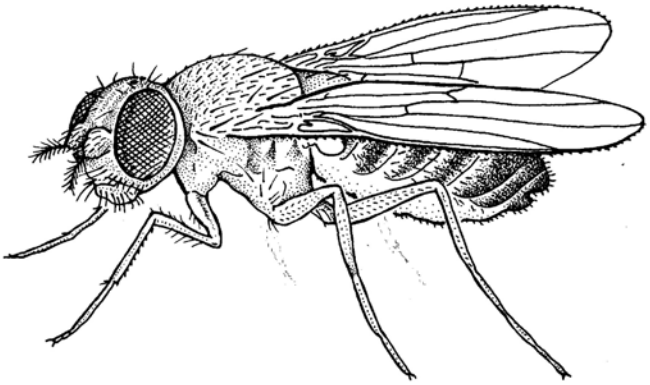
Drosophila Information Service



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Preface

Drosophila Information Service (often called "DIS" by those in the field) was first printed in March, 1934. Material contributed by *Drosophila* workers was arranged by C.B. Bridges and M. Demerec. As noted in its preface, which is reprinted in DIS 75 (1994), Drosophila Information Service was undertaken because, "An appreciable share of credit for the fine accomplishments in *Drosophila* genetics is due to the broadmindedness of the original *Drosophila* workers who established the policy of a free exchange of material and information among all actively interested in *Drosophila* research. This policy has proved to be a great stimulus for the use of *Drosophila* material in genetic research and is directly responsible for many important contributions." Since that first issue, DIS has continued to promote open communication.

The production of DIS volume 94 could not have been completed without the generous efforts of many people. Robbie Stinchcomb, Carol Baylor, and Clay Hallman maintained key records and helped distribute copies and respond to questions. Clay Hallman was also especially helpful in generating pdf copies of early articles in response to large numbers of individual researcher "reprint" requests, and he has worked closely with Jenna Hellack this year to complete the on-line collection of previous DIS volumes. All except the special issues that contained now-dated stock lists and similar material are now freely-accessible from our web site: www.ou.edu/journals/dis.

We continue to encourage all researchers to consider submitting articles that use *Drosophila* for teaching as well as articles that report new techniques, research results, and interesting new mutations. In the interests of honoring the long-standing philosophy of open exchange of ideas, we sometimes accept articles that have unusual perspectives. We thank the many contributors from around the world who sent material for this issue, and we invite your submissions as well as any suggestions you have for maintaining this as a useful *Drosophila* research community resource.

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List of Contributions

General Announcements

Research Notes

Technique Notes

Mutation Notes

Teaching Notes

Other Reports

Research Notes

New record on novel hosts for the Drosophilid pest *Zaprionus indianus.*

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Few species in the Drosophilidae family are known as an active pest on commercial crops (Vilela 1999). One species, *Zaprionus indianus* Gupta, 1970, is recognized as a worldwide pest. Lachaise and Vilela (1983) reported that *Z. indianus* breeds on fallen fruits or fruits on the trees from 74 species in 31 plant families on the African continent. On the other hand, Brazil was the first country in the American continent where *Z. indianus* was collected. In this country, *Z. indianus* caused a substantial commercial damage in orange, peach, and fig orchards (Santos *et al.*, 2003). It seems that *Z. indianus* colonized the entire American continent from Brazil in less than seven years (Castrezana, 2007a). In 2005, the Division of Plant Industry from the Florida Department of Agriculture & Consumer Service issued a pest alert for the presence of *Z. indianus* in several commercial orchards like guava, pond apple, Barbados cherry, cashew, pomegranate, orange, and grapefruit among others (Steck, 2005; Van Der Linde *et al.,* 2006). Nevertheless, Van Der Linde *et al.* (2006) point out that *Z. indianus* may play a major stress factor in local Drosophilid species.

In 2010, our team went to collect *Drosophila* at the tropical deciduous forests in the cape region of Baja California. We were looking for *D. mojavensis* (Patterson 1940) and *D. spenceri* Patterson 1943. *Drosophila spenceri* is a species from the longicornis complex in the mulleri subgroup of the repleta group. The distribution of this Mexican endemic Drosophilidae species encompasses the northwest thorn forest (Sonora and Sinaloa), the Pacific coastal plain (Jalisco, Michoacán, Guerrero, and Oaxaca), the driest central Mexico low lands (Nuevo León, San Luis Potosí, Morelos, and Puebla), and the deciduous forest of the Baja California Sur. The cactus *Pachycereus pectin- aboriginum* (cardón barbón) is the known host for *D. spenceri.* Nevertheless, this *Drosophila* species can use other columnar cacti in the subfamily Pachycereeae like *Carnegiea gigantea* (saguaro), *P. pringlei* (cardón), *P. weberi* (candelabro), *Stenocereus quevedonis* (Pitire), *S. thurberi* (pitaya dulce), *Ferocactus cylindraceus* (California barrel), and *F. emoryi* (Sonoran barrel) (Oliveira *et al.,* 2005).

In my previous field trips to Baja (1995-2003), regardless the season, *D. spenceri* was a frequent species on banana baits at the cape area (Castrezana, 2007b). In addition, I was collecting *D. spenceri* larvae from rotten material and fruits of *P. pringlei* and *P. pecten.* Nevertheless, in June 2010, the temperature in Cape area was over the normal, 42^{o}C @ 12:00 at noon. In addition, the fruit season for *P. pecten* was almost at its end. Despite these problems, our team set up over 15 banana baits along the eastside of the Cape area road (see Markow and O'Grady, 2006, for bait description). We collected more than 2,000 flies. Unfortunately, *D. arizonae* Ruiz, Heed and Wasserman, 1990 was the most abundant species in the collection (catalog number 15081-1271.34A *Drosophila* Species Stock Center). Just about 10% of the flies collected were *D. simulans* Sturtevant 1919 (catalog number 14021-0251.280A in the DSSC) and *D. melanogaster* Meigen 1830; only three *D. mojavensis* females were collected. We didn't find *D. spenceri* on the banana baits.

On the other hand, on the road to the Santa Rosa Town, we visited an area called the cactus sanctuary (23.772837N;-110.132039W). In this area, we found abundant ripened fruits of *P. pecten*, both in the plants and on the ground. Also, we located several *P. pecten* plants with rotten sections. I

recognized Drosophilid larvae in these material pieces. In addition, we got pitaya dulce fruits (*S. thurberi*) with Drosophilid larvae at the San Antonio town (23.800903, -10.109672). So, we took fruits and rotten material samples to the lab. I anticipated that these larvae could be *D. spenceri* and *D. mojavensis.*

Once in the lab, I saw with surprise that pupae had a reddish-brown color without clear horns. When adults emerged, I positively identified the species as *Z. indianus*. Surprisingly, *Z. indianus* didn't arrive to the banana baits. This note is the first report of *Z. indianus* using cacti fruits and rotten cactus tissue. So, I suspect *Z. indianus* could potentially detoxify some of the alkaloids present in the tribe Pachycereeae and if it is not competing, at least *Z. indianus* could potentially disturb some Drosophilidae desert populations.

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Effect of Cyclophosphamide on *hsp70* expression in transgenic *Drosophila melanogaster* (*hsp-70*-lacZ) Bg^9.

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Abstract

In the present study the effect of 0.0035, 0.025, 0.050, 0.10, and 1 µl/ml of cyclophosphamide (CP) was studied on the 3rd instar larvae of transgenic *Drosophila melanogaster* (*hsp70*-lacz) Bg^9 for 6, 24, and 48 hr durations. The treatment of 0.0025 µl/ml of CP did not induce significantly the activity of *hsp70* as compared to control. The treatments of 0.025, 0.050, 0.10, and 1 µl/ml of CP induced a significant increase in the activity of *hsp70* for the different duration of exposure. The results of the present study suggest that the doses of 0.025, 0.050, 0.10, and 1 µl/ml are cytotoxic in the 3rd instar larvae of transgenic *Drosophila melanogaster* (*hsp70*-lacZ) Bg^9.

Introduction

Cyclophosphamide is an alkylating agent (Ren *et al.,* 1998). It is used as a chemotherapeutic agent to treat various forms of leukemia (Shanafelt *et al.,* 2007) and tumors (Young *et al.,* 2006). It

is also used to treat rheumatoid arthritis (Scott *et al.,* 1984), Wegner's granulomatosis (Hoftman *et al*., 1990). All living organisms under stressful conditions respond by synthesizing heat shock proteins (HSPs) (Nover, 1994, 1991). HSPs function as a molecular chaperone that prevents cellular damage (Bennet and Waters, 2000). In recent years, *hsp70* has been considered to be one of the candidate genes for predicting cytotoxicity against environmental chemicals (Bierkens, 2000; Mukhopadhyay *et al.,* 2002, 2003). In the present study, the toxicity of Cyclophosphamide was investigated by the quantification of *hsp70* expression in the 3rd instar larvae of transgenic *Drosophila melanogaster* (*hsp70*-lacZ) Bg^9, for the different doses and hours of exposure.

Materials and Methods

Fly strain

A transgenic *Drosophila melanogaster* line that expresses bacterial β-galactosidase as a response to stress was used in the present study (Lis *et al.,* 1983). The flies and larvae were cultured on the standard *Drosophila* food containing agar, cornmeal, sugar, and yeast (Nazir *et al.,* 2003).

ONPG assay

Cyclophosphamide at 0.0025, 0.025, 0.050, 0.10, and 1 µl/ml of food concentrations were established. The third instar larvae were allowed to feed on them for different time intervals, *i.e*. 6, 24, and 48 hr. The expression of *hsp70* was measured by soluble o-nitrophenyl-β-D-galactopyranoside (ONPG) assay (Nazir *et al.,* 2003; Lakhotia and Singh, 1989). Briefly, after washing in phosphate buffer, the larvae were taken in a microcentrifuge tube (20 larvae/tube, 5 replicates/group), permeabilized for 10 min in acetone, and incubated overnight at 37°C in 600 µl of ONPG staining buffer. Following incubation, the reaction was stopped by adding 300 µl of Na_2CO_3. The extent of reaction was quantified by measuring the absorbance at 420 nm using Systronics UV/VIS spectrophotometer 118 (India).

Statistical analysis

Statistical analysis was carried out by student's "t" test using Commercial Software Statistica Ssoft Inc (2007).

Table 1. β-galactosidase activity measured in transgenic *Drosophila melanogaster* (hsp70-lacZ) Bg^9 third instar larvae exposed to different concentrations of Cyclophosphamide for various time intervals.

Treatments Cyclophosphamide (µl/ml)	After 6 hr O.D. (Mean ± SE)	After 24 hr O.D. (Mean ± SE)	After 48 hr O.D. (Mean ± SE)
0.0025	0.2103 ± 0.0032	0.2036 ± 0.0041	0.1898 ± 0.0082
0.025	0.2433 ± 0.0043*	0.2639 ± 0.0011*	0.2723 ± 0.0054*
0.050	0.2593 ± 0.0071	0.2763 ± 0.0018*	0.2844 ± 0.0063*
0.10	0.2634 ± 0.0023	0.3012 ± 0.0073*	0.2930 ± 0.0034*
1.0	0.1132 ± 0.0011	0.1034 ± 0.0017	-
Untreated	0.2011 ± 0.0039	0.2112 ± 0.0040	0.1994 ± 0.0050

*P < 0.05 compared to control; O.D. = Optical Density; SE = Standard Error.

Results

The treatment of 0.0025 µl/ml of CP did not show any significant increase in the β-galactosidase activity for various time intervals as compared to untreated (Table 1). The treatment of 0.025 µl/ml of CP did not show any significant increase in the β-galactosidase activity for 6 hr of exposure but showed a significant increase in the β-galactosidase activity for 24 and 48 hr of duration of exposure (Table 1). The exposure of 3rd instar larvae to 0.050 and 0.10 µl/ml of CP showed a dose dependent significant increase for each exposure (Table 1). The treatment of 1 µl/ml of CP results in a decrease in the activity of β-galactosidase for 6 and 24 hr of duration (Table 1). After 48 hr of exposure to 1 µl/ml of CP, the ONPG was not performed due to the mortality of larvae.

Discussion

The results of the present study reveal that the CP is not cytotoxic at 0.0025 µl/ml. The metabolite of CP is phosphoramide mustard (Hales, 1982). It forms DNA cross links between and within DNA strands of guanine N-7 positions that lead to the cell death (Benson *et al*., 1988). The higher doses of CP are associated with cytotoxicity (Hales, 1982). Although having protective roles in living systems, HSPs are being exploited by toxicologists (Bierkens, 2000; Mukhopadhyay *et al.,* 2002, 2003; Nazir *et al.,* 2003). Now-a-days, the use of animals for toxicological evaluations has become the fundamental concern for scientists, not only because of protests from animal rights organizations but also because of difficulty in interpreting data due to intra-species variation and exorbitant costs (Benford *et al.,* 2000). This has led researchers to encourage the use of alternative animals in toxicological evaluations. *Drosophila* is a well established animal model for genetics, developmental and molecular biologists. In the past years a significant contribution has been made by successfully employing transgenic *D. melanogaster* as an alternative animal model for toxicological research (Mukhopadhyay *et al.,* 2002). Although there is no comparative data, the studies by Hirsch *et al*. (2003) indicate that flies and humans have similar dose response relationships with lead. At the highest tested dose, *i.e*. 1 µl/ml of CP, the decrease in the activity of *hsp70* expression is due to the tissue damage at this dose, which is also evident by the morality of the larvae after 48 hr of exposure. HSPs are formed in response to stressors like LPO, DNA damage, osmotic imbalance, protein misfolding, membrane perturbation, metals, heat shock, and so forth (Nazir *et al.,* 2003). A dose dependent increase in the activity of β-galactosidase clearly demonstrates the dose dependent toxic effects of CP in transgenic *Drosophila melanogaster* (*hsp70* lacz) Bg^{9} and give support to the utility of *hsp70* expression as a bioindicator of exposure to the environmental chemicals.

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Inconsistent associations between recombination rate and codon bias across *Drosophila* species.

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Abstract

A positive association between recombination rate and codon bias has been observed at broad and fine scales in *Drosophila* species. However, this relationship is complicated by other genomic features that correlate with codon bias. No prior studies have evaluated the relationship between recombination rate and codon bias across multiple species within this genus. Utilizing published recombination maps along with complete genome sequences, we contrasted recombination rate to codon bias and intronic GC across four *Drosophila* species. We did not observe a consistent significant correlation between recombination rate and codon bias across the species examined. Indeed, we did not observe even the previously reported trend of higher codon bias in regions of high recombination, though we saw some evidence this pattern may be affected by differences between centromeric/telomeric regions and central regions. More fine-scale recombination data from more *Drosophila* species is necessary for a comprehensive picture of the relationship between recombination rate and codon bias.

Introduction

Rates of recombination vary across *Drosophila* genomes at broad and fine scales and have been shown to be correlated with codon usage bias (Cirulli *et al*., 2007; Comeron *et al*., 1999; Hey and Kliman, 2002; Kliman and Hey, 1993; Marais *et al*., 2001; Singh *et al*., 2005; Stevison and Noor, 2010). Specifically, regions of high recombination tend to have disproportionately high usage of G- or C-ending codons relative to regions of low recombination. The relationship of recombination rate to codon bias is complicated by other features that also correlate with codon bias

(*e.g.*, expression level) but may nonetheless indicate the intensity of natural selection on translation efficiency (Duret and Mouchiroud, 1999). However, biased gene conversion (BGC), a neutral process, can also occur as a result of high recombination rates and result in strong codon bias, but this force would apply equally in codons as in noncoding (*e.g.*, intronic) regions. Analysis of recombination rate variation and codon bias across several species of *Drosophila* may provide a more comprehensive picture of this relationship.

Several previous studies have documented a positive correlation between recombination rate and codon bias on a genome-wide scale in *Drosophila melanogaster* (Comeron *et al*., 1999; Hey and Kliman, 2002; Kliman and Hey, 1993; Marais *et al*., 2001, 2003; Singh *et al*., 2005). The degree of association between codon bias and recombination varies over the range of recombination, and a much stronger relationship between codon bias and recombination has been observed in genes with the lowest levels of recombination (Comeron *et al*., 1999; Hey and Kliman, 2002; Kliman and Hey, 1993). Further, a strong negative correlation has been documented between recombination rate and codon bias on the X chromosome (Singh *et al*., 2005).

Examination of the association between recombination rate and codon bias has been conducted on a much smaller spatial scale in *D. pseudoobscura* and *D. persimilis* (Cirulli *et al*., 2007; Stevison and Noor, 2010). Cirulli *et al*. (2007) detected a significant positive association between crossover rate and codon bias across 126 full or partial gene CDSs within a 2-megabase (Mb) interval on the XL chromosome arm of *D. pseudoobscura*.

Stevison and Noor (2010) conducted a fine-scale analysis of recombination rate variation on the right arm of the X chromosome (XR) and the entire second chromosome of *D. persimilis* and observed a significant positive correlation between recombination rate and codon bias on chromosome 2 but not on XR. As more frequently used codons in *Drosophila* genomes end in G or C, codon bias is often measured by the GC-content of third-base positions in codons (Akashi and Schaeffer, 1997; Bachtrog, 2007). Stevison and Noor (2010) also examined GC content at fourfold degenerate codons (GC4), which should be less constrained than non-degenerate codons, and GC content in small introns (<100 bp), which appear to be under less evolutionary constraint than longer introns (de Procé *et al*., 2009; Parsch, 2003). They observed strong correlations between recombination rate and GC4 and GC content in small introns in chromosome 2 (Stevison and Noor, 2010). For their analysis, they excluded 5 Mb upstream and downstream from the centromere and telomere—which undergo little crossing over and can potentially disrupt correlations between recombination rate and other genomic features—and one interval of especially high recombination rate (which may have been anomalously inflated) from each chromosome (Stevison and Noor, 2010).

These previous studies have examined the relationship between recombination rate and codon bias in individual *Drosophila* species, but none have evaluated the relationship across multiple species within a genus. The publication of broad and fine scale recombination maps along with complete genome sequences of several *Drosophila* species allow for the evaluation of meiotic recombination and codon bias across this genus. Broad-scale recombination linkage maps have been generated for *D. virilis* and *D. mojavensis* by Huttunen *et al*. (2004) and Staten *et al*. (2004), respectively, using microsatellite markers. More recently, fine-scale recombination maps have been generated for *D. pseudoobscura* and *D. persimilis* by Kulathinal *et al*. (2008) and Stevison and Noor (2010), respectively, using SNP genotyping markers. Complete genome sequences for each of these four species have been made available through the publication of the *Drosophila* 12 genomes sequencing project (Clark *et al.*, 2007). In an effort to understand better the relationship between meiotic recombination and codon bias in *Drosophila*, we utilized these data to contrast recombination rate to GC4 and intronic GC across four *Drosophila* species.

Methods

Recombination linkage maps of microsatellite markers for multiple chromosomes by Huttunen *et al*. (2004) and Staten *et al*. (2004) provide Kosambi recombinational distances between pairs of markers in centiMorgans for *D. virilis* and *D. mojavensis*, respectively. Microsatellite primer sequences obtained from Huttunen and Schlötterer (2002) and Staten *et al*. (2004) were BLASTed against the caf1 *D. virilis* and *D. mojavensis* genome scaffolds, respectively. The physical distance between adjacent markers was determined and recombination rates were calculated in centiMorgans per megabase (cM/Mb) for pairs of markers. For *D. pseudoobscura* (Kulathinal *et al*., 2008) and *D. persimilis* (Stevison and Noor, 2010), the start/end position for each SNP genotyping marker along the scaffolds and Kosambi recombination distances between pairs of markers in centiMorgans were obtained from the Dryad data repository (doi:10.5061/dryad.484; doi:10.5061/dryad.1877), and used to calculate recombination rate in cM/Mb.

Chromosome and scaffold sequences containing the markers used for recombination rate calculations were downloaded from GenBank. GC content at fourfold degenerate codons have been shown to be less constrained than non-degenerate codons (de Procé *et al*., 2009). Parsch *et al*. (2010) showed bases 8 – 30 of introns ≤65 bp in length are under the least selective constraint and can be used as a reference for unconstrained evolution. A series of custom Perl scripts were used to annotate the GenBank files and calculate GC and AT content at third positions in codons for fourfold degenerate codons (GC4 and AT4, respectively) and GC and AT content for bases 8 – 30 of introns ≤65 bp in length (GCi_65 and ATi_65, respectively). The percent of GC4 and GCi_65 were calculated for each recombination interval and regressed against recombination rate in Excel.

Broad-scale microsatellite linkage map data were available for chromosomes 2, 3, 4, and 5 of *D. virilis* and chromosomes 2, 3, 4, 5, and X of *D. mojavensis* (Huttunen *et al*., 2004; Staten *et al*., 2004). Data from *D. virilis* and *D. mojavensis* autosomal chromosomes were combined into a single group for each species for regression analyses. Fine-scale SNP genotype data were available for chromosome 2 of *D. pseudoobscura* and chromosomes X and 2 of *D. persimilis* (Kulathinal *et al*., 2008; Stevison and Noor, 2010). One *D. mojavensis* recombination interval had an especially high recombination rate and was excluded from our regression analysis. Kosambi recombinational distances between microsatellite and SNP genotyping markers varied widely. To obtain similar Kosambi recombinational distances between markers for each of the four species, short recombination intervals of *D. pseudoobscura* and *D. persimilis* were grouped together for further regression analyses.

Results

Table 1 presents regression analyses of recombination rates with %GC4 and %GCi_65 for four species of *Drosophila* at different scales. Data from *D. mojavensis* and *D. persimilis* X chromosome regression analyses are not shown. Analyses of initial recombination intervals reveal a significant correlation between recombination rate and %GCi_65 only in *D. pseudoobscura*, a marginally significant correlation between recombination rate and both %GC4 and %GCi_65 in *D. virilis*, and a marginally significant correlation between recombination rate and %GC4 only in *D. mojavensis* and *D. pseudoobscura*. When recombination intervals of *D. pseudoobscura* and *D. persimilis* were combined to make them more similar in size to *D. virilis* and *D. mojavensis* recombination intervals, no significant correlation was observed between recombination rate and %GC4 or %GCi_65 in either species.

Table 1. Results of regression analysis of recombination rate and %GC4 and %GCi_65 across four *Drosophila* species.

				%GC4 regressed against cM/Mb			%GCi_65 regressed against cM/Mb		
	N	cM range	cM/Mb range	r	Coefficients	*P* value	r	Coefficients	*P* value
D. virilis (Chr 2,3,4,5)	15	10.8 - 43.4	3.1 - 23	0.441	68.486	0.100	0.425	109.745	0.114
D. virilis ≤25 cM	9	10.8 - 24.9	3.1 - 23	0.443	64.085	0.232	0.396	94.221	0.292
D. virilis >26 cM	6	25.4 - 43.4	3.4 - 9.2	0.019	2.255	0.972	0.733	-269.330	0.097
D. mojavensis (Chr 2,3,4,5)	12	1.2 - 37.1	1.4 - 11.7	0.509	52.103	0.091	0.259	31.033	0.417
D. mojavensis ≤10 cM	9	1.2 - 9.9	1.4 - 11.7	0.670	60.031	**0.048**	0.347	38.176	0.360
D. mojavensis >10 cM	3	11.7 - 37.1	1.8 - 11.1	0.846	-475.387	0.358	0.875	-795.930	0.321
D. pseudoobscura (Chr 2)	49	0 - 9.8	0 - 15	0.271	5.399	0.060	0.420	15.462	**0.003**
D. pseudoobscura (intervals combined)	10	5.1 - 17	2.8 - 5.3	0.458	-6.962	0.183	0.112	-4.432	0.758
D. pseudoobscura (intervals combined) ≤10 cM	5	5.1 - 9.5	2.9 - 5.3	0.773	-9.744	0.125	0.801	-30.859	0.103
D. pseudoobscura (intervals combined) >10 cM	5	12.1 - 17	2.8 - 4.8	0.801	29.549	0.103	0.873	36.006	**0.053**
D. persimilis (Chr 2)	129	0 - 25.2	0 - 30	0.104	4.429	0.243	0.035	1.723	0.697
D. persimilis (intervals combined)	13	1 - 30.4	0.4 - 8.3	0.049	-2.404	0.874	0.215	22.713	0.481
D. persimilis (intervals combined) ≤10 cM	8	1 - 8.2	0.4 - 3.6	0.492	-9.368	0.215	0.550	-24.718	0.158
D. persimilis (intervals combined) >10 cM	5	10.6 - 30.4	4.9 - 8.3	0.050	-3.287	0.936	0.299	45.721	0.625

For each species, data were sorted by recombination interval size and separated into small and large intervals to examine correlation between recombination rate and %GC4 and %GCi_65 using similar sized intervals. For *D. mojavensis*, *D. pseudoobscura*, and *D. persimilis*, small recombination intervals were ≤10 cM, and large recombination intervals were >10 cM. The available recombination intervals for *D. virilis* were larger and were separated into small intervals ≤25 cM and large intervals >26 cM. Subsequent regression analyses revealed a significant correlation between recombination rate and %GC4 in *D. mojavensis* ≤10 cM and between recombination rate and %GCi_65 in *D. pseudoobscura* >10 cM; marginally significant correlation was observed between recombination rate and %GC4 and %GCi_65 in D. psedoobscura ≤10 cM, between recombination rate and %GC4 in *D. pseudoobscura* >10 cM, and between recombination rate and %GCi_65 in *D. virilis* >26 cM.

Discussion

We did not observe a consistent significant correlation between recombination rate and codon bias across the four species of *Drosophila* examined. Overall, broader scale recombination intervals showed more significant correlations between recombination rate and %GC4 and %GCi_65 than fine-scale recombination intervals. However, when the fine-scale recombination intervals were grouped together to make larger intervals and reanalyzed, the relationships between recombination rate and %GC4 and %GCi_65 were not significant. Further separation of the data into small and large recombination intervals did not provide any more insight into the relationship between recombination rate and codon bias among *Drosophila* species.

Unlike previous studies, we did not observe a trend of higher codon bias in regions of high recombination. Recombination rate and codon bias were not significantly correlated on a genome-wide scale in *D. virilis* or *D. mojavensis* in our study, as has been observed in *D. melanogaster*. Although the fine-scale regression analysis of only the second chromosome for *D. pseudoobscura* revealed significant correlation between recombination rate and intronic GC and marginally significant correlation between recombination rate and codon bias, analysis of the second

chromosome of *D. persimilis* did not reveal significant correlations between recombination rate and codon bias or intronic GC when all data points were examined.

Our results initially appear inconsistent with those reported by Stevison and Noor (2010) due to differences in the data sets used in each study. However, when we reanalyzed the data using their specified parameters—excluding 5 Mb upstream and downstream from the centromere and telomere and high recombination rates from the analysis—for *D. persimilis*, we observed the same significant correlation between recombination rate and codon bias and intronic GC (results not shown) that they reported. Clearly, excluding centromeric and telomeric genomic regions that undergo little crossing over affects the results of studies examining the correlation between recombination rate and codon bias.

Our study was limited by the scarcity of fine-scale recombination data available for analysis in these *Drosophila* species. Further analyses of fine-scale recombination rates at multiple chromosomes in more *Drosophila* species are necessary to develop a comprehensive picture of the association of recombination rate and codon bias in this genus.

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Drosophilids of Perumalai hills of Kodaikanal (Tamilnadu State, India).

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Significant progress has been made in the field of taxonomy and systematics of the family Drosophilidae (Diptera) in India. The *Drosophila* species are observed in essentially any environment, from the sea level to considerable altitudes, and in temperate as well as in equatorial zones (Throckmorton, 1975). However, Lachaise (1979) suggests that these species are subject to restrictions as regards the habitats they live in. Many factors affect the ability of a species to survive

and reproduce in sufficient numbers in order to persist in a locality. Biotic and abiotic factors may vary in space and time, and for a mobile organism spatial variation often becomes temporal variation (Futuyma, 1998). The belief that organisms are remarkably well suited to the world they live in predates scientific biology; metaphors used to explain the relation between organism and environment usually invoke an external world that has acquired its properties independently of the organism (Lewontin, 2000).

However, a vast area of great ecological interest still either awaits exploration or is poorly explored. Very little is known regarding Drosophilid fauna of Perumal alai peak of Kodaikanal, which is a highest peak at Kodaikanal in the Perumalmalai hills, that are a part of the greater Western Ghat mountain range in Tamil Nadu, India, having an elevation of 2,440 metres (8,005 ft). Meadows and grasslands cover the hillsides. Gigantic Eucalyptus trees and shola forests flourish in the valleys. Kodaikanal has the richest repositories of biodiversity in the world (Dwivedi *et al*., 1979, 1980; Singh and Gupta, 1977; Reddy and Krishnamurthy, 1971).

Table 1. Distribution pattern of different species of *Drosophila* at three different seasons of the year (2010) of Perumalai hills of Kodaikanal, Tamilnadu, India.

Months	Seasons		
	Summer (April)	Rainy (August)	Winter (December)
Temperature (° C)	18° C -22° C	16° C – 20° C	8° C - 11° C
*D. melanogaster*****	**73**	**71**	**44**
D. annanassae	22	21	04
D. bipectinata	39	12	09
D. biarempis	27	13	02
*D. simulans***	**58**	**44**	**22**
D. kikkawai	10	08	03
D. malerkotliana	12	10	00
*D. immigrans****	**61**	**59**	**47**
D. jambulina	02	00	00
D. nigra	16	11	00
D. s. neonasuta	23	17	09
*D. nasuta**	**34**	**39**	**09**
D. rajashekari	12	03	00
D. takahashii	04	00	00
TOTAL	393	308	149

*indicates the order of wide distribution in number.

A preliminary survey on Drosophilids of Perumalai hills has been made as no information is available about this particular peak of Kodai so far. It is situated at latitude 10° 14' 0" N / 77° 29' 0" E'. Perumalai hill has a sub-tropical climate. During summers (March to May) the average temperature ranges between 11°C to 20°C. Winters are very cool with maximum temperature of about 17°C and minimum touches about 8°C. During January, ice formations are seen at night and temperature can drop down to freezing level. The hills station has an average rainfall during monsoons (June to September). Collections were made using fermenting fruits as baits in three different seasons of the year (2010), *i.e* in summer (April), winter (December), and rainy seasons (August). Altogether 15 different *Drosophila* species were collected (Table 1). Total number of flies collected was 850. Maximum number of flies was collected in summer (46.23%) and least in winter (17.32%), and in rainy the percent of flies collected was 36.23%. With decreased temperature many species of the present study have diminished in number. It indicates that temperature is certainly a bias for the *Drosophilids* to survive in nature. The species diversity is increased in dry season rather than the wet condition. Out of fifteen species found, four species, namely, *Drosophila melanogaster*, *D. immigrans*, *D. simulan*, and *D. nasuta* were found to be distributed widely more in number in all the seasons of the present study. It clearly states that these species can withstand the temperature tolerance compared to other species of the present study. Interestingly our results also

compares with similar observation with regard to *D. immigrans* distribution in Nilgiris and Kodaikanal ranges of South thrives well in a humid climate (Sreerama Reddy and Krishnamurthy, 1971) in addition to the other three species.

Thus, climatic variables, such as humidity and rainfall, are determining factors in the occurrence of drosophilid species (Pavan, 1959). Therefore, the composition and structure of a drosophilid assemblage depends on the habitat in which it was established. The recognition of patterns of distribution leads to organizational levels about the ecology and evolution.

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A duplication at the tip of left arm of the second chromosome carrying alpha inversion in *Drosophila ananassae.*

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Chromosomal polymorphism is common in *Drosophila* (Da Cunha, 1960; Dobzhansky, 1970; Sperlich and Pfriem, 1986). *Drosophila ananassae* is a cosmopolitan and domestic species. It exhibits a high degree of chromosomal polymorphism (Singh, 1998a,b). *D. ananassae* in particular is known to carry 78 paracentric inversions, 21 pericentric inversions, and 48 translocations (Singh and Singh, 2007). However, only three paracentric inversions, namely, alpha (AL) in 2L, delta (DE) in 3L, and eta (ET) in 3R, are very common and have been called cosmopolitan inversions. The chromosomal polymorphism has been studied extensively in a large number of natural and laboratory populations of *D. ananassae* (Singh, 1996, 1998b; Singh and Singh, 2008).

Table 1. Observed and expected numbers of different karyotypes in laboratory population (Ranchi) of *D. ananassae.*

	Karyotypes											
	2L			X^2	3L			X^2	3R			X^2
	ST/ST	ST/AL	AL/AL		ST/ST	ST/DE	DE/DE		ST/ST	ST/ET	ET/ET	
Obs	0	28	72	2.65	41	53	6	4.3254*	57	39	4	0.7166
Exp	1.96	24.08	73.96		45.56	43.87	10.56		58.52	35.95	5.52	

df =1, *P<0.05

Table 2. Frequencies (in percent) of different gene arrangements and mean number of heterozygous inversions per individual in laboratory population (Ranchi) of *D. ananassae.*

Gene arrangements						Mean number of heterozygous inversions per individual
2L		3L		3R		
ST	AL	ST	DE	ST	ET	
14.0	86.0	67.5	32.5	76.5	23.5	1.2

D. ananassae flies were collected from Ranchi, Jharkhand in October 2010, and a mass culture stock was established (number of founding females = 21). In this note, we report a duplication at the tip of the left arm of the second chromosome of *D. ananassae*. The duplication was detected during cytological analysis of this mass culture stock. Duplication at the tip is reported for the first time in *D. ananassae*. Duplication was detected in ST/AL (Figure 1b) and AL/AL (Figure 1c) larvae, but it was not observed in ST/ST (Figure 1e) larvae. From the figure it is clear that the duplication has occurred at the tip of 2L carrying AL inversion. Its persistence in the stock suggests that it does not have any deleterious effect.

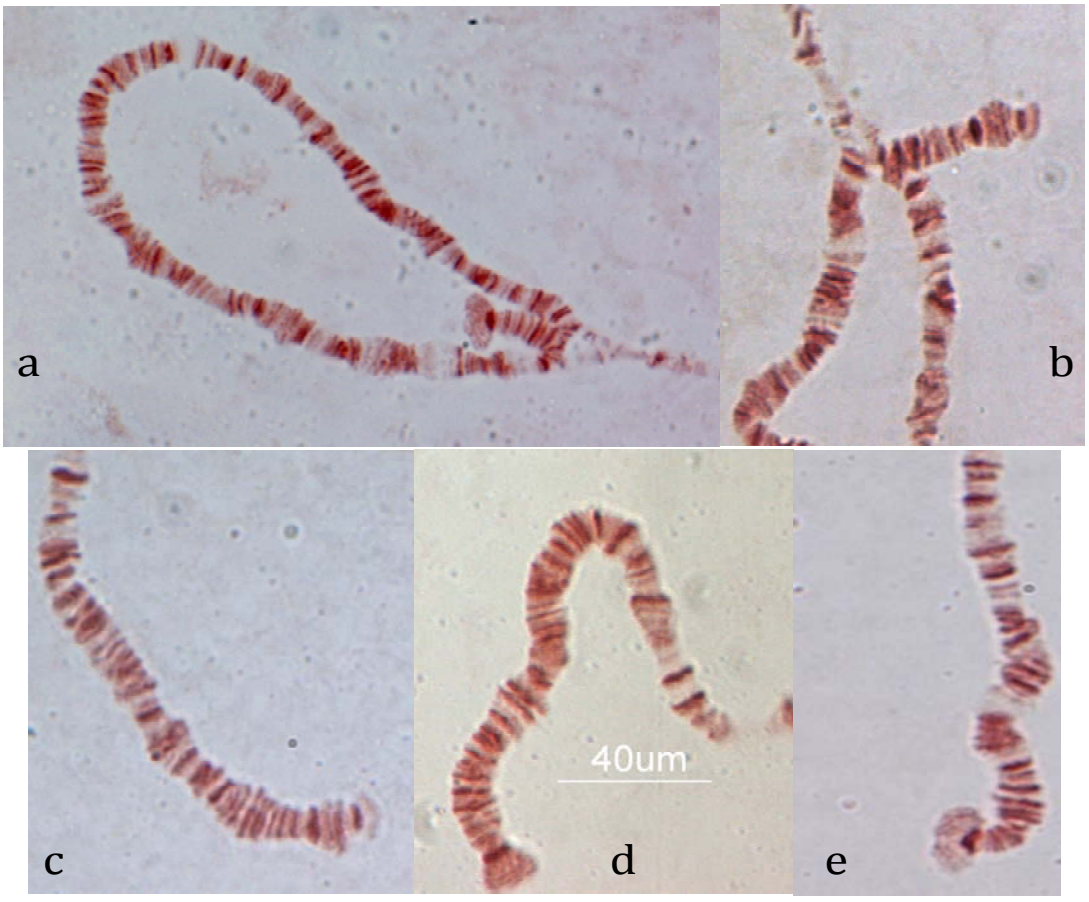

Figure 1. Microphotographs of 2L in *D. ananassae*. a, ST/AL; b, ST/AL with duplication at the tip; c, AL/AL with duplication at the tip; d, AL/AL; e, ST/ST.

Chromosomal analysis of this stock was made by squashing 100 larvae randomly taken from culture bottles by lacto-aceto-orcein method. The observed and expected (via Hardy- Weinberg proportion) numbers of 2L, 3L, and 3R karyotypes are given in Table 1. The data on the frequencies of different gene arrangements in 2L, 3L, and 3R were obtained along with the level of heterozygosity (Table 2). The frequency of AL inversion is nearly 86 percent, while the chromosomes with delta and eta inversions are less frequent than those with the standard sequence. The mean number of heterozygous inversions per individual is 1.2. Hardy-Weinberg equilibrium was tested, and Chi-square values were calculated. The difference between observed and expected numbers of different karyotypes in 3L is statistically significant and insignificant for 2L and 3R. This shows that the population is polymorphic chromosomally, and there is a significant deviation from Hardy- Weinberg equilibrium as the difference between observed and expected numbers of different karyotypes in 3L is statistically significant ($p< 0.05$). This is due to a significant excess of inversion heterozygotes.

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Colored light norms of genotypes of parental strains and hybrids in *D. melanogaster*.

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The object of the present experiment was to study the norms of genotypes in *D. melanogaster* iso-female lines and in hybrids, using the three colored lights as an environment and the average weight as a phenotypic trait.

Materials and Methods

Four iso-female strains (genotypes) were used for the present study. The F_1's and F_2's were made for each strain. The parental, F_1's, and F_2's were tested under three different colored lights: Blue: 25W; White: 25W; and Red: 25W. The males and females were counted and the average weight per male and per female was calculated for each of the parental, F_1's and F_2's.

Results and Discussion

Genetic variation: Table 1a and 1b and the graphic representation, Figure 1a and 1b, show the variation in an average weight from one genotype to another when tested under a given colored

Table 1a and 1b. Average weight in microgram (µg) ± 1 for parental, F_1's and F_2's of each of the four genotypes of *D. melanogaster* tested under three different colored lights: red; blue; and white.

Table 1a: Males

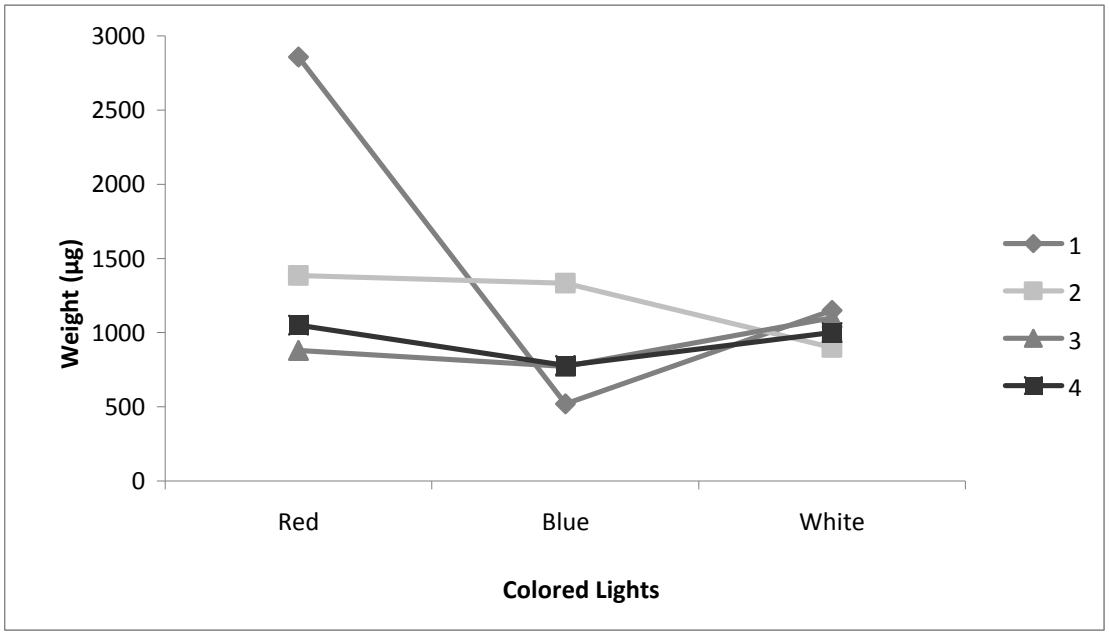
Genotype	Red (19°–20°C)	Blue (20°–21°C)	White (20°–21°C)
1			
Parental	2857.00	520.00	1150.00
F1	1900.00	1750.00	1900.00
F2	1100.00	1500.00	860.00
2			
Parental	1385.00	1333.00	900.00
F1	1800.00	1850.00	1800.00
F2	900.00	1700.00	2500.00
3			
Parental	880.00	772.00	1100.00
F1	2000.00	1800.00	1950.00
F2	900.00	1700.00	1700.00
4			
Parental	1050.00	779.00	1000.00
F1	1750.00	1417.00	1300.00
F2	1000.00	1700.00	1700.00
Average			
Parental	1543.00	851.00	1037.50
F1	1862.50	1704.25	1737.50
F2	975.00	1650.00	1690.00

Table 1b: Females

Genotype	Red (19°–20°C)	Blue (20°–21°C)	White (20°–21°C)
1			
Parental	3714.00	666.00	1450.00
F1	2000.00	2150.00	2150.00
F2	1600.00	1800.00	500.00
2			
Parental	520.00	1185.00	1350.00
F1	2250.00	2150.00	2200.00
F2	1600.00	1900.00	2000.00
3			
Parental	2341.00	397.00	1400.00
F1	2350.00	2250.00	2400.00
F2	1500.00	2100.00	1300.00
4			
Parental	520.00	1000.00	1300.00
F1	1700.00	1626.00	1650.00
F2	1200.00	2000.00	800.00
Average			
Parental	1773.75	812.00	1375.00
F1	2075.00	2044.00	2100.00
F2	1475.00	1950.00	1150.00

light for parental, F_1's, and F_2's. For example, in ***males*** (Table 1a and Figure 1a): i) under ***red*** light for parental class from 880.00 to 2857.00 µg; F_1's from 1750.00 to 2000.00 µg; and F_2's from 900.00 to 1100.00 µg. ii) under ***blue*** light for parental from 520.00 to 1333.00; F_1's from 1417.00 to 1850.00 µg; and F_2's from 1500.00 to 1700.00 µg; iii) under ***white*** light for parental class from 900.00 to 1150.00 µg; F_1's from 1300.00 to 1950.00 µg; and F_2's from 860.00 to 2500.00 µg. In ***females*** (Table 1b and Figure 1b): i): under ***red*** light for parental class from 520.00 to 3714.00 µg

(with the exception of genotypes 2 and 4 having the same weight, Figure 1b); F_1's from 1700.00 to 2350.00 µg; and F_2's from 1200.00 to 1600.00 µg. ii) under ***blue*** light for parental class from 397.00 to 1185.00; F_1's from 1626.00 to 2250.00 µg; and F_2's from 1800.00 to 2100.00 µg; iii) under ***white*** light for parental class from 1300.00 to 1450.00 µg; F_1's from 1650.00 to 2400.00 µg; and F_2's from 500.00 to 2000.00 µg. The data support the hypothesis that the variation among genotypes is *mainly genetic*.



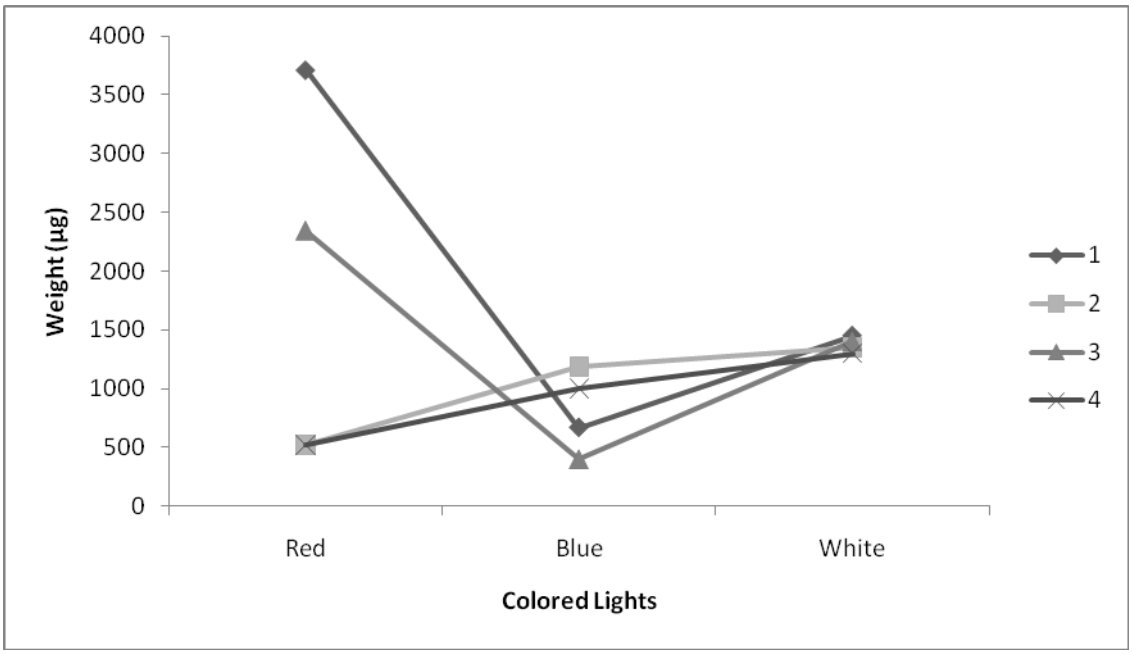


Figure 1a (top) and 1b (bottom): Average male and female weight in microgram ± 1 for four genotypes of *D. melanogaster* tested under three different colored lights.

Environmental variation: The data in Table 1a and Figure 1a demonstrate that the **male** weight varied from one colored light to another, for example, for parental genotype 1 (2857.00 µg for ***red*** light; 520.00 µg for ***blue*** light; and 1150.00 µg for ***white*** light); in case of F_1's (1900.00 µg for ***red*** light; 1750.00 µg for ***blue*** light; and 1900.00 µg for ***white*** light); and while for F_2's (1100.00 µg for ***red*** light; 1500.00 µg for ***blue*** light; and 860.00 µg for ***white*** light). Similar results, in general, were observed for the other three genotypes, F_1's and F_2's (Table 1a and Figure 1a). In case of females (Table 1b and Figure 1b), the **female** weight varied from one colored light to another, for example, for parental genotype 2 (520.00 µg for ***red*** light; 1185.00 µg for ***blue*** light; and 1350.00 µg for ***white*** light); in case of F_1's (2250.00 µg for ***red*** light; 2150.00 µg for ***blue*** light; and 2200.00 µg for ***white*** light); and while for F_2's (1600.00 µg for ***red*** light; 1900.00 µg for ***blue*** light; and 2000.00 µg for ***white*** light). Similar results, in general, were observed for the other three genotypes, F_1's and F_2's (Table 1b). These results provide the experimental evidence that the male and the female weight variation from one colored light to another for the same genotype are *mainly* environmental. (It should be noted that the graphic representation for F_1's and F_2's is not presented here but can be done from the data available from Table 1a for F_1 and F_2 males and from Table 1b for F_1 and F_2 females, to analyze the genetic and environmental variation.)

Statistical analysis: The average male and female weight of four genotypes for parents, F_1's, and F_2's are given in Table 1a and 1b. The 1-sided student paired 't' test was performed to analyze the significant difference in means between: 'red and blue'; 'red and white'; and 'blue and white' lights. It was done for parental males, parental females, F_1 males, F_1 females, F_2 males, and F_2 females separately (calculated values not detailed here but available from A. Gupta* upon request). The 1-sided 't' table value for 3 degrees of freedom is 5.841 at a probability of 0.99 (0.005 percent level of significance), and that is higher than the 't' observed value in parental males and females; F_1 males and F_1 females; and F_2 males and F_2 females, implying the means calculated and tested for the difference between red and blue'; 'red and white'; and 'blue and white' lights were significantly different. The statistical analysis provides the evidence of the effect of colored light on weight in parental males and parental females; F_1 males and F_1 females; and F_2 males and F_2 females.

Genotype and colored light interaction: The genotype × colored light interaction phenomenon is observed for two genotypes (1 and 2) between red and blue light, between blue and white light in case of males (Figure 1a), and in case of females (Figure 1b). The wavelength for *red* light is less than 650nm; and for *blue* light about 450 – 400 nm. The wavelength for white light is an unfiltered incandescent bulb. [However, the wave length (not measured) at which such an interaction is observed differs from males to females.]

Canalization: It is a measure of the ability of a population to produce the same phenotype regardless of variability of its environment or genotype. In the present experiment, genotypes 3 and 4 show the formation of canalization originating from blue light (point of canalization) towards red as well as towards the white light (Figure 1a). This means that the two genotypes becomes phenotypically cryptic under canalizing conditions but uncovered under particular decanalizing environmental or genetic conditions. ["The canalization therefore may, at least temporarily, constrain phenotypic evolution, Flatt (2005)"].

Evolutionary aspect: These data suggest that genetic variation in the four genotypes may allow different levels of successful adaptation to different colored light environments. Thus, over a longer period of time in such environments, the *Drosophila* populations may evolve to favor the most successful genotypes.

Finally, the present experimental data show the effect of colored light due to the difference in *light wavelength* on the development of a phenotypic trait from a genotype. The results are in accordance with those in *Drosophila* by Dobzhansky and Spassky (1944), Gupta and Lewontin (1982), in plants by Clausen *et al*. (1948), in human-beings by De Lorenzo *et al.* (1999), and Van't Veer and Bernards (2008). The present data confirm the results reported by Berry-Wingfield *et al*. (2010) using different colored light and analyzing *only* the *data on parental* iso-female lines of *D. melanogaster*, and *not* the hybrids (F_1's and F_2's). The data suggest that further research should be carried out on an individual genotype basis for the development of a phenotypic trait.

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Evaluation of ethidium bromide effects in the life cycle and reproductive behavior of *Drosophila melanogaster*.

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Abstract

Ethidium bromide (EB) is an intercalating agent of nucleic acids. For this reason it is generally used in molecular biology and in structural studies of DNA and chromatin. Many scientists have demonstrated that this chemical can have mutagenic properties in some living organisms, including *Drosophila melanogaster*. However, most of them used concentrations up to a thousand times higher than those used used in methods of molecular biology for nucleic acid staining after electrophoresis. In the present work we verified the effect of Ethidium Bromide in all phases of development (egg, larva, pupa, and adult) of ten generations of *Drosophila melanogaster* exposed to the chemical treatment (F1, F3, F6, and F10). Moreover, we analyzed the time spent for pre-copulation and copulation. The results show that ethidium bromide interferes in the viability of eggs, larvae, pupae, and adults of *Drosophila melanogaster*. On the other hand, the behavior related to reproduction showed significant differences between the groups exposed to 30 μM EB and 1μ M EMS (ethylmethanesulfonate) and the control group in terms of the time spent in copulation. So, the data suggest on one side that ethidium bromide interfered in developmental genes, causing in some individuals inviability to reach the adult phase, and on the other side that it can interfere in the fruit fly behavior, acting as a neurotoxic agent.

Introduction

Most of the chemical substances do not have a proper hazard classification. In this context, environmental monitoring is essential for identification of toxic products. Because of that, in the last years occurred a significant growth in the interest for studying the effects of substances to which people are daily exposed (Itoyama *et al.*, 1998). The effects of those drugs have been analyzed in several organisms, including bacteria, yeast, plants, and animals, besides humans, whenever it is possible (Timson, 1977; Leonard *et al.*, 1987).

When the organisms cannot avoid the exposure to a poisonous agent, the biological effects in response to these agents always happen after biochemical and cellular events. Accordingly, the cellular and biochemical parameters have a great potential to be used as stress indicators to evaluate the physiological conditions of an organism (Stegman *et al.*, 1992). Organisms try to overcome these stressful conditions by the activation of genes to produce specific proteins (Nazir *et al.*, 2003a), and the expression of such genes can minimize the stress effects (Atkinson and Walden, 1985).

Biochemical changes can often be translated as modifications in the morphology, behavio,r or metabolic pathways analyzed in a species known as a bioindicator, chosen for its sensibility or tolerance to several parameters, such as organic pollution or other kinds of pollutants (Washington, 1984).

In the last decades, the use of animal tissues for toxicological tests involves two fundamental concepts: science and ethics, leading to a search for alternative approaches. Nowadays, species of *Drosophila* are model organisms for toxicological studies, since they are well defined in terms of their genetics, biological development, and genome (Mukhopadhyay *et al.*, 2004). Additionally, fruit flies have high sensibility to toxic substances, and they are insects of easy maintenance in the laboratory, feeding mainly on bacteria and yeast that participate in the fermentation of carbohydrate rich substrates, such as decomposing fruits (Almeida *et al.*, 2001). Besides, the use of *Drosophila* is recommended by the European Center for Validation of Alternative Methods, which promotes the scientific and regular acceptance of alternative experimental methods (Mukhopadhyay *et al.*, 2004).

The use of insects, specifically *Drosophila*, for biomonitoring of genetic damages caused by chemical agents has traditionally been done more than 50 years. However, during the last years, experiments using *Drosophila* are related to the identification of carcinogens and as a model for the study of mutagenicity mechanisms induced by chemicals (Vogel *et al.*, 1999).

Genotoxic substances such as diethylestilbestrol, diphenylhydantoin, imipramine, testosterone, and tolbutamide have shown a high teratogenic potential, whose effects were evident in muscles and neurons in *Drosophila melanogaster* (Bournais-Vardiabasis *et al.*, 1983). The presence of varying ethanol concentrations in the culture medium of *Drosophila* caused the malformation of legs (segments lacking, absence of the legs or deformed), wings, dumbbells, and melted buccal parts (Ranganathan *et al.*, 1987). Cypermethrin, a potent insecticide, promoted the significant increase in DNA damage in the cells of the medium and previous cerebral ganglia (Mukhopadhyay *et al.*, 2004).

The genus *Drosophila* is found in six of the seven zoogeographic areas of the Earth (with exception of Antarctica). *Drosophila melanogaster*, as the other ones from genus *Drosophila*, has a complete metamorphosis, passing through all the developmental stages. The female lays eggs that hatch as larvae. These larvae pass for three stages and then, get into the pupal stage. Pupae stay attached to the glass wall in an artificial system. After a few days**,** from the pupae emerges the flying adult. Among thousands of residues generated by research laboratories, we have chosen to analyze the toxic effects of ethidium bromide (EB). That is the common name for 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide, an intercalating agent used in methods of molecular biology. There are no studies focusing on the effect of EB in the developmental stages of *Drosophila melanogaster***.** However, the effects of this chemical have been tested in other organisms.

Nishiwaki *et al.* (1974) pointed out that in mice EB acts as an inhibitor of RNA-dependent DNA polymerase activity, and for this reason it can be considered as an antitumoral agent. Furthermore, Heinen (1978) showed that EB inhibits cell growth in tissue culture, even at very low concentrations. However, in spite of this, EB is not used as an antitumoral agent because it has mutagenic capacity in some organisms. Results in bacteria show that EB is an effective frameshift mutagen if it is metabolically activated by liver microsomes (McCann *et al.*, 1975). Sea urchin eggs exposed to water containing 50 μM of EB developed chromosomal abnormalities and failed to divide normally (Vacquier and Brachet, 1969). Experiments in bacteria showed that EB is an effective frameshift mutagen if it is metabolically activated by liver microsomes (McCann *et al.*, 1975). Experiments reported by Nass (1972) indicated that the growth of mouse fibroblasts and hamster kidney cells are inhibited by 0.3-13 μM of ethidium, and that mitochondrial, not nuclear, DNA synthesis was inhibited by ethidium.

In *Saccaromyces cerevisiae* EB acts as a strong inducer of *petite* mutants (Slonimski *et al.*, 1968). Its action is based on the inhibition of mitochondrial nucleic acid and protein synthesis and is probably due to specific intercalations between the base pairs of mitochondrial DNA (Perlman and Mahler, 1971).

In previous work (Ouchi *et al.*, 2007), we have analyzed the effect of EB in productivity, protein profile, and phenotypical changes. The present work involved the exposure of ten generations of *D. melanogaster* to EB and intended to analyze its effect in the developmental phases of the insect. For specimens of F10, we also analyzed the effect of EB in sexual behavior, measuring duration of pre-copulation and copulation.

Materials and Methods

Stocks

Specimens of *Drosophila melanogaster* were collected at São José do Rio Preto (State of São Paulo, Brazil) and identified at the *Drosophila* Systematic Laboratory from our Institute. Two lines have been used in this work. One of them originated from one female (isofemale line). The other one was called massal line, because it has been originated by six females, having therefore higher genetic variability. Both stocks were maintained in a temperature-controlled chamber at 24ºC ± 1ºC.

Exposure to ethidium bromide

We used three different concentrations of EB (1, 5, and 30 μM) and two control groups. 1 μM of EB corresponds to the concentration used for visualization of nucleic acids, a solution that is frequently disposed in the drain without chemical neutralization. For the positive control we used 1 μM EMS (Ethylmethanesulfonate – *Acros Organics*), a mutagen, whereas the negative control was fed with uncontaminated culture medium. The chemicals were fully mixed with 50 mL of warm (45ºC) banana-agar medium, and then poured into 250 mL glass bottles. For each treatment, four replicates were prepared; three of them were used for the productivity experiments (Ouchi *et al.*, 2007) and the fourth one for an experiment of larval viability (not shown in this article). Ethidium bromide was purchased from *Promega*.

Maintenance of generations

For each bottle, twelve males of the *Drosophila melanogaster* stock were joined to the same number of virgin females. The culture medium was used as substrate for feeding, and females were allowed to oviposit for 6 days. After that, the adults were removed from the bottle for quantification. Ten days after the parents were added to the glass bottle, the new generation initiated its emergence

(F1). In the fifth day, that corresponds to the maximum emergence of the control group, twelve males and virgin females were isolated from each replicate and then transferred to a new glass bottle, maintaining the same conditions, in order to originate F2 (the second generation). The same procedure was repeated until the tenth generation.

Viability from egg to adult

A couple from the fourth replicate of each treatment of F10 from the isofemale line was separated, keeping the males separated from the females. Each *Drosophila* stayed individually in glass tubes for five days until they reached their sexual maturity. After this period, males and females from each treatment were mixed in the same glass tube where they were allowed to copulate for 24 hours. Afterwards, we removed the male and allowed the female to lay eggs for 24 hours on a spoon containing 3 mL of agar-sucrose medium. The eggs for the four replicates were counted with a stereoscopic microscope (*Carl Zeiss*). The spoons containing eggs were transferred to a 250 mL glass bottle containing 50 mL of banana-agar culture medium, containing or not EB or EMS. These experiments allowed us to count how many pupae and adults were viable.

Viability from larva to adult

The fourth replicate was also used to collect larvae for viability experiments, accomplished for F1, F3, F6, and F10 of the isofemale line. Ten glass tubes containing 7 mL of treated banana-agar culture were used for each treatment, and to each one we added ten larvae. After a few days, adults initiated their emergence and were analyzed quantitatively and morphologically in a stereoscopic microscope.

Viability from pupa to adult

Three replicates were used for experiments of productivity (not shown). After fifteen days (time reserved to collect productivity information), some pupae remained attached to the wall of the glass without emerging as adults. These pupae were counted for F1, F3, F6, and F10 from the isofemale line and for F1 and F10 of the massal line.

Sexual behavior

For each treatment, 24 couples were divided in six glass tubes, containing banana-agar culture medium. Adults from both sexes were maintained isolated for five days in order to reach sexual maturity. For each day, a negative control was analyzed for each treatment. At the time of measurements every day, parameters of temperature, brightness, and period were the same. After females and males were mixed the pre-copulation times were logged. When each couple started to mate, this copulation time was logged, too.

Statistical analysis

The data of pre-copulation and copulation time were analyzed using Student´s T test ($p < 0.05$) by the software BioEstat 4.0 (Ayres *et al.*, 2005; Zar, 1999).

Results

In order to verify the action of EB in the different developmental stages of *Drosophila melanogaster*, we have analyzed the viability from larvae to adults of the isofemale strain (Table 1) and also pupae that did not emerge in the isofemale and massed lines (Table 2).

Table 1. Larval viability (%) from the isofemale strain of *D. melanogaster* after exposition to EB and EMS.

Treatment	Initial number of larvae	Generations				Average
		F1	F3	F6	F10	
Control	100	89	93	92	84	89.5
1µM EB	100	82	94	95	73	86.0
5µM EB	100	86	97	89	79	87.8
30µM EB	100	84	92	81	71	82.0
1µM EMS	100	74	90	86	80	82.5

Table 2. Pupal viability (%) from isofemale and massal strains of *D. melanogaster* after exposition to EB and EMS.

Treatment	Isofemale Strain				Massal Strain	
	F1	F3	F6	F10	F1	F10
Control	98.7	93	99.6	99.5	98.5	99.4
1µM EB	98.1	94	99.4	99.3	94.7	98.1
5µM EB	97.7	97	99.3	99.1	97.9	96.8
30µM EB	98.2	92	99.1	98.7	92.0	93.7
1µM EMS	93.5	90	98.7	97.1	97.4	93.7

Table 3. Egg to adult viability for F10 from the isofemale line from *D. melanogaster* exposed to EB and EMS.

Treatment	F10			
	Eggs laid (24h)	Pupae	Adults	% emerged adults
Control	127	111	111	87.4
1 µM EB	161	139	139	86.3
30µM EB	109	74	74	67.8
1µM EMS	130	43	43	36.2

Table 1 shows that in the first generation, the exposed animals had a lower viability than the control group. Even having small differences in viability, some morphological alterations were found in adults for the groups exposed to 1 and 5 µM of EB: one male with morphological alteration in wings and two males with morphological alterations in wings, respectively. This fact shows that even those insects that emerged might have suffered some type of gene alteration.

In F3, we could notice that the viability from larva to adult was not always higher in the control group, as verified for F1. For F6, a similar result to that described previously for F3: for the group exposed to 1 µM of EB the larval viability was higher than for the negative control. However, this fact was not observed in the same experiment for the 10^{th} generation, where the larval viability was always smaller in the exposed groups. Considering the average of all generations, the larval viability was, in all treated groups, lower than for the control. We also observed that some flies started their emergence, but stopped in the middle of the process. So, we noticed that not only larvae were affected by the chemical treatments, but pupae were, too.

This fact was confirmed by the experiment involving the viability from pupae to adults, showed in Table 2. There we can see that the viability of pupae for the groups treated with EB and EMS were, in all conditions, slightly lower than that from the negative control group, in F1, F6, F10 of isofemale strain and for F1 and F10 of massed strain. If we compare the isofemale and massed lines for F10, we notice that the massed line, for the same concentration of EB and EMS, had a lower viability.

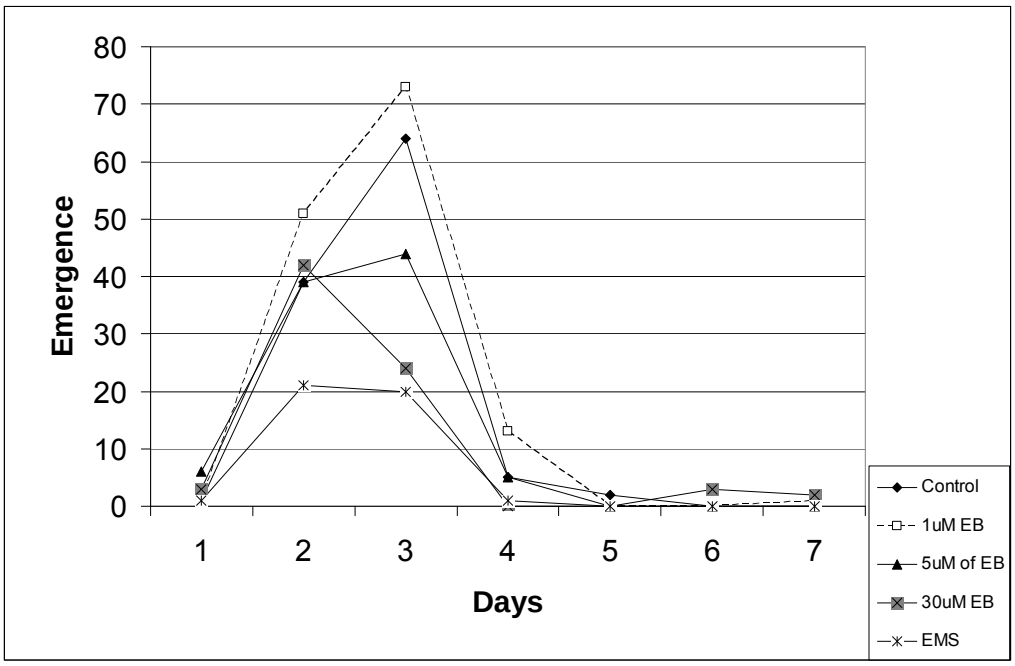

Figure 1. Pattern of daily emergence (egg viability).

Table 4. Average time expressed as minutes (') and seconds (") of pre-copulation and copulation for F10. The asterisks (*) represents the process (pre-copulation or copulation) and the treatment that showed significant difference compared to the control.

F10 isofemale line		
Treatment	Pre-Copulation	Copulation
Control	9'02"	19'36"
1 µM of EB	7'36"	22'22"
Control	13'15"	19'15"
5 µM of EB	10'56"	20'35"
Control	16'14"	18'48"
30 µM of EB	14'08"	21'01" *
Control	17'15"	21'50"
1 µM of EMS	12'15"	20'30" *

In Table 3, we show the effect of the chemical treatment in all the developmental stages. This experiment shows that EB and EMS affected mainly eggs and larvae, since from all the pupae emerged adults. Moreover, we quantified the pupae by the day that emerged in adults' emergence of the groups exposed to 30 µM EB and 1 µM EMS occurred one day before the others treatments, and their productivity was lower than the control, as shown by Figure 1.

The pre-copulation and copulation times are shown in Table 4. We can observe that the group exposed to 30 µM EB had a significantly larger time for copulation when compared to the control. However, for the group exposed to 1 µM EMS, the spent time was smaller than in the control. Both differences were significant ($p \leq 0.05$).

Discussion

Until the decade of 1980, there are no available works related to the action of EB in *Drosophila*. Marcos *et al.* (1981a) tested the genotoxic effects of EB in *Drosophila melanogaster*, using wild-type males. The concentrations that they used were in the range from 0.03 to 3 mM, many times larger than those used in our experiments. Toxicity tests were performed and detected that LC_{50} = 2.16 mM, for a 48h exposure. EB induced a significant increase in sex-linked recessive lethals (1.01% at 3 mM), and induced dominant lethals to a significant extent (Marcos *et al.*, 1981b).

Our results showed that in the experiments of viability from larvae to adults and pupae to adults, in ten generations, some alterations had happened during the development of the insects. These effects were observed by the higher amount of inviable larvae, which did not complete their development or failed to emerge as adults. Moreover, some flies emerged with wing alterations. Ranganathan *et al.* (1987) tested ethanol for teratogenicity in *Drosophila melanogaster* and reported malformations involving the legs and wings. Also, by exposing larvae to ethanol, the developmental stage sensitivity was investigated, showing also harmful effects. Genotoxic effects of griseofulvin, an antimycotic agent widely used in dermatophytoses, were studied by Tripathy *et al.* (1996) in the somatic and germ line cells, on third and second instar larvae of *Drosophila melanogaster*. Second and third instar larvae, exposed to acrylamide, considered to be a carcinogen, displayed genotoxic effects in *Drosophila melanogaster*, by the wing mosaic assay and the sex-linked recessive lethals test. It was observed that acrylamide is both mutagenic and recombinogenic in the wing disc cells and induces sex-linked recessive lethals (Tripathy *et al.*, 1991).

As mentioned above, some pupae started their emergence, but died in the middle of the process, leaving the body partially out of the pupal case. A similar result was observed by Sousa-Polezzi and Bicudo (2004), analyzing the effect of Phenobarbital (PB) in the development of *Aedes aegypti* (Diptera, Culicidae), suggesting that PB may affect the nervous system.

Analyzing the viability of laid eggs for the tenth generation of the isofemale line, we observed that it was lower in the exposed groups (Table 3). Moreover, we can see that there was a decrease of viability as EB concentration increased. Concerning the groups exposed to EMS, viability was lower than for those treated with EB. In the present study, EMS was used as a positive control, since it is a known mutagenic product (Griffiths *et al.*, 1998), being used as a parameter for the data obtained with ethidium bromide. Marcos *et al.* (1981a) carried out similar experiments analyzing the influence of EB and egg viability counting laid eggs and emerged flies. The number of viable eggs was inferior to the number of laid eggs (when compared to the control group), in agreement with our results. Our experiment allowed us to analyze all the stages, and it is possible to verify that the highest effect affected both eggs and larvae, because all the pupae emerged as adults. Several cellular divisions, mitoses and meiosis characterize the egg phase. Marcos *et al.* (1981a) have pointed out that EB act as a mitotic and meiotic poison and it even blocks the process of spermatogenesis.

Some other studies have focused on the effects of some chemical products in adults and in the different stages of development, using *Drosophila* as a bioindicator. Akins *et al.* (1992) revealed that some heavy metals, such as lead and cadmium, caused in *Drosophila melanogaster* a developmental delay at the phase from larva to pupa. In larvae of *D. melanogaster* exposed to sodium azide (a potent mutagenic product), it was observed the induction of somatic mutations and mitotic recombination in the wing cells (González-César and Branch-Morales, 1997). The same effect was observed when the tests were performed with methyl parathion, azametyphos, dichlorvos, and diazinon (Ekebas *et al.*, 2000). Until now, there are no studies focusing on the effect of EB in all developmental stages of *Drosophila melanogaster*.

It is noteworthy that the groups exposed to 30 μM EB and 1 μM EMS had emergence in the 6th and 7th days, whereas the others already stopped at the 5th day.

Itoyama *et al.* (1995) reported similar delays, analyzing the influence of caffeine in *Drosophila prosaltans*. In larvae of *Telmatoscopus albipunctatus* (Diptera – Pshychodidae), Sehgal and Simões (1977) verified that caffeine caused a significant delay of development and high mortality.

In the literature there are some works that verified the reproductive performance through the behavior, since some drugs can act on the nervous system. Itoyama *et al.* (1995) studied the effects of caffeine in mating of *Drosophila prosaltans*, based on the observation of the duration of pre-copulation and copulation. Statistically, they have found a difference only in the pre-copulation time. Nazir *et al.* (2003b) observed that dimethyl sulfoxide in *Drosophila melanogaster* has expressed a toxic effect in hatchability, emergence, fecundity, and in reproductive performance. In order to verify if the behavior could be influenced by EB, we observed the duration of pre-copulation and copulation. Our results showed that all the exposed groups had a smaller duration for pre-copulation than the negative control. The groups treated by EB showed a larger time for copulation, but only for 30 μM EB the difference was significant. In *Drosophila,* the mating movements are complex and follow a characteristic pattern of each species. Previous work (Ouchi *et al.*, 2007) showed that the presence of ethidium bromide caused malformations in *Drosophila*'s body, which could have influenced sexual behavior. Besides, EB could have some influence on the neural system.

In conclusion, our results suggest that EB in low concentrations influenced in phases of development, mainly in eggs, causing in some *D. melanogaster* inviability to reach the adult stage and could have some effects on the neural system.

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Genetic control of color dimorphism in *Drosophila punjabiensis* of montium subgroup.

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Abstract

Drosophila punjabiensis, belonging to the montium species subgroup of melanogaster group, was examined for abdominal melanisation. Females show dimorphism dark or light in coloration, whereas males exhibit monomorphic abdominal melanisation, *i.e*., all are dark. The color dimorphism is regulated by two alleles of a single autosomal locus, and the light allele is dominant. Thus, *D. punjabiensis* exhibits color dimorphism controlled by a single locus, but its ecological significance is not clear.

Introduction

Abdominal melanisation is a conspicuously variable adaptive trait in many insects including *Drosophila* (Wittkopp *et al.,* 2003; True, 2003; Rajpurohit *et al.,* 2008). In different insect taxa, there are diverse patterns of body melanisation, *i.e.,* (a) several black species of Collembola occur in temperate regions, *i.e.,* Pyrenees, Swiss Alps, and Himalayas (Mani, 1968; Rapoport, 1969); (b) in *D. melanogaster*, a cosmopolitan species, the extent of melanism varies with geographical location (Pool and Aquadro, 2007; Parkash *et al.,* 2008a,b); (c) discrete melanic and non-melanic morphs occur as genetic polymorphism in species of the montium species subgroup (Ohnishi and Watanabe, 1985). The color polymorphism in abdominal tergites was first reported by da Cunha (1949) for *D. polymorpha.* Later on color variations in montium species subgroup *D. rufa* (Oshima, 1952), *D. kikkawai* (Freire-Maia *et al.,* 1954), *D. auraria* (Lee, 1963), and *D. jambulina* (Parkash and Sharma, 1978; Parkash *et al.,* 2009) were described.

The present work is a first report showing the genetic basis of color dimorphism in *D. punjabiensis* of montium species subgroup, through Mendelian crosses between dark selected and

light selected strains. Our results exhibit color dimorphism for the last two abdominal segments in females; and the light morph is dominant over the dark morph.

Material and Methods

Cultures

Wild individuals of *D. punjabiensis* (n = 130–160) were collected from lowland localities (~28.5ºN; 219 m) of subtropical parts of the Indian subcontinent. The collections were made in September- October with net sweeping and bait traps from fruit markets and godowns, as well as from nurseries. Based on the T_{ave} data of the sites of origin of populations, cultures were maintained at 25ºC. Density was kept low (30–40 eggs per vial) by limiting the egg laying period for 6–8 h. Climatic data for the sites of origin of populations were obtained from the Indian Institute of Tropical Meteorology (IITM; www.tropmet.res.in).

Dark and light strains

In *D. punjabiensis*, body color polymorphism is limited to females only and is not evident in males. For obtaining true breeding dark and light strains, isofemale lines were established from the wild-caught females (60–80) of each population, and the progeny of each line were checked for 8 successive generations for dark and light phenotypes. Many of the isofemale lines showed segregation for dark and light strains. We isolated virgin flies from such laboratory cultures and made several single pair matings (40–50). The crosses that gave all dark or light progeny were considered as true breeding strains.

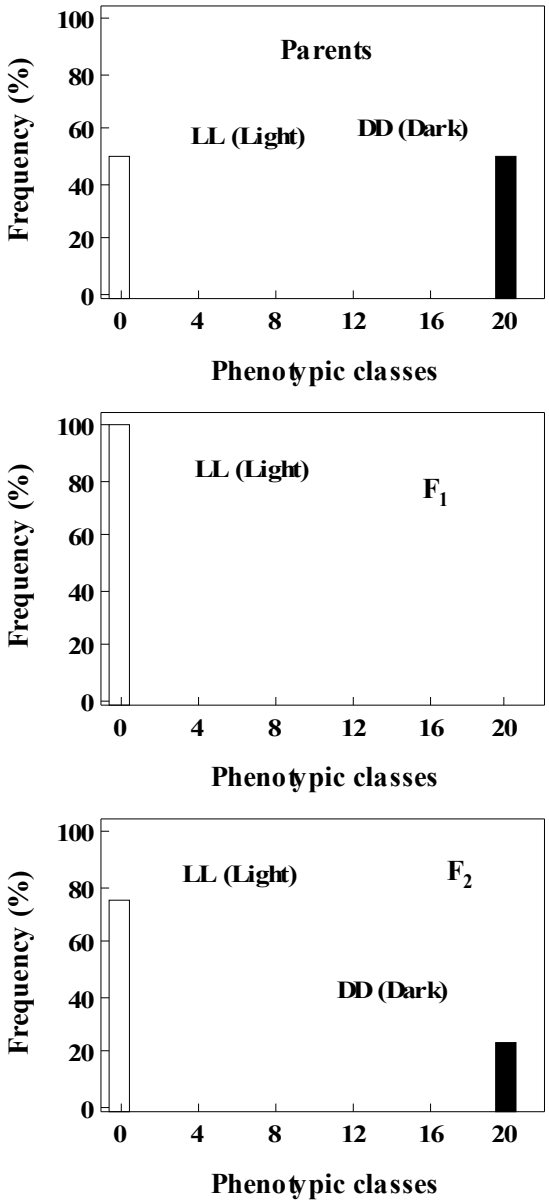
Genetic basis of color dimorphism

In female individuals of *D. punjabiensis,* there are variations in percent melanisation for the last two abdominal segments (6^{th} and 7^{th}), *i.e.,* either these segments are totally dark or light which correspond phenotypically to dark or light morphs. However, males do not show such variations for

Table 1. Results of F_1 and F_2 genetic crosses (5 replicates) between homozygous light and dark strains of *D. punjabiensis* for female progeny scored. Only females demonstrate body color polymorphism.

Genetic Crosses	Type/ Replicate	♀ flies scored* (n)	Light ♀ Morph (Freq. %)	Dark ♀ Morph (Freq. %)	Mendelian Ratio	χ^2 test
(A) Light ♀ * ♂	F_1	200	200 (100 %)	0 (0 %)	----	----
F_{1A} ♀ * F_{1A} ♂	F_2: 1.	125	96 (76.80%)	29 (23.20%)	3.31:1	ns
	2.	146	109 (74.66%)	37 (25.34%)	2.96:1	ns
	3.	112	83 (74.11%)	29 (25.89%)	2.86:1	ns
	4.	134	101 (75.37%)	33 (24.63%)	2.97:1	ns
	5.	128	97 (75.78%)	31 (24.22%)	3.13:1	ns
(B) Dark ♀ * ♂	F_1	200	200 (100 %)	0 (0 %)	----	----
F_{1B} ♀ * F_{1B} ♂	F_2: 1.	130	97 (74.62%)	33 (25.38%)	2.94:1	ns
	2.	152	115 (75.66%)	37 (24.34%)	3.11:1	ns
	3.	140	106 (75.71%)	34 (24.29%)	3.12:1	ns
	4.	115	86 (74.78%)	29 (25.22%)	2.97:1	ns
	5.	129	98 (75.97%)	31 (24.03%)	3.16:1	Ns

F_{1A} = Light ♀ * ♂: F_{1B} = Dark ♀ * ♂, ♂'s appear phenotypically similar; ns = nonsignificant, no significant difference is observed between expectations and the observed numbers through χ^2 test.



the last two abdominal segments. The reason for this sexual dimorphism is not known. Thus, only female progeny of crosses were scored for genetic analysis. Furthermore, in order to ascertain the genetic basis as well as allelic dominance, we carried out Mendelian crosses (F_1 and F_2 crosses) with these true breeding dark and light strains of *D. punjabiensis*. We made 18 single pair matings using 1 ♂ and 1 ♀, of each morph type for obtaining F_1 progeny. We randomly scored 200 ♀ flies (F_1) and all were found to be of light morph. Further, out of the pooled F_1 progeny, 100 ♂ and 100 ♀ were randomly selected and ten replicates with 10 pairs each were used for F_2 progeny. Therefore, the data on male progeny were not included in the tables.

Figure 1. Frequency distributions of phenotypic classes on the basis of the last two abdominal segments (6^{th} and 7^{th}) for percent melanisation in dark and light females in F_1 and F_2 crosses of *D. punjabiensis*. For parents, equal numbers of dark and light flies were taken to perform crosses.

Results and Discussion

Table 1 shows the results of crosses between true breeding strains for dark and light morphs for the female color dimorphism in *D. punjabiensis*. Crosses helped to analyze genetic basis as well as allelic dominance for body color polymorphism of the last two abdominal segments in females. Reciprocal crosses for F_1 progeny always yielded light morph (Table 1). In reciprocal F_2 crosses ($F_1 \times F_1$), dark morph reappeared, and phenotypic ratios of light: dark morphs were close to the Mendelian F_2 ratio of 3:1 (Table 1; Figure 1). Dark and light morphs were discrete and followed Mendelian inheritance of a single locus with two alleles. The data from F_1 and F_2 progeny clearly evidenced lack of any intermediate morph (Table 1; Figure 1). Our results concur with similar genetic analyses of southeast Asian populations of *D. jambulina* (Ohnishi and Watanabe, 1985; Parkash *et al.,* 2009). The dominance of light allele over dark allele may help this species in adaptation to warm and humid environments in the tropics (Parkash *et al.,* 2009). Thus, like its sibling species *D. jambulina*, we hope to find similar evidence in *D. punjabiensis* of montium species subgroup and definitely this study needs further investigation.

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Reduced mating activity and fitness of *Drosophila ananassae* on exposure to valproic acid.

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Abstract

Over the last few decades, *Drosophila* has been used as model for the study of toxic effect of drugs. The drugs play major implication as stress molecules that reduce the overall fitness in general. The present study is aimed to address the exposure of *D. ananassae* to variable doses of Valproic acid an anti epileptic drug (AED). In order to ascertain the biological potentiality in terms of life history traits such as mating propensity, fecundity, fertility and life span mating propensity, fecundity, fertility and life span. Interestingly the observation reveals that flies exposed to the higher dose of VAL have experienced increased courtship duration with low fecundity and fertility, while the differences are insignificant for longevity.

Key words: Valproic acid, *D. ananassae*, Mating propensity, Fecundity, Fertility, life span.

Introduction

Valproic acid (VAL) is an anticonvulsant drug used in the treatment of epilepsy and bipolar disorder, across AEDs, valproate was associated with the highest risk of reproductive toxicity. VAL has the highest risk of birth defects of any of the commonly used antiepilepileptic drugs. However, some epilepsy can only be controlled by valproate, and also the risk of birth defects with valproate is two to five times higher than other frequently used anti-epileptic drugs (Sander, 2010).

D. ananassae is used as a model organism for genetic studies because of its excellent viability, high mutability, and certain peculiarities in its cytological and genetic behavior. With the progress of research, it has become clear that it is unique among the species in the genus *Drosophila* (Singh and Chatterjee, 1985; Singh, 2010; Sisoda and Singh, 2006, 2009). *Drosophila* has emerged as one of the most powerful models for human diseases and toxicological research (Chowdhuri *et al*., 2005). VAL can affect each life stage in a different manner and may depend on a specific stage of life history traits. Pharmacological tools have introduced behavioral alterations in *Drosophila* reminiscent of human behavior. The fly can effectively be used for low- to high-throughput drug screens (Pandey and Nichols, 1982).

Materials and Methods

The fly stocks are routinely cultured in standard wheat cream agar medium. The test flies are cultured in wheat cream agar media along with the different concentrations of epileptic drug valproic acid (VAL) 0.2, 0.3, and 0.4 mg/ml. Both control and treated flies were cultured in un-crowded conditions at 22±1ºC (rearing temperature) and a relative humidity of 70%.

Antiepileptic drug – Valproic acid (*VAL*)

Valproic acid 98% (CAS no: 1069-66-9) was obtained from Sigma-Aldrich, soluble in water added to media. Standardization of lethal concentration was carried out on adult mortality for seven days and sub lethal (0.2 and 0.3 mg/ml) and lethal doses (0.4 mg/ml) were used to treat the flies (Mohammed *et al.*, 2009).

Experimental Crosses

D. ananassae virgin females and unmated males were collected and reared separately for 2 days. Further these flies were fed on wheat cream agar media with different concentrations (0.2, 0.3, and 0.4 mg/ml) of valproic acid and alongside control flies, fed on media for three days.

Four sets of crosses were made using about 30 pairs of flies for each cross, facilitating single pair mating, *i.e*., each pair in a separate vial. These crosses include untreated male × untreated female (Control-C), treated male × untreated female (T_1), untreated male × treated female (T_2), and treated male × treated female (T_3). A total of 120 pairs of flies were used to study mating propensity (courtship duration and copulation duration), reproductive fitness (fecundity and productivity) (Sisoda and Singh, 2009), and longevity (Luckinbill and Clare, 1985).

The mating propensity, *i.e*. courtship duration and copulation duration, was observed from 7 am to 9 am as maximum mating occurs during morning hours (Hegde and Krishna, 1997). Soon after mating, males from each pair were separated and females transferred into separate vial containing fresh food medium. Fecundity was assayed by counting number of eggs laid. Flies were successively transferred into fresh vials containing media every alternate day for 6 days. Eggs were allowed to hatch, and dilute yeast was added until pupation. Further, the same sets of vials were assessed for the emergence of the adult flies and likewise the fertility was recorded for the total productivity (Harini and Ramachandra, 2007). In addition to this, the treated and untreated flies were maintained until death to record lifespan of the flies.

Statistical Analysis

One-way ANOVA was performed for the said life history parameters, namely courtship duration, copulation duration, fecundity, fertility, and longevity. Post-hoc Duncan's multiple range test (DMRT) was conducted to record the significant differences. The analysis was performed using the statistical presentation system software package SPSS 15.0 for MS Windows.

Results

Mating propensity

Figure 1a reveals that the exposure to higher doses the courtship time taken compared to low dose was significantly more and this was similar for both treated females and treated males, *i.e*., T_1, T_2, and T3 than C significant value for sublethal dose $P < 0.001$ and for lethal dose is $P < 0.001$

(Table 1). Insignificant difference in copulation duration was determined (Figure 1b) between control and treated trials in different concentrations.

Table 1. Mean mating propensity of *D. ananassae* treated with Valproic acid

Traits→	Courtship Duration			Copulation duration		
Trials*↓ Doses→	0.2	0.3	0.4	0.2	0.3	0.4
C	5.40 ± 0.54	4.80 ± 0.55	4.80 ± 0.48	4.30 ± 0.98	4.20 ± 0.29	4.30 ± 0.30
T_1	4.34 ± 0.45	7.60 ± 0.70	18.90 ± 2.12	4.30 ± 0.94	4.40 ± 0.37	4.20 ± 0.35
T_2	6.10 ± 0.60	8.80 ± 1.11	21.80 ± 1.94	4.10 ± 0.99	3.82 ± 0.41	3.80 ± 0.41
T_3	5.80 ± 0.42	11.20 ± 1.22	32.80 ± 2.47	4.44 ± 0.88	3.80 ± 0.32	3.28 ± 0.32
ANOVA	F = 115 d.f. = 3,116 P > 0.951	F = 7.98 d.f. = 3,116 P < 0.001	F = 3.302 d.f. = 3,116 P < 0.001	F = 0.229 d.f. = 3,116 P < 0.876	F = 0.717 d.f. = 3,116 P > 0.548	F = 0.564 d.f. = 3,116 P > 0.648
DMRT	C/T_1, C/T_2, T_1/T_2, $T1/T_3$, T_2/T_3	C/T1, C/T_2, C/T_3, T_1/T_2, T_1/T_3, T_2/T_3	C/T_1, C/T_2, C/T_3, T_1/T_2, T_1/T_3, T_2/T_3	C/T_3, T_1/T_2, T_1/T_3, T_2/T_3	C/T_1, C/T_2, C/T_3, T_1/T_2, T_1/T_3	C/T_2, C/T_3, T_1/T_2, T_1/T_3,

*Note: C- Untreated♂ x Untreated♀; T_1-Treated♂ x Untreated♀; T_2- Untreated♂ x Treated; T_3-Treated♂ x Treated♀

Fecundity

The mean fecundity (Table 2) indicates significant reduction in mid dose $P < 0.001$ and high dose $P < 0.001$ of T_3 than T_2 and T_1. When compared to control, fecundity has been reduced in T_1 and T_2 while differences are insignificant at low dose for both treated and control (Figure 2a).

Fertility

Table 2 indicates that the fertility has reduced significantly in mid and high dose treatments. Reduction in the fertility was observed for 0.3 mg/ml and 0.4 mg/ml, $P< 0.001$ and $P < 0.001$, respectively. There was no difference between low dose and controlled treatments. A significant difference was observed in higher dose in T3. A difference was also seen in high dose treatment of T_1 and T_2 than the control experimental trial (Figure 2b).

Longevity

Treated and controlled flies in Figure 2c have not shown differences in lifespan with all three concentrations. Thus, the present study shows that the exposure of flies to valproic acid at variable concentration has left no effect on the lifespan.

Discussion

Fertility is dependent on sexual activity, mating season, and on semen quality. Very few studies have addressed the issue of sexual activity in animals after AED treatment; however, it was reported that sexual desire was reduced in rats treated with VAL at very low doses (Tauboll *et al.,* 2008). The present study confirms that time taken to court the females is maximum in treated flies

Table 2. Mean life history traits of *D. ananassae* treated with Valproic acid.

Traits→ / Trials*↓ Doses→	Fecundity			Fertility			Longevity		
	0.2	0.3	0.4	0.2	0.3	0.4	0.2	0.3	0.4
C	102.40 ± 2.37	98.02 ± 2.73	104.02 ± 2.71	96.80 ± 2.77	98.50 ± 3.14	101.40 ± 2.87	85.06 ± 0.462	89.24 ± 0.802	92.64 ± 0.262
T_1	101.70 ± 4.08	99.60 ± 3.95	98.80 ± 2.86	96.90 ± 3.86	94.30 ± 4.18	76.40 ± 2.56	88.02 ± 0.321	84.24 ± 0.654	82.10 ± 0.942
T_2	95.70 ± 3.66	95.50 ± 4.70	94.10 ± 5.32	90.50 ± 3.95	87.60 ± 4.25	68.60 ± 4.25	82.34 ± 0.824	79.28 ± 0.464	86.98 ± 0.246
T_3	96.30 ± 2.94	86.80 ± 3.16	67.70 ± 3.39	94.17 ± 2.76	65.20 ± 4.11	43.20 ± 5.04	84.12 ± 0.234	89.68 ± 0.456	84.02 ± 0.102
ANOVA	F = 2.016	F = 4.348	F = 17.962	F = 1.617	F = 14.090	F = 14.083	F = 1.265	F = 2.454	F = 0.986
	d.f. = 3,116	d.f. = 3,116	d.f. = 3,116	d.f. = 3,116	d.f. = 3,116	d.f. = 3,116	d.f. = 3,116	d.f. = 3,116	d.f = 3,116
	P > 0.129	P < 0.001	P < 0.001	P > 0.202	P < 0.001	P < 0.001	P > 0.268	P > 0.432	P > 0.864
DMRT	C/T_1, C/T_2, C/T_3, T_1/T_2, T_1/T_3, T_2/T_3	C/T_2, C/T_3, T_1/T_2 T_1/T_3, T_2/T_3	C/T_1, C/T_2, C/T_3, T_1/T_2, T_1/T_3, T_2/T_3	C/T_2, C/T_3, T_1/T_2, T_1/T_3, T_2/T_3	C/T_1, C/T_2, C/T_3, T_1/T_2, T_1/T_3, T_2/T_3	C/T_1, C/T_2, C/T_3, T_1/T_2, T_1/T_3, T_2/T_3	C/T_1, C/T_2, C/T_3, T_1/T_2, T_1/T_3, T_2/T_3	C/T_1, C/T_2, T_1/T_2, T_1/T_3, T_2/T_3	C/T_1, C/T_2, C/T_3, T_1/T_2, T_1/T_3, T_2/T_3

*Note: C- Untreated♂ x Untreated♀; T_1-Treated♂ x Untreated♀; T_2- Untreated♂ x Treated♀; T_3 -Treated♂ x Treated♀

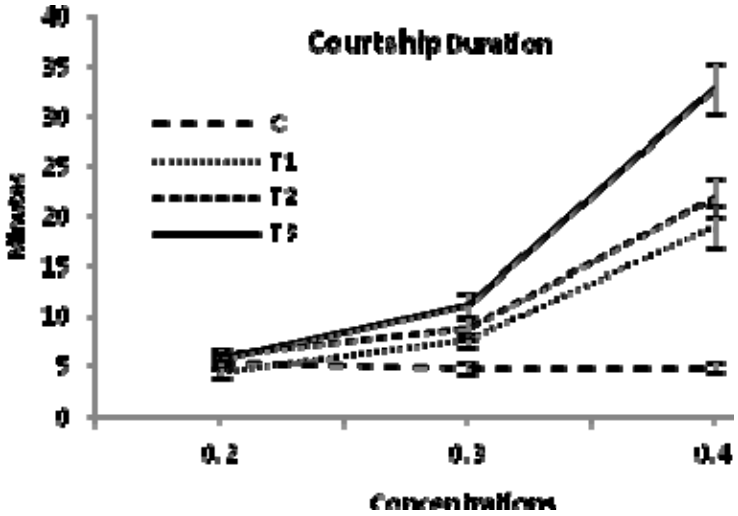

Figure 1a. Mean ± SE of courtship duration for three concentrations of valproic acid in different experimental crosses: C, T_1, T_2, and T_3.

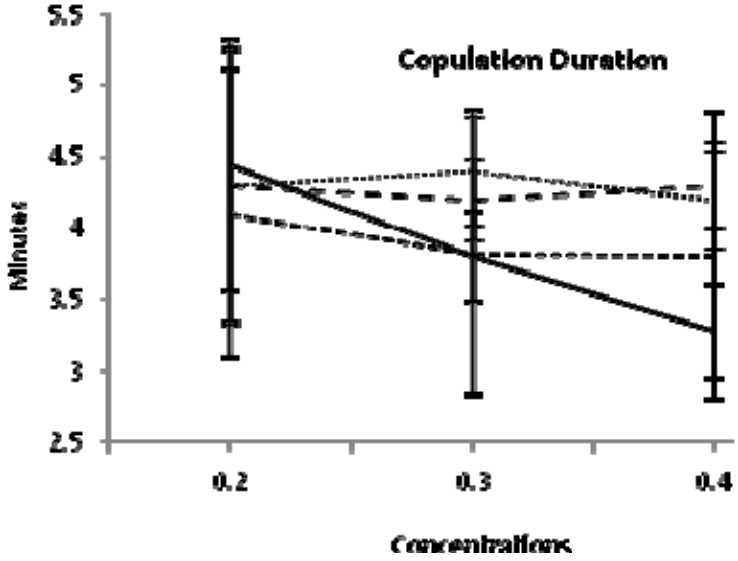

Figure 1b. Mean ± SE of copulation duration for three concentrations of valproic acid in different experimental crosses: C, T_1, T_2, and T_3.

indicated in Figure 1a than control in 0.3 mg/ml and 0.4 mg/ml of drug, while a difference was not observed between 0.2 mg/ml and control. The differences were insignificant for copulation duration between treated and control flies (Figure 1b) for all the three concentrations. It has been claimed that fertility rates have been reduced after long-term treatment with different AEDs; fertility rates in rats after 60 days low-

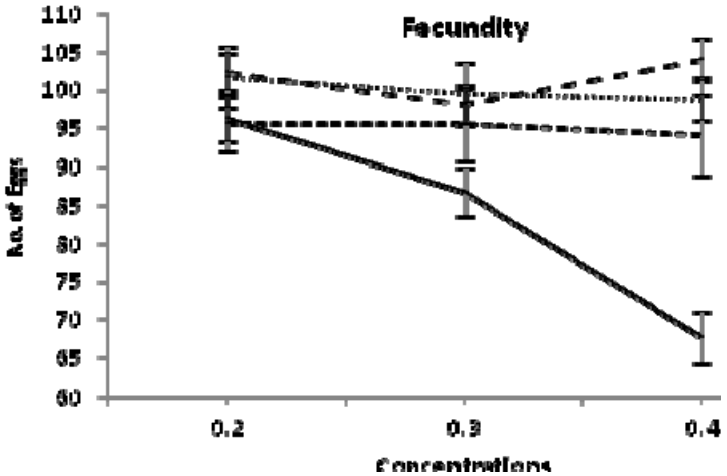

Figure 2a. Mean ± SE of fecundity for three concentrations of valproic acid in different experimental crosses: C, T_1, T_2, and T_3.

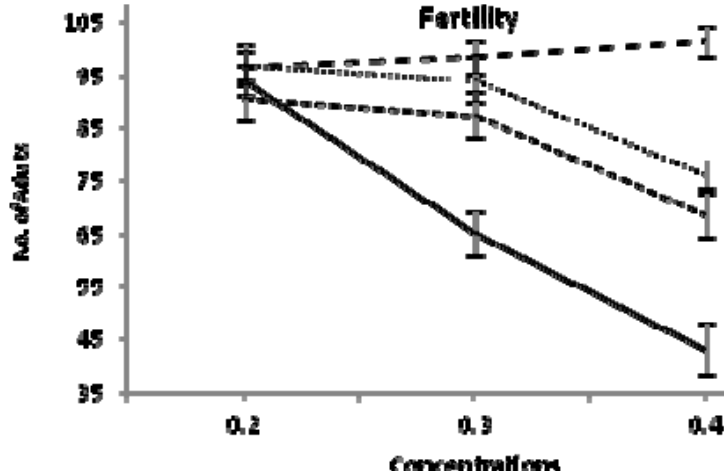

Figure 2b. Mean ± SE of fertility for three concentrations of valproic acid in different experimental crosses: C, T_1, T_2, and T_3.

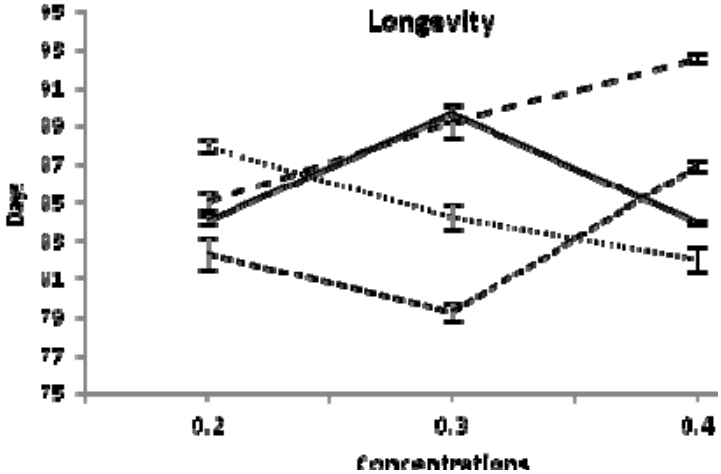

Figure 2c. Mean ± SE of longevity for three concentrations of valproic acid in different experimental crosses: C, T_1, T_2, and T_3.

dose drug treatment with valproate were reduced from 90% in controls to 40% and 30%, respectively. A significantly reduced fecundity and fertility rate was also observed for valproic acid in *D. ananassae* with increased concentration of valproic acid whether male or female is treated (Figures 2a-2b). Valproic acid is more commonly associated with reproductive endocrine disorders characterized by an ovulatory dysfunction than some other AEDs (Morrell *et al*., 2002; Betts *et al*.,

2003). A decrease in the prostate weight was found in the valproic acid treated rats. Sperm content and motility were decreased and the fertility rate diminished by 60%. It is important to remember that valproic acid has been found in the semen of treated rabbits and that chronic toxicity studies in animals showed testicular damage, including degeneration of the interstitial cells (Cohn *et al.,* 1982). VAL caused a significant effect on steroidogenesis in both unstimulated and gonadotropin-stimulated porcine ovarian follicular cells. These findings showed a direct effect of VPA on steroidogenesis, independent of epileptic activity (Aktas *et al.,* 2010).

The present study implies that the males and females of *D. ananassae* are prone to toxic effects with lesser reproductive ability. Fecundity and fertility are the fitness parameter that are used to assess the fitness in different species of *Drosophila*. Valproic acid caused significant effects on mating activity and reproductive fitness with the increase of courtship duration led to significant reduction in fecundity and fertility of treated flies. Thus fecundity and productivity of treated flies showed contrasting results with mating propensity when compared to the control with increased concentration of drug. Courtship duration is less in control and increased for copulation duration. An insignificant difference was observed for copulation duration for both treated and control experimental trial but maximum time consumed for courtship in treated trials. Fecundity and fertility are reduced in treated flies compared to control, while the differences for lifespan are insignificant in both control and treated flies. Statistical analysis and DMRT showed significant differences in mating activity and fitness parameters. Thus, the cost of reproductive success has been significantly reduced with the use of antiepileptic drug valproic acid, but it has left least adverse effect on life span. Therefore, the effect of toxicity on life history traits is dose dependent.

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Anti-stress property of *Rauwolfia serpentine* (Sarpagandha) on stress induced *Drosophila melanogaster.*

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Abstract

Stress is a condition or circumstance (not always adverse), which can disturb the normal physiological and psychological functioning of an individual. In medical parlance `stress` is defined as a perturbation of the body`s homeostasis. This demand on mind-body occurs when it tries to cope with incessant changes in life. A `stress` condition seems `relative` in nature. Anti-stress property of *Rauwolfia serpentina* (Sarpagandha) was evaluated using the *Drosophila melanogaster* (fruit fly) as a model organism. In the first group, control flies were taken as normal flies and were considered as control to compare with that of second group of stress induced flies by different concentrations of MTX, and a third group of flies were reared on media containing plant sample of 0.1g along with MTX, and the last group of flies were reared on the media containing only 0.1g of plant sample. Then the flies were subjected for enzymatic assay using enzymes Catalase and SOD at a time. The result of the present study showed that the plant powder used may have the anti-stress property as it reduced the stress, which was demonstrated by the reduced activities of marker enzymes like SOD and Catalase in stress induced *Drosophila melanogaster.*

Introduction

Life in the 21st Century is infinitely far more complex than it has ever been. It had been never designed to live in this complex, modern world with its many demands. One physiological response to stress is the increased activity of certain enzymes (Sorensen *et al.,* 2003). Oxidative stress is a "privilege" of aerobic organisms. It can be induced by endogenous and exogenous factors (Dallman *et al.,* 2005).

Overproduction of the reactive oxygen species (ROS) superoxide (O_2-) and hydrogen peroxide (H_2O_2) are increasingly implicated in human disease and aging. ROS are also being explored as important modulating agents in a number of cell signaling pathways. Increasing attention has been devoted to developing catalase or peroxidase mimetic as a way to treat overt inflammation associated with the pathophysiology of many human disorders.

It focused on recent development of catalytic scavengers of peroxides and their potential use as therapeutic agents for pulmonary, cardiovascular, neurodegenerative, and inflammatory disorders. Antioxidants, the free radical scavengers, however, are shown to be anticarcinogens. They function as the inhibitors at both initiation and promotion/transformation stage of carcinogenesis and protect cells against oxidative damage (Sun *et al.,* 1993).

The novel antioxidant enzyme was shown to reduce hydro peroxides and, more recently, peroxynitrite with the use of electrons provided by a physiological thiol like thioredoxin. Methotrexate decreases titers of reduced folates, interferes with DNA synthesis, and results in the arrest of rapidly proliferating cells; it results in stress in *Drosophila* flies (Barclay *et al.,* 1982).

Their defense against these free radicals is achieved by natural antioxidant molecules but also by antioxidant enzymes. Three important anti-oxidant enzymes are Cu/Zn- superoxide dismutase (Cu/Zn-SOD), catalase, and selenium-glutathione peroxidase. They are all necessary for the survival of the cell even in normal conditions. In addition, these three enzymes act in a cooperative or synergistic way to ensure a global cell protection. However, optimal protection is achieved only when an appropriate balance between the activities of these enzymes is maintained (Michiels *et al.,* 2005).

Reactive oxygen species (ROS) are defined as oxygen-containing species that are more reactive than $O(_2)$ itself, which include hydrogen peroxide and super oxide. Although these are quite stable, they may be converted in the presence of transition metal ions, such as Fe (II), to the highly reactive oxygen species (hROS). hROS may exist as free hydroxyl radicals (HO), as bound ("crypto") radicals or as Fe(IV)-oxo (ferryl) species and the somewhat less reactive, non-radical species, singlet oxygen (Tiwari *et al.,* 2001).

Catalytic activity is present in nearly all animal cells and organs and in aerobic microorganisms. Catalase activity varies greatly between tissues with highest activities in the liver, kidney, and erythrocyte, and lowest activity present in connective tissues. In eukaryotic cells the enzyme is concentrated in sub-cellular peroxisome organelles.

Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long (Boon *et al.,* 2007). The optimum temperature also varies by species.

Catalase exhibits an unusual kinetic behavior in that it is not possible to saturate the enzyme with H_2O_2 substrate up to 5M catalase concentration, but there is a rapid inactivation of the enzyme at substrate concentrations above 0.1M H_2O_2. Therefore, the activity assay is typically carried out with 10-50 mM H_2O_2. Since H_2O_2 substrate must be present at substantially less than saturated concentration, the enzyme activity is dependent on precise concentration of H_2O_2.

Stress was induced to the flies by adding Methotrexate to check the anti-stress property of the plant. Methotrexate is a structural analog of folic acid and acts by binding and inhibiting dihydrofolate reductase (DHFR), a key enzyme required for intracellular folate metabolism. It is an antimetabolite and antifolate drug, and works by inhibiting the metabolism of folic acid. Methotrexate acts specifically during DNA and RNA synthesis and is cytotoxic during the S-phase of the cell cycle. Methotrexate is commonly used in combination with misoprostol to terminate early pregnancies, *i.e*. pregnancy in the early stages. It may also be used in case of missed miscarriage, in which fetal demise has occurred, but the body has not expelled the fetus (Mol *et al.,* 2008).

Reduced folate is involved in normal synthesis and metabolism of neurotransmitters in central nervous system. Methotrexate may exert a beneficial effect in psoriasis by mechanism other than inhibition of dihydrofolate reductase. Methotrexate inhibits neutrophil chemotaxis (Johnston *et al.,* 2005).

Addition of folic acid to methotrexate therapy should allow dermatologists to use methotrexate in a much better way and enhance patient compliance (Mol *et al.,* 2008). The improved efficacy of high-dose methotrexate as compared to conventional dose methotrexate suggests that osteosarcoma may have intrinsic methotrexate resistance, which can be Sarcoma overcome by achieving a high extracellular drug concentration (Johnston *et al.,* 2005). Intracellular methotrexate undergoes polyglutamylation whereby the polyglutamylated methotrexate is preferentially retained in the cell and ultimately results in DHFR inhibition (Bertino, 1993). As a cytotoxic drug it may slow the rapid growth of cells in the synovial membrane that lines the joints (Sirotnak, 1985). Methotrexate is a chemotherapy drug used to treat leukemia, lymphomas, and osteosarcoma. It is also used in the treatment of AIDS and rheumatoid arthritis. Analogues of folic acid were in development, and by 1950, methotrexate (then known as ametopterin) was being proposed as a treatment for leukemia (Meyer *et al.,* 1950).

The rates of termination for ineffectiveness were lower and the adjusted drug retention rates were better for re-employed courses. With regard to new treatment strategies, including monitoring, co-medication and even the increasingly employed paradigm to change therapy if a state of low disease activity is not reached within few months.

Materials and Methods

The *DROSOPHILA* STOCK CENTRE, Department of Zoology, University of Mysore, provided the stocks of wild type of *D. melanogaster*. Further the stocks were cultured in our laboratory at 26°C. As the temperature decreases, the development time increases (Ashburner and Thompson, 1978; Ashburner *et al*., 2005). At higher temperature around 31 degrees, flies may become sterile and may result in death. They require a controlled temperature and humidity environment. Stocks kept at room temperature were transferred to fresh media every 20 days or the flies (5 male and 5 female flies) were transferred to fresh media when overcrowding occurs.

Culturing of stress induced flies

Methotrexate is an antimetabolite; it interferes with the way cells utilize essential nutrients, so this chemical agent was added to create stress. Into the bottle along with media, methotrexate was added in different concentrations in the range of 5ppm, 10ppm, 15ppm, 20ppm, and 25ppm. The catalase and SOD activity was increased in the flies cultured in the media containing MTX. This was confirmed by comparing the activity of the enzymes with the control flies cultured in normal media.

Enzyme collection

Different groups of flies were taken in different eppendroff tubes as methotrexate flies of different concentrations from 5ppm-25ppm and also theses stress induced flies along with plant sample. These were fully homogenized in a 200 microlitres of fresh phosphate buffer of 50 mM for catalase assay of pH 7.0 and for SOD assay 250mM phosphate buffer of pH 7.8. These were homogenized with the help of tissue homogenizer, which was kept in ice cold condition and centrifuged at 8000rpm for 20 min in a cooling microfuge. After centrifugation supernatant was transferred to fresh eppendroff tube, and 100 microlitres of this supernatant serves as enzyme source for both Catalase and SOD enzymatic assays.

Assay of catalase enzyme

Catalase enzyme (EC 1.11.1.6) activity method is essentially described by Beers and Sizer (1952). 2.9 ml of hydrogen peroxide was taken along with 0.1 ml of enzyme extract, then immediate mixing by inversion, and the absorbance was read at 240 nm in spectrophotometer, the absorbance decreases gradually. Then activity of Catalase was calculated. Then to know the specific activity, protein estimation was done which was expressed in units/mg of protein.

Assay of SOD

SOD enzyme (EC 1.15.1.1) was assayed using a slightly modified procedure originally described by Beauchamp and Fridovich (1971). Mix 3 ml of cocktail solution containing Phosphate buffer (0.8 ml), Methionine (1 ml), riboflavin (0.5 ml), EDTA (0.1 ml), NBT (0.5 ml), and the volume is made up to3 ml by adding distilled water. A blank was set without the enzyme and NBT to calibrate the spectrophotometer having buffer (1.0 ml), Methionine (1 ml), riboflavin (0.7 ml), and EDTA(0.3 ml). Another control was prepared having NBT but no enzyme and is taken as a reference

control, which contains buffer (0.9 ml), Methionine (1 ml), riboflavin (0.5 ml), EDTA (0.1 ml), and NBT (0.5 ml). These colored solutions absorbance was read at 560 nm immediately to know the activity, and later on to know the specific activity protein estimation was done by Lowry's method and units expressed in Units/mg of protein.

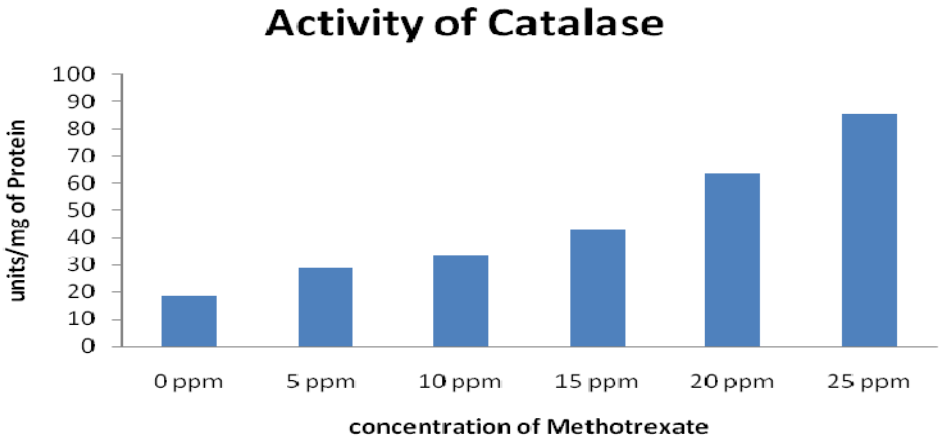

Figure 1. Activity of catalase in flies reared on media containing different concentrations of methotrexate when compared with control flies.

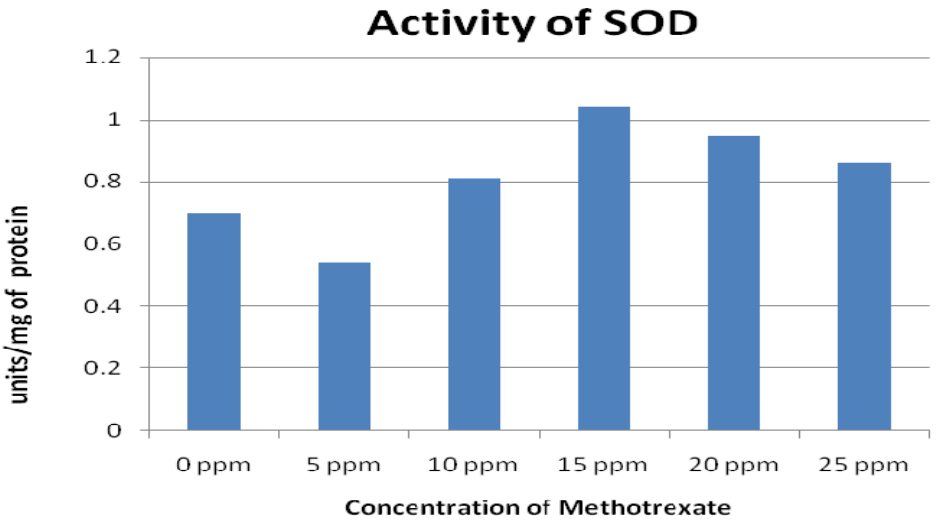

Figure 2. Activity of SOD in flies reared on media containing different concentrations of methotrexate when compared with control flies.

Table 1. Increased Catalase and SOD activity in flies exposed to different concentration of Methotrexate

Concentration of MTX	0 ppm (Control)	5 ppm	10 ppm	15 ppm	20 ppm	25 ppm
Catalase Activity in units/mg of protein	18.32	28.84	33.43	42.69	63.49	85.72
SODActivity in units/mg of protein	0.70	0.54	0.81	1.04	0.95	0.86

Results

Rearing of flies on media containing Methotrexate resulted in the increased activity of SOD and Catalase. These enzymes are the marker enzymes for the oxidative stress. The activity of SOD and Catalase increases, with respect to the increased concentration of Methotrexate in the media

(Figure 1 and 2, Table 1), when compared to control flies on normal media (no Methotrexate, *i.e.*, 0 ppm).

Enzyme activity in stress induced flies treated with plant sample

The activity of SOD is decreased in flies, reared on the media containing different concentrations of Methotrexate in presence of plant sample. The elevated level of enzyme due to Methotrexate was decreased in the presence of plant sample (Figure 3 and 4, Table 2).

The enzyme activity was different in the flies reared on the media containing only 0.5 gm of plant sample. There was increased Catalase activity compared with control flies (Figure 5), and the SOD activity was found to be decreased when compared to the control flies (Figure 6, Table 3).

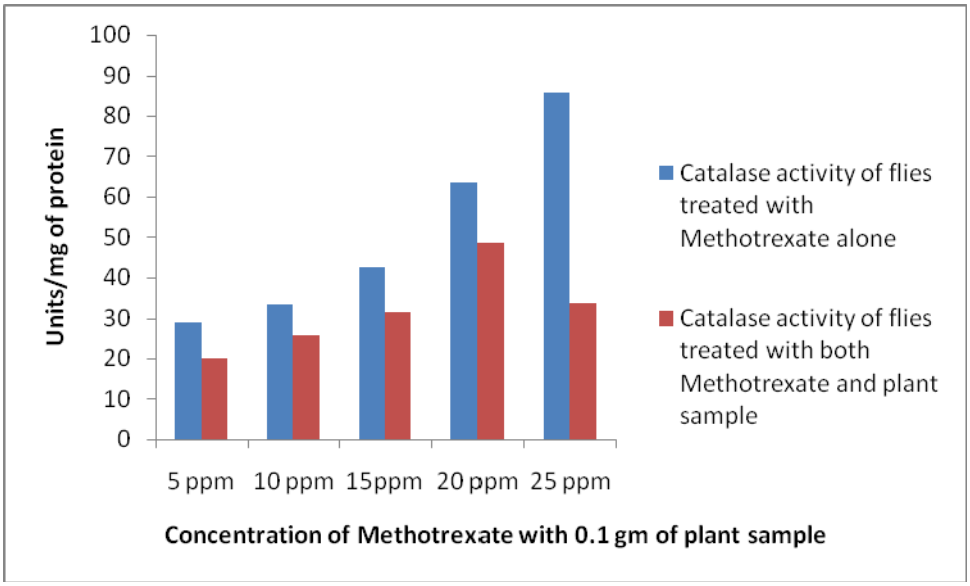

Figure 3. The increased activity of catalase in stressed induced flies is reduced when treated with plant sample.

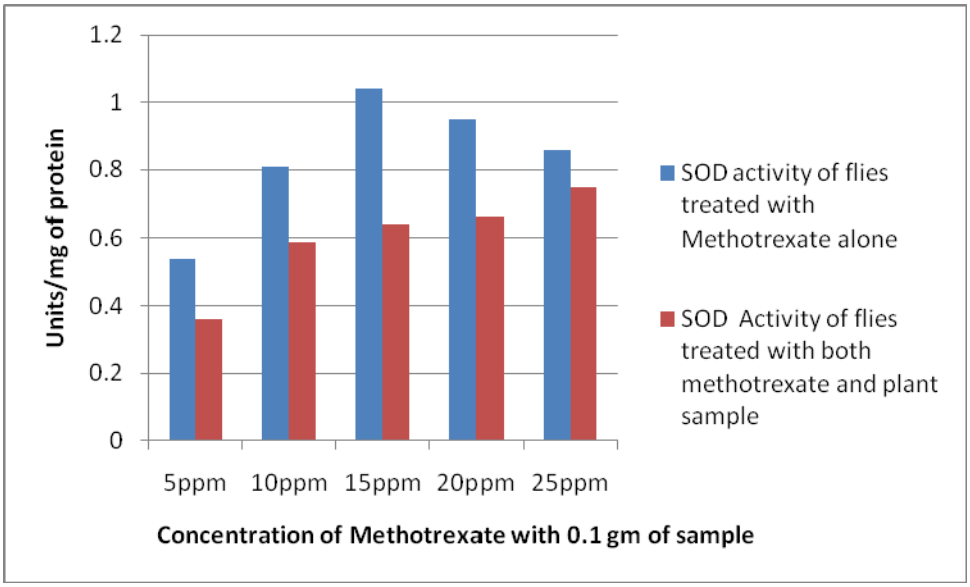

Figure 4. The increased activity of SOD in stressed induced flies is reduced when treated with plant sample.

Table 2. Catalase and SOD activity of alone stress induced flies and MTX + 0.1 gm of plant sample of different concentrations.

	Concentration of MTX	5 ppm	10 ppm	15 ppm	20 ppm	25 ppm
Catalase Activity in units/mg of protein	MTX alone	28.84	33.43	42.69	63.49	85.72
	MTX + 0.1gm plant sample	19.86	25.74	31.57	48.53	33.63
SOD Activity in units/mg of protein	MTX alone	0.54	0.81	1.04	0.95	0.86
	MTX + 0.1gm plant sample	0.36	0.59	0.64	0.66	0.75

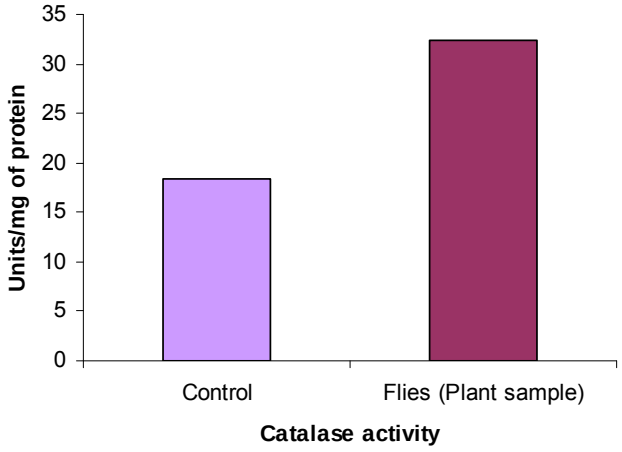

Figure 5. Catalase activity compared with the control flies, and the flies treated with plant sample.

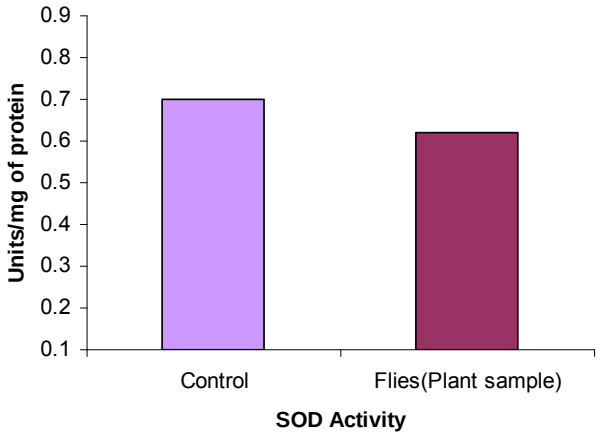

Figure 6. SOD activity compared with the control flies, and the flies treated with plant sample.

Table 3. Enzyme activity variation in normal *D. melanogaster* flies and flies treated with plant sample alone.

	Catalase Activity in units/mg of protein	SOD Activity in units/mg of protein
Control flies	18.32	0.70
Flies treated with plant sample alone	32.42	0.62

Discussion

Catalase and SOD activity was measured in stress induced flies along with the plant sample of 0.1 gm, but there was a decrease in the activity compared to the control flies. Oxidative stress has been implicated to play a role, at least in part, in pathogenesis of many disease conditions and toxicities in animals and overproduction of reactive oxygen species and free radicals due to use of toxic chemicals showed elevated increased catalase and SOD activity. So decrease in the catalase activity showing that plant sample is effective in decreasing catalase activity, but there was not much difference in the SOD activity compared to the control indicating that plant may not be found effective in decreasing SOD activity. SOD dismutases the highly reactive superoxide anion to the

less reactive species H_2O_2 (Teixeira *et al*., 1998). Catalase, a haeme-containing enzyme, scavenges hydrogen peroxide to water and molecular oxygen (Mates and Sanchez-Jimenez, 1999), and non-enzymic ascorbic acid, which is a water-soluble antioxidant forage free radical protect the biological system from oxidative stress (Beyer, 1994).

When flies were treated with plant sample alone, the activity of SOD was increased and the activity of catalase was decreased. The increase in the SOD activity may be because of the additional components present in the plant sample, since the plant sample used was commercially available crude sample of *R. serpentina*, and hence further study has to be achieved to isolate active constituents from the plant that can be used for applied research. Taken together our data suggest that the plant sample we used may have anti-stress property in it.

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New records of Drosophilidae in southern Amazônia.

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Introduction

The records in the literature of Brazilian species of the family Drosophilidae show an evident concentration in the South, Southeast, and Center-West regions of the country (Val *et al*., 1981; Gottschalk *et al*., 2008). These records cover several environments and different resources for

feeding or oviposition (Medeiros and Klaczko, 2004; Chaves and Tidon, 2005; Schmitz *et al*., 2007; Gottschalk *et al*., 2009). A total of 133 species occur in the North region while the South region shows 367 species, with 177 species found in São Paulo State (Gottschalk *et al*., 2008). The concentration of these records and the shortage of geographical information for several species (Chaves and Tidon, 2008) can result in misinterpretations of the species distributions for Drosophilidae.

In the analysis presented by Gottschalk *et al*. (2008), the species records for the entire North region constitute approximately 45% of the Brazilian records. However, approximately 80% of these records are concentrated in Amazonas and Pará States, leaving several gaps in the distribution of Drosophilidae.

The records of Drosophilidae from Rondonia State include three genera and eight species (Table 1). The purpose of this study is to add new records to the species list of Drosophilidae for this Amazonian State.

Table 1. Drosophilidae species list for Rondonia State.

Genus	Subgenus	Group	Subgroup	Species
Drosophila	*Drosophila*	*cardini*	*cardini*	*Drosophila cardinoides* Dobzhansky and Pavan, 1943
Scaptodrosophila		*latifasciaeformis*	-	*Scaptodrosophila latifaciaeformis* (Burla 1954)
Zaprionus	*Zaprionus*	*armatus*	*vittiger*	*Zaprionus indianus* Gupta 1970
Zygothrica		*bilineata*	-	*Zygothrica bilineata* (Williston 1896)
		dispar	*aldrichi*	*Zygothrica joeyesco* Grimaldi 1987
				Zygothrica paraldrichii Burla 1956
				Zygothrica pilipes Hendel 1936
				Zygothrica zygia Grimaldi 1987

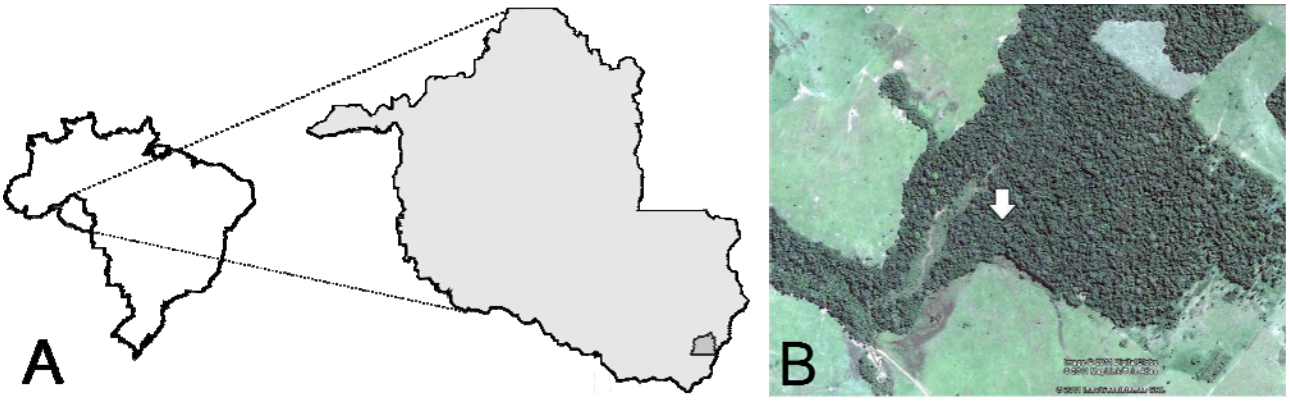

Figure 1. A, Map of Brazil and Rondonia State showing the city of Colorado do Oeste. B, Satellite view of the collection site. Source: Google Earth.

Materials and Methods

Collections were made between January 11th and 14th, 2009, in a fragment of the Amazon Forest (13°00'37.7"S; 60°35'24.9"W) at Colorado do Oeste, in the Southeast of Rondonia State (Figure 1). Five banana-baited traps (Tidon and Sene, 1988) were placed along a transect approximately 100 meters in length.

The individuals were identified through external morphology, identification keys, and comparisons with the literature. The species of the males were identified through analysis of the terminalia using the methodology described by Bächli *et al*. (2004).

Results and Discussion

The collections made in this study included 217 drosophilids belonging to two genera. The genus *Drosophila* was predominant, and only one specimen of *Zaprionus indianus* was found (Table 2).

Burla and Pavan (1953) recorded *D. calloptera* in Porto Velho, Acre State, and Gottschalk *et al*. (2008) cited this record in their revision. However, the record published by Burla and Pavan (1953) should be referred to the location of Porto Velho, in Rondonia state. Therefore, the record of *D. calloptera* obtained in this study is not the first for Rondonia but confirms the occurrence of this species in the state.

Table 2. Abundance of drosophilid species in Colorado do Oeste, state of Rondonia, Brazil.

Species	Number of individuals
Drosophila willistoni subgroup Dobzhansky 1940	177
Drosophila prosaltans Duda 1927	36
Drosophila subsaltans Magalhães 1956	2
Drosophila calloptera Schiner 1868	1
Drosophila malerkotliana Parshad and Paika 1964	1
Zaprionus indianus	1
Total	218

Although this report presents the results of a preliminary collection, it includes five new records for Rondonia State and highlights the need of further development of research in this Brazilian region belonging to the Amazonian biome and shows several gaps remain in the knowledge of the diversity of the Drosophilidae in Rondonia state.

Acknowledgments: The authors thank Paulo Lima dos Santos and Marcielly Daiane de Oliveira for help in collecting and Mauricio de Lazari for permission to access his property.

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Cloning and characterization of β-esterase (Est B) gene in *Drosophila virilis*.

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The nucleotide sequence data reported in this paper appeared in the DDBJ / Gen Bank nucleotide sequence databases with accession number AB679280.

β-esterases of *Drosophila* display marked variation in their patterns and sites of the expression. Est 5 of *D. pseudoobscura* is expressed in the eyes and haemolymph (Lunday and Farmer, 1983). Est 6 of *D. melanogaster* is highly expressed in the sperm ejaculatory duct of the adult male (Sheehan *et al*., 1979). The variation of gene expression in the *Est 5B* of *D. pseudoobscura* and the *Est 6* of *D. melanogaster* has been examined (Tamarina *et al*., 1997). In the β-esterase of *D. virilis*, two types were detected, one of which is specific for the genitalia (Est S) (Sergeev *et al*., 1993) and the other for the adult head and haemolymph (Est B) (Sasaki, 1974). In order to compare *Est 5B* and *Est 6* with *Est B*, I examined in the first place, the DNA sequence and transcripts of the *Est B* gene in *D. virilis*.

The cDNA and genomic libraries of adult flies of *D. virilis* were constructed in lambda gt10 and lambda dash, respectively. The cDNA library was screened with partial *Est 6* gene of *D. melanogaster* as a probe. The genomic library was screened with the *Est B* cDNA of *D. virilis*. The region of the *Est B* gene was subcloned in pBluescript and sequenced on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

mRNA in adult flies of *D. virilis* was prepared by use of a QuickPrep mRNA Purification Kit (GE Healthcare). The primers designed for 5' RACE were as follows: Pesb, 5'- P-ACAGCTCTGACTAG -3'; A1esb, 5'- ATGCGAATCGACTTATC -3'; A2esb, 5'-GGATCAGCAAAGGTAGC -3'; S1esb, 5'- CCGGGCAACTTTGGATTA -3'; S2esb, 5'-TAGACGTGCGTTTGAGCT -3'. For 5' RACE, 1 μg of mRNA was reverse-transcribed into cDNA by using a 5'-Full RACE Core Set (Takara) in the presence of Pesb primer according to the instructions of the manufacturer. The cDNA was amplified by use of an LA PCR Kit (Takara) with A1esb and S1esb primers for the first step and with A2esb and S2esb primers for the second step. PCR amplifications were carried out for 25 cycles under the following conditions: 94ºC, 60ºC and 72ºC for 30 sec for the respective steps.

One positive cDNA clone was isolated by screening of about 8×10^3 plaques of the cDNA library. The sequence analysis indicated that the clone was 1.3 Kbp in length, though it was not a full-length one. Two positive genomic clones were isolated by screening of about 7.2×10^4 plaques by use of the cDNA as a probe. I determined the nucleotide sequences of 3-Kbp stretches of the genomic regions.

Sequence analyses of cDNA and genomic DNA revealed that the *Est B* gene comprised two exons, one is 1381bp and the other 236bp, separated by a short intron of 62bp. Poly A signal was identified 252bp downstream of the stop codon. The exon–intron organization of *Est B* was the same as those of *D. pseudoobscure* and *D. melanogaster*, *Eat 5B* and *Est 6*. Nucleotide sequences in the coding region of *Est B* showed 67.3% and 62.9% similarities to those of *Est 5B* and *Est 6*, respectively. These values are low in comparison with other genes encoding constitutive proteins, suggesting high variability of the *esterase* genes in *Drosophila*. The deduced amino acid sequence of Est B consisted of 538 residues, shorter 7 residues than Est 5B and 6 residues than Est 6. Alignment of amino acid sequences of Est B, Est 5B, and Est 6 (Figure 1) indicated that the N-terminal signal

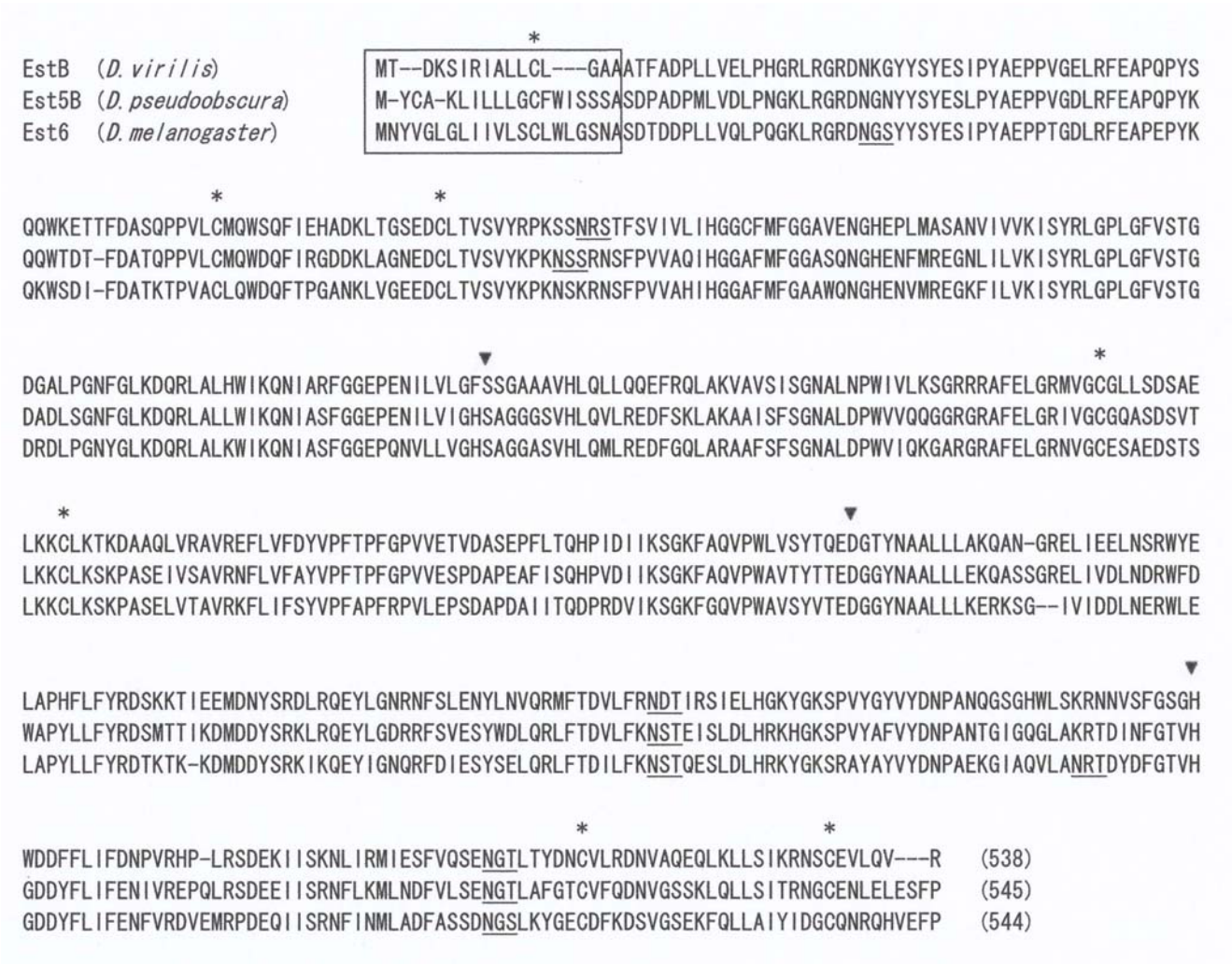

Figure 1. Sequence alignment of the β-esterase of *D. virilis*, *D. pseudoobscura* and *D. melanogaster*. The 3 noncontiguous residues for the catalytic activity are indicated by triangle. The seven cysteine residues conserved among *Drosophila* species are indicated by an asterisk. The putative N-glycosylation sites are underlined. The N-terminal signal sequences are boxed.

peptides, seven cysteine residues, three noncontiguous catalytic residues, and two N-glycosylation sites were found in the same positions. Comparison of hydropathy profile of these proteins among three species showed that the C-terminal region of Est 6 enzyme composed of a monomer was more hydrophilic than those of Est 5B and Est B such as a dimer (data not shown).

A 5'RACE analysis identified two transcriptional start sites (Figure 2). The one transcript started at -32 and the other transcript started at -387, in which the 71 bps (from -321 to -251) in the 5' leader sequence were spliced. The *Est 6* of *D. melanogaster* has multiple transcriptional start sites between nucleotides -38 and -32 (Collet *et al*.,1990). These results showed that the transcripts of these esterases examined are similar to each other, except for the transcriptional pattern in the 5' region. Therefore, it is likely that different transcripts at the 5' region of the gene are useful for specific tissue expression.

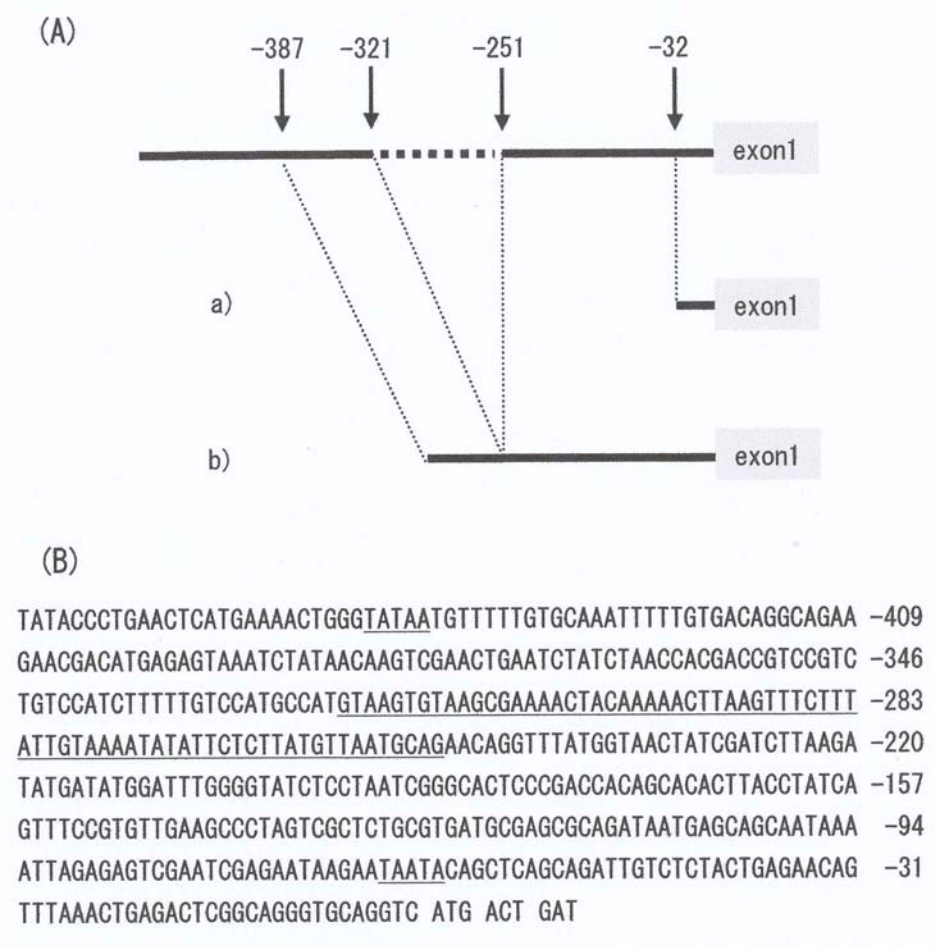

Figure 2. (A) Two transcriptional patterns a) and b) of the 5' region of *EstB* gene. Intron is indicated by dotted line. (B) Nucleotide sequences of the 5' region of *EstB* gene. Transcriptional start sites are boxed. Intron is double-lined. Putative TATA boxes are underlined.

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Guide to Authors

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receipt deadline of 31 December is a firm deadline, due to printer submission schedules. Electronic submissions are encouraged, and may be required for lengthy or complex articles.

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Submission: Articles should be submitted electronically, if possible. Alternatively, we ask that a diskette be included with an article mailed to us. MS Word or Rich Text Formats are preferred. To help minimize editorial costs, proofs will not be sent to authors unless there is some question that needs to be clarified or they are specifically requested by the authors at the time of submission. The editor reserves the right to make minor grammatical, spelling, and stylistic changes if necessary to conform to DIS format and good English usage. If the article contains tables, complex line figures, or half tones, we may ask that a printed copy be mailed to us after seeing the electronic version if we have questions about content or presentation. Color illustrations will appear black and white in the printed version but will be in color in the electronically-accessible version on our web site (www.ou.edu/journals/dis).

Citation of References: Citation should be by name and date in the text of an article (Smith, 1989; Jin and Brown, 1990; Waters *et al*., 1990). At the end of the article, references should be listed **alphabetically** by senior author, listing all authors with initials, date, journal, volume and page numbers. Titles will not be included except for books, unpublished theses, and articles in press. An example format is:

Green, R.L., 1998, Heredity 121: 430-442.

Waters, R.L., J.T. Smith, and R.R. Brown 1990, J. Genet. 47: 123-134.

Note the initials are before each name except for the senior author.

Availability of the University of Texas Publications Dealing with *Drosophila*

Marshall R. Wheeler

From 1940 to 1972 many research articles were published by the University Press in the series, "Studies in the Genetics of *Drosophila*" with J.T. Patterson as editor and later (from 1957-1972) with M.R. Wheeler as editor. In 1960 the series title was changed to "Studies in Genetics." There were also a few special issues. Many of these are now out of print (OOP); all known copies of the remaining issues have been made available by Dr. Wheeler. The copies are available from the office of the Editor, *Drosophila Information Service*; contact Dr. James N. Thompson, jr., (jthompson@ou.edu) for details.

Some issues were given titles and subtitles, but the Publication Number (*e.g*., UTP 4213) is the best reference. This is the complete list of all the publications:

1940: UTP 4032 (OOP). 1942: UTP 4213 (OOP). 1942: UTP 4228 (OOP). 1943: UTP 4313, "Drosophilidae of the Southwest" (OOP). 1944: UTP 4445, with "Drosophilidae of Mexico" (OOP). 1947: UTP 4720, "Isolating Mechanisms" (OOP). 1949: UTP 4920 (OOP). 1952: UTP 5204. 1954: UTP 5422 (OOP). 1957: UTP 5721. 1959: UTP 5914, "Biological Contributions." Dr. Patterson's 80th birthday issue. 1960: UTP 6014. 1962: UTP 6205. 1966: UTP 6615, Morgan Centennial Issue. 1968: UTP 6818. 1969: UTP 6918, W.S. Stone Memorial Issue. 1971: UTP 7103. Final volume, 1972: UTP 7213.

This announcement is reprinted from 2002, *Dros. Inf. Serv*. 85: 106-108.

When does the *spiny legs*^{1} allele of the *prickle* gene cause extra joints?

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Wild-type flies have four ball-and-socket joints that divide each tarsus into five segments (Tajiri *et al.*, 2010). Mutations in genes of the planar cell polarity (PCP) pathway—*e.g.*, *frizzled* and *dishevelled*—can cause extra joints that, curiously, are upside-down (Held *et al.*, 1986). The most extreme phenotype occurs with the *spiny legs*^{1} allele of the *prickle* gene (*pk*^{sple1}) (Tree *et al.*, 2002). This mutation evokes an extra, inverted joint in tarsal segments T2, T3, and T4, plus a partial extra joint in T1. Although much is known about how PCP genes enforce cell polarity (Goodrich and Strutt, 2011), little is known about how they induce extra joints (Bishop *et al.*, 1999; Held, 2005).

The present study investigates the timing of *prickle* gene action in joint formation. Does *pk*^{sple1} induce all of its extra joints simultaneously or sequentially? Does it affect bristle polarity at the same time that it affects joint formation? Does it act before or after puparium formation?

Materials and Methods

The *pk*^{sple1} allele (henceforth called *sple*^{1}) was experimentally suppressed at different times by expressing the wild-type *sple* isoform (here called *spleWT*) of the *prickle* gene (Lin and Gubb, 2009) via the *Gal4-Gal80* technique (McGuire *et al.*, 2003). This isoform was linked to the upstream activating sequence (*UAS*) of the transcription factor Gal4, which was ubiquitously expressed via the *daughterless* locus (*da-Gal4*). Both constructs were inserted into a third chromosome (Gubb *et al.*, 1999) kindly supplied by David Gubb. Expression of the *Gal4* gene was manipulated by a temperature-sensitive allele of *Gal80ts*, which inhibits *Gal4* at 18˚C but not at 30˚C (McGuire *et al.*, 2004).

Because *sple*^{1}/*sple*^{1}; *da-Gal4 UAS-spleWT*/*TM3, Ser* males proved to be infertile, experimental and control individuals were obtained as F_1 offspring by crossing males from a *sple*^{1}/*CyO; da-Gal4 UAS-spleWT*/*TM3, Ser* stock (barely fertile) with females from a *sple*^{1}/*sple*^{1}; *Gal80ts/Gal80ts* stock (quite fertile). Curly-winged (*sple*^{1}/*CyO; da-Gal4 UAS-spleWT/Gal80ts*) siblings were scrutinized for any artifactual side-effects of the *da-Gal4 UAS-spleWT* constructs, but their legs looked wild-type (data not shown). Serrated-wing siblings whose legs had an *sple*^{1} phenotype (*sple*^{1}/*sple*^{1}; *Gal80ts*/*TM3, Ser*) were examined for any extraneous effects of *Gal80ts*, but none were found at either temperature.

Flies were raised on Ward's *Drosophila* Instant Medium plus live yeast. Since females did not oviposit at 30˚C, eggs were collected daily at room temperature (22˚C), whereupon the food vials were placed at the starting temperature (18˚C or 30˚C). Nutrition was optimized (and overcrowding minimized) to prevent retardation that could affect staging. Legs were dissected in 70% ethanol, placed in gum arabic fluid (Faure's) between cover glasses (Lee and Gerhart, 1973), and examined at 200× in a Nikon microscope. Females were used unless stated otherwise. Sample sizes for each time point were 6 legs (left and right midlegs from 3 flies), except the cohorts upshifted at PF (N = 12 legs) and downshifted at -15 hBPF (N = 4 legs). Presence or absence of joints was scored based on the ball and socket components of the hinge alone, without regard to the completeness of any associated intersegmental membrane.

For ease of comparison, all times have been recalibrated to the standard staging scale at 25˚C (Ashburner, 1989) as explained previously (Held, 2010). Specifically, actual times at 18˚C were divided by 2.0 (Held, 1990) to yield the times reported here ("@25˚"). For ease of viewing, the images of left legs (Figures 1a, 1c, 2d, 3a, 3b, and 3f) were flipped (in *Adobe Photoshop*®) so as to appear as right legs. Abbreviations include: PF (puparium formation), BPF (before PF), APF (after PF), h (hours), US (upshifted), DS (downshifted), TSP (temperature-sensitive period), fem (femur), tib (tibia), and bas (basitarsus), which is synonymous with tarsal segment T1. Because the maximal expression of the extra joint in T1 is only partial even in *sple*[1] homozygotes (Held *et al*., 1986), that joint was excluded from this analysis.

For shifts at PF, white prepupae were collected (synchrony = +/- 0.5 h). For shifts BPF, vials of larvae were shifted, and the resulting pupae were harvested at 12-h intervals (actual time), recalibrated as 6-h intervals @25˚C for downshifts (ages varying +/- 3 h) or reported unaltered as 12-h intervals @25˚C for upshifts (ages varying +/- 6 h). For shifts APF, pupae were harvested at 12-h intervals (actual time) at the starting temperature, kept in moist petri dishes, and transferred to the other temperature when the desired age was reached. Larvae and pupae were handled as described previously (Held, 2010).

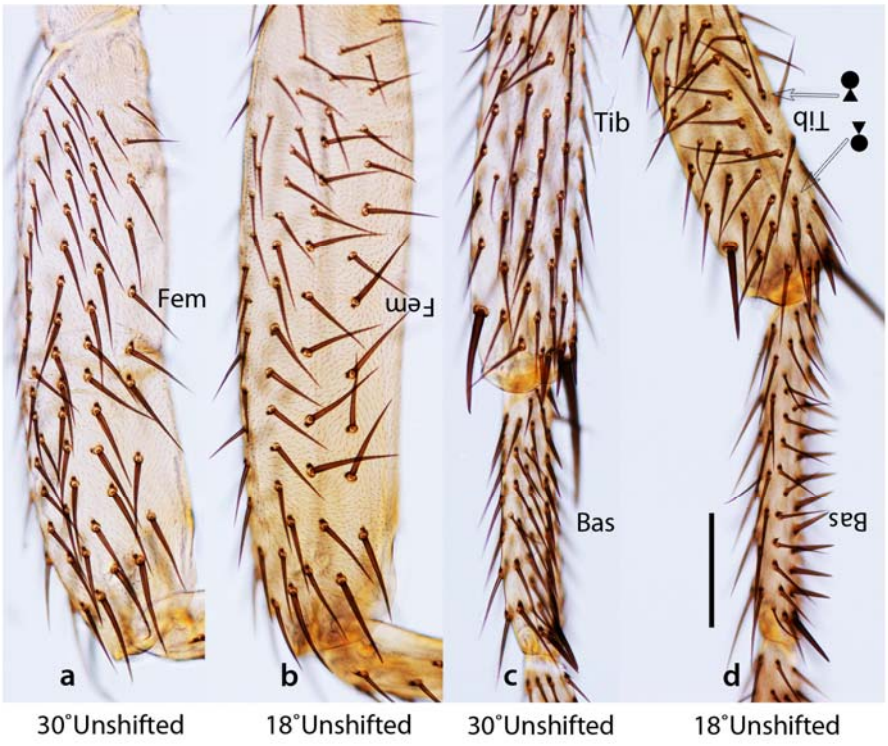

Figure 1. **a**, **c**. Femur (Fem), tibia (Tib) and basitarsus (Bas) from a control female raised at 30˚C. **b**, **d**. Corresponding leg segments from a control female raised at 18˚C. The 18˚C leg displays a patch of reversed bristles on each segment. These misorientations are also evident (**d**) in the polarity of the bristle sockets (solid circles) relative to their associate "bracts" (Hannah-Alava, 1958) (solid triangles). Upside-down segment names (**b**, **d**) are used to denote reversed-bristle phenotypes (Figures 2, 3, and 4). In all panels here and elsewhere, the anterior face is depicted, with proximal at the top and distal at the bottom, dorsal to the left and ventral to the right (except Figure 4, where the leg is rotated 90˚ clockwise). All photos are at the same magnification (scale bar in **d** = 100 microns).

Results

Flies of the genotype *sple*^{1}/*sple*^{1}; *da-Gal4 UAS-spleWT*/*Gal80ts* were exposed to various temperature regimens. Control flies were raised entirely at 18˚C or 30˚C. At 18˚C, *Gal80ts* disables *Gal4* so that the *spleWT* allele linked to *UAS* cannot suppress *sple*^{1}, leading to extra joints and misoriented bristles (Figures 1b, 1d, and 2f). At 30˚C, *Gal80ts* malfunctions, so *Gal4* activates *UAS*, hence expressing *spleWT* and suppressing *sple*^{1}, yielding legs that look wild-type (Figures 1a, 1c, and 2a). The ability of *spleWT* to rescue *sple*^{1} to 100% penetrance and expressivity allows partial phenotypes to be assessed with high precision. Experimental flies of the same genotype were upshifted (18˚C to 30˚C) or downshifted (30˚C to 18˚C) at different times.

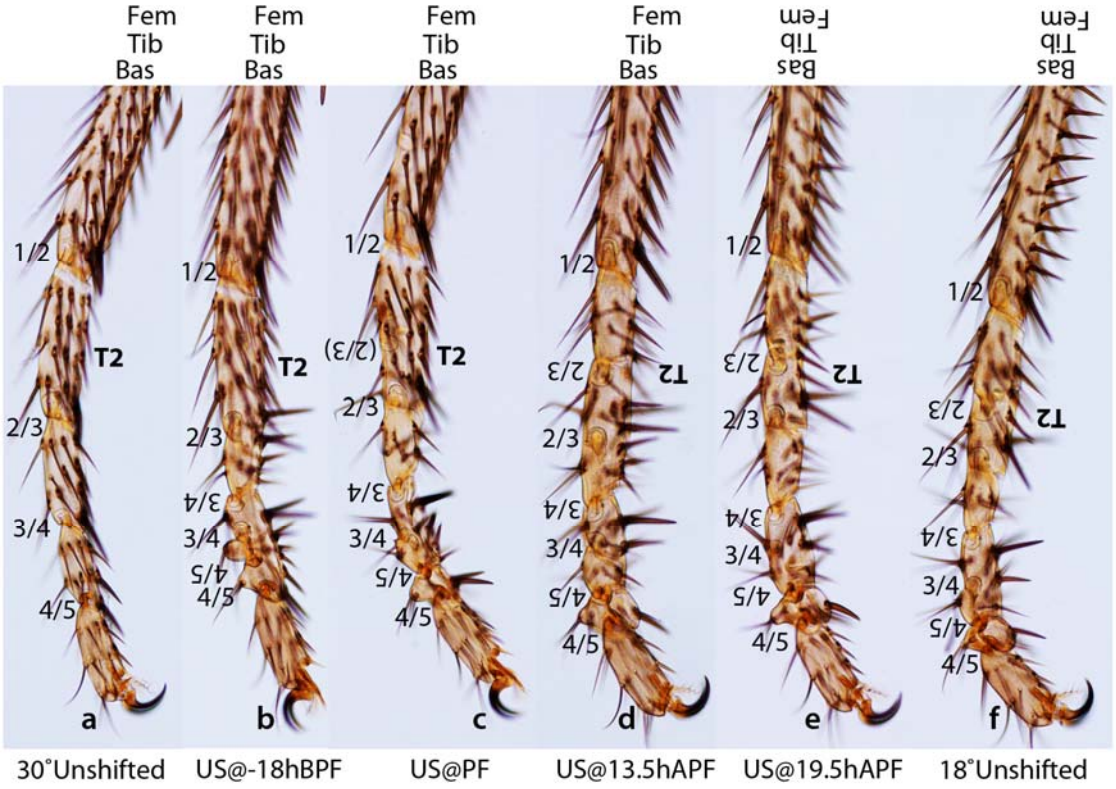

Figure 2. Upshift results. **a**. Tarsus from a control ("unshifted") fly raised entirely at 30˚C. **b-e**. Tarsi from flies upshifted (from 18˚C to 30˚C) at various times (normalized to 25˚C) before (BPF) or after (APF) pupariation (see Materials and Methods). **f**. Tarsus from a control ("unshifted") fly raised entirely at 18˚C. Upside-down numbers denote inverted joints (2/3, 3/4, 4/5), while upside-down names indicate reversed bristles on particular segments (T2, Bas, Tib, Fem).

Upshifts (Figure 2). Upshifts restore *spleWT* function. They rescue T2 to a wild-type state at -18 hBPF (Figure 2b) but not later: this rescuing ability wanes at PF (Figure 2c; extra joint in 75% of legs) and disappears by 13.5 hAPF (Figure 2d). Evidently, the extra joint in T2 is irreversibly induced by *sple*^{1} at or near PF. The extra joints in T3 and T4 must be induced much earlier (before -18 hBPF) because upshifts cannot rescue those segments even at -18 hBPF (Figure 2b). Bristle

polarity can be rescued in the basitarsus, tibia, and femur through 13.5 hAPF (Figure 2d) but no later (Figure 2e), implying that *sple*^{1} firmly reorients bristles there between 13.5 and 19.5 hAPF.

Downshifts (Figure 3). Downshifts remove *spleWT* function. The later the downshift, the more normal the leg, because *spleWT* has had sufficient time to suppress *sple*^{1} in the affected areas. The order in which segments lose their extra joint is: T2 (before -51 hBPF, where 2/6 legs showed an extra joint), T3 (-51 to -27 hBPF), and T4 (-21 to -15 hBPF). Subsequently, segments are cured of their reversed bristles: T2 and basitarsus (-15 hBPF to PF), then tibia and femur (PF to 18 hAPF). Although tibial bristle polarity *per se* is restored at PF, residual bristles in its "hot spot" still display bracts on the distal (abnormal) side of the socket (not shown).

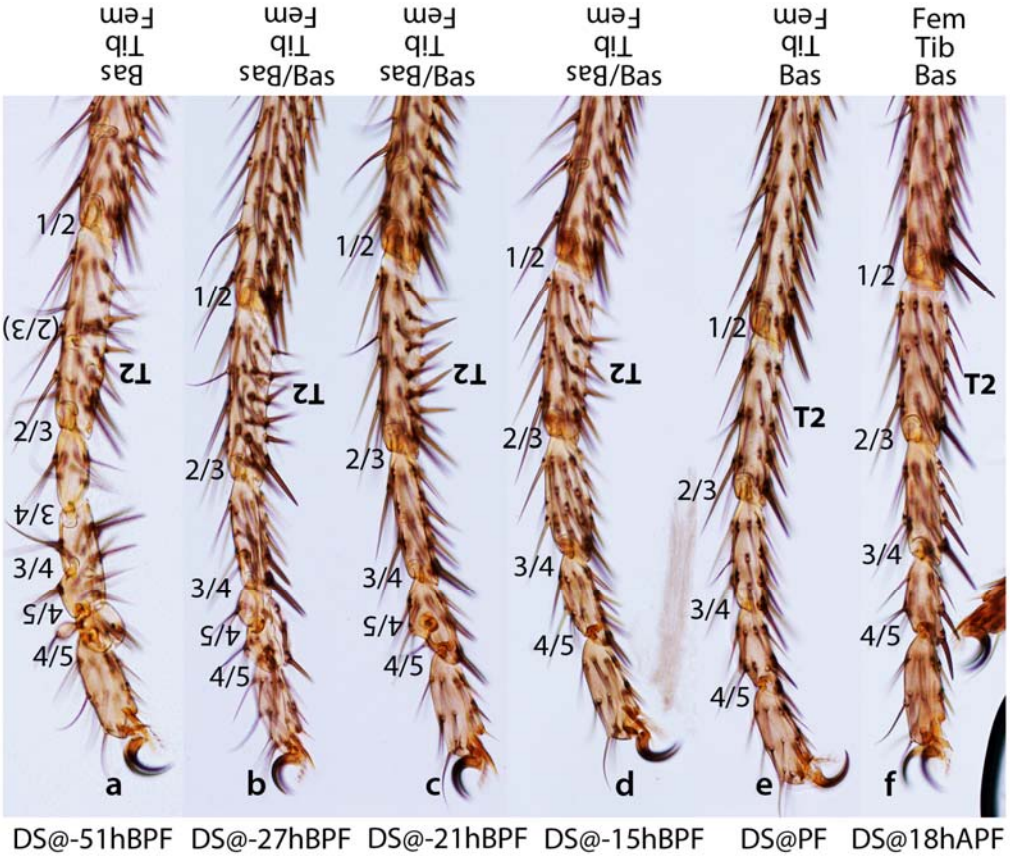

Figure 3. Downshift results. Tarsi from flies downshifted (from 30˚C to 18˚C) at various times (normalized to 25˚C) before (BPF) or after (APF) pupariation (see Materials and Methods). Upside-down numbers denote inverted joints (2/3, 3/4, 4/5), while upside-down names indicate reversed bristles on particular segments (T2, Bas, Tib, Fem). Heterogeneous basitarsal phenotypes (some tarsi normal, others abnormal) are denoted by "inverted Bas/right-side-up Bas".

Discussion

Figure 4 plots these results along a time line. One conclusion to be drawn is that bristles and joints are affected differently in time. Downshifts cure the extra-joint defect (-51 to -15 hBPF) before bristle polarity (-15 hBPF to 18 hAPF). Extra joints are induced more than a day and a half before

overt histological differentiation of normal joints, which occurs at 24-38 hAPF (Mirth and Akam, 2002), but bristle polarity is affected shortly before elongation of bristle shafts (Graves and Schubiger, 1981) and cuticle deposition (Reed *et al.*, 1975) at 36 hAPF. That the extra joint in T2 is initiated before the 3rd instar, which begins circa -48 hBPF, is startling.

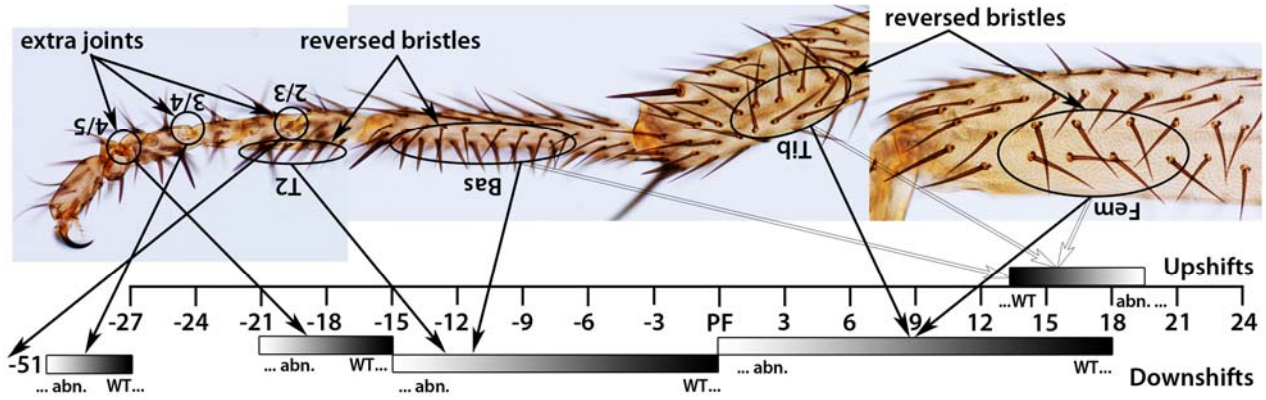

Figure 4. Stages (bars) when traits (circled) start to manifest mutant phenotypes (upshifts) or return to normal (downshifts) plotted along a time axis. Gradient shading shows the transition from abnormal (abn.) to wild-type (WT) or vice versa. Arrows point to bars (*i.e.*, time spans), not to specific times within these ranges. Outlined arrows for upshifts and solid arrows for downshifts. Upside-down numbers denote inverted joints (2/3, 3/4, 4/5), while upside-down names indicate reversed bristles on particular segments (T2, Bas, Tib, Fem). Omitted is the transition from normality to abnormality (upshift) for the extra joint phenotype in T2 (-18 hBPF to PF; see text).

Secondly, bristle polarity is restored in a distal-to-proximal sequence (T2 & Bas before Tib & Fem) that is consistent with the order in which bristles develop along the leg axis (Graves and Schubiger, 1981), whereas extra joints disappear in an opposite, proximal-to-distal, sequence (T2, then T3, then T4). Previous studies failed to establish a clear order for normal joint formation (de Celis *et al.*, 1998; Rauskolb, 2001; Mirth and Akam, 2002), so we cannot know whether this extra-joint sequence conforms to the normal joint sequence.

The temporal uncoupling of bristle polarity from joint formation is further evident in the three time points (-27, -21, and -15 hBPF) when reversed bristles persist in T2 despite a lack of even the slightest remnant of an extra joint in that segment (Figure 3b-d). Of course, a comparable genetic dissociation was already evident in the ability of *sple*^{1} to cause bristle reversals on the femur and tibia in the absence of any extra joints at those locations (Held *et al.*, 1986).

Note that the time spans delineated by bars in Figure 4 are not temperature-sensitive periods (TSPs). Rather, they represent the starts or ends of TSPs. The start of a TSP is classically defined as the first shift that yields the mutant trait, with the endpoint defined as the first shift that restores the wild-type phenotype (Suzuki, 1970). Rephrasing this formula in terms of the *Gal80ts* conditions used here, the start would be the first upshift that yields the *sple*^{1} trait, with the endpoint being the first downshift that restores the wild-type phenotype.

Paradoxically, trying to apply these criteria to the extra joint in T2 yields a start time (-18 hBPF to PF) later than the endpoint (before -51 hBPF). Likewise, trying to define a TSP for bristle polarity on the tibia and femur yields an onset (13.5 to 19.5 hAPF) later than, albeit overlapping with, the terminus (PF to 18 hAPF).

In an effort to resolve this dilemma, pulse experiments were performed. Flies of the usual genotype (*sple*^{1}/*sple*^{1}; *da-Gal4 UAS-spleWT*/*Gal80ts*) were exposed to a 24-h pulse of 30˚ (upshift, then downshift back to 18˚C) at one of three consecutive times (N = 6 legs each). With a pulse in mid-third instar (-36 to -12 hBPF), flies lost their extra joints in T2 and T3 but retained the extra joint in T4. With a pulse that straddled PF (-12 hBPF to 12 hAPF) they lost the extra joint in T2 but retained extra joints in T3 and T4. And with a later pulse (12 to 36 hAPF), all extra joints were present. The first pulse failed to cure any bristle polarity defects, the second rescued T2 and the basitarsus, and the third cured T2, the basitarsus, the tibia and femur.

The bristle-polarity effects of the pulses were reassuring. They conform to the expected sequence based on the downshift bars in Figure 4, though the third pulse (12 to 36 hAPF) should not have cured T2 and the basitarsus if their TSP ends at PF. The main problem is the joint data. They imply that the TSP for T3 ends before that for T2, which contradicts the downshift order (T2 before T3). Moreover, they show that the extra joint in T2 can be eliminated by a pulse (-36 to -12 hBPF) that begins after -51 hBPF, when the TSP for that trait was supposed to have ended.

Part of the explanation for these inconsistencies may be artifactual peculiarities of the *Gal4-Gal80* method itself. When a wild-type allele of *Ubx* is ectopically expressed in the wing using this technique, Ubx protein is detectable by 6 h after an upshift, but its level continues to rise to a plateau at 16 h and does not peak until 24 h (Pavlopoulos and Akam, 2011). If the same is true for upshifts that activate *spleWT*, then the times in Figure 4 must be recalibrated substantially. For downshifts a complicating factor is the unknown perdurance of *spleWT* mRNA and protein. Finally, certain joints may need higher thresholds of the spleWT isoform than others in order to be suppressed. Further work will be required to disentangle these confounding variables.

Acknowledgments: Fly stocks were supplied by David Gubb (*sple* constructs) and Jeff Thomas (double-balancer strains used to make the parental stocks). The manuscript was kindly critiqued by Simon Collier, Juan Pablo Couso, Ibo Galindo, David Gubb, and Jeff Thomas.

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Anthracycline (epirubicin) induced mutation studies in *Drosophila melanogaster*.

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The fruit fly, *Drosophila melanogaster*, is a powerful model genetic organism that has been used since the turn of the previous century to evaluate genotoxins. In this study, the antineoplastic drug Epirubicin was scrutinized for its genotoxic effects in *Drosophila melanogaster*. The end points chosen for this study directly evaluate the genotoxic effects of the drug, which can be extrapolated to humans owing to the homology between the two species. The four concentrations of drug used to estimate genotoxicity in this study include 200µg, 300µg, 400µg, and 500µg. The drug-treated flies were analyzed for any somatic mutations after 48 hour exposure, and F1 analysis was performed to check for germ line mutations. The results of fragmentation assay indicate DNA damage as a result of apoptosis revealing DNA instability when exposed to Epirubicin, which confirms the genotoxic activity of the antineoplastic agent. The wing somatic mutation and recombination test, a one-generation test, makes use of the wing imaginal disc cells in larvae. They are based on the principle that the loss of heterozygosity of suitable recessive markers can lead to the formation of mutant clones of cells that are then expressed as spots on the wings of the adult flies. The two markers employed in this study include multiple wing hair (*mwh*), and flare (*flr3*). Three-day-old larvae, trans-heterozygous for these markers, are treated chronically by oral administration with Epirubicin. After eclosion, the wings of the adult flies were scored for the presence of single and twin spots. The results revealed spots/mutations caused due to different genotoxic events: either mitotic recombination or mutation (deletion, point mutation, or translocation).

Introduction

Genotoxicity Testing

Drosophila melanogaster is a eukaryotic organism linked to the development of the Genetic Toxicology (Alentorn, *et al*., 1995). *Drosophila melanogaster* is a well-established insect model for human diseases and toxicological research due to its well-documented genetics and developmental biology (Buschini, *et al*., 2003).

Chemical carcinogenesis is a multi-step process featuring alteration of genome integrity detected as pivotal gene mutations and chromosome damage, malignant transformation of cells, and ultimately development of cancer after exposure to chemical agents. Because of the association between DNA damage and cancer development, the preclinical safety evaluation paradigm for drugs and chemicals consists of assessing their genotoxicity, *i.e*., their potential to cause DNA damage, and carcinogenicity, the latter comprising the ability to produce tumors in animals upon long-term exposure. The standard genotoxicity testing battery consists of (a) a bacterial gene mutation assay, (b) an *in vitro* mammalian mutation and/or chromosome damage assay, and (c) an *in vivo* chromosome damage assay. The test battery allows for relatively simple, accurate, and economical hazard identification associated with exposure to chemicals, namely a potential to cause DNA damage resulting in mutations at the gene and chromosome levels (Henderson, 2004).

Obtaining positive results in *in vitro* genotoxicity tests is not uncommon. Follow-up studies to determine the biological relevance of positive genotoxicity results are costly, time consuming, and utilize animals. More efficient methods, especially for identifying a putative mode of action like an indirect mechanism of genotoxicity (where DNA molecules are not the initial primary targets), would greatly improve the risk assessment for genotoxins.

Anticancer drugs are the most common genotoxic agents studied on *Drosophila*. There are three goals associated with the use of the most commonly-used anticancer agents.

1. DNA damage
2. DNA synthesis inhibition
3. Termination of mitosis

The majority of drugs currently on the market are not specific, which leads to the many common side effects associated with cancer chemotherapy including genotoxicity.

Antineoplastic drugs generally have a narrow therapeutic index and are delivered at doses close to toxicity. Endogenous factors affecting drug response involve genetic predisposition, disease states, and other factors that influence absorption, distribution, activation, and detoxification of the drug. In particular, the pharmacological activity of any genotoxic anticancer drug is strictly dependent on tumour-specific physiological/biochemical conditions, such as a functional respiratory chain and the presence/absence of drug metabolizing enzymes (Hu *et al*., 2004).

Broadly classifying anticancer based upon their genotoxic ability as:

Direct-acting genotoxins (where DNA is the initial primary target) includes the DNA crosslinking agents, mitomycin C (MMC) and cisplatin (CIS), and an alkylating agent, methyl methanesulfonate (MMS).

Indirect-acting genotoxins includes hydroxyurea (HU), a ribonucleotide reductase inhibitor, taxol (TXL), a microtubule inhibitor, etoposide (ETOP), a DNA topoisomerase II inhibitor, doxorubicin and its analogs (antitumor antibiotics) are microtubule inhibitor and DNA topoisomerase II inhibitor (Lehmanna *et al*., 2003).

The toxicity of most of these drugs is associated with their enzymatic conversion to toxic metabolites (Hu *et al*., 2004).

Many chemicals/drugs are investigated for their genotoxic properties through the wing spot test (SMART) in *Drosophila melanogaster*, which is the gold standard for genotoxicity assessment. Almost 300 chemicals have been evaluated in the wing spot test. These include various antineoplastic drugs, small alkylating agents, bulky adduct-forming compounds, crosslinking agents, clastogenic intercalating and nonintercalating topoisomerase inhibitors, antimetabolites that disturb nucleotide pools, DNA synthesis inhibitors, and nucleoside analogs. The genotoxic effects of these representative compounds are, in general, strong and dose related.

The wing spot test is also well suited for testing complex mixtures, such as airborne aerosol extracts, plant extracts, beverages such as coffee, herbal teas and wine, as well as tannic acid. Although both caffeine and tannic acid were determined to be genotoxic in the wing SMART, they also both showed antigenotoxic activity in combination with several known strong mutagens. Thus, the SMART can also be used to assess the effects of nongenotoxic chemicals, which may act as modulators when combined with genotoxins. Such approaches identified the protective effects of chlorophyllin, ascorbic acid, novo-biocin, antipyretic analgesics, sodium thiosulfate, epigallocatechin, and tannic acid.

The applicability of the SMART to studies of antigenotoxic effects is reinforced by the demonstration that some modulators that decrease the incidence of mutational effects are equally able to increase the occurrence of mitotic recombination. This means that modulating agents must be

evaluated not only in terms of their action on mutagenic events (point and chromosomal mutations) but also in relation to their effects on mitotic recombination.

The broad spectrum of genetic end points monitored as LOH in somatic cells—including point mutations, deletions, unbalanced half-translocations, mitotic recombination, chromosome loss, and nondisjunction—makes the wing SMART a most versatile *in vivo* test. It is also technically simple, quick and inexpensive to do, and allows flexibility in the choice of both route of administration of the test chemical and time of exposure. In addition, it allows analysis of an extensive sample size, because microscopic inspection covers approx 50,000 cells per fly. Moreover, statistical procedures applicable to the SMART are well established, and different statistical tests can be applied according to the peculiarities that specific sets of data may show (Siddique *et al*., 2005).

Doxorubicin and Its Analogs – Anti Tumor Antibiotics

The genotoxic effects of the anthracycline doxorubicin and two of its analogues, epirubicin and pirarubicin, were studied using the wing Somatic Mutation and Recombination Test (SMART) in *Drosophila melanogaster*. These compounds are classified as topoisomerase II poisons and act by stabilizing topoisomerase II-cleaved DNA complex. By using SMART test it was possible to estimate the quantitative and qualitative genotoxic effects of these compounds, comparing the wing spot frequencies in marker- and balancer-heterozygous flies. On exposure to doxorubicin and its analogs, it was found that all the three compounds induce high frequency of spots related to homologous recombination, which is the major event responsible for their genetic toxicity. Pirarubicin was the most genotoxic anthracycline, inducing ~21 times more genetic lesions than doxorubicin, probably due to the presence of a second sugar ring in the amino sugar moiety in its chemical structure. Although the only difference between epirubicin and doxorubicin is the steric position of the amino sugar 4'-OH in the molecule, epirubicin is approximately 1.6 times as genotoxic as doxorubicin.

Genotoxic Potential Attributed to the Mechanism of Action

This experimental study indicates that all three anthracyclines analyzed were capable of damaging the DNA of *D. melanogaster* somatic cells. About 91–100% of the genetic toxicity observed for all drugs was associated with homologous mitotic recombination.

The high recombinational activity of these drugs can be attributed to their mechanism of action.

- The block of DNA replication due to the attachment of topoisomerase II in both strands, leading to non-homologous recombination repair.
- The unfavorable DNA topology related to the inhibition of topoisomerase II function after replication, leading to the occurrence of homologous and non-homologous recombination events.

Doxorubicin and pirarubicin were identified as inducers of both mutagenic and recombinagenic events, whereas epirubicin was considered as a pure recombinagenic compound.

Genotoxic Potential Attributed to the Chemical Structure

Epirubicin or 4'epidoxorubicin differs from doxorubicin in the steric position of the amino sugar 4'OH group. This unique difference confers a genotoxic activity ~56% when compared to doxorubicin. Pirarubicin or 4'-tetrahydropyranyl-doxorubicin is a disaccharide analogue to doxorubicin, which presented a superior genotoxic as substitutions at the 4'-position enhances the biological activity of anthracyclines (Ziegelbauerb and Aubrechta, 2009).

Materials and Methods

Canton-S flies were bred for two generations on corn meal agar. Drug exposure studies were performed in Carolina Formula 4 instant medium. Various concentrations of epirubicin (control, 200µg, 300µg, 400µg, and 500µg) were each mixed with 3g of instant food. Test and duplicates were set up for each concentration. The food was labeled with appropriate drug concentrations, and foiled, and allowed to set for two hours. For phenotypic change analysis, 100 male and 100 female flies were exposed to each of the drug concentrations and analyzed microscopically after 48 hours of exposure. F1 generation analysis was performed on emergence, microscopically. 100 flies, both males and females, were exposed to each of the four concentrations. DNA isolation was performed after 48 hours of exposure, which was followed by fragmentation assay. 100 trans-heterozygous larvae of the *mwh*/*flr3* cross were exposed to each of the four concentrations and adult flies were allowed to emerge. The emerged flies of the *mwh*/*flr3* cross were etherized using 500µl ether. The wings of the flies were dissected and placed on a glass slide. The wings were observed microscopically for any mutations.

Results

The results are tabulated in Table 1, Images 1, 2, 3, 4, 5, 6, 7, 8, 9.

Discussion

The present experimental study indicates that the anthracycline used (epirubicin) was capable of damaging the DNA of *D. melanogaster*. The various concentrations of epirubicin used (200µg, 300µg, 400µg, 500µg) were found to be genotoxic as it induced toxic DNA damage in both the somatic cells and germ cells of the exposed flies.

Epirubicin being classified as topoisomerase II poisons act by stabilizing a topoisomerase II-cleaved DNA complex and enhancing DNA double strand breaks. The genetic toxicity observed for the drug is associated with homologous mitotic recombination as a result of which significant increments are observed in the different end points chosen.

All four concentrations used in this study demonstrated 100% viability. Phenotypic changes revealed somatic mutations, which were observed in both the parental and F1 generations. With increase in the concentration of the drug, more somatic mutations were observed, indicating dose dependent expression of mutations. Abdominal curling and change in the abdominal color was observed in most concentrations and could be attributed to the feeding of the flies on the drug, which causes mutations in the gut cells, which was observed only in the parental generation suggesting that the mutation could be somatic. The color change in the thorax was seen in both the parents and the F1 generation, indicating germ line mutations that were carried to the F1 offspring and expressed. The change in the eye color though not consistent in all concentrations indicates the toxicity of the drug in the omatidium.

DNA fragmentation assay was performed with the adult flies exposed to epirubicin after 48 hours of feeding. The results of the fragmentation assay confirm the genotoxic effects of the drug at the DNA level/molecular level. Series of fragments of size ranging from 3,000 to 1,000 base pairs and significant dense shearing below 1,000 base pairs pose a threat to the stability of DNA when exposed to epirubicin.

Table 1.

END POINTS → / CONCENTRATION ↓	PHENOTYPIC CHANGES	F1 GENERATION	DNA FRAGMENTATION ASSAY	SMART
200 µg	Abdomen curling observed in males and females.	Flies appeared small, round and swollen. Curling was observed in both sexes (100%). Wings appeared small and rounded. Orange discoloration in thorax.	Single intact band at 1kb. A single fragment at 3,000 base pairs. A series of 2 fragments between 2,000 and 1,000 base pairs.	mwh/flr3 mutation. Single spots
300 µg	Orange discoloration in thorax and eyes.	Color change in thorax.	Single intact band at 1kb. A single fragment at 3,000 base pairs. A series of 2 fragments between 2,000 and 1,000 base pairs. Significant dense shearing was observed below 1,000 base pairs.	mwh/flr3 mutation. Single spots
400 µg	Orange discoloration in eyes, thorax and abdomen.100% abdominal curling in males.	Deep orange discoloration in thorax (100%).	Single intact band at 1kb. Series of 2 fragments between 2,000 and 1,000 base pairs- in pellet. A single fragment was observed at 1,000 base pair- in supernatant. Significant shearing was observed below 1,000 base pairs.	mwh/flr3 mutation. Single spots
500 µg	Orange discoloration in eyes, thorax and abdomen.100% abdominal curling in males.	Deep orange discoloration in thorax (100%).	Single intact band at 1kb. Series of 2 fragments was observed below 1,000 base pairs- in pellet. A single fragment was observed at 1,000 base pairs- in supernatant. Significant shearing was observed below 1,000 base pairs.	mwh/flr3 mutation. Single spots

The wing spot test (somatic mutation and recombination test – SMART) in *D. melanogaster* has been shown to be an efficient short term bioassay for the detection of genotoxic or antigenotoxic activity of pure compounds or complex mixtures. It is capable of activating enzymatically, promutagens and procarcinogens, for a quantitative determination of the recombinagenic potential of genotoxic agents.

In this study, SMART was used to investigate the genotoxicity of epirubicin, because of their beneficial effects in treatment for cancers. With SMART test it was possible to estimate the quantitative and qualitative genotoxic effects of these compounds, by comparing the wing spot

frequencies in marker- and balancer-heterozygous flies. The results obtained indicate that epirubicin induced a high frequency of spots (both single spots and *mwh*/*flr3* spots/mutations) related to homologous recombination, chromosomal alterations (mainly deletions), point mutations, or mitotic recombination, which is the major event responsible for their genetic toxicity.

Figure 1. Exposure to epirubicin: 200 µg. Abdominal curling (both sexes) (Left: male; Right, male and female).

Figure 2. Exposure to epirubicin: 300 µg. Orange discoloration in eye and thorax in both sexes (Left: female; Right, male).

Figure 3. Exposure to epirubicin: 300 µg. Orange discoloration in eye, thorax, and abdomen.

Figure 4. Exposure to epirubicin: 500 µg. Orange discoloration in eye, thorax, and abdomen in both sexes (Left, male; Right, female).

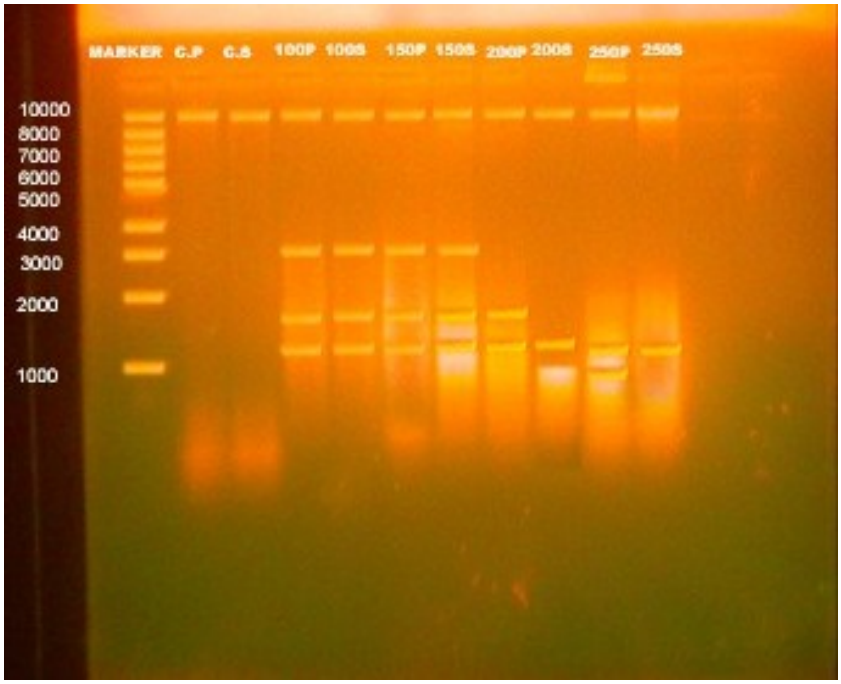

Figure 5. Agarose gel electrophoresis showing DNA fragmentation.

Thus, with the above end points, we are able to conclude the toxicity of epirubicin on both somatic and germ cells. Epirubicin being the synthetic analog of doxorubicin is widely used in the treatment of a variety of malignancies and is known for its reduced cardiotoxicity. The toxicity of epirubicin has also been demonstrated in cultured mammalian cells through SCEs and was found to exhibit dose dependent behavior.

The only reason behind the effective genotoxicity of epirubicin in comparison with doxorubicin arises at the differences in the steric position of the amino sugar 4'-OH in the molecule

(which enhances lipid permeability), thus making it approximately 1.6 times more genotoxic than doxorubicin.

In spite of the similarity concerning therapeutical effectiveness, epirubicin showed increased genotoxic effects expressed as loss of heterozygosity (LOH) in somatic cells of *D. melanogaster* especially in terms of homologous recombination.

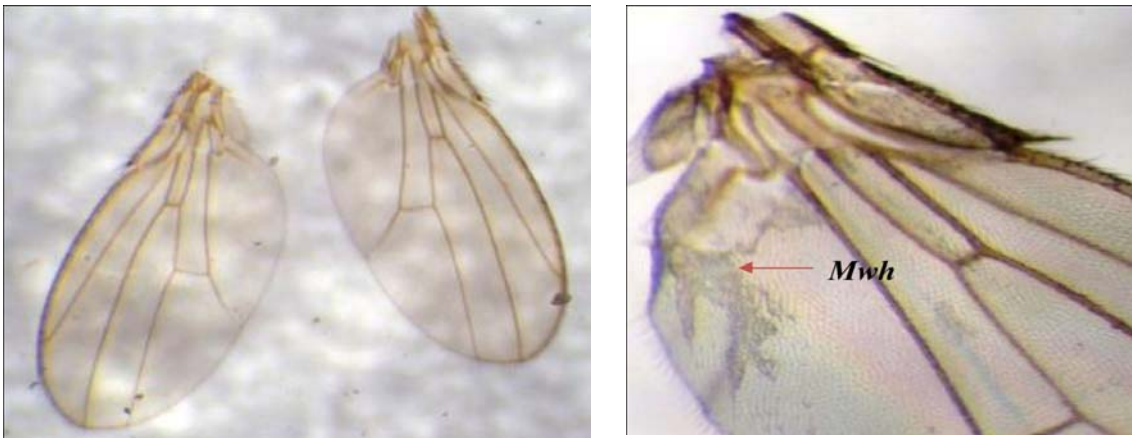

Figure 6. SMART analysis. Control (Left); Exposure to epirubicin: 200 µg (Right). Wing showing *mwh* pattern.

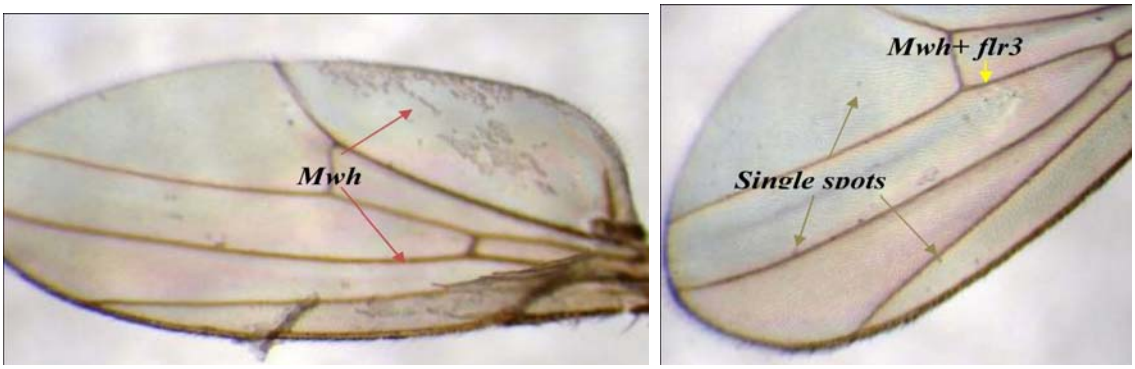

Figure 7. Exposure to epirubicin: 300 µg (SMART analysis).
Left: *mwh* pattern; Right: mwh + *flr3* pattern along with single spots.

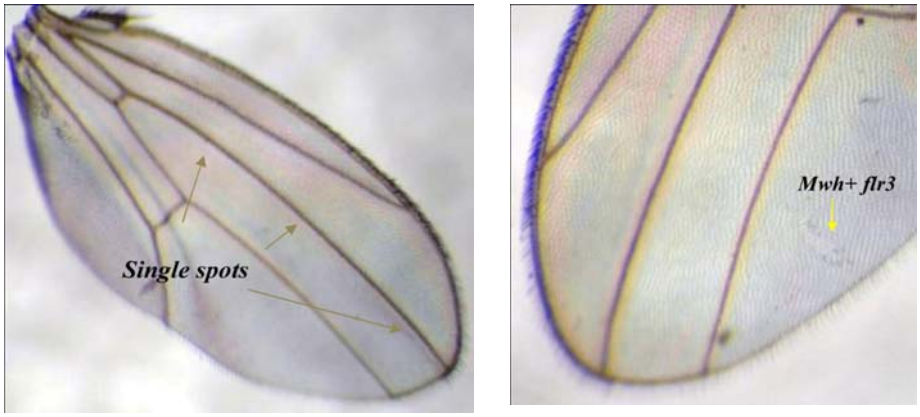

Figure 8. Exposure to epirubicin: 400 µg (SMART analysis).
Left: single spots; Right, *mwh* + *flr3*.

Figure 9. Exposure to epirubicin: 500 µg (*mwh* + *flr3* pattern) SMART analysis.

References: Alentorn, M.B., N. Xamena, A. Creus, and R. Marcos 1995, Mutation Research 341: 161-167; Buschini, A., P. Polil, and C. Rossi 2003, Mutagenesis 18(1): 25–36; Henderson, Daryl S., 2004, Drosophila *Cytogenetics Protocols*. Humana Press, Totowa, New Jersey; Hu, T., D.P. Gibson, G.J. Carr, S.M. Torontali, J.P. Tiesman, and J.G. Chaney 2004, Mutat. Res. 549(1-2): 5-27; Lehmanna, M., A. Franco, and H. Rodrigues 2003, Mutation Research 539: 167–175; Siddique, H.R., D.K. Chowdhuri, Saxena, and A. Dhawan 2005, Mutagenesis 20(4): 285-290; Ziegelbauerb, H.E., and J. Aubrechta 2009, Toxicology Letters 186: 36–44.

Testing gene function in fly head formation using transgenic RNAi.

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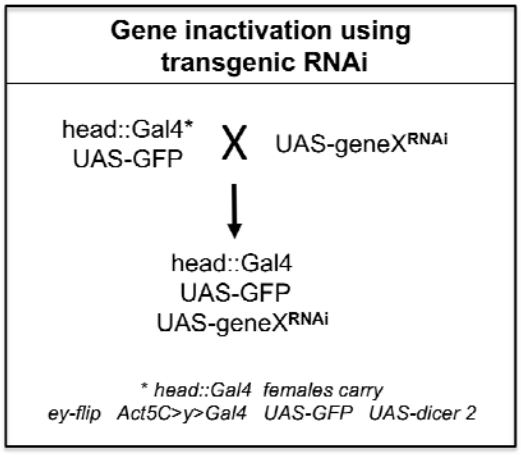
Abstract and Introduction

In this project, we investigated the potential role of 31 genes in eye/head formation in *D. melanogaster*. We tested the function of each gene by using RNAi as a means of knocking-down a single gene at the time within the progenitor tissue that gives rise to the adult head (eye-antennal imaginal disc). The eye/head phenotype was evaluated first at the adult stage and then during development in the larval stage. The range of phenotypes observed included eyeless, reduced eye, bar eye and rough eye, headless, reduced head, and enlarged head. Specific phenotypic categories were also analyzed at the level of imaginal discs in order to understand the nature of the defect better. The mutant phenotypes induced by RNAi-mediated silencing of *Ccn* or *KCNQ* (reduced eye), garz (disorganized neurons), alien or Trn (reduced head), and syx1A, *dock*, *Gdi*, or *drosha* (headless) suggest that these genes play significant roles in the development of the head and/or eye.

Methods

Genes were inactivated by transgenic RNAi (reviewed by Perrimon *et al*., 2010) specifically in the eye-antennal disc by inducing RNAi expression using the Gal4/UAS system (Brand and Perrimon, 1993). Virgin females of the *ey-flip; Actin5C>y>Gal4 UAS-GFP; UAS-dicer-2* genotype, here called "head-Gal4," were crossed to males from UAS-RNAi lines (Figure 1) obtained from the TRiP center collection (see Table 3 for specific TRiP stock numbers; http://www.flyrnai.org/TRiP-TTR.html). All crosses were carried out at 25°C.



Adult F1 progeny were scored using a stereomicroscope. Third instar larvae were dissected and directly mounted to identify eye discs (GFP-positive tissue). To look at neuronal development, anti-ELAV staining was carried out by standard Ab protocol (Sullivan *et al*., 2000) on L3 eye-antennal discs, and images were obtained on a Leica SPE confocal. Images were processed in Adobe Photoshop.

Figure 1. Genetic scheme used to knock-down gene activity specifically in eye/head progenitor cells. Head::Gal4 flies carry *ey-flip* on the X chromosome, *Act>y>Gal4* and *UAS-GFP* on chromosome 2, and *UAS-dicer2* on chromosome 3. All UAS-RNAi transgenes are on chromosome 3.

Results and Discussion

In order to identify genes required for eye and head development, we crossed 31 UAS-RNAi lines with the eye-antennal imaginal disc Gal4 driver line "head-Gal4." The progeny from each cross were first scored at the adult stage and fell into one of the categories shown in Table 1.

The overall summary of the data is shown in Table 2, examples of the HL, EL, and RH phenotypes are shown in Figure 1, and detailed data are provided in Table 3. Among the 31 crosses, 15 gave no viable progeny and 16 did; 12 of the 15 lethals died in the late pupal stage and could be scored from pupal dissection. Out of 31 crosses, 5 crosses gave normal progeny, and 26 led to mutant phenotypes including 16 with abnormal heads (9 HL, 6 RH, and 1 LH), 7 with abnormal eyes (3 Bar, 2 RE, 1 EL, and 1 RR). Three were unscorable due to early death (DD).

The twelve crosses, scored DD (3) and HL (9), raised the question of whether the progeny simply lacked discs, thus making it impossible to form heads and eyes, or whether the discs were present but unable to join and form the head during metamorphosis. Because of this and since in all cases death occurred at the pupal stage, these two categories were selected for analysis at the larval stage.

Third instar larvae were dissected and eye-antennal discs were stained for the ELAV protein to visualize neurons and for the EYA protein to mark eye progenitor cells. In four cases (*brm, dock, Gdi, Syx1A*), no discs (GFP-positive) were found, suggesting that discs were either absent or extremely small in size (not shown). Six other crosses (*skpA, N, Smr, nct, mam, drosha*) showed extremely small discs (not shown), whereas two crosses (*neur, garz*) produced L3 discs that when

stained for ELAV showed plenty of neurons but somewhat abnormal clusters (Figure 3B and not shown).

Table 1. Phenotypic Categories.

CODE	ADULT HEAD/EYE PHENOTYPE
BAR	Bar-like eye
DD	dead (some could be scored as late pupae)
EL	eyeless
HL	headless
LH	larger head than normal (including eyes)
NSS	normal shape and size
RE	reduced eye, smaller eye
RH	reduced head
RR	rough eyes

Table 2. Overview Cross Results.

Crosses with normal progeny	5/16
Crosses with abnormal progeny	26/31
Lethals	15/31
Lethals scored as late pupae	12/15 (3/15 DD RH, 9/15 DD HL)
Lethals unscorable	3/15 (3 DD)
Viable with abnormal heads	16/31 (9/16 HL, 6/16 RH, 1/16 LH)
Viable with abnormal eyes	7/31 (3/7 Bar, 2/7 RE, 1/7 EL, 1/7 RR)

We also analyzed at the larva stage the knock-down crosses resulting in the Bar phenotype: *ato, csw,* and *spitz*. As expected, silencing of the *ato* gene resulted in strong bar-eyed flies and, based on anti-ELAV antibody staining, it had only a few neurons (not shown). The *csw* knock-down also resulted in an extreme bar-eye phenotype at the adult stage, and only a thin line of ELAV-positive cells were seen in the discs (Figure 3C). Spitz-RNAi adults were also bar-eyed; however, at the L3 stage, the discs were large in size and displayed plenty, but disorganized, neuronal clusters along the posterior portion of the disc (Figure 3D). Therefore, in the case of *Spitz*, neuronal development did begin to produce neurons (though abnormally as shown by the disorganization of the neuronal clusters) and then it probably stopped before all ommatidia were formed (hence the bar-like eye).

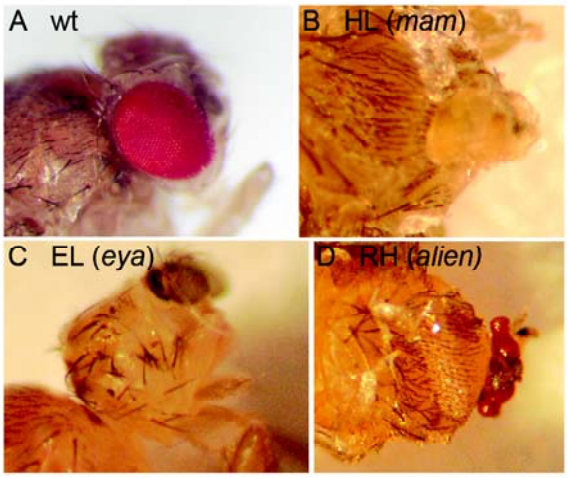


Figure 2. Examples of adult phenotypes. A) wt fly head; B) *ey-flip; Act>y>Gal4 UAS-GFP; UAS-dicer2/UAS-mam*^{RNAi}; C) *ey-flip; Act>y>Gal4 UAS-GFP; UAS-dicer2/UAS-csw*^{RNAi}; D) *ey-flip; Act>y>Gal4 UAS-GFP; UAS-dicer2/UAS-alien*^{RNAi}.

Of the 31 genes tested, 14 of them were previously known to affect imaginal discs and/or eye/head development. In these cases, our findings were consistent with previous reports (as recorded in Flybase). These loci include *Notch, mam, neur,* and *nct* (components of Notch signaling), *spi, Cbl,* and *csw* (components of EgfR signaling), *dpp, eya, ato, trr, SkpA, Pten, and Mib1* (see Table 3 for details of phenotype and molecular function).

Conversely, *SesB* and *burs* were not expected to affect head/eye size or shape based on previous data and/or molecular function, as reported in Flybase (see Table 3), and no abnormalities were observed in our test as well. Knock-down of *SNF4Ag, raptor,* and *dnd* also did not result in any obvious defects. However, in these cases, we cannot exclude that gene silencing simply did not occur because of lack of relevant data. Nonetheless, these results generally support the validity of our test.

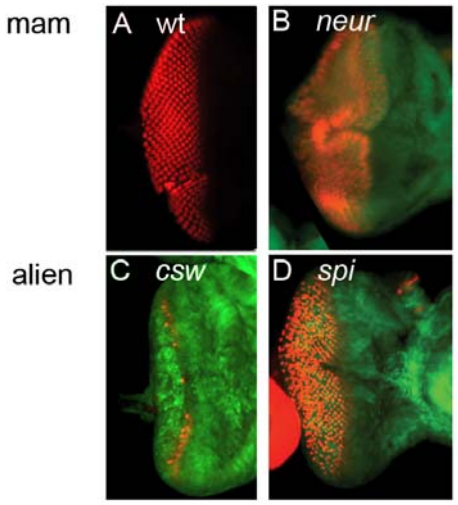


Figure 3. Examples of L3 eye disc phenotypes. L3 eye discs were stained for Elav to mark neurons (red) in all panels and for GFP to mark RNAi expressing cells (green) in panels B-D. A) wt eye disc stained for Elav to mark neurons (red); B) HL fly with proboscis directly attached to thorax, *ey-flip; Act>y>Gal4 UAS-GFP; UAS-dicer2/UAS-neur*^{RNAi}; C) EL fly, *ey-flip; Act>y>Gal4 UAS-GFP; UAS-dicer2/UAS-csw*^{RNAi}; D) RH fly with small collapsed head, *ey-flip; Act>y>Gal4 UAS-GFP; UAS-dicer2/UAS-spi*^{RNAi}.

We report here novel head and/or eye phenotypes for 12 loci. Among these, the biological processes affected by *brm*, *smr*, and *Pp4-19C* (which include chromatin-level regulation of gene expression and control of mitotic cell cycle) agree with the observed phenotypes of DD with no discs (*brm*), HL with small discs (*Smr*), and RH (*Pp4-19C*). The mutant phenotypes induced by knock-down of *Ccn* and *KCNQ* (RE), *garz* (DD with disorganized neurons), *alien* and *Trn* (RH), *syx1A*, *dock*, and *Gdi* (HL with no discs), and *drosha* (HL with small discs) are reported here for the first time and implicate these genes in the formation of the eye (*Ccn*, *KCNQ*, *garz*) and/or head (*alien*, *Trn*, *Syx1A*, *dock*, *Gdi*, *drosha*).

Based on their reported function (Flybase), processes such as vesicle-mediated transport (*garz* and *Gdi*), insulin receptor signaling (*dock*), primary micro-RNA processing (*drosha*), potassium ion transport (*KCNQ*), phagocytosis and/or nuclear import (*Trn*) may play significant roles in the development of the eye and head tissue. Confirmation of these phenotypes using a second, different RNAi, or other reagent, will have to be carried out prior to further studies.

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Table 3 follows:

Table 3. Head/eye phenotypes for 31 genes tested by transgenic RNAi. * entries based on flybase (*http://flybase.org*)

CODE	ADULT HEAD/EYE PHENOTYPE	CODE	ADULT HEAD/EYE PHENOTYPE
BAR	Bar-like eye	NSS	normal shape and size (more subtle defects may have been missed)
DD	dead (some could be scored as late pupae)	RE	reduced eye, smaller eye
EL	eyeless	RH	reduced head
HL	headless	RR	rough eyes
LH	larger head than normal (including eyes)		

TRiP #	CG #	Gene Name	Head/Eye phenotype	Phenotype code	Disc phenotype	Reported eye function*	Molecular function*	Biological process*
HM05119	CG9556	*alien*	Dead (pupa); reduced eyes; reduced head; collapsed head and eyes	DD RH	Not Determined	NONE	ligand-dependent nuclear receptor binding; transcriptional repressor co-factor	protein stabilization
JF02089	CG7508	*ato - atonal*	Strong Bar eyes	BAR	Only a couple of neurons	photoreceptors are completely absent; required for R8	transcription factor	eye morphogenesis; R8 photoreceptor cell fate commitment; neuronal fate and differentiation
HM04019	CG5942	*brm brahma*	Dead (pupal stage)	DD	No discs recovered	Formation of larval head structures is abnormal but the nature of the defects is undetermined.	protein binding, DNA binding, general RNA polymerase II transcription factor activity, regulation of transcription	chromatin-mediated maintenance of transcription; positive regulation of EGFR signaling pathway; cell differentiation
JF02260	CG18419	*burs bursicon*	Normal shape and size	NSS	Not Determined	NONE	hormone activity	chitin-based cuticle tanning
JF02650	CG7037	*Cbl*	Rough eyes	RR	Not Determined	Rough mosaic eyes, larger than wt, with larger ommatidia. block of apoptosis; over-recruitment of all eye cell types	protein binding; signal transducer activity	sensory organ development; regulation of EgfR; neuron differentiation; regulation of cell proliferation

TRiP #	CG #	Gene Name	Head/Eye phenotype	Phenotype code	Disc phenotype	Reported eye function*	Molecular function*	Biological process*
HM04023	CG32183	*Ccn*	Reduced eyes (in some)	RE	Not Determined	NONE	growth factor activity	unknown
HMS00012	CG3954	*csw corkscrew*	Extreme Bar eye, very narrow and oblong	BAR	a thin line of ELAV positive neurons in the disc	loss of photoreceptor R7, & occasional loss of outer (R1-R6) photoreceptors	protein binding; protein tyrosine phosphatase activity	EGFR signaling pathway; cell signaling; cell fate commitment; mitotic cell cycle
JF02638	CG6560	*dnd dead end*	Normal shape and size	NSS	Not Determined	NONE	GTPase activity; GTP binding	cilium assembly; signal transduction
JF02809	CG3727	*dock dreadlocks*	Dead (pupa); headless	DD HL	No discs recovered	photoreceptor cell axons targeting defects	insulin receptor binding	insulin receptor signaling pathway, axon guidance
JF01090	CG9885	*dpp decapentaplegic*	Dead (pupal stage) - reduced head, small head	DD RH	Not Determined	small and/or roughened eyes, consistent with a failure of normal eye morphegenesis	ligand; cell signaling	regulation of organ morphogenesis
JF02784	CG8730	*drosha*	Dead (pupa); headless	DD HL	Small discs	NONE	double-stranded RNA binding; ribonuclease III activity	primary microRNA processing
JF03160	CG9554	*eya eyes absent*	Eyeless	EL	Not Determined	eya mutants have reduced eyes or are eyeless	ser/thr phosphatase; tyr phosphatase; transcriptional activator non-DNA-binding co-factor	regulation of cell fate specification; eye progenitor cells specification
JF01603	CG8487	*garz gartenzwerg*	Dead (pupal stage)	DD	Plenty of neurons but disorganized	NONE	ARF guanyl-nucleotide exchange factor activity	ER to Golgi and intra-Golgi vesicle-mediated transport
JF02617	CG4422	*Gdi GDP dissociation inhibitor*	Dead (pupa); headless	DD HL	No discs recovered	NONE	Rab GDP-dissociation inhibitor activity	neurotransmitter secretion; regulation of Rab GTPase activity; vesicle-mediated transport

TRiP #	CG #	Gene Name	Head/Eye phenotype	Phenotype code	Disc phenotype	Reported eye function*	Molecular function*	Biological process*
JF02562	CG33135	*KCNQ potassium channel*	Abnormal head & very small eye	RE	Not Determined	NONE	voltage-gated potassium channel	potassium ion transport
JF02881	CG8118	*mam mastermind*	Dead (pupa) - headless	DD HL	Small discs	mediates N signaling; Notch pathway required for disc growth	transcription coactivator activity	compound eye development; nervous system development; Notch signaling pathway
JF02629	CG5841	*mib1 mind bomb 1*	Reduced head	RH	Not Determined	small wing and eye imaginal discs	protein binding; ubiquitin-protein ligase activity	compound eye morphogenesis, positive regulation of Notch signaling
JF01356	CG3936	*N Notch*	Dead (pupa) - headless	DD HL	Small discs	required for proliferation of eye disc epithelium	cell-cell signaling receptor; regulator of transcription	sensory organ development, neurogenesis; regulation of cell proliferation
JF02648	CG7012	*nct nicastrin*	Dead (pupa); headless	DD HL	Smaller and deformed discs	formation of supernumerary neurons; Notch pathway; required for disc growth	processing of Notch signaling receptor	cytoskeleton organization; Notch signaling pathway; photoreceptor cell morphogenesis; cell proliferation
JF02048	CG11988	*neur neuralized*	Dead (pre-pupal stage)	DD	Plenty of neurons but disorganized	ommatidia poorly defined, rhabdomeres and lenses severely disrupted, eye bristle missing	ubiquitin-protein ligase activity	regulation of Notch signaling pathway
JF02807	CG32505	*Pp4-19C Protein phosphatase 19C*	Reduced head	RH	Not Determined	NONE	protein serine/threonine phosphatase activity	microtubule-based process; regulation of mitotic cell cycle
JF01859	CG5671	*Pten*	Big head; big eyes	LH	Not Determined	larger eyes than normal when eyes mosaic for mutant	serine/threonine phosphatase activity	insulin receptor signaling pathway; negative regulation of growth, cell size, proliferation, cell cycle
JF01088 - strong	CG4320	*raptor*	Normal shape and size	NSS	Not Determined	NONE	protein binding	response to DNA damage

TRiP #	CG #	Gene Name	Head/Eye phenotype	Phenotype code	Disc phenotype	Reported eye function*	Molecular function*	Biological process*
JF01810	CG16944	*sesB stress-sensitive B*	Normal shape and size	NSS	Not Determined	normal eyes	ATP: ADP antiporter	synaptic vesicle transport
HM05185	CG16983	*skpA*	Dead (pupa); headless; mouth parts directly on shoulders	DD HL	Small discs	imaginal discs are either entirely absent or rudimentary, extensive apoptosis	protein binding	centrosome duplication, chromosome condensation, positive regulation of mitotic cell cycle
JF02413	CG4013	*Smr Smrter*	Dead (pupa) - headless	DD HL	Small discs	NONE	protein and DNA binding; transcription corepressor activity	regulation of mitotic cell cycle
JF02060	CG17299	*SNF4Aγ SNF4/AMP-activated protein kinase gamma subunit*	Normal shape and size	NSS	Not Determined	NONE	AMP-activated protein kinase component	positive regulation of cell cycle; cholesterol homeostasis
JF03322	CG10334	*spi spitz*	Bar eyes	BAR	Discs with disorganized neuron clusters along posterior only	mitotic clones have reduced numbers of photoreceptors and loss of whole ommatidia	epidermal growth factor receptor binding	EGFR signaling pathway; cell signaling; mitotic cell cycle; cell fate commitment
JF01829	CG31136	*Syx1A Syntaxin 1A*	Dead (pupa); headless	DD HL	No discs recovered	disrupted ommatidial array	SNAP receptor activity	cytokinesis; cellularization; exocytosis; neurotransmitter secretion; vesicle-mediated transport
JF02697	CG7398	*Trn Transportin*	Reduced head	RH	Not Determined	NONE	protein transmembrane transporter activity	protein import into nucleus; phagocytosis, engulfment
JF03242	CG3848	*trr trithorax-related*	Dead (pupal stage) - small head	DD RH	Not Determined	morphogenetic furrow progression defects, disorganized ommatidial array	histone methyltransferase activity; transcription coactivator binding	cell differentiation

Further studies on five types of inversions in Japanese and African populations of *Drosophila melanogaster.*

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Many kinds of inversions have been detected on the four major autosome arms in natural populations of *D. melanogaster* through a number of worldwide studies. Usually these naturally occurring inversions are the paracentric type. Most of them are detected only once in a particular population, but some are nearly always found in most populations. In view of the frequency and geographical distribution, Mettler *et al*. (1977) categorized inversions into four classes (*Common Cosmopolitans*, *Rare Cosmopolitans*, *Recurrent endemics*, and *Unique endemics*). In addition, Inoue and Igarashi (l994) added a new class, *Quasi Cosmopolitans*, to the category and a change from *Recurrent endemics* to *Endemics*. *Common Cosmopolitan* inversions are usually maintained in most populations all over the world with higher frequencies, on occasions being more frequent than standard chromosomes, and show frequency clines over large geographical regions. Four inversions, *In(2L)t*, *In(2R)NS*, *In(3L)P*, and *In(3R)P*, are in this category, each of which exists in the right and left arms of two major autosomes. *Rare Cosmopolitan* inversions are also distributed all over the world, but their frequencies are usually low, not enough to show a geographical cline. *Quasi Cosmopolitan* inversions are also widespread in the world, but just enough to say "cosmopolitan" in their distribution. They appeared with very low frequencies and were absent in many populations. *Endemic* inversions are found more than once in a given population but appear in geographically region-limited manner. Occasionally they show higher frequencies than *Rare Cosmopolitans*. *Unique* inversions are usually observed only in a single individual or its brood from a single population and never found in a different population. However, some of *Common Cosmopolitans* showed the tendency to decrease frequencies, and most were sometimes absent in the recent surveys. On the contrary, some *Quasi Cosmopolitans* or *Endemics* are more frequent than *Common Cosmopolitans*. In the present study, we reported the results of recent surveys of eight Japanese and three African populations (Table 1), and of annual surveys of the Osaka population for 10 years (Table 2). Wild caught females were individually transferred to culture vials and allowed to lay eggs. Established strains were kept at 25°C for examination. Inversions were determined through direct observation of the chromosomes of one larva from each female. This method gives two genomes sampled per each female. Cytological analyses were made on salivary gland chromosomes stained with the lactic-acetic orcein method. Breakpoints and nomenclature of inversions were established by comparing with representations of Bridges' standard map (Lefevre, 1976). Frequency of each inversion was calculated per each chromosome arm. Average frequencies of total *Cosmopolitan* inversions per major autosome arm were also calculated to compare the degree of inversion polymorphisms, because significant positive correlations were observed among all four major autosome arms (Inoue *et al*., 1994).

Table 1 shows the frequencies of the *Cosmopolitan* inversions in Japanese and African populations. Four localities with very low frequency were less than 0.100 on an average among five Japanese northern mainland populations (Locality No. 1-5). The highest frequency found in the mainland was the Kanazawa population (No. 3), being 0.115 on an average. In(2R)NS was absent in Katsunuma, and *In(3L)P*, *In(3R)C*, and *In(3R)Mo* were absent or almost absent in populations other

Table 1. Frequencies of Cosmopolitan inversions in Japanese and African populations.

Locality (Year)	N*	In(2L)t	In(2R)NS	In(3L)P	In(3R)P	In(3R)C	In(3R)Mo	Arm Average **
1. Yamagata (2006)	200	0.010	0.005	0	0.050	0	0	0.016
2. Fukushima (2009)	92	0.083	0.022	0	0.065	0	0	0.043
3. Kanazawa (2009)	200	0.155	0.060	0.010	0.180	0.005	0.050	0.115
4. Shiojiri (2008)	200	0.070	0.015	0	0.085	0	0	0.043
(2009)	128	0.086	0.031	0	0.094	0.008	0	0.055
5. Katsunuma (2007)	200	0.080	0	0	0.130	0	0	0.053
6. Tsushima (2008)	100	0.110	0	0.070	0.060	0.040	0	0.070
7. Amami-Oshima (2006)	200	0.270	0.275	0.120	0.285	0.050	0.030	0.258
8. Iriomote-jima(2005)	186	0.388	0.293	0.303	0.723	0.090	0	0.450
9. Uganda (2010)	158	0.133	0	0	0.057	0	0	0.049
10. Zambia (2010)	200	0.235	0.115	0.005	0.105	0.005	0	0.116
11. South Africa (2010)	189	0.328	0.167	0.010	0.061	0.131	0.005	0.176

* Number of genomes examined

** Average frequency of inversions per major autosome arm

Table 2. Temporal change in frequencies of Cosmopolitan inversions in the Osaka population.

Year	N*	In(2L)t	In(2R)NS	In(3L)P	In(3R)P	In(3R)C	In(3R)Mo	Average **
1992***	240	0.175	0.096	0.054	0.096	0.067	0.046	0.134
1999	292	0.236	0.106	0.014	0.158	0.059	0.096	0.167
2000	400	0.150	0.118	0.033	0.100	0.050	0.165	0.154
2001	400	0.170	0.048	0.043	0.118	0.053	0.043	0.119
2002	400	0.243	0.133	0.125	0.095	0.130	0.045	0.193
2003	200	0.215	0.110	0.060	0.070	0.025	0.070	0.138
2004	200	0.275	0.080	0.050	0.080	0.020	0.055	0.140
2005	200	0.225	0.100	0.045	0.105	0.030	0.050	0.139
2006	200	0.180	0.115	0.035	0.085	0.010	0.080	0.126
2007	200	0.220	0.150	0.035	0.080	0.075	0.025	0.146
2008	200	0.215	0.105	0.040	0.060	0.020	0.090	0.133
Average		0.209	0.105	0.049	0.095	0.049	0.070	0.144

* Number of genomes examined

** Average frequency of inversions per major autosome arm

*** Data from Inoue and Igarashi (1994)

than Kanazawa. The small island Tsushima (No. 6) also showed low degree of polymorphisms with absence of *In(2R)NS* and *In(3R)Mo*. Amami-Oshima (No. 7) and Iriomote-jima (No. 8) in the South-west Islands showed significantly higher polymorphisms, with average frequencies of 0.258 and 0.450, respectively. In this region all four Common Cosmopolitans were observed with high frequencies, which were the same results as 1979, 1982, and 1998's surveys in Inoue *et al*. (2002). In the African populations, Uganda (No. 9) showed similar low frequencies to Japanese mainland populations, lacking *In(3L)P*, *In(3R)C*, and *In(3R)Mo*, being 0.049 on an average. The Zambia population (No. 10) had the same level of polymorphisms as Kanazawa with absence of *In(3R)Mo*.

The degree of polymorphisms of the South Africa population (No. 11) was between Kanazawa and Amami-Oshima, being 0.176 on an average.

Table 2 shows the results of annual surveys in each September of 1999-2008. The collection by the banana traps was carried out in the domestic place in Osaka prefecture, the middle region of Japanese mainland. The yearly average frequencies were relatively stable to be 0.144 on a total average, which was more than the case of Kanazawa (No. 3) and less than Amami-Oshima (No. 7) of Table 1. The data of 1992 from the same place showed the similar results. The characteristics of the Osaka population were that all *Common* and *Rare Cosmopolitans* were observed every year.

Quasi Cosmopolitan, *Endemic*, and *Unique* inversions observed in the present survey are listed in Table 3 with their breakpoints, localities, and appearance number. The 17 inversions categorized as *Uniques* were observed in the present study, and they have never been described before. In addition, there are several inversions whose categories are intermediate between *Common Cosmopolitans* and *Uniques*. Five *Endemic* and three *Quasi Cosmopolitan* inversions are as follows:

In(2R)43D;48B was observed twice in the Osaka population; most probably these inversions are selected out soon after they were born and dismissed by a stochastic process. *In(2R)44C;54F* and *In(3L)62C;67E* were found only in African populations. The latter appeared a total of 29 times and twice in Zambia and South Africa populations, respectively. This inversion is the typical case of temporal increasing pattern of Endemics.

In(2R)O 51B;55E was first reported by Inoue *et al*. (2002) from populations of the South-west Island in Japan. The present study showed that it appeared constantly in the populations of the western region of Japan including the South-west Islands. Recently *In(2R)O* was also observed in Hokkaido, the northern part of Japan of 2004, and Hawaiian population of 2005 (Inoue and Watada unpublished data). The naming of "*In(2R)O*" is derived from Rika Ogoshi, one of the authors.

In(2L)W 28C;32C was first found in the middle region of Japan of 1972 (Inoue and Watanabe, 1979). Although its frequencies were always low to be a few percent, the distribution covered all over Japan. Inoue and Igarashi (1994) confirmed its distribution at the five localities in Japanese mainland and the South-west Islands, but *In(2L)W* was found only once in the Osaka population in the present study.

In(3R)K 86F/87A;96F/97A was distributed in the worldwide scale (*Quasi Cosmopolitan*). As Inoue and Igarashi (l994) summarized, it was found in many places in Africa, some places in the USA, Wales, Greece, and Mexico. In Japan *In(3R)K* was first found in the South-west Islands (Inoue 1992). Inoue and Watada (2006) and the present study confirmed its distribution in Japanese mainland. *In(3R)K* was also found in Korea (Paik, 1998) and in the Hawaii of 2005 and in Taiwan of 2011 (Inoue and Watada unpublished data). Especially Table 3 showed its high level of polymorphisms in Africa, being the frequencies of 0.184 in Uganda, 0.200 in Zambia, and 0.076 in South Africa, respectively. In these three populations, *In(3R)K* prevailed over the *Common cosmopolitans*, *In(3R)P*, in the right arm of the third chromosome.

In(3L)Y 68F;75C was first found in a Japanese mainland population in the 1970's (Inoue, 1979). Although its frequencies were always very low, it was present all over Japan in the 1980's (Inoue, 2000). This inversion is also found in regions other than Japan; in Spain (Roca *et al*., 1982) and North Carolina, USA, in 1974 and 1975 (Inoue and Igarashi, l994). However, *In(3L)Y* could not be found in the present study.

In(3L)M 66C-71B showed the worldwide distribution in Florida and Texas in USA, Korea, New Guinea, and Egypt (Inoue and Igarashi, l994). In Japan it was found in the Osaka region of the mainland and the South-west Islands in both 1970's and 1980's with low frequencies (Inoue *et al*., 1994). In the present study, *In(3L)M* was found four times in the Osaka population.

The Katsunuma population (No. 5 in Table 1) in the middle of Japanese mainland has been continuously surveyed for chromosomal polymorphisms since the l 970's (Inoue and Watanabe,

Table 3. *Quasi Cosmopolitan, Endemic* and *Unique* inversions found in the present study.

Chromosome Arm and breakpoints	Locality and collection year (observed number)
Chromosome Arm 2L	
W	Osaka 2007 (3)
23E; 27F	Osaka 2007 (1)
22C; 34	Osaka 2003 (1)
26A; 31F	Katsunuma 2008 (1)
21E; 27C	Fukushima 2009 (1)
26E; 34B	Shiojiri 2009 (1)
25B; 33F	Zambia 2010 (1)
Chromosome Arm 2R	
O	Osaka 1999 (17), 2000 (8), 2001 (33), 2002 (33), 2003 (21), 2004 (25), 2005 (20), 2006 (11), 2007 (17), 2008 (15), Amami-Oshima 2006 (26), Iriomote-jima 2005 (8), Tsushima 2008 (5), Katsunuma 2007 (1), Kanazawa 2009 (5)
43D; 48B	Osaka 2001 (2)
44F; 54B	Osaka 2007 (1)
44F; 55B	Katsunuma 2007 (1)
45A; 53D	Katsunuma 2007 (1)
44F; 56A	Yamagata 2006 (1)
44C; 54F	Zambia 2010 (4), South Africa 2010 (1)
51F; 55D	Zambia 2010 (1)
Chromosome Arm 3L	
M	Osaka 1999 (1), 2000 (1), 2005 (1), 2008 (3)
70C; 76D	Osaka 2007 (1)
62C; 67E	Zambia 2010 (29), South Africa 2010 (2)
71D; 76B	South Africa 2010 (1)
Chromosome Arm 3R	
K	Osaka 1999 (6), 2002 (1), 2004 (4), 2005 (3), 2006 (2), 2008 (1), Amami-Oshima 2006 (1), Shiojiri 2009 (1), Uganda 2010 (29), Zambia 2010 (40), South Africa 2010 (15)
86F; 96A	Osaka 1999 (1)
85D; 92D	Shiojiri 2008 (1)
87F; 96A	Katsunuma 2007 (1)
89E; 93F	Zambia 2010 (1)

1979). Among four *Common Cosmopolitans*, *In(2L)t*, *In(2R)NS*, and *In(3R)P* showed the stable higher frequencies, and *In(3L)P* was always in lower frequencies than the other three inversions. In the 1980's, *In(2R)NS* and *In(3R)P* began to decrease in their frequencies and were absent in some collections during the period. Moreover the present study showed lower frequencies of *In(2L)t* and *In(3R)P* with absence of *In(2R)NS* and *In(3L)P*. The Korean populations surveyed from 1978 to 1992 showed similar low frequencies of *In(2R)NS*, *In(3L)P*, and *In(3R)P* (Paik, 1998). Instead of the decreases of *Common Cosmopolitans*, some *Endemics* such as *In(2R)O* and *In(3R)K* increased their frequencies and expanded their distribution. In some cases they overwhelmed the frequencies of

Common cosmopolitans in a given chromosome arm. Thus, the long-term surveys over several decades reveal the flexible phase of chromosomal polymorphisms in natural populations of *D. melanogaster*.

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Is *Zaprionus indianus* invading a preserved Amazon forest?

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In June 2010 *Zaprionus indianus* Gupta, 1970 (Diptera: Drosophilidae) was registered for the first time in the Caxiuanã National Forest in Pará, Brazil. Caxiuanã is a very preserved conservation area, localized in the center of endemism Xingu (1º 57' 37" S; 51º 36' 55" W) (Silva *et al*., 2005). The Caxiuanã correspond to 80% of primary forest, with 1,013 species of plants, classified in 189 families. The most extensive and diverse environment is the dense terra-firme forest occupying 85% of the area; the rest is occupied by forests of inundation (várzea and igapó), savanna formation (hydromorphic field), secondary vegetation, and residual vegetation on sites of orchards (Lisboa *et al*., 1997). The human presence is perceived by small patches of antique fields in recovery areas that are generally close to the river banks and were previously occupied by small proprietors and riverside populations whose current occupation is much more restricted (Praxedes and Martins, in press).

Drosophilidae species have been monitored in Caxiuanã National Forest since 2000, with the specific use of traps (Martins *et al*., 2008) baited with banana exposed into the forest. In this collection five individuals of *Zaprionus indianus* were recorded from two points (1° 57' 38.2" S; 51 ° 36' 57.8" W and 1° 59' 42" S; 51° 37' 03.6" W) in one total of 49,139 drosophilids collected which represented 0.012% of all drosophilids. Since in very low abundance, these finds indicated the higher dispersive potential of these species. The next question is if *Zaprinous* will be well established in this forest.

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Horizontal transmission of intracellular endosymbiotic bacteria: a case between mites and fruit flies and its evolutionary implications.

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Abstract

Insect intracellular endosymbiotic bacteria of the genus *Wolbachia* are known to play an important role in the evolution of their hosts by influencing the hosts' sex ratios and creating sexual isolation within their populations. As the bacteria are confined to the egg cellular matrix and are inherited maternally, their wide distribution among arthropod taxa remains enigmatic. This ubiquitous dispersal among insects can partially be explained by the existing evidence of lateral transfers of the bacteria in host-parasite systems, usually via direct tissue contact between the agents. However, lateral transfer beyond these systems has never been reported. In the present study, we demonstrate for the first time that *Wolbachia* lateral transmission is possible between fruit flies and the non-parasitic mites *Tyrophagus noxius*. We discuss potential evolutionary implications of the reported phenomenon.

Keywords: endosymbionts; *Wolbachia*; *Tyrophagus noxius*; *Drosophila melanogaster*; lateral transfer

Introduction

Maternally inherited intracellular bacteria of the genus *Wolbachia* are widely spread among invertebrates and infect 16% to 66% of all arthropod species, including all major insect orders (Werren *et al.*, 1995; Werren, 1997; Hilgenboecker *et al.*, 2008). The bacteria are known to affect the host populations via reproductive manipulations, such as parthenogenesis, feminization, and cytoplasmic incompatibility (*e.g.*, Min and Benzer, 1997; Veneti *et al.*, 2003; Weeks *et al.*, 2003; Zchori-Fein and Perlman, 2004). Due to the last effect, individuals of the same host population cannot interbreed if infected with different *Wolbachia* strains. The resulting reproductive isolation effectively splits the population into subunits, entailing what is called *infectious speciation* (*e.g.*, Wade, 2001; Miller *et al.*, 2010). This allows a tentative presumption that *Wolbachia* may have played a crucial role in the rapid diversification of arthropods. However, as the bacteria are confined to the host eggs' cellular matrix and are usually inherited maternally (Werren, 1997), their wide distribution among insects remains largely a mystery.

Meanwhile, there is evidence that *Wolbachia* can be transmitted to a new host species via parasites. For example, such transfers have been reported between the parasitic wasps *Trichogramma kaykai* (Schilthuizen and Stouthamer, 1997; Huigens *et al.*, 2004) and *Leptopilina boulardi* (Heath *et al.*, 1999) and their respective hosts. Woodlice have also been found to acquire *Wolbachia* from conspecifics via a shared source of blood (Rigaud and Juchault, 1995). However, such cases usually imply direct tissue contact between transfer agents and are perhaps unlikely to explain the whole

network of *Wolbachia* lateral transfers that must have happened in nature, as inferred from phylogenetic studies (*e.g.*, Vavre *et al.*, 1999).

In this study, we found the mites *Tyrophagus noxius* to be infected with *Wolbachia pipientis* strain *w*Mel, the strain characteristic of the fruit flies *Drosophila melanogaster*, and experimentally demonstrate the possibility of *Wolbachia* lateral transfer between these species. The mites *Tyrophagus noxius* Zachvatkin, 1935 (*Sarcoptiformes, Acaroidea, Tyroglyphidae*) often cohabit with *Drosophila*, including cultured laboratory flies, sharing the same nutritious media but are not *Drosophila* parasites. We discuss possible evolutionary implications of this phenomenon.

Materials and Methods

Mites and flies

Mites were taken from the nutrient medium in tubes containing a reared *Drosophila* culture and identified (Figure 1) at Schmalhausen Institute of Zoology, NAS of Ukraine, Department of Acarology. Fruit flies were taken from the *Drosophila melanogaster* living laboratory culture at the Department of General and Molecular Genetics of National Taras Shevchenko University of Kyiv.

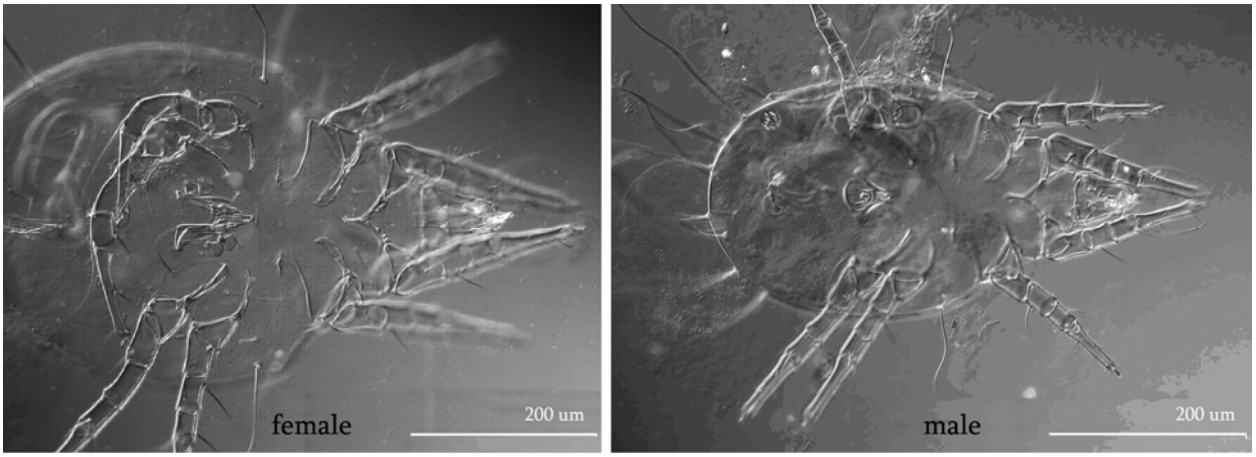

Figure 1. *Tyrophagus noxius*.

Experimental setup

First of all we ensured that the experimental mites and fruit flies were infected with *Wolbachia* by PCR with primers W-Specf and W-Specr amplifying a 438 bp fragment of the bacterial 16 rRNA gene (Werren and Windsor, 2000). Then both flies and mites were separated into two groups so that we had two infected groups of each species. After this, we separately reared two groups of flies and one group of mites on a medium containing 2 mg/ml tetacyclin hydrochloride (Morimoto *et al.*, 2006), which is an efficient antibiotic against *Wolbachia*, and ensured by PCR that these groups got rid of the bacteria. Accumulation of living material in the antibiotic-treated groups took nearly two weeks. Finally, we set up two joint cultures: 1) infected mites with uninfected flies (test culture), and 2) uninfected mites with uninfected flies (control ensuring that no bacterial revival occurs after antibiotic treatment; Figure 2). Both joint cultures were reared for 12 *Drosophila* generations. Bacterial infection status in both flies and mites was checked in both cultures by PCR three times coinciding with the *Drosophila* 2nd, 9th, and 12th generations. During the last PCR check (12th generation), we also amplified a 590-636 bp region of the *Wolbachia* surface protein Wsp gene

using the general primers *wsp81f* and *wsp691r* to identify the *Wolbachia* strain (Zhou *et al.*, 1998). The whole experiment was repeated twice. The *wsp* and *16S rDNA* amplicons from the first experiment obtained at generation 12 were sequenced.

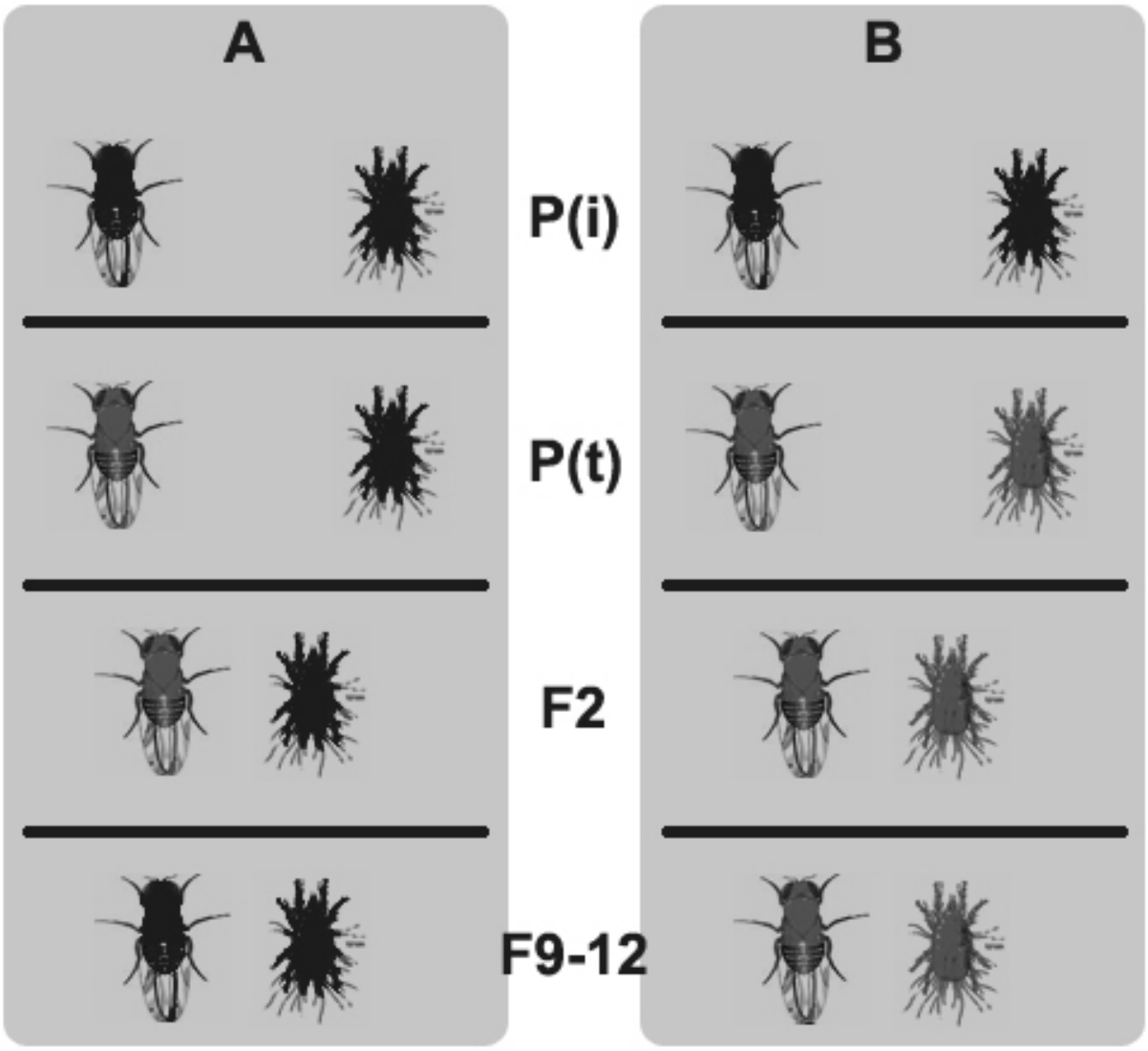

Figure 2. The experimental setup. Black flies/mites – infected, grey flies/mites – uninfected. A – experiment, B – control, P(i) – initial parental generation, P(t) – parental generation after antibiotic treatment (experimental mites were not treated). In the second generation (F2), the infection status remains the same as in P(t). Experimental flies (joint culture) get reinfected in/before the generation F9 and the infection persists through F12. No infection revival in control flies/mites.

PCR profiles

16S rRNA gene: denaturation 93°C/2 min; 30 cycles of denaturation 93°C/30 s, annealing 56°C/30 s, elongation 72°C/45 s; final elongation 72°C/8 min.

wsp gene: denaturation 94°C/2 min 50 s; 35 cycles of denaturation 94°C/30 s, annealing 55°C/40 s, elongation 72°C/50 s; final elongation 72°C/7 min.

Sequence alignment

The *wsp* and *16S rDNA* sequences were verified by BLAST (default parameters) against the NCBI's non-redundant nucleotide database. Local alignment was performed using the Vector NTI Suite 6 software (Invitrogen, USA).

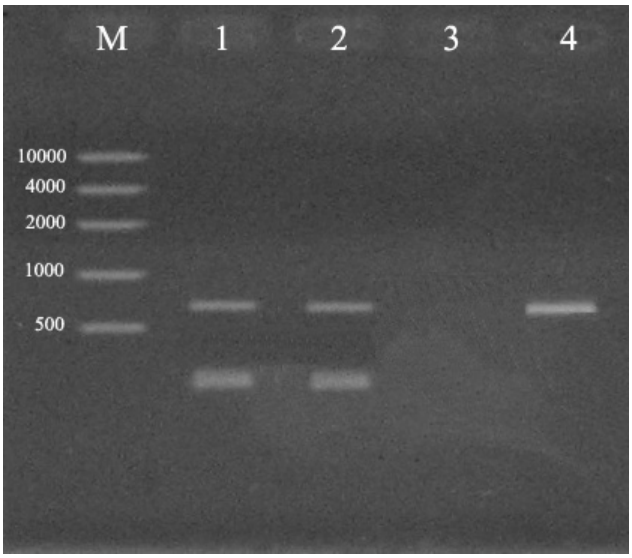

Figure 3. Infection diagnostics of *T. noxius* and *D. melanogaster* (Canton-S) after the 9th generation of cohabitation. M – molecular weight marker FastRuler High Range („Fermentas"); 1 – a fragment of *Wolbachia* sp. *wsp* gene in a *T. noxius* DNA sample; 2 – a fragment of *Wolbachia* sp. *wsp* gene in a *D. melanogaster* DNA sample (Canton-S); 3 – negative PCR control (*D. melanogaster* after tetracycline); 4 – positive PCR control for *Wolbachia* sp. (*D. melanogaster* "Uman" wild-derived lineage).

Results

In the control setups (see Materials and Methods) all three PCR checks in both experiments revealed no presence of *Wolbachia* both in mites and flies past antibiotic treatment, indicating that the treatment was reliable enough to ensure total elimination of the bacteria from tested flies and mites.

Both experiments gave identical results in the experimental setups (uninfected flies jointly cultured with infected mites). We had not detected *Wolbachia* in F2 flies, but the bacteria were present in F9 flies (Figure 3). We further tested F12 flies to check if the infection was stable, and F12 flies were also *Wolbachia*-positive. Therefore, the bacteria had been transferred from mites to flies. The presence of the bacteria in F12 flies indicates that the infection was stable and persisted into further generations.

Alignment of the mite and fly *wsp* sequences from F12 generation PCRs is shown in Figure 4 and demonstrates that both amplicons represented the same *Wolbachia* strain. BLAST alignment indicates that this strain is *w*Mel (aligned to GenBank ID: AF020064; see Zhou *et al.*, 1998), one characteristic of *Drosophila melanogaster*. Interestingly, this implies that the mites had previously (perhaps long before the experiment) acquired this bacterial strain from the flies they lived with, as we did not treat the experimental mites with an antibiotic. Therefore, our experiment just double-proofs that lateral transfer of *Wolbachia* is possible even without any invasive tissue contact between the transfer agents.

Discussion

From the evolutionary perspective, our findings indicate that either of the two players in the *D. melanogaster – T. noxius* symbiotic system has acquired the bacterium from its counterpart some time ago. Theoretically, this could occur with some "mediated" tissue contact, *e.g.*, mites eating fly eggs or vice versa from culture medium or natural nutritious media. *Wolbachia* can remain viable outside the host cell for up to one week and, more importantly, can restart their normal life cycle once back inside a cell (Rasgon *et al.*, 2006). As the mites oviposit their eggs into the medium, *Drosophila* larvae, which are not very fastidious in diet, could eat some of the eggs and thus become the vehicle of horizontal transmissions. As *T. noxius* is not a parasite, the infection gateway through fly tissue damage characteristic of host–parasite systems is unlikely in this case.

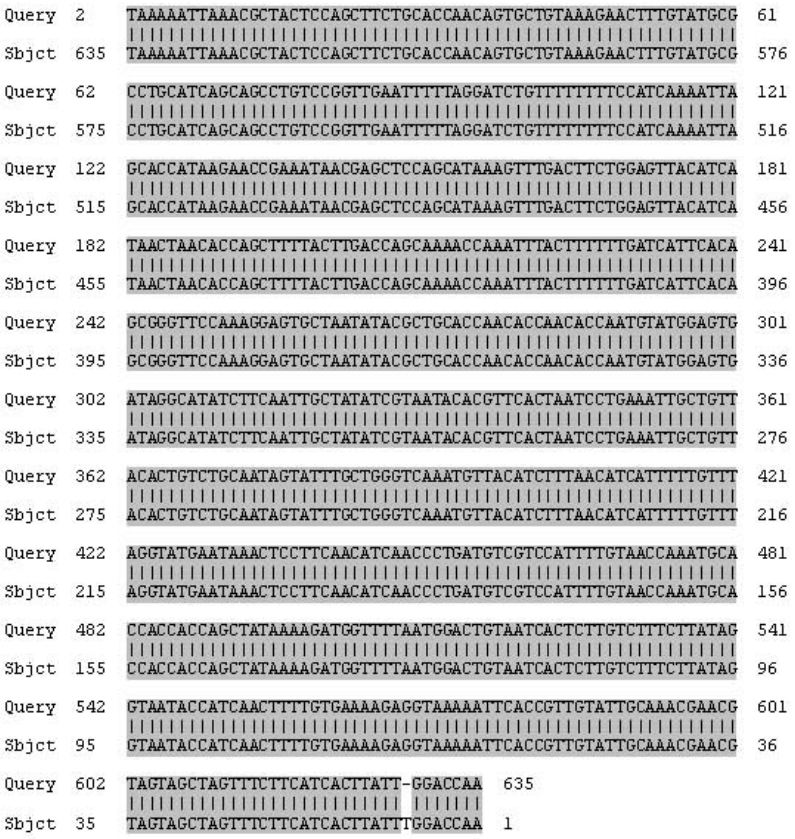

Figure 4. Alignment of *Wolbachia* sp. *wsp* gene sequences extracted from the F12 fly and mite DNA. Query – *Wolbachia* sp. *wsp* gene sequence extracted from the mite DNA; Sbjct – *Wolbachia* sp. *wsp* gene sequence extracted from the fly DNA.

Screening of the 12th generation flies suggests that the newly acquired bacterial infection gets "fixed" in ovaries and transmits further in its normal vertical way.

Although the results of this single experiment cannot be directly extrapolated to nature, such a mode of transmission could potentially explain the ubiquitous occurrence of *Wolbachia* in a wide range of hosts, because the network of species sharing common nutritious media would greatly expand the network of parasite-host and prey-predator interactions within arthropoda.

The possibility of fast *Wolbachia* transmission in nature would perhaps also mean significant effects on host evolution. One of these effects has recently been aptly designated as *infectious speciation* (Wade, 2001; Miller *et al.*, 2010). *Drosophila* flies infected with different *Wolbachia* strains within one population cannot interbreed and produce offspring because of the phenomenon called *bidirectional cytoplasmic incompatibility*, which prevents forming a normal zygote from gametes. In this way, a population infected with different bacteria strains is, in fact, reproductively separated, with the ensuing genetic drift and divergence. This bizarre way of reproductive isolation might in theory have been responsible for the rapid diversification of arthropods.

Besides, if shared laterally between many hosts, *Wolbachia* might potentially have served as a *horizontal gene transfer* (HGT) vehicle. One of the brightest examples of HGT between multicellular eukaryotes is the transfer of transposons between dipteran insects, in particular P element (a DNA transposon) between *Drosophila* species, many occurrences of which have been reported to date (reviewed in Silva and Kidwell, 2000). The most painstakingly studied case, that between *D. willistoni* and *D. melanogaster*, is known to have occurred less than a century ago. Interestingly, the rapid worldwide spread of P element in *D. melanogaster* populations, which ensued immediately upon its transfer, seems to have concurred with the spread of a particular genotype of *Wolbachia pipientis* strain *wMel*, which is now ubiquitous in *D. melanogaster* populations (Riegler *et al.*, 2005). As P element is generally harmful to fly genomes, its wide spread might have potentially benefited from its co-occurrence with *Wolbachia*, as some *Wolbachia* strains are known to give selective advantages to their host flies, particularly in antivirus (Teixeira *et al.*, 2008; Glaser and Meola, 2010) and anti-insecticide (Berticat *et al.*, 2002) defense and reproductive success (Weeks *et al.*, 2007).

HGT between multicellular eukaryotes itself probably requires bacterial and viral vectors to commute between the HGT-involved genomes. *Wolbachia* seems also to be a plausible candidate HGT vector from this perspective, as genetic exchange with its host genomes has yet been demonstrated (discussed in Introduction). A number of bacteriophages, like the phage WO (Kent and Bordenstein, 2010) known to mediate HGTs (Kent *et al.*, 2011) are encoded in the *Wolbachia* genome and might potentially serve as gene transfer vehicles between the *Wolbachia* and arthropod genomes.

This cumulative evidence, together with our results, suggests that *Wolbachia* may be a promising candidate genetic exchange driver, markedly influencing arthropod evolution. Further research is necessary to clarify if and to what extant horizontal transmission of *Wolbachia* occurs in nature, particularly in systems with no direct tissue contact between transfer agents. If proven, this phenomenon would greatly contribute to our understanding of the evolution of many invertebrate taxa and the biology of HGT between multicellular eukaryotes.

Acknowledgments: We are indebted to Prof Timothy A. Mousseau, College of Arts and Science, University of South Carolina for assistance in sequencing; Serhiy Sereda and Dr Olga Zhuk, Department of General and Molecular Genetics of Taras Shevchenko National University of Kyiv for helpful discussion.

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Light-induced retinal degeneration in *Drosophila* with green fluorescent protein (GFP) attached to rhodopsin.

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Quite by serendipity, this laboratory noticed that *w*; *cn bw*; Rh1-GAL4 + UAS-Rh1GFP/TM2 *Drosophila* had degeneration of R1-6 receptors; this white-eyed stock has a transgene with GFP-labeled R1-6 rhodopsin (Rh1-GFP) driven into R1-6 by the promoter of the Rh1 gene (ninaE), so we will nickname this stock "Rh1-GFP." We utilized mostly time-tested optical ("pseudopupil") techniques summarized by Stark and Thomas (2004).

Dark-reared flies have a very obvious pseudopupil darkening, our way to visualize the amount of rhodopsin to metarhodopsin conversion (Figure 1 A *vs*. B); also R1-6's GFP was clear in the fluorescent deep pseudopupil (Figure 1 C), and R1-6's GFP-labeled rhabdomere tips were nicely imaged with optical neutralization of the cornea (Figure 1 D).

Contrast this with flies maintained 5 days in constant room light: there is a substantial decrease in the pseudopupil darkening (Figure 1 E *vs*. F); the fluorescent deep pseudopupil is hazy (Figure 1 G), and the fluorescent rhabdomere tips seen under oil immersion have missing profiles (Figure 1 H). White-eyed control *Drosophila*, without the Rh1-GFP transgene, had never shown any indications of light-induced damage in decades of research by this laboratory.

We used optical neutralization and the confocal microscope to verify the expectation that vitamin A deprived flies do not have fluorescent rhabdomere tips (Figure 1 I), while vitamin A replete flies show R1-6 GFP label (Figure 1 J). We aged vitamin A deprived flies for 8 days in constant room light, then put them in the dark on carrot juice; their GFP-labeled R1-6 rhabdomere tips look beautiful in the confocal microscope (Figure 1 K). We aged vitamin A deprived flies 5 days in constant light, then put them in the dark with carrot juice and obtained substantial recovery in the pseudopupil darkening (Figure 1 L *vs*. M); also R1-6 showed tidy GFP fluorescence in the deep pseudopupil (Figure 1 N). The work with vitamin A deprivation and replacement therapy shows that light is not damaging when Rh1-GFP is greatly reduced.

We present a light micrograph of Rh1-GFP flies maintained 23 days in room light showing cells in the process of degeneration and missing rhabdomeres (Figure 1 O). Control white-eyed flies (without Rh1-GFP), also maintained in room lighting for this same duration, did not have any signs of degeneration (not shown).

Earlier, Stark (2005) showed that white-eyed *Drosophila* with GFP driven into R1-6 had completely normal structure, rhodopsin-metarhodopsin conversions, and electrophysiology. While this may seem contradictory, GFP, in that case, was not attached to Rh1; flies were the F1 from a

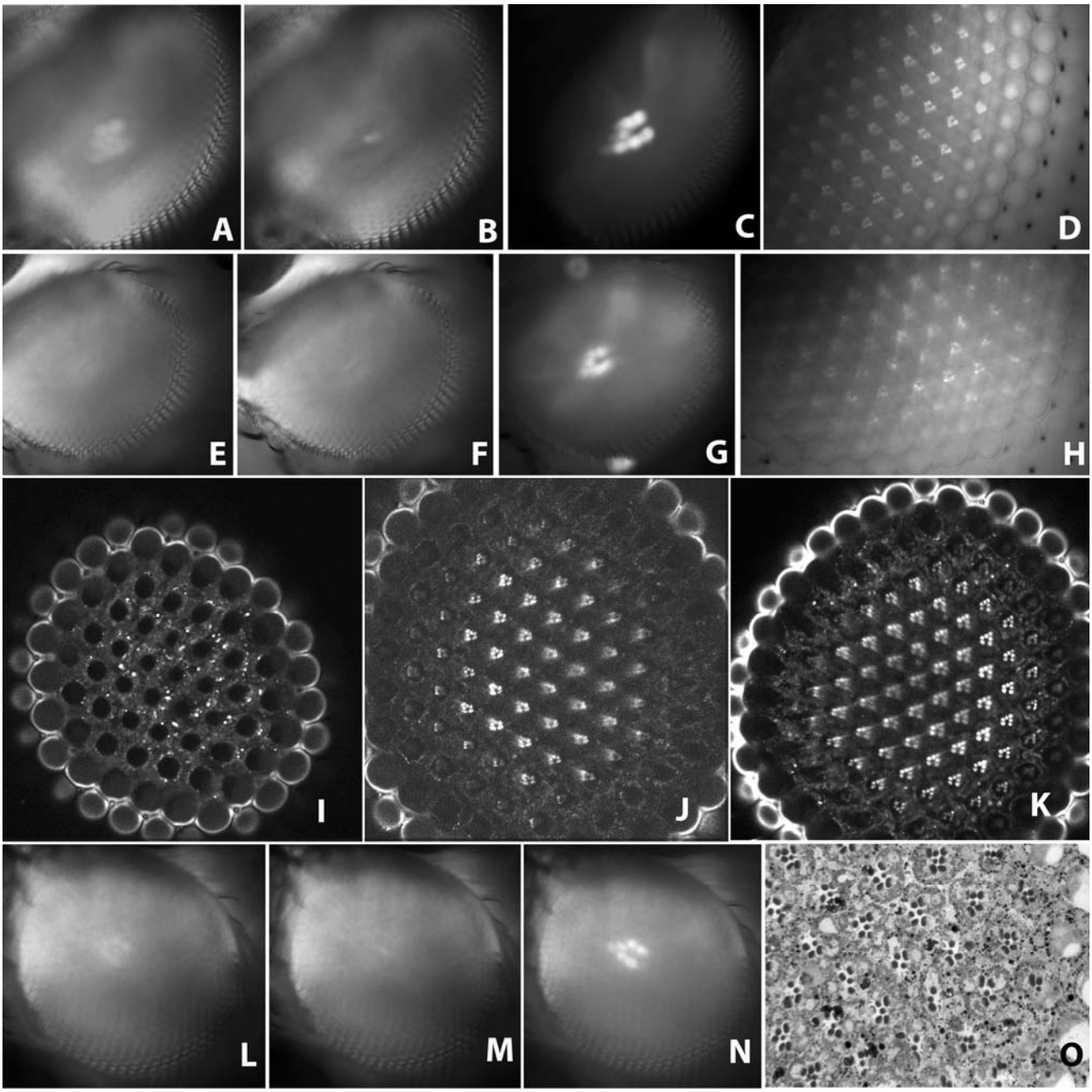

Figure 1.

cross between w^-/w^-; Sp/CyO; Rh1Gal4 p(17)/Rh1Gal4 p(17) (Rh1 promoter driving Gal4 in a rosy plasmid homozygous on the third chromosome) and w^-/w^-; UAS GFP/UAS GFP; UAS GFP/UAS GFP (homozygous for UAS GFP on second and third chromosomes). This suggests that light absorbed by either the rhodopsin or the attached GFP in Rh1-GFP *Drosophila* causes damage, but not light absorbed by GFP that is not linked to rhodopsin. However, it is conceivable that light absorbed by the Rh1 that is not attached to GFP in Rh1-GFP flies is what causes damage; we presume that the native ninaE gene, together with its promoter, is still expressing Rh1 (in addition to the Rh1-GFP expressed in the transgenics).

This laboratory (Selimovic *et al*., 2010) showed that room light decreases rhodopsin in *Drosophila* photoreceptors. We still believe those published quantitative measurements. However, we did present a confocal image of the Rh1-GFP stock we used in this study with very weakly fluorescent rhabdomere tips in flies that had been maintained in the light for 6 days. Now we understand that, in addition to light-induced decreases in rhodopsin, degenerative changes were also contributing to the weak fluorescence we observed.

One question remains. Does Rh1-GFP, as well as Rh1 without GFP attached, contribute to the pseudopupil darkening (Figure 1 A *vs*. B)? Recall that the pseudopupil darkening was our way to visualize the photoconversion of rhodopsin to metarhodopsin. In other words, we wonder whether the attachment of GFP to Rh1 interferes with conversion of rhodopsin to metarhodopsin.

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A comparison between the effect of aqueous and methanolic extract of *Decalepis hamiltonii* on the level of alcohol tolerance in *Drosophila melanogaster*.

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The oxidative damage of biological molecules is an important event in the development of a variety of human diseases. Antioxidants, especially natural ones, have potential applications in prevention or cure of such diseases. *Decalepis hamiltonii* (family: Asclepiadaceae) has been shown to possess potent antioxidant properties.

The present study was carried out to check whether the root extract of *D. hamiltonii* has any neuroprotective potential. For this purpose, Oregon K strain flies were divided into three groups, *viz*., control, *D. hamiltonii* aqueous extract – fed, and *Decalepis hamiltonii* methanolic extract – fed ones. The exposure chamber was made for each group in which 8 flies of same sex were transferred by aspiration. Cotton stubs were coated with 0.5 ml ethanol and subsequently inserted into exposure vials. Numbers of stationary flies were recorded for each minute and the time required for sedation of 50% flies was documented.

Present study revealed that the flies fed on *Decalepis hamiltonii* aqueous extract containing media have relatively 30 percent higher ST50 (50% sensitivity) value compared to control and *Decalepis hamiltonii* methanolic extract – fed ones.

It can be concluded that antioxidant properties of this plant extract, especially aqueous one, can give higher degree of protection to the flies against oxidative stress induced by ethanol.

Neuroprotection and antioxidant evaluation of methanol extract of *Sida glutinosa* using *Drosophila* model system.

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Abstract

Oxidative stress is a harmful condition that occurs when there is an excess of free radicals or a decrease in antioxidant levels. The evidence to date for oxidative stress in Parkinson's disease (PD), Schizophrenia (SCZ), Alzheimer's disease (AD), and other neurodegenerative diseases is strongly persuasive. Clinical studies showed that a number of events associated with Alzheimer's are capable of stimulating production of free radicals and depletion of antioxidant levels. As pointed out, whether oxidative stress is eventually proven to be primary or secondary in etiologic progression, the therapeutic rewards of antioxidants are likely to be substantial. Clearly, strategies aimed at limiting free radical induced oxidative stress and damage may slow the progression of neurodegenerative diseases (Ravindra *et al*., 2004).

Paraquat (1,1-dimethyl-4,4-bipyridynium dichloride) is a quaternary nitrogen herbicide and highly toxic substance for humans and animals; many cases of acute poisoning and death have been reported (Sittipunt, 2005). The toxicity of paraquat is due to the generation of the superoxide anion, which can lead to the synthesis of more toxic reactive oxygen species (ROS) such as hydroxyl radicals and hydrogen peroxide (Suntres, 2002). On the other hand, the oxidation of reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH) as a consequence of paraquat administration results in the disruption of biochemical processes requiring NADPH (Smith *et al*., 1978).

The use of antioxidant compounds provides an easy and convenient way of testing the validity of the free radical theory using *Drosophila melanogaster* as model organism. The organisms are easier to culture and manipulate under laboratory conditions than the mammals. Administration of the test compounds can be done easily by adding the dissolved compounds to the food medium thus ensuring their uptake by the flies (Tapiwanashe *et al*., 2006).

Sida glutinosa plant belongs to family *Malvaceae*, commonly called as "*Sticky Fanpetals*". It is a sub-shrub which is available in the forest at shady areas along Ravines. It was identified in Biligiri Rangana Hills of Chamarajanagar District, Karnataka, India.

In this paper methanol extract of *Sida glutinosa* (**G**) was preliminary screened for Physico-chemical, Phyto-chemical properties, *in vitro*, *in vivo* antioxidant and neuroprotective studies were carried out using *Drosophila melanogaster* (Oregon K) strain adult male flies. *In vitro* antioxidant studies exposed that the extract exhibited concentration dependent activity. The oxidative stress markers employed in the study included lipid peroxidation products malondialdehyde (MDA) and hydroperoxide (HP), reduced glutathione (GSH), and antioxidant enzymes included superoxide dismutase (SOD) and catalase (CAT). The oxidative stress was induced by using paraquat at 15 mM. The concentration of extract for studies was fixed based on LC50 values. There was significant

demolition in the levels of MDA and HP in case co exposure of **G** with Par treated flies homogenate. The level of SOD and CAT were brought to near basal level in the homogenate of flies co exposed with **G** and Par. In negative geotaxis assay it was found that **G** was able to rescue the flies significantly from deteriorating locomotors dysfunctions. The extract **G** showed significant antibacterial property against tested strains.

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Sequence variations in dosage compensation genes and histone deacetylases in *In(1)B^{M2}(reinverted)* of *Drosophila melanogaster.*

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The *Drosophila* strain *In(1)B^{M2}(reinverted)* arose in 1978 as a spontaneous re-inversion in *In(1)B^{M2}* (inversion break points 16A-20F) (Mazumdar *et al*., 1978). This re-inverted strain manifests a sex and chromosome specific alteration of the male X chromosome, which can either be induced through continuous rearing at 18 ± 1^{o}C (Mukherjee and Ghosh, 1986) or through a brief exposure to cold shock at 12 ± 1^{o}C for four hours (Kar *et al.,* 2000). The alteration in the structure of the male X chromosome is associated with a transient, male specific increase in the acetylation of histone H4 at lysine 16 (H4K16) (Kulkarni-Shukla *et al.,* 2008). This male specific acetylation is brought about by the gene *males absent on the first* (*mof*), a histone acetyltransferase that is a component of the multiprotein complex that brings about dosage compensation in *Drosophila melangoster* (Bone *et al.*, 1994). Due to the male sex specific phenotype and the observed hyperacetylation, we investigated whether the structural alteration of the male sex chromosome occurred due to mutations in the genes of the dosage compensation pathway (*i.e*., *mof*, *male- specific lethal 1*, *msl-1*; *male-specific lethal 2, msl-2; male-specific lethal 3*, *msl-3*; *maleless, mle*; *RNA on the X 1, roX1;* and *RNA on the X 2, roX2*) chromatin remodelling genes associated with dosage compensation (*Imitation SWI*, *Iswi*; *Trithorax-like*, *Trl*; *supercoiling factor*, *scf*; and *JIL-1*) or due to mutations perturbing the activity of histone deacetylases (HDAC) (*Rpd3, HDAC6, Histone deacetylase 3* [*Hdac3*], *HDAC4,* and *Sir2*).

The sequences of these sixteen genes were identified using FlyBase (Tweedie *et al.,* 2009), and primers were designed using Primer3 primer design programme (Rozen and Skaletsky, 2000). Amplifications and sequencing were carried out in triplicates. The gene sequence variations were determined by aligning them to the wild type sequences obtained from FlyBase using ClustalW2 with default settings (Larkin *et al.,* 2007). Coding sequences were translated using EMBOSS Transeq (http://www.ebi.ac.uk/Tools/ emboss/transeq / index), and the predicted protein sequences for each gene were aligned to that of the wild type using ClustalW. Nucleotide variations were categorized as synonymous, non-synonymous, conserved or semi conserved depending on the resultant amino acid change.

Of the sixteen genes only one, HDAC, *Rpd3* showed 100% homology to the wild type sequence reported in the FlyBase. Table 1 shows the variations reported for the other fifteen genes.

Table 1. Nucleotide variations in the sequenced genes of *In(1)B^{M2}(reinversion).*

Gene	Gene Size (bp)	Total changes observed	Unique variations	Type of variation	Gene	Gene Size (bp)	Total changes observed	Unique variations	Type of variation
mof	3017	9	7	3 synonymous 1 non-synonymous 3 non coding	*Iswi*	4011	31	31	6 non coding 25 synonymous
msl-1	5077	5	4	1 conserved 1 non-synonymous 2 non coding	*Trl*	13121	26	9	3 synonymous 6 non coding
msl-2	3899	51	4	2 synonymous 2 non coding	*scf*	1772	19	8	5 non coding 3 synonymous
msl-3	2314	36	22	10 non coding 6 conserved 2 semi conserved 3 synonymous 1 non-synonymous	*Rpd3*	2751	-	-	-
mle	6035	2	-	-	*HDAC6*	7401	4	4	3 non coding 1 non-synonymous
roX1	3792	2	2	2 non coding	*Hdac3*	1789	2	2	1 synonymous 1 non coding
roX2	1377	2	1	1 non coding	*HDAC4*	22433	45	40	34 non coding 6 synonymous
JIL-1	14391	11	4	4 non coding	*Sir2*	4001	8	8	5 non coding 2 synonymous 1 non-synonymous

Table 2. Non-synonymous changes in genes of *In(1)B^{M2}(reinversion).*

Gene	Position	Nucleotide change(s)	Amino acid Position	Amino acid Change
mof	2580	C→G	791	His→Pro
msl-1	2446-2448	3 bp deletion	672..673	Del of 2aa
msl-3	418	T→G	67	Val →Gly
HDAC6	5535	A→G	973	Glu→ Gly
Sir2	3023	G→T	802	Asn→Asp

There were 253 nucleotide changes which were not reported in either the FlyMine (Lyne *et al.*, 2007) or The FlySNP Project databases (http://flysnp.imp.ac.at/flysnpdb.php). After performing a BLAST search (Altschul *et al.*, 1990) for the variations, 107 variations found matching hits in the *Drosophila melanogaster* Nucleotide database of NCBI. Thus, there were 146 variations that were unique to *In(1)B^{M2}(reinverted)*. Of these, 21 were structural variations (insertions/deletions), 48 were synonymous variations, seven were conserved, two were semi-conserved, and 64 variations were in the non-coding regions. Only four single nucleotide variations were non-synonymous and were present within the coding regions of *mof*, *msl-3*, *HDAC6*, and *Sir2* genes (Table 2). Except for MSL-3, none of the amino acid substitutions were within the functional domains of the proteins. For MSL-3, the valine to glycine change observed at position 67 was within the chromo related domain (CRD) of this protein. Of the 21 structural changes, only one was present in the exonic region of *msl-1*. This change involved a 3bp deletion (TCA), resulting in the deletion of valine and asparagine at

672..673aa and addition of aspartic acid (Table 2). The mechanism by which the sequence variations of *In(1)B^{M2}(reinverted)* may affect the phenotype is being currently studied.

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Circadian rhythm of locomotor behavior of *D. agumbensis* and *D. rajasekari* collected from Sakleshpur.

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Abstract

Circadian clock regulates physiological and behavioral processes in a wide variety of organisms ranging from unicellular organism to human beings. The present study is aimed to investigate circadian rhythm of locomotor behavior of *D. agumbensis* and *D. rajasekari* collected from Sakleshpur, Karnataka, India at 910m altitude. Locomotor activity behavior was assayed by using *Drosophila* activity monitor (Trikinetics IV) under laboratory conditions. Both the species exhibited unimodal activity. The range of activity was 126-851 in *D. agumbensis* and 114-964 in *D. rajasekari*. The period of activity of both the species was closer to 24h. Statistical analysis revealed that there is a significant difference in the locomotor behavior of the two species. Keywords: locomotor activity; *D. agumbensis*; *D. rajasekari.*

Introduction

Circadian rhythms characterize the physiological processes of organisms ranging from the unicellular dinoflagellate *Gonyaulax polyedra* (Hastings and Sweeney, 1958) to human beings (Wever, 1979; Aschoff, 1981; Brady, 1981). It is now reported that besides eukaryotes, even cynobacteria among eubacteria possess circadian clocks (Ouyang *et al.,* 1998). The ubiquitous occurrence of circadian clocks at various levels of organization and complexity suggests that they may be of adaptive value (Aschoff, 1994; Aschoff *et al.,* 1982; Hastings *et al.,* 1991; Pittendrigh, 1993; Sharma, 2003a). It is believed that circadian clocks benefit organisms by efficiently timing various behavioral and metabolic processes to appropriate times of the day in accordance with cyclic external and internal environments (Aschoff, 1964; Aschoff *et al.,* 1982; Hastings *et al.,* 1995;

Pittendrigh, 1993; Sharma, 2003a). Previous studies on fruit flies (*D. melanogaster*) (Pittendrigh and Minis, 1972), bollworm (*Pectinophora gossypiella*) (Von Saint Paul and Aschoff, 1978), and Cynobacteria (Ouyang *et al.,* 1998) have demonstrated that survival of organisms regularly maintained under light/dark (LD) cycles (12:12h) is enhanced considerably if their periods of activity closely match those of the (LD) cycles.

Several studies on the altitudinal variation in circadian physiology of *Drosophila* are rather few as compared to that of latitudinal variation. Altitude dramatically altered the basic parameters of eclosion rhythm of the Japanese strains (Pittendrigh and Takamura, 1989), *D. suboobscura* (Lankinen, 1993) in the locomotor and eclosion rhythms of *D. ananassae* from Srilanka. Molecular polymorphism in *period* gene of different latitudinal strains of *D. melanogaster*, *D. littoralis,* and *D. simulans* collected from Europe, Africa, and Australia was ascribed to natural selection (Costa *et al*., 1992; Sawyer *et al*., 1997; Weeks *et al*., 2006; Vanlhriatpuia *et al*., 2007). Consistent with the idea that there may be a link between circadian genes and latitudes, there is a significant latitude cline in the distribution of the Th/Gly polymorphisms in Europe and North Africa (Costa *et al*., 1992; Sawyer *et al*., 1997). Genetic components for the altitudinal differences in oviposition rhythm were analyzed by carrying out crosses within and between populations of *D. buzzatii* that originated from different altitudes in Argentina (Dahlgaard *et al*., 2002). Recently several aspects of circadian physiology of *Drosophila* have been investigated, such as the latitudinal variation in the circadian rhythm of eclosion (Joshi and Gore, 1999), adult locomotor activity (Joshi, 1999; Palaksha *et al.,* 2011), and altitudinal variation in Himalayan strains of *D. helvetica* (Vanlalhriatpuia *et al*., 2007). In view of this, the present study has been undertaken to investigate the circadian rhythm of locomotor behavior of *D. agumbensis* and *D. rajasekari.*

Materials and Methods

The species used in this experiment were *D. agumbensis* and *D. rajasekari,* collected from Sakleshpur, Karnataka, India at 910m altitude during the month of April 2011. Isofemale lines were maintained, and from the first generation flies adult locomotor activity was assayed. Activity was recorded under 12L/12D conditions.

Locomotor activity was analyzed by using *Drosophila* activity monitor (Trikinetics IV). The activity of an individual fly was monitored by placing the fly into a glass tube (6.5 cm length × 1.5 cm diameter). The tube was inserted into a ring detector. One end of the tube was provided with culture media (2 g agar and 2 g sucrose and yeast), and the other end was plugged with cotton. The tube was placed in the path of an infrared beam. The culture medium was replenished once each two days. Every interruption of the infrafred beam by fly movement triggers all or none electric signal that was counted and registered. Every time the fly moves is referred as bout. It was summed at 10 minute intervals known as bins (10 min = 1 bin). This procedure was continued without interruption for ten days or 240 hrs.

Statistical analysis: The data were subjected to Student 't' test, and period was calculated using Refinetti's software, version 3.5.

Result

Circadian rhythms of locomotor behavior of *D. agumbensis* and *D. rajasekari* were depicted in Figures 1 and 2. Locomotor activity behavior of both species showed unimodal activity. Activity continued throughout the day (photophase) and exhibit rhythmic activity pattern. The peak of

activity was observed at 14 h in *D. agumbensis* and 10 h in *D. rajasekari* (Figures 1 and 2). The period of activity was 23.8 h and 23.7 h, respectively, in *D. agumbensis* and *D. rajasekari*. The range of activity was 126-851 in *D. agumbensis* and 114-964 in *D. rajasekari*. The Student 't' test revealed that activity between two species was significant ($t = 13.3$, $P < 0.05$).

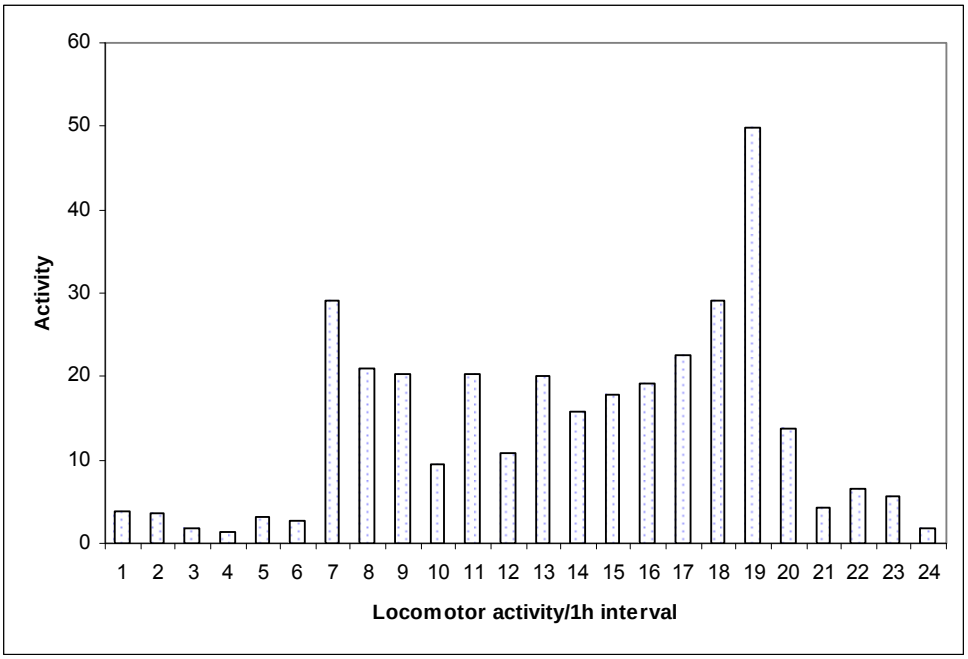

Figure 1. Average locomotor activity of *D. agumbensis* from Sakleshpur at 910m altitude.

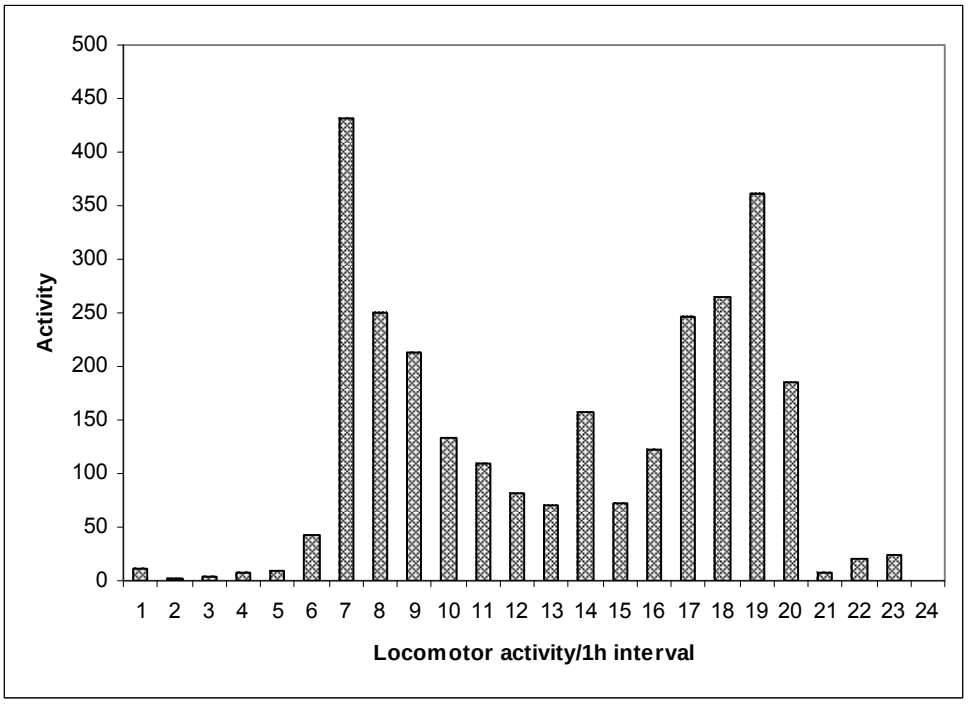

Figure 2. Average locomotor activity of *D. rajasekari* from Sakleshpur at 910m altitude.

Discussion

Circadian rhythm helps the organism to adapt to daily light/dark cycles and environmental cues such as light, moisture, temperature and can entrain the clock cells that affect rhythmic behavior

(Klarsfeld and Rouyer, 1998; Zheng and Sehgal, 2008; Cong *et al*., 2010). The wild type *D. melanogaster* flies are extensively studied and exhibit regular locomotor rhythm under 12L/12D light cycles. It had two peaks, one at dawn and the other at dusk (Wheeler *et al*., 1993; Forster, 2001; Sharma, 2003). Organisms that live in an environment in which they experience alternating times of constant conditions and diurnally changing physical and social conditions are useful as they enable researchers to examine the plasticity of circadian rhythms in response to the change in the environment (Sharma, 2003). Flies from Sakleshpur populations, such as *D. agumbensis* and *D. rajasekari*, showed unimodal activity. Activity was observed throughout the day in both the species. Simunovic and Jaenike (2006) have reported locomotor behavior of a few species of *Drosophila* from higher latitude active throughout the day and species from lower latitude activity restricted to a particular time of the day. Locomotor behavior of a high altitude Himalayan strain of *D. helvetica* showed unimodal activity pattern with a single delayed peak that commenced in the forenoon and continued till evening, while the low altitude Himalayan strain of *D. helvetica* had bimodal activity pattern with an early morning peak that was separated from the evening peak (Vanlalhriatpuia *et al*., 2007). Altitudinal strains of *D. bipectinata* exhibited bimodal activity with morning and evening peaks, and *D. malerkotliana* showed unimodal activity with evening peak (Palaksha *et al.,* 2011).

In the present study both the species of *Drosophila* showed activity throughout the day. This might be due to the flies experience of low desiccation stress in high altitude regions. They inhabit in the higher altitude where temperature is much less and relative humidity is very much higher than the lower altitude. Thus, in a physically permissive environment, selection may favor those individuals that continue their activities, mating, and egg laying throughout the day.

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The effect of number of founder females on inversion polymorphisms in laboratory populations of *Drosophila melanogaster.*

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Natural populations of *Drosophila melanogaster* are known to be chromosomally polymorphic, the paracentric inversions being the most common. When these populations were transferred to laboratory cages with several thousand flies, all kinds of polymorphic inversions found at high frequencies even more than 50% of average inversion frequencies in nature, were rapidly eliminated in less than three years (Inoue and Watanabe, 1992). The first several generations were the most critical period for the prediction of inversion extinction in the cage. This "cage effect" was observed irrespective of the geographical origin of the population or the initial frequency of each inversion. The decrease and elimination of inversions in the cage was not overcome by changing conditions such as medium, temperature, or the number (40-600) of isofemale lines initially introduced (Inoue, l979). On the other hand, isofemale lines derived from the same geographical origins as the cage populations kept inversion polymorphisms at significantly high levels, though some of the isofemale lines lost inversions. Moreover, inversion polymorphisms were maintained in individual vials of isofemale lines in most cases. Isofemale lines were established by individually transferring the females inseminated in nature to the glass vial cultures (3 cm diameter × 10 cm height), and they were allowed to lay eggs. These lines were maintained by mass transfer of about 30-60 flies every generation. The cage populations established with one or two isofemale lines also maintained the inversion polymorphisms as high as vial cultures (Inoue and Watanabe, 1992). So, the maintenance or elimination of inversions in the laboratory populations may depend on the number of isofemale lines used. In the present study, we examined inversion polymorphisms in the vial culture established with 2-60 isofemale lines, and in the cage populations established with 2-20 isofemale lines. These lines were sampled from Ishigaki-jima of 1976, one of the Japanese South-west Islands, and maintained for 14 years in the laboratory. The laboratory populations started by mixing flies of equal numbers of females and males from each isofemale lines. The laboratory cages were bucket type (22 cm diameter × 20 cm height) with 12 food cups of the standard medium. Three old food cups were replaced with new ones every four days. The number of generations on the cage population was calculated according to Crow and Chung (1967); a period of 15 days at 25ºC was estimated to represent one generation. For cytological analysis, the salivary gland chromosomes from third instar larvae were stained with lactic-acetic orcein. One larva was considered as analysis of two genomes from each sampled females in the experimental populations. All experiments were carried out at 25ºC. The designations of polymorphic inversions followed the nomenclature of Lindley and Grell (l968). The frequency of each inversion was recorded as frequency per chromosome arm, and the average frequency of polymorphic inversions per major autosome arm was also calculated for each sampling.

In the first preliminary experiment, the inversion polymorphisms in each of 35 isofemale vial cultures were examined individually. A total of 59 chromosome arms among 140 arms (35 vial cultures × 4 chromosome arms) were confirmed to be heterozygous for the inversions and standard chromosomes in the beginning of the experiments, and only one arm among them was found to lose the inversion after 24 generations of laboratory maintenance. Almost all arms were found to

maintain inversions in the balanced condition with the standard chromosomes. These results were the same as those of Inoue and Watanabe (1992). In the second preliminary experiment, the four glass bottle cultures (5 cm diameter × 14 cm height) established with one hundred isofemale lines showed significantly lower frequencies than the initial frequencies after 45 generations of laboratory maintenance. The number of flies or space of laboratory maintenance may not suppress the inversion decrease (data not shown).

Table 1. Number of homozygous chromosome arms in vial cultures initiated by mixing 2-60 isofemale lines and maintained for 24 generations in the laboratory; the 16 replication cultures were made for each class*.

Classes for the used line number	2L	2R	3L	3R	Total
2	2	0	0	0	2
5	9	3	2	0	14
10	14	5	3	0	22
20	11	12	5	0	28
40	8	15	6	0	29
60	9	13	10	1	33

*Final homozygous arms were all found to be with the standard gene arrangement.

Table 1 shows inversion polymorphisms after 24 generations of the vial cultures established with 2, 5, 10, 20, 40, and 60 isofemale lines. The left and right arms of the second and third chromosomes were abbreviated as 2L, 2R, 3L, and 3R, respectively. Sixteen replications of vial cultures were made in each class. In the beginning of the experiment, all vial cultures were confirmed to be heterozygous conditions with the inversion and standard arrangement for all four autosome arms. Among all observed 384 chromosome arms (6 classes × 16 cultures × 4 arms), a total of 128 arms were found to be homozygous after 24 generations. The homozygous conditions were decided by the analyses of 20 larvae (40 genomes) per vial culture. The homozygous cases were found to be all with the standard arrangement. The class of two lines vial culture showed two homozygous chromosome arms, and the class of 60 lines vial culture showed 33 homozygous chromosome arms. The number of homozygous chromosome arms increased gradually in relation to the number of the initially used isofemale lines. When the vial cultures were established with a few number of isofemale lines, they seemed to suppress the inversion decrease.

Table 2. Inversion frequencies in cage populations established with 2, 5, 10 and 20 isofemale lines. All cages were maintained for 48 generations in the laboratory. See text for explanation.

Cage Class	Generation	2Lt	2RNS	3LP	3RP	Average
2 lines cage	Initial	0.335	0.450	0.470	0.495	0.438
	Cage A	0.150	0.650	0.475	0.510	0.446
	Cage B	0.075	0.545	0.365	0.405	0.348
5 lines cage	Initial	0.155	0.290	0.290	0.525	0.315
	Cage A	0.025	0.270	0.085	0.320	0.175
	Cage B	0.015	0.250	0.015	0.280	0.140
10 lines cage	Initial	0.255	0.265	0.140	0.425	0.271
	Cage A	0.010	0.180	0.150	0.275	0.154
	Cage B	0.010	0.145	0.065	0.320	0.135
20 lines cage	Initial	0.350	0.230	0.105	0.575	0.315
	Cage A	0.085	0.165	0.010	0.395	0.164
	Cage B	0.070	0.155	0	0.505	0.184

The cage populations established by mixing many isofemale lines lost the inversions, while the cages established by one or two isofemale lines basically maintained inversions throughout generations. We examined frequencies of inversions from the cages established with 2, 5, 10, and 20 isofemale lines and maintained for 48 generations. Each inversion frequency was obtained by the 200 genomes analysis and was calculated per chromosome arm. The results of cage experiments are shown in Table 2. Four inversions, In(2L)t, (In2R)NS, In(3L)P, and In(3R)P, are categorized as Common Cosmopolitans, each of which exists in the right and left arms of two major autosomes, respectively. They are usually maintained in most natural populations all over the world with high frequencies. The inversions of other categories (Rare Cosmopolitans, Quasi Cosmopolitans, Endemics, and Uniques) were generally in much lower frequencies than Common Cosmopolitans (Inoue and Igarashi, 1994). The number of inversions in each population sample was thus represented by frequencies of the four Common Cosmopolitans and their average frequency. Two replication cages (A and B) were made for each class. In two lines, cages of the initial average frequency of 0.438, In(2L)t decreased and instead In(2R)NS increased, and final average frequencies of A and B were 0.446 and 0.348, respectively. Similar results were reported by Inoue and Watanabe (1992), where one of two cages established with two isofemale lines maintained the initial inversion frequency on the average and the other cage showed lower average frequency than the initial. In both cage experiments established with two isofemale lines, some inversions increased or decreased compared with each initial frequency. All four inversions were decreased in both A and B, especially In(2L)t and In(3L)P in the five lines cages of the initial average frequency of 0.315. The final average frequencies of In(2L)t and In(3L)P were 0.175 and 0.140, respectively. In the 10 lines cages of the initial average frequency of 0.271, all cases showed the inversion decreases other than In(3L)P of cage A. The final average frequencies were 0.154 and 0.134, respectively. In the 20 lines cages of the initial average frequency of 0.315, all cases showed the inversion decreases other than In(3R)P of cage B. The final average frequencies of A and B of 20 lines cages were 0.164 and 0.184, respectively. All six cages other than the two lines cages did not suppress the inversion decreases, showing similar final average frequencies ranging between 0.135 and 0.184.

In the present study, the vial cultures established with a few number of isofemale lines kept more heterozygous condition than those established with many number of isofemale lines. In addition, laboratory cage populations started with over five isofemale lines decreased inversion polymorphisms after 24 generations. These results confirmed the importance of initial number of the founder females on inversion polymorphisms in laboratory populations. It is interesting that observation of the cellar habitat population showed lower frequencies of the third chromosome inversions than those of vineyard population outside (Gonzalez and Mensua, 1987). The behavior of inversions in the cellar may be analogous to that of the laboratory populations with the mixture of many isofemale lines. However, a mechanism for high inversion polymorphisms in cage populations established with one or two isofemale lines has not been clear by the studies of inversions only. If one type of inversion, such as In(2L)t, has several different genetic constitutions in a population, one of the mechanisms we supposed is that vial cultures and cage populations established with a few number of isofemale lines can keep more heterozygous condition by over dominance effect without any breakdown of genetic constitution of inversions. On the other hand, laboratory populations established with a large number of isofemale lines will decrease inversion polymorphisms by low fitness of heterogeneous genetic constitution after recombination between the same inversions type with different genetic constitution. The above hypothesis will be confirmed by the combinational studies of inversions and their DNA sequences on effects of initial number of the founder females of inversion polymorphisms in laboratory populations.

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Studies on *Drosophila* biodiversity of Harangi Forest: Coorg District, Karnataka.

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Drosophila has been used as a model organism for research for almost a century. It has richly contributed to our understanding of the pattern of eco-distribution, biodiversity (Guru Prasad *et al.,* 2010), and altitudinal variation (Guru Prasad and Hegde, 2006). The studies have also been made on the population genetics of different species of this genus. The *Drosophilid* family is composed by 65 genera and more than 3500 described species that occur in a number of ecosystems all over the world (Bachli, 1998). However, various studies have been carried out in the laboratory and field by many workers. Though early studies on *Drosophila* in India were mainly concerned with taxonomy, 1970 onwards studies in other fields have also been initiated such as biodiversity. The taxonomical and population genetical studies have progressed little due to lack of interest of people in it. Although many workers feel that taxonomical work shall not be neglected, people show little interest because of the hardship during work and lack of opportunity in the field. To fill up this gap at least partially, we took this work for the study *Drosophila* population and altitudinal variation of *Drosophila* and their species diversity in give Harangi hill. Three months survey was conducted to analyze the altitudinal variation in diversity of *Drosophila* in Harangi hill of Kushalnagar, Coorg district Karnataka state, India.

To study the altitudinal variation of *Drosophila* and their community, the collection was done in the Harangi hill during June-Aug 2009. The Harangi hill is a famous tourist spot with altitude 900 meters, 8 km from the Kushalnagar City, Coorg, Karnataka, India. The altitude of the hill from the foot (base) is 500 meters, the temperature ranges from 16°C to 35°C, and relative humidity varies from 19% to 75%. The collections of flies were made during monsoon season (June to August once in 15 days of the months). For this method flies were collected by using sweeping and bottle trapping methods from the altitudes 500m, 600m, 700m, and 800m (500m base of the hill) such as lower altitude of Harangi hill. 1) Bottle trapping method, 2) Net sweeping method. In bottle trapping method regular banana baits in quarter pint 250 ml milk bottles sprayed with yeast were tied to the twigs of trees at two and half feet above the ground in cool shaded areas covered by scrubs. Next day flies were attracted by the bait and thus the bottles were collected during early morning by plugging with cotton to the mouth of the bottles.

In net sweeping methods rotting fruits are spread usually beneath shaded areas of the bushes of plantation various fruits such as *Musca paradisca* (banana), *Ananas comuses* (pineapple), *Vitas vanifera* (grape), *Artcarpus hetrophylles* (jack fruit), *Pyrus malus* (Apple), *Carica papaya* (papaya), *Arthras* (guava), and *Citrous auranthium* (lime) are mixed and used for spreading. After one day of spreading, the flies are swept using fine net. This is done in all the altitudes, 500m, 600m, and 700m, and 800m height of the hill. The flies are transferred to the bottles containing wheat cream–agar medium and then brought to the laboratory isolated, sexed, and identified according to the Texas Publication 1975 records. Then they were examined under microscopy.

Analysis of species diversity of flies collected in monsoon was assessed by Berger-Parker (1/d) indices (Mateus *et al.,* 2006). Berger-Parker index (1/d) which shows the relative abundance was calculated using the formula,

$$1/d = \frac{N}{N_{Max}}$$

Where N = Number of individuals of all species; N_{Max} = Number of individuals in the most common species.

Table 1. Showing the list of species of *Drosophila* and their numbers collected at different altitudes of Harangi hill during June-Aug 2009 (m-Meters).

Sl.No	Species	500m	600m	700m	800m	Total
	Subgenus Sophophora					
1	*D. anomelani*	24	25	30	39	118
2	*D. coonorensis*	00	00	15	15	30
4	*D. jambulina*	150	209	200	200	759
5	*D. kikkawai*	11	05	03	11	30
6	*D. malerkotliana*	482	686	880	1003	3051
8	*D. punjabiensis*	32	27	33	40	132
9	*D. rajasekari*	780	740	237	236	1993
10	*D. suzukii*	16	10	09	20	55
11	*D. takahashii*	05	33	11	31	80
12	*D. bipectinata*	260	180	150	142	732
	Total	1760	1915	1568	1737	6980
	Subgenus Drosophila					
1	*D. nasuta*	901	913	1123	2017	4654
2	*D. neonasuta*	834	581	819	950	1850
3	*D. repleta*	95	96	61	74	326
4	*D. immigrans*	00	00	96	154	250
	Total	1830	1590	2099	3195	7080
	Subgenus Dorsilopha					
1	*D. buskii*	145	136	111	127	519
	Total	145	136	111	127	519
	Subgenus Scaptodrosophila					
1	*D. brindavani*	470	570	564	783	2387
2	*D. nigra*	200	204	289	309	1002
3	*D. mundagensis*	70	80	79	89	318
	Total	740	854	932	1181	3707
	Total	4475	4495	4710	6240	19920
1	Berger-Parker index	1.109	1.051	1.036	1.023	

The results of our *Drosophila* survey are shown in Table 1. This table shows as altitude increases there was an increase in number of *Drosophila* species. Totally 19,920 flies were encountered during the collection, which belong to four subgenera, namely *Sophophora, Drosophila, Dorsilopha, Scaptodrosophila* with twenty species. The 500m altitude comprises 4475, 600m (4495); 700m (4710); and 800m (6240) of *Drosophila* in numbers. The *Sophophora* comprises more

numbers of flies and species in the caught compared to other genera. The subgenus *Dorsilopha* is least in the number and as well as species. *D. nasuta* and *D. malerkotilana* species are the common species found in the hill and all the altitudes. So this is regarded as the common and abundant species in the hill. Another most important finding is all species were not found in all altitudes, and *D. nasuta, D. neonasuta, D. malerkotliana, D. rajasekari, D. jambulina,* and *D. bipectinata* were common species found in all altitudes. There are some species such as *D. coonorensis, D. suzuki, D. immigrans* found only in one or two altitudes. The community and biodiversity was big in higher altitude compared to lower altitudes. These results are entirely reverse to our own studies in Chamundi hill during 2007 (Guru Prasad and Hegde, 2006). These results were due to micro and macro climatic conditions, which are different from the location to other locations. The highest number and species of flies were found in 800m altitude with numbers. Further our intention is not only to study the taxonomy of *Drosophila,* but also the biodiversity using the index called Berger-Parker. The result of the Berger-Parker index according to the altitude was depicted in Table 1, where it shows the lower number in higher altitude (800m).

According to Ludwig and Reynold (1988), the greater the value of 1/d, the lower is the diversity. Application of these indices to the collection data of different altitudes of hill demonstrates that higher altitude of 800 m with lower value of 1/d has higher biodiversity than other altitudes (Table 1). This may be more easily understood if we observe the quantity and dominance of each species in each altitude, since the index combines two functions: number of species and uniformity, *i.e*., the number of individuals present in each species (Ludwig and Reynold, 1988; Torres and Ravazzi, 2006). Thus, from the present eco-distributional and population analysis of *Drosophila* in hill, it is clear that the distributional pattern of a species or related group of species is uneven in space and time. *D. malerkotliana* and *D. nasuta* emerged as champion species, as they are registered in all altitudes. *D. immigrans* is completely absent in the lower altitudes. In a nutshell, it can be said that the *Drosophila* community of hill is highly diverse and depends on several environmental factors like flora, which provides the habitat for flies in addition to the genetic structure of the species present in it.

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Analysis of inversion polymorphism and new inversion recorded in *Drosophila polymorpha* in the South of Florianopolis, Santa Catarina, Brazil.

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Drosophila polymorpha, described by Dobzhansky and Pavan (1943), belongs to the *cardini* group within the genus *Drosophila*. Species of this group inhabit different areas of Neotropical America. In Brazil, *D. polymorpha* has reports of a wider distribution in the Southeast (Da Cunha *et al.*, 1953), while in the south of the island of Santa Catarina, taxonomic studies of *Drosophila* communities consider this to be one of the species most often found (De Toni and Hoffmann, 1994).

Studies involving chromosomal inversions in the group *cardini* point to *D. polymorpha* as showing the highest number of polymorphisms of the group, both in relation to pigmentation and chromosomal inversions (Da Cunha *et al*., 1953; Rohde and Valente, 1996a; De Toni *et al.* 2001a). These inversion polymorphisms are one of the most studied systems in population genetics. Paracentric inversions are a common form of this polymorphism, restricted to chromosomal arms. This does not include centromeric regions, which are commonly observed in *Drosophila* (Ananina *et al.*, 2004). To analyze these inversions, the banding pattern present in polytene chromosomes, illustrated in a reference photomaps, allows one to identify the breakpoints (De Toni, 2001).

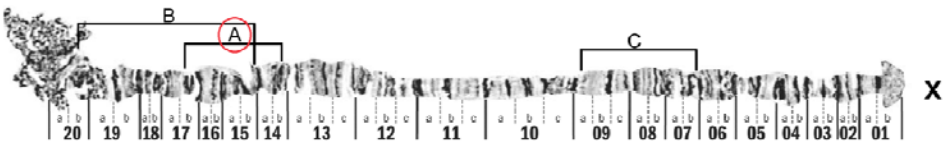

Figure 1. Indication in red represents the break points (14 b distal, 17 b proximal) XA, present in *D. polymorpha* isoline *1*.

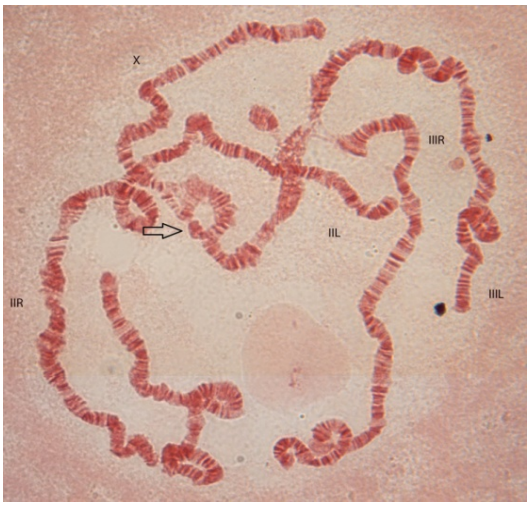

Figure 2. *Polytene chromosomes* hybridized. The arrow points to the paracentric inversion XA in heterozygous state found in the sample of *D. polymorpha 1*.

Heed and Krishnamurthy (1959) developed genetic studies on the *cardini* group of island populations of the West Indies. Heed and Russell (1971) also contributed to studies of chromosomal inversion polymorphisms of the group, observing a high proportion of fixed inversions in the chromosomes of *D. polymorpha*. Further study of chromosomal polymorphism in this species was made by De Toni *et al.* (2001) in mainland and island communities in southern Brazil, in which seven different inversions were found in the communities of Santa Catarina, (six of them found and described for the first time).

Continuing with this study, we collected samples from populations of *Drosophila* in the south of the island, in an island region called Caiera da Barra Sul (S 27° 48'S; The 48° 33'), of Florianópolis, Santa Catarina, an area with formation of secondary Atlantic Forest in advanced stages of regeneration.

Samples of populations of *Drosophila* were obtained as adults flying around rooting fruit and banana baits left for at least three days in the area of collection.

The taxonomic identification of species, maintenance of collected samples, production of isofemale lines, cytological preparation, and cytogenetic analysis were all performed at the *Drosophila* Laboratory at the Federal University of Santa Catarina, Brazil.

Even though few strains have been analyzed so far, there has been a fairly high number of inversions. Out of the six strains analyzed, two inversions occurred, one of which was undescribed before now.

According to the photomap of the polytene chromosomes of this species, proposed by Rohde and Valente (1996) modified by Cordeiro and De Toni (unpublished data), the inversion detected in isoline named as *D. polymorpha 1,* corresponds to an inversion X (Figure 1), since it has the same breakpoints described by De Toni *et al.* (2001).

The strain called *D. polymorpha 2* shows a new paracentric inversion in the arm of chromosome IIR, now named IIRE, with break points set in the sessions 50a distal and 48c proximal (Figure 3).

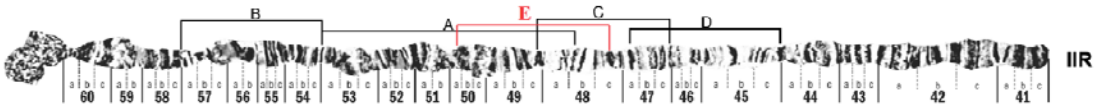

Figure 3. Indication in red represents a new inversion, IIRE found in *D. polymorpha* isoline *2.*

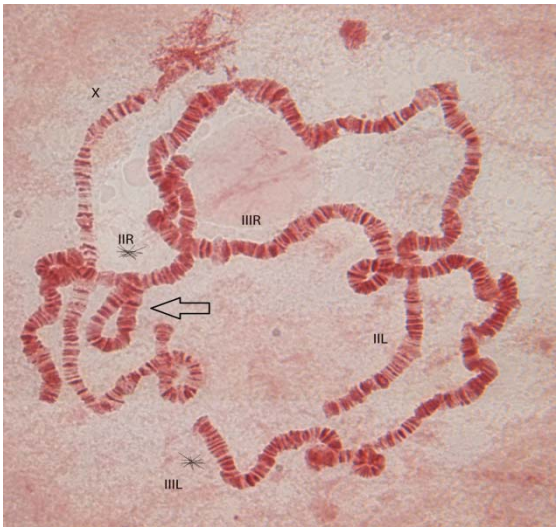

Figure 4. Polytene chromosomes hybridized. The arrow points to the new paracentric inversion IIRE in heterozygous state found in the sample of *D. polymorpha* isoline *2.*

The study of chromosomal inversions through the polytene chromosomes in *Drosophila* allowed us to assess evolutionary aspects of the genus, for example, the numerous chromosomal rearrangements as inversions of segments, which were fixed in each species (Wasserman, 1986).

As can be seen in the references (Dobzhansky, 1943; Tiniakov and Dubinin, 1945; Dobzhansky and Levene, 1948, 1951), the nature of chromosomal polymorphism in flies, especially in *Drosophila*, is clearly adaptive and balanced.

Thus, according to Da Cunha *et al.* (1950, 1959) and Da Cunha and Dobzhansky (1954), the amount of polymorphism present in a species is related to the variety of ecological niches they occupy by their species.

The hypothesis presented in this paper largely meets the data available in literature, which possibly explains the polymorphisms of this species, even with only such a low sampling in the area.

The results presented in this paper will contribute to future analysis in greater detail of the evolution of the *D. cardini* group.

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Radio-protective effect of piperine on reproductive organs of *Drosophila* model by induction of electron beam radiation.

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Introduction

Piperine is a major pungent substance and active component of black pepper (*Piper nigrum* Linn.) and long pepper (*Piper longum* Linn.). Both plants are used worldwide as household spices and condiments. They are also used as important ingredients in folklore medicine in many Asian countries. Piperine significantly enhances the absorption rate of nutrients such as Beta-Carotene, Vitamin B6, and Selenium. Selenium and Vitamin B6 levels increased from 30% to 40% percent, while Beta-Carotene increased by sixty (60%) percent. Study on piperine influence on chromosomes in rat bone marrow cells was analyzed in which male Wistar rats were orally administered piperine, then treated with cyclophosphamide by intraperitoneal injection. The results of chromosomal analysis demonstrated that piperine, at a dose of 100 mg/kg body weight, gave a statistically significant reduction in cyclophosphamide-induced chromosomal aberrations, thus indicating that piperine can have antimutagenic potential. Aqueous ethanolic extracts obtained from *Piper* species showed potent inhibitory activity for testosterone 5α-reductase. Piperine also showed *in vivo* anti-androgenic activity. It is suggested that piperine inhibits lipid droplet accumulation in mouse macrophages and especially inhibited cholesteryl ester (CE) synthesis. Studies on curcumin administered rats showed markedly elevated activity of the antioxidant enzymes malondialdehyde (MDA), catalase, and glutathione S-transferase (GST) in the cerebrum and cerebellum of epileptic rats due to PTZ-induced oxidative stress. Piperine (20 mg/kg orally) administered along with curcumin enhanced the bioavailability of the latter up to 20-fold more. Administration of piperine inhibited lipopolysaccharide (LPS), induced endotoxin shock, leukocyte accumulation and the production of tumor necrosis factor-alpha. Black pepper and its constituents like hot pepper, exhibit anti-inflammatory, antioxidant, and anticancer activities which are showed by using proinflammatory transcription factor NF-kappaB, COX -1 and -2 enzymes, human tumor cell proliferation, and lipid peroxidation (LPO) studies. Piperine, the compound of black pepper, can cause a significant decrease of blood pressure in normotensive rats possibly via calcium channel blockade, a pathway that is known to be effective in prevention of L-NAME (N (G)-nitro-L-arginine) methyl ester induced hypertension.

Black pepper, *Piper nigrum* L. (Piperaceae), has insecticidal properties and could potentially be utilized as an alternative to synthetic insecticides. Treatment of *D. melanogaster* with *P. nigrum*

extract led to a greater than 2-fold upregulation of transcription of the cytochrome P450 phase I metabolism genes *Cyp 6a8, Cyp 9b2*, and *Cyp 12d1* as well as the glutathione-S-transferase phase II metabolism gene *Gst-S1*. Therefore, any agent that can protect against such alterations can provide protection against radiation damage. In the present investigation, the flies were exposed to electron beam irradiation at 1.5 Gy. It was found that there was severe decrease in the size of gonads of unfed, irradiated *Drosophila* flies.

Materials and Methods

Drosophila melanogaster

D. melanogaster (Oregon K) adult flies (8-10 days old) were obtained from *Drosophila* stock centre, Department of Studies in Zoology, University of Mysore, Manasagangotri, Mysore, Karnataka, India.

Preparation of compounds for feeding the flies

Piperine was dissolved in 0.5% dimethyl sulfoxide (DMSO) was used as control. The concentrations of compound used were 100, 150, and 200 µg/ml. The test compound was introduced into the medium at semisolid state and mixed well and allowed to solidify. 50 adult flies were introduced into the vials containing media.

Evaluation of compounds

Studies were carried out to find out whether the compounds are mutagenic or cause any abnormality in the experimental batches. In this set of experiments, the male and female flies (test) were fed separately on a medium containing piperine (100, 150, and 200 µg/ml that are below LD_{50} concentration), while control flies (Batch I) were fed with regular wheat cream agar medium for 7 days. Lethality due to compounds was monitored by counting dead flies every 24h up to 7 days, and data were expressed in terms of percentage mortality.

Irradiation

Using Microtron Accelerator at Mangalore University, 2-3 days old flies taken in 2 mm thick polypropylene tubes of 65×25 mm were exposed to 1.5 Gy electron beam radiation. Just before irradiation, the flies were introduced into fresh vials containing standard wheat cream agar medium.

Anatomical investigations

Flies were mildly anesthetized using diethyl ether, and their gonads dissected out in saline solution by fine needles.

Results and Discussion

In the present study, our primary focus was to examine the radioprotective nature of piperin using electron beam as radiation source in *Drosophila*, which is a widely employed eukaryotic model organism for genetic studies. This model organism offers several advantages. The first one is that it allows rapid screening of potential therapeutic agents and physiochemical.

The results of our study showed decrease in the size of gonads in both sexes of both control and tested groups. But the hallmark point was that treated flies showed much less decrease in their gonad size compared to untreated ones.

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NBAD-hydrolase processing in brain and epidermis of *Drosophila melanogaster*.

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In recent years, research on N-β-alanyl-derivative metabolism in insects has shed new light on its physiological relevance. While NBAD (N-β-alanyldopamine), the first conjugate studied in this metabolism, was originally described as the main sclerotization precursor of insect brown cuticles (Hopkins and Kramer, 1992), new roles have been proposed in neural tissue (Pérez *et al*., 2004, 2010, 2011; Schachter *et al*., 2007). Another studied N-β-alanyl-derivative is carcinine (N-β-alanylhistamine, NBAHA), which has been suggested as of physiological importance for the visual system (Borycz *et al.*, 2002; True *et al.*, 2005; Wagner *et al.,* 2007). Both NBAHA and NBAD have been proposed as shuttle/recycling agents of histamine (HA) and dopamine (DA), between glial and neuronal cells (Borycz *et al.*, 2002; True *et al*., 2005; Pérez *et al*., 2010).

NBAD and NBAHA are both synthesized by the same enzyme: NBAD-synthase, also known as Ebony protein in *Drosophila melanogaster*. This enzyme shows rather wide substrate specificity, since tyramine, octopamine, norepinephrine, tyrosine, and serotonin can also be conjugated to β-alanine (Pérez *et al*., 1997, 2002, 2004, 2010; Richardt *et al*., 2003; Schachter *et al*., 2007).

The hydrolysis of these conjugates is catalyzed by NBAD-hydrolase (also known as Tan), which is encoded in *D. melanogaster* by the gene *tan* (Wright, 1987; True *et al.*, 2005) and has recently been partially characterized in *C. capitata* and *D. melanogaster* (Badaracco *et al*., 2009; Aust *et al*., 2010; Pérez *et al*., 2011). As the synthase, it shows a wide substrate specificity, since it hydrolyses at least NBAD, NBAHA, and NBANE (Wright, 1987; True *et al*., 2005; Pérez *et al*., 2011). The study of NBAD-hydrolase has revealed a constitutive expression in neural tissue and epidermis throughout the *Drosophila* life cycle (True *et al*., 2005; Badaracco *et al.*, 2009; Pérez *et al*., 2011).

By expression in *E. coli*, Tan was described as a homo-dimeric protein with subunits of around 30 and 15 kDa apparent molecular weight (aMW). Apparently, these subunits arise from self-processing of a precursor polypeptide of around 45 kDa (Wagner *et al*., 2007; Aust *et al*., 2010). A Gly-Cys motif, at position 121, was crucial for this self-processing, and the Tan^1 mutant protein, with

an Arg for Pro mutation (at position 217), showed no processing in *E. coli*. We decided to study this enzyme and its expression further in the t^1 mutant.

To our knowledge, this is the first *Drosophila*-expressed study of this protein. Moreover, it is the first study suggesting different tissue-specific expression/processing of the Tan protein.

Materials and Methods

D. melanogaster were reared in commercial fly medium: Formula 4.24 Instant Drosophila Medium (Carolina Biological Supply). Both wild type Canton S (CS) and *tan*^{1} (t^1) mutant strains were from the Bloomington Stock Center. Flies of 1-2 weeks were anesthetized with ice, decapitated under binocular magnifying glass, and the bodies frozen in liquid N_2. Brains were dissected (n = 100 each experiment) by separating both the eyes and lamina and stripping away as many tracheas as possible. The resulting brains and "head carcasses + eyes" were immediately frozen in N2 and stored at -80ºC. All dissections were carried out in Petri-dishes on top of ice, at 4ºC and with pre-chilled buffer. We have recently shown that homogenates from beheaded bodies show similar levels of hydrolase enzymatic activity as carcasses (which had been stripped of internal organs), whereas the activity in internal organs was negligible (Pérez *et al*., 2011). Thus, we considered decapitated bodies as epidermis-tissue material (in contrast to the brain/neural tissue material). Protein extracts were prepared by homogenizing the tissues in 70 mM Tris/HCl pH 7.5 buffer, containing 10% Glycerol, 20 mM EDTA-Na_2 pH 8, 0.01 mM Pepstatin A, 1 mM PMSF and saturated with phenylthiourea. These homogenates were cleared by centrifuging (20000 × g, 15 min, at 4ºC) and the supernatants used for western blots. Protein concentration was from 1 mg/ml to 10 mg/ml (Bradford method). Protein extracts were boiled in Laemmli buffer, loaded (50 µg) in a 12.5% polyacrylamide gel (Laemmli, 1970) and separated in a minigel apparatus (BioRad). Protein transfer was done by electroblotting onto PVDF membrane (Termo Scientific) at 400 mA for 1 hour 30 minutes, using Towbin transfer buffer (192 mM Glycine, 25 mM Tris/HCl pH 8, 20% methanol). Western blots were performed as described in Wittkopp *et al.* (2002). The membranes were temporarily stained in 0.02% Ponceau S (to confirm even protein loading and running), washed until all the Ponceau was removed, blocked with 3% non fat milk in phosphate buffered saline, 0.15% Tween 20 (PBST) for 1h at room temperature. Membranes were then incubated with primary antibody (1:500 rat anti-Tan, in PBST 3% non-fat milk) overnight at 4°C and washed (15, 20, and 25 minutes) in PBST. Finally, membranes were incubated with goat anti-rat horseradish peroxidase (Jackson ImmunoResearch) secondary antibodies (1:1000) in PBST containing 3% non fat milk for 2 hours at room temperature, washed in PBST (10, 20, and 30 minutes), and developed with ECL (G&E, Healthcare). The affinity-purified Tan antiserum was a generous gift by Dr. B. Hovemann (Rhur University, Bochum), the description of which is found in Wagner *et al*. (2007) and a personal communication. Rat pre-immune serum was used as a control (not shown). ImageJ was used to measure Rfs by measuring the peak value of intensity for each band and to measure the total intensity for each band. Relative intensity distribution was then calculated by dividing the intensity from each line by the total intensity of all the peaks with aMW of 51 kDa or less (aMWs are calculated to the nearest 0.5 kDa).

Results and Discussion

Wild-type bodies showed three immunoreactive bands of aMW 43 kDa, 28.5 kDa, and 14.5 kDa species. The latter seem to correspond to the previously described 30 and 15 kDa processing subunits of *E. coli*-expressed Tan protein (Aust *et al*., 2010). The t^1 mutant carries a point mutation

that probably changes the spatial structure of the protein, which could explain its inability to hydrolyze NBAD and has been proposed to inhibit processing (Aust *et al*., 2010; Pérez *et al*., 2011). It shows a new, heavier, peptide of 51 kDa aMW, the 43 kDa band, and the 14.5 kDa aMW band and no trace of the 28.5 kDa subunit. This shows the first difference observed with the data from expression in *E. coli*: Both the novel 51 kDa peptide and the 14.5 kDa band were previously not described for the Tan[1] protein mutation. This is important to point out since it shows a specialized processing of the Tan protein. We have no further information about the 51 kDa peptide, which is almost 8 kDa heavier than the expected full-lengh peptide (43.7 kDa). We hypothesize that this could be a post-translational modification (*e.g*., Ubiquitination), possibly targeting the non-functional protein for degradation. More research is needed to clarify this hypothesis. With regards to the 14.5 kDa peptide, we have no current explanation apart from the possibility that it might be a degradation product or an experimental artifact.

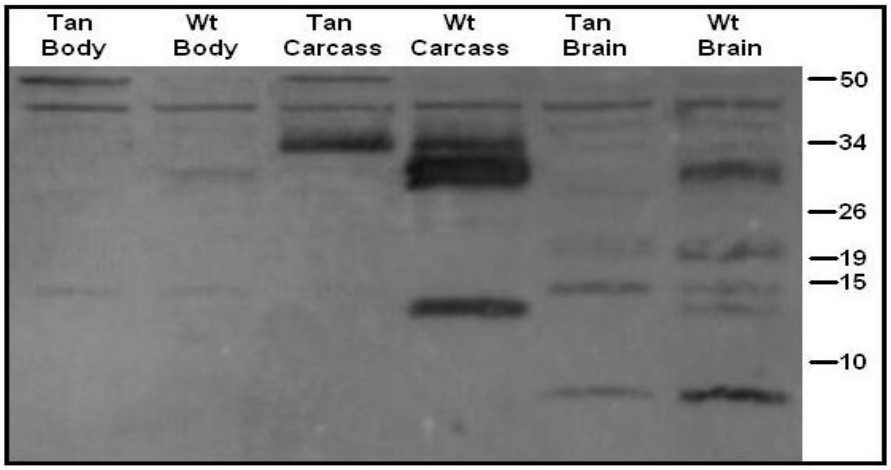

Figure 1. From left to right, the first two lanes show the homogenates from beheaded bodies, then head carcasses (lanes 3 and 4) and dissected brain ganglia (lanes 5 and 6) from mutant t^1 and wt, respectively. Numbers on the right side indicate molecular weight markers.

The analysis of the wt "head carcasses" shows a novel, more complex processing profile. In addition to the 43 and 28.5 kDa aMW bands (the latter of much higher intensity than in bodies), a new 34.5 kDa band was visualized, and the 28.5 kDa band showed a "double" appearance. This can be due to physiological processing but also to artifacts provoked by the unspecific proteases eventually activated during dissection, in spite of the protease-inhibitor cocktail present. Surprisingly, a band of 13 kDa aMW, which was clearly distinguished from the 14.5 aMW band, was recorded, suggesting further processing. This might also be interpreted as an artifact. The difference in processing between bodies and heads might eventually be sustained by observation of the head carcass t^1 profile (lane 3) since, in addition to the pre-precursor (51 kDa) and the precursor (43 kDa) proteins present in body extracts, one of the novel bands (34 kDa) present in wt heads was also detected, whereas the canonic 28.5 kDa product and the novel 13 kDa peptide were absent. Were these peptides the product of degradation by unspecific proteases, it is probable that they would be present in wt and mutants alike.

Finally, analysis of wt brains (lane 6) showed, in addition to the canonical 43, 28.5, and 14.5 KDa bands, a new band of aMW 18 kDa and a faint band of the 13 kDa species as well as a small (less than 10 kDa aMW) peptide. Again, these "new" peptides can be the result of physiological processing (or post-translational modifications) of the Tan peptide as well as unspecific degradation. The t^1 brain showed the 43 kDa and the 14.5 kDa bands as well as the unusual (albeit very faint) 18 kDa peptide and maintains the mutant characteristic of the absence of a 28 kDa peptide as well as the 13 kDa band present in the wt head. Noteworthy is the absence of the 51 kDa band observed in other tissues and the strong presence of the 14.5 kDa peptide.

In order to visualize the relative amount of peptide distributed among the different processing intermediates and post-translational modifications, we graphed the percentage of the total intensity for each peptide species. Both graphs show a shift in the distribution from heavier (*i.e.*, non-processed) proteins, in body tissue, towards lighter (processed) peptides in head and neural tissue. This, together with the almost uniform expression of the 43 kDa peptide in all tissues of both wt and mutant, suggests an important post-translational control of Tan protein processing and, thereby, activity.

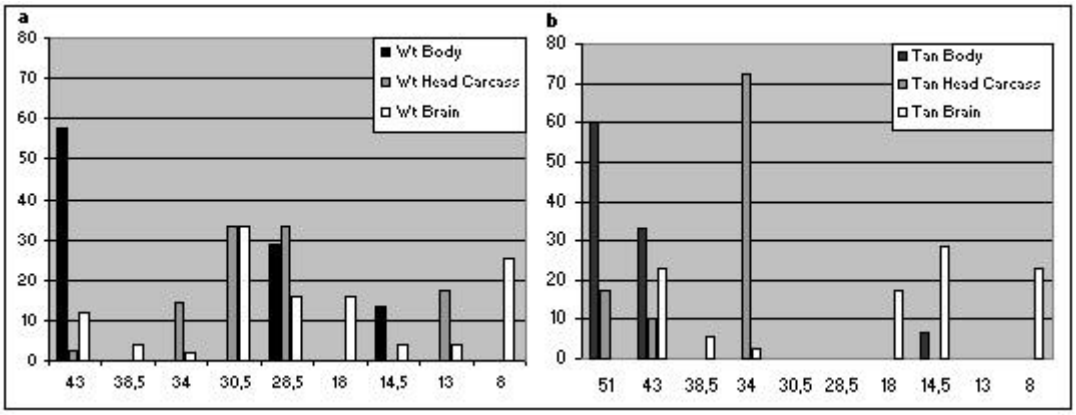

Figure 2. Relative intensity distribution of protein species for wt (a) and tan[1] (b).

The overall picture indicates that a previously overlooked processing of the Tan protein seems to occur, mainly demonstrated by the wide variety of novel peptide species present in head carcasses and brain tissue. In addition, the Tan[1] protein, which was previously suggested to be unable to produce the 28.5 (30) and 14.5 (15) kDa subunits, seems able to be at least partially processed to the 14.5 subunit. The complete absence of the other subunit might be the result of rapid degradation, since, theoretically, the production of the 14.5 kDa "half" should be accompanied by the complementary 28.5 kDa peptide. The anomalous "extra" weight of the Tan[1] protein is also a mystery. Ubiquitination as a consequence of its inactivating mutation seems plausible but does not explain the absence of this protein in brain tissue.

Further studies will be required to pinpoint exactly which of the peptides reported here are degradation artifacts, which are part of a *bona fide* physiological processing of the Tan protein, and which are post translational modifications. Apart from the proposed ubiquitination, the unusually high amount of phosphorilable residues (67, more than 17%) may provide an alternate explanation for the high variety of peptides, also adding to the hypothesis of a post-translational control of the protein's activity.

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Preliminary results of a forward genetic screen for X chromosomal dominant modifiers of *Drosophila melanogaster dfmr1*.

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Introduction

Fragile X syndrome is a neuro-developmental disease in humans. It is caused by a mutation in the gene *Fmr1,* which expands abnormally a CGG-repeat in its promoter region, thus leading to a subsequent hypermethylation and transcriptional inactivation of the gene (Sutcliffe *et al.*, 1992). *Fmr1* encodes the fragile X mental retardation protein (FMRP).

The most important clinical symptoms of the disease include mental retardation, sleep disturbances, autism, and impaired motor coordination. They reflect the key role of FMRP in the brain, where it is predominantly expressed (Devys *et al.*, 1993).

The lack of this protein is accompanied by defects in synaptic maturation and morphology, synaptic connectivity disturbances, and dysfunction throughout the nervous system (reviewed in Tessier and Broadie, 2009; Pfeiffer and Huber, 2009; Gatto and Broadie, 2011).

Research on animal models confirmed the main characteristics of fragile X syndrome – neuronal defects, synaptic abnormalities in synaptic development and function, circadian rhythms disturbances, impaired long term plasticity, abnormal mGluR signaling, and learning and memory deficits (reviewed in Bassell and Warren, 2008; Gatto and Broadie, 2009; Mercaldo *et al.*, 2009; Pfeiffer and Huber, 2009).

Drosophila studies have shown that dFMRP functions in axon growth, path finding, and activity dependent pruning and refinement of synaptic elaborations(Dockendorff *et al.*, 2002; Morales *et al.*, 2002; Michel *et al.*, 2004; Pan *et al.*, 2004; Tessier and Broadie, 2008).

FMRP is a selective mRNA-binding protein (with two KH domains and an the RGG box), which is a negative regulator of protein synthesis of its mRNA targets at synapses (Laggerbauer *et al.*, 2001; Li *et al.*, 2001; Sung *et al.*, 2003; Zalfa *et al.*, 2003; Qin *et al.*, 2005; Antar *et al.*, 2006; Price *et al.*, 2006). Accumulating evidence shows a role of FMRP in mRNA transport (Dictenberg *et al.*, 2008; Estes *et al.*, 2008) and in the regulation of mRNA stability (Zalfa *et al.*,2007; Gantois *et al.*, 2006; Miyashiro *et al.*, 2003; D'Hulst *et al.*, 2006; De Rubeis and Bagni, 2010).

Numerous candidate m-RNA targets, interacting with FMRP, were obtained by different approaches, though only a few of them have been validated *in vivo* (see the reviews: Zalfa and Bagni, 2004; Bassell and Warren, 2008; Callan and Zarnescu, 2011).

In order to exercise its multiple functions and to participate in different processes from the nucleus to the synapses, FMRP is thought to shuttle between nucleus and cytoplasm and to form different protein complexes. Models were created, suggesting that FMRP might take part in DNA

regulation, RNA transport, and translation by interacting with different subsets of mRNAs and proteins and by forming different RNP-complexes and protein-protein complexes (Zalfa and Bagni, 2004; Zarnescu *et al.*, 2005).

One way to identify functional partners of FMRP is to induce mutations in random genes and to screen for those of them, which interact with the gene, encoding the protein. In our study, we used the *Drosophila* Fragile X- model, which has a single FMRP ortholog - dFMRP, encoded by a single gene - *dfmr1* (Wan *et al.*, 2000). We designed a forward genetic screen to look for EMS-induced dominant enhancers and suppressors in a sensitized genetic background, where normal *dfmr1* function was disrupted by its GAL-4 over-expression in the wings. Such genetic interaction screens are a straightforward approach in identifying genes functioning in common biological pathways.

In our work we searched for X chromosomal interactors of *dfmr1*, which dominantly modified – enhanced or suppressed its mutant over-expression wing phenotype. We isolated 7 enhancers and 11 suppressors with recessive lethal effects on viability and determined their rough map locations on the X-chromosome.

Materials and Methods

Drosophila stocks and fly rearing

In our work we used the following *Drosophila* stocks:
w[1118]; P{w[+mc] = UAS – Fmr.Z}3, w[*];P{w[+m]=GAL4vg.M}2;TM2/TB6B,Tb[1];
w[67c23]P{w[+mC]=lacW}dlg1[GO456]/FM7c; C96-GAL4; *UAS-MamN; y ct v*; *y f* .
They all were obtained from the Bloomington Drosophila Stock Center at Indiana University. Additional information on the above stocks can be found at the website of the Bloomington Drosophila Stock Center (www.flybase.org).

We also used the stocks: *y* w^a N^{54l9}*//FM6* and rst^{CT} N^{54l9} *rb//C(1)DX y w f; Dp(1;2)w+51b//+*, which were kindly gifted to us by Prof. S. Artavanis-Tsakonas.

They all were maintained on corn meal/ yeast extract/raisins at the standard temperature of 25°C.

Genetic interaction experiments were performed at two temperatures: at 21°, when we looked for dominant enhancers of the over-expression wing phenotype of *dfmr1*, and at 27°, when we looked for dominant suppressors of the same phenotype (see below).

UAS/GAL4 system and over-expression phenotype of the gene dfmr1

In our study we used the UAS/GAL4 system, which enables expression of a particular gene, driven by a selected promoter in a tissue of choice (Brand and Perrimon, 1993).

We induced over-expression of *dfmr1* in the wing imaginal discs of *D. melanogaster* by means of the following cross:

$$\text{P: } \female \ \frac{w}{w}\frac{GAL4-vg.M}{GAL4-vg.M}\frac{TM2}{TM6B} \times \male \ \frac{w}{+}\frac{+}{UAS-fmr1}\frac{UAS-Fmr1}{}$$

$$\downarrow$$

$$F_1: \quad \frac{w}{w}\frac{GAL4-vg.M}{+}\frac{UAS-Fmr1}{TM2}$$

The F_1 progeny was maintained at a set of temperatures - 18º; 21º; 25º, and 27º, in order to determine the optimal *dfmr1* over-expression phenotype, designated by us as control wing phenotype or control phenotype.

EMS mutagenesis and screening for dominant modifiers

To induce mutations dominantly modifying the over-expression phenotype of *dfmr1*, we applied the mutagen EMS (ethyl-methane sulfonate) according to the method of Lewis and Bacher (1968) with modifications. The principle of the method is in directly exposing the *UAS*- bearing stock to mutagenesis and then in expressing the trangene in F1 generation (Guichard *et al.*, 2002; Penton *et al.*, 2002).

We used 0-48 hours old males of the genotype *w[1118]; P{w[+mc] = UAS – Fmr.Z}3*, which were starved for 12 hours. Cohorts of 20 flies were put into vials with a piece of kitchen paper, soaked with a 25 mM EMS in 1% sucrose and were exposed to the mutagen for 6 hours. After the recovery of the treated flies on fresh media for one hour, each male was crossed individually to several virgin females of the genotype *w[*]; P {w [+m] = GAL4-vg.M}2; TM2/TB6B, Tb[1]* and allowed to mate for 3 days.

The progeny of these crosses were kept either at 21º or at 27º and screened for wing phenotypes different from the *dfmr1*-over-expression phenotype in the control crosses, cultured at the same temperatures.

These new phenotypes were considered to result from genetic interactions between *dfmr1* and the EMS-induced dominant modifier mutations.

Selection of X chromosomal enhancers and suppressors with recessive lethality

In order to select for X chromosomal modifiers - suppressors and enhancers with recessive lethal effects, only female mutants from F_1, bearing the mutation either on the autosomes or on the X chromosome, were used for further analysis.

Each newly emerged female fly with a modified wing phenotype was crossed individually to several males from the stock *w[1118]* and their progeny was inspected for a male/female ratio and for the segregation of the mutant and the control wing phenotypes. During this isogenization step, only families where the progeny (F_2) showed a sex ratio 2:1 in favor of the females, instead of the regular 1:1, were taken for further studies. We assumed that they contained an X chromosomal mutation, which dominantly affected the *dfmr1*-over-expression phenotype and recessively influenced viability.

Such mutations were put over the balancer chromosome *FM7c*, and balancer stocks were generated [*w* l(Su or Eh)/FM7c; +/+; +/+*, where "*l*" is the lethal mutation].

The ability of the isolated mutations to modify specifically the over-expression wing phenotype of *dfmr1* was tested by using the stocks *C96-GAL4* and *UAS-MamN* (previously described in Helms *et al.*, 1999). When these stocks are crossed, their progeny exhibits a similar wing nicking phenotype, due to over-expression of the dominant negative MamN – a truncated version of Mam (Notch transcriptional co-activator mastermind) at the wing margins.

Meiotic mapping

Meiotic mapping of each X-linked modifier mutation with a recessive lethality was performed by crossing females from the balanced mutant stock to male flies *y ct v*. Some modifier stocks were crossed in another experiment to males from the stock *y f*.

In the F_1 generation females *w* l(Su or En)/y ct v* were mated to their brothers *FM7c//Y* from the same generation, and in F_2 only the male progeny was analyzed. Over 1200 male flies from F_2 generation were scored, and recombinant flies were counted for each mutation.

Recombination distances were calculated between the lethal mutation "*l*" and the recessive markers "*y*" and "*ct*" or "*y*" and "*f*", and these distances served to determine the region of its map position.

Drosophila wing processing

Wings were dissected from adult flies of interest and mounted in DePeX - Mounting medium for histology (SERVA). Pictures were taken using Stereomicroscope *CARL ZEISS JENA TECHNIVAL* with Nikon Coolpix L10 5MP Digital Camera with 3× Optical Zoom.

Results and Discussion

Screening for EMS-induced dominant modifiers of dfmr1

In our work we used the *UAS/GAL4* system for targeted gene expression (Brand and Perrimon, 1993). We crossed flies from the stocks *GAL4-vg.M* and *UAS – Fmr.Z* to drive in their F_1 - progeny over-expression of dFMRP in the wing imaginal discs of developing *Drosophila* larvae. As a result, we observed notched wing phenotypes, due to apoptotic cell loss at the wing margins, where the *vg*-promoter functions (Wan *et al.*, 2000).

On the background of these wing phenotypes we looked for EMS-induced mutations, dominantly modifying them. We were interested in those of them, which were X-linked, as there are so far no data on experimentally induced *dfmr1*- modifier mutations.

We first optimized the conditions for the application of the *GAL4-vg.M/UAS – Fmr.Z* and investigated the penetrance of the over-expression notched wing phenotype (control phenotype) at different temperatures, as it is known that transcription activation by *GAL-4* is strongly influenced by temperature (Brand *et al.*, 1994). On the other hand, expression of genes downstream of the *UAS*-sequence influences *Drosophila* development/viability, and this also correlates with the temperature of rearing the flies (Allemand *et al.*, 2001).

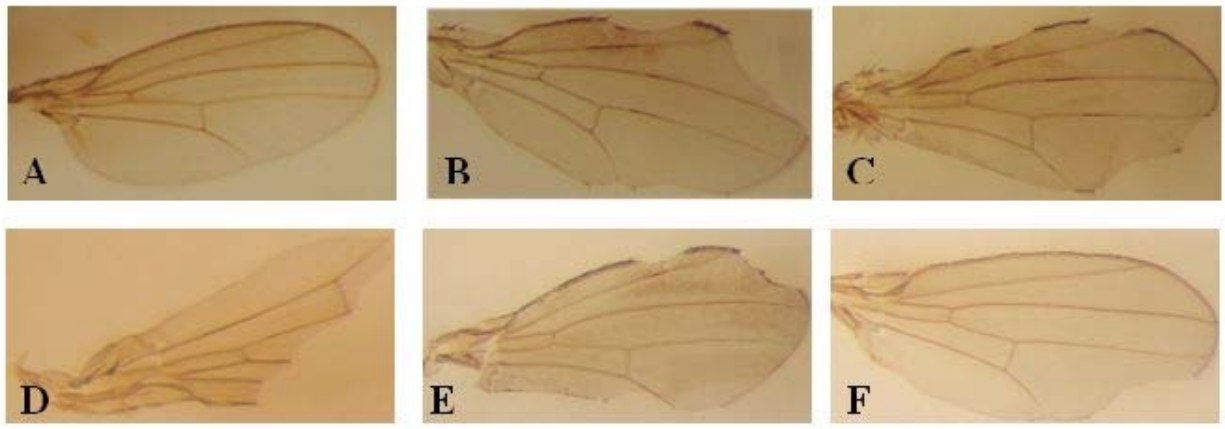

Figure 1. Wing phenotypes of flies. A – wild type wing (♀ and ♂); B - over-expression of *dfmr1* in the adult wings at 21°(♀, control phenotype); C, over-expression of *dfmr1* in the adult wings at 27° (♀ and ♂, control phenotype); D - strong enhancer wing phenotype; E – moderate enhancer wing phenotype; F- weak enhancer wing phenotype. *Note: Suppressor wing phenotype is not shown, as it looks like the wild type.*

We reared *GAL4-vg.M/+; UAS – Fmr.Z/+ flies* (F_1- generation, see the cross in Materials and Methods) at several different temperatures and found that the notched wing phenotype, induced by the over-expression of *dfmr1,* was temperature sensitive. It showed a complete penetrance in the male flies from F_1, reared at 21°C and no penetrance in their sibling females. At 27°C both sexes had notched wing phenotypes (Figure 1).

We also observed, that these two temperatures ensured about 70 – 80% adult eclosion and viability, which was high enough to allow a large-scale genetic screen.

The difference in the wing phenotype penetrance between the male and female F_1 progeny at 21°C, as well as the uniform over-expression wing phenotype at 27°C, was used by us to set up a two-part screen for *dfmr1* dominant modifier mutations induced by EMS-mutagenesis (for details of the EMS-procedure, see Materials and Methods).

In one part of it we set up 1250 individual crosses, which consisted of one mutagen-treated *UAS – Fmr.Z* - male and several *GAL4-vg.M*- females, and cultured the progeny at 21°C. As we screened for X-linked modifier mutations, according to our cross procedure, we needed to isolate from F_1 only female flies with modified phenotypes. At this temperature, we easily visualized and scored for an appearance of female flies with notched wing phenotypes. We defined such phenotypes as enhancer phenotypes. They represented "worsened" control wing phenotypes, the latter being indistinguishable from the wild type at this particular temperature. Mutated genes, causing this "worsened" phenotype, were designated as enhancers.

Altogether we examined about 18,000 female flies over-expressing *dfmr1* under the control of *GAL4-vg.M* and found 126 enhancer mutations, dominantly modifying the control wing phenotype at 21°C.

We divided them into four groups, according to an arbitrary scale of their modifying effect on the control wing phenotype: very strong, strong, moderate, and weak enhancers. As very strong enhancers (7) we considered those having extremely "worsened" wing phenotype – one or two wings were completely absent. The strong enhancers (53) showed missing wing blades. As moderate enhancers (57) were determined those having one or two small notched areas- anterior and posterior wing margin loss on both wings, and the weak enhancers (9) had only one small notched area on one of the wings. Enhancer wing phenotypes are presented in Figure 1.

In the second part of our screen, we set up 1900 individual crosses, designed as above, but reared the progeny at 27°C. At this temperature, we scored for an appearance of female F_1 flies with normal wings among the uniformly present notched wing phenotypes. Such flies with an "improved" wing phenotype were considered as having suppressor phenotypes, and their mutated genes were designated as suppressors.

We scored about 21,000 female flies, over-expressing *dfmr1* in the developing wing tissue and found 128 suppressor mutations, which dominantly modified the over-expression wing phenotype at 27°C. To avoid false weak suppressors, we isolated only those of them which modified this phenotype to wild type appearance.

Meiotic mapping of the modifier mutations with recessive lethality

Each enhancer or suppressor mutation was isogenized, by crossing the mutant female to male flies *w[1118]*, and the male/female ratio was determined as well as the segregation of the notched wing and the control wing phenotypes. In this way, we were able to define and select only those modifier mutations, which were X-linked and recessively lethal, so that we could further map them genetically.

Among the 126 modifier enhancer mutations isolated in our screen, we found 8 X-chromosomal lethal mutations. We tested them for their specificity to modify the specific abnormal wing phenotype induced by the gene *dfmr1*. For that we combined each mutation in a common

genotype with the trans-genes *C96-GAL4* and *UAS-MamN,* which produce a wing phenotype similar to our control wing phenotype but driven by a different gene and expressed under a different *GAL4* promoter. Neither of our mutations was able to modify the latter wing phenotype, in contrast to a control experiment with the mutation *y* w^a N^{54l9}*//FM76*, which enhanced the MamN-driven wing phenotype (Helms *et al.*, 1999).

Among the 128 suppressor mutations we found 11 to be located on the X-chromosome and to be recessively lethal. They specifically modified the control over-expression wing phenotype at 27°C.

All these modifier mutations were mapped by means of the recombination analysis, and their positions on the X chromosome were calculated by their recombination distances from the genes *y* and *ct* (see Materials and Methods). For those of them, which showed a closer proximity to *y* than to *ct,* genetic distances from *y* and *f* were determined as well.

The results of our recombination analysis are shown in Table 1.

Table 1. Recombination analysis and map position of the X-linked modifier mutations.

Mutation signature	Total number of F_2 – males scored	Map region
Eh 5	1705	1 – 53.0 - 59.0
Eh 25	1175	1 – 49.8 - 55.8
Eh 40	1244	1 – 31.7 - 37.7
Eh 44	1175	1 – 01.0 - 05.0
Eh 50	1380	1 – 00.0 - 04.1
Eh 53	1364	1 – 35.0 - 41.0
Eh 109	1546	1 – 37.9 - 44.9
Eh 134	1382	1 – 38.2 - 44.2
Su 1	1320	1 – 34.6 - 40.6
Su 5	1202	1 – 52.7 - 58.7
Su 10	1340	1 – 48.9 - 54.9
Su 37	1319	1 – 45.2 - 51.2
Su 55	1381	1 – 38.3 - 44.3
Su 71	1238	1 – 46.1 - 52.1
Su 87	1396	1 – 53.0 - 59.0
Su 104	1319	1 – 03.1 - 10.1
Su 121	1263	1 – 31.9 - 37.9
Su 123	1283	1 – 00.0 - 05.0

Note: As recombination distances to known chromosomal markers give only a crude location of a gene, we determined in this way the map region of each lethal mutation, which acted as a dominant enhancer/suppressor of the over-expression dfmr1 wing phenotype.

We undertook our large scale forward genetic screen in order to find new genes, which interact with *dfmr1* and function in a common biological pathway.

So far most modifier screens are conducted by over-expression the gene of interest in the adult retina. Zarnescu and co-authors looked for functional partners of *dfmr1* and performed a dominant modifier screen for the autosomal *Drosophila* genome (Zarnescu *et al.*, 2005). They found 19 mutations in the tumor suppressor gene *l(2)gl* and 90 mutations in other autosomal loci, which modified the retinal over-expression of *dfmr1* in *Drosophila.* Later, Cziko and co-authors screened mutations in 43 candidate genes for their ability to modify the "rough" eye phenotype, induced by over-expression of *dfmr1* in the adult retina, and identified several suppressors of this mutant phenotype (Cziko *et al.*, 2009). In their experiments, new translational repressor proteins were characterized, which interacted with the protein dFMRP.

In our work we drove over-expression of *dfmr1* in the wing imaginal discs. Previous studies have shown that such over-expression leads to notched wing phenotypes, due to apoptotic cell loss at the wing margins (Wan *et al.*, 2000).

We have chosen the wing tissue as a place to conduct our screen, as it very easy to score and save for the organism's viability and fertility. *Drosophila* wing is also a suitable model to study genetic interactions for another reason. Differentiation of distinct cell types within the growing wing tissue depends on their interactions with adjacent cells, and mutations in specific genes affect differently behavior of the different cell subpopulations (Craig *et al.*, 1997). Important signaling pathways - Notch, Wingless, Decapentaplegic, and others take part in the wing development (reviewed in Artavanis-Tsakonas *et al.*, 1995; Klingensmith and Nusse,

1994). Looking for dominant modifiers of *dfmr1* that function in these pathways would elucidate novel developmental roles of this gene, which might be related to the wing tissue (or to other tissues as well).

In our study we found modifiers – enhancers and suppressors, interacting with *dfmr1*, whose product levels were crucial for the signal transduction efficiency in the adult wings, so that their mutations were recessively lethal. To identify and characterize these mutations we plan to carry out fine duplication/deficiency genetic mapping and complementation analysis with selected candidate genes from the same cytological map locations.

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Genetic analysis of body color polymorphism in *Drosophila melanogaster* through selection experiment.

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Abstract

Drosophila melanogaster is a widespread species that exhibits enormous variation in abdominal melanisation throughout its range. To gain insight into this variation, present work involves selection for abdominal melanisation. In 38 generations of selection for melanisation, an increase of ~2.5 fold in dark selected strain and a decrease of ~6-7 folds in light strain was observed in both the sexes as compared to control populations of *D. melanogaster*. Genetic crosses between dark and light strains obtained through selection produced intermediate offspring, but a clear maternal effect differentiated the reciprocal F_1's. F_1 flies showed higher plastic effect as compared to selected dark and light strains across various growth temperatures. Our results are novel in the occurrence of five body color phenotypes in ratio of 1:4:6:4:1 (two-gene) in both sexes of *D. melanogaster*.

Keywords: Abdominal melanisation, dark and light selected strains, *Drosophila melanogaster,* selection experiment.

Introduction

Body melanisation exhibits a large amount of variability in Drosophilids, resulting either from genetic polymorphism or phenotypic plasticity (Gibert *et al.,* 1998; Rajpurohit *et al.,* 2008; Parkash *et al.,* 2011). The color polymorphism in abdominal tergites was reported by da Cunha (1949) for *D. polymorpha.* In this species, both males and females show any one of the three types of abdominal tergite coloration: dark, intermediate or light. In four species of montium subgroup, discrete polymorphism for 6^{th} and 7^{th} abdominal segments occurs only in female individuals, but the dominance level of darker and lighter phenotypes vary between species (Ohnishi and Watanabe, 1985). Recent studies in montium species *D. jambulina* and *D. punjabiensis* have shown genetic polymorphism for body color morphs (Parkash *et al.,* 2009; Singh, 2011). *D. melanogaster* is known for its sexual dimorphism. Males possess a black abdomen (tergites 5 and 6) while females exhibit yellow tergites with black stripe at their posterior margin.

Several studies on melanisation include analyses of phenotypic plasticity (David *et al.,* 1990; Gibert *et al.,* 1998, 2000; Parkash *et al.,* 2011), evolutionary developmental basis of intra- and interspecific differences (Kopp *et al.,* 2000; Wittkopp *et al.,* 2002a, 2002b), a phylogenetic and

speciation context of trait evolution (Hollocher *et al.*, 2000a,b), and traditional quantitative trait loci approaches (Llopart *et al.*, 2002; Kopp *et al.*, 2003; Wittkopp *et al.*, 2003).

Abdominal melanisation has been extensively studied in *D. melanogaster* (Wittkopp *et al.*, 2003). Present work involves selection for melanisation in cosmopolitan *D. melanogaster* for the first time. The selected dark and light strains help in carrying out genetic crosses and thereby, report five body color phenotypes in ratio 1:4:6:4:1 for both sexes in *D. melanogaster*. Crosses between dark and light strains show clear maternal effect between reciprocal F_1's.

Materials and Methods

Selection for melanisation: Preparation of homozygous dark and light strains

The aim of selection experiment was to determine the extent to which the phenotype for melanisation might change as a result of selection. The flies used to start the selection experiment were collected in September-October months of 2007 from a highland locality Shimla. Thirty isofemale lines were initiated from flies collected with net sweeping and bait traps from fruit markets and godowns. The cultures were maintained at a constant growth temperature of 21°C in a biological oxygen demand incubator. The density was controlled by limited egg laying period (6-8 hours) on cornmeal-yeast-agar medium. An equal number of offspring from each isofemale line were pooled to produce a population of at least 3000 individuals in order to generate maximum variability. A mass population of this size was maintained on laboratory medium for five generations and then subdivided into 9 lines, each with ~300 individuals. Three of the lines were maintained on laboratory medium serving as controls, and the remaining six lines were subjected to selection for melanisation, *i.e.*, extreme dark and light female flies were assorted. From the assorted flies, 60 dark (D) and light (L) flies were used for egg laying separately in 5 replicates each. They were labeled as Dark (D_1, D_2, D_3, D_4, and D_5) and Light (L_1, L_2, L_3, L_4, and L_5) lines. Thereafter, every generation extreme dark and light flies were assorted out and other phenotypes obtained were discarded. For about 38 generations, this procedure was followed; thereafter, melanisation did not increase but selection was continued to stabilize the phenotype. Thus, very dark and very light flies obtained were used as parents to establish crosses (Figure 1).

Figure 1. Dark and light body color phenotypes obtained as a result of selection of melanisation for 38 generations in both sexes of *D. melanogaster*. These flies were further used as parents for carrying out genetic crosses.

Drosophila *strains and crosses*

Furthermore, in order to ascertain the genetic basis as well as allelic dominance, we carried out Mendelian crosses (F_1 and F_2 crosses) with these true breeding dark and light strains of *D. melanogaster.* We made 15 single pair matings using virgin 1 ♂ and 1 ♀ each of very dark and very light morph for obtaining F_1 progeny. For

investigating plastic effects of body melanisation, two replicates each of the above pair matings were then transferred to different growth temperatures (14, 17, 21, 25, and 28ºC). Six day-old flies from these different developmental temperatures were scored for melanisation. We randomly scored 100 ♀ as well as 100 ♂ flies (F_1); results differed greatly according to sex. Further, out of pooled F_1 progeny, 50 ♂ and 50 ♀ were randomly selected, and five replicates with 10 pairs each were used for F_2 progeny. F_2 progeny showed segregation for 5 types of body color phenotypes, *i.e*., very dark, dark, intermediate, light, and very light morphs.

Table 1. Data on female progeny scored for F_1 & F_2 genetic crosses between true breeding dark and light strains of *D. melanogaster*. Similar results were obtained for males (data not shown)

Genetic Crosses	Type/ Replicate	♀ flies scored x (n)	V. Light	Light	Intermediate	Dark	V. Dark
(A) Light ♀ * Dark ♂	F_1	200	0 (0 %)	0 (0 %)	200 (100%)	0 (0 %)	0 (0 %)
	F_2: 1.	300	20	69	119	70	22
	2.	270	19	57	124	53	17
$F_{1\,A}$ ♀ * $F_{1\,A}$ ♂	3.	340	22	78	130	85	25
	4.	315	25	80	110	77	23
	5.	294	27	66	103	73	25
(B) Dark ♀ * Light ♂	F_1	200	0 (0 %)	0 (0 %)	200 (100%)	0 (0 %)	0 (0 %)
	F_2: 1.	286	25	62	102	70	27
	2.	308	27	71	110	75	25
$F_{1\,B}$ ♀ * $F_{1\,B}$ ♂	3.	320	30	74	115	78	23
	4.	260	19	58	92	66	25
	5.	255	17	53	100	64	21

$F_{1\,A}$ = Light ♀ x Dark ♂; $F_{1\,B}$ = Dark ♀ x Light ♂

Results and Discussion

Melanisation is one of the most variable traits in the genus *Drosophila*: differences in body color are common among individuals within a population, among populations of the same species, and among closely related species (True, 2003; Wittkopp *et al.,* 2010). Present work involves selection for melanisation (38 generations) in *D. melanogaster* from a highland locality. The dark flies were ~96% and light flies ~4-5% melanic after 38 generations of selection (Figure 1). Table 1 shows results of genetic crosses between true breeding dark and light strains obtained after selection for melanisation in females of *D. melanogaster*. Similar results were obtained for males (data not shown). Crosses helped to analyze genetic basis as well as allelic dominance (if any) for body color polymorphism in this species (Table 1).

Figure 2 summarizes the five body color phenotypes obtained in F_2 generation, close to 2-gene ratio of 1:4:6:4:1 in both sexes of *D. melanogaster.* Mean ± SD for % body melanisation of F_1 flies obtained through genetic crosses (at 21°C) in *D. melanogaster* across five growth temperatures are given in Table 2. We observed significant differences across growth temperatures for F_1 reciprocal crosses. F_1 progeny obtained from genetic crosses with light female parents are less pigmented as compared to F_1 flies from dark female parent (Table 2). F_1 individuals from light ♀ × dark ♂ cross show higher plasticity than F_1 flies from dark ♀ × light ♂ cross. The selected strains were investigated over a range of growth temperatures. Flies of dark selected stains (although quite

dark) show slight decrease in melanisation at higher temperatures, whereas flies of light selected strain (very light) show some increase in melanisation at lower temperatures.

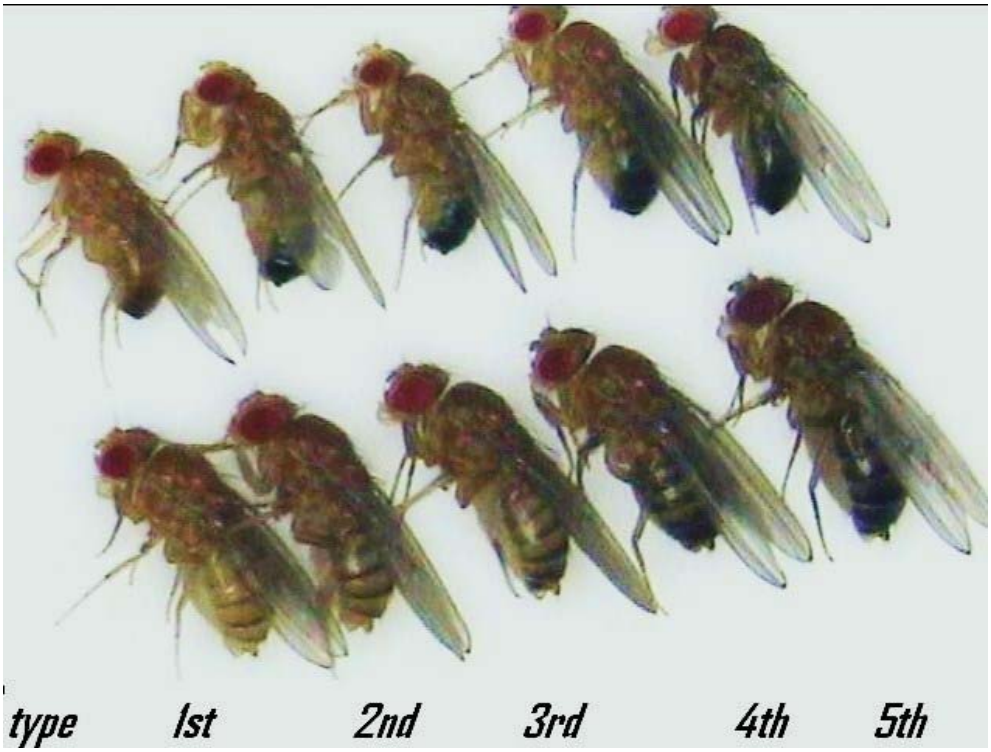

Figure 2. Five body color phenotypes (1st – very light; 2nd – light; 3rd – intermediate; 4th – dark and 5th – very dark) for F_2 crosses in both the sexes of *D. melanogaster*.

Table 2. Mean ± SD for % body melanisation of F_1 progeny obtained through *genetic crosses between true breeding dark and light strains of *D. melanogaster* (Dark ♀ x Light ♂; Light ♀ x Dark ♂) across five growth temperatures.

Temperature	Dark ♀ x Light ♂		Light ♀ x Dark ♂	
(°C)	♂	♀	♂	♀
14 (°C)	70.93 ± 5.22	84.50 ± 4.61	52.72 ± 2.68	68.10 ± 3.21
17 (°C)	56.70 ± 4.30	72.34 ± 3.82	49.20 ± 2.93	60.00 ± 2.56
21 (°C)	48.00 ± 4.71	59.60 ± 4.80	27.50 ± 4.22	41.32 ± 5.10
25 (°C)	36.20 ± 5.03	50.92 ± 5.65	19.00 ± 5.97	33.04 ± 4.27
28 (°C)	24.15 ± 3.17	31.00 ± 4.39	12.78 ± 3.96	14.00 ± 3.42
% Change	2.94	2.72	4.12	4.86

*Crosses were performed at 21 °C and thereafter eggs were transferred to different growth temperatures for obtaining F_1 flies.

The goal of the present study was to examine the striking variation in abdominal pigmentation displayed by cosmopolitan *Drosophila melanogaster*, to develop an understanding of why this trait is so variable in this species. Further investigation of present work will help *Drosophila* workers to

understand many more unknown queries about melanisation in cosmopolitan *Drosophila melanogaster.*

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Comparing selection schemes in BAC-recombineering method of tagging a novel *Drosophila* gene, *DmCSAS.*

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Abstract

Sialylation plays an important role in the *Drosophila* nervous system. However, the regulation of sialylation pathway remains poorly understood. We focused our analysis on a novel gene, CMP-Sialic acid synthetase (CSAS), that is predicted to be a key player of the pathway. In order to investigate its expression, we decided to introduce a tag sequence into the CSAS genomic locus within a BAC clone using recombineering strategy. We wanted to introduce the tag without any additional extraneous sequences in order to minimize the influence of the insert on the gene's function. We sought to modify existing recombineering protocols and test different selection and screening methods during recombineering. Our results confirmed the general utility of positive/negative selection approach using *rpsL*$^{+}$*-kana* marker. However, they also revealed the limitation of this strategy, as it did not allow unambiguously to identify recombinant clones, while resulting in enrichment rather than selection for desired recombineering events.

Introduction

Sialic acid is a nine carbon, negatively charged sugar that can be attached to the termini of carbohydrate modifications of glycoproteins and glycolipids. Sialylation is abundant and universally present in vertebrates. The sialylation of cell-surface molecules is involved in cell signaling, inflammatory response, immune response, and development, while sialylation defects have been linked to cancer and autoimmune diseases (Varki, 2008). Unlike vertebrates, invertebrate organisms, including *Drosophila*, appear to have a tightly controlled sialylation pathway limited to some cells and developmental stages (Repnikova *et al.,* 2010). *Drosophila* has homologues of most of the essential components of vertebrate sialylation pathway, which suggests that *Drosophila* can be used a model system to reveal mechanisms of sialylation in vertebrates. Recent studies revealed that *Drosophila* sialyltransferase (DSiaT), a key enzyme of the sialylation pathway, plays an important role in the regulation of neural transmission in the nervous system (Koles *et al.,* 2004; Repnikova *et al.,* 2010). The pattern of DSiaT expression is restricted to neurons within the CNS. At the same time, little is known about other components of *Drosophila* sialylation pathway, including enzymes functioning upstream of DSiaT, such as CMP-sialic acid synthetase (CSAS) that generates activated sugar donor for DSiaT-mediated sialylation. *Drosophila* CSAS protein was shown to have an unusual Golgi localization in cell culture experiments (Viswanathan *et al.,* 2006). However, the expression and function of CSAS *in vivo* have never been characterized. Detailed understanding of the expression of this protein could lead to a better grasp of the pathway itself and any interactions among the functional components within the pathway. Here we describe the generation of BAC construct with tagged CSAS gene using recombineering approaches.

Table 1. Oligonucleotide primers used in recombineering experiments.

Primer #	Primer name	Template used	Primer sequence (5'→3')
1	CSAS-kana-f	PL452	CGATCTGACTCTAGCCAAATACATCTTAAGTAGTGAAACAAAAACCGAGggtctgaagaggagtttacgtcc
2	CSAS-kana-r	PL452	CAAGATCAATGAACTTTACACTTTCTTTGATGAATAGCATATCTATTTCaagggttccgcaagctctagtc
3	CSAS-rpslkan a-f	pSK+RpsL-kana	CGATCTGACTCTAGCCAAATACATCTTAAGTAGTGAAACAAAAACCGAGtgggcggttttatggacagca
4	CSAS-rpslkan a-r	pSK+RpsL-kana	CAAGATCAATGAACTTTACACTTTCTTTGATGAATAGCATATCTATTTCgtgggcgaagaactccagcat
5	CSAS-gnmc-f	CSAS BAC	GAGTCCCATGACTGTGTTTTTGCCGCTAAGAGG
6	CSAS-gnmc-r	CSAS BAC	TGTGTGTCGCTCGGTTTGGCAGGACTTGCTTGG

Materials and Methods

The BAC clone for CSAS tagging (*CH322-158A02*) was obtained from BACPAC Resource bank at Children's Hospital Oakland Research Institute (CHORI). The clone includes 22.1 kb genomic insert containing *CSAS* locus. The *PL452* plasmid was obtained from Koen Venken (Baylor College of Medicine, Houston), *pSK+RpsL-kana* was purchased from Addgene. DY380 cells were obtained from NCI-Frederick Biological Resources Branch. Primer sequences used in recombineering experiments are shown in Table 1. Except for the modifications to protocols

discussed in the text, we used previously published recombineering methods described in (Venken *et al.*, 2008; Wang *et al.*, 2009).

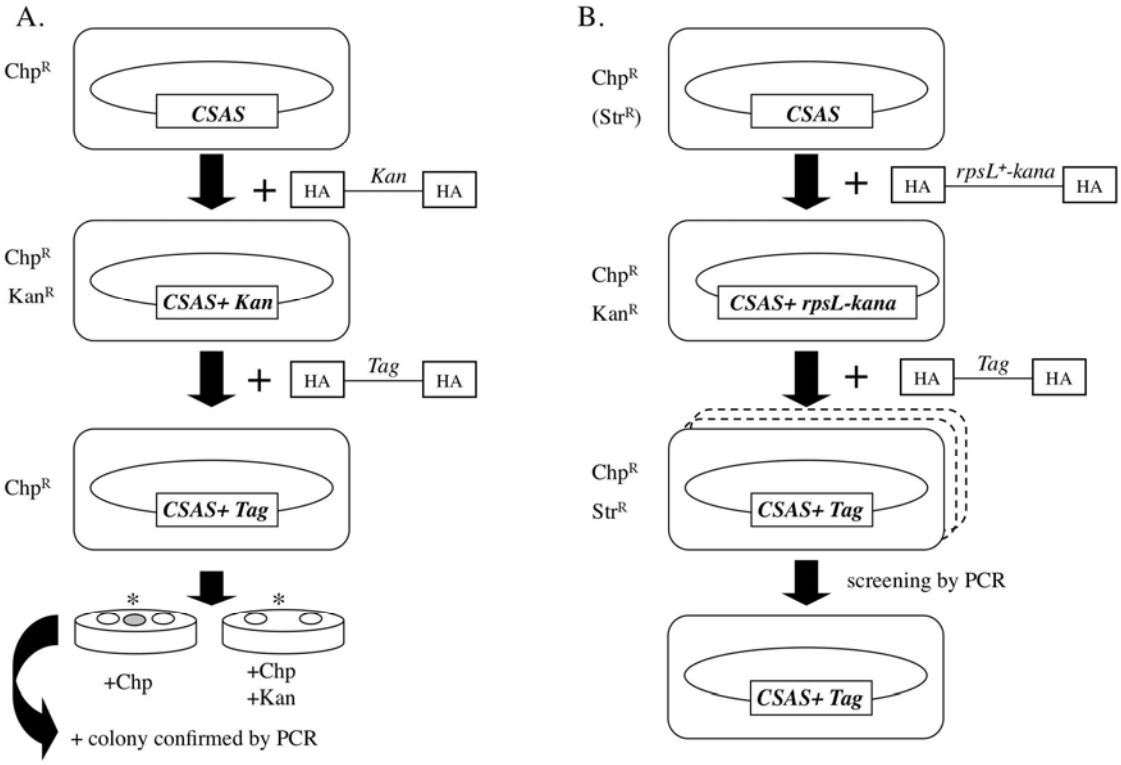

Figure 1. BAC recombineering-mediated tagging of *CSAS*. BAC constructs are shown inside DY380 cells containing λ-*cI857* repressor. A, A one-step positive selection strategy with replica plating. B, A two-step positive/negative selection strategy. In both cases inserts contained flanking homology arms (HA), with either selection or gene of interest sequences. The tag insert encodes 3FLAG protein tag. Chp^R, Kan^R, and Str^R indicate chloramphenicol, kanamycin, and streptomycin antibiotic resistance markers, respectively.

Results and Discussion

In order to study CSAS *in vivo* expression, we sought to generate a BAC-based transgenic construct that contains the entire *CSAS* gene along with a short sequence insert encoding the 3FLAG protein tag. Our rationale was based on the fact that BAC clones can contain gene loci including the majority of important regulatory elements that determine a spatiotemporal pattern of gene expression. The expression of genes included in transgenic BAC constructs was shown to reflect closely the endogenous expression pattern of these genes (Poser *et al.*, 2008; Venken *et al.*, 2008). Several methods for modifying sequences within BAC clones have been described, while most popular approaches commonly rely on a two-step recombineering strategy, with the first step including the introduction of desired changes along with an antibiotic marker (*e.g.*, kanamycin) to a genomic location. In the second step, this marker is removed by site-specific recombinase (*e.g.*, using Cre-Lox system) (Venken *et al.*, 2008). However, this strategy leaves behind some irrelevant insertions (*e.g.*, a LoxP site) that may affect gene functions. Several techniques that result in a "clean" introduction of a tag sequence or a mutation have been developed, including the *galK*

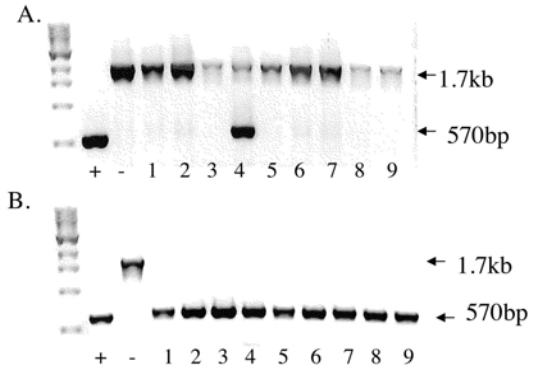

Figure 2. Confirmation of 3FLAG insert into CSAS BAC following recombineering. A, Colony PCR of selected colonies appearing to have lost streptomycin sensitivity. Most retained the selection cassette (~1.7kb). Positive clone is in lane 4 (570 bp insert). B, Individual colonies obtained from positive culture (lane 4) were confirmed by PCR to have 3FLAG insert. + and –, positive and negative controls for PCR amplification of the 3FLAG and selection cassette. The left lane is a DNA ladder.

positive/negative selections method and the *rpsL*$^+$*-kanamycin* scheme (Warming *et al.,* 2005; Wang *et al.,* 2009). The second step in these positive/negative selection schemes appears to be a bottleneck of the approaches that limits their efficiency and sometimes results in a low success rate. This problem arises from high frequency of false-positive colonies due to the loss of selection marker because of inactivating mutations (Wang *et al.,* 2009). Thus, we wished to modify the two-step protocol to simplify the scheme and decrease the background of false-positive clones. To this end, we decided to modify the second step of the procedure while leaving the first one essentially unchanged. Similarly to the *rpsL*$^+$*-kanamycin* scheme, our protocol started with introducing an antibiotic marker into the CSAS coding region that we wished to modify with tag-encoding sequence. Just the kanamycin marker alone was inserted into the 3' end of *CSAS* open reading frame. As described for other similar recombineering approaches (Venken *et al.,* 2008; Wang *et al.,* 2009), this step was very efficient and resulted in hundreds kanamycin-resistant clones. When analyzed by PCR, virtually all tested clones (>95%) included the desired insertion. The second step was designed to replace the antibiotic marker gene by a 3FLAG-encoding sequence (~70 bp). The efficiency of recombineering in a similar system without selection was estimated as 1.7×10^{-3}, or 1 out of approximately 600 induced DY380 cells electroporated with a targeting fragment including two 45 bp homology arms (Lee *et al.,* 2001). We reasoned that this frequency of targeting events should allow us to identify successful replacements of antibiotic marker with a tag-encoding sequence by comparing colonies on replica plates with and without antibiotic selection (Figure 1). To enhance the efficiency of recombination, we increased the length of homology arms to 300 bp. Using this approach, we screened approximately 7,000 individual colonies each replicated on two sets of plates, with and without kanamycin selection. We identified 3 colonies that lost the antibiotic marker; however, they all turned out to be false-positive as reveled by further PCR analysis. Thus, the efficiency of targeting in our case was estimated as at least an order of magnitude lower ($<1.4\times10^{-4}$) than in previously described experiments (Lee *et al.,* 2001). To compare these results to the efficiency of a two-step recombineering scheme with selection applied at both steps, we used the same homology arms as in our previous experiment while employing recombineering system with *rpsL*$^+$*-kana* cassette as a selection marker (Wang *et al.,* 2009). In order to isolate individual streptomycin-resistant colonies, we used several serial dilutions when plating electroporated cells during the second step of the procedure. In addition to 1:25 dilution recommended by the original protocol (Wang *et al.,* 2009), we used higher dilutions of 1:250, 1:625, or 1:2500. However, no positive results were obtained with higher dilutions. The colonies that had incorporated the tag-encoding insert must have lost the selection cassette containing *rpsL*$^+$ and, thus, they should be able to grow more robustly on the plates containing streptomycin. However, after 16-hour incubation, the time used in the original protocol, there was no distinguishable difference in size or morphology between colonies. After increasing the incubation time to 24 hours, a slight difference in colonies

could be detected on 1:25 dilution plates, but still no positive colonies were found on the plates with higher dilutions. This indicated that the efficiency of targeting events was rather low, which was consistent with conclusions from our previous attempt of targeting using no selection during the second step. By focusing on the 1:25 dilution plates, we found colonies that grew on the lawn and appeared larger and denser than surrounding areas, indicating that the selection step resulted in enrichment rather than a strict selection for desired recombineering events. These more dense areas were picked up and tested further by colony PCR. Out of 40 tested cultures, one positive clone carrying the tag insert was isolated (Figure 2A). In order to insure that the corresponding culture contained only one colony (the positive clone was picked from a lawn), cells from the colony PCR media were streak plated and grown overnight at 32°C, then retested by colony PCR again to confirm results (Figure 2B). Finally, the insert was sequenced using primers located outside of the region of the targeting sequence and found to include the correct insertion of 3FLAG-encoded sequence at the 3' end of the *CSAS* coding region (Figure 3). Our results from this experiment using the two-step selection scheme with *rpsL*$^+$*-kana* cassette were similar to previously published data (Wang *et al.,* 2009). The previous work estimated the efficiency ranged from 1-29% positive for the loss of the *rpsL*$^+$*-kana* cassette, but only <1-18% efficiency when considering the accuracy of inserted sequence (Wang *et al.,* 2009). Our two-step selection scheme resulted in 2% efficiency with no unwanted rearrangements as confirmed by sequencing.

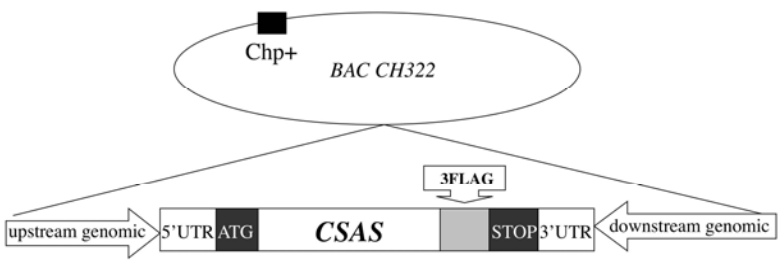

Figure 3. The CSAS BAC construct with 3FLAG-encoding insertion at the 3'-end of *CSAS* coding region. The insertion was confirmed by sequencing of the *CSAS* region of the clone encompassing the targeted region using primers #5 and 6 (Table 1).

In summary, we generated a BAC construct for transgenic expression of 3FLAG-tagged CSAS protein. This construct will be used to analyze CSAS expression *in vivo* and to investigate regulatory mechanisms of *Drosophila* sialylation. In our experiments, we compared two recombineering schemes for generating a BAC construct. We modified a two-step protocol by replacing the second negative selection step with the step based on comparing colonies on two sets of replica plates, with and without selection. This approach relies on a relatively high efficiency of targeting. However, despite the fact that the homology arms were increased in our approach to 300 bp, we found that the efficiency of targeting was significantly lower than the one previously reported for a similar recombineering system. This result can be potentially explained by a decrease efficiency of transformation of targeting fragment in our case, or by a peculiar property the DNA locus used in our experiments. Overall, our experiments indicated that a modified positive/negative selection scheme worked best when the efficiency of targeting is low. However, when the targeting efficiency is relatively high (> 0.1%) the scheme without selection at the second step may still provide some advantages, such as simplicity and speed.

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Preliminary data on the *Drosophila* fauna in the city of Tandil, Buenos Aires Province, Argentina.

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Introduction

It is said that the drosophilid fauna in temperate regions is better known than that of the tropical regions (Val *et al.,* 1981; Wheeler 1986). However, when we take into consideration what is known about the fauna of these insects in Argentina, we find that the published knowledge is precarious. The majority of the studies on drosophilids available there are centered on genetic and evolutionary questions of determined species (Barker *et al.,* 1985; Iriarte *et al.,* 2009; Soto *et al.,* 2010).

In temperate and cold regions the climatic factors have considerable influence on the drosophilid populations and limit the occurrence of many species. Because of this, Dobzhansky and Pavan (1950) stated that the number of species found dropped as they proceeded from the heat of the tropical areas to the colder regions. This indicates that it is a rare feature for a drosophilid to be adapted to life at low temperatures.

The objective of this study is to contribute to better knowledge of the Argentinean drosophilid diversity in Tandil city (located about 400 km south of Buenos Aires), an area never studied on this question up to the present.

Materials and Methods

Adult drosophilids were collected in Tandil city (37°19´S; 59°09´W), in the province of Buenos Aires, Argentina (Figure 1). This province is situated in the center-east of the country and is steppe-land mainly covered by herbaceous grass and known as the Pampas. A large part of the Pampas is at sea level, with the exception of two major mountain systems denominated Ventania and Tandilia. Tandil city itself is located in the Tandilia mountain foothills, in the southwest of Buenos Aires at about 200 meters above sea level. The climate in Tandil is temperate or humid meso-thermal with average annual temperature of 13.9°C, an average maximum of 20.1°C, and average minimum

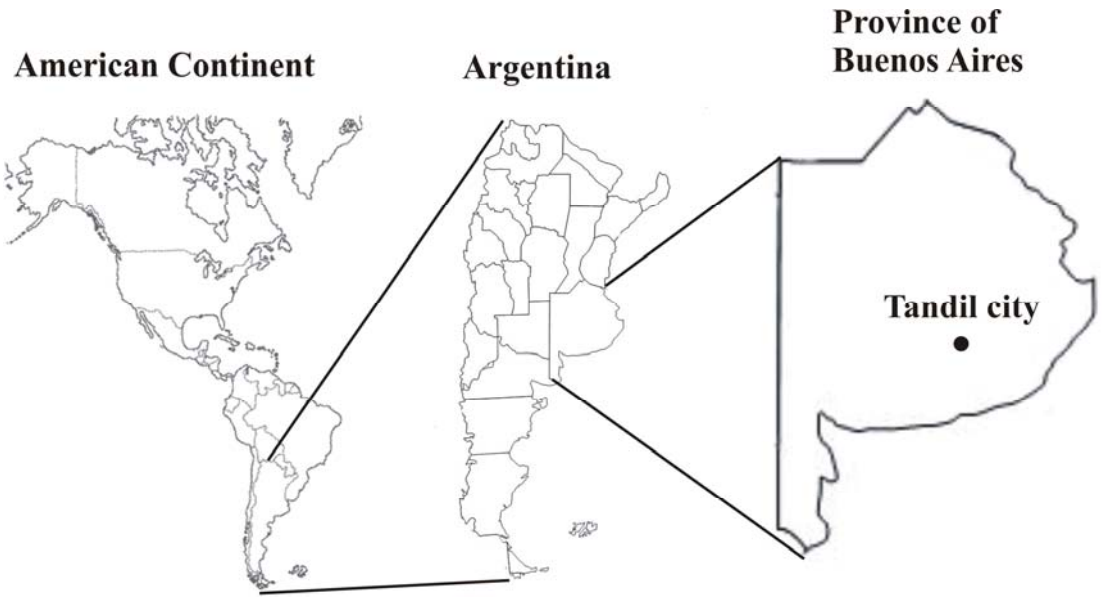

Figure 1. Location of Tandil city.

of 6.9°C. Annual average precipitation is 900 millimeters (http://www.tandil.gov.ar/notas/destacados/movediza/pdf/AnexoI.pdf).

The drosophilids were collected in the *Parque Independencia* during the Summer Season (January 2008) when the average temperatures were around 21°C and rainfall was about 82 millimeters (http://www.tutiempo.net/clima/Tandil_Aerodrome/01-2008/876450.htm).

All insects were collected with the model traps proposed by Tidon and Sene (1988) using banana bait. The traps were left at the sites for three days and thereafter the insects were maintained in ethanol and identified at the species level by reference to the specialized literature.

Table 1. Absolute abundance of the *Drosophila* collected in the city of Tandil, Argentina.

Species	N
D. gaucha	711
D. buzzatii	88
D. subobscura	73
D. immigranns	26
D. simulans	23
D. nigricruria	10
D. hydei	8
D. melanogaster	4
D. nebulosa	1
D. busckii	1
Total	945

Results and Discussion

For this study we collected a total of 945 drosophilids of 10 species (Table 1). This study is the first record of *D. nigricruria* for the province of Buenos Aires and the southernmost record for *D. nebulosa* and *D. buzzatii* (Bächli, 2011).

Drosophila gaucha was the most abundant species found, representing 75% of the collected insects. This is a neotropical species widely distributed in South America (Brncic, 1987; Iriarte and Lopez, 1995) and tolerant to low temperatures.

Among the collected species, six are cosmopolitan: *D. subosbscura*, *D. immigrans*, *D. simulans*, *D. hydei*, *D. melanogaster,* and *D. busckii*. With the exception of *D. melanogaster*, all of these species had previously been registered in Mar del Plata, a city located approximately 200 km distant from Tandil city (Iriarte and Lopez, 1995).

Of the other species sampled, *D. buzzatii* was the second

most abundant corresponding to approximately 9% of the drosophilids collected in Tandil. This is probably a species originally from the Argentinean Chaco which was distributed by humans – together with its host *Opuntia ficus-indica* – to many parts of the world (Tidon-Sklorz and Sene, 1999).

Although this is still a preliminary study of the drosophilid fauna in Tandil, the diversity of the sampled species (10) was greater than that observed in areas near to those where we made our study. Iriarte and López (1995), evaluating during the four seasons of the year the fauna of these insects in the city of Mar del Plata, registered the occurrence of only seven species of which six were sampled in the present study. Data such as this suggest that further sampling of drosophilids should be made, during the different seasons of the year in Buenos Aires province.

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***Curcumin longa* and *Emblica officinalis* increase lifespan in *Drosophila melanogaster*.**

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Introduction

Every living organism ages with time. However, every individual wants to stay healthy, look younger, and live longer. In recent years, scientists are interested to discover the scientific clues to the aging process and to determine if the process of ageing is genetically or environmentally controlled, or by both. To this respect, different food components have been shown to increase life-span of many organisms; however, no conclusive evidence in favor of any particular food component has yet been established. Principally, oxygen free radicals or reactive oxygen species (ROS) are known to cause aging. Aerobic cells generate ROS as a by-product of oxidative metabolism. The primary assumption of this theory is that normal antioxidant defense levels are not sufficient, so that some ROS escape elimination causing molecular damage, some of which is irreparable and accumulates with age. If ROS cause aging, then enhanced defense against ROS

should reduce oxidative stress, slow down aging process, and ultimately extend the lifespan. These theoretical assumptions can be possible to test practically in model organisms like *Drosophila* as a baseline work which could further be extended to other organisms including humans.

Emblica officinalis (common Indian name "amla") and *Curcumin longa* (common Indian name "haldi", in English "turmeric") are very familiar plant derivatives in India and are considered to contain medicinal properties. Turmeric is commonly used in everyday life as a component of spices, and "amla" is very commonly taken as common practice. Both amla and turmeric are known to have high antioxidant property. Amla is known to contain Vitamin C and a number of tannins, whereas turmeric has curcuminoids known to be potential antioxidants. Earlier report has provided evidence of the fact that Amla (*Emblica officinalis*) extract increases life span of tumor bearing animals by up to 60% (Jose *et al*., 2001). However, such evidence is based on very limited studies with small number of animal models (Wiegant *et al*., 2009; Sowjanya Sree and Padmaja, 2008). It is well known that *Drosophila* has served as an important model organism for gaining information in genetical and biological aspects (including gerontological) that have direct relevance to human health (Birney, 2007; Bier, 2005). We hypothesize that if amla and turmeric bear any component that alters lifespan, it can very well be experimented with the *Drosophila* model. Since *Drosophila* share about 70% of its genome similarities with humans, any information from this research could be directly applied in human benefit.

Materials and Methods

Flies of *Drosophila melanogaster* used in the present experiments have been derived from a mass culture already being maintained in our lab since 2009. The flies were grown on standard Maize-yeast medium in the laboratory. For experimental set ups, the regular food media was mixed with turmeric extract in different concentrations (0.25 g/100 ml, 0.5 g/100 ml, and 0.7 g/100 ml). Similarly, for amla, two concentrations were taken, 20 ml/100 ml and 30 ml/100ml. In each case, virgin males and females (of similar age) were collected from the mass culture and three pairs (three males and three females) were transferred to a food vial with different concentrations of either amla or turmeric. In each experimental food vial these three pairs of flies were kept for five days, after which they were transferred to fresh food vials of similar concentrations. Each vial was minutely monitored and the numbers of dead flies were recorded. Each experiment was replicated 35 times (216 flies), and a controlled experiment (without any amla or turmeric) was also run simultaneously. For each experiment, life span of each fly was noted by simply noting the survivability of flies. All these experiments were conducted inside a BOD incubator with constant temperature (25˚C) and other environmental conditions.

Results

Life span with turmeric supplement

Variations in the rate of survivability of *D. melanogaster* flies in each category (with normal and supplemented food) were found and noted down in term of the number of days. For the control and the treated categories (for all the concentrations), the observations were tabulated (Table 1) and plotted (Figure 1). For the food supplemented with different concentrations of turmeric, a marginal increase in mean lifespan of the flies (~3%) at the turmeric concentration of 0.25 gm/100 ml of food in comparison to the controlled experiments was observed. However, at a turmeric concentration of 0.50 gm/100ml of food, a significant increase in life span was observed (Table 1, Figure 1). An

increasing trend of lifespan was also observed in the experimental flies supplemented with 0.7 gm/100 ml of food. However, the increase was not that linear as observed from 0.25 gm/100 ml to 0.50 gm/100 ml of turmeric supplement (Table 1, Figure 1).

Table 1. Survivability rate of *Drosophila melanogaster* flies under different concentrations of food supplements of turmeric and amla.

Survivability	Life span (in days)					
	Control	Turmeric			Amla	
		(0.25g/100 ml)	(0.5g/100 ml)	(0.7g/100ml)	(20 ml/100ml)	(30 ml/100ml)
Minimum	30	32	35	36	33	33
Maximum	36	38	43	44	37	38
Mean	34	35	40	40	36	36

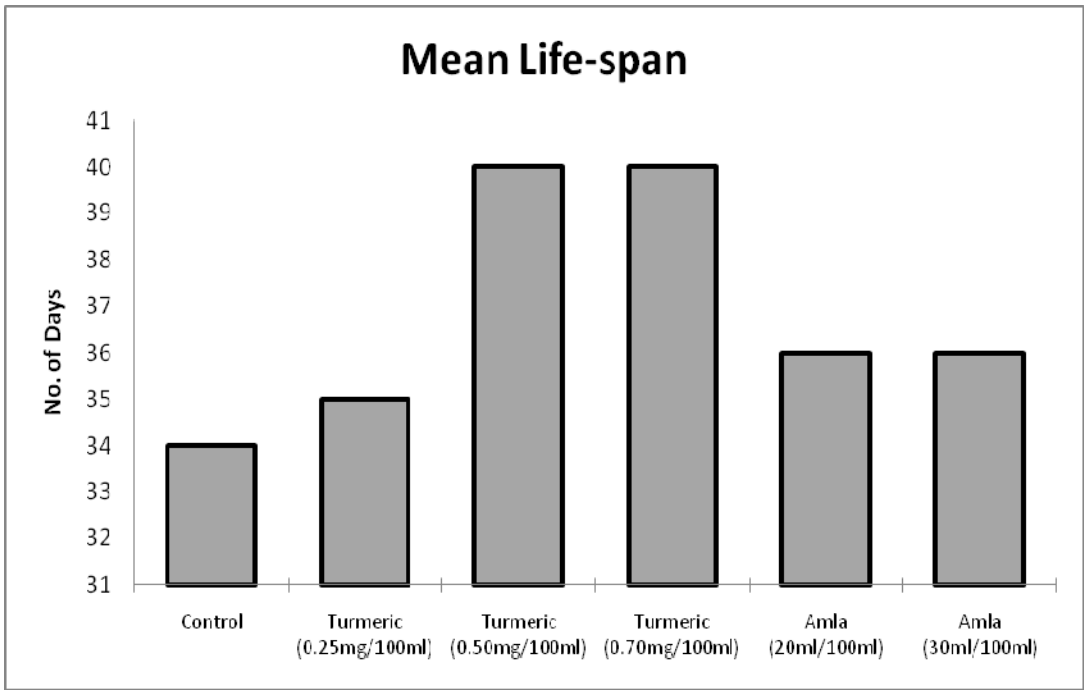

Figure 1. Mean lifespan of *D. melanogaster* flies exposed to different concentrations of turmeric and amla.

Life span with amla supplement

Like in the case of different concentrations of turmeric (see above), *D. melanogaster* flies fed with different concentrations of amla extract were found to affect the rate of survivability but at a lesser extent. At amla concentration of 20 ml/100 ml of food, there was a small increase in lifespan in comparison to the corresponding control lines (Table 1, Figure 1). For a concentration of 30 ml/100 ml, however, no visible increase in the average lifespan could be detected (Table 1, Figure 1). Thus, it is clear from the dataset that while turmeric has a high impact on the survivability rate of *D. melanogaster*, in comparison, amla does not increase the average lifespan of *D. melanogaster* flies to any significant extent.

Discussion and Conclusion

Various concentrations of turmeric and amla were tested for their effect on lifespan of *Drosophila melanogaster*. At lower turmeric concentrations, there was a marginal increase in lifespan of the flies. Higher concentrations increased the life span of flies significantly. Increasing the concentrations further did not show more increase in lifespan. This indicates that there is a particular concentration of turmeric which causes maximum lifespan extension (around 0.5 mg/100 ml of food). Concentrations beyond that do not further increase the lifespan, which may be due to the absorption threshold of turmeric and so, higher concentrations may not get absorbed completely, thereby, having no further effects on the lifespan. The increase in lifespan of the flies due to turmeric can be attributed to the high anti-oxidant properties of turmeric, as has been already documented before. However, observation of a very little effect of different concentrations of amla on the lifespan of *D. melanogaster* signifies that although amla was found to enhance the longevity of *D. melanogaster* flies, it does not bear the same property as of turmeric to drastically enhance the longevity.

Although the exact mechanisms of the action of turmeric and amla by which these plant products are able to enhance the survivability of *D. melanogaster* could not be ascertained from this study, it can be presumed that the results might hold of some significance in humans too. Turmeric and amla are two most commonly used plant products in India and are regularly used in preparation of many traditional herbal-based medicines (Ayurveda). However, enhanced lifespan cannot be attributed to just because of turmeric/amla, as several other factors, like genetic as well as environmental factors (living conditions, life-style, demographic location, pollution and contaminant levels, etc.), might have profound effect on this parameter. Whatever the case may be, it is shown here that turmeric has significant effect on the enhancement of longevity, at least in *D. melanogaster*.

Acknowledgments: We thank the Vice Chancellor, JIIT for extending facilities in the department for carrying out research on *Drosophila*.

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Technique Notes

Climbing assay.

Klosowski, Rafael, and Bernhard T. Hovemann. Ruhr-University-Bochum, Department of Chemistry and Biochemistry, NC5/170, 44780 Bochum, Germany.

Climbing performance is a means to assay overall locomotion either purely driven by geotaxis in red light or by a combination of geotaxis and phototaxis in daylight. We employ a single glass cylinder for the test and videotape the whole process of climbing for 30 seconds with internal time exposure. It avoids falsifying the result from stalling of flies at the interface of the two tubes, which are usually placed on top of each other to separate flies that arrived in the upper part from those which did not. Tiny marks on the glass at fixed distances allow easy calculation of the proportion of flies, which reached a given distance within a time of choice. In addition we cut the wings to prevent flight.

Flies needed

1. Prepare several vials of flies (*e.g.*, 15 age-matched males/vial) for each genotype to be tested. Flies are stored at room temperature inside the test box until ready to test.

Equipment needed

1. Use one Plexiglas test tube stand for 6 glass tubes (height = 20 cm, opening width = 2-3 cm).
2. Parafilm to cover the cylinder opening
3. Black experiment box
4. Light source above the test tube stand
5. Red marker (Edding) for marking climbing distances (we use 10 or 15 cm)
6. Video camera with internal time recording
7. Timer

Climbing assay

1. Cold anesthetize flies and cut wings off.
2. Prepare 15 flies on fresh food the day before.
3. One hour before testing load glass tube with 15 flies, cover the opening with Parafilm and place it into the test tube stand inside the experiment box (Figure 1).

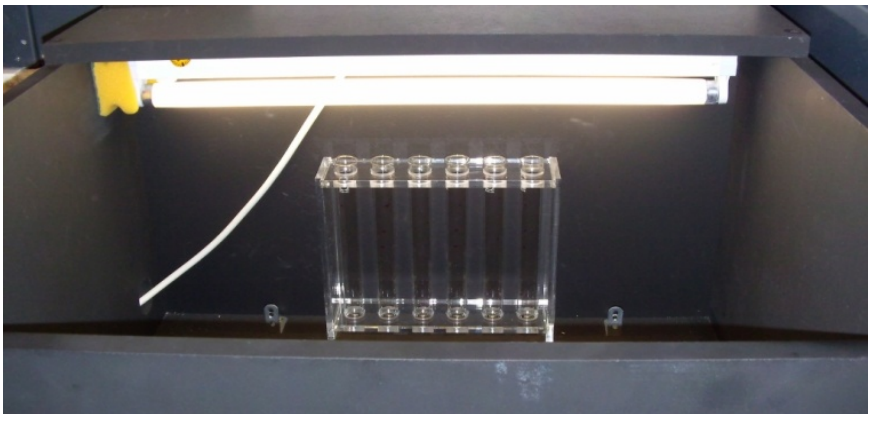

Figure 1.

4. Place the video camera at the appropriate distance to have all 6 vials in focus. Start the recording.
5. Bang flies down to the bottom of the cylinders and start your timer.
6. At 30 seconds, bang the flies down again and repeat this for a total of 6 climbing opportunities. The flies should have 15-20 seconds to climb within each 30-second recording time frame.
7. After a 5 min break, another test run is performed with the same parameters.
8. The camcorder records are fed into a computer for movie presentation.
9. Climbing evaluation is performed by visually counting the flies climbing over the distance mark within a chosen time frame that is visible in the camcorder movie.
10. Data of repeated test series are calculated for statistical relevance.

A convenient method for supplying food to *Drosophila*.

Perry, Catherine[*], Ashley Boseman[*], and Rongsun Pu[#]. Department of Biological Sciences, Kean University, Union, NJ 07083. [#]author for correspondence. Tel: (908)737-3663; E-mail: rpu@kean.edu; * undergraduate student authors and equal contributors to this work.

Commonly used food for *Drosophila* is supplied in the form of dry flakes (for example, Instant Medium by Carolina Biological Company). Cool water is added to the flakes before use in the culture vials. The disadvantage of this method is that the food tends to become very dry in a month or so, and the vials need to be washed extensively before reuse or have to be discarded altogether.

We have found that *Drosophila* food can be supplied in a liquid form to prevent it from drying out. We used cotton balls to soak up highly moistened fly food and put the cotton balls directly in the bottom of culture vials. This method has the advantage of maintaining an adequate amount of moisture in the fly food for up to two weeks without disturbing normal fly development. When the cotton balls get dry, they can be easily taken out with forceps and replaced with new cotton balls soaked with freshly moistened food. This method thus enables the continuing use of the same culture vials for an extensive period of time.

Additionally, if one is concerned about mold and bacteria growth, a low concentration of propionic acid (0.5% by weight) or sodium azide (0.001% by weight) can be included in the moistened food.

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Submissions to Drosophila Information Service are welcome at any time. The annual issue now contains articles submitted during the calendar year of issue. Typically, we would like to have submissions by mid-December to insure their inclusion in the regular annual issue. but articles can be accepted for this volume until 31 December. Details are given in the Guide to Authors or on the DIS web site: www.ou.edu/journals/dis. Very early submissions may be uploaded as “prepublication” files on this web site.

Comparative methodologies for estimating *mariner* activity using *white-peach* assay in *Drosophila simulans.*

Jardim, S.S., and E.L.S. Loreto. PPG Biodiversidade Animal, Biology dep. UFSM, Brazil. sinarajardim@gmail.com.

Introduction

Transposable elements are DNA segments that can be mobilized within and among genomes. These elements have been pointed out as having great impact on the host genome evolution by their capacity to insert into coding or regulatory regions and induce chromosome rearrangements (Giraud and Capy, 1996; Biémont and Vieira, 2006; Feschotte and Pritham, 2007).

The transposable element *mariner* was originally discovered in the promoter region of the *white* gene of *D. mauritiana* causing an attenuated colorless eye phenotype: this mutant has *white-peach* color eyes (Jacobson and Hartl, 1985; Jacobson *et al.*, 1986). That original *mariner* element, called *wpch* (short term for *white-peach*), is an inactive copy, and it is not able to mobilize itself. Posteriorly, it was experimentally transferred to *mariner*-free strains of *D. simulans* and *D. melanogaster* (Garza *et al*., 1991; Bryan *et al*., 1990). The *D. simulans wpch* strain, containing a *wpch* mutation, does not show evidence of excision in somatic cells or germline, because only this inactive *mariner* copy is present in the genome (Hartl, 2001). In the presence of an autonomous *mariner* copy, which could provide the transposase enzyme, the nonautonomous *wpch* element can be removed from the *white* gene promoter causing reversion of mutation to wild condition in some cells, generating a mosaic pattern of eye pigmentation (Lohe and Hartl, 1996).

Once a transposase source can be provided by crossing these *wpch* mutants with flies carrying potentially active copies of *mariner,* the extend of mosaic formation can be utilized for quantification of *mariner* transposition activity (Capy *et al*., 1990; Bryan *et al*., 1990; Woodruff and Thompson, 2001; Picot *et al*., 2008). This method has been used to describe latitudinal clines of *mariner* transposition activity in *D. simulans* natural populations (Giraud and Capy, 1996; Russell and Woodruff, 1999; Picot *et al*., 2008). In general, these studies use the percentage of mosaic males (PMM) observed in the F1 of crosses between *wpch* female and males of strains to be tested.

Another system for quantification of transposable activity has been proposed. It uses a predefined categorization where the number and size of red-color spots are considered, and flies are classified into a determined category (Figure 1). The larger the red area in mosaic eyes, more active the *mariner* elements from testing strains are considered to be (Giraud and Capy, 1996; Bryan *et al.*, 1990; Medhora *et al*., 1988; Hartl, 1989). However, this classification has been done only by visual inspection and can result in imprecise quantification of areas affected by reversions. Another aspect to be considered is that spot size may reflect the moment of reversion during the eye development more than the degree of activity from certain *mariner* element. When an event of *wpch* excision takes place in early developmental stages within a single cell, many other cells will be produced from that reverting cell, forming a great spot (Haymer and Marsh, 1986). Flies bearing those great spots are classified by the pattern methodology as high activity, but in fact may result from only transposition event.

The objective of this study was to compare methodologies used to estimate the *mariner* activity in *wpch* assays using: i) predefined classes; ii) total red area in the mosaic eyes; iii) number of red spots. Additionally, the effect of temperature was evaluated in generating the mosaic eye pattern.

Materials and Methods

For testing, in this study we used *D. simulans* strains (wild phenotype) collected in Brasília (15º44'47"S; 47º 55' 47"O; Brazil) as transposase source, besides the *D. simulans wpch* lineage. Crosses were performed among 10 males (wild phenotype) and 10 females *D. simulans* (*wpch* phenotype), and three temperatures were analyzed. After crossing, vials were stored at 14°C, 20°C, or 28°C until F1 emergence. 8, 25, and 29 replicas for 14°C, 20ºC, and 28°C were done. F1 generation males carrying *wpch* mutation are expected to show mosaic eyes (Jacobson and Hartl, 1985). The rate of mosaic male formation was evaluated (for PMM analysis), and the mosaic flies were classified into the categories shown in Figure 1. Also, both eyes of each mosaic male were photographed, and the total area occupied by red spots was measured using the software Image Manipulation Program – GIMP 2.6.7. In addition, the number of red spots was also quantified.

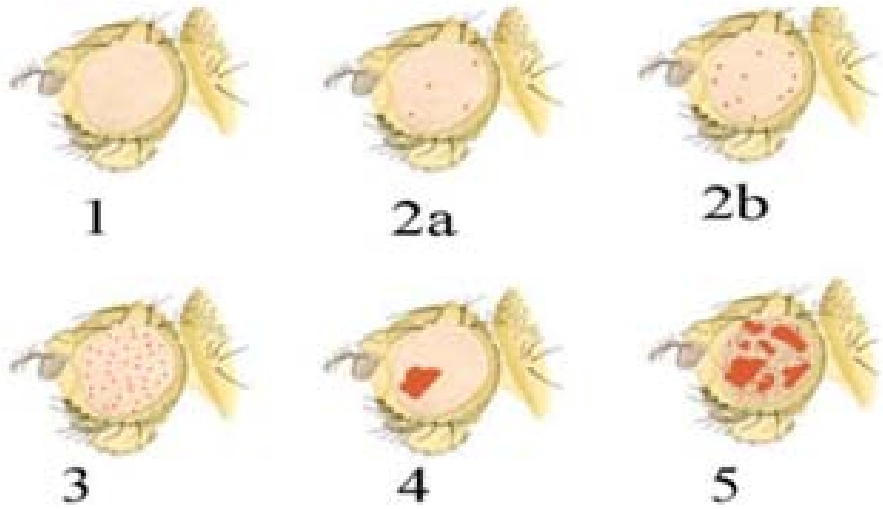

Figure 1. Classification of mosaic males according to standard methodology in six categories.

Results and Discussion

The PMM observed at the analyzed temperatures was: 20.88% at 14°C, 61.47% at 20°C, and 66.96% at 28°C. These results agree with other previous studies that showed the influence of temperature in the rate of *mariner* transposable element transposition (Giraud and Capy, 1996; Russell and Woodruff, 1999; Picot *et al*., 2008). Once high transposition rate occurs in higher temperature, as confirmed by our temperature test assay, all other experiments were performed at 28°C. Every male fly emerged from crossing experiments was classified into one of six categories using the pattern system showed in the Figure 1. Alternatively, the entire red pigmented area was measured for both eyes. As shown in the Figure 2, the standard deviation observed for the pigmented area among classes encompasses the average observed in all categories. These findings show that the use of conventional mosaic categories is not totally effective for quantifying the *mariner* activity in *wpch* crossing assay, once the classes form a continuous array. Besides which, the major critics to both methodologies either the visual classification using the mosaic pattern or the measurement of pigmented area is that the size of spots is seriously affected by the moment of *mariner* excision during fly development.

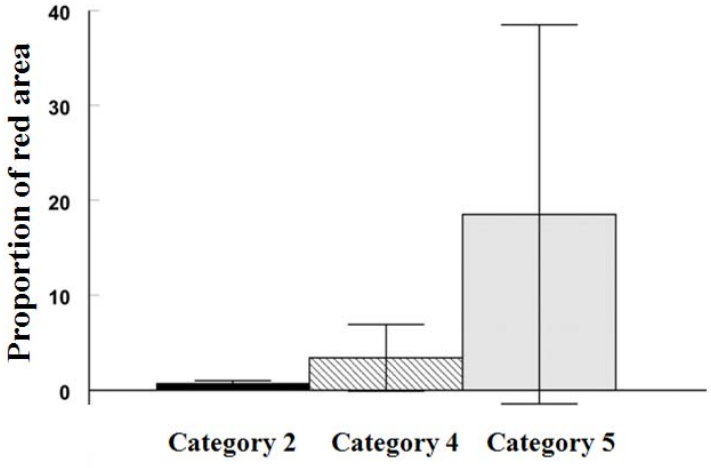

Figure 2. Proportion of red area in mosaic eyes classified in three categories based on pattern methodology, at 28°C.

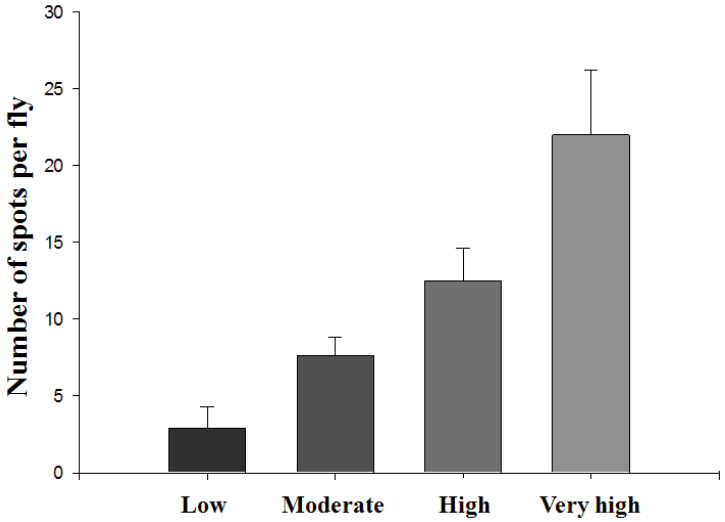

Figure 3. Average number of spots according to the four classes proposed by the new methodology.

An alternative method for quantification of transposition rate was done by counting the number of red spots and comparing it with the standard categorization analysis. The data show that the spot number is more accurate for quantification of transposition activity than area analyzes, because the variation in spot number within categories is relatively smaller than red size area (Figure 3).

We propose that *mariner* transposable activity can be classified into four categories using the number of spots in both eyes. Flies with 1 to 5 spots are classified as low activity. Flies with 6 to 10 spots are classified as moderate activity. Flies with 10 to 15 spots are classified as high activity, and finally flies with more the 15 spots are classified as very high activity. A similar classification was proposed by Capy *et al*. (1990).

When the reversion of *wpch* mutation occurs in developmental early stages, few spots appear, but they form a large pigmented area. When the reversion occurs in later stages, few cells revert back to wild condition per reversion event, giving the small spots (Giraud and Capy, 1996; Bryan and Hartl, 1988; Hartl, 1989). Thus, the number of spots reflects more accurately the transposable activity level of the element, allowing a more detailed understanding of their behavior.

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An instant fly medium and a convenient method to dispense it.

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Introduction

Most small research and teaching laboratories use instant media to grow fruit flies, with Carolina Formula 4-24 being a popular choice, although simpler and less expensive media such as mashed bananas (Bennett, 1961) or instant mashed potatoes (Duenas *et al*., 2002) can also be used. Other than the higher price relative to cooked versions and other types of media (Formula 4-24 is currently $5.13 per lb from Carolina Biologicals in a 50 lb bag), the principal disadvantage of instant medium comes from how it is commonly mixed in the laboratory: the dry food powder and water are added separately to individual vials or bottles, a procedure that will result in variable ratios of dry media to water across the bottles or vials (unless each bottle or vial is individually weighed after the sequential additions of dry medium and water). This is not a trivial concern: dietary depletion – produced by varying the amounts of macronutrients relative to agar – is well known to increase the lifespan of fruit flies, as well as to affect the expression of behaviors, including locomotion. Although the mechanism underlying the lifespan extension produced by dietary depletion is controversial, the end result – longer lived and differently acting flies – is not.

On the practical side, filling even a relatively small number of vials (50-75) in this manner is tedious. To address this problem, Laverty (1986) fabricated a device that will concurrently fill multiple vials with approximately the same amount of dry media, to which water is then added with a repeater pipette, reducing the labor associated with filling vials. Since the amount of dry medium added to each vial will still vary with this apparatus, however, the food concentrations will vary across individual vials. Rather than adding the dry ingredients and water separately into individual vials, mixing the dry ingredients and water prior to dispensing ensures that all vials and bottles will contain media of the same concentration. Described here is a recipe for an inexpensive medium similar to that described by Duenas *et al*. (2002) with physical properties similar to Formula 4-24, as well as a simple and efficient method to dispense rapidly this or any other instant medium by using a vertical screw-type sausage stuffer.

Recipe for potato/yeast/dextrose instant medium for fruit flies.

The dry ingredients are (by weight) potato flakes (75%), yeast flakes (15%), glucose (9.3%), and methylparaben (0.7%). To prepare the media, the dry ingredients and water at 4 times the weight of the dry ingredients are mixed in a large bowl to form a thick paste. Depending on the hydration of the ingredients, more or less water might be needed. Once mixed, the media is dispensed into vials or bottles as described below. At the time of this writing, the total cost of the ingredients is $2.35 per pound, which is substantially less than that of Formula 4-24 and compares very favorably with the cost of a typical cooked medium. An advantage of this instant medium relative to proprietary formulations is that the exact constituents are known, which allows the specific nutritional properties of the diet to be varied as needed, by changing the ratio of yeast and glucose to potato flakes, for example. If freezer space is available, the media could be mixed and frozen in containers, then thawed prior to use. Bulk storage of the mixed dry ingredients is not recommended unless individual use aliquots are made, as there will be separation of the ingredients with time.

The use of a vertical screw-type sausage stuffer to dispense fly food.

Dispensing requires a device with a large enough diameter to allow the dough-like medium to be easily scooped in, a capacity large enough to fill a reasonable number of vials and bottles, and, most importantly, sufficient power to dispense this or another viscous medium. Manual or electric caulking guns and similar devices have been used with simple media, such as mashed bananas (Bennett), but unless the media can be mixed directly inside the cylinder of the gun (difficult given the small diameter of the tubes) filling them with medium is problematic. Also because of their relatively small size, most caulking guns will require repeated refillings as the vials are filled, detracting from any added efficiency afforded by the gun. The Drosophila Species Center (http://stockcenter.ucsd.edu) mixes a saguaro potato media in a resealable plastic bag, cuts off a corner, and dispenses the media into bottles directly from the bag. A similar approach might be used to fill a caulking gun or other device with media, but would probably not work well for the direct filling small bottles or vials, since the media would not be forced to the bottom of the vials by the bag, which would result in food adhering to the sides of the container.

An alternative to these dispensing approaches is the use of a screw-type sausage stuffer, which is normally used to force chopped meat into sausage casings. To dispense with the stuffer, the medium is mixed in a bowl and then scooped into the stuffer cylinder. Turning the hand crank lowers a plunger into the cylinder, forcing the medium out of a dispensing tube into the bottoms of vials or bottles. Since this or any other instant media is very viscous, each turn of the crank results in a fairly continuous rate of extrusion, allowing a single operator to use the device while managing the empty and filled vials and bottles. The author can easily fill ~15 standard 30 mm vials/min with ~12 g media using this device. At the time of this writing, a 5-lb capacity, vertical sausage stuffer (Lem model #606) can be purchased new for about $130. Other models of stuffers would also work, but screw-type stuffers, including the model used here, are easier for a single operator to use than other types.

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Pigmentation scoring method for *Drosophila.*

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Abstract

Melanization in *Drosophila* is complex and varies from species to species. Several methods in practice today quantitate pigmentation in *Drosophila* species, but these existing methods are compromised at certain levels. The majority of drosophilid body tergites show a range of yellow to dark brown pigmentation as well as segment to segment variation, making it difficult to score correctly. We present a method which accounts for these ranges of pigmentation and segmental variability. This method is able to score melanization as well as the area for each abdominal tergite. This method is also applicable to score wing pigmentation features. We hope our method will inspire the fly community to attempt to score species with complex pigmentation patterns.

Introduction

Melanism is diverse across insect taxa and has been reported to be adaptive in numerous fitness related traits (Parkash *et al.*, 2009; Rajpurohit *et al.*, 2008; True, 2003; Wittkopp and Beldade, 2009). There are several techniques used to quantitate melanization, and they are complex and subjective. One such technique developed by David *et al.* (1985, 1990) scores body tergite pigmentation in cosmopolitan *Drosophila melanogaster*. Their method is based on manual scoring. The fly is positioned laterally under a dissecting microscope so the observer can see lateral to dorsal midline. Then a trained scorer assigns a pigmentation score for each segment ranging between 0-10 (0 for non-pigmented segment and 10 for completely pigmented segment). Even though their method shows high repeatable correlations among independent scorers, it requires a highly trained scorer. The method works well with species like *D. melanogaster* and *D. simulans*, which have easily visualized linear patterns across lateral views. Unfortunately other *Drosophila* species do not have such simple patterns.

To avoid these species-specific constraints, abdomen whole mounts spread on glass slides have been used to score pigmentation (Gibert *et al.*, 1998; Hollochor *et al.*, 2000). One particular method developed by Pool and Aquadro (2007) scores only a certain rectangular region in the fourth tergite. The scores are based on pixel density of the region selected. In cases where the total body pigmentation with respect to total surface area is needed for comparison, the Pool and Aquadro method cannot be used.

The methods described so far (David *et al.*, 1985, 1990; Hollochor *et al.*, 2000; Parkash and Munjal, 1999; Parkash *et al.*, 2008; Pool and Aquadro, 2007) either neglect the segment area or total body pigmentation. The existing methods are also inefficient in performing segment size corrections across the samples or populations, which is very important when looking at within species variations (Gibert *et al.*, 1998). There are several cases in *Drosophila* literature where total body area is equally important to segment pigmentation. Populations spread along latitudinal or altitudinal gradients vary in their body size as well as in total body pigmentation (Parkash and Munjal, 1999; Parkash *et al.*, 2008, 2009). In some species each body tergite shows a different kind of temperature reactivity (Gibert *et al.*, 1999).

Here we describe a method to determine pigmentation for a given body tergite, an entire dorsal surface, along with their total area measurements. The user can also target within a segment to obtain gray score and area measurements, while accounting for the variable shades of the segments.

Method

With forceps and a needle remove both wings and then detach the head and thorax from the abdomen. Place the abdomen on a glass slide, dorsal side up, and apply a drop of whole mount material covering the entire abdomen. Place a glass cover slip over the abdomen, allowing the cover slip to fall in the posterior to anterior direction. Press abdomen firmly until all the viscera squeeze out from the open abdominal cavity and all the segments spread smoothly parallel to the glass slide. You can place multiple abdomens on a single glass slide with practice. For wing spot melanin scoring, remove the wing, place it on a glass slide and follow the steps discussed above. Once the slides are dry they are ready to use for imaging. Images can be taken using a digital camera attached to microscope with 2.5× objective lens. On the same magnification an image of a reference scale coded glass slide is needed. Now go to 'ImageJ' software (http://rsbweb.nih.gov/ij/). Open scale image file first and select the distance between any two given points on the scale. Set those measurements as reference, open abdomen image file, and start selecting the segmental areas according to your research interest (Figure 1). When the area is selected in the image, the software simultaneously calculates the actual area and gray score quantitatively.

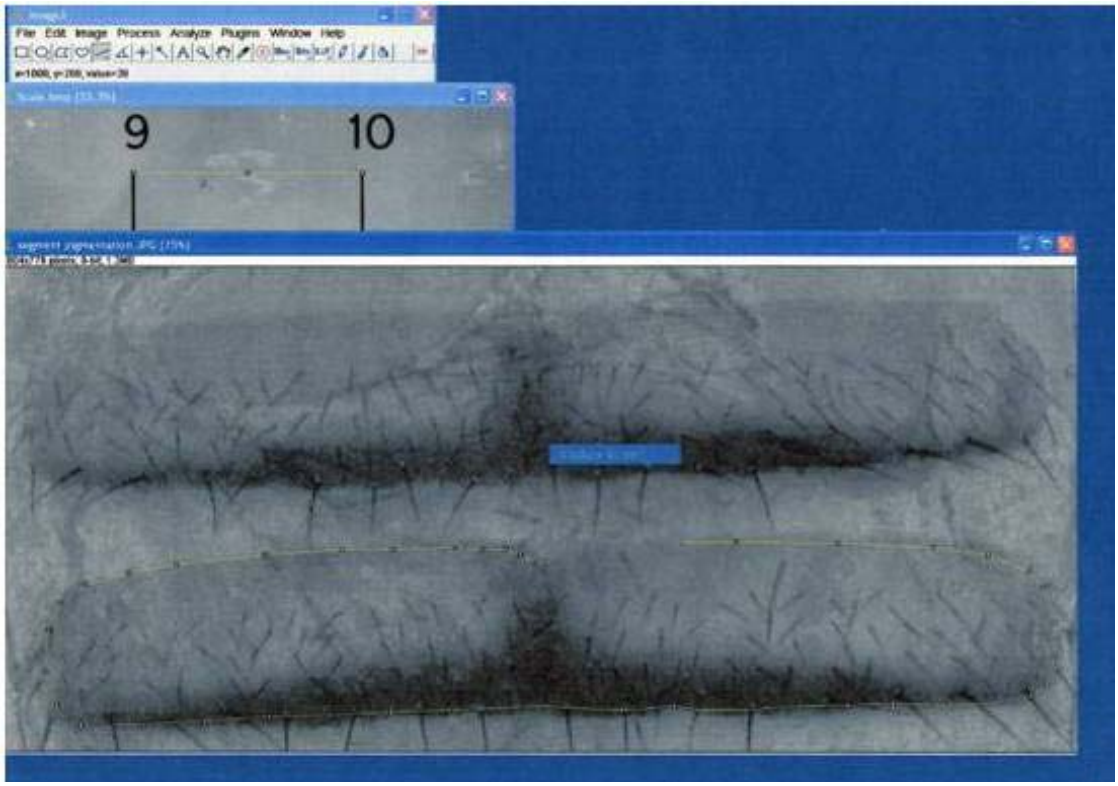

Figure 1. Image J working window and applets showing an area selection for 4th abdominal segment of *D. melanogaster* female (broader view of the actual image is shown in Figure 2a). Selected area is shown in yellow line.

Case Examples

In the Drosophilidae, pigmentation traits, including abdominal pigmentation, wing pigmentation, and sexual dimorphism, have evolved independently in several distantly related evolutionary lineages. Evolution of pigment patterns involves both divergent and convergent changes. In this section we will cover abdominal and wing pigmentation particularly where this technique could be applied for a variety of purposes.

Body tergite melanization

Abdominal melanization constitutes stripes of dark pigment at the posterior region of each abdominal segment; these stripes may be continuous or interrupted, or the pigmentation may appear as spots. Pigmentation patterns also vary between segments (Figures 2a and b). Thus, diverse patterns of pigmentation occur in a variety of evolutionary lineages within the Drosophilidae.

In cosmopolitan *D. melanogaster* each body tergite segment has a black melanin strip, which starts from the posterior region of every segment and covers none to the complete part of the segment. In *D. melanogaster* females, posterior body tergites are darker than their anterior segments (Figure 2a). The data presented in Table 1 show the gray score and area for each body tergite where only dark portion (in a segment) is selected (*D. melanogaster* female). The sum values for gray score and dorsal segmental area for all body tergites (1-7) have also been presented in the Table 1. In *D. melanogaster* females a total of 32.06% of abdominal segmental area is pigmented (example image, Figure 2a). Analyses made on image (shown in Figure 2a) conclude segment 4 as the largest and segment 6 as darkest segment (the lower the gray score, the darker the segment).

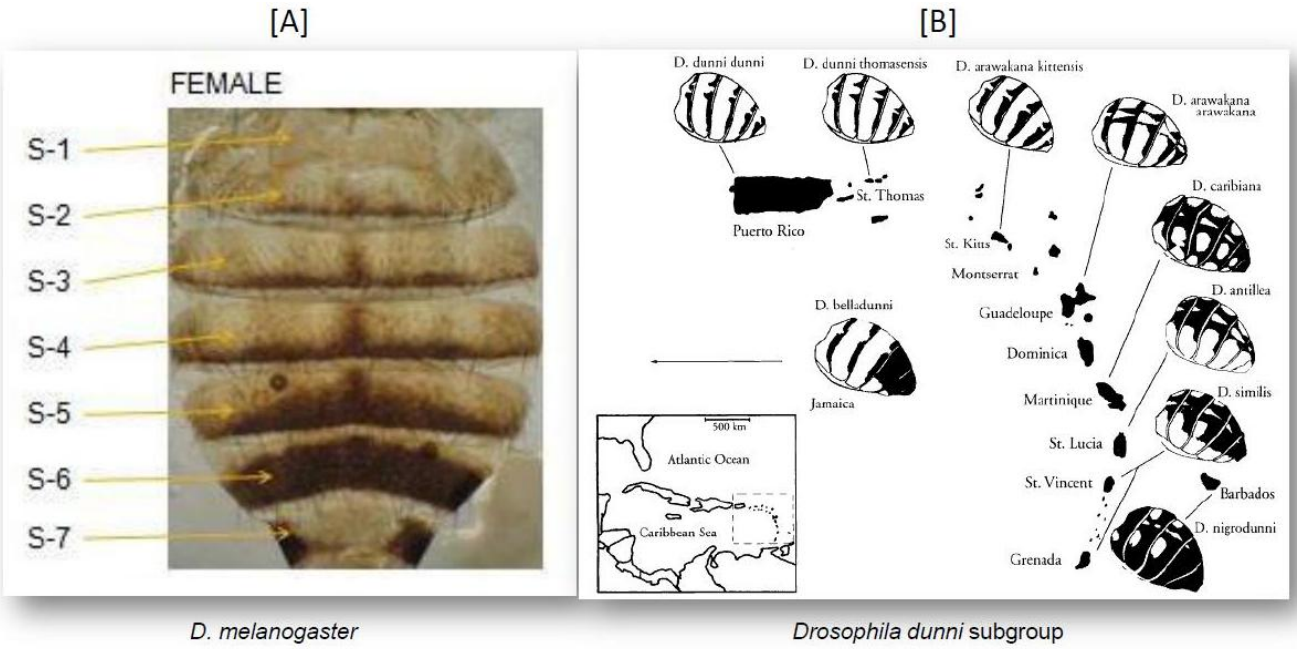

Figure 2. Abdominal pigmentation for females of *D. melanogaster* (A) and each species in the *Drosophila dunni* subgroup (B; adapted from Hollocher *et al.*, 2000).

Table 1. Data on abdominal segment area and abdominal pigmentation for *D. melanogaster* female. Scale of 10380 pixels/mm shown (known distance 0.1 mm). GS = Gray Score; A = Area (mm^2).

Segment	Parameter	Entire segment	Pigmented part	Non-pigmented part
1	GS	103.5	94.8	
	A	0.0015	0.0001	0.0013
2	GS	100.8	79.2	
	A	0.0028	0.0007	0.0020
3	GS	111.3	79.8	
	A	0.0042	0.0010	0.0031
4	GS	111.6	74.5	
	A	0.0048	0.0012	0.0031
5	GS	107.1	68.7	
	A	0.0039	0.0011	0.0027
6	GS	89.2	65.7	
	A	0.0033	0.0019	0.0013
7	GS	108.1	96.6596	
	A	0.0014	0.0006	0.0007
Total	GS	732.0	559.6	
	A	0.0216	0.0069	0.0141

Table 2. Data on total abdominal segmental surface area and their gray score in eight species of *D. dunni* species subgroup. Values are based on a hypothetical reference scale (81 mm) defined between tips of anterior and posterior abdominal region of *D. dunni dunni* female on the acquired image (Figure 2b; from Hollochor *et al.*, 2000). Scale of 9.37 pixels/mm is shown (known distance 81 mm). For David *et al.'s* (1985, 1990) method percent darkness was calculated after adding the values obtained for every segment (sum values are provided). TV = Trained Volunteer.

Species	Method Presented		Based on David *et al*. (1985, 1990) method		
	Gray Score	Area (mm^2)	Sum of body tergites (% Darkness)		
			TV-1	TV-2	TV-3
D. dunni dunni	173.9801	0.4963	20.00	08.57	22.85
D. dunni thomasensis	158.7276	0.4987	21.42	12.85	22.85
D. arawakana kittensis	151.1759	0.4848	28.57	20.00	27.14
D. arawakana arawakana	144.6642	0.5309	30.00	27.14	28.57
D. caribiana	142.9036	0.5838	55.71	54.28	58.57
D. antillea	105.7496	0.5242	25.71	25.71	35.71
D. similis	93.7803	0.5088	58.57	54.28	60.00
D. nigrodunni	50.3080	0.6087	78.57	78.57	75.71

In *D. dunni* subgroup species, the dark strip is not as regular as in the case of *D. melanogaster* females (Figure 2b, adapted from Hollocher *et al.*, 2000). The patterns are irregular and complex and are difficult to measure efficiently with routine available methods discussed in the introduction section. One could score the same parameters (as explained for *D. melanogaster*, Table 1) for the *D. dunni* subgroup or any other species with such complex patterns using our method. The selection scheme remains the same as explained in Figure 1. In Table 2 data on gray score and total abdominal

segmental area is given for eight species of *D. dunni* subgroup which demonstrates a striking variation for pigmentation across the islands of the Caribbean (Hollocher *et al.*, 2000). In this analysis (Figure 2b, Hollocher *et al.*, 2000), *D. nigrodunni* emerges as the darkest species (with lowest gray scores). One can also focus segment-wise gray score and area for *D. dunni* subgroup species (in Table 2, sum values for all segments are given) as presented for *D. melanogaster* in Table 1. Using this method a difference in gray score as minimum as 1.76 pixels/mm (which is statistically significant; data not shown) could be analyzed between *D. arawakana arawakana* and *D. caribiana* (Table 2).

Wing spot

Wing pigment spots occur in highly reproducible, species-specific, two-dimensional patterns, and their genetics and development are beginning to be understood (Edwards *et al.*, 2007). True *et al.* (2003) found that wing spot patterns have two main components: a vein-independent "prepattern" formed during wing development prior to eclosion, and vein-dependent melanization that forms after eclosion. These studies provide the platform required for understanding the evolution of complex pigment patterns in the *Drosophila* species where wing spots are present and their ecological significance is yet to be discovered. Analysis of pattern variation using color-coded overlays of wing photos suggested by Edwards *et al.* (2007) could be more extensively exploited and embedded in our method. We hope our method will inspire the fly community to attempt Hawaiian flies and other species with the same kind of wing-spot patterns to gain further molecular insights into morphological evolution which is deeply associated with behaviors like producing a camouflage or the role of wing-spot display during courtship in some species (*e.g.*, *D. elegans*) (Wray, 2006). The proximal and distal borders of spots (Figure 3) can vary independently, as shown by Edwards *et al.* (2007) in their Hawaiian drosophilids picture database. This suggests that wing patterning genes somehow exert a very flexible, fine-scale control over the pigmentation process. The intensity, size, and numbers of spots may vary among individuals and populations. These minor morphological deviations from spot to spot and species to species can be easily handled using our technique.

Analysis based on Figure 3 wing spots gray score measurements suggest that *D. planitibia* females are significantly different from *D. differens* females ($p < 0.05$) where they do not differ in the area ($P = 0.45$) covered by spots. No other available method can attempt measurements for such spot variation across *Drosophila* species.

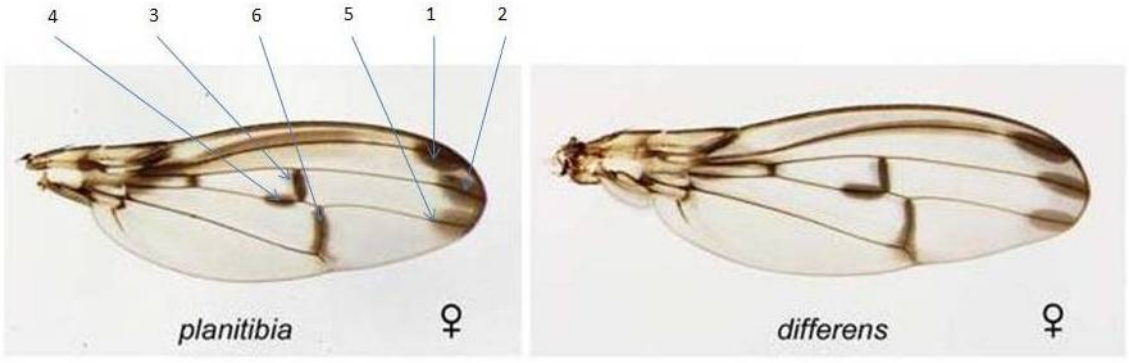

Figure 3. The planitibia subgroup of picture wing species from Hawaiian islands shows spot to spot variation in a single wing as well as species to species variations. Image was acquired from Edwards *et al.*, 2007.

Validation With Existing Methods

In order to validate our method with other available methods, we compared gray score data obtained for eight species of *D. dunni* subgroup (shown in Figure 2b; Hollocher *et al.*, 2000) with David *et al.*'s (1985, 1990) procedure. For this we trained three volunteers in both the techniques (David *et al.*'s, 1985, 1990; ours) and provided the hardcopy for David *et al.*'s method as well as the softcopy for our method of Figure 2b (from Hollochor *et al.*, 2000). The volunteers were asked to score pigmentation for eight species mentioned in Figure 2b. The data obtained from trained volunteers (TV-1, TV-2, and TV-3) are presented in Table 2. The gray score data using our method do not differ across users (Table 2; r = 0.98; authors of manuscript) but significantly differ across the users using David *et al.*'s method. This clearly indicates that user to user variation for assigning a score (0-10) for a given segment significantly affects the analysis. David *et al.*'s method works well (r = 0.97; between two trained scorers) with *D. melanogaster* where body tergite pigmentation is not as complicated as in other *Drosophila* species.

Materials Required

Here we list the materials required for the execution of the method: forceps, needles, glass cover slips, glass slides, dissecting microscope, mounting material, ImageJ software installed on a PC, a reference scale, and a microscope with camera attached.

Limitations

This method gives the user better results in yellow, brown, and black body surfaces, but it is less promising when one is looking at different species for comparisons which may have completely different patterns and colors. Secondly our method can better handle dorsal abdominal pigmentation and wing patterns.

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Technical adaptations of retention traps used to catch drosophilids.

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Introduction

In Brazil, one of the current methods most utilized for the capture of live adult drosophilids in the field is the use of traps based on the model proposed by Tidon and Sene (1988). However, this model presented methodological problems that led to the implementation of structural changes by Klaczko and Medeiros (1999). Nevertheless, some problems remain. In this study, we present innovations aimed to resolve these problems improving the practicality and efficiency of drosophilid retention traps.

Trap Description

For constructing of the proposed model, we use two joined PET two liter soft-drink bottles (Figure 1-A). For a perfect fit, the bottles should have the same format without curves.

The flies retention compartment consists of a whole bottle (Figure 1-B). In the bottleneck there is a wire to hang the traps in the sampling sites (usually trees). Inside the bottle cap there is another wire that anchors a cotton roll wrapped in gauze used by the flies as a landing site (perch). One millimeter holes located next to the bottleneck help eliminate excess moisture inside the bottle (Figure 1-C). The bottom half of this bottle is painted black to induce drosophilids to move toward the translucent region of the bottle, since they have positive phototaxis. In the painted part, there are holes (0.5 cm diameter) arranged in sets of three parallel series allowing for the flies entry (Figure 1-D). These holes are closed with adhesive tape when the traps are removed from the field to prevent the escape of captured specimens. In addition, in the same part of the retention compartment, a wire is traversed from one side of the bottle to the other and tied at each end forming nodes. At the base of the retention compartment, there are five holes about 2.0 cm in diameter, covered by thin mesh screens and fixed with PVC or styrofoam glue. These holes allow for the passage of the bait smell, which is isolated in a specific compartment. This eliminates the direct contact of the flies with the bait (Figure 1-E).

The bottom of the trap is the bait storage compartment. This compartment is completely painted black and it is the bottom half of another bottle. On each side of the bottle there is a wire that is going to be tied to the nodes of the retention compartment when joining the bottles (Figure 1-F).

Advantages of This Model Compared to Previous Models

One of the problems with the Tidon and Sene (1988) model is the flies contact with the bait, since the entry holes are located in the bait compartment. Medeiros and Klaczko (1999) made entry

holes in the retention compartment, and they covered the bait compartment with a piece of panty hose. However, this material proved to be inefficient due to damage caused by coleopterans. In our model, the retention compartment is totally isolated from the bait. Thus, captured flies do not adhere to the bait and oviposition chances outside of laboratory cultures are minimized.

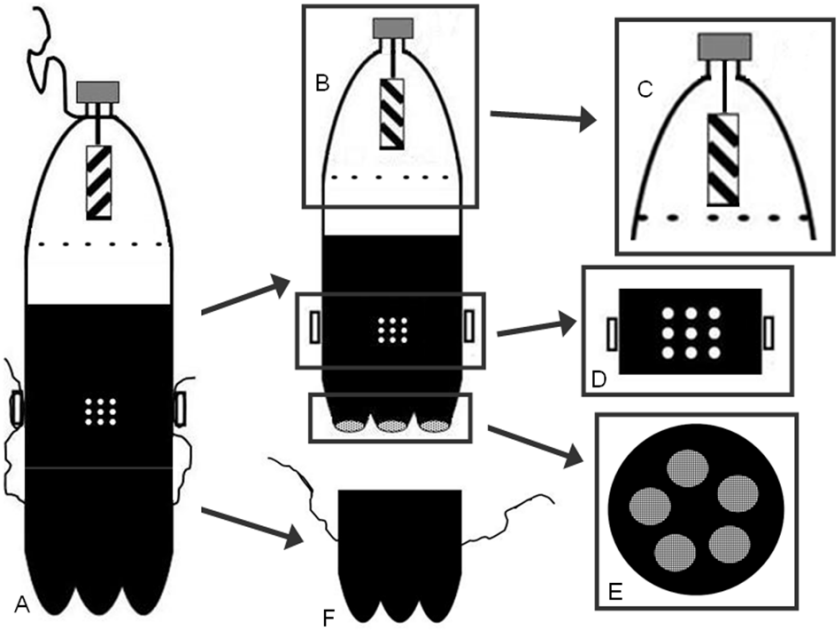

Figure 1. Improved trap model for live drosophilid retention. A: complete trap; B: flies retention compartment; C: perch flies and small holes for moisture elimination; D: holes for flies entry; E: holes for bait smell passage; F: bait storage compartment.

In the previous two models, many specimens escaped due to inadequate joining of the compartments. In our model, there is a tight fit that prevents the flies entry directly into the bait compartment and reduces bait predation by small mammals (*e.g.*, capuchin monkeys, coatis, and marsupials).

Another disadvantage of the previous models is the loss of flies that stay trapped between the funnel and the wall of the upper bottle. In our model, the funnel is unnecessary and its absence minimizes such losses. This still decreases the number of bottles required for the traps manufacture, which saves time and financial resources.

Our trap model is very efficient in retaining drosophilids: Roque and Tidon (2008) obtained 1,050 individuals in a single trap. We recommend its use for field studies collecting drosophilids, considering the abundance of flies caught using our model as well as its technical advantages.

Acknowledgments: We are grateful to the Sobradinho Garbage Collectors Association for the acquired bottles, to F. Chaves for our trap model illustration, to *Universidade de Brasília* for logistical support, and to Capes and CNPq for financial support.

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Submissions to Drosophila Information Service are welcome at any time. The annual issue now contains articles submitted during the calendar year of issue. Typically, we would like to have submissions by mid-December to insure their inclusion in the regular annual issue. but articles can be accepted for this volume until 31 December. Details are given in the Guide to Authors or on the DIS web site: www.ou.edu/journals/dis.

Eyer: Automated counting of ommatidia using image processing techniques.

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Introduction

The *UAS/GAL4* ectopic expression system (reviewed in Brand *et al.,* 1994) is a widely used tool for the overexpression of transgenes in *Drosophila melanogaster*. This bipartite expression system allows for the expression of transgenes in specific tissues, including the *Drosophila* eye using the *glass multiple reporter (GMR)-GAL4* driver line (Freeman, 1996). During this expression, it is of interest to measure differences in morphological characteristics such as ommatidia number, ommatidial area, and overall area of the eye. This measurement is both time consuming and labor intensive given the number of *Drosophila* required for analysis and the limitations of currently available programs. Here, we describe a novel program designed for simple, morphological analysis such as counting and area measurements. Although initially designed for analysis of the *Drosophila* eye, this program could be used for analysis of various tissues, and could be used for other applications apart from expression studies.

Design of Program

Software

The Eyer software was developed as the user interface for a segmentation algorithm optimized for the *Drosophila* eye. It was written for the Windows platform using Visual Studio C# and WPF. The image processing routines used by Eyer are performed by the AForge.NET open-source imaging library and the Vincent-Soille immersion watershed algorithm. The program was developed using images at a resolution of 1.33 pixels per micrometer. This equates to ommatidia that are approximately 20 pixels in diameter at the center of the eye in adult *Drosophila*.

User interface

The Eyer software is a single purpose application, which allows for a completely tailored interface to make the eye analysis task as quick and easy as possible. Instead of opening individual image files, a folder is selected for editing, and all images are shown as a list of thumbnails. The user can switch between active images by clicking on its thumbnail. An image can be easily panned and zoomed via mouse or keyboard. All measurements carried out on an image are automatically recorded in an associated text file and stored with the image in the original folder.

Algorithm

There are several challenges presented to standard segmentation algorithms by the images of *Drosophila* eyes. The convex nature of the eye results in ommatidia size and shape variations across the eye leading to partially obscured ommatidia around the edge. The lighting used when taking

SEM images can vary over the surface of the eye and also from image to image. Light spots and shadows cause the intensity of an ommatidium to be unpredictable. The bristles that grow between *Drosophila* ommatidia reflect intense light, creating further challenges for a segmentation algorithm. These bristles, from an image processing perspective, appear to split many of the ommatidia while following along the edge of others. For these reasons, the goal set for the development of the segmentation algorithm was not to segment the eye completely, but to segment the largest area possible with the highest accuracy. The portion of the eye not segmented by the software is then completed manually.

The solution to the segmentation problem described above required a set of processes that remove color variation and highlight the ommatidia edges uniformly. Once that had been accomplished, an immersion watershed algorithm was used to separate and mark each ommatidium. The complete algorithm can be described in five steps. (1) *Contrast Enhancement:* This is achieved with a combination of histogram equalization and contrast stretching. (2) *Edge Detection:* Both horizontal and vertical edges are detected with a standard edge detection routine. The edge detection results are added to the contrast-improved image to further distinguish the lines around the ommatidia from the centers. (3) *Local Thresholding:* Removal of all color intensity to create a black and white image. The typical conversion method uses a threshold value, where intensities above that value are white, and below are black. Since the intensity of the ommatidia varies over the surface of the eye, an adaptive local threshold has to be used, where the value of the threshold changes for each pixel, dependent on the surrounding area. After this step most edges are visible; however, many ommatidia still appear joined. (4) *Watershed:* The watershed algorithm is able to separate connected blobs. This step processes the image as if it is a topographic relief, identifying locations where water would pool if poured onto the image. (5) *Blob Analysis:* The resulting watershed lines are then used to segment the eye. The list of segmented objects is filtered by size and shape by a blob analysis package to determine which objects are valid ommatidia.

Sample Analysis and Discussion

Drosophila

Flies of various genotypes were aged three days past eclosion and frozen at –80 °C. Flies were mounted, desiccated overnight, and coated in gold before photography at 170 times magnification with a Hitachi S-570 scanning electron microscope as per standard methods.

Area

To measure area, or to segment an image for counting purposes, the region of interest (ROI) must be defined by the user. The ROI tool creates a point based polygon where points can be easily added and removed while defining the area. The polygon can be made with as many points as needed, resulting in a clearly defined area without the labor intensive process of using a freehand tool. The total area of the eye is calculated in real time and recorded in the associated text file as mentioned.

Counting

The resolution of the image affects the performance and speed of the segmentation required for counting. Higher resolutions will result in a higher segmentation percentage, with a reduction in speed.

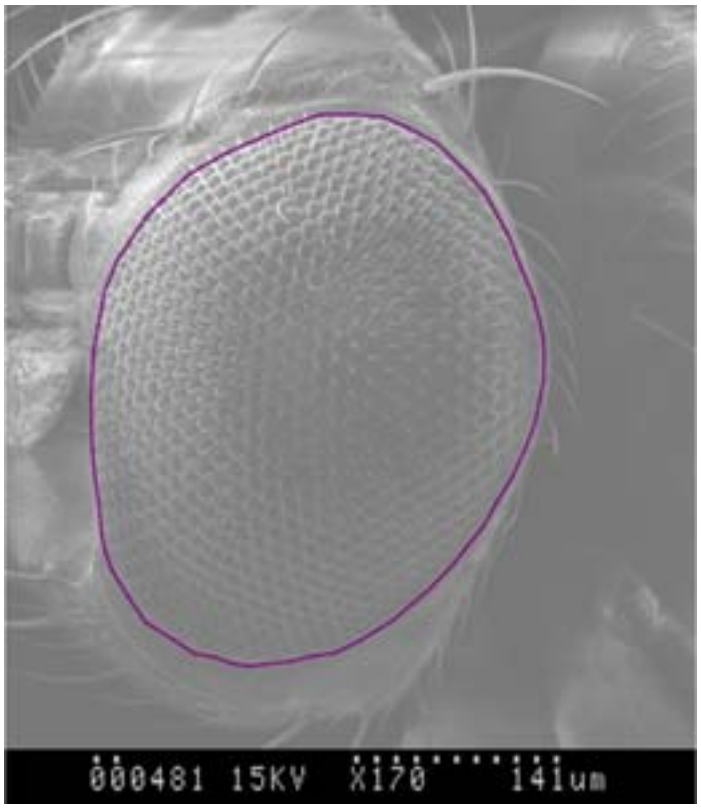

Figure 1. Original image with user-defined ROI.

The contrast enhancement performed by Eyer can be applied to the entire image separately to improve the visibility of detail on the subject. This was found to be useful when examining features such as the overall area of the eye.

After the segmentation process, identified ommatidia are marked with red dots. In this example, segmentation took 4.8 seconds to complete, and it completed with 561 of 688 ommatidia correctly marked. Four dots were placed incorrectly. The four false positives and remaining unmarked ommatidia can be completed manually before the results are saved.

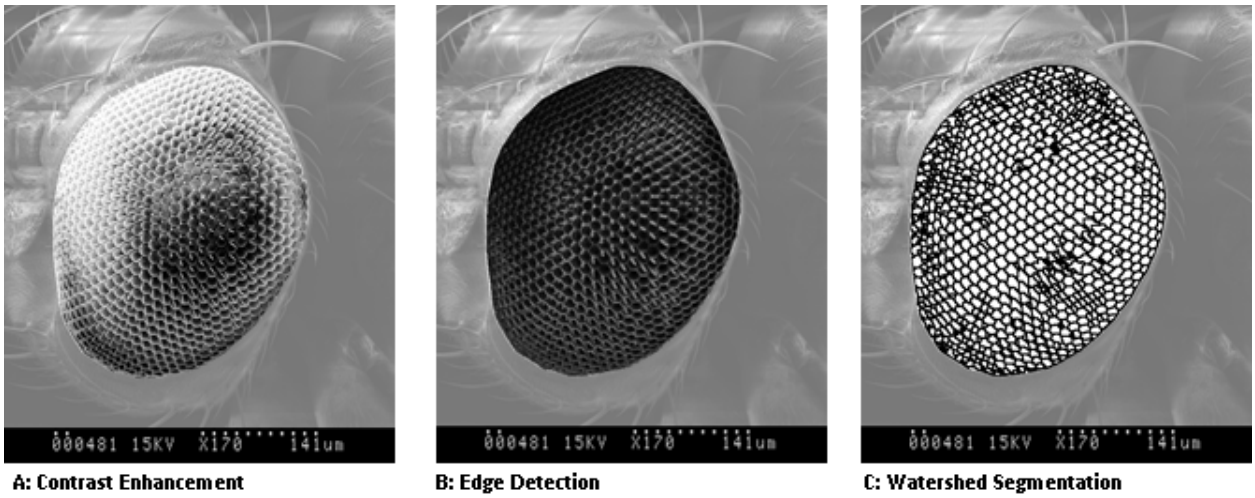

Figure 2. Image processing stages.

Conclusions

Morphological analysis in *Drosophila* is widely used during expression studies to investigate gene and protein function. In the *Drosophila* eye, measurement of characteristics such as ommatidia number, ommatidial area, and area of the eye are often time consuming and labor intensive. Our novel program, Eyer, has been shown to be a valuable tool for simple morphological analysis of the

Drosophila eye. This program could reduce the time needed for morphological analysis of various *Drosophila* tissues, as well as tissues in other organisms.

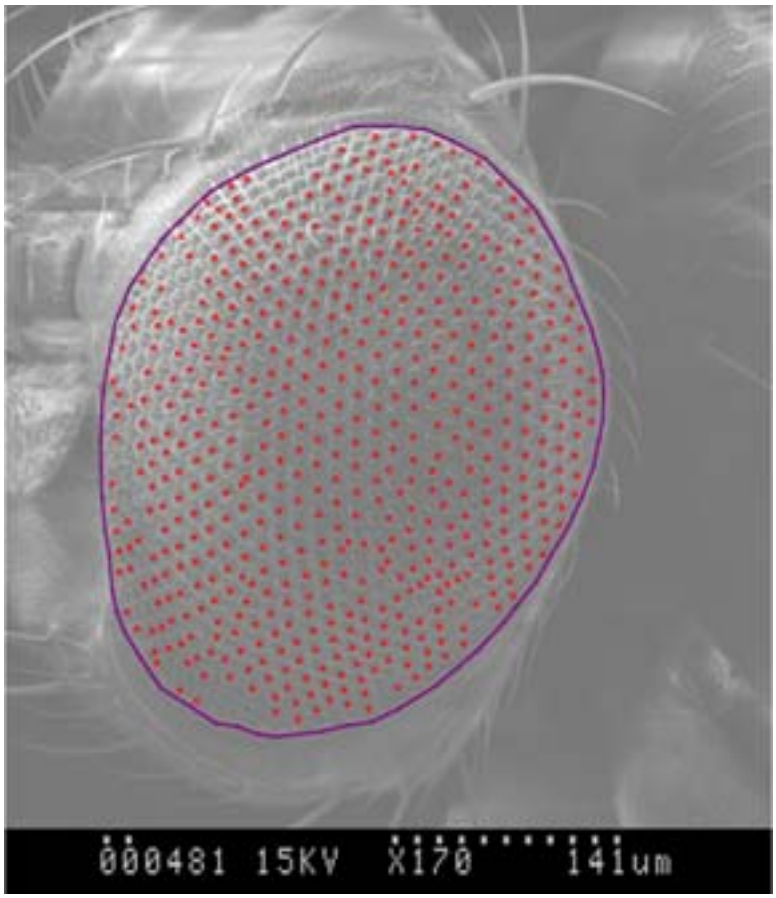

Figure 3. Identified ommatidia marked by Eyer.

References: Kirillov, A., 2011, Aforge.NET: Framework. Retrieved from http://www.aforgenet.com/framework; Brand, A.H., A.S. Manoukian, and N. Perrimon 1994, Methods Cell Biol. 44: 635-654; Freeman, M., 1996, Cell 87: 651-660; Prajdić, M., 2008, Watershed Image Segmentation in C#. Retrieved from http://weblogs.sqlteam.com/mladenp/archive/2008/02/11/Watershed-Image-Segmentation-in-C.aspx; Vincent, L., and P. Soille 1991, IEEE Transactions on Pattern Analysis and Machine Intelligence 13(6): 583-598.

Call for Papers

Submissions to Drosophila Information Service are welcome at any time. The annual issue now contains articles submitted during the calendar year of issue. Typically, we would like to have submissions by mid-December to insure their inclusion in the regular annual issue. but articles can be accepted for this volume until 31 December. Details are given in the Guide to Authors or on the DIS web site: www.ou.edu/journals/dis.

Novel method for determining chromosome compaction and DNA content of salivary gland nuclei in *Drosophila*.

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Introduction

For years the endoreplicating nuclei of *D. melanogaster* salivary glands has been a useful tool for researchers interested in investigating the mechanisms of chromosome condensation and DNA replication. These nuclei complete ten successive rounds of replication by the time they are third instar larvae reaching an average ploidy of 1024n (Edgar, 2001). In addition to endoreplication, salivary gland nuclei achieve their distinctive banding pattern by aligning multiple copies of sister chromatids along their lengths and adopting a level of condensation similar to interphase chromosomes (Lee, 2009). The ability to quantitate easily the level of chromosome compaction in normal and polyploid tissues is lacking in the literature. Techniques developed previously to examine chromosome compaction include fluorophore hybridization to chromosome arms (Dej, 1999) or simply categorizing defects by level of severity (Pflumm, 2001). These methods, while useful, are labor intensive and potentially ambiguous.

Described in the following is a novel technique capable of quantitating the compaction ratio of salivary gland polytene chromosomes. Using the Qubit® dsDNA assay kit in combination with our novel method for determining the volume of DNA, we are able to establish the quantity of DNA per unit volume ($pg/\mu m^3$) – compaction ratio. This technique could be useful for molecular biologists in the quest to understand the dynamics of DNA replication and chromatin formation.

Materials and Methods

Drosophila Stocks

The w^{1118} line was obtained from the Bloomington Stock Center (Flybase ID: FBst0006326). The PCNA (Proliferating Cell Nuclear Antigen) mutant strain *mus209*^{2448}*/CyO* was also obtained from the Bloomington Stock Center (Flybase ID: FBgn0005655). Stocks were maintained at 25°C on *Drosophila* Diet Medium K12 (US Biological Cat # D9600-07B).

Tissue Acquisition

Wandering third instar larvae were collected and placed in a 16 well dissecting dish containing 100 µl of 1× PBS (140 mM NaCl, 2.7 mM KCl, 1.4 mM Na_2HPO_4, 1.8 mM KH_2PO_4). Salivary glands were isolated using No.5 tweezers (Electron Microscopy Sciences, Hatfield, PA). Once salivary glands were dissected, they were transferred to a separate holding well containing 100 µl 1× PBS.

Fixing Tissue and DAPI Staining

After acquiring the desired number of salivary glands, glands were transferred into a new well containing 100 µl of 4% formaldehyde in 1× PBX (1× PBS with 1% Triton X-100) and allowed to incubate for 20 minutes at room temperature. After 20 minutes, salivary glands were moved into another well containing fresh 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) solution (diluted from 3

µg/ml 100× DAPI stock with 1× PBS) for 5 minutes in order to stain the DNA. After 5 minute incubation, salivary glands were removed, washed twice immediately in 1× PBX for 5 minutes, followed by one 45 minute wash, and one 10 minute wash at room temperature. During the final 10 minute wash, prepare slide as directed below:

Slide Preparation and Tissue Mounting

Using a 20cc syringe equipped with a 22 gauge blunt fill needle filled with Vaseline®, two lines of Vaseline® were dispensed the width of the slide (Fisherbrand® 25 × 75 × 1.0 mm, Cat. No. 22-034-486) about an inch and a half apart. In the space between the two lines of Vaseline®, 30 µl of Vectashield® Mounting Medium (Cat. No. H-1000, Vector Laboratories, Burlingame, CA) were dispensed along the length of the slide. When the final 10 minute wash was complete, the salivary glands were transferred to the Vectashield®. A coverslip (Fisherfinest®, 22×50-1, Cat. No. 12-548-5E) was gently placed on top of the slide being careful to avoid air bubbles. With the coverslip on the slide, the two lines of Vaseline® were gently tapped to lower the coverslip making sure the entire area between the two lines of Vaseline® was taken up by Vectashield®.

Imaging Salivary Gland Whole Mounts

Slides were imaged using 20× magnification on an Olympus IX81 Motorized Inverted Microscope with Spinning Disk Confocal equipped with Slidebook™ software (Slidebook™ V4.2). Salivary gland nuclei are three dimensional structures and because of this, it was necessary to create a three dimensional image using the Z-stack feature of the microscope. The Z-stack images were created using 18-20 1.5 µm steps depending on the thickness of the salivary gland. Salivary glands are also rather large and take up multiple fields of view requiring a montage to accommodate the entire gland in one image. Each image was acquired using epi-fluorescence with a DAPI filter. Each field of view was then aligned using Slidebook™ software and a maximum projection image was created. Images were saved as .tiff files for analysis using Adobe® Photoshop® elements CS5.

Salivary Gland Nuclei Size Analysis

The first step to determining salivary gland nuclei volume is to set the appropriate parameters in Photoshop®. To account for the difference in pixel length between Slidebook™ and Photoshop®, the measurement scale in Photoshop® was adjusted. In the measurement scale setting pixel length was set to 1 and the logical length was set to .8 to give a scale factor of 1.25. The wand tool sensitivity (used to select individual salivary gland nuclei) was set to 80. With the parameters set, individual nuclei in a gland were selected and the measurements recorded. Measurements were exported as .txt files and transferred into Excel® spreadsheets.

Determining Average Volume of Salivary Gland Nuclei

Statistical analysis was performed using Minitab® 14 Statistical Software. Area and circularity measurements for each genotype were imported into Minitab® and the mean, standard deviation, and N were recorded. To control for false points resulting from the wand tool analysis, data points lying more than one standard deviation above and below the mean area were removed. With respect to circularity, any data points lying more than one standard deviation below the mean were removed. This middle 68% of the data is a representation of the true mean area and circularity. The mean, standard deviation, and N of the data was then recorded and used to determine DNA volumes. Using the area of a circle equation ($A = \pi r^2$) the area of each data point was converted into a radius. Next, using the volume of a sphere formula ($V = 4/3\ \pi r^3$) values for each radius were

converted into volumes. Volume measurements were then transferred into Minitab® where the mean, standard deviation, and N were calculated.

Salivary Gland Nuclei Counts

Using the counter tool available in Photoshop®, nuclei counts were taken from ten individual salivary glands. The number of nuclei for each gland was then averaged.

Salivary Gland Digestion and DNA Extraction

The salivary glands of third instar wandering larva were dissected in 150 µl of HyQ® Graces's Unsupplemented Insect Cell Culture Medium (Cat. No. 30610.01, HyClone, Logan, UT) and transferred to a holding well also containing 150 µl of Grace's. Once the desired number of salivary glands were acquired in the holding well, glands were transferred to PCR tubes (Fisherbrand®, Cat. No. 14230225) prefilled with 3-5× 1 mm glass beads (BioSpec Products, Inc., Cat. No. 11079110) along with 300 micron glass beads (Sigma®, 212-300microns Unwashed, Lot. No. 033K1546) and 25 µl of squishing buffer (20 µg/ml proteinase K, 10 mM Tris-base, 25 mM NaCl, and 1 mM EDTA). Each tube received one pair of glands. PCR tubes were then vortexed at max speed for 15 seconds and spun to collect liquids. PCR tubes were then placed in a thermocycler (C1000™ Thermo Cycler, Biorad®) and incubated at 37°C for 30 minutes then heated to 85°C for 10 minutes. After incubation, the PCR tubes were vortexed for 15 seconds and centrifuged at 12,000 rpm for 2 minutes.

Note: At this point extracts can be frozen at -20°C until desired number of digestions has been completed.

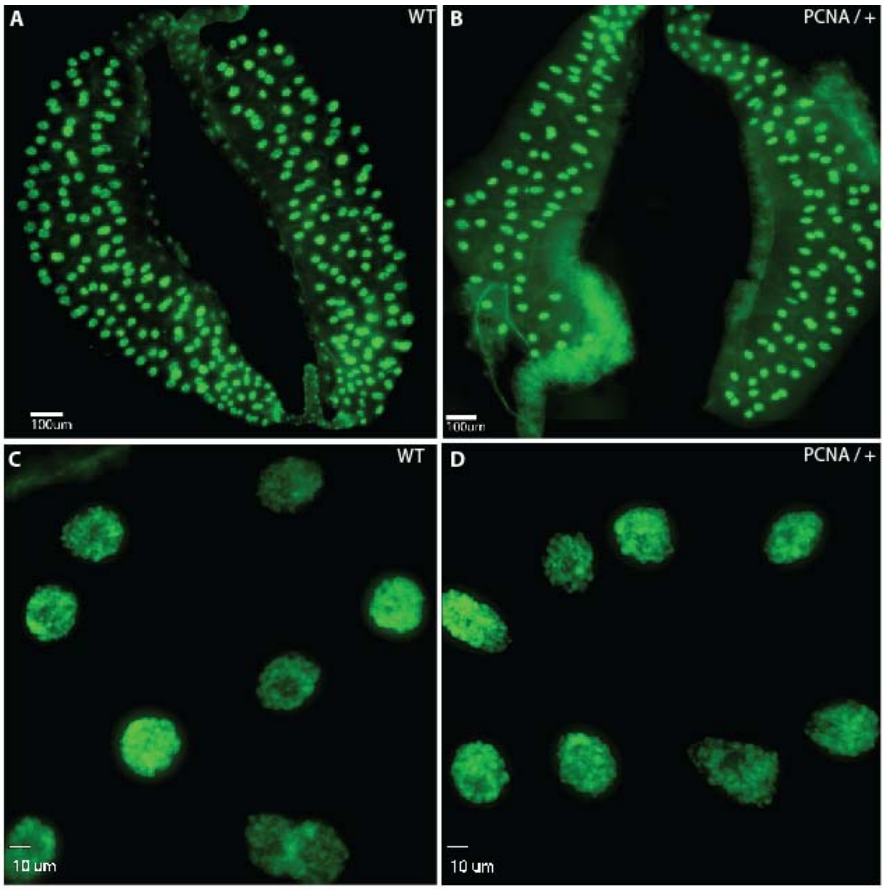
Salivary Gland DNA Quantitation

DNA content values were determined using the Qubit® dsDNA HS Kit (Qubit® dsDNA HS Assay Kit, Invitrogen™, Cat. No. Q32854) along with the Qubit 2.0 Fluorometer™ (Invitrogen™, Cat. No. Q32866). Qubit® working solution was prepared by diluting the Qubit® reagent 1:200 in Qubit® buffer. 190 µl of Qubit® working solution was transferred to Qubit® assay tubes (Invitrogen™, Cat. No. Q32856) along with 10 µl of salivary gland DNA extract. After Qubit® working solution and salivary gland DNA extract was loaded, each tube was gently vortexed to mix and spun for 10 seconds to collect liquid at the bottom of each tube. DNA content values were determined using the Qubit® 2.0 Fluorometer. The Qubit® 2.0 Fluorometer was standardized using two standard solutions provided in the Quant-iT™ dsDNA HS Kit.

Results

Epi-fluorescence imaging and Photoshop® analysis is sufficient to determining nuclear DNA volume and total salivary gland nuclei

In order to establish the compaction ratio of polytene chromosomes, it was first necessary to determine the volume of DNA present in each nucleus. Epi-fluorescence images of WT and PCNA/+ salivary gland whole mounts stained with 4',6-diamidino-2-phenylindole (DAPI) were acquired using z-stack and montage features available in Slidebook™ (Figure 1A and B). Representative images of the two dimensional projection image used for size analysis can be seen in Figure 1C and D. Using the wand tool feature available in Photoshop®, the area of DNA for each nucleus was recorded (data not shown). As described in the materials and methods, the volume of nuclear DNA was derived using the area of a circle equation to determine the radius followed by the volume of a sphere equation. Using this method, we were able to determine that there is an 81.21% reduction in the average volume of PCNA/+ nuclear DNA compared to WT (Figure 1F). Volumes for PCNA/+ and



E

	Genotype	
	WT	PCNA / +
Volume of Nuclei	13116 μm^3 ± 209	10652 μm^3 ± 152
# of Nuclei Per Gland	116.4 ± 5.34	117.6 ± 4.23
DNA Content (ng / gland Pair)	22.04 ± 2.23	14.24 ± 1.10
DNA / Nuclei (pg)	94.6 pg	60.05 pg
Packing Ratio (pg/μm^3)	0.0072	0.0056

F

	Volume of Nuclei (μm^3)	# of Nuclei Per Gland	DNA Content (ng/gland pair)	DNA (pg) Per Nuclei	Packing Ratio (pg/μm^3)
PCNA / + relative to WT	81.21% (p < .000)	101% (P = .852)	64.6% (P = .003)	63.50%	78%

Figure 1. Data derived from salivary gland polytene chromosome size analysis and DNA content quantitation. Panels A and B: representative images of salivary gland montages. Panels C & D: two dimensional maximum projection images of nuclei used for size analysis. Panel E (top): average volume of nuclei DNA for each genotype (T = 9.53, P < 0.000, N = 356 WT and 357 PCNA/+). Panel E (second from top): the total number of nuclei in each gland averaged from glands (T = -.18, P = 0.862, N = 10). Panel E (third from top): DNA content (pg) per nucleus. Panel E (bottom): compaction ratio of polytene chromosomes. Panel F: percents relative to WT (p-values depict significance).

WT were 10,653 μm^3 and 13,116 μm^3, respectively (Figure 1B upper panel). The results of a two-sample t-test confirmed the difference in volume to be significant with T = 9.53 and P < 0.000. The N for WT was 356 and PCNA/+ 357. Total nuclei counts were completed using the montages as well (Figure 1B, second panel from top). There was no significant difference in total number of nuclei between the two genotypes (T = -0.18, P = 0.852).

Determining salivary gland DNA content

Spectroscopic analysis using A260/A280 is usually sufficient in determining the quantity of DNA in a sample. Unfortunately, the results of this method are easily influenced by the presences of interfering molecules like proteins. As an alternative to performing chloroform DNA extractions on each pair on salivary glands (which would be both costly and time consuming), we used the Qubit® dsDNA HA kit. This kit is selective for dsDNA over RNA and is not influenced by salts, free nucleotides, or protein that remained in the sample after extraction. Our results showed PCNA/+ mutants have an average of 14.24 ng of DNA per gland pair compared to 22.04 ng seen in WT; a 63.5% reduction (Figure 1E, third panel from top). T-tests show the reduction in DNA content to be significant: T = 3.12 and a P = 0.003 (N = 49 for both genotypes). Having established the average number of nuclei per gland and the DNA content per gland pair, we were able to infer the average DNA content per nucleus. On average, PCNA/+ nuclei contain 60.05 pg of DNA per nucleus, while WT contain 94.6 pg, a reduction of 63.5% (Figure 1E and F). Using volumes derived from the size analysis in conjunction with DNA content data, we are able to deduce the compaction ratio of the polytene chromosomes. The packing ratios of PCNA/+ and WT polytene chromosomes were determined to be 0.0056 pg/μm^3 and 0.0078 pg/μm^3, respectively, a 78% reduction.

Discussion

Endoreplicating tissues provide a unique system to study defects in DNA replication. In endoreplicating tissues (such as the salivary glands in *D. melanogaster*), cells undergo successive rounds of DNA replication to reach an average ploidy of 1024n (Edgar, 2001). Using this ploidy, we were able to calculate the theoretical quantity of DNA present in salivary gland nuclei. The *Drosophila* genome (n) is estimated to be around 180Mb. Of this 180Mb, 70% is euchromatin that reaches an average ploidy of 1024n and 30% is tightly packed heterochromatin that remains at 2n (Gall, 1973). Using these ploidy values and multiplying by the average atomic mass of a nucleotide (330Da), we determined the theoretical mass of salivary gland nuclei to be 1.2×10^{11} Daltons or 135.3 pg. This number probably represents an overestimate as it has been demonstrated that within a particular gland there are different levels of DNA content. Nuclei near the distal tip tend to contain more DNA as compared to those near the duct (Park, 2008). Our estimation of 94.6 pg of DNA/nucleus in WT is in good agreement (30% different) with the theoretical estimates of DNA content of 135.3 pg per nucleus. Put another way our estimates put the average ploidy of WT nuclei at ≈716n which is right in between the 512n and 1024n that would compromise the last two rounds of endoreplication.

Techniques previously described for measuring DNA content involved the A260/A280 ratio derived from spectroscopic analysis (Yanjuan Xu *et al.*, 2009). In our analysis, using the A260/A280 ratio resulted in inflated DNA content values due to the presence of interfering proteins (our unpublished data). As an alternative to labor intensive and costly chloroform purification to remove proteins, we found a method for quantitating DNA concentrations not influenced by the presence of common contaminants. The Qubit® dsDNA HS kit uses a fluorophore that binds selectively to

dsDNA eliminating interference from free nucleotides, salts, and proteins. Using this method to quantitate the amount of DNA per gland pair then dividing by the total number of nuclei, we determined that on average wild type nuclei contain 94.6 pg of DNA. Testing the validity of our methods, we chose to perform the analysis on *Mus209*^{2448}, a PCNA mutant.

PCNA is a protein clamp that functions as a processivity factor allowing DNA polymerases δ and ε to move with the replication fork (Scalfini, 2007). Using BrdU incorporation assays, recent research has shown *Mus209* to be defective for DNA replication (Pflumm, 2001). Using the methods described, we have been able to further the findings of Pflumm *et al.* and quantitate the reduction in DNA synthesis in endoreplicating salivary gland nuclei attributed to the PCNA/+ mutant. We show PCNA/+ mutants to have a 63.5% reduction in DNA content per nucleus and an 81.21% reduction in average volume relative to wild type.

It is generally accepted that organisms can grow either by increasing their cell size or cell number. In the terminal tissues of *D. melanogaster* salivary glands, the short term need for copious amount of saliva has been met by increasing the ploidy of nuclei. Research in plant cells has shown a direct link between ploidy levels and cell size (Kondorosi, 2000). Here we show our technique is sensitive enough to detect minor differences in both DNA content and volume. We confirm and extend the findings of Pflumm *et al.* where they analyzed homozygous *Mus209* mutant salivary glands and showed a decreased DNA synthesis, by showing that salivary glands from heterozygous larvae are also defective for endoreplication.

The overall reduction in nuclei size observed in the PCNA/+ larvae is not solely due to a reduction in DNA content. If it were the case, then we would expect that the compaction ratio would be unchanged compared to WT. Instead we observe a lowered compaction ratio that would be indicative of condensation defects. It is interesting to note that in addition to its role as a processivity factor, PCNA has been shown to be involved in chromatin restoration and *de novo* histone deposition on newly synthesized DNA (Groth, 2007). As part of the histone restoration process, PCNA directly interacts with Caf-1 which recruits histone methyltransferases (HMTase) responsible for the methylation of histones tails, which are usually a determinant in the formation of transcriptionally inactive euchromatin and heterochromatin (Weaver, 2008). The reduced compaction ratio seen in the *Mus209* mutant is possibly a consequence of the failed recruitment of HMTase's resulting in improperly condensed chromatin.

In summary, we have created a novel technique capable of quantitating minute differences in the DNA content and volume of individual nuclei. These two values in combination allow us to ascertain the compaction ratio of chromosomes. This technique provides an easy, highly reproducible, and cost effective alternative to similar methods for quantitating levels of condensation. Moreover, this technique can be easily adapted to other research models where tissues with a finite number of nuclei can be dissected.

Acknowledgments: We would like to thank the Imaging Core Facility house in the East Carolina University Department of Biology.

References: Dej, K.J., and A.S. Spradling 1999, Development 126: 293-303; Edgar, B.A., and T.L. Orr-Weaver 2001, Cell 105: 297-306; Gall, B.B., 1973, PNAS 70(5): 1359-1363; Groth, A.R., 2007, Cell 128: 721-733; Kondorosi, E., F. Roudier, and E. Gendreau 2000, Curr. Opin. Plant Biol. 3: 488-492; Lee, H.D., 2009, Genes and Development 23: 2461-2477; Park, S.Y., 2008, PNAS 105: 12343-12348; Pflumm, M.F., and M.R. Botchan 2001, Development 128: 1697-1707; Scalfini, R.H., 2007, Annual Review of Genetics 41: 237-280; Weaver, R.F., 2008, *Molecular Biology*, 4th edition. New York: McGraw-Hill; Yanjuan Xu, *et al.*, 2009, PLoS ONE 4(7): 1-13.

Protocol: Salivary Gland Whole Mounts with Size Analysis

Reagents

1× PBS (140 mM NaCl, 2.7 mM KCl, 1.4 mM Na_2HPO_4, 1.8 mM KH_2PO_4)
4% formaldehyde in 1× PBX (1× PBS with 1% Triton X-100)
1 µg/ml DAPI in 1× PBS

Equipment

Slides (Fisherbrand® 25 × 75 × 1.0 mm, Cat. No. 22-034-486)
Vectashield® Mounting Medium (Cat. No. H-1000, Vector Laboratories, Burlingame, CA)
Coverslip (Fisherfinest®, 22×50-1, Cat. No. 12-548-5E)

Dissecting Salivary Glands and DAPI Staining

1. Using a 16 well dissecting dish or two well depression slide, dissect 3rd instar wandering larva salivary glands in 100 µl of 1× PBS.
2. Dissect five pairs of salivary glands for a total of 10× glands. Try to extract salivary glands as a pair – keeping them attached to each other.
3. After dissections, move salivary glands into a second well filled with a 4% formaldehyde solutions in PBX (1× PBS with 1% Trition X-100) for 20 minutes (make fresh).

***Helpful Hint**: Be careful. After fixation the salivary glands become very rigid and will break easily when transferring from well to well from this point on.*

4. After 20 minutes, move salivary glands into another well with 1 µg/ml DAPI solution for 5 minutes (make fresh).
5. After 5 minute DAPI incubation, move salivary glands into a well with 1× PBX and wash twice for 5 minutes each wash.
6. After two immediate 5 minute washes, move salivary glands into a well with 1× PBX and wash for 45 minutes followed by a final 10 minute wash.
7. During final 10 minute wash, prepare slide as follows:
   a. Use a syringe to dispense two lines of Vaseline® the width of the slide, about an inch and a half apart.
   b. In the middle of the space between the two lines of Vaseline®, dispense 30 µl of Vectashield® mounting media along the length of the slide.
8. When the final 10 minute wash is complete, transfer the salivary glands into the Vectashield®.
9. Gently place coverslip on the slide and tap the two lines of Vaseline until the Vectashield takes up the entire area between the two lines of Vaseline.
10. Seal the coverslip around the edges with nail polish to prevent Vectashield® from evaporating.
11. Image, using procedure below.

Imaging Salivary Gland

1. Salivary gland preps were imaged on an Olympus IX81 Motorized Inverted Microscope with Spinning Disk Confocal.
2. Locate salivary glands using 10× objectives and DAPI eyes filter.

3. Once the first gland you are attempting to image is in the field of view, move to the 20× objective and focus on a nucleus.

4. Change scope setting to either confocal mode or epifluorescence mode with a DAPI filter and put the central most part of the salivary gland in the field of view.

5. Since the salivary gland is a three dimensional image, it is necessary to take an image from multiple focal planes. This type of image is usually referred to as a z-stack.

**Helpful Hint: Defining the step of your z-stack between 1-1.5 µm, is generally a good idea. Steps sizes lower than 1 µm result in too large of a file for some imaging software.*

6. With the three dimensional parameters of the z-stack set, it is necessary to utilize the montage feature of your imaging software, since salivary glands take up multiple fields of view.

7. After the montage parameters are sent, move the field of view back to central most portion of the salivary gland.

8. Set the ND filter and Intensity to settings that allow for low exposure times.

**Helpful Hint: When imaging several different slides, make sure you use the same ND filter setting and Intensity settings.*

9. Adjust the exposure time and take the picture.

10. Save each montage as its own file for analysis later.

Analyzing Salivary Gland Pictures Using Adobe® Photoshop®

1. Use the wand tool to select individual nuclei. You want to make sure there are no gaps around the parameter of the nuclei. Not having a good fit on every nucleus will artificially inflate the area of your nuclei resulting in larger volumes.

**Helpful Hint: In our attempts, we find that a wand tool setting of 80 provides the best results.*

2. With all of the nuclei of one salivary gland selected, record measurements and export data.

a. Data for each measurement will appear. You will need to delete the first data point each time as it is a total of all the data points.

b. I recommend setting up a folder dedicated solely to exported files. Photoshop® will export both a data folder and .txt file.

**Attention: Be sure to adjust the measurement scale in Photoshop® to account for the pixel length used by your imaging software.*

Determining Average Area and Volume for Nuclei

1. Compile the data points for area and circularity into one spreadsheet.

2. Determine the mean, standard deviation, and N for both area and circularity.

3. With the parameters above defined, remove points that are beyond one standard deviation away from the mean area; remove points that lie one standard deviation below the mean circularity.

- This will allow us to select the middle 68% of the data each time in addition to eliminating false points that can be acquired during the size acquisition.

4. After you have eliminated points for each of the genotypes, again determine the mean, standard deviation, and N.

5. With the area of each nucleus determined, use the area of a circle formula ($A = \pi r^2$) to turn the area into a radius for each point. With a radius for each point, you can use the volume of a sphere formula ($V = 4/3\ \pi r^3$) to determine the volume of each nucleus.

Protocol: Salivary Gland DNA Extraction and Quantitation

Reagents

100 ml Squishing buffer (- Proteinase K) (10 mM Tris-Base, 25 mM NaCl, 1 mM EDTA, dH_2O to 100 ml)
Proteinase K Stock solution (200 µg/ml dissolved in dH_2O)
HyQ® Graces's Unsupplemented Insect Cell Culture Medium (Cat No. 30610.01, HyClone, Logan, UT)
Qubit® dsDNA HS Kit (Invitrogen™, Cat. No. Q32854)

Equipment

0.2 ml PCR tubes (Fisherbrand®, Cat. No. 14230225)
1 mm glass beads (BioSpec Products, Inc., Cat. No. 11079110)
212-300 micron glass beads (Sigma®, 212-300microns Unwashed, Lot. No. 033K1546)
Thermocycler (C1000™ Thermo Cycler, Biorad®)
Qubit® assay tubes (Invitrogen™, Cat. No. Q32856)
Qubit® 2.0 Fluorometer (Invitrogen™, Cat. No. Q32866)

DNA Extraction

Preparation

1. Obtain desired number of 3rd instar larvae and wash them in a 16 well dissecting dish with dH_2O to remove food particles.
2. Mix enough squishing buffer with Proteinase K to aliquot 25 µl to each of the dissected salivary glands.
3. Store squishing buffer at 4°C until ready to use.

**Attention:* *Squishing buffer should be stored in the 4°C refrigerator without Proteinase K. Proteinase K stock solution should be stored at -20°C. Dilute stock solution of Proteinase K to 20 µg/ml with squishing buffer.*

4. Place ~3-5 1 mm glass beads along with 300 micron glass beads (just enough to cover the bottom) in a PCR tube.

Dissection and Salivary Gland Digestion

5. Transfer larva from the washing well to a new well containing 150 µl Grace's dissecting medium.
6. Fill a separate "holding well" with 100 µl of Grace's.
7. Dissect out the salivary glands from each larva and transfer to the "holding well."
8. Once you have dissected out the desired number of salivary glands, aliquot 25 µl of squishing buffer into each PCR tube containing glass beads.

****Helpful Hint****: Limit the amount of time from the start of the dissection of the salivary glands to their placement into PCR tubes to the number of salivary glands that can be dissected in 25 minutes.*

9. Place one set of salivary glands into each PCR tube.

****Helpful Hint****: Make sure that the salivary glands come off of the tip of the tweezers. If salivary glands sit in Grace's for too long they tend to become sticky.*

10. Vortex the PCR tubes at max speed for ~10 seconds (pulse).
11. Briefly spin PCR tubes to collect the entire sample in the bottom.

12. Place the PCR tubes in thermocycle and run cycle (heat block will also work).
    a. Incubate at 37°C for 30 minutes followed by 10 minute incubation at 85°C.
13. Remove PCR tubes from thermocycler and vortex again for ~10sec (pulse w/ vortex); spin to collect sample.
14. At this point you can freeze samples at -20°C until desired number of digestions have been completed.
15. After thawing, or if you continue from Step 12, centrifuge samples for 2 min. at 12,000 rpm.

DNA Concentration Quantitation using Qubit® dsDNA HS Kit

1. Prepare the proper amount of Qubit® working solution by diluting the Qubit® reagent 1:200 in Qubit® buffer.
    a. 190 µl of working solution is required for each sample and standard.
    b. 1 µl × N # of samples = X µl of Qubit® Reagent
    c. 199 µl Qubit® buffer × N # of samples = X µl of Qubit® Buffer
2. Aliquot 190 µl of Qubit® working solution into Qubit® ultra-clear assay tubes.
3. Dilute DNA extract 1:20 in Qubit® working solution.
    a. 10 µl of DNA extract into 190 µl of Qubit® working solution.

****Attention**:** *Be sure to make samples with Standard #1 and Standard #2 to standardize the Qubit® Fluorometer each time.*

1. Vortex all tubes for 2-3 seconds to mix and spin to collect sample at the bottom of the tube.
2. Incubate the tubes for ~2 minutes at room temperature.
3. Select proper program, dsDNA HS, on Qubit® Fluorometer and standardize machine.
4. Read tubes in the Qubit® Fluorometer.

****Helpful Hint**:** *You can automatically calculate the concentration of your original sample by selecting "calculate sample concentration" → GO → 10µl → GO.*

The Citric-Arabic-glycerated gum: An alternative to Fourè gum.

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Introduction

The conservation of biological material for analysis of results in different areas of biology is a process of great importance. Making a microscopic preparation involves enclosing the material of study between two sheets of glass: the slide serving as a support, and the coverslip, used to cover the material.

The material can be mounted in air or in a liquid medium. In the first case material is solidified by cooling or by evaporation. Another side, when the material is put in liquid medium, the preparation has to be locked to avoid mounting fluid evaporation and subsequent drying of the material. In all cases, the object must be placed between the slide and coverslip so that the preparation remains uniformly flat and the front lens of high magnification objectives do not touch

the liquid. The flattening of the material also improves the quality of the image and ease of approach. One of the important features of the mounting medium is the visibility index or index of refraction, which changes according to degree of product purity and origin of the constituents (Sheehan and Hrapchak, 1980; Locquin and Langeron, 1985).

The mounting types are classified into temporary and permanent. There are different hydrophilic mounting media depending on the kind of ingredientes used: glycerinated gelatin, gum chloral, chloral gelatin, and gum syrup of Apathy among others.

Chloral gum, also known as Faure liquid or liquid glycerine Hoyer, has been used for the preservation of different structures and agencies (Upton, 1943). In 1983, Graf *et al*. recommended the use of the Faure's solution (gum arabic 30 g, glycerol 20 ml, chloral hydrate 50 g, water 50 ml) to mount the wings from adults of the fruit fly, in order to analyze the induction of somatic mutation and recombination in the wing cells.

Currently, chloral hydrate is a compound with restricted access so that its availability has been affected. Due to the known preservative properties of the chloral hydrate, we look for an alternative ingredient that remains unchanged in the mounting gum characteristics.

The Citric Acid Monohydrated is a preservative broadly used in industry and pharmacy (Loutit *et al*., 1943; Nielsen and Arneborg, 2007).

Materials and Methods

Faurè – Modified Recipe

Arabic gum 30 g (CAS 9000-01-5, Reasol, IT), Higroscopic glycerin 20 ml (CAS 5681-5, Omnichem, IL), Citric Acid Monohydrate granular 50 gr (CAS 5949-29-1, Omnichem, IL), Distilled Water 50 ml.

The ingredients are weighed separately and mixed dry. Water is added and all is put under constant stirring for 24 h (Rheostat Corning, model PC 351) in the absence of light. The content is then stored in amber bottles.

This mounting liquid has been used for preserving different parts of the flies' body: genital plates, head, thorax, legs, and other structures.

Actually, we make the wing's slides for the Somatic Mutation and Recombination Test (Graf *et al*., 1983) using this gum and after two years, the slides are kept in perfect condition.

References: Graf, U., H. Juon, A.J. Katz, H.J. Frei, and F.E. Würgler 1983, Mutation Research 120: 233-239; Locquin, M., and M. Langeron 1985, *Manual de Microscopia*. Editorial Labor, S. A. Mexico, 372 pp.; Loutit, J.F., P.L. Mollison, and I.M. Young 1943, BMJ 2: 744; Nielsen, M.K., and N. Arneborg 2007**,** Food Microbiology 24(1): 101-105; Sheehan, C.D., and B.B. Hrapchak 1980, *Theory and Practice of Histotechnology*, The C.V. Mosby Company. 480 pp.; Upton, M.S., 1943, Experimental Physiology 32: 183-202.

Mutation Notes

A recessive lethal gene, *l-Cy*, found in a natural population of *Drosophila melanogaster*.

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The second chromosome balancers, SM1, SM5, and SM6, have been used as a most useful tool to study the genetic variations in natural populations of *Drosophila. melanogaster* (Ives, 1945; Wallace, 1946; Mukai, 1964; Kosuda and Moriwaki, 1971). These balancers suppress recombination in almost the whole region of the second chromosome, since they have complicated inversions both in the right and left arms. They are lethal in the homozygous condition, and they also have a dominant mutant, *Cy*, and several recessive mutations (Lindsley and Zimm, 1990). Homozygous lethality has been explained by the presence of In(2LR) or *Cy* itself. However, the cause of lethality is not necessarily clear. Recently, the author has found a recessive lethal gene in a natural population, which is allelic to these balancer chromosomes. In this short communication the author reports that the lethality of these balancers in the homozygous condition is due to a recessive lethal gene.

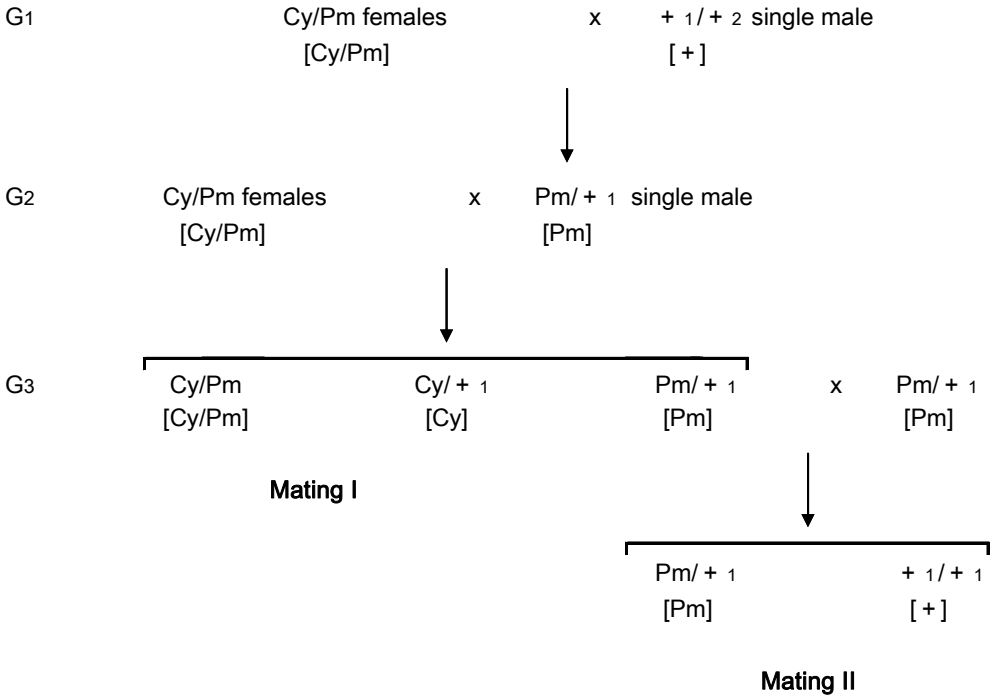

Figure 1. Mating scheme. If + 1 chromosome is bearing a recessive lethal, *l-Cy*, all the progenies in the G3 of Mating I are only *Cy*/*Pm*, and the progenies in G4 of Mating II are only *Pm*, since *Cy*/+ 1 is dead.

Table 1. Progeny from Mating-I, Cy/Pm x Pm/C-1367

	Cy/Pm	Pm	Cy
	86	106	0
	40	41	0
	22	40	1
	113	129	1
	82	84	0
	112	112	0
	125	126	0
	104	109	0
	67	87	1
	153	143	2
	142	150	0
	116	125	0
	132	136	0
	71	83	2
	101	115	1
Total	1466	1586	8

Table 2. Progeny from Mating II, Pm/C-1367 x Pm/C-1367.

	Pm	+
	59	2
	60	0
	52	1
	70	0
	71	0
	72	1
	134	0
	141	1
	111	3
	100	3
	155	0
	138	1
	146	0
	163	2
Total	1472	14

Mating-I in Figure 1 was repeated and the number of progenies in generation 3 is given in Table 1. As clearly shown in Table 1, one of second chromosomes sampled from a natural population in Szentendre, Hungary, was found to be lethal in combination with the second chromosome balancers. This lethal chromosome and lethal gene were tentatively named C-1367 and *l-Cy*, respectively. It should be noted that it was impossible to detect *l-Cy*, if *Cy* males were singly mated with *Cy/Pm* females in generation 2. The result clearly shows that C-1367 is carrying a recessive lethal gene, although it is not complete. *Pm* males in generation 3 of Mating-I were also mated with *Pm* females repeatedly. The result shown in Table 2 indicates the same conclusion, that *Cy*/*l-Cy* is substantially lethal and the viability of Cy*/l-Cy* is less than 0.005 of *Pm* /*l-Cy* heterozygote. Several wild type phenol-types appeared in the progenies in this mating, because *Pm* chromosome is not complete as a balancer. These results indicate that C-1367 has a lethal allele, *l-Cy*, and this lethal allele is concealed in the second chromosome balancers in the heterozygous condition, and that *l-Cy* is completely linked with *Cy*.

References: Ives, P.T., 1945, Genetics 30: 167; Kosuda, K., and D. Moriwaki 1971, Genetics 67: 287; Lindsley, D.L., and G.G. Zimm 1990, *The Genome of* Drosophila melanogaster; Mukai, T., 1964, Genetics 50: 1; Wallace, B., 1956, J. Genetics 54: 280.

Ovary phenotype and expression of *bab1* and *bab2* paralogs in the larval ovary of two mutants of the *bab* locus in *Drosophila melanogaster*.

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Introduction

The *bric-à-brac* locus (*bab*, Godt and Laski, 1995) is composed of two evolutionarily related genes, *bab1* and *bab2* (Couderc *et al.*, 2002), that play important roles in various developmental processes and sex-specific differentiation (Sahut-Barnola, *et al.*, 1995; Godt, *et al.*, 1993; Barmina and Kopp, 2007; Randsholt and Santamaria, 2008; Kopp *et al.*, 2000; Williams, *et al.*, 2008). In

particular, *bab* function is necessary for organogenesis of the *D. melanogaster* ovary (Godt and Laski, 1995), involving both *bab1* and *bab2* paralogs (Couderc *et al.*, 2002). However, *bab1* and *bab2* have different expression patterns in the larval ovary, since *bab2* is expressed in all somatic cells whereas *bab1* is restricted to the terminal filament (TF) cells that form the tip of the ovariole (Couderc *et al.*, 2002).

In an attempt to assign specific functions to each paralog, *bab* alleles were classified according to associated defects in legs as well as in ovarian morphogenesis and fertility, in correlation with expression levels of *bab1* and *bab2* gene products (Couderc *et al.*, 2002). Two mutants of the *bab* locus, $bab1^{P}$ and $bab2^{E1}$, were shown to affect specifically *bab1* and *bab2*, respectively (Couderc *et al.*, 2002), and to display a strong phenotype in the ovary. The $bab1^{P}$ allele corresponds to a *P*[lacZ] insertion in the first intron of *bab1*, whereas $bab2^{E1}$ is an EMS-induced mutation. Available antibodies allow the detection of BAB1 and BAB2 by tissue immunostaining, and only BAB2 on Western blot. In $bab1^{P}$ mutants, no BAB1 protein was detected and BAB2 protein levels were unaffected. In $bab2^{E1}$ mutants, BAB2 was undetectable by tissue immunostaining and almost absent on Western blot, and BAB1 levels were unaffected (Couderc *et al.*, 2002).

The recessive ovary phenotype of $bab1^{P}$ and $bab2^{E1}$ mutants is characterized by a reduction of the size of the ovary (Couderc *et al.*, 2002), which is stronger in $bab2^{E1}$ than in $bab1^{P}$ mutants. This result suggested that the two paralogs are necessary for organogenesis of the ovary. Results of rescue experiments of *bab* mutants using transgenes expressing either *bab1* or *bab2* cDNAs suggested that the two paralogs play redundant functions, *bab2* being, however, more important than *bab1* in ovary morphogenesis (Bardot *et al.*, 2002).

Aiming to characterize further the possible differences between *bab1* and *bab2* functions, we compared the structure of the ovary in $bab1^{P}$ and $bab2^{E1}$ mutants and found that the reduced ovaries in these two mutants are in fact very different structurally. In parallel, we quantified the levels of BAB1 and BAB2 proteins in $bab1^{P}$ and $bab2^{E1}$ mutant larval ovaries by immunofluorescent confocal microscopy and showed that both alleles affect the expression of both paralogs.

Results and Discussion

The ovary phenotype of $bab1^{P}$ and $bab2^{E1}$ mutants was compared to that of bab^{AR07}, a deficiency that covers both *bab1* and *bab2*. As previously described (Couderc *et al.*, 2002), the size of the ovary was reduced in all three mutants (Figure 1B-D) compared to that of the wild-type Canton-S control flies (Figure 1A). However, the structure of the ovary differed significantly between the three mutants. In wild-type Canton-S flies, ovarioles are characterized by the presence of a germarium at their distal tip (Figure 1A', arrowheads). In both $bab1^{P}$ and bab^{AR07} ovaries, the whole structure of the ovary was affected. In $bab1^{P}$ mutants, germaria and ovarioles were in most cases indistinguishable, though egg chambers at various stages of oogenesis and even mature eggs were observed in rare cases (Figure 1B'). In bab^{AR07} mutants, ovarioles were not distinguishable (Figure 1D'). In addition, mature eggs were never observed, although disorganized egg chambers appeared to be present (Figure 1D' and inset). In contrast, $bab2^{E1}$ mutant ovaries contained about 20 ovarioles (Figure 1C'), as is observed in Canton-S flies. However, $bab2^{E1}$ mutant ovarioles appeared to contain only early stages of oogenesis (before stage 9) and mature eggs were rarely observed (Figure 1C'). These results clearly differentiate the phenotype of $bab1^{P}$ and $bab2^{E1}$ mutant ovaries: both are reduced in size, however likely through different mechanisms, since $bab1^{P}$ mutant ovaries lack ovarioles, whereas $bab2^{E1}$ mutant ovaries contain a wild-type number of ovarioles in which oogenesis is blocked early. If the previous characterization of these two *bab* mutations is taken into

account, the results of the present phenotypical analysis would lead us to conclude that *bab1* is required for the formation of ovarioles and *bab2* for oogenesis.

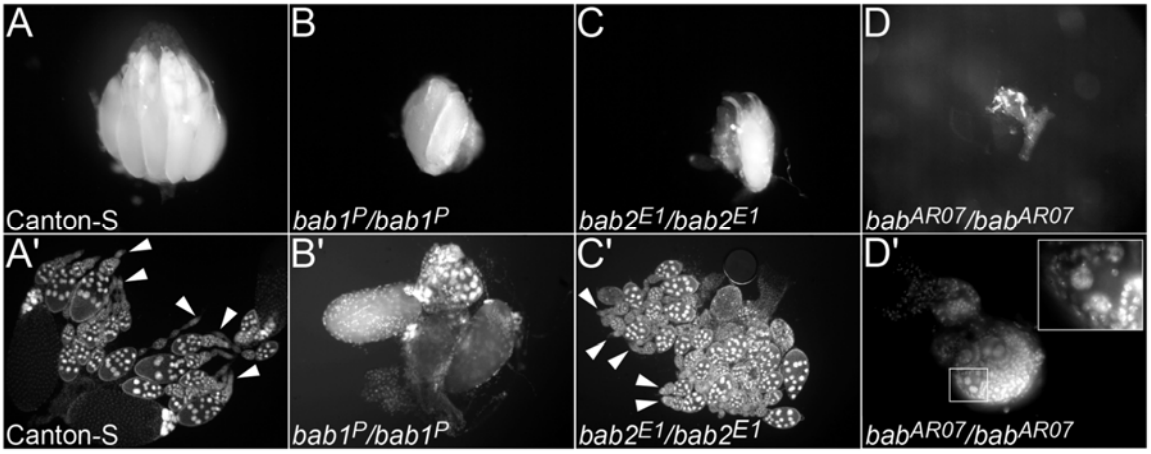

Figure 1**.** Morphology of whole adult ovaries visualized using bright field microscopy (A-D) and ovariole structure revealed by DAPI staining and epifluorecence microscopy (A'-D') of Canton-S (A, A'), *bab1*^{P} homozygotes (B, B'), *bab2*^{E1} homozygotes (C, C'), and *bab*^{AR07} homozygotes (D, D') characterized by a layer of nuclei embedding (inset).

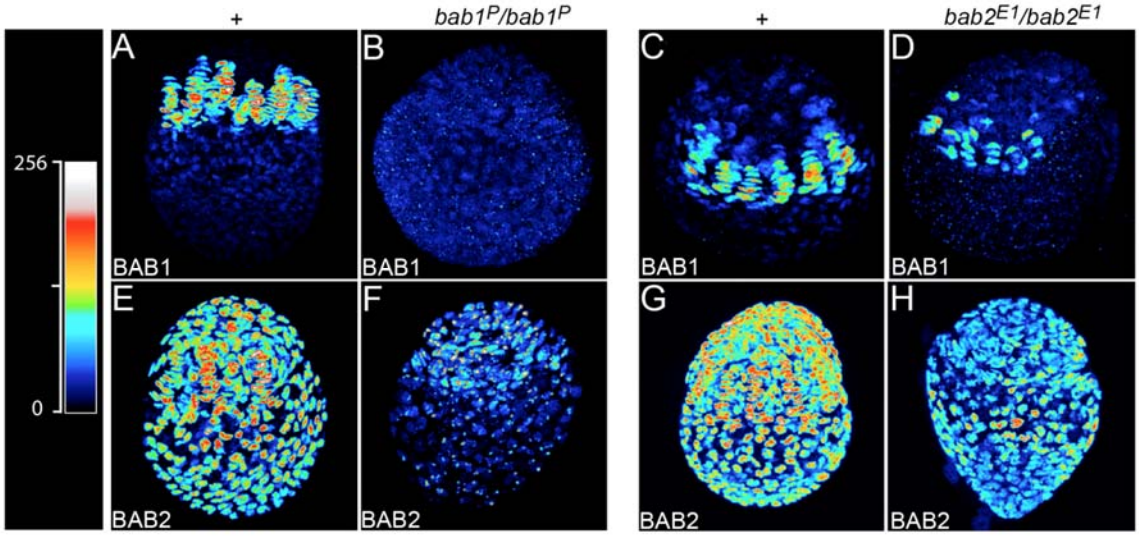

Figure 2**.** Expression of *bab1* and *bab2* in wandering third instar larval ovaries. Comparison of BAB1 and BAB2 protein levels between control (A, E, C, G) and *bab1*^{P} (B, F) or *bab2*^{E1} (D, H) homozygotes as measured by immunostaining and confocal analysis followed by quantification using the lookup table shown on the left of the panel. BAB1 positive cells in A, C and D correspond to TF cells.

The latter conclusion is, however, challenged by the results of the present analysis of *bab1* and *bab2* expression in the *bab1*^{P} and *bab2*^{E1} mutant ovaries. BAB1 and BAB2 protein levels were measured by immunofluorescent confocal microscopy in homozygous *bab1*^{P} and *bab2*^{E1} mutant

larval ovaries. As expected from previous work (Couderc *et al.*, 2002), BAB1 was undetectable in *bab1*^{P} mutant ovaries (Figure 2B, compare to wild-type in 2A). BAB2 was reduced in *bab2*^{E1} mutant ovaries (Figure 2H, compare to wild-type in 2G), whereas it was undetectable in Couderc *et al.* (2002). This difference is not surprising, since low levels of BAB2 had been detected on Western blot and since fluorescent immuno-detection with confocal microscopy is more sensitive than classical enzymatic immunostaining as was previously used. However, BAB2 levels were also strongly reduced in *bab1*^{P} (Figure 2F, compare to wild-type in 2E), and BAB1 levels were reduced in *bab2*^{E1} mutant ovaries (Figure 2D, compare to wild-type in 2C). These results suggest that the *P*-element insertion in *bab1*^{P} as well as the EMS-induced mutation in *bab2*^{E1} affect the expression of both *bab* paralogs in the larval ovary. Therefore, it is impossible to conclude on the specific implication of each *bab* paralog in ovarian morphogenesis and oogenesis based solely on the analysis of the phenotype of these mutants.

The *bab1* and *bab2* genes may interact in *trans*, regulating each other at the transcriptional level. In fact, it was shown that the *bab2* gene product binds to both *bab1* and *bab2* sequences (Lours *et al.*, 2003). If binding of BAB2 on *bab1* gene sequences normally activates transcription, the reduction of BAB2 in *bab2*^{E1} ovaries may lead to a reduction of *bab1* transcription and, consequently, to a reduction of BAB1 protein levels in the ovary, as observed in our data (Figure 2D). Since the BAB1 protein is restricted to TF cells, whereas *bab2* is expressed in all somatic cells of the ovary, direct transcriptional control of *bab2* by the BAB1 protein is unlikely to occur outside TF cells. However, BAB2 protein levels are strongly reduced in all somatic cells in *bab1*^{P} ovaries. A non autonomous and thus indirect regulation of *bab2* by *bab1*, involving long-distance signaling between *bab1*-expressing cells and other somatic cells in the ovary, cannot be excluded.

Alternatively, *cis*-regulatory effects between *bab1* and *bab2* may also be responsible for the effect of the *bab1*^{P} mutation on *bab2* expression or for that of *bab2*^{E1} on *bab1* expression. It was shown that at least one *cis*-regulatory element (CREs) located in *bab1* sequences is shared between the two *bab* paralogs for the control of *bab1* and *bab2* expression in the abdomen (Williams *et al.*, 2008). This CRE, or other yet unidentified CREs, may also co-regulate the two paralogs in the ovary. The *bab1*^{P} allele contains a *P*-element inserted in the first intron of *bab1*. This insertion may disrupt the function of an unidentified ovary-specific CRE, thereby also affecting the expression of *bab2* in the ovary. This *cis*-regulatory effect may not occur in other tissues, since BAB2 protein levels were unaffected in larval imaginal discs and brains as detected by Western blotting (Couderc *et al.*, 2002). Since the *bab2*^{E1} allele has not been characterized at the sequence level, *cis*-regulatory effects as well as alteration of *bab1* sequences in this mutation, leading to *bab1* down-regulation in TF cells, may also explain the low level of *bab1* expression in *bab2*^{E1} mutant ovaries.

Taken together, our results show that the presence of reduced ovaries in *bab* mutants may be due to different mechanisms depending on the mutation and that, due to possible *cis*- and *trans*-regulatory effects, specific functions of *bab1* and *bab2* cannot be assigned using the two strongest *bab* locus alleles previously characterized as affecting only one of the two paralogs. Other approaches such as RNAi silencing of the individual *bab* paralogs will be necessary to address these questions.

Material and Methods

Fly stocks and analysis of adult ovaries

Flies were grown on standard corn-agar medium under uncrowded conditions at 25°C. *bab* mutant stocks were kind gifts from J-L. Couderc (*bab2*^{E1}), D. Godt (*bab1*^{P}), and M. Boube (*bab*^{AR07}), and *hh-lacZ* from A.-M. Pret. Female flies were dissected 24 to 48 hours after eclosion in phosphate-

buffered saline 0.66× (PBS tablets, Sigma). Ovaries were fixed in PBS 1× 3.7% formaldehyde during 30 min at room temperature and stored at 4°C. General morphology of the ovary was observed under a stereoscopic microscope (Leica MZFL III). Ovaries were labeled in DAPI (5 µg/ml in PBS) and observed under epifluorescence (Leica DMRD).

Immuno-fluorescence and imaging

bab mutant stocks were balanced over *TM6Tb*. Female wandering third instar larvae of the Tb+ phenotype were dissected in PBS and whole fat bodies to which ovaries are attached were collected, fixed in PBS containing 2% BSA (Sigma A2058), 3.7% formaldehyde (Sigma), 1% Triton during 30 min at room temperature, washed in PBS 0.3% Triton (PBT), and blocked in PBT, BSA 1% (PBTA). Fixed larval ovaries were incubated in PBTA with the appropriate combination (see Figure 2 legend) of rabbit anti-BAB1 (1 :1000, (Williams *et al.*, 2008)), rat anti-BAB2 (1 :1000, (Couderc *et al.*, 2002)), and mouse anti ß-galactosidase (DSHB) overnight at 4°C. Ovaries were incubated during 2 hours at room temperature with the appropriate Alexa 488- and Alexa 568-coupled secondary antibodies (1:500 in PBTA, Molecular Probes). Ovaries were mounted in Citifluor (AF1, Biovalley, FR) and directly observed under an inverted confocal microscope NIKON TE2000-U. Fixation and immuno-fluorescence of ovaries of mutant and control genotypes were performed in the same tube, thus allowing direct comparison of signal levels between genotypes. Control and mutant ovaries were genotyped using *lacZ* reporters. Control ovaries were Canton-S for $bab1^{P}$, and *hh-lacZ* for $bab2^{E1}$. Genotypes were identified after imaging: $bab1^{P}$ carries a *lacZ* reporter that is expressed in TF cells, allowing to positively discriminate the $bab1^{P}$ homozygotes from Canton-S controls; $bab2^{E1}$ mutants were identified by the absence of anti-betaGalactosidase immunostaining, and *hh-lacZ* controls by the presence of anti-betaGalactosidase immunostaining in TF cells (data not shown). The bab^{AR07} deficiency that covers both *bab1* and *bab2* was used as a control for the specificity of BAB1 and BAB2 signals in a separate experiment (data not shown). Confocal images were analyzed using ImageJ (NIH) and Photoshop CS2 (Adobe) softwares, using identical settings for all samples of the same experimental series.

References: Bardot, O., D. Godt, F.A. Laski, and J.L. Couderc 2002, Genesis 34: 66-70; Barmina, O., and A. Kopp 2007, Developmental Biology 311: 277-86; Couderc, J.L., D. Godt, S. Zollman, J. Chen, M. Li, S. Tiong, S.E. Cramton, I. Sahut-Barnola, and F.A. Laski 2002, Development 129: 2419-33; Godt, D., J.L. Couderc, S.E. Cramton, and F.A. Laski 1993, Development 119: 799-812; Godt, D. and F. A. Laski 1995, Development 121: 173-87; Kopp, A., I. Duncan, D. Godt, and S.B. Carroll 2000, Nature 408: 553-9; Lours, C., O. Bardot, D. Godt, F.A. Laski, and J.L. Couderc 2003, Nucleic Acids Res 31: 5389-98; Randsholt, N.B., and P. Santamaria 2008, Evolution & Development 10: 121-33; Sahut-Barnola, I., D. Godt, F.A. Laski, and J.L. Couderc 1995, Dev. Biol. 170: 127-35; Williams, T.M., J.E. Selegue, T. Werner, N. Gompel, A. Kopp, and S.B. Carroll 2008, Cell 134: 610-23.

Mutants in *D. simulans* and *D. sechellia.*

Sousa-Neves, Rui, and Youngmin Chu. Department of Biology Case Western Reserve University

Here we report the isolation of nine new spontaneous mutants in *D. simulans* identified this year, as well as notes on mutants described in Sousa-Neves *et al*. (2009). We also report the genetic

and molecular position of the recessive mutation *zinfandel* (*zn*[1]) of *D. sechellia* and *small wings*[1] (*swg*[1]) of *D. simulans*.

The new mutants of *D. simulans* described in this report are:

1- *Minute 3 Super Los Angeles* (*M(3)SLA*[1])

ORIGIN: Isolated in February 2011 from the stock Super Los Angeles.
PHENOTYPE: *M(3)SLA*[1] is haplo-insufficient, homozygous lethal like most *Minutes* and because of that it has to be selected every generation. Heterozygotes can be easily scored and have excellent viability and fertility.
LINKAGE: *M(3)SLA*[1] was located on the 3[rd] chromosome and mapped using *scarlet* (*st*) and *ebony* (*e*) as references on this chromosome. In *D. simulans st* and *e* appear 13.7 units apart. *M(3)SLA*[1] maps 26.9 units to the left of *st*.

2- *Minute of Rincón* (*M*[R])

ORIGIN: Isolated in the September 2011 from the Stock Rincón de la Vieja.
PHENOTYPE: Haploinsuficient. Strong Minute with excellent viability as heterozygote
LINKAGE: not yet determined.

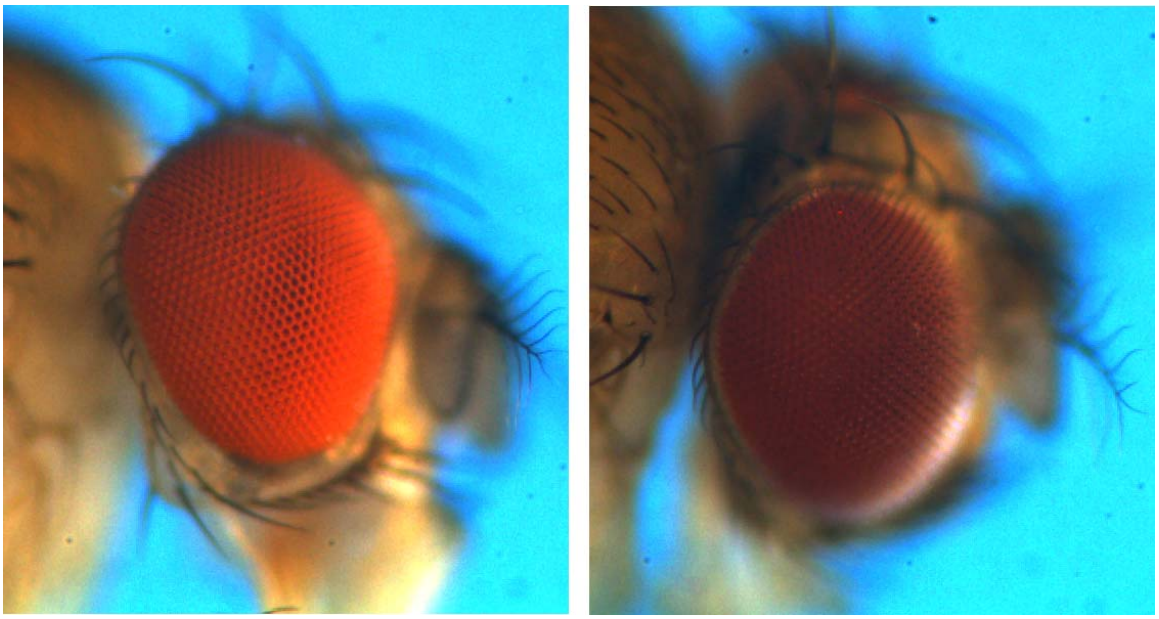

Figure 1. *purple maternal* (*pr-m*[1]) mutant. Wild type eye (left) and mutant *pr-m*[1] (right).

3- *purple maternal* (*pr-m*[1])

ORIGIN: Isolated in February 2011 from the stock Tabacón.
PHENOTYPE: Recessive. *pr-m*[1] flies have eyes with a dark Port wine color, smooth appearance and faint pseudopupils (which appear white or gray in young flies, as opposed to black). Ocelli colored. With age, this color progresses to a thick reddish brown. Males homozygous from *pr-m*[1]

are viable and fertile. However, homozygous females lay fertilized eggs that die as embryos with extreme pattern defects. Very rare escapers can be obtained and those appear absolutely normal.
LINKAGE: 3

4- *singed*^{3} (*sn*^{3})

ORIGIN: Isolated in June 2011 from the Stock Super Los Angeles.
PHENOTYPE: Strong allele of *singed* with macrochaeta extremely curled. Complements the *D. simulans f* ^{66} and fails to complement *sn*^{X2} from *D. melanogaster* carried in the balancer Binscy. *sn*^{1} and *sn*^{2} were isolated by Sturtevant in the 1920's and are presumably lost. Thus, *sn*^{3} may be the only existing allele of *sn* in *D. simulans.*
LINKAGE: X

Figure 2. *singed*^{3} (*sn*^{3}) mutant. Note the severely distorted thoracic bristles.

5- *scarlet* (*st*^{3})

ORIGIN: Isolated in February 2011 from the Stock Super Los Angeles.
PHENOTYPE: Recessive. Eyes with a bright red color like *vermilion, cinnabar,* and *scarlet.* Ocelli colorless. Allelic to the *D. simulans st*^{1}.
LINKAGE: 3L

6- *curly of Rincón*^{1} (*cyR*^{1})

ORIGIN: Isolated in June 2011 from the Stock Rincón de la Vieja.
PHENOTYPE: Recessive. Wings curled. In addition to curly wings, *cyR*^{1} often exhibit a loss of humeral hairs. At this point it is not clear whether the lack of humerals is separable from the wing phenotype.
LINKAGE: Not yet determined.

Figure 3. *curly of Rincón*^{1} (*cyR*^{1}) mutant female. Note the curled wings and the lack of humeral bristles (white arrow). For comparison, see the humerals of the individuals in Figure 5.

7-*Ultrabithorax-like*^{1} (*Ubx-l*^{1})

ORIGIN: Isolated in November 2011 from the Stock *sn*^{3}; *st*^{3}.
PHENOTYPE: Recessive. Halteres flat with irregular lobes and partial transformations to wings. Flies are flightless and often exhibit divergent wings.
LINKAGE: Not yet determined.

8-*rough-forked*^{1} (*rof* ^{1})

ORIGIN: Isolated in November 2011 from the stock Rincón the la Vieja.

PHENOTYPE: Eyes rough with ommatidia distorted. In addition to the eye phenotype the posterior dorsocentrals appear gnarled. The gnarling is usually less extreme than *sn*^{3}. Other bristles may appear distorted. Frequently *rof* ^{1}flies exhibit divergent wings.
LINKAGE: Autosomal, not yet determined.

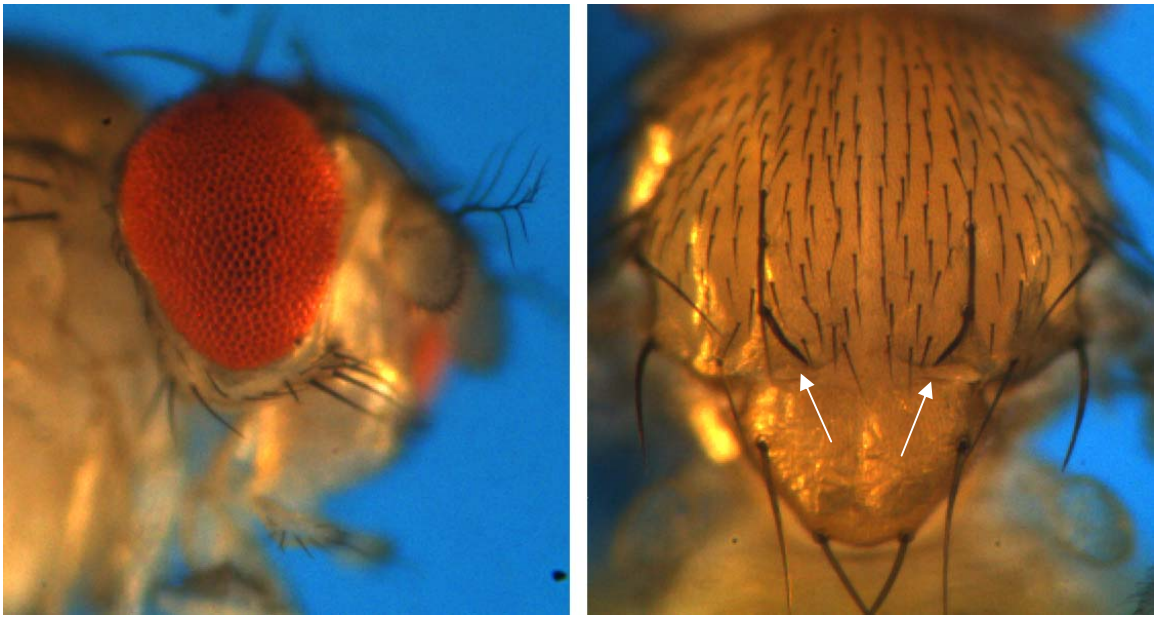

Figure 4. *rough-forked*^{1} (*rof* ^{1}) mutant. (Left) the eye of a *rof* ^{1} male. (Right) the thorax of the same individual. Compare the disorganized ommatidia of the mutant above with the wild type of Figure 1. The crystalline structure of the wild type ommatidia resembling a mesh is replaced by an irregular tissue. Note the abnormal thoracic bristles (white arrows).

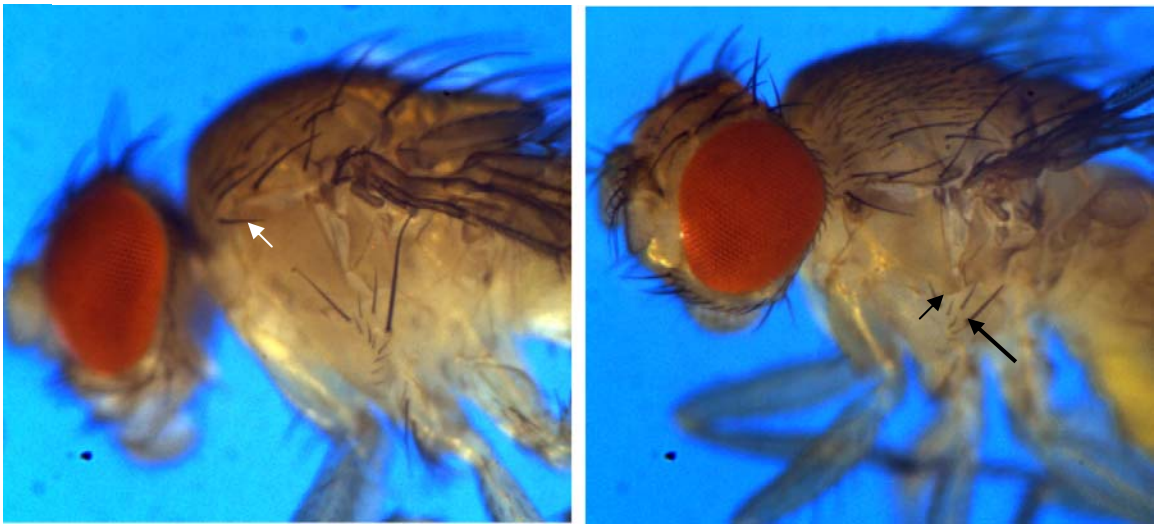

Figure 5. *sternopleurals reduced* (*sr*) mutant. (Left wild type and right *sr*). Observe the extreme reduction of the sternopleural bristles (black arrows). The white arrow points to humeral bristles, which appear wild type in *sr* mutants.

9-*sternopleurals reduced* (*sr*)

ORIGIN: Isolated from the Super Los Angeles stock in December 2011.
PHENOTYPE: Sternopleural bristles reduced. Post-scutellar bristles also reduced to a great extent. Body color is not as shinny and with the waxed appearance as the wild type.
LINKAGE: not yet determined.

Updates of mutations previously described in D.I.S. (Sousa-Neves *et al*., 2009):

Genetic and physical position of the *D. simulans small wings* (*swg*)

Previously we reported that *swg* is an X-linked recessive mutation. Recombination mapping in *D. simulans* showed that *swg* is 5.4 units to the right of *v*. This result suggested that *swg* might correspond to the *D. melanogaster dusky* (*dy*) or *minitature* (*m*). However, *swg* complements *m*. We tested whether the *D. melanogaster* deletion Df(1)BSC876 that deletes *dy* disrupts *swg* and found that this deletion fails to complement *swg*.

Genetic and physical position of the *D. sechellia zinfandel* (zn^1)

zn^1 is an X-linked recessive mutation that affects eye color. We mapped it by recombination in hybrids *D. simulans*/*D. sechellia* at position 23.0. Since the position and phenotype of zn^1 is very similar to the *D. melanogaster* gene *carmine* (*cm*) located at the physical position 6.9Mb, we tested whether zn^1 corresponds to *cm* in heterozygous hybrids *D. sechellia*/*D. melanogaster* zn^1/Df(1)BSC867. We find that Df(1)BSC867 uncovers the recessive zn^1. Thus, it seems likely that zn^1 corresponds to *carmine*, which is located between the *D. melanogaster* molecular coordinates (X: 6875892..6935548).

References: Sousa-Neves, R., J. Schinaman, and J. Cater 2009, Dros. Inf. Serv. 92: 143-147.

Teaching Notes

The identification of nearly neutral mutations in *Drosophila melanogaster*: The *bw*^{75} and *bw*^{1} alleles of Buri.

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The identification of nearly neutral mutations with only small differences in fitness is difficult in higher organisms. In a classical experiment Buri (1956) was able to follow changes in the *bw*^{75} and *bw*^{1} alleles at the second-chromosome brown locus of *Drosophila melanogaster* over 19 generations due to drift, because he estimated that the two alleles were neutral, *i.e.,* the *bw*^{75}/*bw*^{75}, *bw*^{75}/*bw*^{1}, and *bw*^{1}/*bw*^{1} flies (that were also homozygous for the third-chromosome mutant scarlet, *st/st*) had equal fitness. "No evidence consistent with an hypothesis of selection appeared in either series when the sample frequencies were grouped according to donor frequency classes. From this standpoint gene frequency changes could safely be attributed wholly to accidents of sampling." (Buri, 1956).

We synthesized the Buri *bw*^{75}/*bw*^{1}*;st/st* stock from stocks of *bw*^{75}/*bw*^{75}, *bw*^{1}/*bw*^{1}, and *st/st*, and tested the fitness (viability) of the genotypes *bw*^{75}/*bw*^{75}*;st/st* (orange eyes), *bw*^{75}/*bw*^{1}*;st/st* (yellow eyes), and *bw*^{1}/*bw*^{1}*;st/st* (white eyes). The *st/st* genotype will be assumed in subsequent crosses. We mated single *bw*^{75}/*bw*^{1} virgin females with single *bw*^{75}/*bw*^{1} males in vials and counted the three possible genotypes in F1 progeny. The results of two experiments are shown in Table 1.

The P values indicate that the results are not significantly different from the Hardy/Weinberg expectations (317, 634, and 317 in experiment A; and 305, 610, and 305 in experiment B) if *bw*^{75} and *bw*^{1} were neutral alleles. Hence, as in the study of Buri (1956) it seems that the *bw*^{75} and *bw*^{1} alleles are neutral, or nearly neutral, mutations.

Table 1. Frequencies of brown locus genotypes.

Experiment (vials)	bw^{75}/bw^{75}	bw^{75}/bw^{1}	bw^{1}/bw^{1}	P value
A(25)	330	620	318	0.81
B(28)	310	606	304	0.97

Buri (1956) also tested the fitness of the three genotypes of the brown locus using population cage experiments and observed no difference in fitness. "Considering the group of cage experiments as a whole, there is no indication of a difference in relative selective value between the two alleles …". Hence, we ran two population cage experiments beginning each cage with a frequency of bw^{75} and bw^{1} of 0.5; the cages were each started with 200 bw^{75}/bw^{1} heterozygous flies (100 virgin females and 100 males). The cages contained 15 population cups, and every seven days the five oldest cups were replaced with new cups and the first 300 flies that eclosed from the five older cups were identified as bw^{75}/bw^{75}, bw^{75}/bw^{1}, or bw^{1}/bw^{1}every fifth generation up to generation 25. We then determined the frequencies of the bw^{75} and bw^{1} alleles. The results are shown in Table 2. The changes in the frequency of the bw^{1} allele over time are shown in Figure 1.

Table 2. Frequencies of brown locus alleles in two population cages over time.

	Generation	bw^{75}/bw^{75}	bw^{75}/bw^{1}	bw^{1}/bw^{1}
Cage 1:	5	87	144	69
	10	85	161	54
	15	88	141	71
	20	83	158	59
	25	93	152	55
Cage 2:	5	95	147	58
	10	93	152	55
	15	119	135	46
	20	109	153	38
	25	103	143	54

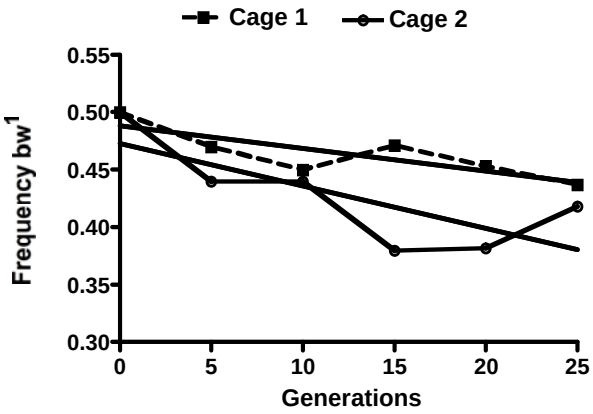

Figure 1. Frequency of the bw^{1} allele over time in two population cages.

The two slopes are not significantly different (P = 0.10). In both population cages the frequency of bw^{1} went down with time. The slope of cage 1 was significantly different from zero (P = 0.04), whereas the slope of cage 2 was not significantly different from zero (P = 0.07).

If we assume the following fitness model for the three brown genotypes, with s being the selection coefficient,

	bw^{75}/bw^{75}	bw^{75}/bw^{1}	bw^{1}/bw^{1}
Fitness	1	1	1 –s

then one can estimate the s value by using the following equation (Hedrick, 2011, equation 3.6c),

$$s = \frac{1}{n}\left[\left(\frac{q_o - q_n}{q_o q_n}\right) + \ln\left(\frac{q_o(1-q_n)}{q_n(1-q_o)}\right)\right]$$

where q_0 is the frequency of *bw*^{1} at the beginning of the population cage experiment (0.5), q_n is the frequency of *bw*^{1} at the end (generations 25) of the population cage experiment (0.43 from an average of Cages 1 and 2), and n is the number of generations (25). Hence,

$$s = \frac{1}{25}\left[\left(\frac{0.5 - 0.43}{(0.5)(0.43)}\right) + \ln\left(\frac{0.5(1 - 0.43)}{0.43(1 - 0.5)}\right)\right]$$

$$\mathbf{s = 0.024}$$

The *bw*^{1}/*bw*^{1} flies have 98% of the fitness of the *bw*^{75}/*bw*^{75} and *bw*^{75}/*bw*^{1} flies. This supports *bw*^{1} as a nearly neutral allele.

Since *bw*^{1} and *bw*^{75} are almost neutral alleles, we can also get an estimation of the effective population size (N_e)(the ideal population size in which all parents have an equal expectation of being the parents of any progeny; Hedrick, 2011) of the flies in the population cages by measuring the drop in the frequency of heterozygotes over time (Hedrick, 2011, page 197), with $H_0 = 0.5$, t = 25, and the Cage 1 and Cage 2 combined change in the frequency of heterozygotes from 0.5 to 0.49 (295/600) in 25 generations was 0.49 ($H_t = 0.49$):

$$\frac{H_t}{H_0} = e^{-t/2N_e}$$

$$0.98 = e^{-25/2N_e}$$

$$0.98 = e^{-12.5/N_e}$$

$$N_e = \frac{-12.5}{\ln 0.98}$$

$$\mathbf{N_e = 618}$$

Since we estimated that there were at least 2,000 flies in each cage each generation after the beginning generation, the effective population size (N_e) was about 31 percent of the census population size (N). Malpica and Briscoe (1981) have estimated that the effective population size in cages with about 5,000 flies is as low as 190. Frankham (1995) has estimated that N_e/N is on average about 1/10 in natural populations of higher organisms.

A class discussion of the results of this teaching exercise could include 1) a discussion of the genetics of neutral alleles, including how the data of the population cage experiments would have differed over time if the three genotypes (*bw*^{75}/*bw*^{75}, *bw*^{75}/*bw1*, and *bw*^{1}/*bw*^{1}) had the same fitness, 2) how does the observation that N_e is much smaller than N influence the conservation of endangered species (see discussions in Frankham *et al.*, 2010), and 3) since Buri's experiment is considered a classical experiment showing genetic drift, how do the results of this study alter his study. Keep in mind that the *bw*^{75}/*bw*^{1}*;st/st* stock used in this study would have a different genetic background from the stock used by Buri.

References: Buri, P., 1956, Evolution 10: 367-402; Frankham, R., 1995, Genet. Res. 66: 95-107; Frankham, R., J.D. Ballou, and D.A. Briscoe 2010, *Introduction to Conservation Genetics*. Cambridge University Press, Cambridge; Hedrick, P.W., 2011, *Genetics of Populations*. Jones and Bartlett Publishers, Sudbury, MA; Malpica, J.M., and D.A. Briscoe 1981, Experientia 37: 947-948.

Utilizing *Drosophila* Activity Monitors (DAMs) in an undergraduate teaching and research setting.

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The fruit fly model system is suitable for efficient genetic, neural, and behavioral analysis (Mackay and Anholt, 2006), including analyzing the circadian rhythm. *Drosophila melanogaster* has been a widely used model for studying circadian mutants since the isolation of the *period* (per) mutants (Konopka and Benzer, 1971). Further explorations have also concluded that there exists a high degree of homology between the molecular circadian clock mechanism of *Drosophila* and mammals (Helfrich-Foster, 2004), which makes *Drosophila* an excellent model for examining circadian clock function.

As Klarsfeld *et al.* (2003) reports, studies have shown that different devices were invented to observe the behavioral circadian locomotor activity rhythm in fruit flies (Hamblen *et al.*, 1986; Helfrich 1986), but currently, the most widely used are the *Drosophila* Activity Monitors (DAMs), such as the devices built by the company Trikinetics (Waltham, MA) (Klarsfield *et al.*, 2003) (Figure 1a). Previous articles describe the actual process of setting-up the computers and monitors, and placing the flies into the individual monitor tubes (Rosato and Kyriacou, 2006) (Figure 1b) – in particular, Chiu *et al.* (2010) provides detailed instructions beginning with incubator set-up to data analyses. Essentially, the DAMs monitor the activity of individual flies by counting the number of infrared beam crossings within a 10-min time period or bin, and subsequently compiling the activity in a raw data text file (Klarsfield *et al.*, 2003; Rosato and Kyriacou, 2006; Chiu *et al.*, 2010; Pfeiffenberger *et al.*, 2010). It is then necessary to utilize a separate data-analysis program, such as ClockLab (Actimetrics, Wilmette, IL) or VitalView (Minimitter, Bend, OR), in order to extract the information from the raw-data file and to produce an actogram, which is a graphical representation of the individual fly's activity.

Figure 1a (left) and 1b (right). a) An example of a DAM, specifically model DAM2. The DAM2 connects to the computer and power source via the standard 4-wire telephone jack in the bottom left corner. b) Thirty-two individual flies are placed into tubes with an agar-sucrose food, covered with a black cap (shown), and capped off on the other end with cotton (not shown).

Monitoring the locomotor activity in individual flies can uncover altered behavior, including the free-running rhythm, over the course of several days or even weeks (Pfeiffenberger *et al.*, 2010). Observing individual flies allows the investigator or student to easily discern rhythmic from arrhythmic, and mutant from non-mutant individuals, through quick examination of the actogram (Figure 2). This is in contrast to eclosion experiments in which the population will be interpreted as arrhythmic if constituted of rhythmic, albeit unsynchronized individuals (Konopka and Benzer, 1971; Klarsfeld *et al.*, 2003). Indeed, recording the locomotor activity of an individual fly was, and still is, an extremely powerful tool that helps identify circadian rhythm mutants (Klarsfeld *et al.*, 2003; Zordan *et al.*, 2007; Pfeiffenberger *et al.*, 2010). Therefore, I submit that the DAMs, similar to the monitors from Trikinetics, are an excellent, although unfortunately underutilized, tool in teaching undergraduate students concepts of the circadian clock, behavior, genetics, and neuroscience.

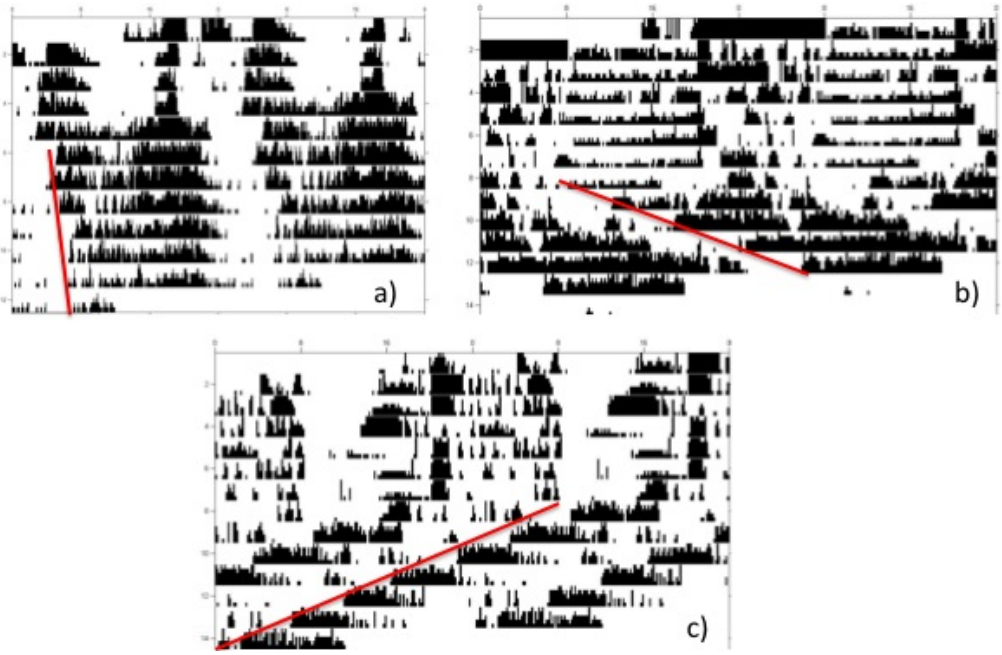

Figure 2. Three representative, double-plotted actograms showing the free-running periods from wild-type and *period* mutant *D. melanogaster*. All three actograms were generated using the program ClockLab. The red line indicates the slope of the free-running period. a) Canton-S wild-type, b) per^L, c) per^S. The wild-type fly has a period approximately 24.5 h, while the long and short mutants (per^L and per^S) have average periods of 28.6 h and 19.5 h, respectively. The per^L and per^S are the mutant flies from the Konopka and Benzer (1971) study and were provided generously by Dr. Michael Rosbash.

The teaching and research projects conducted in an undergraduate setting should fulfill the following criteria: 1) be in accordance with the curriculum and reinforce the theoretical concepts learned in the classroom, 2) train students to conduct a series of experiments that achieve a set of objectives in a timely manner, 3) prepare them for future scientific careers as graduate or medical students and beyond, and 4) be feasible in terms of available funds. The DAMs fulfill all of these conditions as described through the following.

1) Research with undergraduates should reinforce the concepts learned in their upper-level elective courses (*i.e.*, 300- or 400-level), regardless of their sub-discipline in biology. As both a

neuroscience instructor and a circadian rhythm researcher at a primarily undergraduate institution, I rely heavily on the use of DAMs as an important teaching tool in both the classroom and research setting. In both my Neurobiology and Biological Clocks classes, students design group research projects with only directional input from me. These projects investigate simple yet informative questions posed by the students and serve as the final project in each of my two upper-level electives. I highly encourage the students to use the DAMs in their work, because in doing so they can easily observe a significant and quantitative behavior in the flies**.** I have observed that when given the opportunity, my undergraduate students develop extremely creative and original projects that utilize: a) easily accessible pharmacological agents (*i.e*., over-the-counter solutions, supermarket items, chemicals the department already possesses, and so forth), b) the plethora of *Drosophila* mutants available from Bloomington Stocks (Bloomington, IN) and c) the DAMs, for their laboratory-class assignments.

2) The students can design experiments that may reveal the activity behavior of certain genetic backgrounds or even crosses of their own design, including some potentially unusual ones. Furthermore, they can test the effects of environmental or pharmacological agents on the following circadian parameters: a) free-running period, b) the capacity to adapt to day-night cycles with photoperiods of differing length (or perhaps use a skeleton photoperiod), and/or c) the phase of the circadian activity cycles with respect to the stimulus (Zordan *et al.*, 2007). In addition to the activity monitors, Trikinetics also sells devices that can examine other aspects of the fly's behavior by which students can further explore their interests. Examples of projects students may investigate can include showing how different pharmacological agents can alter the fly's ability to maintain balance on a rotarod, or how sleep deprivation affects the activity of the fly. Students can subsequently create projects that utilize multiple tools and determine if different agents or genetic factors affect multiple behaviors. The DAMs also have the advantage of collecting relatively large amounts of data in a very small amount of time; the students can gather results for their in-class projects in about two weeks. In a class-laboratory setting, the instructor can illustrate how to use the DAMs during the first lab week, and then have the students complete different lab assignments during the second week while the activity is being monitored. During the third lab week, the instructor can focus on data analysis instruction. Since the DAMs and the other tools are simple and efficient to use, the students can focus on designing creative experiments, rather than concentrating primarily on the technical aspects of the study.

3) Since the DAMs allow for continuous recordings of several flies (32 per activity monitor), they can be used as a "high-throughput" way to study circadian rhythm and locomotor activity (Pfeiffenberger *et al.*, 2010). In fact, many journal articles have been published from prestigious research institutions that utilize the DAMs in their research. These studies include recent publications from both sleep (Parisky *et al.*, 2008; Wu *et al.*, 2008; Harbison *et al.*, 2009; Weber *et al.*, 2009) and circadian laboratories (Zhang *et al.*, 2009; Beaver *et al.*, 2010). A major advantage of circadian rhythm research is the minimal physical maintenance required after the flies are placed into the monitors. The undergraduate students are not relegated to performing only "grunt-work", such as cleaning equipment or waste, preparing solutions, labeling and preparing petri dishes, and so forth. They are afforded the opportunity to participate in authentic scientific inquiry, by designing the project, maintaining the fly stocks, which can include crossing different genetic strains, and anesthetizing the flies which are the essential technical skills required for participation in any *Drosophila* laboratory at the graduate level. Not only can the students create the protocols for the experiment, they can also play an important role in the data analyses and form conclusions based on their results. After the initial setup, a computer program collects the circadian activity data, offering the students the opportunity take part in calculating the circadian parameters themselves and running statistical analyses on the results, which ultimately could lead to student publication.

4) The DAMs from Trikinetics are also extremely cost effective. Four activity monitors, 200 re-usable monitor tubes and caps, as well as all of the wiring accessories (which have the capability to record the activity from 128 flies) can be purchased from Trikinetics for approximately $2,500. The only additional required equipment would be a *Drosophila* incubator, which all fly-laboratories would have. In addition, Trikinetics provides all of the computer software for recording the activity of the individual flies free of charge. Also needed is a computer program that can analyze the text files. Some simple programs that can produce an actogram or calculate the free-running period can be downloaded from the Internet at no cost (http://www.circadian.org/softwar.html), or more advanced software can be purchased from either Actimetrics or Minimitter. Once the initial system is assembled, instructors can utilize this paradigm year-after-year in the lab-section coursework of a Neuroscience, Biological Clocks, Animal Behavior, or Genetics class for no more than the cost of maintaining the fly stocks.

In using the DAMs, simple experiments can be conducted providing an excellent medium to teach undergraduates a scientifically sound method that is currently used in the fields of genetics and neuroscience. The students can address a number of questions in which they are interested, and these devices can allow the students a tremendous amount of creativity and flexibility in designing the experiments for their courses or in collaboration with their research advisor. These reasons make the *Drosophila* Activity Monitors excellent models and tools for examining activity behavior and circadian clock function in a classroom or small research setting.

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Inbreeding depression, loss of genetic variation, and survival in a high salt diet.

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Since Darwin (1859), it has been assumed that genetic variation (phenotypic variation for Darwin) is essential for adaptive evolution. This has been shown to be true in *Drosophila melanogaster* experiments where selection response is reduced in inbred lines with no genetic variation compared to outbred lines that have genetic variation (Falconer and Mackay, 1996; Clayton and Roberson 1955; Woodruff and Thompson, 2008). Here we have tested if inbreeding, with a slow loss of genetic variation over time and the associated inbreeding depression caused by the homozygosis of deleterious mutations (Hedrick 2005; Lynch and Walsh, 1998), reduces the potential for populations to adapt to a new stressful environment. Onasch and Woodruff (2008) have shown that inbreeding does cause a significant decrease in viability (progeny numbers) in *D. melanogaster*. This experiment, however, was performed in a non-stressful environment.

In a modification of Frankham *et al*. (1999) and Zhang *et al*. (2011), we have measured the ability of outbred and inbred lines of *D. melanogaster* that were originally isolated from nature to survive and produce progeny on increasing concentrations of dietary NaCl, up to a toxic level (6%). Twelve mated females were captured from nature (Perrysburg, Ohio) on July 30, 2010. From these females, twelve isofemale lines were maintained by sibling matings (a single virgin female and a single sib male were mated in a vial each generation). In addition, three outbred lines (OBA, OBB, and OBC) were set up by mixing the progeny of four isofemale lines per outbred line in generation two and maintaining these lines by mass transfers each generation into bottles. These outbred lines were controls that contained genetic variation.

At generation three, five females and five males from each of the outbred lines were placed in vials with 0% NaCl, 2% NaCl, 4% NaCl, and 6% NaCl (mixed into Carolina Instant Drosophila Food). In addition, five females and five males of each of the seven inbred lines (IBC, IBE, IBI, IBJ, IBM, IBP, and IBU), which had been brother/sister mated each generation, were placed in vials with the same NaCl concentrations. All vials were placed at 25ºC and the parents were removed after seven days. Furthermore, the progeny were counted per vial for a total of 14 days from the day the vials were initiated. The same procedure was repeated for generations six and nine. It was expected that the outbred lines would continue to have genetic variation throughout this study, while the inbred lines, which were maintained each generation by single brother/sister matings, would with time lose genetic variation and show inbreeding depression.

The results (number of progeny per vial) for generation three are shown in Table 1, for generation six in Table 2, and for generation nine in Table 3.

In all generations, 6% NaCl was toxic to the outbred (OB) and inbred (IB) lines; no progeny were recovered in any line. Frankham *et al* (1999) and Zhang *et al* (2011) saw similar results.

Inbreeding caused a significant decrease in the number of progeny in all the concentrations of NaCl, whereas the outbred lines had a reduction in offspring numbers only at 4% NaCl. From unpaired t tests, the outbred (OB) lines had a significant reduction in progeny at 4% NaCl as compared to 0% at generation six ($P = 0.0001$) and at generation nine ($P = 0.03$), but not in generation three ($P = 0.14$). Hence, 4% salt reduces viability even in lines with genetic variation. The outbred lines, however, did not have a significant decrease in offspring in 2% NaCl.

On the other hand, the inbred (IB) lines had a significant decrease in progeny at 2% and 4% saline. In inbred generation three, there was a significant reduction ($P = 0.0007$) in the in 0% *vs*. 4%

NaCl vials. In addition, for the inbred lines there were significant decreases in progeny in 0% *vs*. 2% in generation six (P = 0.018), and generations nine (P = 0.0079), as well as decreases in progeny numbers in 0% *vs*. 4% in generation six (P = 0.0016) and generation nine (P < 0.0001).

Table 1. Progeny numbers after three generations.

Line	0% NaCl	2% NaCl	4% NaCl	6% NaCl
OBA	57	10	14	0
OBB	18	21	29	0
OBC	49	40	2	0
OB Avg.	41.33	23.67	15.00	0
IBC	31	22	14	0
IBE	29	34	2	0
IBI	36	32	2	0
IBJ	18	28	12	0
IBM	38	16	11	0
IBP	14	14	4	0
IBU	19	23	11	0
IB Avg.	26.43	24.14	8.00	0

Table 2. Progeny numbers after six generations.

Line	0% NaCl	2% NaCl	4% NaCl	6% NaCl
OBA	38	51	12	0
OBB	36	49	13	0
OBC	38	20	9	0
OB Avg.	37.33	40.00	11.33	0
IBC	30	17	5	0
IBE	25	18	6	0
IBI	20	5	1	0
IBJ	22	11	3	0
IBM	67	23	2	0
IBP	34	19	2	0
IBU	77	9	8	0
IB Avg.	39.29	14.57	3.86	0

Table 3. Progeny numbers after nine generations.

Line	0% NaCl	2% NaCl	4% NaCl	6% NaCl
OBA	68	47	18	0
OBB	97	36	40	0
OBC	132	75	45	0
OB Avg.	99.00	52.67	34.33	
IBC	46	26	2	0
IBE	33	18	0	0
IBI	59	45	21	0
IBJ	49	14	0	0
IBM	39	21	5	0
IBP	39	27	13	0
IBU	28	5	4	0
IB Avg.	41.81	22.29	6.43	

In conclusion, inbreeding, with loss of genetic variation and inbreeding depression can decrease the ability of populations to adapt to a new stressful environment, such as an increase in the concentration of NaCl in the diet. In this study the inbred lines produced significantly lower numbers of progeny in two and four percent NaCl after three, six, and nine generations. In contrast, the outbred lines, which contained genetic variation, only had a reduction in offspring numbers at 4% NaCl.

Others have also observed that there is an increased sensitivity to stressful environments in inbred lines of *D. melanogaster* and other organisms (Miller, 1994; Armbruster and Reed, 2005). This is one reason why in conservation biology it is so important to avoid reductions in population sizes, which cause inbreeding and could lead to an inability of a population or species to respond to a changing environment, such as new parasites or new environmental stresses (Frankham *et al*., 2002).

Questions that could be asked of students include: 1) how much genetic variation is expected to be lost each generation if the variation is neutral (not deleterious or beneficial)? The amount is 1/(2N) per generation, with N being the effective population size. Discussion of this topic can be found in Hedrick (2005). 2) Is inbreeding depression and the loss of genetic variation a problem for organisms in nature? Yes. See a discussion in Crnokrak and Roff (1999). 3) Is there evidence that inbreeding depression occurs in humans? The answer is yes. Infant mortality is higher in the offspring of matings of first cousins than in matings between unrelated individuals (Freeman and Herron, 2007; Hedrick, 2005).

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Lethal mutations and their elimination by selection in natural populations of *Drosophila melanogaster*.

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Lethal mutations are surprisingly frequent in all organisms. For example, up to 70% of *Drosophila melanogaster* in nature carry at least one recessive lethal mutation (Crow, 1993a,b; Lynch *et al*., 1999; Azad *et al*., 2003), and new lethal mutations arise in about six percent of these flies (Simmons and Crow, 1977; Woodruff *et al*., 1983, 1984, 1996; Fu and Huai, 2003; Gao *et al*., 2011). In humans, recessive mutations that can cause death in homozygotes or hemizygotes (X-linked in males) before reproductive maturity are numerous (Morton, 1981; Strachan and Read, 2004; also see Online Mendelian Inheritance in Man (OMIM) at the National Center for Biotechnology Information at http://www.ncbi.nim.hih.gov.omim). Examples of such mutations in humans include Duchenne muscular dystrophy, Lesch-Nyhan syndrome, congenital erythropoietic porphyria, and cystic fibrosis (Morton, 1981; Cummings, 2009).

In addition, most lethal mutations are not completely recessive (Muller, 1950; Simmons and Crow, 1977; Crow and Simmons, 1983; Crow, 1993a,b; Garcia-Dorado and Caballero, 2000). Organisms that are heterozygous for a recessive lethal mutation (*Ll,* with *l* being the lethal mutant allele) have a fitness that is lower than those with homozygous dominant alleles (*LL*). This can be modeled as follows where *L* is the wild- type allele, *l* is the recessive deleterious mutant allele, *s* is the selection coefficient (with s = 1 for lethals), and *h* is the dominance coefficient.

	For autosomal mutations			For X-linked mutations		
	LL	*Ll*	*ll*	*LL female*	*Ll female*	*lY male*
Fitness =	1	1-*hs*	1- *s*	1	1-*hs*	1-*s*
Fitness =	1	1-*h*	0	1	1-*h*	0

A completely recessive mutant allele would have $h = 0$ in heterozygotes, making the fitness of the heterozygotes the same as the *LL* homozygotes. Most lethal mutations, however, have *h* values greater than zero, *i.e*., they are not completely recessive and the heterozygotes (*Ll*) have a fitness that

is less than the *LL* homozygotes. In addition, lethals that occur on X chromosomes are eliminated almost entirely in hemizygous males (one X and one Y chromosome), whereas, almost all autosomal lethal mutations, which are rare, are eliminated in heterozygotes.

What do we know of the values of *h* for lethal mutations in higher animals? For lethal mutations in *D. melanogaster*, the *hs* value on average is about 0.025; hence, *h* is also about 0.025. This makes the fitness of *Ll* flies about 98% of *LL* flies (Crow, 1993a,b). In addition, the genomic (X, second, and third chromosomes) lethal mutation rate per gamete is about 0.016 (Woodruff *et al*., 1983, 1984, 1996; Fu and Huai, 2003; Hedrick, 2011; Gao *et al*., 2011). This rate comes from the lethal rate for X chromosomes of about 0.001 and the autosomal mutation rate of about 0.015 for the second and third chromosomes. Hence, about one fly in 15 will have a new genomic lethal mutation (a mutation can occur either in the female or male parent) and a typical recessive autosomal lethal will persist for about 40 generations ($1/hs = 1/0.025 = 40$) (Crow, 1993a,b). In addition, since it has been estimated that there are about 1,200 genes that can mutate to lethality in *D. melanogaster* (Abrahamson *et al*., 1980; Gao *et al*., 2011), the per gene mutation rate for lethals (*u*) in *D. melanogaster* is about 1.3×10^{-5} ($0.016/1200 = 0.000013$).

Estimations of dominance for autosomal lethals are especially important because these mutations are usually low in frequency and are, therefore, almost always in the heterozygous state. Hence, their mutation/selection equilibrium frequencies are dependent almost entirely on their dominance (Muller, 1950; Crow 1993a,b). For autosomal recessive lethals ($h = 0$), the expected mutation/selection equilibrium value for the frequency of the recessive deleterious allele *l* is $(u/s)^{1/2}$, or $(u)^{1/2}$ for lethals, whereas for mutations with dominance ($h > 0$) the equilibrium value for *l* is $u/(hs)$, or u/h for lethals (Hedrick, 2011). For example, using the *s* and *h* values given above for *D. melanogaster* lethals (1 and 0.025), and *u* of 0.000013 per gene, the mutation/selection equilibrium frequency for lethals with dominance ($u/hs = 0.000013/0.025 = 0.00005$) would be about eight times lower than that for completely recessive ($h = 0$) deleterious mutations [$(us)^{1/2} = (0.000013)^{1/2} = 0.004$]. The expected frequency of X-linked lethals in nature would be much lower.

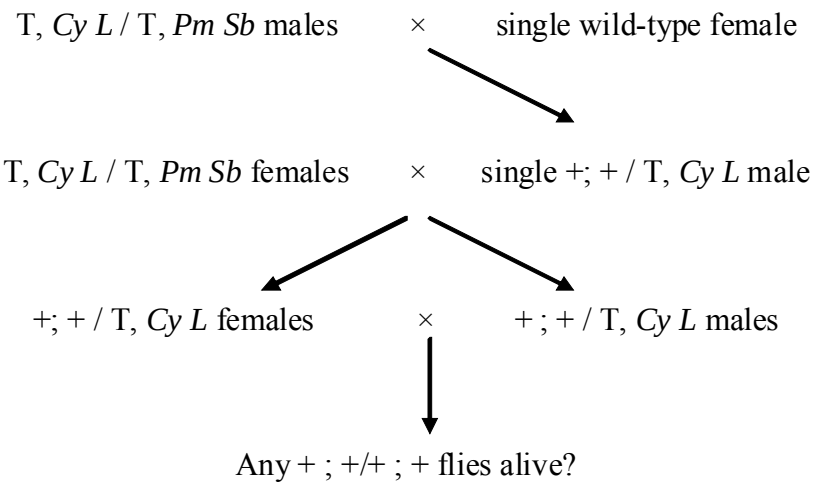
For X-linked lethals, where selection will occur in hemizygous males, the equilibrium frequency for *l* is $3u/s$ (Crow and Kimura, 1970) and will be about 0.00004 (3*0.00013/1). Hence, the expected equilibrium frequency of recessive X-linked lethals in nature will be about 13 times lower than autosomal lethals with dominance (0.0005/0.00004) and 100 times lower than completely recessive autosomal lethals (0.004/0.00004). Because of hemizygous selection for X-linked lethals in males *vs*. selection in heterozygotes for autosomal lethals, one would expect, therefore, a much lower frequency of X-linked lethals in nature compared to autosomal lethals. Is this true?

In this study we attempted to determine if the frequency of X-linked lethal mutations is significantly lower than the frequency of autosomal lethal mutations in natural populations of *D. melanogaster*, as predicted by population genetic theory. Female *D. melanogaster* were captured by sweeping bananas in Perrysburg, Ohio on November 5, 2009 and July 30, 2010. From these flies, isofemale lines were set up from single flies, and, after one generation, single virgin females from each line were tested for preexisting second and third recessive lethal mutations and single virgin sibling females were tested for preexisting X-linked lethal mutations by the following two sets of crosses.

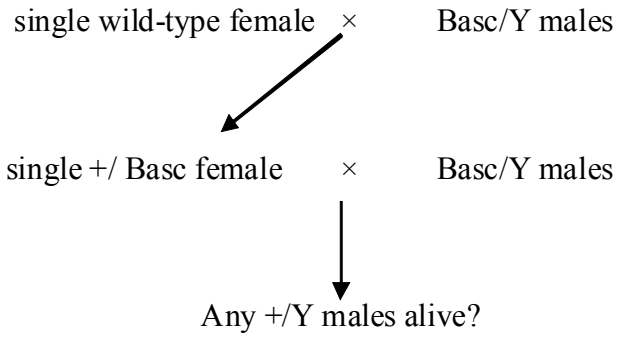
In these crosses, + = a wild-type X, second, or third chromosome from nature; T(2;3)A1-W, *Cy L* is a translocation for the second and third chromosomes that contains the dominant visible markers *Cy* (curly wings) and *L* (lobed eyes) (*Cy* and *L* are also recessive lethals), plus multiple inversions that make it a balancer for the second and third chromosomes; T(2;3)B18, *Pm Sb* is a translocation for the second and third chromosome that contains the dominant visible markers *Pm* (plum eyes) and *Sb* (stubble bristles) (*Pm* and *Sb* are also recessive lethals); Basc is a balancer for the X chromosome that contains the dominant marker *B* (bar eyes), the recessive white-apricot mutation

(with the symbol *a*, for w^a, which causes white eyes), and multiple inversions associated with the *sc* (scute) mutation. See Lindsley and Zimm (1992) for descriptions of these mutants, chromosomal rearrangements, and balancer chromosomes. Since T(2;3)A1-W, *Cy L* and Basc are balancer chromosomes, recombination events in heterozygotes will be eliminated (see a discussion of this topic in Klug *et al*. 2010). This keeps new lethal mutations from being moved from the wild-type chromosomes by recombination. In these crosses, the T(2;3) chromosomes will be given the symbol T and all females are virgins.

For the identification of preexisting second or third chromosome lethal mutations:



For the identification of preexisting X-linked lethal mutations:



The absence of +;+/+;+ (wild-type, non-*Cy* and non-*Sb*) flies or the absence of +/Y (wild-type, red eyed) males in the final generations indicate the presence of a preexisting recessive lethal mutation on the autosomes or X-chromosome, respectively. Additional crosses to confirm lethals were made by mating +; +/T, *Cy L* females and males or by mating +/ Basc females with Basc/Y males. A presumptive lethal was declared a lethal if no wild-type (+;+/+;+) flies or no wild-type (+/Y) males were observed among at least 100 progeny.

The results of the screens for autosomal and X-linked lethals are shown in Table 1.

As predicted by population genetic theory, in nature there was a significantly higher frequency (30%) of autosomal lethals as compared to X-linked lethals (0%). Part of the reason for the high frequency of autosomal lethals in nature may be due to some lethals within a collection being the same mutation (are allelic). For example, the mutations could be in siblings or the original

mutation could have arisen as a premeiotic cluster (see Woodruff *et al*., 1996, for a discussion of this topic). Hence, by interline crosses we also tested the allelism of the five lethals from the 11/05/2009 collection and the five lethals from the 7/20/2010 collection. All of the lethals in each collection were unique (non-allelic). Hence, all of the autosomal lethals were probably of independent mutational origin. Again, because of selection against X-linked lethals in hemizygous males and autosomal lethals in heterozygotes, the results of this study clearly show that X-linked lethals are in a lower frequency than autosomal lethals in nature.

Table 1. Frequencies of autosomal (2nd and 3rd) and X-linked recessive lethal mutations in a natural population (Perrysburg, Ohio) of *Drosophila melanogaster*.

Collections	Autosomal Lethals/ Total	X-Linked Lethals/Total	P
11/05/2009	5/17	0/29	0.011
7/20/2010	5/13	0/21	0.015
Total	10/30	0/50	0.0001

A classroom discussion of the results of this teaching exercise could include the following questions. 1) If the expected equilibrium frequencies of autosomal and X-linked lethal mutations were similar in *D. melanogaster* and humans, what would be the expected frequency of humans that are homozygous for an autosomal lethal mutation that t kills in young children? For autosomal lethals, each parent would have to carry the recessive mutation and then there would be a one-fourth chance that their offspring would be homozygous for the recessive lethal mutations. Hence, for a completely recessive autosomal lethal: $0.004 \times 0.004 \times 0.25 = 0.000004$ or one in 250,000 humans would be homozygous. For an autosomal lethal mutation with a dominance of 0.025 ($h = 0.025$), $0.00005 \times 0.00005 \times 0.25 = 0.0000000006$ or one in 1,600,000,000 humans would be homozygous. 2) What is the expected frequency of human males that are hemizygous for a recessive lethal mutation that t kills in young children? For a preexisting sex-linked lethal to appear in a male, there would need to be a female parent that is heterozygous for the lethal, and then she would have a one-half chance of having a son with the recessive lethal. Hence, $0.00004 \times ½ = 0.00002$ or one in 50,000 human males. 3) This teaching exercise has only considered lethal mutations. How many deleterious mutations, that are not lethal, occur in each human? The answer is that there may be as many as ten new deleterious in each human (Reed and Aquadro, 2006). 4) James Crow (Crow, 1999) has stated that we now live in an environment with improved living conditions that reduces selection against deleterious mutations. What impact would this have on future humans? Would the frequency of humans with homozygous lethal and deleterious mutations increase?

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Cummings, Boston; Lindsley, D.L., and G.G. Zimm 1992, *The Genome of* Drosophila melanogaster, Academic Press, New York; Lynch, M., J. Blanchard, D. Houle, T. Kibota, S. Schultz, L. Vassilieva, and J. Willis 1999, Evolution 53: 645-663; Morton, N.E., 1981, Mutation rates for human autosomal recessives. In: *Population and Biological Aspects of Human Mutation*, (Hook, E.B., and I.H. Porter, eds.), pp. 65-89, Academic Press, New York; Muller, H.J., 1950, Amer. J. Human Genet. 2: 111-176; Reed, F.A., and C.F. Aquadro 2006, Trends in Genetics 22: 479-484; Simmons, M.J., and J.F. Crow 1977, Ann. Rev. Genet. 11: 49-78; Strachan, T., and A.P. Read 2004, *Human Molecular Genetics*, Garland Science, New York; Woodruff, R.C., B.E. Slatko, and J.N. Thompson, Jr. 1983, Factors affecting mutation rates in natural populations. In: *The Genetics and Biology of Drosophila*, (Ashburner, M., H.L. Carson, and J.N. Thompson, Jr., eds.), Vol. 3C, pp. 37-124. Academic Press, New York; Woodruff, R.C., J.M. Mason, R. Valencia, and S. Zimmering 1984, Envir. Mut. 6: 189-202; Woodruff, R.C., H. Huai, and J.N. Thompson, Jr. 1996, Genetica 98: 149-160.

The role of sexual reproduction and recombination in adaptive evolution.

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The evolution and maintenance of sexual reproduction that leads to recombination of chromosomes at meiosis is a evolutionary puzzle. Why should a fit organism give up half of its genes and experience possible harm (for example, sexually transmitted diseases and increased risk of predation) when it could reproduce asexually? With asexual reproduction all of the genes of an individual are passed to its offspring, and there is no harm that occurs in the search for a mate (Muller, 1964; Maynard Smith, 1978; Ridley, 1993; Michod, 1995; Barton and Charlesworth, 1998).

There are two main hypotheses for the evolution and maintenance of sexual reproduction: 1) Sexual reproduction brings together favourable alleles of different genes by recombination, increasesing the fitness of offspring and the rate of adaptation to new environments; 2) Sexual reproduction can bring together deleterious alleles of different genes by recombination. These deleterious alleles can then be eliminated from the population in bunches by negative selection more quickly than can a combination of deleterious alleles that are removed one at a time in the absence of recombination (for reviews of this topic see Crow and Kimura, 1965; Barton and Charlesworth, 1986; Kondrashov, 1988; Otto and Lenormand, 2002; Rice, 2002; Gillespie, 2004).

The objective of this proposed study is to test the first hypothesis listed above (combining favourable genes by recombination) by measuring the rates of selection response in the presence and absence of recombination in the model system *Drosophila melanogaster*. Rice and Chippindale (2001) have shown that beneficial alleles that increase offspring numbers in *D. melanogaster* accumulate faster in populations with recombination than in populations without recombination.

We took advantage of the natural lack of recombination in *D. melanogaster* males and the aviability of balancer chromosomes with multiple inversions that eliminate recombinant gametes in *D. melanogaster* females. With the appropriate crosses, as shown below, we tested a model for adaptive evolution, selection response for bristle numbers, in the presence and in the absence of recombination. As part of this model, it was assumed that flies with decreased or increased bristle numbers are more fit. This proposed study is partially based on the materials, methods, and results in

McPhee and Robertson (1970). Figure 1 shows the sternoplueral bristles (eleven here) that will be counted in this study.

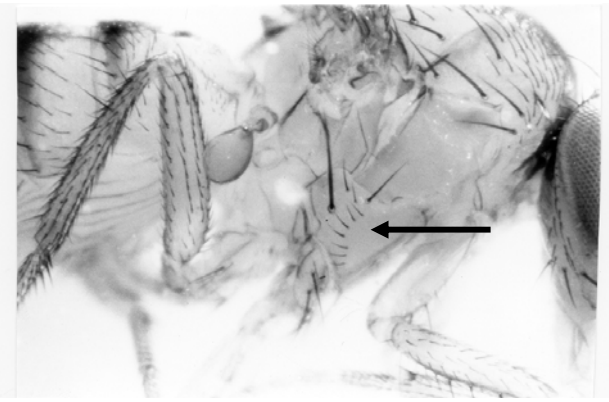


Figure 1. Sternopleural bristles of *Drosophila melanogaster*.

The following mating scheme includes two crosses (Rec with recombination and NoRec without recombination) to measure the role of recombination in evolution (rate of response to selection). In this mating scheme, "+" stands for a wild-type chromosome, T(2;3)A1-W, *Cy L* is a translocation for the second and third chromosomes that is also a balancer (eliminates recombinant gametes) for the second and third chromosomes, *Cy* = curled wings (dominant visible and recessive lethal mutation), and *L* = lobed eyes (dominant visible and recessive lethal mutation) (after the first generation below, this translocation will be given the symbol A1); T(2;3)B18, *Pm Sb* is a translocation for the second and third chromosomes, with *Pm* = plum eye color (*Pm* is also called bw^{V1}) and *Sb* = stubble bristles; and +/+;+/+ is a wild-type stock that was derived from a mixture of four isofemale lines captured from nature (Perrysburg, OH) on July 30, 2010. For details on the mutant genes, rearrangements, and balancer chromosomes used in this study, see Lindsley and Zimm (1992). Again, we emphasize that recombination does not occur in the males of these crosses. Virgin females were used in all crosses.

+/+;+/+ wild-type females × T(2;3)A1-W, *Cy L*/T(2;3)B18, *Pm Sb* males

F1: select for +;+/T(2;3)A1-W females × +;+/T(2;3)A1-W males

Cross Rec Cross NoRec

F2: +/+;+/+ females × +;+/A1-W males (recombination in +;+/+;+ females)

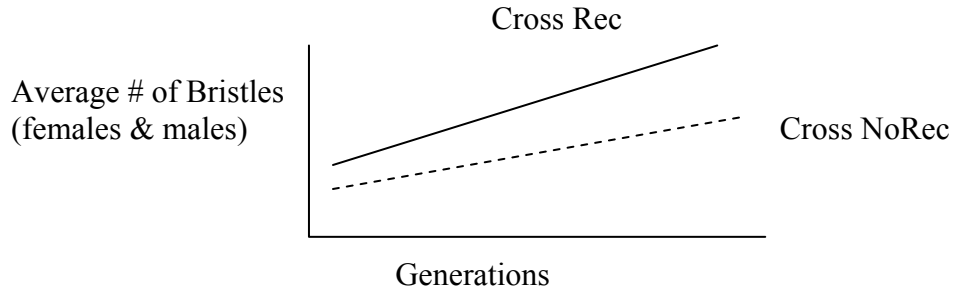
F2: +;+/A1-W females × +/+;+/+ males (no recombination in +;+/A1-W females)

Selection for Lower Bristle Numbers: For Cross Rec and NoRec, we picked eight of 20 F3 +/+;+/+ virgin females and eight of 20 F3 +;+/A1-W males with the lowest number of bristles and

mated them for the next generation. We then repeated these crosses and bristle counts for ten generations.

Selection for Increased Bristle Numbers: For Cross Rec and NoRec we picked eight of 25 F3 +;+/A1-W virgin females and eight of 25 F3 +;+/+;+ males with the highest number of bristles and mated them for the next generation. We then repeated these crosses and bristle counts for six generations.

It is our hypothesis that recombination in Cross Rec will increase the coupling of beneficial alleles of different genes on autosomes that will increase the rate of selection response above that of Cross NoRec, where there is no recombination. Hence, the response to selection will be faster in the flies in Cross Rec compared to flies in Cross NoRec as shown below for an increased bristle number experiment.



We will determine if the slopes of the lines from Cross Rec and Cross NoRec are significantly different using the Prisim Statistical Program.

The results of the two selection experiments for decreased bristle numbers in the presence and absence of recombination are shown in Figure 2, whereas the results of the two selection experiments for increased bristle numbers in the presence and absence of recombination are shown in Figure 3. There was a significant (P = 0.002) decrease in bristle numbers in the presence of recombination in one experiment (Figure 2a), and a significant (P = 0.01) increase in bristle numbers in the presence of recombination in one experiment (Figure 2b). These results support the theory that adaptive evolution (selection response in this study) is faster in the presence of recombination.

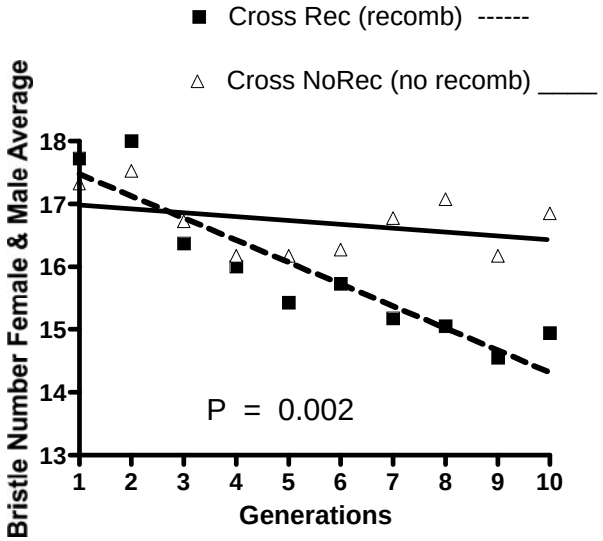


Figure 2a. Response to selection for decreased bristle numbers in the presence and absence of recombination.

In relation to a teaching exercise, the increased bristle number experiment might be more appropriate in a teaching environment, since the positive response occurred in fewer (six) generations. Part of the reason this experiment gave a faster significant increase in bristle numbers than that observed in the decreased bristle number experiment was because there was a larger selection differential in the

increased bristle experiment (eight of 25 flies were selected each generation) as compared to the decreased bristle experiment (eight of 20 flies were selected each generation). In a teaching environment with a shorter time frame, one could also increase the selection differential.

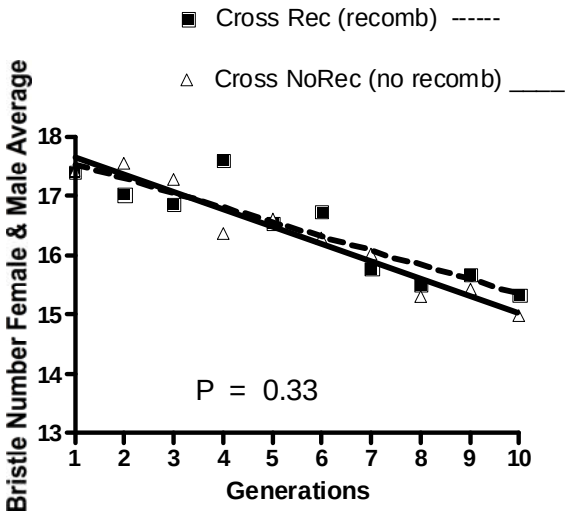
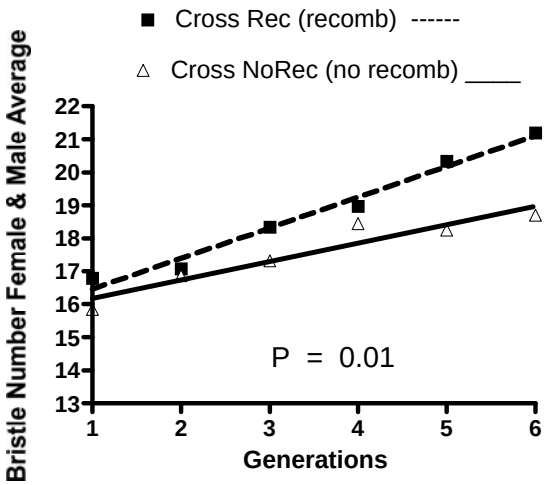


Figure 2b. Response to selection for decreased bristle numbers in the presence and absence of recombination.



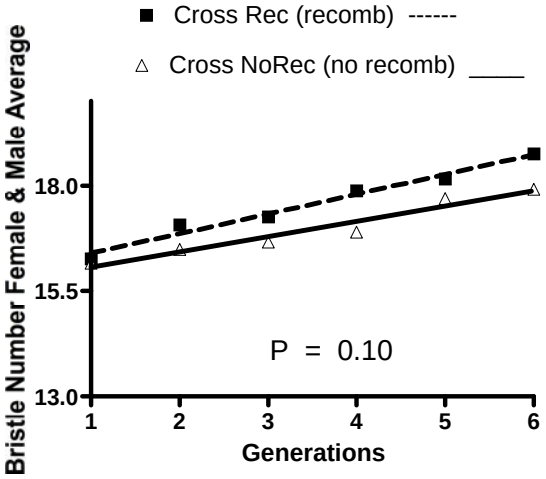


Figure 3a and b. Response to selection for increased bristle numbers in the presence and absence of recombination.

Part of a class discussion of the results of this teaching exercise could include: 1) What would have been the expected results of this experiment if a highly inbred stock with no genetic variation had been used instead of the wild-type line? All responses to selection over time would have been non-significant (see Woodruff and Thompson, 2005). 2) The class might be asked to read Goddard *et al*. (2005), which shows that sex increases the rate of adaptation of yeast to a new harsh environment. 3) The mutants *Cy*, *L*, *Pm*, and *Sb* used in this experiment are homozygous dominant visible mutations and recessive lethal mutations in *D. melanogaster*. Are there similar acting mutations in humans? One might ask students to go to National Center for Biotechnology Information (NCBI) (www.ncbi.nim.nih.gov) and then to Online Mendelian Inheritance in Man (OMIM) (www.ncbi.nim.nih.gov/omim) and search for achondroplasia (#100800). This form of short-limb

dwarfism is caused by a dominant autosomal mutation that also has more drastic effects in homozygotes.

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Spontaneous and gamma ray induced chromosome breakage in *Drosophila melanogaster*.

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Chromosomal rearrangements are more frequent in humans than previously thought. For example, the diploid genome sequence of J. Craig Venter (his company, Celera Genomics, and the Human Genome Sequencing Consortium first sequenced the human genome) contained 292,102 heterozygous insertion/deletion events (1 to 571 base pairs), 559,473 homozygous indels (insertions and deletions of one to 82,711 base pairs), 90 inversions, and numerous duplications (Levy *et al.*, 2007). The rate of new chromosome aberrations in humans is about 4/1000 live births (Sankaranarayanan and Wassom, 2005), with one in 500 humans carrying a new reciprocal translocation (Gajecka *et al*., 2008). In addition, many chromosome rearrangements are associated with human genetic defects and cancer (Strachan and Read, 2004; Lupski 2007; Hastings *et al*., 2009). Hence, it is important to identify spontaneous and induced chromosome breakage events and to estimate their rates in a model organism such as *Drosophila melanogaster*.

It is the objective of this study to measure spontaneous and gamma ray induced X-chromosome breakage events in an F1 assay in *D. melanogaster*. This hyperploidy chromosome breakage assay involves the identification of breakage events that delete segements of the X chromosome in males, which are then recovered as extra chromosomal fragments (hyperploidy) in F1 females. This assay is shown in Figure 1, and is discussed in Auerbach (1962) and Blount and Woodruff (1986). The C(1)DX, *y w f* chromosome is two X chromosomes attached to a single centromere and containing the recessive markers *y* (yellow, yellow body color), *w* (white, white eyes), and *f* (forked, short bristles) (Lindsley and Zimm, 1992); *D. melanogaster* that have two X chromosomes and a Y chromosome are fertile females. In this cross, y^+, w^+ and f^+ denote the wild-type alleles of the three genes; Canton-S is a wild-type stock (containing a y^+ w^+ f^+ X chromosome), and O is a centromere. It should be noted that some exceptional C(1)DX, $y\ w\ f\ /\ y^+\ w^+\ f^+$ triplo-X female progeny occur in older cultures in this breakage assay. These XXX females have grey bodies, red eyes, and long bristles, plus they usually have slightly deformed wings, move slowly, have slow

development, and are sterile (see Lindsley and Zimm, 1992, for more details). Since these triplo-X females do not contain breakage events, they will not be counted in this experiment.

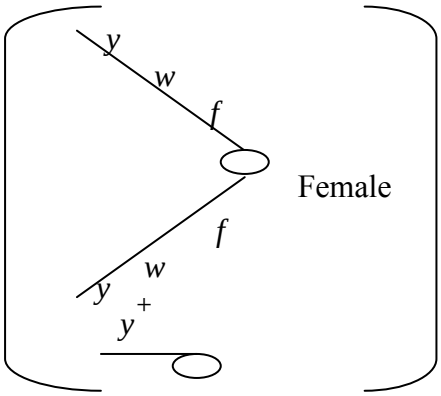
C(1)DX, *y w f*/Y females X Canton-S/Y (y^+ w^+ f^+/Y) males
Untreated (controls) or gamma ray trea

↓

F1 No chromosome breakage — Possible X-Chromosome breakages

C(1)DX, *y w f*/Y females
yellow body color
white eyes
short (forked) bristles

1. C(1)DX, *y w f*/y^+ females
grey body color
white eyes
short (forked) bristles



2. C(1)DX, *y w f*/w^+ females
yellow body color
red eyes
short (forked) bristles

3. C(1)DX, *y w f*/f^+ females
yellow body color
white eyes
long bristles

4. C(1)DX, *y w f*/y^+ w^+ females
grey body color
red eyes
short (forked) bristles

Figure 1. Hyperploidy chromosome breakage assay for X-chromosomes in males that are identified as chromosome fragments in F1 females.

In this study, the spontaneous rate of chromosome breakage will be measured and added to the historical spontaneous rate at Bowling Green State University of two breakage events among 88,161 scored F1 females (0.00002) (74,959 are from Blount and Woodruff, 1986, and 13,202 are from subsequent control runs). In addition, males were treated with 2,010 rads of gamma rays from a cesium source at the University of Toledo Medical Center (treatments were performed by Eddie

Brentlinger, Radiation Safety Officer). This is a positive control, because gamma rays are known to cause chromosome breakage in *Drosophila* and humans (Alexander and Bergendahl, 1962; Ganetzky, 1977; Gubb *et al*., 1984, 1985; Hilliker and Trusis-Coulter, 1987; Ashburner, 1989; Sankaranarayanan and Wassom, 2005).

The chromosome breakage results for this study are shown in Table 1.

Table 1. Rates of chromosome breakage in *Drosophila melanogaster* males.

	Breakage Events	Total F1 Females	Rate
Spontaneous			
a. Historical	2	88,161	0.00002
b. This Study	0	7,613	0
c. Total	2	95,774	0.00002*
Gamma Ray Treated (2,010 rads)	2	1,521	0.0013*

*P < 0.0001

The hyperploidy assay has been used to identify chemical-induced, X-ray-induced, and transposable DNA element-induced chromosome breakage in *D. melanogaster* (Auerbach, 1962; Blount and Woodruff, 1986). In this study, this assay was used to identify gamma ray-induced chromosome breakage. A significant ($P < 0.0001$) increase in chromosome breakage was caused by gamma rays. The two gamma ray-induced breakage events observed in this study were C(1)DX, $y\ w\ f\ /\ y^+\ w^+$ females that had grey body color and red eyes. These X-chromosome fragments, therefore, contained the yellow and white loci and a segment of the chromosome that contained a centromere, *i.e.*, a two-break deficiency occurred that removed a part of the X chromosome between the white locus and the centromere.

The hyperploidy assay could be used in a teaching environment to screen other possible chemical and physical agents for their ability to cause chromosome breakage, including ultraviolet light (see Toth *et al*., 2007) and tobacco products that are mixed into *Drosophila* food. The students could be asked to suggest possible chromosome breakage agents that could be tested using the hyperploidy assay.

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Rapid immunofluorescent staining of spectrin in cultured *Drosophila* S2 cells for use in teaching undergraduate cell biology lab.

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Introduction

Immunofluorescent staining is a commonly used method in biology to study cellular structures. Immunofluorescence utilizes antibody binding to a target antigen within a cell. Indirect immunofluorescence is often used, where a primary antibody attaches to the target antigen and a fluorescently labeled secondary antibody attaches to the primary antibody. The fluorescent dye allows for the visualization of specific components of a cell using a fluorescence microscope.

Traditional immunostaining protocols usually take a minimum of 5 hours and can take more than 24 hours if an overnight antibody incubation is used. These lengthy protocols make the immunostaining procedure impractical for the undergraduate teaching laboratory that only meets a couple of hours per week. The most time-consuming portions of the procedure are the antibody incubations, so reducing these incubation times is critical. Previous studies have shown the utility of microwave irradiation to reduce the antibody incubation time in the staining of tissue sections (Leong and Milios, 1986; Hite and Huang, 1996; Hatta *et al.*, 2006). It is thought that microwaves increase molecular vibrations that speed up antibody to antigen interactions. Therefore, we sought to develop a rapid immunostaining procedure for fixed *Drosophila* S2 cells by using microwave incubation. We tested this new protocol in an undergraduate cell biology laboratory using α-spectrin antibodies and found that it produced robust staining of the spectrin cytoskeleton. The staining procedure can be performed in less than 30 minutes and is easily adapted for use in undergraduate settings.

Materials and Methods

Preparation of Drosophila S2 cells for immunofluorescence (1 hour)

S2 *Drosophila* cells were cultured in sterile petri dishes at room temperature in M3 medium supplemented with 12% fetal bovine serum and antibiotics. S2 cells adhere to the bottom of the culture dish and also create cell clumps. In order to create a homogeneous suspension, the cells were gently pipetted up and down to dislodge them from the plate and to break up cell aggregates. The cells were counted using a hemocytometer and diluted to 200,000 cells/mL with serum free medium (Thermo Scientific HyClone CCM3, Catalog #SH30065.01) before being added to each well of a PFTE printed microscope slide (Electron Microscopy Sciences, Catalog No. 63424-06). 50 µL of diluted cells were pipetted onto the microscope slide using a clean micropipette tip. The cells were allowed to attach to the treated microscope slide for 30 minutes in a humidity chamber to prevent the samples from drying out. A humidity chamber was created using a sealed pipet tip box with a damp paper towel in the bottom. After cell attachment, the medium was drawn off with a micropipette, being careful not to scrape any cells off of the slide. Using a fresh tip 30 µL of fixing solution (4% formaldehyde in PBS) was applied to each sample for 10 minutes. Next, the fixing solution was drawn off, and the cells were rinsed three times with 30 µL of PBS (phosphate buffered saline) for 2

minutes each. 30 µL of wash buffer (TBS + 0.05% Tween-20) was applied to each well for 2 minutes, three times each. 30 µL of blocking solution (wash buffer + 10% normal goat serum) was applied to each sample. For convenience the slide was placed in the refrigerator inside the humidity chamber at 4°C for one week until the next lab session. The cells are now ready for rapid immunostaining.

Immunostaining procedure (30 minutes)

A plastic tray was filled with 2 liters of cold tap water and placed inside of a microwave oven (1250W Panasonic, Model NN-T945SF). The water is used to absorb excess microwave radiation to prevent the cells from overheating. The blocking solution from the previous treatment was drawn off. A secondary antibody staining control was included by not adding any spectrin antibody to the first sample (see Figure 1A). 20 µL of diluted α-spectrin antibody (Developmental Studies Hybridoma Bank, 3A9, diluted 1:20 to 3 µg/mL in blocking solution) was applied to the cells of the second sample (See Figure 1B). The slide was then placed on a platform in the water tray and microwaved for 3 minutes at 50% power. The slide was left to stand for 2 minutes inside the microwave. Once removed, the liquid was drawn off from each sample, and the samples were washed three times with 30 µL of wash buffer, 1 minute each. The remaining washing solution was drawn off and 20 µL of the diluted secondary antibody (Alexa Fluor 488 goat anti-mouse, Invitrogen #A-11029, diluted 1:1000 to 2 µg/mL) was applied to both samples. The water in the microwave was replaced with 2 liters of fresh tap water. The slide was again placed on platform in the water tray. The samples were microwaved for 3 minutes at 50% power, then allowed to stand for 2 minutes inside the microwave. 30 µL of DAPI solution (100 ng/mL) was applied to each sample for 5 minutes. Next, the samples were washed twice in 30 µL of wash buffer for 1 minute each. Finally, two drops of Vectashield (Vector Labs, Catalog #H-1000) was added on each end of the slide and a coverslip (Electron Microscopy Sciences, Catalog #63769-01) was placed over the slide. The coverslip was sealed using clear top coat nail polish. Fluorescent staining was imaged using a fluorescence microscope (Motic BA410).

Results and Discussion

Immunofluorescence allows for the visualization of specific structures within the cell. This technique is useful in many fields of science, such as developmental biology and pathology. Immunofluorescence can be a very long and tedious process, making it prohibitive for use in the undergraduate teaching laboratory. Therefore, developing a rapid and effective method of immunostaining would be very advantageous to teaching this method to students. We used microwave irradiation to shorten the antibody incubation steps, since microwave radiation is thought to speed up the formation of antigen-antibody complexes (Hatta *et al.*, 2006). Our new procedure is much faster compared to the standard immunofluorescence protocol. Since it is fast and uses common laboratory equipment, the procedure is practical to use in any undergraduate cell biology lab.

Spectrin is an important component of the cytoskeleton. For example, spectrin gives flexible, structural support to the shape of red blood cells (Delaunay, 2007). Spectrin is located at the periphery of the cell near the plasma membrane. In our rapid antibody staining procedure, spectrin clearly localized to the outer regions of the cell, and the staining fluoresced very brightly (Figure 1B). DAPI stained DNA was used as a reference to distinguish the nuclei from the membrane-localized spectrin. In contrast, the secondary antibody control showed very little green fluorescence, demonstrating the specificity of the spectrin antibody staining (Figure 1A).

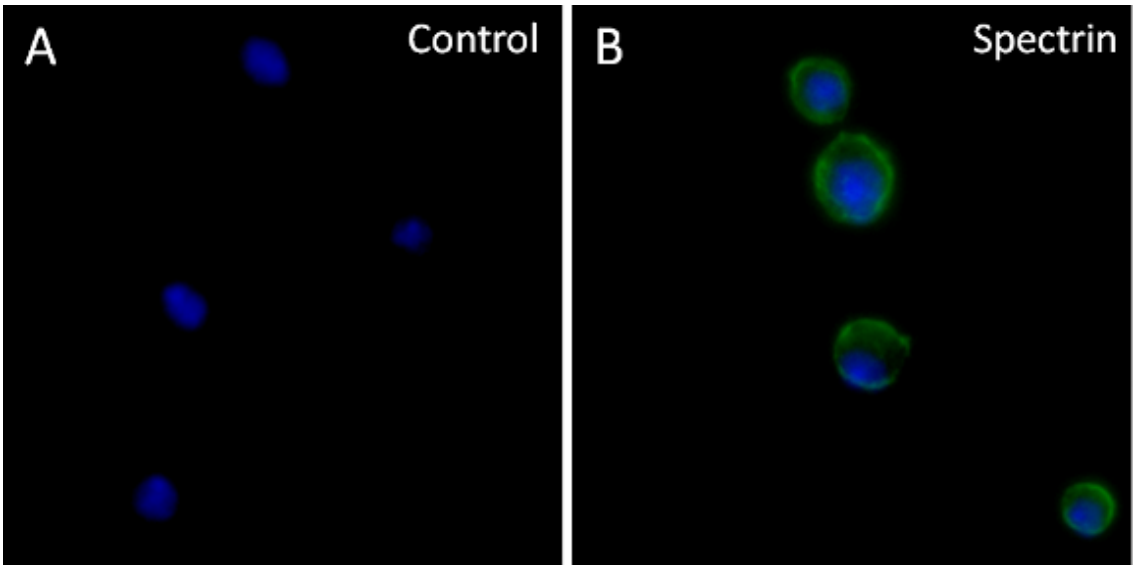

Figure 1. Immunofluorescent labeling of spectrin in *Drosophila* S2 cells. Cells were fixed with 4% formaldehyde and blocked with 10% normal goat serum before the rapid immunostaining protocol. A, To ensure that nonspecific staining of the green fluorescent secondary antibody was minimal, we performed a control stain where the primary antibody was omitted. No green fluorescence was seen in the control samples. Nuclei are in blue stained with DAPI. B, Inclusion of the α-spectrin antibody during the rapid staining procedure resulted in bright fluorescence at the periphery of the cell near to the plasma membrane.

Immunoflourescence is an excellent technique to help undergraduate cell biology students to see the location of spectrin in the cell with their own eyes and relate it to material learned in lecture. Along with being time efficient, our rapid immunofluorescence procedure gives every student useful practice and hands on learning of this important technique. Repetitive steps also allow the students to further develop micropipetting skills. Students are given the opportunity to learn about fluorescence microscopy and to further develop microscopy techniques. Importantly, students learn how to interpret their observations, which gives the student insight into what they are viewing with a fluorescence microscope. Rapid immunofluorescent staining of spectrin also gives the students exposure to DNA staining with DAPI, which is commonly used in fluorescence staining of cells to show nuclei. A practical understanding of the immunofluorescence technique will benefit students by helping them to better understand the biology of the cell. This technique is also an important aspect of scientific training, as many of these students will become future scientists and medical professionals who will be better able to interpret scientific data and perform this technique in a laboratory setting.

In conclusion, we have developed a rapid immunostaining protocol for the visualization of spectrin in cultured *Drosophila* S2 cells. This staining protocol may be used for other antibodies, although the incubation conditions may need to be optimized for each antibody. Furthermore, the protocol could also be developed to include multiple antibodies for the detection of two or more antigens. The efficiency of performing immunofluorescence in 30 minutes and the simplicity of this protocol makes our rapid immunofluorescent staining protocol ideal for any undergraduate cell biology course.

Acknowledgments: We would like to thank the Department of Natural Sciences and the College of Science and Health Professions at Northeastern State University for providing support for this study. We would also like to thank the Cell Biology students during the 2011 Fall semester for their enthusiasm and willingness to try new techniques.

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Protein trap lines of *Drosophila* to demonstrate spatio-temporal localization of proteins in an undergraduate lab.

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Abstract

The objective of this teaching note is to generate a laboratory exercise, which allows students to get a hands-on experience of a cell biology technique. The short duration of the laboratory classes is the biggest challenge with the development of a cell biology lab for an undergraduate curriculum. Therefore, it is necessary to design a laboratory exercise that enables the students to carry out cell biological assays in the desired time. This laboratory exercise focuses on tracking protein expression levels along a spatial (space) and temporal (time) axis in developing *Drosophila melanogaster* organ primordium. Here we use the protein trap model developed in *Drosophila* to demonstrate the sub-cellular localization of proteins. The protein trap transgenic flies have Green Fluorescent Protein (GFP) reporter tags to the full-length endogenous proteins that allow observation of their cellular as well as sub-cellular distribution. Since the life cycle of *Drosophila* is short, it is easy to rear them in the lab and also use them as an excellent model for an undergraduate lab curriculum. The goal of this exercise is to train undergraduate students and teach them the use of one such powerful tool which enables the localization of proteins.

Introduction

The present day undergraduate pedagogy puts great emphases on quantitative reasoning and inquiry-based activities in a laboratory experience. It is widely accepted across the teaching community that promotion of intellectual development through habit of enquiry is an important pillar of learning in undergraduate education. We have been introducing new lab exercises in our Cell Biology (Bio-440 Lab) lab curriculum by exploiting the tools available in *Drosophila melanogaster*, a.k.a. fruit fly, model system. The short generation time, ease of handling, high reproductive ability, and wide array of genetic tools make *Drosophila* an excellent choice for demonstration of biological phenomena in the undergraduate labs (Tare and Singh, 2009; Tare *et al.*, 2010).

There are several approaches available to analyze gene expression in the tissues. The commonly used methods are visualizing gene expression by enhancer trapping, epitope tagging,

antibody staining, or gene trapping. These techniques like enhancer trapping and gene trapping essentially involve generation of transgenic animals. Since *Drosophila* has proved to be a versatile organism for transgenics, it can be used for the study of gene expression and protein localization. The protein trap strategy is a modification of gene trapping which allows epitope tagging of the endogenous proteins of interest. The principle of this approach is to tag proteins by an epitope. Protein trapping is achieved by fusing the endogenous messenger of a gene with the DNA sequence encoding the reporter genes like Green Fluorescent Protein (GFP) (Morin *et al.,* 2001; Buszczak *et al.,* 2007; Kelso *et al.,* 2004; Quiñones-Coello *et al.,* 2007). Thus, protein trapping allows spatio-temporal localization of the protein of interest. A transposable artificial exon, which encodes a GFP protein and is flanked by both splice acceptor and donor sites, can get inserted into the endogenous coding region of a gene. It may result in a chimeric protein harboring the GFP reporter, which allows the localization of endogenous proteins. The full-length endogenous proteins resulting from protein trap in *Drosophila* can be seen as GFP fusion proteins from their endogenous promoters. The fusion of reporter serves as an excellent tool to study cellular and sub-cellular localization of the proteins.

Some protein traps lack the initiation codon and are fused to the N-terminal region of endogenous proteins and are flanked by acceptor and donor sites, which are inserted into an intron separating the exons coding for a chimeric protein in which the GFP is fused with the amino and carboxyl terminal of the trapped protein. So the reporter is expressed only if it gets integrated into the region of the target gene causing GFP expression, hence called Protein Trapped lines (Morin *et al.,* 2001; Buszczak *et al.,* 2007; Kelso *et al.,* 2004; Quiñones-Coello *et al.,* 2007). These protein trap lines have been generated by various labs and are available on request.

The potential of this technology has been extensively documented in yeast, as evident from large collections of protein trap strains generated by using transposable elements or by homologous recombination. Protein trapping has also been employed in cultured embryonic stem (ES) cells.

Protocol

Protein-trap transgenic flies were procured from *Carnegie* protein trap library (http://flytrap.med.yale.edu/index.html). These flies were reared on yeast-cornmeal-agar fly medium at room temperature. Fly food rearing medium is also commercially available (http://lab-express.com/). *Drosophila* life cycle comprises of 24 hours of embryonic development, followed by three larval instar stages. The larva develops into pupa, and adults emerge from pupa upon metamorphosis. *Drosophila* is a holometabolous insect. In *Drosophila*, the precursors of adult organs are housed inside the larva as a monolayer epithelium called imaginal disc. The imaginal disc is a favored model system to study patterning, growth, and differentiation. Imaginal discs are monolayer epithelium comprising of the groups of epithelial cells housed inside the larva that will give rise to adult appendages and cuticle after metamorphosis. We have used the eye-antennal imaginal disc, wing imaginal disc, and leg imaginal disc for this study. The eye-antennal imaginal disc gives rise to eye and the antenna in the adult fly, the wing imaginal disc forms the wing, and the leg imaginal disc forms the leg in the adult fly.

The third instar larvae were selected based on their size and were dissected in Phosphate Buffered Saline (PBS, containing 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4; Dulbecco and Vogt, 1954) using sharp Dumostar forceps (Electron Microscopy Sciences Cat. No. # 72707-01). The salivary glands and imaginal discs attached with mouth parts and brain were dissected. We isolated the leg, wing, and eye-antennal imaginal discs to study the protein trap expression.

Sample preparation (staining)

The larvae were dissected in PBS, and imaginal discs were fixed for twenty minutes with 4% paraformaldehyde (EMS Cat. No. # 15710) in PBS. The fixation is required to preserve and maintain the morphology of the tissue. After fixation, the fixative was removed by rinsing the tissue with ice cold PBS, which was followed by three washes (of ten minutes each) with PBST [PBS+ 0.2% Triton X-100 (Sigma Aldrich Cat No. # T100)]. The PBST washes allow the permeabilization of the tissue. The tissue was then incubated with dyes in PBS for twenty minutes at room temperature in the dark. The dyes used in this study are – Phalloidin Texas Red (Molecular Probes, Invitrogen, Cat. No. # T7471) and DAPI (1, 4', 6-diamidino-2-phenylindole; Molecular Probes, Invitrogen, Cat. No. # D 1306). Phalloidin specifically marks the actin filament meshwork (Small *et al*., 1999). The DAPI stains the nuclear material within the cell by binding to AT clusters in the minor groove of double stranded DNA (Mikael *et al*., 1987). Tissue after treatment with dye was washed in PBST. Each wash was ten minutes long on a nutator and finally the tissue was mounted on glass slides using Vectashield mountant (Vector labs, Cat. No. # H-1000). Vectashield is a glycerol based mountant that does not solidify. It serves as an antifade agent and prevents rapid loss of fluorescence during examination of the sample under microscope. Thus, Vectashield allows anti-fading ability for long term storage.

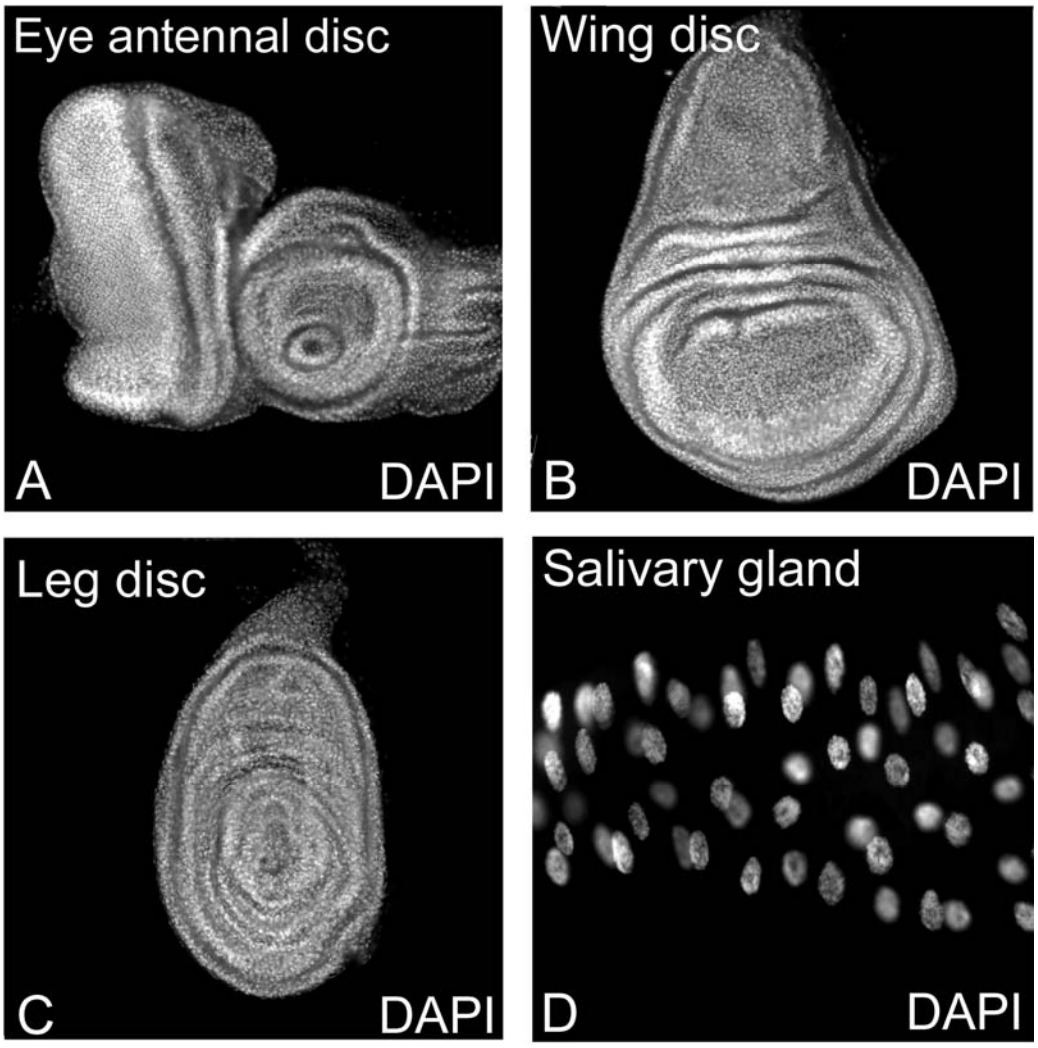

Figure 1. DAPI, a nuclear dye, marks the nuclei in (A) Eye antennal-, (B) Wing-, (C) Leg-imaginal discs, and (D) Salivary gland.

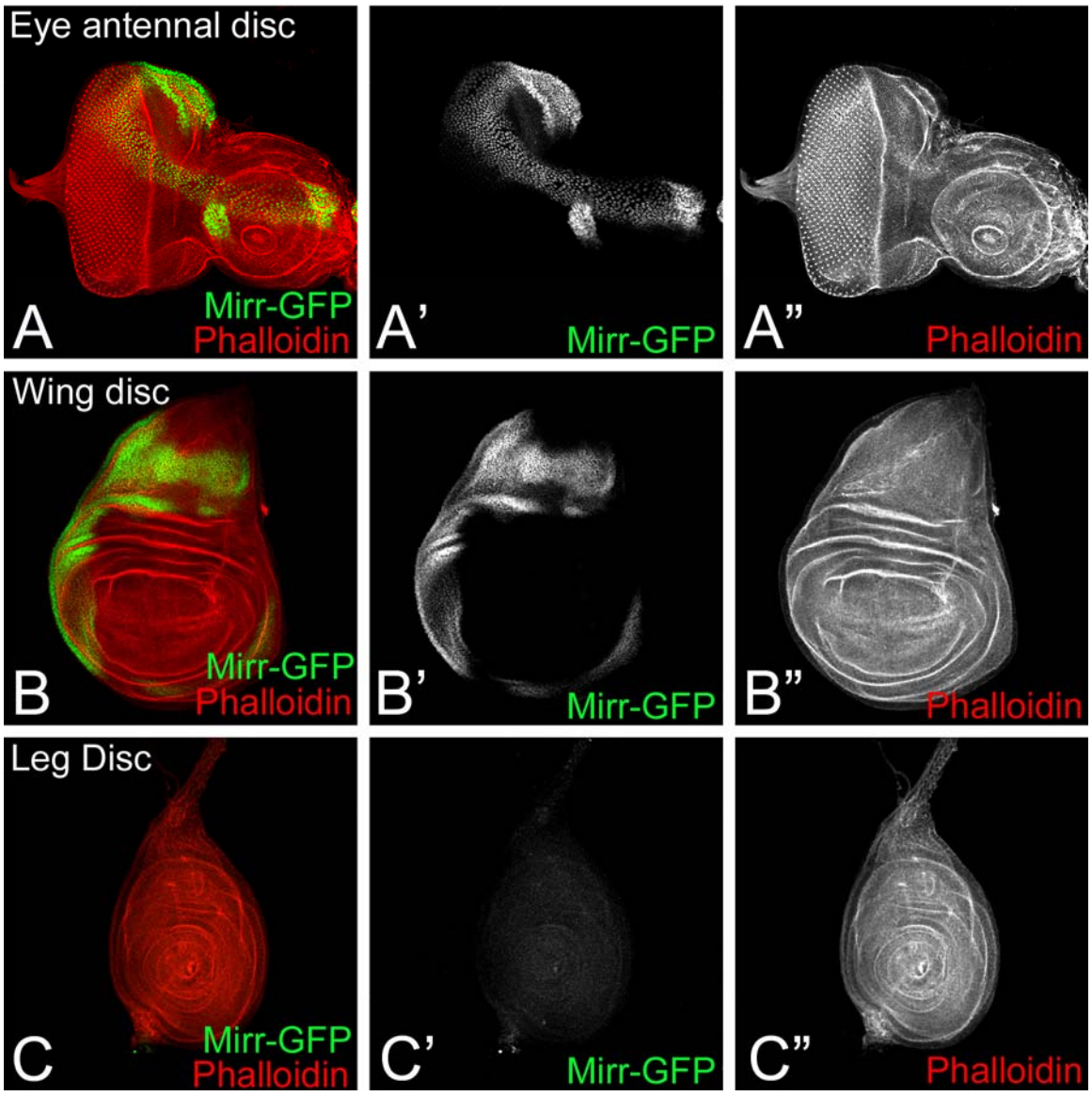

Figure 2. Expression of Green Fluorescent Protein (GFP) reporter marks the localization of Mirror (Mirr) and GFP chimeric protein using the protein trap line. Localization of Mirr and GFP chimeric protein in (A) Eye-antennal-, (B) Wing-, and (C) Leg-imaginal disc. Note that phalloidin (red) marks the actin cytoskeleton and provides the outline of the disc. Both Mirr-GFP (Figures 2A', B', C') expression and phalloidin (Figures 2A", B", C") are also shown in separate channels.

Imaging

Olympus BX51 epifluorescence microscope was used to take DAPI images, whereas GFP and Phalloidin staining images were taken using Olympus Fluoview 1000 Laser Scanning Confocal Microscope and edited using Adobe Photoshop 5.5 software.

The discs stained for nuclear marker DAPI (Kapuscinski, 1995) can be seen using DAPI filter that has an excitation wavelength of 358 nm and emission wavelength of 461 nm. A strong nuclear

staining was observed using Olympus BX51 Olympus epifluorescence microscope in eye-antennal (Figure 1A), wing- (Figure 1B), leg-imaginal disc (Figure 1C), and salivary gland (Figure 1D).

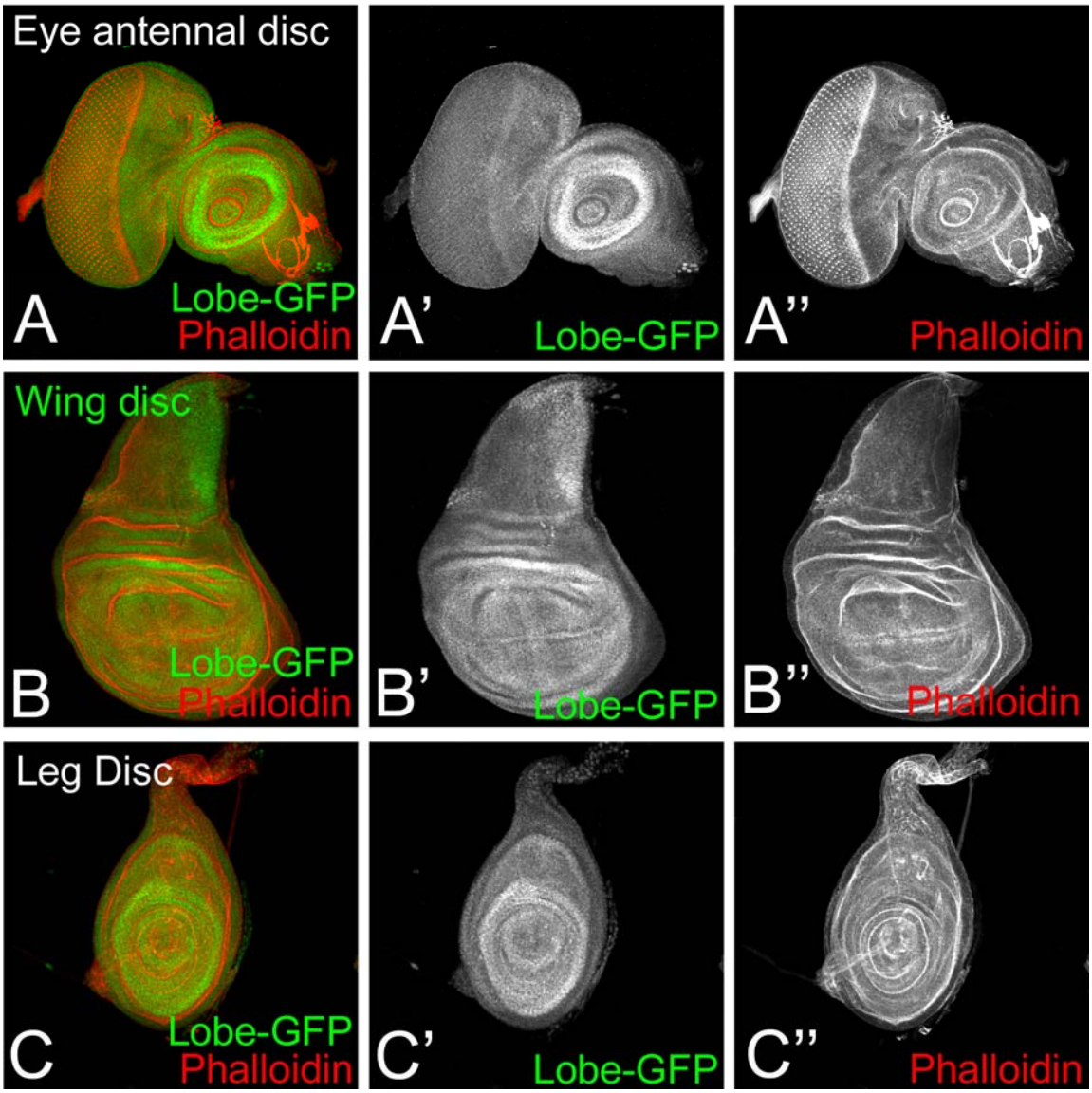

Figure 3. Expression of Green Fluorescent Protein (GFP) reporter marks the localization of Lobe (L) and GFP chimeric protein using the protein trap line. Localization of L and GFP chimeric protein in (A) Eye-antennal-, (B) Wing-, and (C) Leg-imaginal disc. Note that phalloidin (red) marks the actin cytoskeleton and provides the outline of the disc. Both L-GFP expression (Figures 3A', B', C') and phalloidin (3A'', B'', C'') are also shown in separate channels.

The protein trap transgenic lines used in this study are Mirror (Mirr) and Lobe (L), which are part of the protein trap lines collection. The database of these protein trap lines is called Flytrap (http://flytrap.med.yale.edu/index.html) (Kelso *et al*., 2004). Mirror is a homeodomain transcription factor which belongs to the Pbx (pre-B cell leukemia homeobox) class of genes, which is expressed in the dorsal half of the eye (Figure 2). Lobe, a PRAS40 homolog in fly, is expressed uniformly in

the developing imaginal discs (Figure 3). The protein trap approach employs the use of Green Fluorescent Protein (GFP) reporter (Chalfie *et al.*, 1994). GFP can be seen using fluorescein isothiocyanate (FITC) filter having an absorption maximum at 495 nm and excitation wavelength of 488 nm. The GFP reporter expression marks Mirror and Lobe protein expression in eye-antennal (Figures 2A, 3A), wing (Figures 2B, 3B), and leg imaginal discs (Figures 2C, 3C). Note that split channels in Figure 2 and Figures 3 show the expression of GFP and phalloidin separately.

Advantages

1. The protein trap approach can be used to study the sub-cellular localization of proteins.

2. The protein traps are useful to analyze protein localization in the live tissue and can reveal dynamic processes within a cell or multi-cellular organ/ organism.

3. Antibody staining provides more specificity but is more time consuming when compared to dyes. Since antibody staining procedure requires two days to finish, it is profitable to use the protein trap lines and dyes to demonstrate reporter assays and protein localization within the allocated time of a single lab session.

4. These laboratory techniques will allow instructors to depend less on the expensive experimental demonstration kits that are supplied commercially.

5. The variation of markers used and protein trap lines will allow instructors to generate a database of expression profiles, which can be used as a teaching tool for Cell Biology lectures.

Conclusion

Images are considered a powerful means of presenting and communicating scientific results. A high-resolution image can validate an experimental result more effectively than a statement. However, a poor resolution image may raise doubts on any result or conclusion. The majority of the laboratory exercises taught in the undergraduate laboratories are either from commercially developed expensive kits or other conventional experimental labs that do not emphasize communication skills through the use of images. Our laboratory exercises (Tare and Singh, 2009; Tare *et al.,* 2010) including this one, address this problem and are designed to expose students to basic lab skill sets (a) involving epifluorescence microscopy and Laser Scanning Confocal Microscopy, (b) imaging, (c) processing and analyzing the images.

Additional Resources

Protein-trap lines are available from (http://flytrap.med.yale.edu/index.html). The protein trap stocks listed in the Fly Trap website are available for distribution upon request.

Acknowledgements: Authors are thankful to the Flytrap project for the flies, and Dr. Madhuri Kango-Singh and Meghana Tare for the comments on the manuscript. This laboratory exercise was designed in the Department of Biology, at the University of Dayton. ORP is supported by the graduate program at the University of Dayton. AS is supported by the NIH grant (1R15 HD064557-01), and start-up support from the University of Dayton.

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Effect of *hsp*83 activation on cell death as quantified using phenotypic variation of Bar eye in *Drosophila melanogaster*.

Thompson, James N., jr., Clayton N. Hallman, Frank Blixt, Laura Bock, Nam Do, Greg Emde, Daniel Mach, Kymberly May, Valerie Miller, Diana Nguyen, Kayla Owens, Lucas Toho, Lane Donaldson, Joseph Lykins, Barbara Safiejko-Mroczka, and Jenna J. Hellack. Department of Zoology, University of Oklahoma, Norman, OK 73019.

Quantifying the phenotypic variation in a trait is a sensitive way to assess the role a genetic or environmental factor has on a targeted developmental process. Thompson *et al*. (2009) used this approach to evaluate heat shock effects on cell death. Scanning electron micrographs allowed us to measure phenotypic changes very precisely. The current study draws upon that same experimental design to measure the influence of heat shock protein 83 (*hsp*83) on the expression of cell death in the *Drosophila* eye.

The Experimental Genetics and Cell Biology Lab (ZOO 4970) course used this genetic system to study the effect of an *hsp*-defective allele on cell death under normal room temperature (control) or heat shock (37°C) conditions. The hypothesis was that a significant increase in cell death would occur in the *hsp*-defective genotype when stressed by elevated temperature. The normal heat shock system, represented by a dominantly-marked balancer chromosome, would have significantly reduced cell death.

The *hsp*83 mutation was balanced over the dominant Tubby, which causes a shortening of the body (Bloomington Stock Center #5696, w*; *Hsp*83^{e6D}/TM6B, *Tb*^{1}). By crossing Bar females with males from this stock, F1 flies that are either *hsp*83 or Tubby are easily distinguishable as 3rd instar larvae. There were four treatments: third instar larvae (*hsp*83 or Tubby) treated for 40 minutes at 37°C and the same two genotypes raised at room temperature. The 37°C exposure activates the heat shock activity, although it is defective in the *hsp*83 strain.

Treatment was done by selecting F1 larvae of each genotype and placing them in 1.5 ml microfuge tubes containing 0.5 ml of yeast-glucose medium. These were then placed in baggies and either submerged in a 37°C water bath for 40 minutes or left on the lab bench for the same period. Tubes were then uncapped and inserted into a normal food tube, where the flies were allowed to pupate and eclose. Heads were removed from Bar-eyed males eclosing from each of the four conditions. In addition to touring the Electron Microscope Facility and developing this experimental plan, the students benefitted from the microdissection practice needed for successful mounting of

heads on SEM plugs, a process that requires care but which can be learned quickly. Each heads was cut in half with a razor blade so the eyes could be mounted flat for ready visualization in the SEM. Preparations were dried and sputter-coated as described in Thompson *et al*. (2009). Eyes were viewed and photographed with a Zeiss DSM-960A scanning electron microscope. This yields images that allow very accurate counting of facet number (Figure 1).

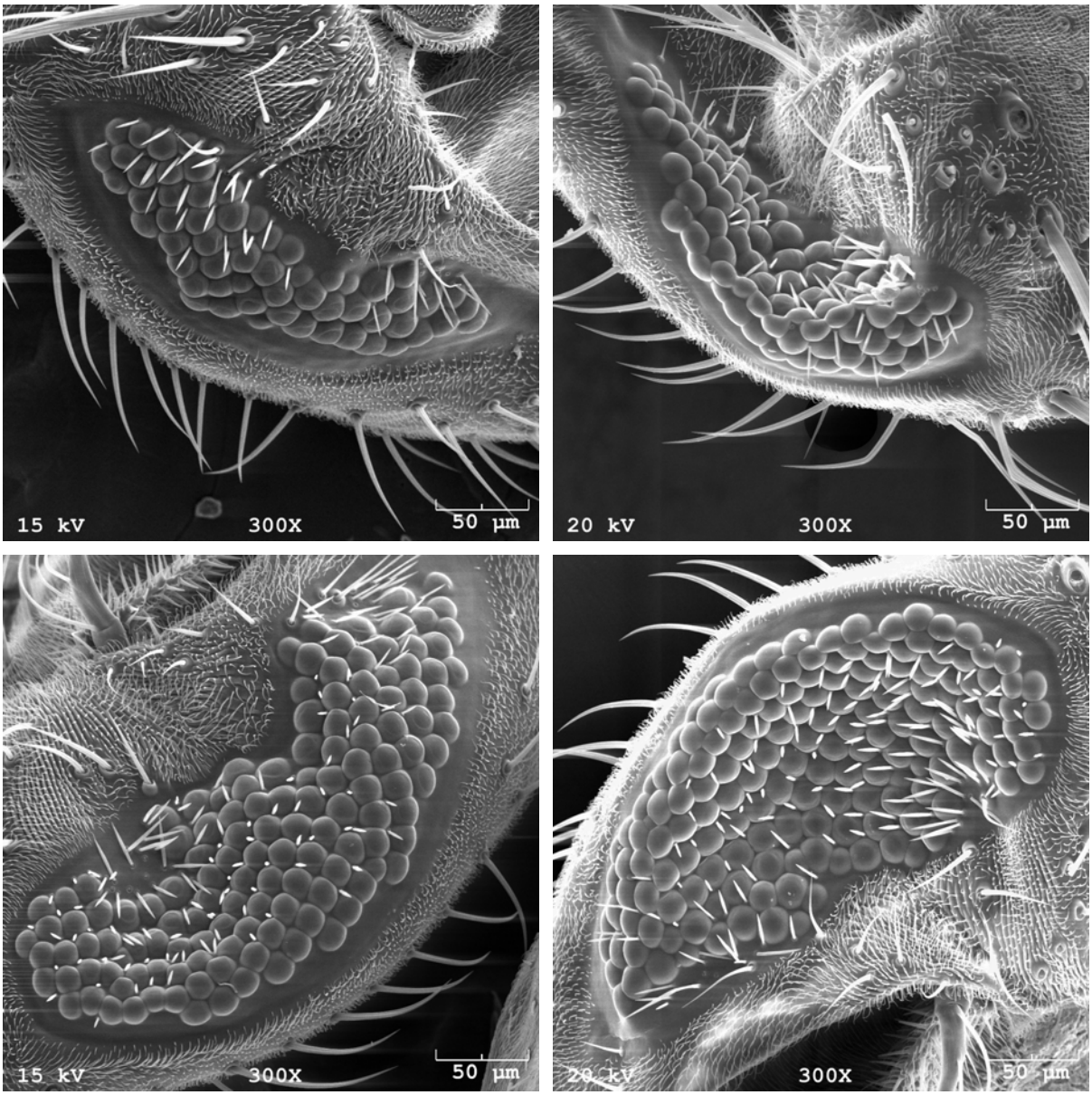

Figure 1. Bar eyes from four different genotypes and treatments: Top left, *hsp*83 mutant at 37°C; bottom left, control at 37°C; top right, *hsp*83 mutant at room temperature; bottom right, control at room temperature.

Representative eyes of each genotype and treatment are shown in Figure 1. The challenge posed by shape of some eyes is shown in Figure 2. Although replicate numbers differed slightly due to genotype survival, the main source of sample variation was in the successful mounting of heads

and eyes. Often it is difficult to know if a good quality image can be produced until the plug is dried, sputter-coated, and examined in the SEM. Since this was a classroom experiment with defined time available, the opportunity to repeat treatments was limited. But the project still showed the students the steps that would be needed to produce a larger dataset and provided an estimate of the data yield for one treatment cycle.

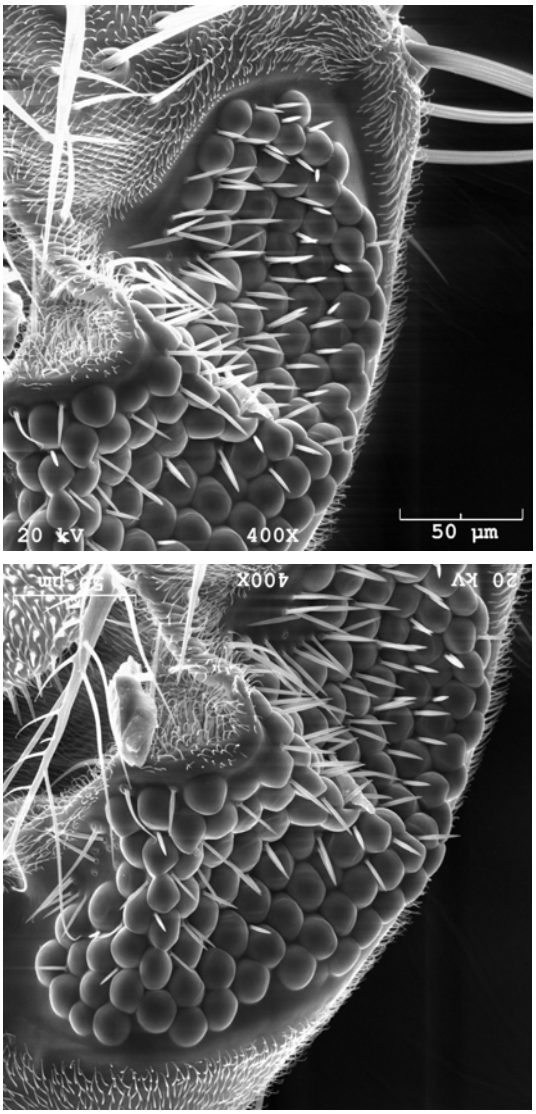

Figure 2. Bar eye showing the challenge to accurate counts caused by folds in the eye surface that can occur during specimen drying or microdissection. Note that the lower image is rotated 180°.

For 37°C treatments and averaging over the replicated counts by eight students, there were significantly fewer eye facets in the Bar-eyed flies with *hsp*83 background genotypes (84.47 ± 1.46) than with normal *hsp*83 activity (116.64 ± 3.67). From five replication counts of the genotypes raised at room temperature, Bar-eyed flies with *hsp*83 background genotypes had fewer facets (84.14 ± 0.30) than did those with normal *hsp*83 activity (146.24 ± 1.05). These differences can be evaluated more in-depth with a larger number of samples and an appropriate ANOVA, but this study at least points to an initial conclusion. Cell death in the Bar eye of *Drosophila* is greatest when *hsp*83 activity is defective. Even when "activated" at 37°C, the phenotype is the same for *hsp*83 deficient samples.

Taking both eyes from most heads also allowed us to measure developmental homeostasis as reflected in symmetry, or more precisely the deviations from symmetry (fluctuating asymmetry (FA) = |L – R| / (L + R)/2). In this case, the data do not show a significant trend (FA: 37°C treatments for 7 heads, *hsp*83 = 0.114 ± 0.078; control for 6 heads = 0.090 ± 0.087; room temperature treatments, *hsp*83 for 9 heads = 0.062 ± 0.049; control sample too small). The sample sizes are clearly too small for any conclusion, but the observations so far can help students consider new alternative hypotheses.

Acknowledgments: This experiment was developed with student input as part of a laboratory course, Experimental Genetics and Cell Biology Lab, taught by JNT and BS-M with the teaching assistance of CNH. JJH provided additional professional contributions to the data analysis. It could not, however, have been accomplished without the excellent assistance of Greg Strout and Preston Larson of the Noble Electron Microscopy Laboratory at the University of Oklahoma. We also thank the Bloomington Stock Center for providing the *hsp*83 strain.

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52nd Annual *Drosophila* Research Conference

The 52nd Annual *Drosophila* Research Conference was held on 30 March – 3 April 2011 at the Town & Country Resort and Conference Center, San Diego, CA. The 2011 Organizing Committee was Daniel Barbash, Giovanni Bosco, and Leslie Griffith. The conference was sponsored by The *Drosophila* Board in association with the Genetics Society of America, 9650 Rockville Pike, Bethesda, MD 20814-3998. The Program and Abstracts Volume lists 958 presentations, including 156 platform session talks and 802 posters (www.drosophila-conf.org).

Historical Lecture, introduced by Leslie Griffith (Brandeis University, Waltham, MA); moderated by Michael Rosbash (Brandeis University, Waltham, MA)

Featuring: Stephen Goodwin, Ulrike Heberlein, Michael Rosbash, and Scott Waddell

Plenary Lectures (In Presentation Order)

Linda Partridge (Gen, Evolution & Environment, University College London, UK). Ageing flies.

Paul A. Garrity (National Center for Behavioral Genomics, Brandeis University, Waltham, MA). From the Cambrian to the sushi bar: TRPAI and the evolution of thermal and chemical sensing.

Brian P. Lazzaro (Cornell University, Ithaca, NY). Pleiotropy, environment, and non-immunological determinants of defense against infection in *Drosophila*.

Lawrence S.B. Goldstein (University of California, San Diego). Genetic analysis of vesicle movement.

Patricia J. Wittkopp (University of Michigan, Ann Arbor). Genetic basis of divergent pigmentation.

Igor F. Zhimulev (Institute of Chemical Biology & Fundamental Medicine, Novosibirsk, Russian Federation, Department of Molecular and Cellular Biology). Chromosomal organization of *Drosophila melanogaster* genome.

Kami Ahmad (Harvard Medical School, Boston, MA). Nucleosome dynamics and gene regulation.

Therese A. Markow (University of California, San Diego). The secret lives of flies: What nature means for the laboratory.

Vivian Budnik (Neurobiology, University of Massachusetts Medical School, Worchester). Autoregulatory and paracrine control of synaptic and behavioral plasticity by octopaminergic signaling.

Eric C. Lai (Sloan Kettering Institute, NY). Biogenesis and function of microRNAs.

Anna Dornhaus (University of Arizona, Tucson). Understanding the "Why?": Evolution of behavior in social insects.

Eric. F. Wieschaus (HHMI, Department of Molecular Biology, Lewis-Sigler Institute for Integrative Genomics, Princeton, NJ). The mechanics of shape change in the *Drosophila* embryo.

Workshops (in order of presentation)

Ecdysone Workshop

Organizers: Christen Mirth (Instituto Gulbenkian de Ciencia) and Arash Bashirullah (University of Wisconsin).

Cell Competition
Organizers: Wu-Min Deng (Florida State University), Tatsushi Igaki (Kobe University), and Eduardo Moreno (CNIO).

Apoptosis, Autophagy, and Other Cell Death Mechanisms
Andreas Bergman (MD Anderson Cancer Center) and Eli Arama (Weizmann Institute)

Chemical Genetics and Drug Screening
Tin Tin Su (University of Colorado) and Claudio Sunkel (IBMC)

Mechanisms and Functions of Chromosome Pairing
Jeff Sekelsky (University of North Carolina) and Giovanni Bosco (University of Arizona)

Confocal Imaging
Steve Paddock (HHMI/University of Wisconsin)

Drosophila Research and Pedagogy at Primarily Undergraduate Institutions (PUI)
Jason Duncan (Willamette University) and Janet Rollins (College of Mount Saint Vincent)

Quantitative Biology of Cell Signaling and Pattern Formation
David Arnosti (Michigan State University), Stas Shvartsman (Princeton University), and Thomas M. Onorato (Laguardia Community College/CUNY)

Emerging Model Arthropods
Molly Duman-Scheel (Indiana University School of Medicine)

Everything You Ever Wanted to Know About Sex
Mark Van Doren (Johns Hopkins University)

modENCODE Workshop
Susan Celniker (Lawrence Berkeley National Laboratory) and Gary Karpen (Lawrence Berkeley National Laboratory)

The North American *Drosophila* Board

The Board's duties include: overseeing community resource centers and addressing other research and resource issues that affect the entire *Drosophila* research community. The Board also administers the finances for the annual North America *Drosophila* Research Conference and its associated awards, and it chooses the organizers and the site of the annual meeting. The Board consists of eight regional representatives and four international representatives, who serve 3-year terms. The three elected officers are President, President-Elect, and Treasurer. In addition, the Board has *ex officio* members who represent *Drosophila* community resources or centers. For more information about the Board and the summaries of the annual Board meetings, see: the FlyBase web site: flybase.bio.indiana.edu.

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