shown below led us to conclude that the endogenous *tefu* gene was altered in four of these events. The fifth was a non-targeting event. Southern blot analyses were performed on *Xho*I-digested genomic DNA from wild-type or homozygous mutant animals. When hybridized with *tefu* probes, the mutant lanes showed a 13 kb band in addition to the 9.5 kb normal band (Figure 1D). Furthermore, only the 13 kb band was detected by re-hybridization with *w* probes (data not shown). Southern blot analyses using two other enzymes separately (*Eco*RV or *Nco*I) verified the structure of the targeted *tefu* locus as well. In addition, inverse PCR followed by sequencing was carried out to demonstrate that the w^+ gene had been inserted into the *tefu* region (data not shown). The presence of the two point mutations was verified by PCR using allele-specific primers and inverse PCR (see Materials and Methods).

We recovered two classes of alleles that showed different severities of the mutant phenotypes (see below). Animals from three of the four *tefu*-targeted events showed strong defects, whereas individuals from the fourth line showed similar but weaker mutant phenotypes. One allele from each class was chosen for further analyses. They are hereby referred to as $tefu^{strong}$ ($tefu^{stg}$) and $tefu^{weak}$ ($tefu^{wk}$) alleles. The same alleles have been previously called atm^{stg} and atm^{wk}, respectively, (Bi *et al.,* 2004). Both alleles retain the STOP as well as the frame-shift mutations. Further sequencing analyses, sampled over the 8 kb region used for targeting, revealed a sequence variation between the alleles. At the position that corresponds to the I-*Sce*I cut site insertion, there was a small insertion at the left-hand copy of *tefu* in Figure 1B. During the targeting process, 13 bp of the endogenous *tefu* sequence were deleted and replaced with a 45 bp insertion that consisted of part of the I-*Sce*I cut site and small repeats originated from the region adjacent to the cut site (data not shown). These small duplications are a hallmark for DNA junctions generated by the non-homologous end-joining repair of a DNA double strand break (DSB) (Rong and Golic, 2003). This insertion led to a frame-shift of the *tefu* reading frame eliminating the last 1527 residues (Figure 1B).

We performed a second step to reduce the duplication in $tefu^{stg}$ into a single copy as described (Rong *et al.,* 2002). By Southern blot and allelic PCR analyses, we verified that some lines had a single *tefu* gene with the frame-shift mutation (Figure 1B and data not shown). These lines are 100% homozygous lethal with identical adult structural defects as $tefu^{stg}$ homozygotes (see below).

Targeted deletion of the Drosophila *mre11 locus*

We deleted *mre11* by making a targeting construct in which the entire Mre11 coding region was replaced by a *GFP* transgene driven by the *Drosophila armadillo* promoter (*arm-GFP*) (Figure 1C). A total targeting homology of 5.3 kb was used to flank *arm-GFP* for ends-out targeting (Gong and Golic, 2003). Since *arm-GFP* has adequate embryonic expression but minimal maternal contribution (Vincent *et al.,* 1994, our unpublished data), we used it as a marker to recover potential targeting events as embryos. We then used w^+ as a negative marker to conduct a secondary screen in adults. We mass-mated ~3000 females and recovered ~3200 GFP-positive individuals from ~300000 embryos screened. The majority of these embryos retained w^+. Twenty of such embryos were randomly chosen for further analyses. None of them had a targeting event (data not shown). Eleven lines were established from GFP^+ but w^- flies. Molecular and genetic analyses led us to conclude that eight of these lines were *mre11*-targeted events. Since the targeting crosses were conducted *en mass*, some of these events could have been non-independent.

The genomic structure of a targeted *mre11* locus is shown in Figure 1C. DNA from wild-type and mutant heterozygotes was digested with *Nde*I for Southern blot analyses. When hybridized with probes from outside of *mre11*, the mutant showed an 8.4 kb endogenous band and an additional 9.1 kb band (Figure 1D). This larger band was also detected with a *GFP* probe (data not shown).

Southern blot analyses with another enzyme digestion (*Sac*I) confirmed the genomic structure (data not shown). In addition, inverse PCR followed by sequencing was carried out to demonstrate that the *arm-GFP* gene had been inserted into the *mre11* region (data not shown).

Materials and Methods

Constructs for gene targeting

An 8 kb fragment from the *tefu* locus was PCR-amplified and cloned into a modified pBluescript II KS (+) from Strategene using the endogenous *Eco*RV and *Xho*I sites of the *atm/tefu* locus. In this vector, a *Kpn*I site in the polylinker was converted to a *Not*I site. A linker containing stop codons in all six reading frames was cloned into a unique *Nsi*I site ~400 bp from *Xho*I. A linker containing the 18 bp I-*Sce*I cut site was cloned into a unique *Cla*I site ~4 kb from *Xho*I. The unique *Hin*DIII site ~3 kb from *Eco*RV was cut and filled by Klenow. The plasmid was then re-ligated giving rise to a four-bp-insertion, hence a +1 frame shift. This 8kb targeting fragment, which contained all three elements that we had engineered, was cloned into the *Not*I site of the ends-in targeting vector pTV2 (Rong *et al.,* 2002). This gave rise to the donor vector for *tefu* shown in Figure 1A.

To construct the *mre11* targeting vector, a one kb fragment containing an *EGFP* gene and the SV40 3'UTR was PCR-amplified from the plasmid pEGFP-N1 (Clontech). It was cloned into the *Pst*I and *Eco*RI sites of a pBluescript II SK (+) vector that contained a 1.8 kb *armadillo* (*arm*) promoter cloned between *Bam*HI and *Pst*I (a generous gift from Dr. P. O'Farrell, UC San Francisco). This gave rise to pBS(arm-GFP). A 2.5 kb fragment immediately upstream of the start codon of Mre11 was PCR-amplified and cloned into the *Spe*I site in pBS(arm-GFP). A 2.9 kb fragment downstream of the stop codon of Mre11 was PCR-amplified and cloned into the *Hin*DIII site in pBS(arm-GFP). The final 8.2 kb targeting fragment that contained the *mre11*-upstream and downstream fragments as well as the *arm-GFP* marker was cloned into the *Not*I and *Xho*I sites of the ends-out targeting vector pW30, which carries the *white*$^+$ (w^+) marker gene (Gong and Golic, 2003).

Gene targeting scheme

Crosses to recover gene targeting events were performed as described (Rong and Golic, 2001) for *tefu*. A similar procedure was followed for *mre11* targeting with the only exception that embryos instead of adult flies were initially screened for targeting events. All GFP-positive embryos were allowed to mature into adults. Their eye color was scored. Lines were established from GFP-positive but white-eyed adults. Upon verification of GFP expression in progeny, the line was subjected to molecular and genetic tests described below.

The reduction of the *tefu* tandem duplication to a single copy was performed as described (Rong *et al.,* 2002). Individual lines were established that had lost the w^+ marker. Southern blot analyses with either *Xho*I or *Xba*I single digestion were performed to verify that there was a single copy of the *tefu* locus.

Molecular characterization of the mutants

Southern blot analyses were performed with standard protocols. The positions of probes for *tefu* and *mre11* are illustrated in Figure 1. For inverse-PCR analyses of *atm/tefu* targeting events, fly DNA was digested with *Nco*I, which cut once inside the w^+ gene and once outside of the targeting homologous fragment. Ligation was setup under conditions that promote intra-molecular ligation, which would generate circular DNA molecules. One primer from *w* and one primer from *tefu* were used for the PCR reaction. Both were aimed toward the *Nco*I site. The 0.8 kb product recovered was sequenced to verify the junction between *w* and *tefu*. For inverse-PCR testing of *mre11* targeting

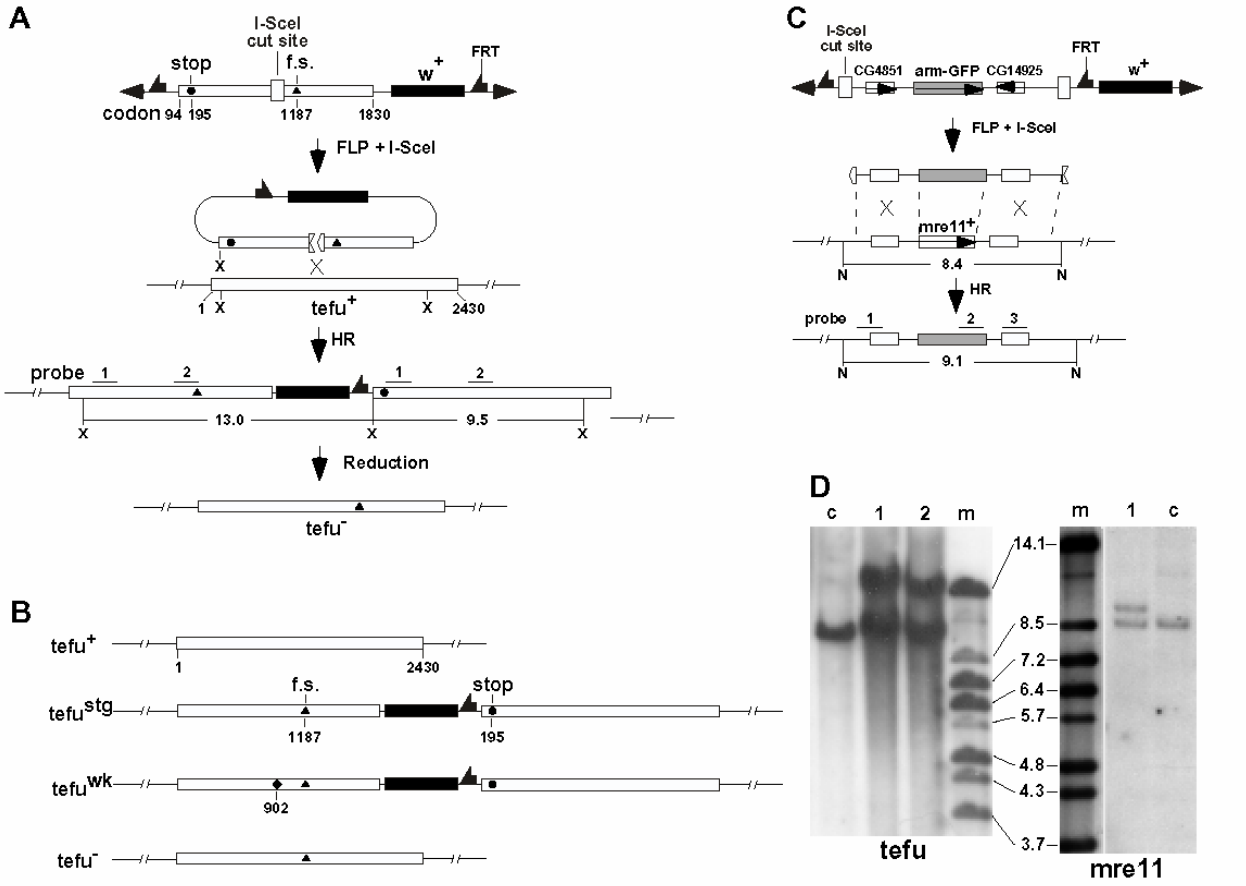

Figure 1. Gene targeting of the *tefu* and *mre11* loci. A. The ends-in targeting scheme for *tefu*. At the top are the *P* element donor constructs with the arrowheads representing the ends of the *P* element. The white rectangular box represents the *tefu* genomic fragment used for targeting. The positions for the "*stop*" (filled circle) and "*frame shift*" (*f.s*., filled triangle) mutations are indicated. The codon numbering was based on the predicted ATM protein sequences. The positions for the I-*Sce*I cut site and the *FRT*s are indicated. In the middle, the FLP/I-*Sce*I-generated targeting donor pairs with the endogenous *tefu* locus in which the start ("1") and stop ("2430") codons are indicated. X: *Xho*I. After homologous recombination (HR), the targeted *tefu* locus consists of a tandem duplication. The probe positions for Southern blots are indicated. The sizes of *Xho*I fragments are shown in kb. At the bottom, the duplication is reduced into a single *tefu* copy without any exogenous sequences except the *f.s.* mutation. B. Different *tefu* alleles in this study. The codons numbered are the last normal ATM codons in the alleles. In $tefu^{stg}$ and the reduced $tefu^{-}$ alleles, the *f.s*. mutation resulted in a terminator 31 codons downstream of normal codon 1187. In $tefu^{wk}$, a small sequence insertion (filled diamond) resulted in a terminator 15 codons downstream of normal codon 902. C. The ends-out targeting scheme for *mre11*. At the top is the *P* element donor construct with targeting homologous fragment flanked by two I-*Sce*I cut sites. Two genes with CG designations flank *arm-GFP*, which was used to replace *mre11* and served as a marker. The directions of transcription are indicated by the arrows. In the middle, an ends-out recombination is shown between the excised donor and the endogenous *mre11* locus. The size of the endogenous *Nde*I (N) fragment is shown. At the bottom is the replaced *mre11* locus with a new *Nde*I fragment. The positions of the probes are shown. D.

Southern blot analyses of targeting events. DNA was digested with *Xho*I for the *tefu* Southern (left). Lanes, c: w^{1118} control, 1: $tefu^{stg/stg}$, 2: $tefu^{wk/wk}$, m: markers with sizes in kb. DNA was digested with *Nde*I for *mre11* Southern (right). Lanes, m: markers, 1: $mre11^{+/-}$, c: w^{1118}.

events, DNA was cut with *Xba*I, once within *GFP* and once at a region outside of the targeting homology. The 460 bp PCR product recovered was sequenced. The linker cloned into the *Nsi*I site of the *tefu* targeting fragment could form a hairpin structure so that traditional PCR was unable to traverse the linker region (data not shown). We employed an inverse PCR method to verify the presence of the STOP mutation in our *tefu* mutants. DNA was double-digested with *Spe*I, which cut at the linker but not at the wild-type sequence, and *Xba*I, which cut once at the *FLP recombination target* (*FRT*) element of the targeting vector (Figure 1A). There are no additional *Spe*I or *Xba*I sites between the above sites. DNA was ligated and PCR-amplified with a primer from *tefu* and one from *w* to generate a 300 bp fragment, which was confirmed by sequencing. The presence of the frame shift mutation in *tefu* mutants was verified by allelic PCR using one primer specific for the mutation and one outside primer. A control PCR was also performed in parallel in which the mutation-specific primer was replaced with a wild-type-specific primer. Both PCR reactions gave rise to a 500 bp fragment.

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Toxicity parameters of 4-nitroquinoline-1-oxide, urethane and methyl methanesulfonate in the *flare* and Oregon-*flare* strains of *Drosophila melanogaster* .

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Introduction

Drosophila melanogaster has proved to be a good model for genotoxicity studies (Vogel *et al*., 1999). Antigenotoxicity studies have also been conducted in the fruit fly to identify chemopreventive components that could contribute to diminish the mutagenic and/ or carcinogenic effects of certain compounds. Toxicological studies for the determination of toxicity parameters of experimental compounds in *D. melanogaster* (Galvão *et al*., 1999; Osaba *et al*., 1999; Ogawa *et al*., 1994; Graf *et al*., 1990) help establish non-toxic or sub-toxic concentrations (Munerato *et al*., 2005) and can also show slight differences in dose response curves in different *D. melanogaster* strains (Castañeda *et al*., 2001).

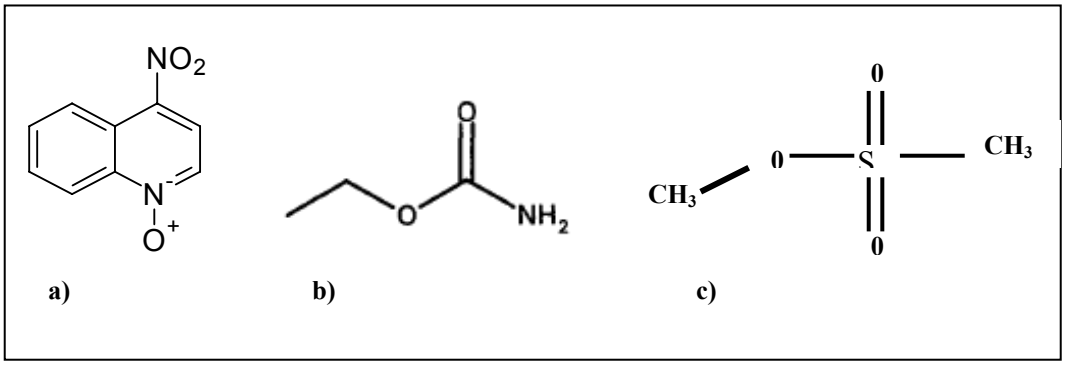

Figure 1. Chemical structures of mutagens. a) 4-NQO, b) URE, and c) MMS.

4-nitroquinoline-1-oxide

The pluripotent carcinogen 4-nitroquinoline-1-oxide (4-NQO) (Figure 1a) increases radical oxygen species (ROS) which can produce DNA single strand breaks (SSBs) and alkali labile sites as UV light does (Friedberg *et al*., 1995). The CYP450 metabolism of 4-NQO produces purine adducts eliminated by excision repair (Mirzayans *et al*., 1999). Recently, our group demonstrated mainly mutagenic effects of 4-NQO in the standard (ST) cross and recombinogenic effect in the high bioactivation (HB) cross of the *Drosphila* wing spot test (Heres-Pulido *et al*., 2004). These crosses are done with *flare* and Oregon-*flare* virgin females with regulated or highly constitutive CYP450 levels, respectively, which can generate differences in results.

Urethane

Urethane (URE) or ethyl carbamate (Figure 1b) is a multipotential animal carcinogenic pro-mutagen. More than 90% is hydrolized to CO_2, CH_3CH_2OH and NH_3 by liver microsomal esterase in rodents and less than 0.5% is biotransformed by CYP450 enzymes to vinyl carbamate that after epoxidation produces DNA adducts *in vivo.* This CYP450 metabolism also transforms 0.1% of URE into *N*-hydroxyurethane. Non enzymatical oxidation generates a nitrose radical and esterase enzymes produce hydroxylamine that finally increases O_2^-. The metabolites of URE induce carcinogenesis through oxidation, and in minor quantities by depurination (AP sites) of DNA (Sakano *et al*., 2002). Frölich and Würgler (1990) showed genotoxicity of URE in *Drosophila* larvae of the HB and ST crosses of the wing spot test finding strong strain differences due to constitutive and regulated CYP450 enzyme levels in these crosses, respectively.

Methyl methanesulfonate

Methyl methanesulfonate (MMS) (Figure 1c) is a small direct monofunctional alkylating agent that methylates DNA nitrogen atoms (N7-MeG, N3-MeA) producing clastogenicity (Vogel *et al*., 1990). It also has a SN2 mechanism of methylation and a high nucleophilic selectivity (*s* value = 0.83) that generates single (SSBs) and double strand breaks (DSBs). MMS damage is repaired by methyltransferases, alkyltransferases, base excision repair (BER), and recombinational repair of double strand breaks (DSBs) (Jenkins *et al.,* 2005).

Genotoxicity screening of 4-NQO, URE and MMS at different concentrations has been done in the past (Table 1). In order to determine the toxicity parameters of these mutagens for future genotoxicity and antigenotoxic protocols with the *Drosophila* wing spot test (Graf and Singer, 1992), we used the two mutant strains of this bioassay: *flare* and Oregon-*flare* with marker flr^3. This marker, a recessive mutation, is located on the left arm of chromosome 3: *flare-3* (flr^3, 3-38.8). The flr^3 allele

is kept over a balancer chromosome carrying multiple inversions [*In(3LR)TM3*] and a dominant marker which is homozygous lethal (Bd^S, *Beaded-Serrate*, serrate wings) (Graf and Singer, 1992). The Oregon-*flare* strain carry chromosomes 1 and 2 of the DDT resistant Oregon strain (Frölich and Würgler, 1989).

Table 1. Previous concentrations (mM) of 4-NQO, URE and MMS tested in chronic treatments in the *Drosophila* wing spot test.

4-NQO		URE		MMS	
mM	Reference	mM	Reference	mM	Reference
2, 3, 8	Batiste-Alentorn *et al*., 1995	5, 10, 20, 30	Frölich and Würgler, 1990	0.5, 1, 2	Rodríguez-Arnaiz *et al*., 1996
3	Kaya *et al*., 2002	5, 10, 20	El Hamss *et al*., 1999	1.25	Santos *et al*., 1999
2.5, 5	Heres-Pulido *et al*., 2004	10, 20	Osaba *et al*., 1999	2.5	Lehman *et al*., 2000
		5	Idaomar *et al*., 2002	0.05	Sánchez-Lamar *et al*., 2002
		20	Coelho de Sousa *et al*., 2003		
		10	Pimenta and Nepomuceno, 2005		
		5, 10, 20	Rojas-Molina *et al*., 2005		

Materials and Methods

Strains

flare ($flr^3/In(3LR)TM3, Bd^S$) with regulated cytochrome P450 (CYP450) levels. Oregon-*flare* (ORR(1)/ ORR(2); $flr^3/In(3LR)TM3, Bd^S$) with highly constitutive CYP450 levels.

Toxicity parameters

Adult flies were grown at 25ºC and aged in culture bottles containing mashed potato flakes medium (Dueñas *et al*., 2001). Eggs were collected from these flies by shaking them without anesthesia into bottles (250 ml) containing an approximately 5 cm layer of fermenting fresh baker's yeast supplemented with sucrose (Graf *et al*., 1991). The egg collection bottles were then kept undisturbed in the dark for 8 h at 25ºC. After removing the parental flies, the egg collection bottles were taken back to 25ºC where they remained at a relative humidity of 65% and dark conditions. Three days later, the 72 h ± 4 h larvae were washed out of the bottles with tap water at room temperature through a fine-meshed stainless steel strainer, collected and thoroughly washed free of yeast while still in the strainer. Immediately after that, 10 or 20 larvae were transferred to vials containing 0.5 g of *Drosophila* Instant Medium (Carolina Biological Supply Co, NC, USA) prepared with 2 ml of solution of the test compounds: MMS (Fluka CAS Nº 66-27-3, 98% purity) at 0, 0.03, 0.09, 0.18, 0.37, 0.75, 1.5, 3.0 and 6.0 mM in distillated water; 4-NQO (Fluka CAS Nº 56-57-5, 99% purity) at 0, 0.625, 1.25, 2.5, 5.0 and 10.0 mM in a mixture of 3% Tween 80 (SIGMA CAS Nº 9005-65-6) plus 3% ethanol (Merck, Darmstadt, Germany); URE (Fluka CAS Nº 51-79-6) at 0, 15, 20, 25, 30, 35 and 40 mM) in distillated water. Three to five replicates were made for each concentration in three independent chronic experiments for each mutagen. The treatment vials were kept at 25ºC and a relative humidity of 65%. The surviving flies were counted from the vials on days 10 to 12 after egg laying to quantify mortality. Mortality results were plotted and the toxicity parameters LC_{50}, potency (lineal slope), and sensivity (origin ordinate) were calculated for each strain and mutagen fitting

better in second order polinomial regressions. Results were analyzed with one-way analysis of variance (ANOVA) where F test was calculated for statistically significant differences between LC_{50}, strain, potency and sensivity.

Results

4-NQO

Figure 2a shows 4-NQO mortality percentages fitting a non linear dose-response relationship. The LC_{50} for 4-NQO (Table 2) was 3.05 mM in Oregon-*flare* and 2.10 mM in *flare*. One-way ANOVA results showed statistically significant differences only in potency between strains ($p < 0.05$).

Table 2. LC_{50} (oral), potency (lineal slope), sensivity (origin ordinate) and r^2 from quadratic equations obtained for the *flare* and Oregon-*flare* strains of *Drosophila melanogaster* after 48 h feeding of 72 ± 2 h larvae with 4-NQO, URE and MMS solutions.

Mutagen	**Strain**	$LC_{50\ (oral)}$ (mM)	Potency	Sensivity (mortality %)	r^2	Equation
4-NQO	OR(R)-*flare*[3]	3.05	15.89	8.82	0.94	$y = -0.7772x^2 + 15.897x + 8.8212$
	flare[3]	2.10	22.89	8.95	0.93	$y = -1.4214x^2 + 22.894x + 8.9515$
URE	OR(R)-*flare*[3]	28.65	-0.55	0.41	0.82	$y = 0.0794x^2 – 0.5555x + 0.4124$
	flare[3]	28.42	-0.49	-2.05	0.85	$y = 0.0817x^2 – 0.4974x -2.0582$
MMS	OR(R)-*flare*[3]	0.90	38.87	19.16	0.64	$y = -4.2431x^2 + 38.871x + 19.168$
	flare[3]	0.87	52.33	10.76	0.82	$y = 6.3138x^2 + 52.338x + 10.768$

URE

Figure 2b shows URE mortality percentages fitting a non linear dose-response relationship. The LC_{50} for URE (Table 2) was 28.65 mM in Oregon-*flare* and 28.42 mM in *flare*. One-way ANOVA results showed no statistically significant differences between LC_{50}, strain, potency and sensivity ($p > 0.05$).

MMS

Figure 2c shows MMS mortality percentages fitting a non linear dose-response relationship. The LC_{50} for MMS (Table 2) was 0.90 mM in Oregon-*flare* and 0.87 mM in *flare*. One-way ANOVA results showed no statistically significant differences between LC_{50}, strain, potency and sensivity ($p > 0.05$).

Discussion

Results for 4-NQO, URE and MMS showed mortality percentages that fit a non linear dose-response relationship. Second order polinomial dose-response curves for 4-NQO, MMS and URE showed r^2 values in a relation 4-NQO > URE > MMS (Table 2 and Figure 2).

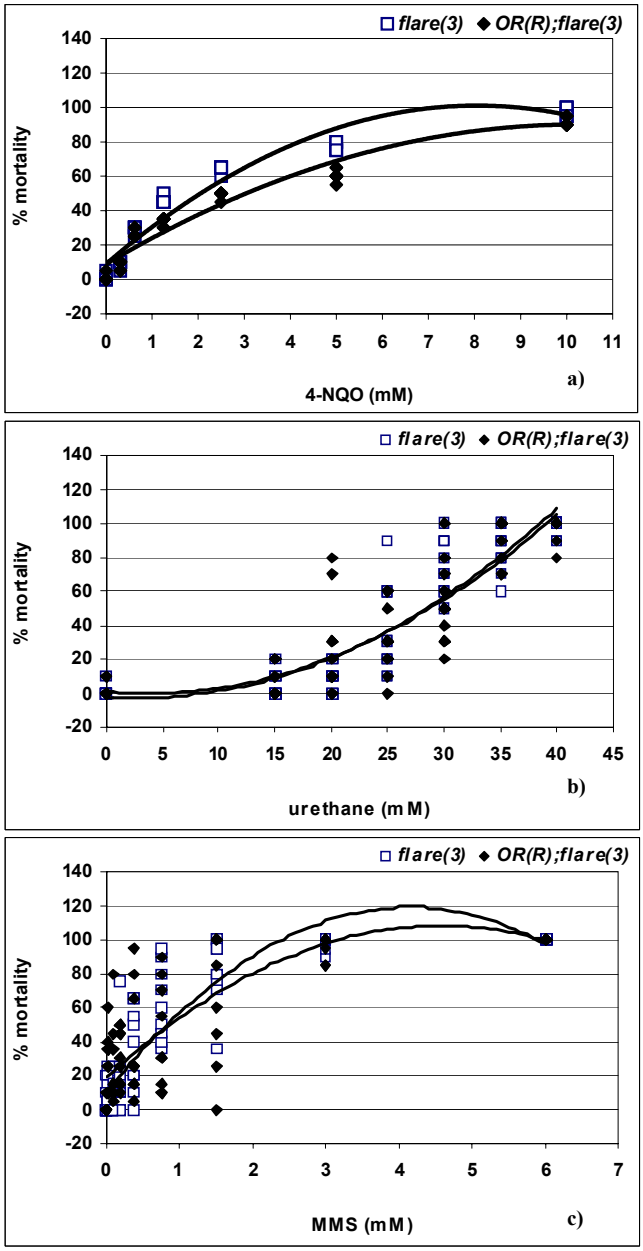

Figure 2. Mortality of *flare* and Oregon-*flare* strains of *Drosophila melanogaster* after 48 h feeding of 72 ± 2 h larvae with: a) 4-NQO; b) URE; c) MMS.

4-NQO

There were no statistically significant differences between 4-NQO LC_{50}, strain, and sensivity ($p > 0.05$). However, significant differences were found for potency ($p < 0.05$) between strains showing that mortality increases in the *flare* strain with a potency higher than in the Oregon-*flare* strain. As we reported before (Heres-Pulido *et al*., 2004) 4-NQO has mainly mutagenic effects in the standard (ST) cross and recombinogenic effects in the high bioactivation (HB) cross of the *Drosophila* wing spot test. It is well known that post-replication recombinational repair of DSBs is part of cellular strategies to repair DNA damage (Friedberg *et al*., 1995). This repair mechanism is possible in both strains, with exception of the region (61A2 and 65E) in the TM3 balancer chromosome (Zordan *et al*., 1994). Considering CYP450 levels in the Oregon-*flare* strain we propose that the lower mortality found in this strain could be related to 4-NQO xenobiotic metabolites that could increase recombinational repair.

URE

The polynomial curves and toxicity parameters determined for promutagen URE showed no statistically significant differences between strains (Table 2). The LC_{50} values were slightly below the concentration of 30 mM reported as toxic by Frölich and Würgler (1990) for *D. melanogaster* larvae from the HB and ST crosses of the wing spot test fed with URE for 48 h. All the parameters values for the *flare* and Oregon-*flare* strains were very similar. These results can be explained considering that more 90% of URE metabolism produces ethanol, ammonia, and CO_2 (Sakano *et al*., 2002), which are toxic compounds *per se*. Therefore, although it is well known that CYP450 enzymes activate this promutagen (Pimenta and Nepomuceno, 2005) our results show that the toxicity of URE to 72h larvae chronically fed (48 h) with URE could be independent of these enzymes.

MMS

In rodents DNA repair, clastogenic efficiency, type of mutation spectra, and carcinogenic potency allow predictions of the genotoxicity of alkylating agents (Vogel *et al*., 1999). It has been

previously demonstrated in the *Drosophila* wing spot test that MMS exhibits greatly enhanced genotoxic activity and toxicity in chronic treatments of 48 h (Rodríguez-Arnaiz *et al*., 1996). The LC_{50} for potency and sensivity obtained for MMS (Table 2) showed no statistically significant differences between strains. We found a great dispersion in both strains in each one of the three independent experiments. This could be caused by individual responses to MMS cytotoxicity, genotoxic effects (Vogel *et al*., 1999), sensivity to MMS mediated by 28 genes (FlyBase, 2005) and the base excision repair mechanism (Jenkins *et al*., 2005). It is noteworthy that regardless this disperssion the LC_{50} values were similar in both strains. This agrees with the fact that MMS is a direct alkylating agent and, therefore, the differences in CYP450 enzymes between the *flare* and Oregon-*flare* strains do not play a role in its metabolism.

Conclusions

Differences between potencies obtained for the *flare* and Oregon-*flare* strains larvae fed with 4-NQO indicate statistically significantly less toxicity of 4-NQO in the Oregon-*flare* strain. We propose that the effect could be related to recombinational somatic events increases that occur when CYP450 enzymes are highly constitutive in *D. melanogaster*. We demonstrate that the LC_{50} values determined for URE in this protocol are above concentrations reported for genotoxicity protocols in the *Drosophila* wing spot test. The LC_{50} for MMS was the same for both strains. Therefore, in order to avoid toxicity and selection of resistant flies in genotoxic treatments with 4-NQO, MMS and URE, we propose the convenience that experimental concentrations must be close or below the LC_{50} values reported here.

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Mating propensity: an indicator of interracial divergence in the *nasuta-albomicans* complex of *Drosophila*.

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Introduction

Interracial hybridization between *Drosophila nasuta* and *Drosophila albomicans* belonging to *nasuta* sub group of *Drosophila immigrans* species group has led to introgression of their genomes, with parental chromosomes being differentially represented in different hybrids (Ranganath, 1978; Ranganath, 2002; Tanuja *et al*., 2003). Over years, varied hybrid lines of *D. n. nasuta* and *D. n. albomicans* with stable karyotypic composition were recognized and subsequently named as Cytoraces. Cytoraces along with parental races are referred to as *nasuta-albomicans* complex (Ramachandra and Ranganath, 1996). This hybridogenetic complex constitutes allo-sympatric populations, which provides a ground for understanding of racial divergence in an artificial hybrid zone. Apart from karyotypic divergence (Tanuja *et al*., 1999a, b, 2003), differences have been reported for different parameters like morphophenotypic traits (Harini and Ramachandra, 1999a, b, 2000), fitness parameters (Ramachandra and Ranganath, 1988), differential mating preferences (Tanuja *et al.,* 2001), and analyses of isozyme (Aruna and Ranganath, 2004) and have shed light on the genetic differences among the members of the *nasuta- albomicans* complex.

Mating propensity is a complex trait based on interaction of both sexes. The mating propensity is defined as the proportion of flies mated during an observation period (Koepfer, 1987). Studies on mating propensity in various species of *Drosophila* are well documented. It is widely recognized that deviation from random mating can be caused by two different biological factors, namely discrimination and mating propensity (Ringo, 1986). Differentiating between these two factors is of paramount importance for the evolutionary implication of these tests, since differences in mating propensity between mutants, strains, and wild populations occur very often (Spiess, 1970). Thus mating propensity was taken to measure sexual receptivity of females and mating ability in males among six races of the *nasuta-albomicans* complex.

Material and Methods

During the course of the present study, we tested the mating propensity in six races of the *nasuta-albomicans* complex of *Drosophila*, namely *D. n. nasuta*, *D. n. albomicans*, Cytorace 1, Cytorace 2, Cytorace 3, and Cytorace 4. All these flies were maintained at 22 ± 1°C under uniform conditions. For experimental purposes, virgin females and males were collected from synchronized cultures and aged for seven days. By using these flies, experiments were carried out by direct observation in an empty vial plugged with cotton, between 7-11 A.M. For each race (intra-racial crosses) five replicates were set up. In each replicate fifteen males and fifteen females were placed, and the number of matings was recorded for 60 min. When a pair commenced mating, it was aspirated out. [Intra crosses involve 6 crosses; these 6 crosses were grouped into homo-parental (2 sets of crosses) and homo-cytoraces (4 sets of crosses)]. Mating propensity was also recorded in mixed cultures (inter-racial crosses) of six races; this involves 30 crosses. For each cross, five replicates, each with fifteen males and fifteen females, were used. These 30 crosses are grouped into hetero-parental (2 sets of crosses), hetero-cytoraces (12 sets of crosses) and hetero-mixed (16 sets of crosses) (Tanuja *et al.,* 2001). The mean values were subjected to one-way ANOVA and also diallel analysis were done by following procedure of Singh (1999), to measure sexual receptivity of females and male mating ability.

Results

During the course of the present study, we tested the mating propensity in six races of *nasuta-albomicans* complex. Of the six races, *D. albomicans* and Cytorace 2 have the highest (13.0 ± 0.7) and the lowest (8.0 ± 1.0) mean number of matings, respectively (Table 1). For testing variation in mean number of matings in different races, analysis of variance was performed, which indicates significant variations among six races (F = 5, 4.53, P = 0.005).

The results are subjected to another type of analysis. Based on mating type, the 36 crosses were grouped in to five groups (Tanuja *et al*., 2001). For testing variation in mean number of matings in five groups, analysis of variance was performed, which indicates, significant variations among five groups (F = 4, 7.75, P = 0.000) (Table 2). Further, among five groups, pair-wise comparisons showed hetero-cytoraces have the least mating propensity (8.6 ± 0.3), while homo-parentals have the highest mating propensity (11.9 ± 0.5).

Table 1. Mean numbers of matings along with standard error in 36 crosses of the *nasuta-albomicans* complex. Note: (N-*D. nasuta*; A- *D. albomicans*; C1-Cytorace 1; C2-Cytorace 2; C3-Cytorace 3; C4-Cytorace 4)

Races of female parent	Races of male parent						
	NN	AA	C1	C2	C3	C4	Total
NN	10.8 ± 0.5	9.6 ± 0.4	6.8 ± 0.4	11.2 ± 0.4	10.6 ± 0.6	8.4 ± 0.4	57.4
AA	13.8 ± 0.7	13.0 ± 0.7	12.6 ± 0.5	9.2 ± 0.3	12.4 ± 0.6	12.0 ± 0.6	73.0
C1	10.0 ± 0.4	9.4 ± 0.5	11.6 ± 0.8	10.2 ± 0.2	5.6 ± 0.5	7.8 ± 0.4	54.6
C2	10.6 ± 0.8	7.6 ± 0.6	8.8 ± 0.3	8.0 ± 1.0	3.0 ± 0.3	4.6 ± 0.4	42.6
C3	9.6 ± 0.6	9.2 ± 0.5	11.2 ± 0.5	11.6 ± 0.2	10.0 ± 0.3	8.2 ± 0.3	59.8
C4	8.0 ± 0.4	9.4 ± 0.6	11.8 ± 0.8	11.0 ± 0.5	9.6 ± 0.4	11.6 ± 1.1	61.4
Total	62.8	58.2	62.8	61.2	51.2	52.6	

Table 1 presents the results of diallel crosses, which were carried out to assess the relative sexual activity of males and females. Analysis of variance was carried out to measure the degree of variation in male mating ability and female receptivity in different races. ANOVA shows highly

Table 2. Mean values along with standard of mating propensity for the pooled data of 36 crosses based on different types of homo-and heterogamic matings among six races of the *nasuta-albomicans* complex, along with the summary of tukey's test.

Categories	Total pairs	Mating propensity (Mean ± SE)
Homogameic		
Homo-parental	150	11.9 ± 0.5[a]
Homo-Cytorace	300	10.3 ± 0.5[b]
Heterogamic		
Hetero-parental	150	11.7 ± 0.7[c]
Hetero-Cytorace	900	8.6 ± 0.3[d]
Hetero-mixed	1200	9.8 ± 0.2[e]
F-value	7.138*	
df	4	

Following pair-wise comparisons showed significant different at 5 % level
Mating propensity: d/c; d/a

significant differences in sexual activity of both sexes. However, variation is greater for females (F = 5, 17.95, P = 0.000) than in males (F = 5, 3.528, P = 0.005). Thus, females of newly evolved races of *nasuta-albomicans* complex contribute more variation in sexual receptivity than males.

By using the data [marginal total: A method generally used to estimate mating propensity (Anderson and Ehrman, (1969)] of diallel analysis, 15 pair-wise comparisons between races have been made to test the differences between male mating ability and female receptivity in different races. These pair-wise comparisons are presented in Table 3, which shows the range of variations and differences for male sexual activity and female receptivity. Out of 15 comparisons, 10 showed greater variation in female receptivity than in male sexual activity. Only in 4 comparisons was there greater variation in male mating ability than in female receptivity. However, differences in male mating activity and female receptivity are nearly identical only in one comparison.

Discussion

During the course of the present investigation, 6 races of *nasuta-albomicans* complex were tested for mating propensity. There is significant variation in mean number of matings among the races tested, which is attributable to genetic heterogeneity among the races resulting from hybridization and genetic drift during laboratory rearing. To assess the relative sexual activity of the two sexes, among six races, diallel crosses were also made. Based on the analysis of data of diallel crosses by ANOVA and pair-wise comparisons, it was demonstrated that the contribution of females is greater to the observed variation.

It is known that male activity and female receptivity are the main factors responsible for successful mating in *Drosophila*. Intra-specific variation in mating activity has been reported in *D. pseudoobscura*, *D. ananassae*, and *D. montana.* The dependence of successful mating on a particular sex varies between species and within species between genotypes, such that males may often be more important if mating is so rapid, while if mating is slow, females play a progressively more important role. In *D. pseudoobscura*, mating is so rapid that variation in female receptivity may be relatively unimportant (Parsons, 1973). Kesseler (1968) has shown that the contribution of females to the total variance of mating propensity was greater than males in *D. pseudoobscura*; Singh (1999) has shown greater variation in female receptivity than males in few strains of *D. ananassae*; and Suvanto *et al.* (2000) have shown that mating propensity was influenced more by the females than males in a few strains of *D. montana.* In *D. persimilis* it has been found that females are critical over a one hour period, because of an interaction between copulation and avoidance tendencies. Thus, the results concerning the contribution of a particular sex to variation in sexual activity as well as dependence of mating success on a particular sex may vary within a species depending upon the genetic constitution. In different species it has been demonstrated that sexual activity of males and female receptivity have genetic basis.

Table 3. Pair wise comparisons to test the differences between male activity and female receptivity based on the marginal total of mean number of matings in diallel crosses.

Pairs of races	Range of variation		Differences
N V/s A	Male activity	62.8-58.2	4.2
	Female receptivity	57.4-73	15.6
N V/s C1	Male activity	62.8-62.8	0
	Female receptivity	57.4-54.6	2.8
N V/s C2	Male activity	62.8-61.2	1.6
	Female receptivity	57.4-42.6	14.8
N V/s C3	Male activity	62.8-51.2	11.6
	Female receptivity	57.4-59.8	2.4
N V/s C4	Male activity	62.8-52.6	10.2
	Female receptivity	57.4-61.4	4
A V/s C1	Male activity	58.2-62.8	4.2
	Female receptivity	73-54.6	18.4
A V/s C2	Male activity	58.2-61.2	2.6
	Female receptivity	73-42.6	30.4
A V/s C3	Male activity	58.2-51.2	7.0
	Female receptivity	73-59.8	13.2
A V/s C4	Male activity	58.2-52.6	5.6
	Female receptivity	73-61.4	11.6
C1 V/s C2	Male activity	62.8-61.2	1.6
	Female receptivity	54.6-42.6	12.0
C1 V/s C3	Male activity	62.8-51.2	11.6
	Female receptivity	54.6-59.8	5.2
C1 V/s C4	Male activity	62.8-52.6	10.2
	Female receptivity	54.6-61.4	6.8
C2 V/s C3	Male activity	61.2-51.2	10
	Female receptivity	42.6-59.8	17.2
C2 V/s C4	Male activity	61.2-52.6	8.6
	Female receptivity	42.6-61.4	18.8
C3 V/s C4	Male activity	51.2-52.6	1.4
	Female receptivity	59.8-61.4	1.6

Note: (N-*D. nasuta;* A- *D. albomicans*; C1-Cytorace 1; C2-Cytorace 2; C3-Cytorace 3; C4-Cytorace 4)

The 'Sexual selection' model predicts that the females could be selective about their mates (Anderson, 1994; Bateman, 1948), since females have a high proportion of their total reproductive effort at stake and so should avoid unfit matings with conspecifics and heterospecifics alike. Thus, one would predict that female would show a stronger conspecific mating preference than males. Clearly the prediction of greater female choosiness under sexual selection model supports our data, where female receptivity had played the main role than male mating ability in mating propensity analysis.

The present study has revealed the existence of genetic variability for the trait mating propensity among six races and greater divergence among females receptivity, which plays a predominant role during mating. Thus, the analysis of mating propensity has shown yet another facet of inter-racial divergence among the closely related members of the *nasuta-albomicans* complex of *Drosophila*.

Acknowledgements: We are grateful to Department of Science and Technology, New Delhi for funding.

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Glue protein pattern specificity in *Drosophila nasuta* and *D. albomicans* of *nasuta* subgroup of *Drosophila*.

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Introduction

Glue proteins are developmental stage specific and tissue specific secretory proteins, synthesized by larval salivary gland cells in *Drosophila* (Lane *et al.,* 1972). Just prior to pupation, they are ejected to the exterior and have been suggested to have a role in attaching the puparium to a solid surface (Fraenkel and Brooker, 1953; Korge, 1977; Rideliford, 1993). Glue protein patterns in *D. nasuta* subgroup members are simple, and the major glue fractions follow X-linked pattern of inheritance in a co-dominant fashion (Ramesh and Kalisch, 1988, 1989a). *D. nasuta* (2n = 8) and *D. albomicans* (2n = 6), belonging to the frontal sheen complex of *nasuta* subgroup of *Drosophila immigrans* species group are morphologically almost identical, allopatric sibling species. They are karyotypically diverged but cross fertile and hence termed as chromosomal races (reviewed in Ranganath, 2002). Further, Glue protein pattern analysis in different geographic strains for *D. nasuta* and *D. albomicans* was taken up.

Material and Methods

For the present study three different geographic strains of *Drosophila nasuta nasuta* and *D. n. albomicans*, namely: (1) *D. n. nasuta* (2n = 8; Coorg strain, India, 201.001); (2) *D. n. nasuta* (2n = 8, Kenya, Taiwan University collections, 0252.21); (3) *D. n. nasuta* (2n = 8, Seychelles, Taiwan University collections, 0252.22); (4) *D. n. albomicans* (2n = 6, Okinawa, Univ. of Texas collections, 3045.11); (5) *D. n. albomicans* (2n = 6, Taiwan, Taiwan University collections, 0215.1) and (6) *D. n. albomicans* (2n = 6, Thailand, Taiwan University collections, 0161.08) were employed. The culture maintenance, sample preparation, electrophoresis and documentation of gels, the pattern and volume analysis procedures are given in detail in Aruna and Ranganath (2005).

Results

Pattern analysis

Three strains of *D. nasuta* as well as three strains of *D. albomicans* were analyzed to get an overview of the pattern variations of major glue protein fractions. Overall 12 major glue protein fractions were noticed with 4 variant phenotypes among the six strains analyzed, with each member showing five fractions (Figure 1). Following Ramesh and Kalisch (1989), these fractions were grouped into four Domains (Table 1). There are no sex-specific differences as well as intra population variations in the glue protein patterns.

Domains I and IV were monomorphic with no variation in molecular weight of the fractions across the six members, whereas the fractions of Domains II and III were polymorphic at both, the inter-strain as well as inter-racial comparisons. Domain II was represented by a single fraction in all the strains/races, with a 43 kD fraction in four of the six strains analyzed, while it was 40 kD in

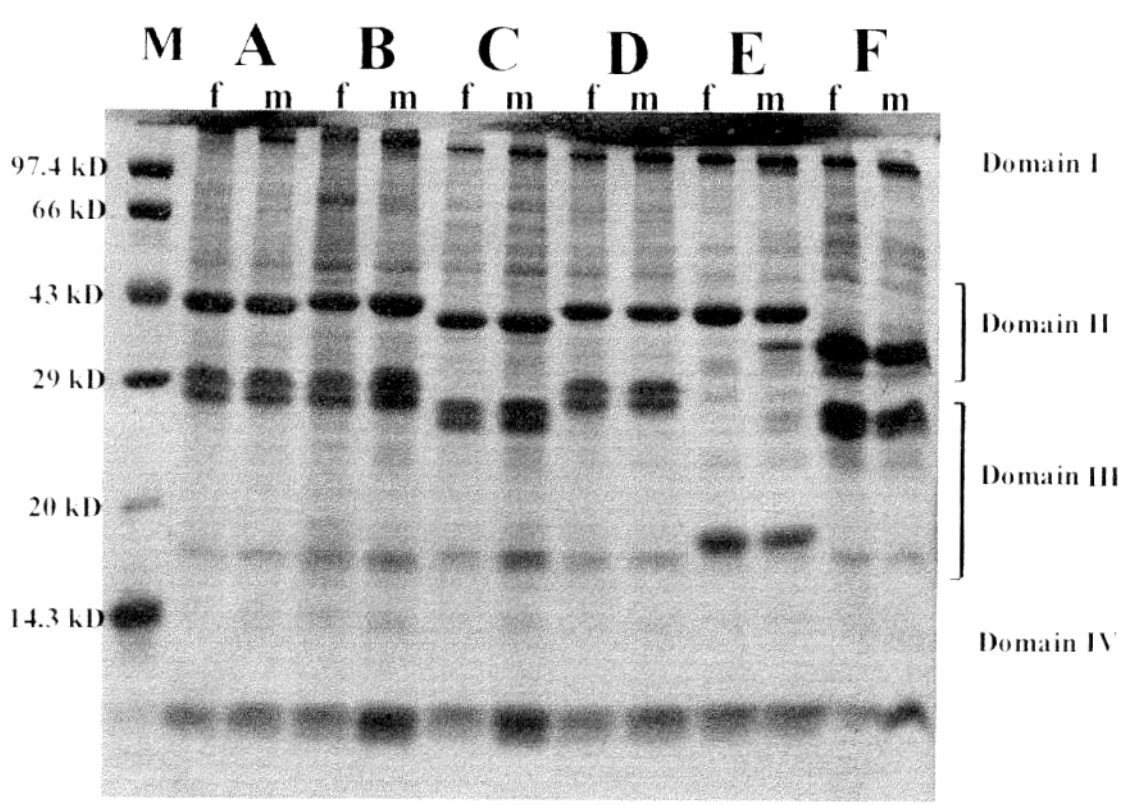

Figure 1. Glue protein patterns among different geographic strains for *D. n. nasuta* and *D. n. albomicans*. A, *D. n. nasuta* (Coorg); B, *D. n. nasuta* (Kenya); C, *D. n. nasuta* (Seychelles); D, *D. n. albomicans* (Taiwan); E, *D. n. albomicans* (Thailand); F, *D. n. albomicans* (Okinawa); M, Marker; f, female; m, male.

Table 1. Glue protein fractions among different geographic strains of *D. n. nasuta* and D. *n. albomicans*..

Strains	Total No. of Major fractions	Domains and respective kD values			
		I (>100 kD)	II* (<60 kD to <30 kD)	III* (<30 kD to <20 kD)	IV (14 kD)
D. nasuta (Coorg)	5	<100	43	30, 28	14
D. nasuta (Kenya)	5	<100	43	30, 28	14
D. nasuta (Seychelles)	5	<100	40	26.5, 25.5	14
D. albomicans (Okinawa)	5	<100	35	25.5, 23	14
D. albomicans (Taiwan)	5	<100	43	30, 28	14
D. albomicans (Thailand)	5	<100	43	18.5, 18	14

Seychelles (*D. nasuta*) and 35 kD in Okinawa (*D. albomicans*). Domain III was represented by a doublet in each strain/race, with 30 kD and 28 kD fractions among three of the six members analyzed, namely Coorg (*D. nasuta*), Kenya (*D. nasuta*) and Taiwan (*D. albomicans*), whereas 26.5 kD & 25.5 kD, 25.5 kD & 23 kD and 19 kD & 18 kD fractions were noticed in Seychelles (*D. nasuta*), Okinawa (*D. albomicans*), and Thailand (*D. albomicans*), respectively. Thus *D. n. nasuta* (Seychelles) had different glue protein patterns compared to *D. n. nasuta* (Coorg) and *D. n. nasuta*

(Kenya), whereas all the *D. n. albomicans* strains namely, Taiwan, Thailand, and Okinawa, had different patterns.

Overall, Coorg (*D. nasuta*), Kenya (*D. nasuta*), and Taiwan (*D. albomicans*) have identical patterns of major glue protein fractions and hence were clustered together in the dendrogram. Thailand (*D. albomicans*) forms an independent lineage from them, whereas Seychelles (*D. nasuta*) and Okinawa (*D. albomicans*) which share one of the Domain III fractions apart from the common >100 kD and 14 kD fractions clusters together (Figure 3). Hence, there is no strain/race specific glue protein pattern for *D. nasuta* or *D. albomicans*.

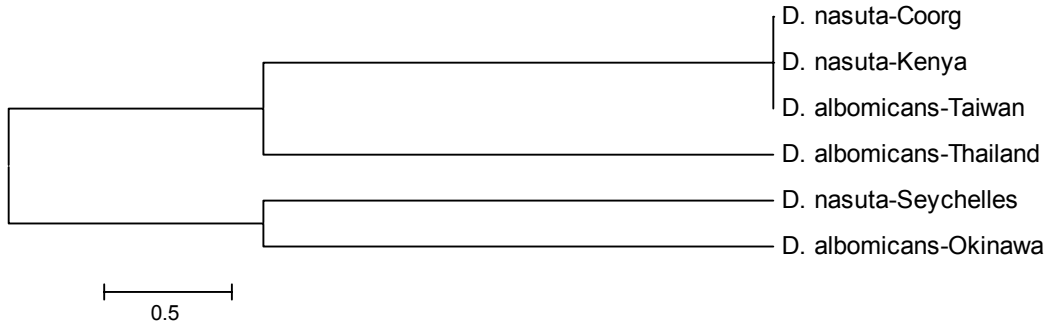

Figure 2. Dendrogram displaying the relationship among different geographic strains for *D. n. nasuta* and *D. n. albomicans* based on number of differences.

D. n. albomicans (Thailand)	*D. n. albomicans* (Taiwan)	*D. n. nasuta* (Kenya)	*D. n. nasuta* (Seychelles)	*D. n. nasuta* (Coorg)	*D. n. albomicans* (Okinawa)

Figure 3. DMRT clusters for the one way ANOVA analysis (SPSS 10.0) based on the values obtained through volume analysis of the major glue protein fractions among three strains of *D. n. nasuta* and *D. n. albomicans*. The members within the same cluster are non-significant, while those in separate clusters are significant. ($F = 169.847$; $df = 5, 294$).

Ramesh and Kalisch (1989) have suggested that glue protein patterns are species and strain specific based on their study involving different strains of *D. nasuta* and *D. albomicans*. On the contrary, though the strains employed have origins to those geographic regions that were employed by Ramesh and Kalisch (1989), our screening revealed that not only two strains (Coorg and Kenya) of *D. nasuta* were identical with respect to glue protein pattern but also one of the *D. albomicans* strains (Taiwan) showed a similar pattern. Here it is essential to note that the electrophoretic patterns of Coorg and Seychelles strains of *D. nasuta* and also the Okinawa strain of *D. albomicans* are identical to those established by Ramesh and Kalisch (1989). The present results clearly show that glue patterns are not strain/race specific thereby contradicting the suggestion of Ramesh and Kalisch (1989).

Volume analysis

A comparative semi quantitative analysis of overall five major glue protein fractions among the strains of *D. nasuta* and *D. albomicans* revealed that the sum intensity (total OD/mm of all the 5

fractions) values are highest in the Okinawa (*D. albomicans*) and least in the Thailand (*D. albomicans*) strain (Table 2). Among *D. n. nasuta* strains, sum intensities of glue protein fractions of Coorg was significantly higher than Kenya as well as Seychelles, while the latter two were non-significant among themselves. However, all the three *D. n. albomicans* strains, namely, Taiwan, Thailand, and Okinawa, were found to be significantly different from each other in their sum intensities of glue protein fractions (Figure 3). Among inter-racial comparisons, the quantitative differences of two *D. n. albomicans* strains (Thailand and Okinawa) were significantly different from all *D. n. nasuta* strains, while *D. albomicans* (Taiwan) was non-significant with Kenya and Seychelles strains of *D. nasuta*.

Table 2. OD values of the overall 5 major glue protein fraction in *D. nasuta* and *D. albomicans*.

Races	Female	Male	Glue protein	T-value
D. n. nasuta (Coorg)	35.00 ± 0.69[a]	32.85 ± 0.65[a]	33.93 ± 0.37[a]	1.879*
D. n. nasuta (Kenya)	32.82 ± 0.65[b]	29.03 ± 0.63[b]	30.92 ± 0.35[b]	3.550
D. n. nasuta (Seychelles)	33.37 ± 0.99[b]	30.0 ± 0.69[b]	31.68 ± 0.5[b]	2.843
D. n. albomicans (Taiwan)	31.67 ± 0.62[b]	29.72 ± 0.52[b]	30.7 ± 0.6[b]	3.207
D. n. albomicans (Thailand)	26.12 ± 0.63[c]	23.88 ± 0.77[c]	25.0 ± 0.4[c]	3.012
D. n. albomicans (Okinawa)	45.80 ± 0.5[d]	43.81 ± 0.44[d]	44.81 ± 0.5[d]	3.127
F- value	85.65*	112.274*	164.813*	
df	5, 144	5, 144	5, 294	24

Note: 1. Strains with same alphabet for each OD fraction are not significantly different at 5% level according to DMRT. 2. A total of 25 replicates for each fraction were screened for each race. 3. * : denotes significant. 4. T value is for the OD of X-linked fractions

The volume analysis suggests that the quantities of the major glue protein fractions are much more variable among *D. albomicans* geographic strains as compared to those of *D. nasuta*. Thus, consistent with pattern analysis, even the quantities of glue proteins are not strain/race specific. Further, the comparisons between the male and female OD values of X-linked fractions has shown that the OD values of major X-linked fractions in males are not 50% of the females, but they are synthesized almost to the levels of females (Table 2). In all the cases except *D. nasuta* (Coorg), there is no significant difference between male and female OD values and even in *D. nasuta*, males have almost 93.8% levels of their counterparts. Therefore, these fractions are dosage compensated.

Thus, both the qualitative (pattern analysis) as well as quantitative (volume analysis) results of the major glue protein fractions clearly indicate the lack of strain/race specificity, because of the overlapping patterns recorded in *D. nasuta* and *D. albomicans*.

Acknowledgments: We thank Prof. S. R. Ramesh and Dr. Ravi Ram, K for their help and advice. The authors acknowledge the financial assistance from DST, New Delhi.

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First record of subgenus *Phloridosa* of *Drosophila* in southern Brazil, with notes on breeding sites.

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Introduction

Flies from the genus *Drosophila* are very widespread throughout the world and are primarily consumers of the micro-organisms associated with the initial stages of decay of plant materials (Carson, 1971). The most common species feed and breed on decaying fruits, but some specialised drosophilids are not attracted to that kind of resource and remain undetected by the traditional banana-baited traps. Among them, there are several poorly studied flower-breeding species. *Phloridosa* is the only subgenus of *Drosophila* in which all species are exclusively flower-breeding (Brncic, 1983), comprising seven species distributed in the Neotropics. The geographical distributions of these species are inadequately known, especially because the lack of proper collections, which focus mainly in fruit-feeders species.

The study of the Brazilian drosophilid fauna increased greatly in the 1940's, with work conducted by Dobzhansky and Pavan (1943, 1950) and others. However, even after decades of researches, the biogeography of *Drosophila* species in the Neotropics is incompletely known.

Santa Catarina and Rio Grande do Sul, the two southernmost states of Brazil, have been target of several studies on drosophilids assemblages (Brncic and Valente, 1978; Araújo and Valente, 1981; Franck and Valente, 1985; Valente and Araújo, 1991; Saavedra *et al.*, 1995, in Rio Grande do Sul; De Toni and Hofmann, 1995; De Toni *et al.*, 2001; Döge *et al.*, 2004, in Santa Catarina). However, research on flower-breeding drosophilids in southern Brazil is restricted on *flavopilosa* group of *Drosophila* (Brncic, 1978; Napp and Brncic, 1978; Hofmann and Napp, 1984). It is also well-known the presence of *D. bromelioides* Pavan and da Cunha, but specific studies still are required. No previous work has reported the presence of subgenus *Phloridosa* in these states.

Materials and Methods

Flowers of *Brugmansia suaveolens*, *Cucurbita pepo* and six species of *Ipomoea* were collected between 27 October 2003 and 21 November 2004 from which the drosophilids were reared. The collection sites are listed in Table 1, as well as the plants collected in each site. The collection sites Itacorubi and *Campus* of UFSC are located in Santa Catarina Island. Armação and Antônio Carlos are continental points nearby the island. Porto Alegre is the unique site situated in the state of Rio Grande do Sul, and the flowers were collected in the surroundings of Arroio Dilúvio and Lago Guaíba.

The flowers were collected directly from the plants, placed at glass vials with the bottom covered with damp paper and closed with foam stoppers. After emergence, the imagines were identified. The number of flowers collected, the dates of collection and some additional information are available in the results.

Table 1. Collection sites and species of plants collected in each of them.

Collection site (Municipality, State)	Coordinates	Environment type	Plants collected
Itacorubi (Florianópolis, SC)	27°34'34"S; 48°31'23"W	Border of a mangrove forest	*I. cairica*, *I.* aff. *Chiliantha*
Campus of UFSC (Florianópolis, SC)	27°35'26"S; 48°30'49"W	Urban	*B. suaveolens*, *I. cairica*, *I. carnea fistulosa*, *I.* aff. *chiliantha*, *I. congesta*
Armação (Governador Celso Ramos, SC)	27°21'16"S; 48°32'21"W	Beach	*I. pes-caprae*
Antônio Carlos (Antônio Carlos, SC)	27°28'02"S; 48°51'01"W	Orchard	*C. pepo*, *I. batatas*, *I. congesta*
Porto Alegre (Porto Alegre, RS)	30°03'00"S; 51°13'60"W	Urban	*B. suaveolens*, *I. cairica*

SC: Santa Catarina; RS: Rio Grande do Sul.

Ipomoea flowers (Convolvulaceae), the morning glories, were the main target of the present investigation. *Ipomoea cairica* is a common creeper that grows spontaneously in open areas, as well as *I. congesta* and *I.* aff. *chiliantha*. *Ipomoea carnea fistulosa* is found as a cultivated plant in gardens, while *I. pes-caprae* is an inhabitant of frontal dunes of the beaches. *Ipomoea batatas* is the common sweet potato, and *Cucurbita pepo* (Cucurbitaceae), the pumpkin, familiar to vegetable gardens. *Brugmansia suaveolens* (synonym: *Datura suaveolens*) (Solanaceae), the angel's trumpet, is commonly found in riversides and swampy areas.

The drosophilids were identified by their external morphology and by genitalia features. Some flies were dissected after preparation with potassium hydroxide (KOH) 10% and acid fuchsine (Wheeler and Kambysellis, 1966). Aedeagus of *D. bromelioides* Pavan and da Cunha were compared to the paper of Val (1982), while aedeagus and surstylus of *D. denieri* Blanchard were checked with Vilela (1986), and aedeagus, surstylus and spermathecae of *D. lutzii* Sturtevant with Vilela (1984) and Vilela and Bächli (1990).

Results and Discussion

Two species of subgenus *Phloridosa*, never recorded in southern Brazil before, were found. *Drosophila lutzii* was collected in Santa Catarina and Rio Grande do Sul, from flowers of all the six species of *Ipomoea* surveyed and from *Brugmansia suaveolens*. *Drosophila denieri* was also collected in both states, emerged from flowers of *B. suaveolens* and *Cucurbita pepo*. In all the cases, they were associated with *D. bromelioides*, a common flower-breeding drosophilid in Brazil, that belongs to *bromeliae* group of subgenus *Drosophila*. The collections and number of imagines emerged from the flowers are shown in Table 2 (for *D. lutzii*) and Table 3 (for *D. denieri*).

The distribution of *D. lutzii* currently known includes Florida, California, Arizona (Sturtevant, 1942), Hawaii (Montague, 1984), Mexico (Patterson and Mainland, 1944; Chassagnard and Tsacas, 1992), Guatemala (Chassagnard and Tsacas, 1992), El Salvador (Heed, 1957), Costa Rica, Cuba, Jamaica, Puerto Rico (Sturtevant, 1942), Guadalupe (Chassagnard and Tsacas, 1992), Colombia (Wheeler, 1981) and Formosa (Argentina) (Vilela, 1986). Hsu (1949) showed a single specimen of *D. lutzii* from Brazil, but Vilela and Bächli (1990) stated that it was "probably misidentified, not *D. lutzii*". The specimens collected in the present work correspond to the redescription done by these authors; then, now it is secure to affirm that this species is really present in Brazil. In addition, the present record also extends its geographical range southwards, down to Porto Alegre (30°03'00"S).

Table 2. Collections in which emerged imagines of *D. lutzii* and *D. bromelioides*.

Date of collection	Number of flowers collected	*D. lutzii*	*D. bromelioides*
Brugmansia suaveolens (*Campus* of UFSC)			
21 Nov. 2004	3	5	10
Ipomoea batatas (Antônio Carlos)			
13 Jun 2004	30	3	1
02 Oct 2004	30	5	6
Ipomoea cairica (*Campus* of UFSC)			
16 Dec 2003	10	1	12
Ipomoea cairica (Itacorubi)			
27 Oct 2003	30	11	42
25 Nov 2003	12	24	9
25 Nov 2003	60*	42	34
29 Dec 2003	30	19	11
21 Jan 2004	30	35	21
Ipomoea cairica (Porto Alegre)			
28 Jun 2004	15	75	16
Ipomoea carnea fistulosa (*Campus* of UFSC)			
29 Dec 2003	30	5	31
Ipomoea aff. *chiliantha* (*Campus* of UFSC)			
29 Dec 2003	30	5	29
Ipomoea aff. *chiliantha* (Itacorubi)			
21 Jan 2004	30	20	6
Ipomoea congesta (*Campus* of UFSC)			
29 Dec 2003	30	13	36
Ipomoea congesta (Antônio Carlos)			
06 Jan 2004	30	10	1
Ipomoea pes-caprae (Armação)			
18 Jan 2004	30	7	37

*****In this collection, the flowers were taken from the ground, in an early stage of decaying.

Table 3. Collections in which emerged imagines of *D. denieri* and *D. bromelioides*.

Date of collection	Number of flowers collected	*D. denieri*	*D. bromelioides*
Brugmansia suaveolens (Porto Alegre)			
28 Jun. 2004	4	5	8
Cucurbita pepo from (Antônio Carlos)			
17 Dec. 2003	5	18	9

As seen, the distribution of *D. lutzii* is fragmented, which suggests that this fly is also present in other areas. For example, it remains undetected in the huge area between Formosa (Argentina), Santa Catarina and Rio Grande do Sul (Brazil), in the south, and Colombia, in the north. As its breeding sites are present in that region and there is no evident climatic restriction, it may be predicted that, if proper collections be carried out, *D. lutzii* will be found in a great part of South America. It also may be introduced in some regions, as happened in Hawaii, where it probably arrived by way of rotting squash plants and found suitable resources (Montague and Kaneshiro, 1982).

This species was already known to breed in flowers of *Cucurbita pepo* (Sturtevant, 1942; Patterson and Mainland, 1944) and in other species of *Brugmansia*, *B. arborea* and *B. candida* (Sturtevant, 1942; Chassagnard and Tsacas, 1992; cited as *Datura arborea* and *D. candida*). Sturtevant (1942) and Patterson and Mainland (1944) also reported it in *Ipomoea*, but the species of the plants were not identified. After that, Montague (1984) reared it from *I. acuminata* in Hawaii and Chassagnard and Tsacas (1992) from *I. tiliacea* in Mexico. It was also collected in flowers of *Hibiscus* sp., *Cucurbita maxima* (Patterson and Mainland, 1944), *Gossypium* sp., *Zantedeschia* sp. (Sturtevant, 1942), *Solandra nitida*, *Allamanda* sp. (Chassagnard and Tsacas, 1992), melon and fruits of tomato (Sturtevant, 1921). *Brugmansia suaveolens* and the six species of *Ipomoea* collected in the present work represent new breeding sites known for *D. lutzii*.

On the other hand, *D. denieri* seems to have a more restricted distribution. It was described by Blanchard (1938), with flies collected in Formosa, Argentina, and after that, it was collected by only two other works. Frota-Pessoa (1952) found it in Rio de Janeiro, Brazil, and Goñi *et al.* (1998) in some localities in Uruguay. Southern Brazil is located among those localities, and this species was not collected before probably merely because the lack of appropriate collections.

Few breeding sites are known for *D. denieri*. In the original description (Blanchard, 1938), the imagines were caught in flowers of *Gossypium* sp. The specimens collected by Frota-Pessoa (1952) were found in *B. arborea*, *C. pepo* and *Cordia superba* as preadults, while Goñi *et al.* (1998) also found them in *C. pepo* and as adults flying over "mixed natural and cultivated vegetation". So, *B. suaveolens* represents a new breeding site known for the species.

D. bromelioides, the species of fly that, in this work, was always found sharing the flowers with the *Phloridosa* species, had already been collected in Santa Catarina (De Toni *et al.*, 2001) and Rio Grande do Sul (Petersen, 1960), the southernmost area of its known distribution. It is a generalist flower-breeding fly, making use of a great variety of flowers (Frota-Pessoa, 1952). Nonetheless, *B. suaveolens* and the six *Ipomoea* species surveyed here are new additions to the roll of known breeding sites for the species. Besides them, a single specimen of *D. mediostriata* Duda emerged from the decaying flowers of *I. cairica* (from Itacorubi, in 25 Nov, 2003), one individual of *D. mercatorum* Patterson and Wheeler developed in *I. pes-caprae* (from Armação, in 18 Jan, 2004) and one individual of *Zygothrica* sp. from *I. batatas* (from Antônio Carlos, in 13 Jun, 2004). These occurrences may be considered as occasional.

It is well-known that flowers of *Brugmansia*, *Cucurbita* and *Ipomoea* are utilized by drosophilids in many regions. *Brugmansia* and *Cucurbita* are used especially by *D. florae* Sturtevant (Sturtevant, 1921), *D. bromelioides* (Frota-Pessoa, 1952), species of subgenus *Phloridosa* (Sturtevant, 1921; Brncic, 1962), *Scaptomyza* (Brncic, 1983) and *Scaptodrosophila aterrima* complex (Burla, 1954; Graber, 1957). *Brugmansia* is also utilized by *Laccodrosophila* (Wheeler, 1968) and *Zapriothrica* (Wheeler, 1956, 1959).

With the information obtained by the present work, we can suggest that *D. lutzii* and *D. bromelioides* are the South American ecological equivalents of the Hawaiian *Scaptomyza* species (Hardy, 1966; Heed, 1968) and of the African *Scaptodrosophila aterrima* complex (Lachaise and Tsacas, 1983), occupying the niche offered by *Ipomoea* flowers. Furthermore, something like twenty

species of drosophilids of five genera were already found making use of *Ipomoea* species in America, Africa, Australia, and Hawaii. The six species of *Ipomoea* treated here raise to at least 14 the number of species of this genus that are utilized by drosophilids. This number may be significantly higher, not only if one considers the unidentified species of *Ipomoea* seen in literature, but also looking upon a lot of other species still never surveyed. The same assumption may be done in respect to the number of drosophilid species that feed and breed in these flowers, with different levels of specialisation.

Acknowledgments: We are sincerely grateful to Prof. Daniel de Barcellos Falkenberg, from Departamento de Botânica of Universidade Federal de Santa Catarina (UFSC), for the identification of *Ipomoea* species.

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Non toxicity of phenylpropanoid verbascoside from a *Buddleja scordioides* extract in *Drosophila melanogaster flare* and Oregon-*flare* strains and toxicity of caffeic acid in the *flare* strain.

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Introduction

The UV-C (100-290 nm), UV-A (320-400 nm) and the increasing intensity of UV-B (290-320 nm) radiation have been reported to damage human skin, producing carcinogenic and immunosuppressive effects (Kerr, 1998). This can be avoided with sunscreens made from plant extracts that protect from UV radiation. In Mexico's Chihuahua desert (Rzedowski and Rzedowski, 1985) the community of Doctor Arroyo, Nuevo León use aerial parts from the shrub *Buddleja scordioides* HBK (Buddlejaceae) to prepare water-alcohol extracts as a traditional sunscreen (Ávila-Acevedo *et al*., 2005). The methanolic extract of *B. scordioides* contains the phenylpropanoid glycoside verbascoside (VER), a molecule composed of 2-(3, 4-dihydroxyphenyl) ethanol and a cafeoil group each one joined to one β-glucose with an ester link in C1 and C4, respectively. The glucose residue is linked to L-rhamnose in C3 position (Figure 1).

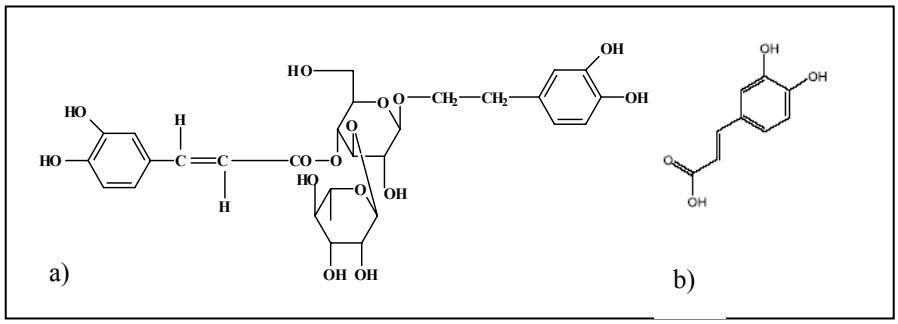

Figure 1. Chemical structures of: a) verbascoside (VER), and b) caffeic acid (CA).

VER exhibits potent *in vitro* activity against respiratory syncytial virus (Chen *et al*., 1998; Kernan *et al*., 1998). It exerts antibacterial and antimicrobial activity against *Staphylococcus aureus* and *Proteus mirabilis*, respectively (Didry *et al*., 1999). Its lethal effect on *S. aureus* is due to protein synthesis inhibition and has also shown to be toxic for bacteria in a mean concentration of 800 µl/ mL (Ávila-Acevedo, 1999a, b).

Structure-activity studies have revealed that the ortho-dihydroxy aromatic systems of phenylpropanoid glycosides are necessary for their cytotoxic and cytostatic activities. Phenylpropanoid glycosides containing caffeic acid, such as VER, show cytotoxic and cytostatic activities against several kinds of cancer cells; however, they do not affect primary-cultured rat hepatocytes (Saracoglu *et al*., 1995). VER shows a tumor growth inhibition activity mediated by

epidermal growth factor-R (EGF-R) tyrosine kinase inhibition (Kunvari *et al*., 1999). The antitumor activity *in vitro* might be due at least in part to inhibition of protein kinase C (PKC) (Herbert *et al*., 1991) and it has been proposed it could reverse MGc80-3 cells' malignant phenotypic characteristics and induce redifferentiation (Li *et al*., 1997). On the other hand, it has been demonstrated that VER inhibits telomerase activity in tumor cells (Zhang *et al*., 2002) and has been determined as a neurosedative drug (Seidel *et al*., 1998).

VER is a strong inhibitor of Cu^{2+} induced LDL oxidation (Seidel *et al*., 2000), it has a potent radical scavenging activity (Gao *et al*., 1999) and photoprotective effect against UV rays (Ávila-Acevedo *et al*., 2005, 1999; Ávila-Acevedo, 1999). Until now, the anti-carcinogenic, anti-mutagenic and anti-oxidant mechanisms of VER are not clear, but it was recently demonstrated it exerts protection against Fenton reaction on plasmid pBR322 (Zhao *et al*., 2005). At present we do not know data available on its *in vivo* toxicity effects on any metazoan.

In vitro chemical hydrolysis or *in vivo* metabolism esterases and glucosidases hydrolysis of each VER molecule could generate one caffeic acid (3, 4-dihydroxycinnamic acid) (CA) molecule, one 2-(3, 4 dihydroxyphenyl) ethanol, one glucose and ramnose. CA has been reported to have anticarcinogenic activities (Tanaka *et al*., 1993), but it is also considered a potential carcinogenic compound to humans, listed in Group 2B by IARC (1993). Furthermore, it has shown tumorigenic activities at low dietary levels in rats (Hirose *et al*., 1997). In rat hepatocytes, cytochromes P450 (Cyp1A1/2) catalyze the detoxification route of *O*-demethylation of ferulic acid, a CA product of *O*-methylation by catechol-*O*-methyltransferase. Also, the NADPH/P450 is in the pathway of *o*-quinone formation, but Cyp2E1 catalyzes glutathione conjugate formation (Moridani *et al*., 2002).

It has been demonstrated that *Drosophila melanogaster* Cyp450 enzymes are similar to those in the S9 fraction of mammalian liver (Hällstrom *et al.,* 1984). Danielson *et al*. (1998, 1997) have also shown there are strong regional homologies between the Cyp6 family of *D. melanogaster* and the Cyp3 family of vertebrates, and Saner *et al*. (1996) characterized the *Cyp6A2* gene of *D. melanogaster* Oregon-*flare* strain used in the *Drosophila* wing spot test HB cross (Graf and Singer, 1992).

Considering VER could be used as a good sunscreen because its SPF is higher than 15 and it has antioxidant and wound healing properties (Mensah *et al.,* 2001), we decided to screen for toxicity of VER and CA. *Drosophila melanogaster* larvae from the *flare* and Oregon-*flare* strains, with regulated and highly constitutive levels of Cyp450 enzymes, respectively, were fed with different concentrations of VER or CA to determine mortality percentages. Afterwards, we performed genotoxicity experiments with the *Drosophila* wing spot test (Graf *et al.,* 1984; Graf *et al.*, 1989; Graf and van Schaik, 1992) (data not published). Surprisingly, when wings were being dissected under a stereo microscope, we observed dark brown spots in the anteroabdominal region of flies emerged after larvae were chronically fed (48 h) with VER. Such spots could represent the VER parental compound. Therefore, in order to reveal the molecular identity of the spots, HPLC determinations were done. This paper shows the toxicity and HPLC results.

Materials and Methods

Plants material

The aerial parts of *B. scordioides* were collected in the vicinity of Huizache, San Luis Potosí, México in April 2001 and identified by Professor Guillermo Ávila-Acevedo. A voucher specimen (IZTA 26140) was deposited in Izta Herbarium, FES-Iztacala-National University of Mexico.

Chemicals

Ethanol (C_2H_5OH), methanol (CH_3OH), hexane [$CH_3(CH_2)_4CH_3$], dichloromethane (CH_2CL_2), Silica gel 60 F_{254} (particle size 0.063-0.200 mm, mesh 70-230 ASTM) and aluminium sheets silica gel 60 F254 precoated (0.2 mm layer thickness) were purchased from Merck, (Darmstadt, Germany). Caffeic acid (99%, CAS No 331-39-5) was purchased from Sigma (St Louis, MO), and *Drosophila* Instant Medium was purchased from Carolina Biological Supply (Burlington, NC. USA).

Verbascoside isolation

Dried and powdered aerial parts of *B. scordioides* (2200 g) were extracted four times by maceration with methanol for 5 days. The methanolic portion was evaporated to obtain a crude syrup (303.10 g), which was chromatographed over silicagel, eluting with CH_2Cl_2-CH_3OH (19:1) followed by increasing concentration of methanol; fraction collected with CH_2Cl_2-CH_3OH (8:2) afforded a pure amorphous powder (10.2 g), $[\alpha]_D^{+22^o}$ -41.5 (MeOH; *c* 1.1). UV $\lambda_{máx}^{MeOH}$ (nm): 208 (4.30), 217 (4.45), 292 (4.10), 329 (4.25). IR $V_{máx}^{KBr}$ (cm^{-1}): 3400 (OH), 2932 (C-H), 1701 (conj. ester), 1631 (C=C), 1604, 1521 (aromatic ring). ^{1}H NMR (DMSO-d_6, TMS) δ ppm: 0.97 (*d*, *J* = 6 Hz, 3H, Me of Rha), 2.70 (*t, J* = 7 Hz, 2H, Ar-C<u>H</u>$_2$-CH$_2$), 4.36 (*d*, *J* = 7.2 Hz, 1H, H-1 of Glc), 4.72 (*t*, *J* = 9.6 Hz, 1H, H-4 of Glc), 5.03 (*d*, *J* = 1.1 Hz, 1H, H-1 of Rha), 6.21 (*d*, *J* = 15.8 Hz, 1H, Ar-CH=C<u>H</u>), 6.49-7.01 (6H, aromatic H), 7.47 (*d*, *J* = 15.8 Hz, 1H, Ar-C<u>H</u>=CH), 8.69, 8.75, 9.19, 9.62 (4 x OH). Analytical data were identical to those published for verbascoside (Zimin and Zhongjian, 1991).

VER and CA concentrations

Although the optimal application for a sunscreen is 2.0 mg/cm^2 (Griffin *et al.,* 1997), real-life sunscreen application thickness averages from 0.5 to 1.0 mg/cm^2 (Wulf *et al*., 1997). Considering a reapplication every 30 minutes, the average 70 kg person (Scherschun and Lim, 2001) would take 6.25 to 12.5 g to cover all skin surface. If VER were to be used as a sunscreen for two hours protection (namely, four applications) total average amounts will represent a skin direct exposition equivalent to 25 to 50 mg/cm^2 of VER. Therefore, treatments were designed to test toxicity in a concentration range from 0 to 93.6 mg/mL of VER and CA.

***Drosophila melanogaster* mutant strains**

Three independent experiments with three replicates/ concentration were done with two mutant strains: *flare* and Oregon-*flare,* carrying the *flr*^{3}marker. This marker, a recessive mutation, is located on the left arm of chromosome 3: *flare-3* (*flr*^{3}, 3-38.8). The *flr*^{3} allele is kept over a balancer chromosome carrying multiple inversions [*In(3LR)TM3*] and a dominant marker which is homozygous lethal (*Bd*^{S}, *Beaded-Serrate*, serrate wings) (Graf and Singer, 1992). The Oregon-*flare* strain carries chromosomes 1 and 2 of the DDT resistant Oregon strain (Frölich and Würgler, 1989).

Larvae treatment

Adult flies were grown at 25ºC and aged in culture bottles containing mashed potato flakes medium (Dueñas *et al*., 2001). Eggs from the two strains were collected for 8 h in vials (250 mL) containing a thick layer of fermenting live baker's yeast supplemented with sucrose at 25°C, relative 60% humidity and dark conditions (Graf *et al*., 1991). Three days later, the larvae (72 ± 4 h) were washed out of the vials with tap water at room temperature through a fine-meshed stainless steel strainer, collected and thoroughly washed free of yeast while still in the strainer. Equal amounts of

larvae were fed until pupation (48 h) at 25°C and 60% humidity with 2 mL of 2 % ethanol, solutions (0, 0.39, 3.9, 39, 62.4, 93.6 mg/mL) of VER extract from *B. scordioides* diluted in 2% ethanol or CA plus 0.5 g of *Drosophila* Instant Medium in culture vials. The treatment vials were kept at 25ºC and a relative humidity of 60%. After emerging, living adult flies were collected from the feeding vials. To determine toxicity parameters: LC_{50}, potency (lineal slope) and sensitivity (origin ordinate) in the *flare* and Oregon-*flare* strains, polynomial regression analysis were done for both compounds. The results were analyzed with one-way analysis of variance (ANOVA) where F test was calculated for statistically significant differences in LC_{50}, strain, potency and sensitivity between strains and compounds.

Molecular identification by HPLC of compounds from dark spots in HB cross flies midgut

To assess genotoxicity of VER with the *Drosophila* wing spot test (Graf *et al.*, 1989; Graf and van Schaik, 1992) (data not published), chronic feeding treatments until pupation (48 h) were made by adding equal batches of larvae to vials with 2 mL solution of 0, 16.9, 50.5 or 84.2 mg/mL of VER diluted in 2% ethanol and 0.5 g of *Drosophila* Instant Medium. The adult living flies were fixed in 70% ethanol. While inspecting them under a stereo microscope, we observed that all flies from the HB cross (Figure 2) and some from the ST cross (not shown) presented dark spots in the anteroabdominal region, probably indicating the presence of the VER parental molecule. High Pressure Liquid Chromatography (HPLC) was done in a HP series 1100 (with a diode-array detector) to detect the presence of VER and traces of CA, its putative metabolite, in fly samples from the HB cross fed with VER. Thirty emerged adults from each VER treatment (0, 16.9, 50.5, 84.2 mg/mL) in the HB cross were homogenized in ethanol (HPLC grade) and centrifuged (Eppendorff 5415C) at 14,000 rpm for 10 min; supernatant was dried in vacuum during three days and diluted in 200 µl of methanol (HPLC grade). Standards of 20 µl were prepared from VER (1.02 mg/ mL = 20 µg), CA (3.15 mg/ mL = 63 µg, HPLC grade) and methanol (HPLC grade). Detection was done at 330 nm and the range was of 200 to 400 nm; the mobile phase used was methanol:phosphate (30:70) buffer 60 mM, pH 6.7; the column was Discovery C18 (25 cm × 4.6 mm x 5 µm) and the flux of 1 mL/ min.

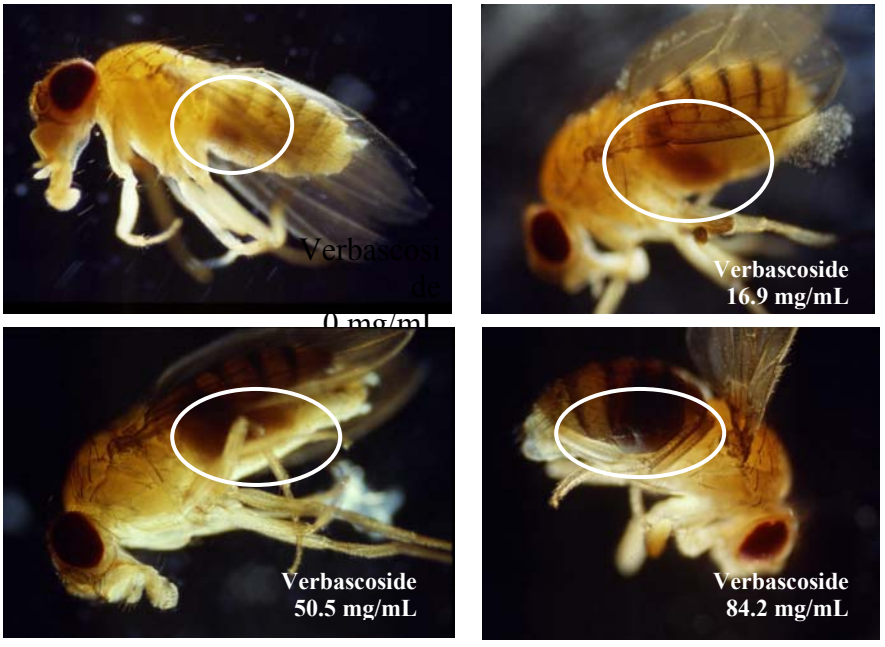

Figure 2. Dark spots found in the HB cross flies emerged after larvae were fed (48 h) with VER (16.9, 50.5, 84.2 mg/mL).

Results and Discussion

Toxicity parameters

Results fit polynomial curves of second order equations for all treatments and toxicity parameters were obtained from them (Table 1). Oregon-*flare* F test results did not show statistically

significant differences between compounds ($p > 0.05$). F test results for the *flare* strain fed with CA were statistically different from VER treatment and from the Oregon-*flare* strain treatment with VER and CA ($p < 0.05$). The lineal slope analysis (potency) of the *flare*/caffeic acid regression (Figure 3a) showed statistical significant differences ($p = 0.0039$). However, the Oregon-*flare*/caffeic acid (Figure 3b), *flare/*verbascoside (Figure 3c) and Oregon-*flare*/ verbascoside (Figure 3d) regressions showed no statistically significant differences between them. On the other hand, the origin ordinate analysis (sensitivity) did not reveal statistically significant differences ($p > 0.005$) between strains and compounds.

Table 1. LC_{50} (oral), origin ordinate (sensivity), lineal slope (potency), and r^2 from quadratic equations obtained for the *flare* and Oregon-*flare* strains of *Drosophila melanogaster* after 48 h feeding of 72 ± 4 h larvae, with VER and CA solutions. Nr = not reached.

	Origin ordinate	Lineal slope	CL_{50}	Equation	r^2
Verbascoside					
flare	1.658	0.0364x	Nr	$y = -0.0002x^2 + 0.0364x + 1.658$	0.369
Oregon-*flare*	1.948	0.0387x	Nr	$y = -0.0002x^2 + 0.0387x + 1.948$	0.931
Caffeic acid					
flare	1.090	- 0.0216x	70.5 mg/mL	$y = 0.0005x^2 - 0.0216x + 1.090$	0.936
Oregon-*flare*	0.633	0.0279x	Nr	$y = -8E\text{-}05x^2 + 0.0279x + 0.633$	0.989

CA: In the *flare/*caffeic acid treatment we obtained a LC_{50} of 70.5 mg/mL (Table 1). The mortality percentage could be explained, because Cyp450 enzymes are regulated in the *flare* strain. In contrast, the low mortality percentage caused by CA in the Oregon-*flare* strain did not allow to reach any LC_{50} value. This indicates that the highly constitutive Cyp450 enzymes could have been enough to detoxify this strain. Now, considering there are homologies between the Cyp6 family of *D. melanogaster* and the Cyp3 family of vertebrates (Danielson *et al*., 1998, 1997), and taking into account that in rat hepatocytes Cyp1A1/2 catalyze the CA detoxification route of *O*-demethylation and that Cyp2E1 catalyzes *o*-quinone-glutathione conjugate formation, we could also explain the differences between the mortality percentages in both strains.

VER: The r^2 value = 0.369 in the *flare* strain does not show a dose response polynomial curve of second order (Figure 3c) (Table 1), therefore, LC_{50} was not reached. In contrast, the mortality percentage in the Oregon-*flare* strain (Figure 3d) fit a second order equation, but LC_{50} was not reached either (Table 1). These results point out no toxicity of VER in these *D. melanogaster* strains.

Supposing that VER hydrolysis generates CA, we would expect the same response for both compounds in the same strain. This was only the case in the Oregon-*flare* strain maybe due to its highly constitutive levels of Cyp450s which metabolized both compounds efficiently or because enzymatic hydrolysis did occur. On the contrary, when comparing the toxicity results of the *flare* strain, no evidence of VER hydrolysis was found.

HPLC data analysis

The VER standard solution showed an absorption peak of 330 nm (Figure 4a). In the range of detection (200 to 400 nm) the CA standard solution showed an absorption peak in the UV spectroscopy at 290 nm (trace not shown). The CA standard trace at 330 nm (Figure 4b) was different from any other traces obtained from the 0, 16.9, 50.5 and 84.2 mg/ml homogenized fly samples. So, the absence of this compound was confirmed. This might be explained in two ways: 1) CA was

generated and quickly metabolized, which agrees with Moridani *et al*. (2002), or 2) VER metabolism did not generate CA, which agrees with our toxicity results for the *flare* strain.

Other absorption peaks at 270 nm were observed in HPLC plots corresponding perhaps to other unidentified compounds. Less retention time absorption peaks found in fly samples at 330 nm (left in Figure 4d) were amplified (Figure 4c) and we confirmed they corresponded to methanol used to dilute fly samples. Absorption peaks of 16.9, 50.5 and 84.2 mg/mL flies samples were superimposed to compare them (Figure 4d). In the 16.9 mg/mL sample, VER traces were observed, showing that it was metabolized during treatment. VER clear absorption peak at 330 nm was present only in the 50.5 and 84.2 mg/mL samples. The 84.2 mg/mL sample had a relative proportion area bigger than the other, as expected, considering the VER solutions added to the *Drosophila* feeding media and the dark spots intensity found in flies of each treatment.

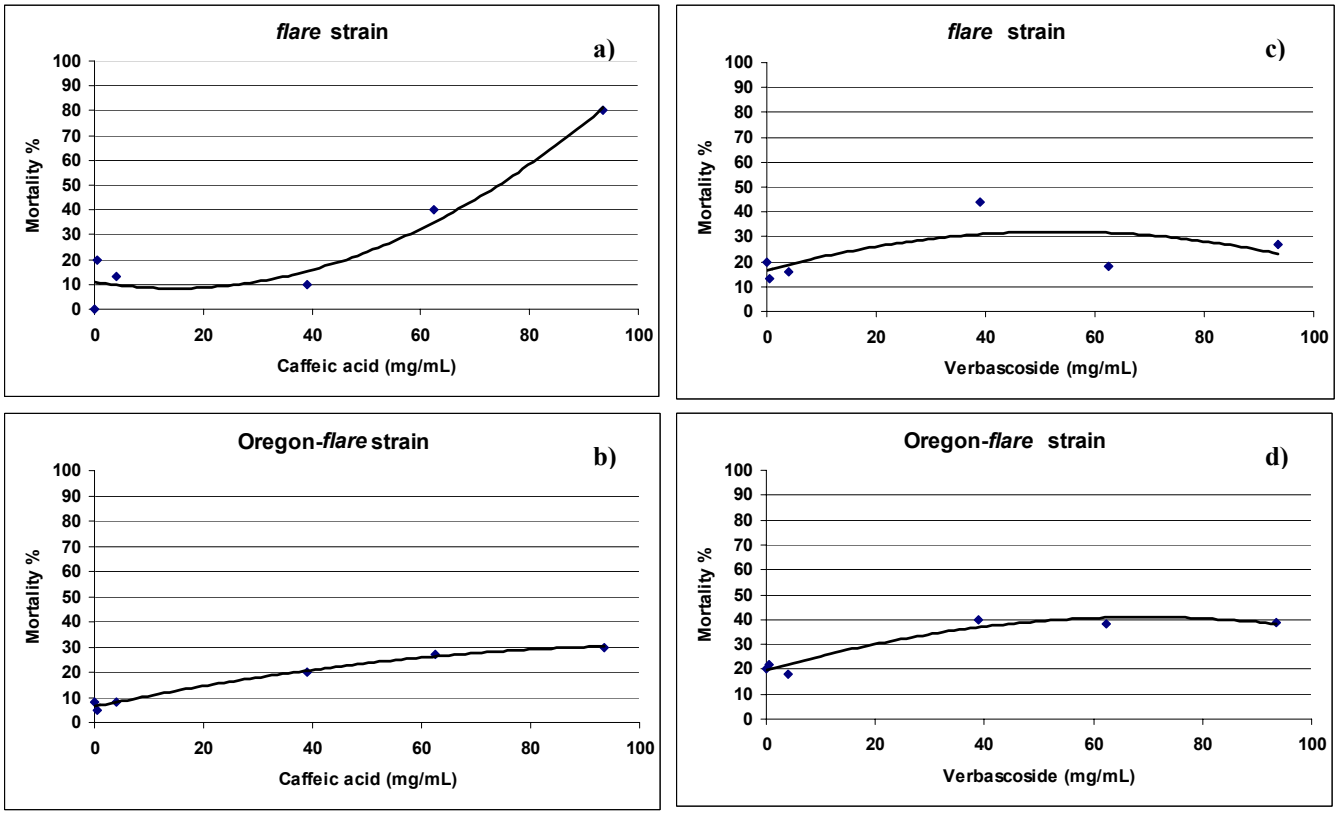

Figure 3. Mortality percentages of the *flare* and Oregon-*flare* strains of *Drosophila melanogaster* after 48 h feeding of 72 ± 4 h larvae with VER or CA. Each point is the average of three independent experiments and replicates.

Conclusions

Although VER showed low toxicity in the *flare* and Oregon-*flare* strains of *D. melanogaster*, presenting Cyp450 enzymes similar to those in the S9 fraction of mammalian liver (Hällstrom *et al.,* 1984), it is necessary to assess its genotoxicity in a wider range of VER and CA concentrations using the standard (ST) and high bioactivation (HB) crosses of the *Drosophila* wing spot test. CA did not show a clear dose response in the *flare* strain, with regulated Cyp450s, which in turn supports the idea of an efficient xenobiotic metabolism of CA in the Oregon-*flare* strain. We did not find enough

evidence to prove the alleged generation of CA by VER hydrolysis. The anteroabdominal dark spots in the emerged flies from the wing spot test HB cross corresponded to VER. The HPLC results demonstrated the absence of traces of CA in the HB cross flies fed with VER. We conclude that maybe due to their big size, undigested VER molecules accumulated in the larvae´s midguts, in a *relative* correlation with the VER concentrations added to the *Drosophila* feeding media. In accordance with the fact that low mortality was shown in the toxicity experiments for VER using the Oregon-*flare* and *flare* strains, HB cross emerged flies seem to have accumulated it without evident damage.

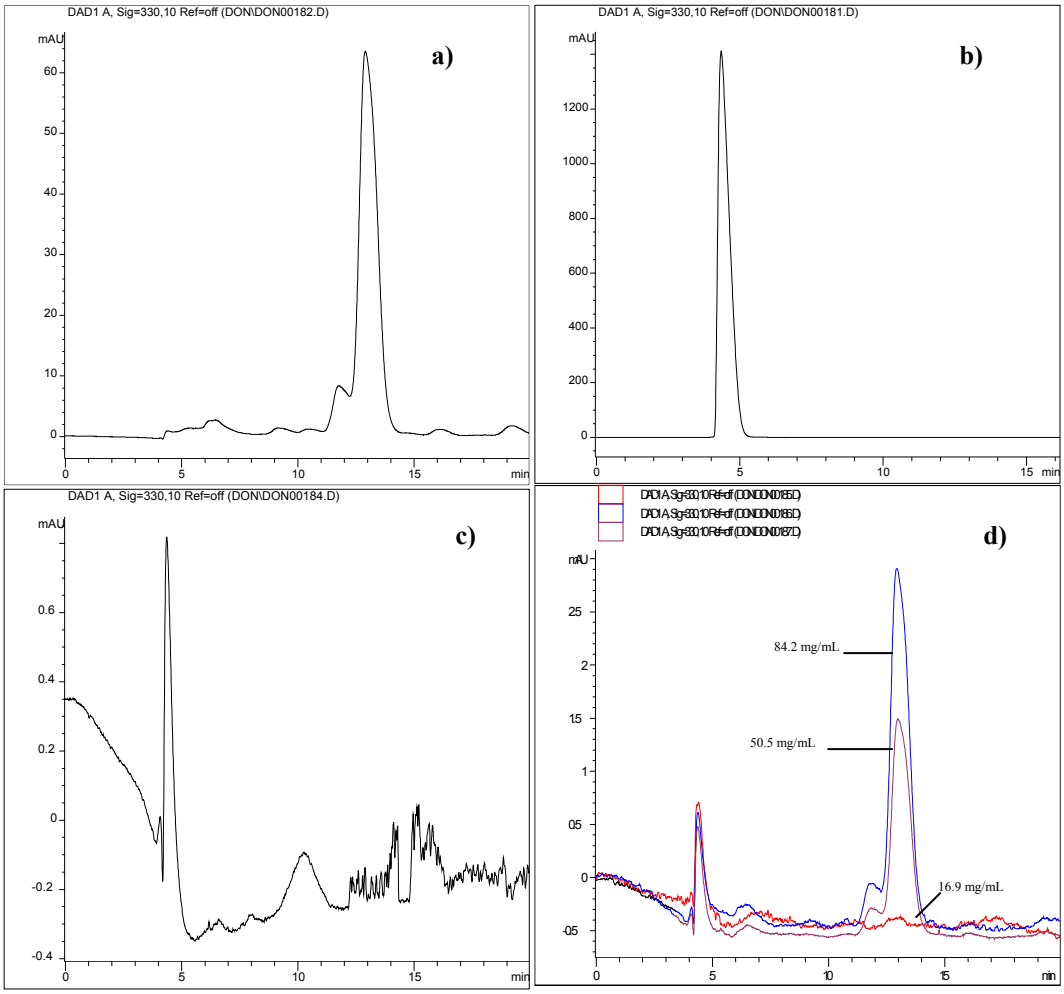

Figure 4. HPLC profiles of the methanolic-extract of emerged flies after feeding larvae 72 ± 4 h from the HB cross of the *Drosophila* wing spot test. Experimental conditions: column, Discovery C_{18} (25 cm x 4.6 mm, 5µm); mobile phase: methanol:phosphate (30:70) buffer 60 mM, pH 6.7; sample injection volume: 20 µL; flow rate: 1.0 mL/ min; detection: 330 nm. a) VER standard 1.02 mg/mL; b) CA standard 3.15 mg/mL; c) methanol; d) VER detection in flies samples from the HB cross fed with 16.9, 50.5 and 84.2 mg/mL.

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Two cryptic Cys3His zinc fingers in the alternative splice *muscleblind* C transcript.

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Muscleblind (Mbl) proteins are tissue-specific alternative splicing regulators that bind pre-mRNA transcripts through evolutionarily conserved tandem Cys3His zinc finger domains (herein

abbreviated as CCCH; reviewed in Pascual *et al.*, 2006). Muscleblind proteins are required for the terminal differentiation of muscle and photoreceptor cells in *Drosophila* (Begemann *et al.*, 1997; Artero *et al.,* 1998) and in mice (Kanadia *et al.*, 2003). In humans, Muscleblind orthologs (MBNL1, MBNL2 and MBNL3) aberrantly bind to mutant mRNAs containing large non-coding CUG and CCUG repeat expansions, which are etiological agents in neuromuscular disorders such as myotonic dystrophies. Through mechanisms not completely identified yet, expression of transcripts containing non-coding CUG/CCUG repeat expansions mimics *muscleblind* loss-of-function phenotypes in humans, leading to missplicing events that explain several symptoms of myotonic dystrophies.

Drosophila muscleblind primary transcripts undergo alternative splicing, giving rise to four mRNAs encoding protein isoforms MblA to MblD. MblA, MlbB and MblC show two CCCH-type zinc fingers with a typical spacing between the zinc-binding moieties of $CX_7CX_6CX_3H$ (referred to as zinc fingers type 6, or "ZF6"), whereas MblD contains only one zinc finger. Human MBNL proteins, in contrast, contain two pairs of CCCH-type zinc finger motifs, each pair consisting of motifs with zinc-binding residues spaced $CX_7CX_6CX_3H$ and $CX_7CX_4CX_3H$ (the last one referred to as zinc finger type 4, or "ZF4"). Previously, we reported that Muscleblind orthologs from protostome species contained one pair of zinc fingers of a ZF6-ZF6 organization, while deuterostomes contained two pairs, each of them with a ZF6-ZF4 organization. It has been suggested that the second pair of CCCH-type zinc fingers might have appeared by duplication of the first pair, which is currently detected in protostomes. Alternatively, ancestral Muscleblind proteins might have had four CCCH-type domains that protostomes reduced to two by deletion of the intervening sequences (Pascual *et al.*, 2006).

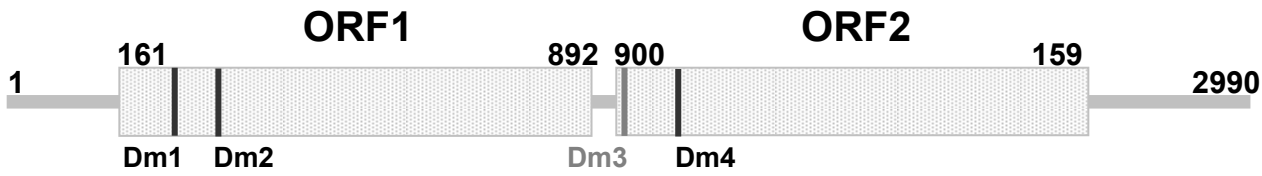
Results and Discussion

Here we show that *Drosophila mblC* transcripts, in addition to their canonical open reading frame (ORF1), contain a second ORF immediately downstream (herein referred to as ORF2; Figure 1A). Because ORF1 ends with two consecutive stop codons, and a +1 frameshift separates both ORFs, ORF2 does not seem to be actually translated. Abnormal pre-mRNA splicing or editing events that would let translation take place are unlikely since sequencing of several cDNA clones and analysis of available EST (GenBank CO318773) all included the frameshift (Begemann *et al.*, 1997, and this work). ORF2, however, potentially encodes 232 amino acids. ORF2 protein is characterized structurally by an additional ZF4-type zinc finger, similar to those found in vertebrate MBNL proteins, as well as several low-complexity regions including alanine, glutamine and serine-rich regions (Figure 1B). In addition, we recognized that ORF2 protein started with two cysteine residues spaced four-amino acids apart, and one histidine residue three-amino acids apart from cysteine (CX_4CX_3H). Since some MBNL zinc finger motifs show a similar structure ($CX_7CX_4CX_3H$), we hypothesized that this sequence was part of a ZF4-type domain.

To shed light into the evolutionary history of the Muscleblind proteins, we searched for ORF2-like sequences in evolutionarily close species using tblastn searches. Previously, we determined that *Drosophila* MblC is the most ancient protein isoform since it is readily recognizable within protostome species, whereas other Muscleblind protein isoforms showed no homology (Monferrer *et al.*, 2005). Most *mblC*-like transcripts from several of the organisms studied do show homology to ORF2, although restricted mainly to the regions encoding the zinc fingers themselves. Sequence conservation within ORF2, however, is lower than the conservation observed within the canonical *mblC* ORF1, thus suggesting that ORF1 and ORF2 have different rates of evolutionary change, perhaps because they are under different selective pressures.

Remarkably among the species showing ORF2-like sequences is *Anopheles gambiae*. We identified an expressed sequence tag (EST; GenBank BM619051) encoding two complete ZF4-type

A



B

MANVVNMNSLLNGKDSRWLQLEV**C**REFQRNK**C**SRQDTE**C**KFA**H**PPANVEVQNGKVTA**C**YDSIKGR**C**NRDK

PP**C**KYF**H**PPQHLKDQLLINGRNHLALKNALMQQMGIAPGQPVISGQVPAVATNPYLTGIPANSYSPYYTT

GHLVPALLGPDPVTSQLGPVVPQTVQVAQQKIPRSDRLEMDVKTVGSFYYDNFQFSGMVPFKRPAAEKSG

IPVYQPGATAYQQLMQPYVPVSFTGHPPGVPRFtagtagatctM**C**SRLN**C**RFV**H**LTEDDKVEVIDQRVAV

CRDHANSQ**C**RRKQ**C**KYY**H**IPIVLPPANIMAAMITSPSDQQPAVPVATTTANPAAAAAAAAAATAAGYNGT

SNLIIIEPQQQQQQPPQQQQQFSYHSNGSNNNYYHSNASKQAAHASNMYQQQQQQHQQQQQHQQHLQHQQ

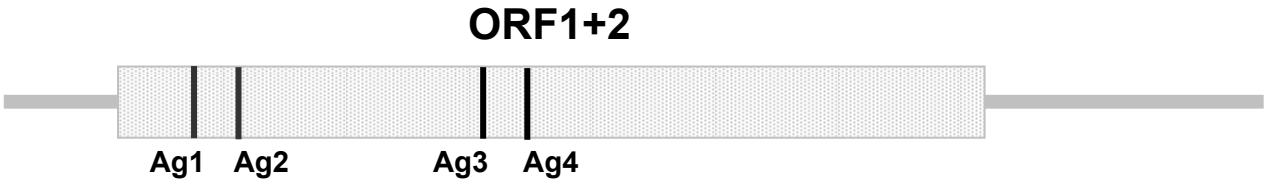
HAQQQTHITYHCSSPYSPSSASSSSSCSIATSPTLSSATTLSTTSTATMPTPQQQYQGPAARRPFtag

Figure 1. Schematic representation of the coding potential of *Drosophila mblC* transcripts and sequence of the proteins encoded. (A) In addition to their canonical open reading frame (ORF1), which includes two CCCH zinc fingers (labelled Dm1 and Dm2), *Drosophila mblC* transcripts contain a second open reading frame (ORF2) with recognizable $CX_7CX_4CX_3H$ zinc fingers (Dm3 and Dm4). Dm3 is shown in grey, because it lacks the first conserved cysteine residue (CX_4CX_3H). Coordinates refer to accession number sequence AF001536. (B) Grey boxes highlight the conceptual translation of ORF1 and ORF2 with intervening cDNA sequence in lower case. Complete CCCH zinc finger domains are shown underlined (solid lines). Conserved zinc-binding moieties are indicated in bold. Within ORF2, alanine, glutamine and serine-rich regions are shown underlined (discontinuous lines), respectively.

CCCH zinc fingers in-frame with the canonical *mblC* ORF1-like sequence (AgORF1+2; Figure 2). Taking into account the high degree of conservation between *Drosophila* and *Anopheles* Muscleblind ORF1 protein, we hypothesized that the *Drosophila* genome should contain a similar organization of zinc finger domains within ORF2. A detailed sequence analysis of the *mblC* transcript identified a thymine insertion in position 857, which puts out of frame *mblC* sequences that actually encode Ag3 and Ag4-like zinc finger domains (Figure 3A,B). *Anopheles mblC*-like transcripts, instead, included a 63 nucleotide insertion in-frame with the *mblC* ORF1-like sequence. Alternative splicing might potentially regulate expression of a Muscleblind ORF1+2 protein in insects through the use of alternative exons that place in-frame or out-of-frame downstream sequences in the mature transcript.

To our knowledge, this is the first description of invertebrate *muscleblind* transcripts that can potentially encode four CCCH-type zinc fingers. What is the relevance of this observation for the understanding of the *muscleblind* evolutionary history? In order to shed light into this question we carried out a phylogenetic analysis of Muscleblind zinc finger motifs present in humans (MBNL1 to

A



B

MAAMVNMTNLLNGKDSRWLQLEV**C**REYQRNK**C**SRPDTE**C**KFA**H**PPANVEVQNGRVTA**C**YDSIKGR**C**NREK

PP**C**KYF**H**PPQHLKDQLLINGRNHLALKNALMQQMGISPGQPVLPGQVPAVATNPYLASMPASTYSPYFQP

GHLVPTLLGPVSDPSSVSQLGPVVQQAVVSTQQKIPRSDRLEVSVPSVFPGMVPFKRSAGEKSGIPVYQP

GATYQQLMQLQQPFVPVSSPSANMPLNAPYVVYTDSNGQLLDTLPV**C**QDFNRSM**C**TRPT**C**RFV**H**LMECDK

VEVCDQRVAV**C**RDHAKGM**C**KRKQ**C**KYY**H**IPIVLPPANVMAATAKLAENL

Figure 2. Schematic representation of the *Anopheles* Muscleblind protein ortholog showing vertebrate-like structure. (A) EST evidence indicates that *Anopheles* can express Muscleblind-like proteins with four zinc fingers (denoted as Ag1 to Ag4). *Anopheles* Muscleblind was reconstructed from EST BM619051.1 and tblastn searches using *Drosophila* MblC as query. Total protein length is 329 amino acids. (B) Grey boxes highlight *Anopheles* counterparts of *Drosophila* ORF1 and ORF2. In contrast to *Drosophila*, the *Anopheles* ORF2 counterpart does not include low-complexity regions downstream Ag4, at least in EST BM619051.1. Ag3 and Ag4 show spacing between zinc-binding moieties and individual zinc fingers typical for vertebrate Muscleblind proteins ($CX_7CX_4CX_3H$ and 16 residues, respectively). Conserved zinc-binding residues are indicated in bold.

3), *Drosophila* (including the two cryptic zinc fingers Dm3 and Dm4), and *Anopheles* (including those encoded by EST BM619051). The similarity tree obtained showed that the first insect zinc finger clusters with the first and third vertebrate zinc finger domains, whereas the second clustered with the second and the forth vertebrate zinc finger domains. Moreover, zinc fingers Dm3/Ag3 and Dm4/Ag4, although more divergent to *Drosophila* Dm1 and Dm2, respectively, than vertebrate zinc fingers, still showed similarity to the corresponding vertebrate zinc fingers (Figure 4). That is, Dm3 and Ag3 are more closely related to vertebrate zinc fingers 1 and 3, while Dm4 and Ag4 are more closely related to 2 and 4. Taken together, these data support the notion that the second zinc finger pair in vertebrate and invertebrates derive from the first pair. Presence of four CCCH-type zinc fingers in *Drosophila* and *Anopheles* supports the hypothesis that the duplication that led to the second pair of zinc fingers happened in a common ancestor before the divergence between invertebrate and vertebrate lineages. Worth noting is that spacing between the last two zinc fingers in invertebrates is identical to vertebrates (16 amino acids). However, cryptic zinc fingers in insects show a ZF4-ZF4 structure, which is different from both vertebrates (ZF6-ZF4) and canonical insect zinc fingers (ZF6-ZF6).

A

```
Frame +2

Ag: 1   MAAMVNMTNLLNGKDSRWLQLEVCREYQRNKCSRPDTECKFAHPPANVEVQNGRVTACYD 60
        MA +VNM +LLNGKDSRWLQLEVCRE+QRNKCSR DTECKFAHPPANVEVQNG+VTACYD
Dm: 161 MANVVNMNSLLNGKDSRWLQLEVCREFQRNKCSRQDTECKFAHPPANVEVQNGKVTACYD 340

Ag: 61  SIKGRCNREKPPCKYFHPPQHLKDQLLINGRNHLALKNALMQQMGISPGQPVLPGQVPAV 120
        SIKGRCNR+KPPCKYFHPPQHLKDQLLINGRNHLALKNALMQQMGI+PGQPV+ GQVPAV
Dm: 341 SIKGRCNRDKPPCKYFHPPQHLKDQLLINGRNHLALKNALMQQMGIAPGQPVISGQVPAV 520

Ag: 121 ATNPYLASMPASTYSPYFQPGHLVPTLLGPVSDPSSVSQLGPVVQQAVVSTQQKIPRSDR 180
        ATNPYL  +PA++YSPY+  GHLVP LLGP  DP + SQLGPVV Q V   QQKIPRSDR
Dm: 521 ATNPYLTGIPANSYSPYYTTGHLVPALLGP--DPVT-SQLGPVVPQTVQVAQQKIPRSDR 691

Ag: 181 LEVSVPSV---------FPGMVPFKRSAGEKSGIPVYQPGAT-YQQLMQLQQPFVPVS   228
        LE+ V +V         F GMVPFKR A EKSGIPVYQPGAT YQQLM   QP+VPVS
Dm: 692 LEMDVKTVGSFYYDNFQFSGMVPFKRPAAEKSGIPVYQPGATAYQQLM---QPYVPVS   856

Frame +3

Ag: 250 LLDTLPVCQDFNRSMCTRPTCRFVHLMECDKVEVCDQRVAVCRDHAKGMCKRKQCKYYHI 309
        LLDTLPVCQDFNRSMC+R  CRFVHL E DKVEV DQRVAVCRDHA   C+RKQCKYYHI
Dm: 858 LLDTLPVCQDFNRSMCSRLNCRFVHLTEDDKVEVIDQRVAVCRDHANSQCRRKQCKYYHI 1037

Ag: 310 PIVLPPANVMAA 321
        PIVLPPAN+MAA
Dm: 1038PIVLPPANIMAA 1073
```

B

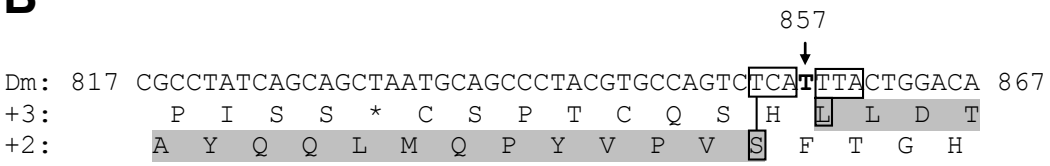


Figure 3. *Drosophila mblC* transcripts encode complete Dm3 and Dm4 zinc fingers in +3 frame. (A) Pairwise alignment between *Anopheles* Muscleblind protein ortholog (reconstructed sequence shown in Figure 2) and dynamically translated *Drosophila mblC* transcript AF001536. Whereas *mblC* frame +2 encodes canonical zinc fingers, frame +3 encodes complete CCCH zinc fingers equivalent to Ag3 and Ag4. Zinc finger domains are denoted in bold. Dm3/Ag3 and Dm4/Ag4 are nearly as conserved as canonical zinc finger are. (B) A thymine base inserted in position 857 puts out of frame Dm3 and Dm4 in *mblC* transcripts. EST evidence (CO318773.1) corroborates a thymine in position 857 of *mblC* (data not shown).

In summary, here we show for the first time that two insect *muscleblind* genes have the potential for encoding proteins with a vertebrate structure, thus providing important clues as for the evolutionary history of Muscleblind proteins. Finding the details of their phylogenetic relationships will require further bioinformatics and experimental work.

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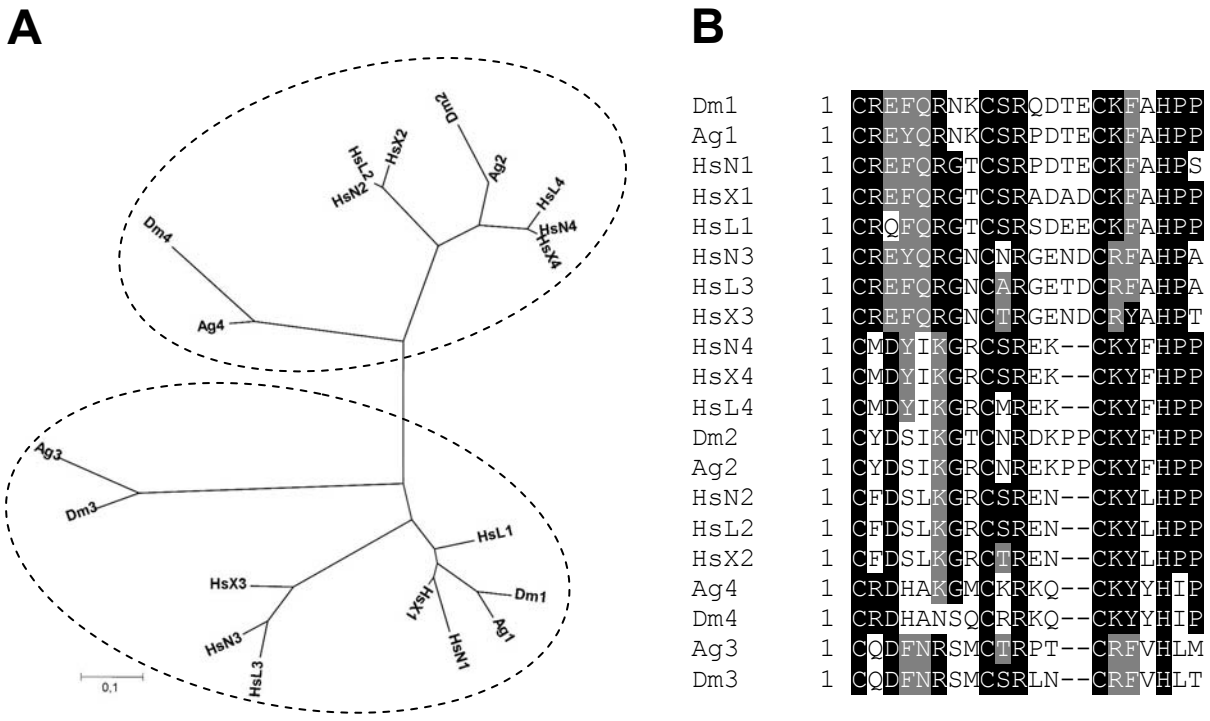

Figure 4. Phylogenetic relationship between *Drosophila* and *Anopheles* third and forth zinc fingers of Muscleblind protein orthologs, and the three human MBNL proteins. (A) Similarity tree between zinc finger sequences of Muscleblind protein orthologs from Human (Hs), *Drosophila* (Dm), and *Anopheles* (Ag). For human MBNL proteins, N, L, and X following the Hs acronym indicate MBNL1, MBNL2 and MBNL3, respectively. Numbers refer to the position of zinc fingers within the corresponding protein, beginning from N-terminus. Third and fourth insect zinc fingers cluster with vertebrate fingers one/three and two/four, respectively. Ag3/Dm3 and Ag4/Dm4, however, are distantly related to canonical *Drosophila* and vertebrate fingers. Scale shown equals 10% sequence difference. (B) Multiple sequence alignment that served as basis for similarity tree. Sequences were aligned using a multiple sequence alignment program (ClustalX) from which a neighbour-joining tree was derived. Multiple alignment was shaded according to the degree of similarity within the alignment with Boxshade program.

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