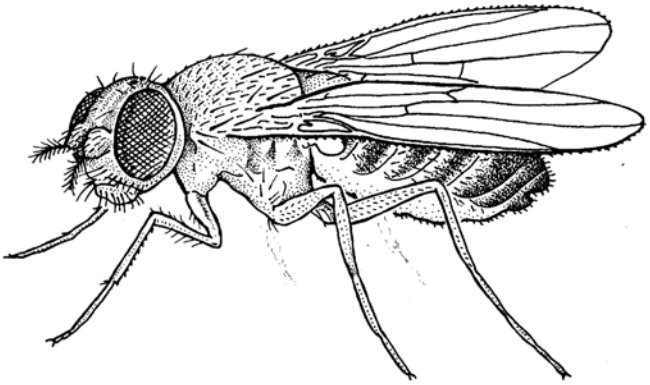
# Drosophila Information Service



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Department of Zoology
University of Oklahoma
Norman, OK 73019 U.S.A.

# Preface

Drosophila Information Service 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." During the more than 70 years since that first issue, DIS has continued to promote open communication.

The production of DIS 90 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. Beginning with volume 84 (2001), the official annual publication date is December, with the contents including all submissions accepted during the calendar year. New issues are available on our web page (www.ou.edu/journals/dis) during the year after publication, and earlier issues are being archived on this site as server allocations permit. For this we thank Jason Glass. We have also added a link to the new *Drosophila* Proteome Atlas.


We continue to encourage all researchers to consider submitting articles that use *Drosophila* for teaching as well as articles that report new techniques and research results. 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.

James N. Thompson, jr., Editor
Department of Zoology
University of Oklahoma, Norman

Jenna J. Hellack, Associate Editor
Department of Biology
University of Central Oklahoma, Edmond

**Contributions, Orders, and Inquiries for the regular annual DIS issue should be sent to:**

James N. Thompson, jr., Department of Zoology, University of Oklahoma, Norman, OK 73019; Phone: (405) 325-2001, FAX (405) 325-6202, email: jthompson@ou.edu

# List of Contributions

## General Announcements



## Research Notes

## Technique Notes



## Teaching Notes



## Mutation Notes

## Other Reports

Research Notes

**Characterization of novel repetitive element *Leviathan* in *Drosophila pseudoobscura*.**

**Garfield, David A., and Mohamed A.F. Noor.** Biology Department, Duke University, Durham, NC, USA; noor@duke.edu.

## Introduction

Repetitive sequences, such as transposable elements, may often contribute to the formation of chromosomal inversions or translocations via ectopic exchange (Charlesworth *et al.*, 1994; Coghlan *et al.*, 2005; Noor and Chang, 2006). The pattern of a strong association between rearrangement breakpoints and repeat sequences has been detected in plants, fungi, and various animals (Coghlan *et al.*, 2005). This association has been demonstrated in many *Drosophila* species (Lim, 1988; Caceres *et al.*, 1999; Evgen'ev *et al.*, 2000) and was particularly striking in the comparison of the published *D. melanogaster* and *D. pseudoobscura* genome sequences, in which at least 60% of all break-point junctions in *D. pseudoobscura* showed significant similarity (by BLAST) to at least one other breakpoint within the genome (Richards *et al.*, 2005). However, this pattern was not apparent at the breakpoints distinguishing *D. melanogaster* from its closer relative *D. yakuba* (Ranz *et al.*, 2007), suggesting a possible burst in transpositional activity in the lineage leading to *D. pseudoobscura*.

Previous analysis of the *D. pseudoobscura* genome sequence assembly identified two particular "breakpoint motifs" that together were associated with nearly half of the almost 1000 inversion breakpoints separating it from *D. melanogaster* as well as both breakpoints of an intraspecific polymorphic inversion (Richards *et al.*, 2005). This strong association is particularly impressive given that these species diverged close to 55 million years ago (Tamura *et al.*, 2004) yet this sequence has no significant similarity to any known *Drosophila* transposable elements (Richards *et al.*, 2005). As such, it seems to have arisen within the *D. pseudoobscura* lineage yet still played a major role in genome rearrangement.

Recently, we localized the inversion breakpoints between *D. pseudoobscura* and its sibling species *D. persimilis* (Ortiz-Barrientos *et al.*, 2006; Machado *et al.*, 2007; Noor *et al.*, 2007) which diverged only within the last 500,000-1,000,000 years (Aquadro *et al.*, 1991; Hey and Nielsen, 2004; Leman *et al.*, 2005). At the breakpoint regions of the XR-chromosome arm inversion separating these species, we identified a tandemly repeated sequence (Machado *et al.*, 2007), and like the previously described "breakpoint motif," we find that this 319-bp motif also appears to be both abundant across the *D. pseudoobscura* genome and absent from genomes of other distantly related *Drosophila* species. As such, it, too, may have been an architect for recent genome structural changes in *D. pseudoobscura* and perhaps led to an increase in the divergence between the closely related *D. persimilis* and *D. pseudoobscura* (*e.g.*, Noor *et al.*, 2007).

In this study, we took three computational steps to characterize this motif, named "Leviathan," in addition to experimentally looking for evidence of transcriptional activity. First, because different molecular mechanisms of replication leave different signatures in the genome (Burt and Trivers, 2006), we examined the abundance and chromosomal distribution of Leviathan across the *D. pseudoobscura* genome, and examined the history of its spread through the genome using sequence divergence between repeat elements. We also tested the "master gene" model (Deininger *et*

*al.*, 1992), wherein one or a few elements within a genome act as "templates" for other copies in the genome, which cannot themselves be used to produce further copies, regarding the spread of this element. The number of templates for a repetitive element provides not only a clue for the mechanisms by which the element replicates (Burt and Trivers, 2006) but also an estimate of the selective costs of replication (Charlesworth and Charlesworth, 1983) and allows for a more thorough characterization of a repetitive element. Finally, we compared the distribution of Leviathan elements between *D. pseudoobscura* and *D. persimilis*, looking for differences in divergence across different parts of the genome as well as for differences in the evolutionary dynamics of Leviathan in one lineage as compared to the other.

**Methods**

Initial characterization of Leviathan:

We performed initial searches for sequences homologous to those flanking the fixed inversions between the two species using BLAST (Altschul *et al.*, 1990) to search the genome of each species (Gilbert, 2007) as well as the NCBI databases. Full genome sequences for *Drosophila pseudoobscura* (comparative analysis freeze 1, genome sequence version 2.0.1) and *D. persimilis* (also CAF1) were downloaded from Baylor College of Medicine (BCM-HGSC, 2006) and FlyBase (Grumbling and Strelets, 2006), respectively. We searched for the presence of Leviathan within annotated CDS and introns using annotations downloaded from FlyBase as well as our own annotation of the *D. persimilis* genome (Noor *et al.*, 2007). We processed several contigs containing multiple BLAST hits using the program Dotter (Sonnhammer and Durbin, 1995) with default parameters.

Tandem Repeat Finder:

To delineate arrays of repetitive sequence, and to assess the distribution of the repetitive sequence across the genome, we ran the Tandem Repeat Finder Program (aka TRF: Benson, 1999) on both the *D. pseudoobscura* and *D. persimilis* sequences using the suggested default settings: matching weight = 2, mismatching penalty = 7, indel penalty = 7, match probability = 80, indel probability = 10, minimum alignment score = 50, maximum period size = 500. We assembled the results into a single, searchable database using custom scripts written in Python.

TRF uses a probabilistic model of tandem repeats to identify candidate tandem repeats in a sequence and uses statistics similar to those employed by BLAST to evaluate the significance of those candidates. Because TRF employs a probabilistic model, running the program with different parameters can yield different results. To test the robustness of the results from the TRF analysis, we repeated the TRF analysis on a subset of the contigs containing Leviathan sequence, changing, in turn, each parameter by 20% of its original value and then rerunning the program. We also ran the program using sets of parameters found by other labs to produce reliable, reproducible results in searches for tandem repeats in other species.

We manually inspected the repeat regions generated by TRF to ensure that individual arrays were not in fact connected by sequence too degenerate to be picked up by the TRF algorithm. To ensure that arrays of repetitive sequences were counted only once in our analysis of the distribution of repeats within the genomes (below), we merged the database entries corresponding to connected arrays.

*Estimating proportion of Repeat Elements in each genome:*

The proportion of a genome made up a particular repetitive element was estimated in a two-step process. First, the repeat database constructed above was screened using BLAST, and the total length of any repeat arrays with a significant match (e-value < -25) to the query sequence was recorded. Second, a BLAST database was constructed from the genome after masking all of the tandem arrays identified by TRF. BLAST was used to identify all significant matches to the query sequence (e-value < -25). Because BLAST can truncate good matches separated by regions of dissimilarity, BLAST may underestimate the total length of an actual match. Therefore, each match identified by BLAST was extended by 100bp on either side and the extended sequence and the query were realigned using a custom implementation of the Smith-Waterman algorithm in Python. The lengths of the resulting alignments were added to the lengths of the arrays identified in the first step. To estimate the proportion of each genome consisting of a particular repetitive element, the total lengths found above was divided by the total number of sequenced bases per chromosome in each species.

*Phylogenetic trees:*

We simultaneously estimated phylogenetic trees and the associated model parameters using genetic algorithms as implemented in GARLI (Zwickl, 2006). We estimated the significance of our results by generating 1000 bootstrap replicates and then constructing a majority-rule consensus tree from the resulting trees using PAUP* (Swofford, 2003).

Test of the Master Gene Hypothesis:

We assessed whether spread of this element was consistent with the master gene hypothesis through the method of Johnson and Brookfield (Johnson and Brookfield, 2006) using the sequence identified by RT-PCR as the putative master gene. Briefly, we compiled a list of the consensus repeat sequence from each repeat region in the *D. pseudoobscura* genome as determined by TRF and aligned them using ClustalW (Chenna *et al.*, 2003) followed by manual corrections of the alignment. Each sequence from this compilation was then compared with each other sequence along with an out-group sequence (the most divergent sequence from the database) and the putative master gene to generate $\binom{n}{2}$ clusters of four sequences.

Under the master gene hypothesis, each observed repeat sequence should share a common ancestor with the putative master gene more recently than with the outgroup. Following Johnson and Brookfield, we assigned each variable site in each cluster as either supporting this hypothesis or better supporting, under the criterion of parsimony, an alternative evolutionary scenario. Supporting sites are sites at which the putative master gene shares a variable site with one or both observed sequences. Alternative sites are those in which the putative master gene shares a variable site with the outgroup or none of the other sequences. We assigned an X,Y coordinate to each set of four sequences based on the number of informative sites compatible with the master-gene hypothesis (Y) and the number of sites that were not (X). We then performed a linear regression on the resulting points.

To assess the statistical significance of the regression, we compared our result to results generated from four sets of 1000 simulated datasets generated by a model corresponding to the

master gene hypothesis using HyPhy (Kosakovsky-Pond *et al.*, 2005). The parameters describing each of the four sets were chosen to mimic those used by Johnson and Brookfield (Johnson and Brookfield, 2006) and span a range of potential distributions each consistent with the master gene hypothesis.

In the first set (A), we assumed a clock-like rate of evolution on all sequences, including the master gene, such that all branch lengths were equal. In the second set (B), branch lengths were taken from the actual dataset. In the third set (C), we assumed a clock-like rate of evolution, but held the master gene constant, simulating strong constraint on the master gene sequence. In the fourth dataset (D), we repeated the first analysis except we used a gamma distribution fitted to the original data to model the distribution of rates within a sequence and to model the possibility of multiple substitutions at some sites.

Empirical confirmation of Leviathan transcription via RT-PCR:

Using a consensus repeat sequence identified by Tandem Repeat Finder, we designed primers to amplify Leviathan from genomic DNA and cDNA, the latter of which was to confirm its current expression. Genomic DNA from the Flagstaff 1993 strain of *D. pseudoobscura* was isolated using the method of Gloor and Engels (Gloor and Engels, 1992). Total RNA was isolated from 25 seven-day posteclosion adult females of the same strain using the Qiagen RNeasy kit. The RNA was reverse transcribed in a reaction containing 5 mM $MgCl_2$, 50 mM KCl, 10mM Tris-HCl, 1mM dNTPs, 20 units RNasin, 20 units reverse transcriptase, and 2.5 µM of primer LeviathanR (5'-TCACGATTTTCGCAAAAAATCATGATGGTTACATC -3'). PCRs were then performed on the cDNA generated above, the genomic DNA, the total RNA, and a negative control using primers LeviathanR and LeviathanF (5'- GCAAATCACAATCTTCGGGAGGC -3'). PCR products were visualized on a TBE agarose gel, and the cDNA amplification product was sequenced using ABI BigDye Terminator version 3.1.

BLAST was used to make sure that the resulting sequence was not found in any annotated coding sequence.

**Results**

Description of Leviathan:

We identified numerous significant BLAST (Altschul *et al.*, 1990) matches to the repetitive sequence ("Leviathan") identified from the breakpoint region of the fixed inversion difference between *D. pseudoobscura* and *D. persimilis* on the right arm of chromosome X (XR). These matches were found on all four major chromosomes in the two species, both in intergenic regions and within the introns of annotated genes. No significant hits were found in the available genome sequences of other *Drosophila* (Gilbert, 2007) or in NCBI databases, nor were we able to identify similar sequence in Repbase (Jurka *et al.*, 2005) or the RepeatMasker (Smit *et al.*, 1996-2004) server. Manual investigation of regions of the *D. persimilis* and *D. pseudoobscura* genome sequences with multiple hits showed that the individual elements identified by BLAST were often parts of long swaths of repeated units up to more than nine kilobases in length.

To better visualize any patterns within the arrays of repetitive sequence, we ran several contigs containing repeat arrays through the program Dotter (Sonnhammer and Durbin, 1995). The results show a clear periodicity within the repeat regions of ~319bp. These 319bp repeats are highly palindromic, contain motifs reminiscent of the TATA box, transcription factor binding sites, and polyadenylation.

Using Tandem Repeat Finder (TRF) (Benson, 1999, and see Methods), we identified several dozen regions containing repetitive sequence with high similarity to the sequence flanking the inversion on chromosome XR and to each other. These repeat regions are found throughout the genomes of both *D. pseudoobscura* and *D. persimilis*, making up 0.1% to 0.15% of the assembled genome of the two species and nearly 4% of all repetitive sequence, and consist of between 2 and 16 repetitions of Leviathan, often surrounded by and interspersed with degenerate Leviathan sequence (see Methods). We were unable to locate any singleton copies of the element. For contrast we calculated that the two repetitive elements associated with chromosomal rearrangements identified by Richards *et al.* (2005) makes up a total of 0.2% of the assembled genome of *D. pseudoobscura* and 8.7% of all repetitive sequence.

While we were unable to locate singleton copies of the element, TRF still identified the same arrays of ~319bp repeats under a variety of different parameters (see Methods). The robustness of these results, along with the figures produced by Dotter, suggest that, while we cannot rule out longer sequences, 319bp is likely the fundamental size of a single repeat unit.

Empirical confirmation of expression:

Because tandem arrays of Leviathan sequence are often surrounded by degenerate Leviathan sequence, it is difficult to determine exactly where an array begins or ends. Thus, we were unable to determine the exact frame of Leviathan. Nonetheless, we were able to successfully amplify a 184-bp portion of Leviathan sequence from *D. pseudoobscura* cDNA using primers designed from the consensus repeat sequences identified by TRF. Amplification from genomic DNA was also successful, but no amplification from RNA or our negative control was obtained, demonstrating that our amplification from cDNA reflects transcription of the Leviathan sequence and not DNA contamination. There was no single perfect match to the sequence we amplified (GenBank accession EU081847) in either genome sequence, perhaps reflecting the difficulties of incorporating highly repetitive regions into genomic assemblies or a potential heterochromatic origin of the isolated transcript.

As the sequence of the RT-PCR product does not match that of genes predicted to contain the repetitive element (data not shown), our results may constitute evidence for an actively transcribed mobile element. We cannot, however, rule out the possibility that the sequence identified is the result of the transcription of Leviathan sequence proximal to a coding or non-coding gene or transcribed as a part of mechanisms inducing heterochromatin (Grewal and Moazed, 2003, and see Discussion).

Genomic distribution and mechanism of spread:

The distribution of Leviathan can be analyzed by looking both at the distribution of individual 319bp elements and at the sequences arrays (consisting of swaths of 319bp elements of nearly identical sequence) in which they are found. We hypothesize (see Discussion) that the two distributions give different views of how Leviathan spreads in the genome. Individual elements within an array likely owe their existence to mechanisms such as unequal crossing over, whereas disjoint repeat arrays originate with a transpositional or other replicative event. Biases in either distribution thus shed light on the different molecular mechanisms by which Leviathan spreads.

We compared the distribution of arrays within and between the two genome sequence assemblies using the filtered results of TRF (see Methods). We saw no evidence for a non-random distribution of number of Leviathan sequence arrays within the genome of either species after correcting for the differences in lengths between the different chromosomal segments, ($\chi^2$ test, NS).

We did, however, find that the proportion of total Leviathan sequence varied widely between chromosomes within each species, with an excess of Leviathan sequence in the XR and, to a lesser extent, 4th chromosomes ($\chi^2$, $p < 2.2e\text{-}16$, Table 1). We found no evidence for differences in the distribution of either total Leviathan sequence or the number of Leviathan sequence arrays between the genomes of the two species (Kolmogorov-Smirnov Test, NS).

Table 1. The Proportion of Leviathan Sequence per Chromosome Per Species. The total size of each chromosome for each species is given as well as the total length of Leviathan sequence in each chromosome and the total proportion of each chromosome made up of Leviathan sequence. Proportions that differ significantly from the others (see text) are marked with asterisks.

| Chromosome | *D. pseudoobscura* | | | *D. persimilis* | | |
|---|---|---|---|---|---|---|
| | Total | Repeat | Proportion | Total | Repeat | Proportion |
| Ch2 | 30,751,037bp | 10,528bp | 0.034% | 31,203,717bp | 5545bp | 0.0178% |
| Ch3 | 19,758,590bp | 3906bp | 0.0198% | 19,649,734bp | 3159bp | 0.0161% |
| Ch4 | 27,194,902bp | 91,392bp | 0.337%* | 27,622,741bp | 69,377bp | 0.251%* |
| ChXL | 24,728,506bp | 21,967bp | 0.0889% | 25,734,256bp | 23,870bp | 0.0928% |
| ChXR | 24,578,333bp | 147,472bp | 0.60%* | 24,376,180bp | 77,779bp | 0.319%* |

Individual repeat arrays had nearly identical repeat sequences, but repeat sequences differed slightly more between arrays. We estimated phylogenetic trees using the consensus repeat sequences identified by TRF in each genome to see if related sequences were clustered in the genome of either species. Our trees do not show any evidence for clustering within or between genomes besides that within arrays, though the presence of extensive polytomies suggests that there may not be sufficient variation between the sequences to detect clustering were it to exist.

We also estimated a tree using sequences from both genomes to evaluate the extent to which Leviathan may be introgressing between the two genomes. The resulting tree does not support clustering by species, though there is some clustering of elements on the XR, indicating a greater divergence of elements on the XR from the arrays on other chromosomes. The lack of clustering by species, coupled with the similarities in the distributions of Leviathan within each of the two species, suggests that interspecies gene flow may be sufficient to homogenize these genomes with respect to this element.

*Master gene test:*

Finally, we assessed whether spread of this element was consistent with the master gene hypothesis through the regression method of Johnson and Brookfield (Johnson and Brookfield, 2006; see also Materials and Methods). We found a strong negative relationship in our regression, which is evidence *against* the master gene hypothesis (most sites do not support the master gene hypothesis). Because the points used in the regression are not independent of one another, we cannot directly assign a probability value to our results. To do this, we compared our result to results from simulated data using three different models consistent with the master gene hypothesis (see Methods). Three datasets from set A were found to have a more negative relationship than that observed in the actual data ($p = 0.001$). In none of the other simulations was a single more negative slope observed ($p < 0.001$). Similar results were obtained when other Leviathan sequences were treated as the putative master gene (results not shown).

**Discussion**

Our results suggest that Leviathan is an actively transcribed element in the genomes of *Drosophila pseudoobscura* and *D. persimilis* and that its extensive presence in the genomes of these two species may be due both to transcription via an RNA intermediate and an unknown mechanism (perhaps unequal crossing over) that increases the size of a Leviathan element array once it has been inserted in the genome. We cannot definitively rule out that Leviathan is transcribed because of its proximity to a coding or non-coding gene, but its presence on multiple chromosomes of these species, but not related species, suggests recent transcriptional, and later retro-transcriptional, activity.

The similarity of the distributions of Leviathan within the two genomes and patterns of variation seen within Leviathan sequences suggest that Leviathan may introgress between the two genomes. *D. persimilis* and *D. pseudoobscura* are known to hybridize in the wild, and substantial introgression has been detected at some loci (Hey and Nielsen, 2004). Alternatively, the observed pattern may be due to the recent divergence of the two species. In either case, Leviathan is likely to exert the similar evolutionary pressures in both lineages.

Characterization of Leviathan and its spread:

The size and composition of the 319bp repeat constrains the possible classifications of Leviathan. 319bp is likely too short to encode a functional protein, suggesting that Leviathan is a satellite, MITE, or parasitic fragment of a DNA transposon. Because the sequence contains several pol-III termination sequences in both orientations, Leviathan is unlikely to be a SINE. Further characterization must await future genome assemblies in which the terminal ends of a Leviathan array can be better identified and analyzed.

Recent work on the role of tandem repeats in RNAi-based mechanisms of heterochromatin formation (Grewal and Moazed, 2003; Martienssen, 2003; Lippman *et al.*, 2004) raises the intriguing possibility that Leviathan is a lineage-specific component of epigenetic control of gene expression. While we have no evidence to support this claim and consider it unlikely given that Leviathan is not found in related, sequenced genomes, it would explain the presence of an RNA transcript and is intriguing enough to warrant investigation in future studies.

One of the most striking features of Leviathan is the size of the tandem arrays in which it is found and the long stretches of degenerate sequence often seen within arrays. It is likely that while Leviathan acts as some sort of mobile element, much of the total Leviathan sequence in these species owes its existence to ectopic exchange or unequal crossing over rather than transposition *per se*. Such a pattern has been observed in previous studies of repetitive sequence (Cox and Mirkin, 1997), and supports the contention that Leviathan is responsible for the generation of at least one of the fixed chromosomal inversions between these two species. Our finding that the XR contains a greater proportion of Leviathan elements than other chromosomes and that these arrays are often more divergent than arrays on other chromosomes (and thus likely older), further supports this contention.

To definitively categorize and fully characterize Leviathan, we would need to identify the frame as well as the insertion sites to look for characteristic signals such as inverted repeats, poly-A tails, etc. (see review in Kazazian, 2004). We attempted to identify such signals at the ends of the repeat arrays in this study. However, despite extensive search, we were unable to identify single units and, in most cases, were unable to delineate these array ends due both to the degeneracy of the primary sequence at the ends and incomplete coverage of the genomes. In cases where we identified plausible flanking regions to the element insertion, we failed to identify any consistent patterns. In at least one case, we did observe the same sequence, but in opposite orientations, at both ends of the

insertion. This sequence did not BLAST to any other mapped regions of the genome (chromosome 4, group 2, position 850593). However, other forces besides transposition (or retro-transposition) likely affect the spread and sequence of this element (*e.g.*, unequal crossing over). As a result, the signatures of the origin of most Leviathan arrays may be obscured.

We found no evidence to support the Master Gene Hypothesis in our explicit test, nor do our phylogenetic trees support the comb-like topology expected under the master gene hypothesis (Johnson and Brookfield, 2006). This suggests that Leviathan replicates in a manner similar to LTR retroelements or endogenous retroviruses rather than a LINE or Alu elements (Shen *et al.*, 1991; Kass *et al.*, 1995), but no definitive statement is possible without detailed laboratory-bases characterization. Our results are, however, consistent with Leviathan having multiple origins of replication. Previous studies (reviewed in Burt and Trivers, 2006) suggest that repetitive elements with multiple origins of replication generally have negative fitness consequences for their hosts (though see Torkamanzehi *et al.* [1992] for a possible exception). Leviathan is likely no exception, showing that potentially important drivers of evolutionary change and speciation can have negative fitness consequences.

Approach and prospects:

To validate our technique, we looked for the presence of Leviathan in a recently assembled collection of computationally predicted and experimentally verified transposable elements from all twelve sequenced species of *Drosophila* (Quesneville *et al.*, 2005; H. Quesneville, personal communication). This collection identified Leviathan-like sequence on several unassigned contigs in *D. pseudoobscura*, but not in any sequence assigned to a chromosome. This is likely due to the removal of tandem repeat sequence, and other repetitive sequence arrays, in this analysis that, while necessary to avoid false-positives in large scale-surveys, would remove unusual repetitive sequences like Leviathan. Our finding demonstrates the value of computational analyses of individual repetitive elements in conjunction with large-scale searches that, by their very nature, may screen out unusual sequences like Leviathan. We have shown that basic computational tools can be used effectively in identifying new repetitive elements, and other repetitive sequences, when combined with and when validated by empirical methods like RT-PCR. Phylogenetic techniques can be especially powerful for examining how repeat sequences spread in the genome when used in conjunction with methods, such as TRF, for identifying repetitive sequence *ab initio*. In applying TRF to the whole genomes of both *D. persimilis* and *D. pseudoobscura,* we identified a second, 170bp repeat that appears to be unique to *D. persimilis* and is distributed non-randomly in the genome ($\chi^2$ test, $p < 0.0001$). We did not follow up further with this element, but this result does support a promising future for computational searches for many more interesting repetitive elements.

## Conclusions

We identified a novel repetitive element, Leviathan, which is found at the XR inversion breakpoints, appears to be unique to the *D. pseudoobscura* and *D. persimilis* genomes, and may have contributed to the divergence of these species from each other. Leviathan appears to increase in abundance in the genome via both unequal crossing over, which increases the size of arrays of nearly identical Leviathan sequences, and through transposition likely via an RNA intermediate, which leads to the creation of new arrays in new genomic locations. We characterized this element using novel computational tests in additional to traditional laboratory techniques, thus demonstrating the value of

adding computational genomic characterization of repetitive elements to traditional laboratory studies to better understand both the molecular and evolutionary dynamics of repetitive sequence.

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References: Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman 1990, J. Mol. Biol. 215: 403-410; Aquadro, C.F., A.L. Weaver, S.W. Schaeffer, and W.W. Anderson 1991, Proc. Natl. Acad. Sci. USA 88: 305-309; BCM-HGSC. (URL: http://www.hgsc.bcm.tmc.edu/projects/drosophila/, Houston, TX, 2006); Benson, G., 1999, Nucleic Acids Res 27: 573-580; Burt, A., and R. Trivers 2006, *Genes in Conflict: The Biology of Selfish Genetic Elements*. Belknap Press of Harvard University Press, Cambridge, Mass.; Caceres, M., J.M. Ranz, A. Barbadilla, M. Long, and A. Ruiz 1999, Science 285: 415-418; Charlesworth, B., and D. Charlesworth 1983, Genet. Res. Camb. 42: 1-27; Charlesworth, B., P. Sniegowski, and W. Stephan 1994, Nature 371: 215-220; Chenna, R., H. Sugawara, T. Koike, R. Lopez, T.J. Gibson *et al.*, 2003, Nucleic Acids Res 31: 3497-3500; Coghlan, A., E.E. Eichler, S.G. Oliver, A.H. Paterson, and L. Stein 2005, Trends Genet. 21: 673-682; Cox, R., and S.M. Mirkin 1997, Proc. Natl. Acad. Sci. USA 94: 5237-5242; Deininger, P.L., M.A. Batzer, C.A. Hutchison, 3rd, and M.H. Edgell 1992, Trends Genet. 8: 307-311; Evgen'ev, M.B., H. Zelentsova, H. Poluectova, G.T. Lyozin, V. Veleikodvorskaja *et al.*, 2000, Proc. Natl. Acad. Sci. USA 97: 11337-11342; Gilbert, D.G., 2007, Nucleic Acids Res. 35: D480-D485; Gloor, G.B., and W.R. Engels 1992, Dros. Inf. Serv. 71: 148-149; Grewal, S.I., and D. Moazed 2003, Science 301: 798-802; Grumbling, G., and V. Strelets 2006, Nucleic Acids Res. 34: D484-D488; Hey, J., and R. Nielsen 2004, Genetics 167: 747-760; Johnson, L.J., and J.F. Brookfield 2006, Mol. Biol. Evol. 23: 235-9; Jurka, J., V.V. Kapitonov, A. Pavlicek, P. Klonowski, O. Kohany *et al.*, 2005, Cytogenet. Genome Res. 110: 462-467; Kass, D.H., M.A. Batzer, and P.L. Deininger 1995, Mol. Cell. Biol. 15: 19-25; Kazazian, H.H., Jr., 2004, Science 303: 1626-1632; Kosakovsky-Pond, S.L., S.D. Frost, and S.V. Muse 2005, Bioinformatics 21: 676-679; Leman, S.C., Y. Chen, J.E. Stajich, M.A.F. Noor, and M.K. Uyenoyama 2005, Genetics 171: 1419-1436; Lim, J.K., 1988, Proc. Natl. Acad. Sci. USA 85: 9153-9157; Lippman, Z., A.V. Gendrel, M. Black, M.W. Vaughn, N. Dedhia *et al.*, 2004, Nature 430: 471-476; Machado, C.A., T.S. Haselkorn, and M.A.F. Noor 2007, Genetics 175: 1289-1306; Martienssen, R.A., 2003, Nat. Genet. 35: 213-214; Noor, M.A.F., and A.S. Chang 2006, Curr. Biol. 16: R890-R892; Noor, M.A.F., D.A. Garfield, S.W. Schaeffer, and C.A. Machado 2007, Genetics, in press; Ortiz-Barrientos, D., A.S. Chang, and M.A.F. Noor 2006, Genet. Res. Camb. 87: 23-31; Quesneville, H., C.M. Bergman, O. Andrieu, D. Autard, D. Nouaud *et al.*, 2005, PLoS Comput. Biol. 1: 166-175; Ranz, J.M., D. Maurin, Y.S. Chan, M. von Grotthuss, L.W. Hillier *et al.*, 2007, PLoS Biol. 5: e152; Richards, S., Y. Liu, B.R. Bettencourt, P. Hradecky, S. Letovsky *et al.*, 2005, Genome Res. 15: 1-18; Shen, M.R., M.A. Batzer, and P.L. Deininger 1991, J. Mol. Evol. 33: 311-320; Smit, A.F.A., R. Hubley, and P. Green. (URL: http://www.repeatmasker.org, 1996-2004); Sonnhammer, E.L., and R. Durbin 1995, Gene 167: GC1-10; Swofford, D.L. 2003, Sinauer Associates, Sunderland, MA.; Tamura, K., S. Subramanian, and S. Kumar 2004, Mol. Biol. Evol. 21: 36-44; Torkamanzehi, A., C. Moran, and F.W. Nicholas 1992, Genetics 131: 73-78; Zwickl, D., 2006, in Integrative Biology, University of Texas, Austin, TX.

**Relative mating success of hybrid and pure species males to highly discriminating *D. pseudoobscura* females.**

**Allen, E. Carley, and Mohamed A.F. Noor.** Biology Department, Duke University, Durham, NC, USA; noor@duke.edu.

## Introduction

In nature, it is often advantageous to mate preferentially within one's species because of negative consequences associated with heterospecific mating. This discrimination against heterospecifics is often driven by female choice rather than male choice due to the large investment of females into small numbers of eggs relative to that of males into large numbers of sperm. Thus, female eggs are wasted on heterospecific matings when the resultant offspring are unfit, and her reproductive fitness is significantly decreased. In contrast, in many species, males can waste many sperm without a significant cost to their potential reproductive output. In the North American species *Drosophila pseudoobscura* and *D. persimilis*, the cost of interspecies mating is that $F_1$ male progeny are sterile; therefore, females have a great incentive to discriminate against mating with heterospecific males.

The extent to which females discriminate against heterospecifics is related to their proximity to other species (Howard, 1993; Noor, 1999; Servedio and Noor, 2003). Reinforcement is the process whereby mate discrimination is strengthened via natural selection to prevent maladaptive hybridization. As a result, while species with geographically overlapping ranges may need high mating discrimination, those with disjunct ranges can be less discriminating. *D. pseudoobscura* females from Mather, California, are hypothesized to be highly discriminating, because they co-occur with *D. persimilis* (Noor, 1995; Noor and Ortiz-Barrientos, 2006), and indiscriminate mating by the females could result in wasteful interspecific matings, because they produce sterile male offspring.

Identifying the phenotypic cues associated with species discrimination can help us understand how it is mediated in nature. Mate discrimination by *D. pseudoobscura* females is thought to originate from two sources: male courtship song parameters and olfactory cues (Ortiz-Barrientos *et al.*, 2004). Male courtship songs are thought to be responsible for some basal mate discrimination in these species (Williams *et al.*, 2001), and olfactory cues (*e.g.*, cuticular hydrocarbons) may be responsible for the enhanced discrimination used in reinforcement (Ortiz-Barrientos *et al.*, 2004). Thus, if true, in highly discriminating Mather females, both male courtship songs and cuticular hydrocarbons would be involved in recognition of conspecific mates.

The hypothesis above predicts that allopatric Flagstaff *D. pseudoobscura* strains use only male courtship song to discriminate conspecifics from heterospecifics. In support of this hypothesis, $F_1$ males (Flagstaff males × *D. persimilis* females) mate with Flagstaff females as well as do pure-species *D. pseudoobscura* males, despite the $F_1$ males bearing a cuticular hydrocarbon profile similar to *D. persimilis* (Noor and Coyne, 1996). A comparable mating study of the highly discriminating *D. pseudoobscura* Mather 17 strain and its $F_1$ hybrids with *D. persimilis* was not conducted.

The $F_1$ progeny of *D. pseudoobscura* Mather 17 females and *D. persimilis* males have a cuticular hydrocarbon similar to *D. persimilis* (Noor and Coyne, 1996). If Mather 17 females use both male courtship songs and cuticular hydrocarbons to distinguish between conspecifics and heterospecifics, they may mate less frequently and have longer copulation latencies with $F_1$ males than with conspecific Mather 17 males, because the $F_1$ males will seem to have olfactory cues similar

to *D. persimilis*. Mather 17 females should also be still more discriminating against mating with *D. persimilis* males, and thus these males should have the lowest mating frequency and the longest copulation latencies. Hence, for matings with *D. pseudoobscura* Mather 17 males, we predict the success to be Mather 17 males > $F_1$ males > *D. persimilis* males.

**Methods**

Mating Trials:

*Drosophila pseudoobscura* Mather 17 (Mather 17) virgin females were mated with *D. pseudoobscura* Mather 17 males, $F_1$ (Mather 17 females × *D. persimilis* Mount St. Helena 1993 males) males, and *D. persimilis* Mount St. Helena 1993 (MSH 1993) males each day possible from January to April 2007. Matings were conducted in the morning between 9:00am and 10:30am, coinciding with "lights-on" in the incubator's 12-hour light-dark cycle. Approximately equal numbers of flies of each type were assayed each day, hence reducing artifacts of day-to-day variation in environmental factors.

For each mating that was conducted, an eight-day post-eclosion Mather 17 virgin female was placed into a food vial with one virgin male, which could be Mather 17, $F_1$, or MSH 1993 male, and a cotton plug was pushed down until the flies had only ½ inch of open space. This ensured that the flies came into contact with one another. For each mating trial, the start time, the time of courtship, and the time of copulation were recorded using the timer, and the courtship and copulation latencies (in seconds) were calculated using these data. If the male fly did not court or mate, that was recorded. Courtship was indicated by the males' wing vibrations toward the female, and the pair was only considered to be mating if the copulation lasted more than thirty seconds (though the initiation of the copulation was recorded as the mating time in the data). If the male fly had not courted ten minutes after being put into the vial with the female, the pairing was recorded as "no courtship". If the male fly had not mated ten minutes after the first bout of courting the female, it was recorded as “no copulation”.

Statistical Analysis:

The results were analyzed via Chi Square test and Mann-Whitney U-tests. The Chi Square test was used for categorical data, such as matings (Y/N) to particular types of males (Mather 17 vs. $F_1$ vs *D. persimilis*). The non-parametric Mann-Whitney U-tests were used for continuous data, such as the length of time it took for each type of male to either recognize and court (courtship latency) or succeed in copulating with (copulation latency) a female.

**Results**

There was a significant difference between the number of *D. persimilis* MSH 1993 males that mated with *D. pseudoobscura* Mather 17 virgin females and the number of Mather 17/$F_1$ males that mated with Mather 17 virgin females (Chi Square P-Value < 0.0001: Table 1). Thus, as reported previously, Mather 17 females are highly discriminating against heterospecific males.

However, contrary to our prediction, there was no significant difference between the number of Mather 17 and $F_1$ males that mated with Mather 17 virgin females after courting (Chi Square P-Value = 0.4224: Table 2). Mather 17 males mated 47 times and did not mate 10 times after courting, and $F_1$ males mated 44 times and did not mate 6 times (Table 2). There was also no significant difference in the copulation latency between Mather 17 and $F_1$ males using the Mann-Whitney U test (P-value = 0.0728) or mean copulation latency in seconds (Tables 3 and 4). Indeed, the opposite trend was observed: $F_1$ males mated slightly more and slightly faster.

Table 1. The number of *D. pseudoobscura* Mather 17, $F_1$, and *D. persimilis* MSH 1993 males that did and did not mate Mather 17 virgin females. Only those males that courted the females were included in the tally. (Ch Square P-Value < 0.0001 for Mather 17/$F_1$ males vs MSH 1993 males).

| Mated? (Y/N) | Mather 17 Males | $F_1$ Males | MSH 1993 Males |
|---|---|---|---|
| N | 10 | 6 | 31 |
| Y | 47 | 44 | 6 |
| Totals | 57 | 50 | 37 |

Table 2. The number of Mather 17 and $F_1$ males that did and did not mate Mather 17 virgin females. Only those males that courted the females were included in the tally. (Chi Square P-Value = 0.4224).

| Mated? (Y/N) | Mather 17 Male | $F_1$ Male |
|---|---|---|
| N | 10 | 6 |
| Y | 47 | 44 |
| Totals | 57 | 50 |

Table 3. Mann-Whitney U information for copulation latency of Mather 17 and $F_1$ males to Mather 17 virgin females. Only those males that courted the females were included in the tally. (P-Value = 0.0728).

| Male Type | Count | Sum Ranks | Mean Rank |
|---|---|---|---|
| Mather 17 | 56 | 3279.5 | 58.6 |
| $F_1$ | 50 | 2391.5 | 47.8 |

Table 4. The mean copulation latency, in seconds, for those Mather 17 and $F_1$ males that both courted and mated Mather 17 virgin females.

| Male Type | Count | Mean (s) | Std. Dev. | Std. Err |
|---|---|---|---|---|
| Mather 17 | 47 | 78.7 | 118 | 17.2 |
| $F_1$ | 44 | 46.7 | 102 | 15.4 |

Table 5. Mann-Whitney U information for courtship latency of Mather 17 and $F_1$ males to Mather 17 virgin females. (P-Value = 0.0044).

| Male Type | Count | Sum Ranks | Mean Rank |
|---|---|---|---|
| Mather 17 | 58 | 2788 | 48.1 |
| $F_1$ | 54 | 3540 | 65.6 |

Table 6. The mean courtship latency, in seconds, for Mather 17 and $F_1$ males that courted Mather 17 virgin females.

| Male Type | Count | Mean (s) | Std. Dev. | Std. Err |
|---|---|---|---|---|
| Mather 17 | 56 | 41.2 | 58.9 | 7.9 |
| $F_1$ | 50 | 81.5 | 111.9 | 15.8 |

This pattern could have resulted from an experimental artifact associated with inbreeding: despite being sterile, $F_1$ males may be slightly more vigorous than Mather 17 males and were thus able to secure more copulations. However, contrary to this hypothesis, $F_1$ males were significantly slower to court *D. pseudoobscura* females than were Mather 17 males (Tables 5 and 6).

**Discussion**

As predicted, *D. persimilis* MSH 1993 males are very unsuccessful at mating with *D. pseudoobscura* Mather 17 females. Given that males of these species do not exhibit any species discrimination (Noor, 1996; Kandul *et al.*, 2006), this finding almost certainly results in large part from the high level of species mate discrimination by Mather 17 females.

However, contrary to our prediction, Mather 17 females are no more discriminating against $F_1$ males relative to Mather 17 males. Indeed, the opposite was true: $F_1$ males were slightly (but not significantly) more successful at mating with Mather 17 females than were Mather 17 males. We further failed to find evidence that this pattern was an artifact of inbreeding.

Ortiz-Barrientos *et al*. (2004) suggested that these females used olfaction as part of their species discrimination, because they identified several olfaction-related genes in the region of the genome to which a mate preference QTL mapped. At least three explanations can reconcile the inconsistency of this suggestion with our findings. One possibility is that the hypothesis of Ortiz-Barrientos *et al*. (2004) was incorrect, and highly discriminating *D. pseudoobscura* females are not necessarily using olfactory cues. Alternatively, *D. pseudoobscura* females may use olfactory cues, but they are prioritized below auditory cues such as courtship song. $F_1$ males from the crosses we conducted would have courtship songs very similar to *D. pseudoobscura* (Noor and Aquadro, 1998), and perhaps these females needed the combination of song and olfactory cue to discriminate. Finally, olfactory cues besides the cuticular hydrocarbons studied previously may mediate species discrimination in this population.

This experimental design does not mimic natural environmental conditions, and there is a concern with overinterpreting such results. It would also be useful to control for inbreeding depression that may have existed in the Mather 17 or MSH 1993 stocks by using flies one generation removed from nature. Further, detailed observations of the process of courtship itself, as done by some classic studies in these species (Brown, 1964; Brown, 1965), may identify specific points at which courtship breaks down in interspecies pairings.

**Conclusion**

*D. pseudoobscura* Mather 17 females do not show any discrimination towards $F_1$ males in terms of mating frequency or copulation latency, potentially in contrast to the prediction that cuticular hydrocarbons are responsible for secondary reinforcement-based discrimination in *D. pseudoobscura* females. Further studies should be conducted to confirm the results reported here.

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References: Brown, R.G.B., 1964, Behaviour 23: 61-106; Brown, R.G.B., 1965, Behaviour 25: 281-323; Howard, D.J., 1993, In: *Hybrid Zones and the Evolutionary Process* (Harrison, R.G., eds.), pp. 46-69. Oxford University Press, New York; Kandul, N.P., K.M. Wright, E.V. Kandul, and M.A.F. Noor 2006, BMC Evol Biol. 6: 54; Noor, M.A., 1995, Nature 375: 674-675; Noor, M.A.F., 1996, Anim. Behav. 52: 1205-1210; Noor, M.A.F., 1999, Heredity 83: 503-508; Noor, M.A.F., and C.F. Aquadro 1998, Anim. Behav. 56: 115-125; Noor, M.A.F., and J.A. Coyne 1996, Genet. Res.

68: 117-123; Noor, M.A.F., and D. Ortiz-Barrientos 2006, Behav. Genet. 36: 322-327; Ortiz-Barrientos, D., B.A. Counterman, and M.A.F. Noor 2004, PLoS Biol. 2: e416; Servedio, M.R., and M.A.F. Noor 2003, Annu. Rev. Ecol. Evol. Syst. 34: 339-364; Williams, M.A., A.G. Blouin, and M.A.F. Noor 2001, Heredity 86: 68-77.

**Tolerance adaptation of *Drosophila melanogaster* to increased salt concentration due to new beneficial mutations.**

**Azad, Priti,[1, 2] and R.C. Woodruff.[1]** [1]Department of Biological Sciences, Bowling Green State University, Bowling Green, OH 43403; [2]Current address-School of Medicine, University of California San Diego, La Jolla, CA 92093.

## Abstract

Mutation and selection are major evolutionary forces that help organisms to adapt to novel environments. Although numerous laboratory experiments with model organisms and observations from nature have demonstrated adaptations to new environmental conditions, it is difficult to distinguish whether adaptation has occurred due to new beneficial mutation or by selection on preexisting genetic variation. By using a highly inbred homozygous stock we have demonstrated adaptation of *Drosophila melanogaster* to increased tolerance to previous toxic levels of dietary salt as a result of new beneficial mutations. The adaptation occurred quickly (seven generations) suggesting that a small number of genes might have been involved and that new mutations can play an important role in adaptive evolution.

## Introduction

Mutation and selection are important interactive forces responsible for major evolutionary changes in all organisms. Yet, whether evolution is driven by natural selection acting on new mutations or on preexisting genetic variation is an ongoing debate (Nei, 1987; Lande, 1988; Gillespie, 1991; Li, 1997; Lynch, 1996; Barton, 1998; Orr and Betancourt, 2000; Kim and Stephan, 2002; Orr, 2005). There are numerous examples of rapid adaptations in natural populations, including insecticide resistance, adaptive melanism in populations of rock pocket mice, pelvic armor loss in fresh water sticklebacks, evolution of speech in humans, metal tolerance in plants, and HIV resistance in humans, that may be caused by new advantageous mutations (Wood and Bishop, 1981; Macnair, 1993; Stephens *et al.,* 1998; Toma *et al.*, 2002; Daborn *et al*., 2002; Nachman and Hoekstra, 2003; Shapiro *et al*., 2004). It is difficult, however, to disentangle the effects of new mutations from preexisting genetic variation. A number of studies have shown that adaptations can occur quickly due to preexisting genetic variation (Moya *et al.,* 2005; Peichel, 2005; Hartley *et al*., 2006; Zhan *et al*., 2006). New beneficial mutations have been studied experimentally in lines of microorganisms, including adaptation of clones of *Escherichia coli* to high and low temperatures (Bennett *et al*., 1992) and yeast to a low phosphate chemostat environment (Francis and Hansche, 1972). Few experiments, however, have been performed with multicellular organisms to measure directly the influence of new beneficial mutations on fitness and adaptation (Francis and Hansche, 1972; Batallion, 2000).

*Drosophila* is also a widely used model system to study adaptations to new environmental conditions such as new food source, temperature fluctuations, osmotic stress, hypoxia, and starvation

(Powell *et al.,* 1982; Dodd, 1989; Lenski *et al.,* 1991; Huey *et al.,* 1991; Frankham *et al*., 1999; Haddad, 2000; Misener *et al.,* 2001; Anderson *et al.,* 2005). Experiments involving new food sources vary from adding salts, such as sodium chloride and copper sulfate, insecticides, and toxins to regular food, plus replacing regular food with a new food source such as starch and dextrose (Powell *et al.,* 1982; Frankham *et al.,* 1999; Wilson, 2001). *Drosophila* quickly adapt to such drastic changes (Wilson, 2001). To eliminate the effect of preexisting genetic variation in the founding populations in such experiments, it is essential to start with homozygous chromosomes, isogenic stocks or clonal lines. The adaptation in such experiments depends on the rate of emergence of new beneficial mutations and their subsequent selection, which in some cases can be rapid (Mackay *et al.,* 1994; Fuller *et al.,* 2005). Determining the number of genes involved in adaptive changes, their dominance and selection effect, and the molecular changes involved, could help us to understand the mechanisms underlying adaptive evolution and speciation (Nachman, 2005; Dodd, 1989).

In this study we observed that homozygous populations of *D. melanogaster* can quickly adapt by increased tolerance to previous toxic levels of dietary salt as a result of new beneficial mutations.

## Materials and Methods

*Drosophila Stocks*

A highly inbred *sepia* (*se*, 3-26.0) stock of *D. melanogaster* with dark eyes (Lindsley and Zimm, 1992) was used to start the experiment. This inbred stock was generated by single-pair matings for 118 generations and was expected to be essentially completely homozygous (Hedrick, 2005). The stock was maintained on instant medium (Carolina Biological Supply) supplemented with yeast in a large population consisting of 10 bottles, transferring 100 males and 100 females per bottle each generation.

*Determination of Initial Extinction Concentration of NaCl*

To determine the initial concentration of salt that caused extinction of the inbred *sepia* stock, 15 sets of vials per treatment (with five pairs of flies in each vial) were tested at increasing NaCl concentrations (1%, 1.5%, 2%. 2.5%, 3%, 3.5%, 4%, 4.5%, 5%, 5.5%, and 6%). The vials contained instant *Drosophila* medium, except that salt solutions were used instead of water. Extinction concentration was defined as the concentration of NaCl at which no larvae or adult flies survived. The initial extinction concentration was tested two times with 15 sets of vials per treatment, and consistent results were obtained.

*Experimental Setup*

After determining the initial extinction concentration for the inbred *sepia* stock, four cages were started by placing 200 pairs of flies in each cage as shown in Figure 1. Two experimental cages (EI, EII) were provided with instant food containing increasing concentrations of NaCl (1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5%, 5%, and 5.5%) each generation (two weeks). Seven food cups were replaced with new salt food every week. Two cages were maintained as controls (CI, CII) by replacing seven food cups, among 15 total cups per population cage, with new regular food every week. All the cages were kept at 25°C in an illuminated incubator (set at 12 hours dark and 12 hours light cycles).

Separate lines were also founded from all cage populations at the end of each generation as shown in Figure 1. For example, at the end of generation one, lines were formed from the two experimental cage populations maintained on 1% NaCl food and the two control cage populations maintained on regular food. Flies from these lines and from the original inbred *sepia* stock were raised simultaneously in bottles containing regular food. These lines were used to verify if the

populations in the cages had evolved by performing the terminal salt-tolerance test as described below.

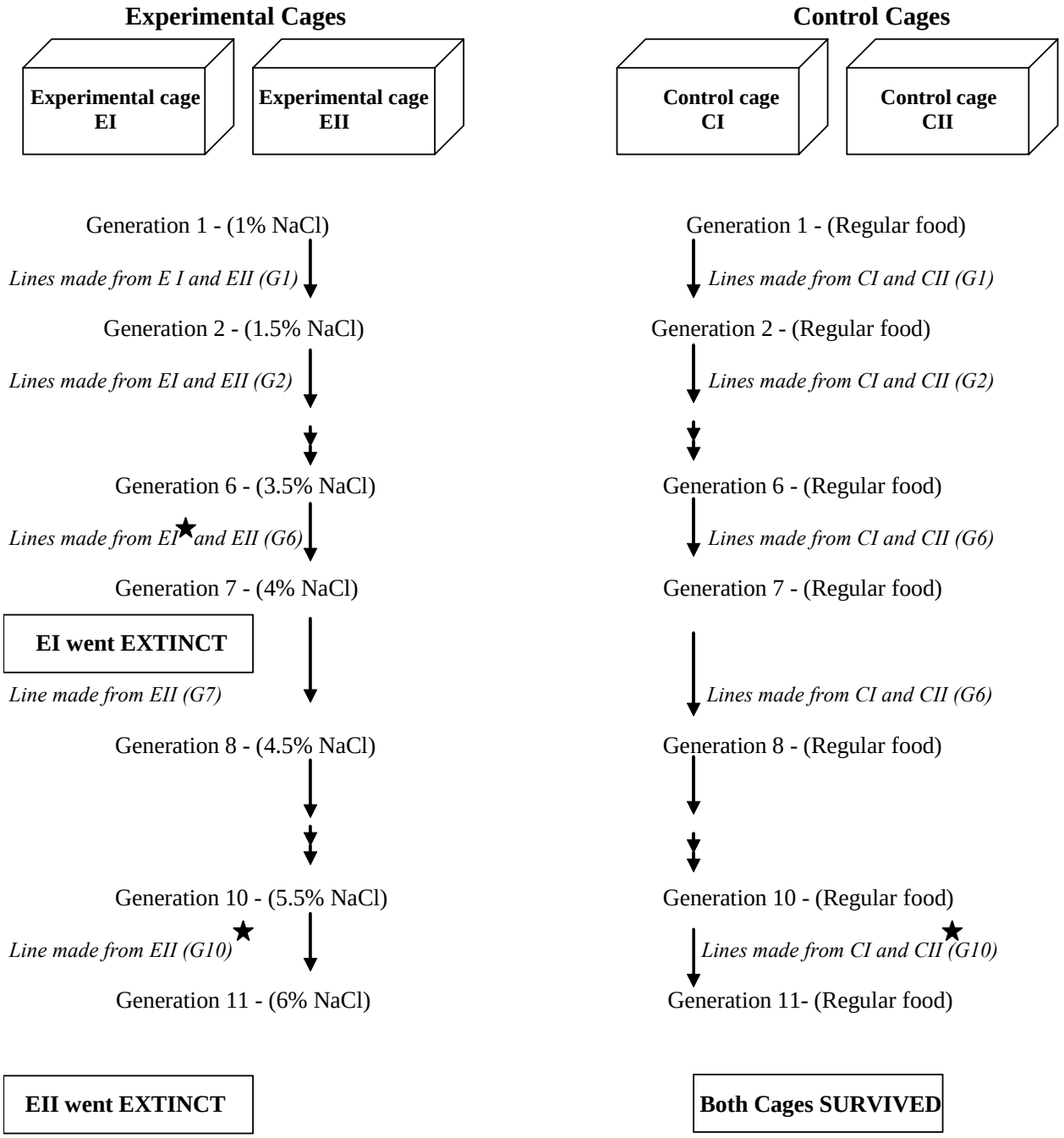


Figure 1. Experimental design. Two Experimental Cages, EI and EII (with NaCl), and two Control Cages, C I and CII (no NaCl), were started with 200 pairs of flies. Experimental and control lines were set up each generation from the previous generation population cage flies. ★ indicates that the G6 line of EI and the G10 lines of EII, CI and CII were used for performing the terminal salt-tolerance test.

*Terminal Salt-tolerance Test*

At the end of the experiment, flies from the lines generated from cages (G6 line of EI, G10 line of EII, and G10 lines of CI and CII) and from the inbred *sepia* stock were tested again to verify that the populations in experimental cages had evolved higher salt tolerance. Fifteen sets of five pairs of flies for each population type (original stock, and lines from control and experimental cages) were reared on regular instant food and instant food with 5.5% NaCl and the number of offspring per vial was recorded.

*Confirmation of Homozygosity by Microsatellite Analysis*

To check the homozygosity of the initial inbred *sepia* stock and to rule out contamination by extraneous flies, microsatellite analysis was performed at the beginning and end of the experiment using primers for the amplification of microsatellite markers on the second (DROYANETSB, DMAC9, DROPAD), third (DROABDB, DRONANOS, DMU1951) and X chromosomes (DMWHITE, DROACS2) of *D. melanogaster*. Ten individual flies from each stock (original inbred *se* stock, G10 lines of CI and CII control population cage lines, G6 line of EI, and G10 line of EII experimental population cage lines) were selected randomly at the beginning and end of the experiment and were genotyped for the eight microsatellites. PCR primer sequences and amplification conditions are described in Schug *et al.* (1998). The PCR products were run on Spreadex 300 gels using the SEA 2000 electrophoretic system (Elchrom Scientific, Switzerland). The gels were stained with SBYR Green and viewed using a Storm Phosphoimager (Amersham Biosciences, U.S.A.). The inbred *sepia* stocks, control lines, and experimental lines were homozygous initially and remained homozygous for the microsatellite markers during this study.

*Statistical Analyses*

Contingency $\chi^2$ tests were performed to determine whether the experimental cage populations had evolved increased tolerance to NaCl. Comparisons were made between the number of flies emerging without NaCl and with 5.5% NaCl from the original stock with the G10 lines of CI and CII, the G6 line of EI, and the G10 line of EII. The number of flies emerging without NaCl and with 5.5% NaCl from the G10 lines of CI and CII were also compared with the G6 line of EI and the G10 line of EII.

**Results**

The initial salt concentration that caused extinction of the inbred *se* stock was consistently 4% NaCl. The flies in the control cages (CI and CII) (regular food without NaCl) survived throughout the entire experiment (11 generations). For the two experimental cages, cage EI flies went extinct at 4% salt in 7 generations, whereas the cage EII flies survived up to 5.5% NaCl at generation 10.

*Terminal Salt-tolerance Test*

As an additional test to confirm that the experimental cage populations evolved increased tolerance to NaCl, terminal salt-tolerance tests were performed on the original inbred *se* stock, the control lines (G10 lines of CI and CII) and the experimental lines (G6 line of EI and G10 line of EII). The results of the terminal tests are shown in Table 1. At 5.5% NaCl no flies emerged from the original inbred stock, the G10 line of control cage CII or the G6 line of experimental cage EI, and only one fly emerged from the G10 line of control cage CI. The G10 line from the experimental cage EII yielded adults that were 3.3% of the number of flies in vials without NaCl. There were highly

significant differences between the original inbred *sepia* stock and experimental cage EII ($P < 0.0001$), and between the experimental cage EII and control cages (CI and CII) ($P < 0.0001$). There were no significant differences between the control cages (CI and CII) and the original inbred *sepia* stock ($P = 0.36$), experimental cage EI and the original inbred *sepia* stock ($P = 0.1146$), or between experimental cage EI and control cages (CI and CII) ($P = 0.3786$).

Table 1. Results of terminal salt-tolerance test of experimental populations compared to the original *sepia* inbred stock and the control population cages. Fifteen vials of five pairs of flies for each population type were tested on regular instant food and instant food with 5.5% NaCl.

| Population Type | Number of flies emerging (# of vials that produced progeny) No NaCl | Number of flies emerging (# of vials that produced progeny) 5.5% NaCl |
|---|---|---|
| Original Stock (O.S.)[a,f,j,l,m] | 929 (15) | 0 (0) |
| Experimental Cage I (G6 line of EI)[a,b,c,d,e] | 745 (15) | 2 (2) |
| Experimental Cage II (G10 line of EII) [b,f,g,h,i] | 909 (15) | 31 (9) |
| Control Cage I (G10 line of CI) [c,e,g,i,j,k,m] | 788 (15) | 1 (1) |
| Control Cage II (G10 line of CII)[d,e,h,i,k,l,m] | 702 (15) | 0 (0) |

[a] E1 vs O.S: $\chi^2= 2.49$, df=1, P=0.1146; [b] EI vs EII: $\chi^2= 19.927$, df=1, P<0.0001;
[c] EI vs CI: $\chi^2= 0.391$, df=1, P=0.5316; [d] EI vs CII: $\chi^2= 1.882$, df=1, P=0.1701;
[e] EI vs (CI&CII); $\chi^2= 1.943$, df=2, P=0.3786; [f] EII vs O.S.: $\chi^2= 31.154$, df=1, P<0.0001;
[g] EII vs CI: $\chi^2= 23.746$ df=1, P<0.0001; [h] EII vs CII: $\chi^2= 23.597$, df=1, P<0.0001;
[i] EII vs (CI &CII): $\chi^2= 46.373$, df=2, p<0.0001; [j] CI vs O.S.: $\chi^2= 1.178$, df=1, p=0.2777;
[k] CI vs CII: $\chi^2= 0.890$, df=1, P=0.3454; [l] CII vs O.S.: Fisher exact P value>0.9999;
[m] O.S. vs (CI & CII): $\chi^2= 2.068$, df=2, P=0.3556. For all the contingency $\chi^2$ comparisons, similar P values were obtained with the Fisher exact tests

## Discussion

This study demonstrates that new mutations and selection can cause rapid adaptation of *D. melanogaster* to increased tolerance to a previous toxic level of NaCl in seven generations, supporting the important role of new beneficial mutations in adaptive evolution. Recent studies, have suggested that a larger fraction of mutations are beneficial than was previously believed (Joseph and Hall, 2004; Shaw *et al*., 2002; Wloch *et al*., 2001; Zeyl and Devisser, 2001). This study supports this view and suggests that new mutations along with strong selection are capable of causing adaptations in a short period of time. Epistatic interactions among various genes could also be responsible for some of these rapid adaptations. It is known that new mutations can interact with other genes and influence many traits (Anholt *et al.*, 2003).

A number of genes have been shown to be involved in adaptation to osmotic stress in *Drosophila*. Some of these include transcription factors, members of mitogen-activated protein family, heat shock proteins, and various osmolyte transporters (Sano *et al.*, 2005; Inoue *et al*., 2001; Huang *et al.,* 2002). The morphological and physiological basis of adaptive responses for salt tolerance is less clear. Various mechanisms such as reducing the area of contact by small anal papillae, the induction of osmolyte transporters, and loss of taste reception have been proposed to adapt *Drosophila* to the higher concentration of salt, but the genetic basis for the adaptations remains to be identified (Velde and Scharloo, 1988; Huang *et al.,* 2002; Ishimoto and Tanimura, 2004). Identifying the number of genes involved in salt adaptation could be helpful in addressing some of

the aspects of the debate of whether adaptation is a result of many mutations of small effects or few mutations of large effects (Fisher, 1930; Orr and Coyne, 1992; Daborn *et al.,* 2002; Nachman, 2005). In this study, adaptation due to new mutations occurred quickly, which might suggest that the adaptation involved mutations in a few genes. There are numerous examples of mutations in one or few genes that cause major morphological and behavioral changes (Carroll, 2000; Daborn *et al*., 2002).

What is clear from this study is that new mutations are capable of causing rapid adaptations to a novel environment. These results give support to the hypothesis that new beneficial mutations can have a major impact on adaptive evolution.

References: Anderson, A.R., A.A. Hoffmann, and W.M. Stephen 2005, Genet. Res. Camb. 85: 15-22; Anholt, R.R.H., C.L. Dilda, S. Chang, J.J. Fanara, N.H. Kulkarni, I. Ganguly, S.M. Rollmann, K.P. Kamdar, and T.F.C. Mackay 2003, Nat. Genet. 35: 180-184; Barton, N.H., 1998, Genet. Res. 72: 123-133; Bataillon, T., 2000, Heredity 84: 497-501**;** Bennett, F., R.E. Lenski, and J.E. Miltler 1992, Evolution 46: 16-30; Carroll, R.L., 2000, Trends Ecol. Evol. 15: 27-32; Daborn, P.J., J.L. Yen, M.R. Bogwitz, G. Le Goff, E. Feil, S. Jeffers, N. Tijet, T. Perry, D.G. Heckel, P. Batterham, R. Feyereisen, T.G. Wilson, and R.H. Constant 2002, Science 297: 2253-2256; Dodd, D.M.B., 1989, Evolution 43: 1308-1311; Fisher, R.A., 1930, *The Genetical Theory of Natural Selection*. Oxford: Oxford University Press; Francis, J.E., and P.E. Hansche 1972, Genetics 70: 59-73; Frankham, R., K. Lees, M.E. Montgomery, P.R. England, E.H. Lowe, and D.A. Briscoe 1999, Animal Conserv. 2: 255-260; Fuller, R.C., C.F. Baer, and J. Travis 2005, Integr. Comp. Biol. 45: 391-404; Gillespie, J.H., 1991, *The Causes of Molecular Evolution*. Oxford: Oxford University Press; Haddad, G.G., 2000. J. Appl. Physiol. 88: 1481-1487; Hartley, C.J., R.D. Newcomb, R.J. Russel, C.G. Yong, J.R. Stevens, D.K. Yeates, and J. La Salle 2006, Proc. Natl. Acad. Sci. USA 103: 8757-8762; Hedrick, P.W., 2005, *Genetics of Populations.* MA: Jones and Bartlett Publishers; Huang, X., Y. Huang, R. Chinnappan, C. Bocchini, M.C. Mustin, and M. Stern 2002, Genetics 160: 561-569; Huey, R.B.L., L. Partridge, and K. Fowler 1991, Evolution 45: 751-756; Inoue, H., M. Tateno, K.K. Fujimaua, G. Takaesu, A.T. Yamada, T.J. Ninomiya, K. Irie, Y. Nishida, and K. Katsumoto 2001, EMBO J. 20: 5421-5430; Ishimoto, H., and T. Tanimura 2004, Cell. Mol. Life Sci. 61: 10-18; Joseph, S.B., and D.W. Hall 2004, Genetics 168: 1817-1825; Kim, Y., and K. Stephan 2002, Genetics 160**:** 765-777; Lande, R., 1988, Science 241: 1455-1460; Lenski, R.E., M.R. Rose, S.C. Simpson, and S.C. Tadler 1991, Am. Nat. 138: 1315-1341; Li, W.H., 1997, *Molecular Evolution*. Sunderland, MA: Sinauer; Lindsley, D.L., and G.G. Zimm 1992, *The Genome of* Drosophila melanogaster. New York: Academic Press, Inc; Lynch, M., 1996, A quantitative-genetic perspective on conservation issues. In: *Conservation Genetics.* (Avise, J.C., and J.L. Hamrick eds). Princeton, NY: Princeton University Press, 471-501; Macnair, M.R., 1993, New Phytol. 124: 541-559; Mackay, T.F.C., J.D. Fry, R.F. Lyman, and S.V. Nuzhdin 1994, Genetics 136: 937-951; Misener, S.R., C. Chen, and V.K. Walker 2001, J. Insect Physiol. 47: 393-400; Moya, A.F., E. Costas, E. Banares-Espana, L. Garcia Villada, M. Altamirano, and V. Lopez-Rodas 2004, New Phytol.166: 655-661; Nachman, M.W., and H.E. Hoekstra 2003, Mol. Ecol. 12: 1185-1194; Nachman, M.W., 2005, Genetica 123: 125-136; Nei, M., 1987, *Molecular Evolutionary Genetics*. New York: Columbia University Press; Orr, H.A., and J.A. Coyne 1992, Am. Nat. 140: 725-742; Orr, H.A., and A.J. Betancourt 2000, Genetics 157: 875-884; Orr, H.A., 2005, Nat. Rev. Genet. 6: 119-127; Peichel, C.L., 2005, Devel. Dynam. 234: 815-823; Powell, J.R., and M. Andjelkovic 1983, Genetics 103: 675-689; Sano, Y., H. Akimaru, T. Okamaura, T. Nagao, M. Okada, and S. Ishii 2005, Mol. Bio. Cell 16: 2934-2946; Schug, M.D., K.A. Wetterstrand, M.S. Gaudette, R.H. Lim, C.M. Hutter, and C.F. Aquadro 1998, Mol. Ecol. 7: 57-70; Shapiro, M.D., M.E. Marks, C.L. Peichel, B.K. Blackman, K.S. Nereng, B. Jónsson, D. Schluter, and D.M. Kingsley 2004, Nature 428: 717-723; Shaw, F.H., C.J. Geyer, and R.G. Shaw 2002, Evolution 56: 453-463;

Stephens, J.C., D.E. Reich, D.B. Goldstein, H.D. Shin, M.W. Smith, M. Carrington, C. Winkler, G.A. Huttley, R. Allikmets, L. Schriml, B. Gerrard, M. Malasky, M.D. Ramos, S. Morlot, M. Tzetis, C. Oddoux, F.S. di Giovine, G. Nasioulas, D. Chandler, M. Aseev, M. Hanson, L. Kalaydjieva, D. Glavac, P. Gasparini, E. Kanavakis, M. Claustres, M. Kambouris, H. Ostrer, G. Duff, V. Baranov, H. Sibul, A. Metspalu, D. Goldman, N. Martin, D. Duffy, J. Schmidtke, X. Estivill, S.J. O'Brien, and M. Dean 1998, Am. J. Hum. Genet. 62: 1507-1515; Toma, D.P., K.P. White, J. Hirsch, and R.J. Greenspar 2002, Nat. Genet. 31: 349-353; Velde, J.H., and W. Scharloo 1988, J. Evol. Biol. 2: 155-164; Wilson, T.G., 2001, Annu. Rev. Entomol. 46: 545-571; Wloch, D.M., K. Szafraniec, R.H. Borts, and R. Korona 2001, Genetics 159: 441-452; Wood, R., and J. Bishop 1981, *Insecticide Resistance: Population and Evolution. The Genetic Basis of Man-made Change.* London: Academic Press; Zeyl, C., and J. Devisser 2001, Genetics 157: 53–61; Zhan, J., F.L. Stefanato, and B.A. Mcdonald 2006, Mol. Plant Pathol. 7: 259-268.

## A revision of the *tumiditarsus* group of the subgenus *Drosophila* and its relation to the genus *Zaprionus*.

**Yassin, Amir.** Laboratoire Evolution, Génomes et Spéciation, CNRS, Bât 13, BP1, av. de la Terrasse, 91198 Gif-sur-Yvette Cedex, France. E-mail: yassin@legs.cnrs-gif.fr.

Both molecular and morphological analyses have confirmed the paraphyly of the genus *Drosophila*, being defined mainly upon non-derived traits (Ashburner *et al.*, 2005). However, many attempts were made to establish groups of species and radiations of groups and to assign other drosophilid genera to them (*e.g.*, Throckmorton, 1975). Nonetheless, species groups were usually arbitrarily defined with no general taxonomical criterion, resulting in some groups including only one species as well as others encompassing more than 150 species. An example is the *tumiditarsus* species group to which a single Oriental species, *Drosophila repletoides*, belongs. The phylogenetic positioning of this species, and thus of this group, had a very long debatable history. At times, it was considered a member of the *virilis-repleta* radiation, at others of the *immigrans-Hirtodrosophila* radiation (see list of synonyms below). A recent molecular phylogenetic study of the *virilis-repleta* radiation (Tatarenkov and Ayala, 2001) using alpha-methyldopa-hypersensitive protein (*amd*) and dopa-decarboxylase (Ddc) genes discarded *D. repletoides* from the analysis due to its high degree of divergence from other species groups. Unfortunately, the same workers did not include the species in a later work using both genes aiming to analyze the phylogenetic relationships among genera of the subgenus *Drosophila* (Tatarenkov *et al.*, 2001). More recently, the species was used in a molecular phylogeny study of the Drosophilidae based on the *Amyrel* gene (Da Lage *et al.*, 2006). According to their results, *D. repletoides* was placed within the *immigrans-Hirtodrosophila* radiation, close to the genus *Zaprionus*, with a Bayesian posterior probability of 96%. The genus *Zaprionus* has long been considered a member of the *D. immigrans* group (Throckmorton, 1975). In order to test the relation of *Zaprionus* to both *D. immigrans* and *D. repletoides*, I used the three sequences available on the GenBank for the latter species (*i.e.*, *amd*, *Ddc,* and *Amyrel*) in a four-taxon analysis, taking the genus *Hirtodrosophila* (an ancient subgenus of *Drosophila*) as an outgroup. As shown in Figure 1, the result obtained from the *Amyrel* gene was further confirmed from the combined analysis: *D. repletoides* is more related to *Zaprionus*, than is *D. immigrans*, although very distant to be included within it.

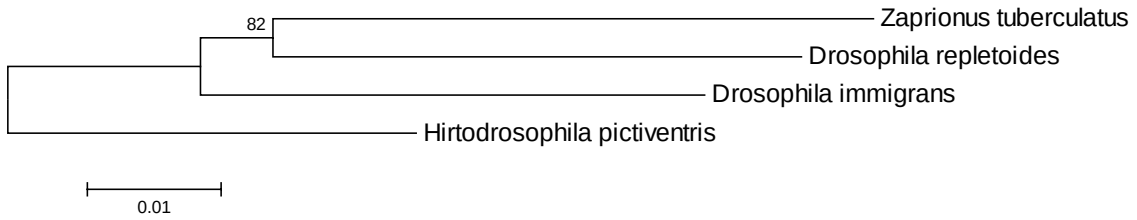


Figure 1. Neighbor-joining (NJ) phylogenetic tree inferred from 1217 amino acid sequences of 3 concatenated genes (AMD, DDC and AMYREL) for 4 species of the *immigrans-Hirtodrosophila* radiation. The number above the node connecting *Zaprionus* to *D. repletoides* represents the bootstrap value after 1000 iterations.

This result prompted me to morphologically revise both *D. repletoides* and the genus *Zaprionus* (especially species belonging to the Oriental subgenus *Anaprionus*). Although *D. repletoides* has a longitudinally striped thorax, which is the most diagnostic feature of the genus *Zaprionus*, it differs greatly from the latter by the thoracic stripes being blackish in color with the median one bifid posteriorly (silvery-white and straight in *Zaprionus*), by bearing 8 rows of acrostichal hairs (6 in *Zaprionus*), having a minute anterior reclinate orbital and no prescutellars (both well-developed in *Zaprionus*), and by having the phallus apically bifid (fused with a characteristic ruff in *Zaprionus*). Figure 2 shows the difference in thoracic ornamentation among *D. repletoides*, a member of the Oriental subgenus *Anaprionus* of *Zaprionus* with the subgeneric characteristic of the presence of a median stripe (*Z. bogoriensis*) and a member of the Afrotropical subgenus *Zaprionus s. str.* with the subgeneric characteristic of the absence of the median stripe (*Z. tuberculatus*).

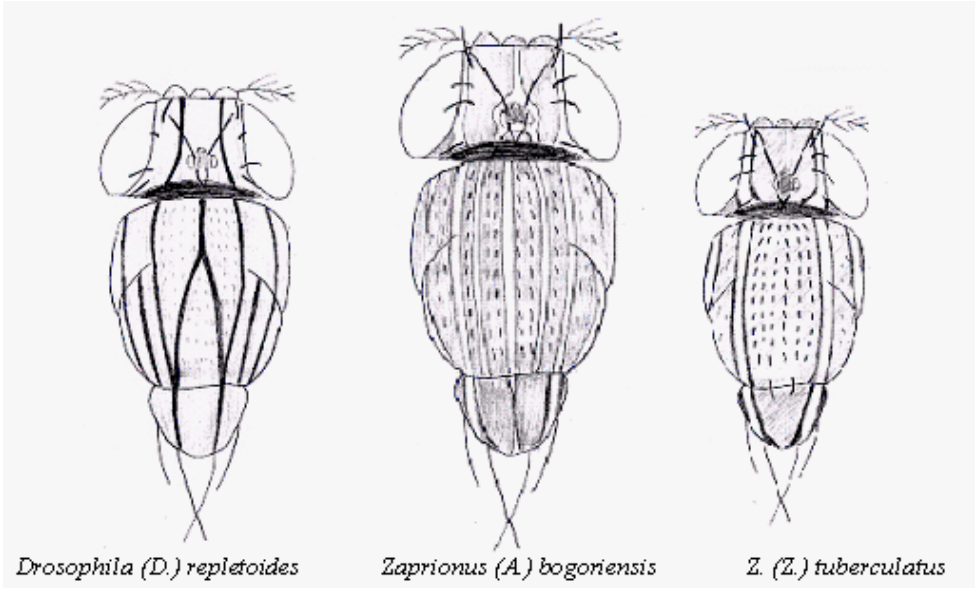


Figure 2. A compare-ison of frons and thoracic ornamentation among *Drosophila repletoides*, *Zaprionus bogoriensis* and *Z. tuberculatus* showing the difference in color and bifurcation of the longitudinal stripes. (Note also the disposition of orbital bristles, the number of rows of acrostichal hairs, and the presence or absence of the prescutellar bristles). Drawings are scaled to the relative sizes of species.

Nonetheless, 3 species classified within the Oriental subgenus *Anaprionus* (Bäechli, 1999-2006) share the same thoracic ornamentation as *D. repletoides*, as well as all the above characteristics distinguishing it from *Zaprionus*. Those are *Z. multistriatus* Duda (1923), *Z. flavofasciatus* Takada *et al.* (1979) and *Z. cercociliaris* Gupta and Gupta (1991). Their position within the genus *Zaprionus* is

no longer justified and they have to be placed in the genus *Drosophila* within the *tumiditarsus* group. In fact, the first two species were first described as *Drosophila* species.

In addition, the widespread *Z. (Anaprionus) bogoriensis* Mainx (1958) should also change its name. This species had a long history of synonyms (see below) of which the oldest is *Z. multistriatus* Sturtevant (1927), preoccupied by *Z. multistriatus* Duda (1923). Since Duda's species is now incorporated into *Drosophila* according to this study, the synonym problem disappears, and *Z. bogoriensis* should recover its older name of *Z. multistriatus* Sturtevant.

In conclusion, the major nomenclatural changes I made in this paper can be summarized as follows:

**Genus *Drosophila*** Fallén, 1823, Diptera sveciae. Geomyzides: 4
**Subgenus *Drosophila*** Fallén
***tumiditarsus* group** Clayton and Wheeler, 1975, In: King, R. C. (ed.), Handbook of Genetics 3: 490
***D. cercociliaris*** Gupta and Gupta, 1991, Proc. zool. Soc., Calcutta 44: 119 **comb. nov.**
***D. flavofasciata*** Takada, Beppu and Toda, 1979, J. Fac. gen. Educ., Sapporo Univ. 14: 122 **nomen protectum**
***D. multistriata*** Duda, 1923, Annls hist.-nat. Mus. natn. Hung. 20: 57 **nomen protectum**
syn. *lineata* de Meijere, 1911, Tijd. Ent. 54: 420 (*Stegana*: preocc.)
***D. repletoides*** Hsu, 1943, Kwangsi Agric. 4: 162
syn. *tumiditarsus* Tan *et al.*, 1949, Univ. Texas Publs 4920: 205
syn. *hayashii* Okada, 1953, Zool. Mag. Tokyo 62: 285
syn. *chinoi* Okada, 1956, Syst. Study of Drosophilidae and Allied Families of Japan, 162
**Genus *Zaprionus*** Coquillett, 1901, Proc. U. S. natn. Mus. 23: 31
**Subgenus *Anaprionus*** Okada, 1990, Jpn. J. Ent. 58: 154
***Z. multistriatus*** Sturtevant, 1927, Philipp. J. Sci. 32: 365 **nomen protectum**
syn. *bogoriensis* Mainx, 1958, Zool. Anz. 161: 126
syn. *argentostriata* Bock, 1966, Univ. Qld. Pap., Dep. Zool. 2: 273 (*Drosophila*)

The taxonomic status of the four species of the *tumiditarsus* group remains to be determined in light of more detailed molecular, morphological and behavioral analyses.

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References: Ashburner, M., K. Golic, and S.H. Hawley 2005, *Drosophila: A Laboratory Handbook*, Cold Spring Harbor Laboratory Press, New York; Bäechli, G., 1999-2006, TaxoDros: The database on Taxonomy of Drosophilidae (http://taxodros.unizh.ch); Da Lage, J-L., G.J. Kergoat, F. Maczkowiak, J-F. Silvain, M-L Cariou, and D. Lachaise 2006, J. Zool. Syst. Evol. Res. (*in press*); Tatarenkov, A., and F.J. Ayala 2001, Mol. Phyl. Evol. 21: 327-331; Tatarenkov, A., M. Zurovcova, and F.J. Ayala. 2001, Mol. Phyl. Evol. 20: 321-325; Throckmorton, L.H., 1975, *In*: *Handbook of Genetics* (R.C. King, ed.). Plenum Press, vol. 3: 421-469.

## *Drosophila* collection in Baja California, México: New records for four species.

**Castrezana, Sergio.** *Drosophila* Tucson Stock Center, University of Arizona, Tucson, AZ 85705, USA; E-mail: castreza@email.arizona.edu

*Drosophila* flies were collected on the Baja California Peninsula in January 2001 in 39 stations covering 22.94°N, 109.99°W to 31.66°N, 116.53°W. *Drosophila* were obtained from natural cactus rots and artificial baits of cardón *Pachycereus pringlei*, senita *Lophocereus schottii*, agria *Stenocereus gummosus,* organ pipe *Stenocereus thurberi,* prickly pear *Opuntia engelmanni,* and banana. For artificial baits, about 20 kilograms of fresh cactus tissue was cut in small cubes, placed in a 10 gallon container cover with water, and inoculated with natural rot liquid. Then, almost 300 grams of prepared rotten tissue was placed in a two-liter green soda container and local vegetation was added for perching purposes. Baits were recovered after a 3-10 day period, flies were sorted on site, and isofemale lines were set up. Specimen identities were confirmed at the University of Arizona, either by external morphology, genitalia morphology, polytene chromosomes squashes, and/or molecular analysis by the author. A total of 5251 *Drosophila* flies in 17 species were collected from 25 rotten cacti plants, 95 baits in 39 localities. Table 1 shows the species and numbers collected by state. *Drosophila* flies were more abundant at the south of Baja Peninsula during collection time. Percentage of species composition per bait type is presented in Table 2. In general, prickly pear baits were not attractive for *Drosophila* flies; only ten individuals were collected over eight baits.

Table 1. Species collected by state in Baja California Peninsula, January 2001.

| Species | Baja North | Baja South | Total |
|---|---|---|---|
| *D. mojavensis* | 90 | 1424 | 1514 |
| *D. pachea* | 219 | 664 | 883 |
| *D. aldrichi* | 124 | 671 | 795 |
| *D. mettleri* | 218 | 347 | 565 |
| *D. simulans* | 57 | 441 | 498 |
| *D. nigrospiracula* | 4 | 356 | 360 |
| *D. pseudoobscura* | 124 | 104 | 228 |
| *D. arizonae* | 1 | 116 | 117 |
| *D. hydei* | 35 | 55 | 90 |
| *D. busckii* | 83 | 1 | 84 |
| *D. spenceri* | 0 | 70 | 70 |
| *D. repleta* | 0 | 14 | 14 |
| *D. eremophila* | 0 | 13 | 13 |
| *D. azteca* | 0 | 8 | 8 |
| *D. mainlandi* | 0 | 6 | 6 |
| *D. melanogaster* | 1 | 3 | 4 |
| *D. mathisi* | 1 | 1 | 2 |

Upon request, the author can provide specific collection data such as flies per bait, locality, and sex of the sample. According to the book by Markow and O'Grady (2006), four species, *D. azteca*, *D. mathisi*, *D. pseudoobscura, D. spenceri*, are new records in Baja California peninsula.

Table 2. Percentage species composition per bait type in Baja California collection, January 2001. Data presented by column.

| Species | | Banana | Agria | Cardon | Senita | Organ pipe |
|---|---|---|---|---|---|---|
| | Total baits | 11 | 28 | 23 | 21 | 10 |
| | Total flies | 566 | 1055 | 1022 | 630 | 374 |
| *D. mojavensis* | | 24.9% | 33.6% | 29.2% | 4.9% | 26.5% |
| *D. aldrichi* | | 6.5% | 24.8% | 19.7% | 15.9% | 35.0% |
| *D. pachea* | | ---- | 1.2% | 2.5% | 54.1% | 8.3% |
| *D. mettleri* | | 7.2% | 4.6% | 16.0% | 12.5% | 11.0% |
| *D. simulans* | | 17.8% | 21.0% | 11.8% | 3.5% | 6.4% |
| *D. nigrospiracula* | | 0.7% | 2.4% | 7.8% | 8.4% | 5.9% |
| *D. pseudoobscura* | | 14.5% | 4.5% | 7.4% | 0.3% | 4.5% |
| *D. arizonae* | | 3.0% | 1.2% | 1.7% | 0.2% | 1.3% |
| *D. hydei* | | 4.9% | 4.5% | 1.0% | ---- | 0.8% |
| *D. busckii* | | 13.3% | 0.8% | ---- | 0.2% | ---- |
| *D. spenceri* | | 2.8% | 0.5% | 2.0% | ---- | 0.3% |
| *D. repleta* | | 1.1% | 0.8% | ---- | ---- | ---- |
| *D. eremophila* | | 1.4% | ---- | ---- | ---- | ---- |
| *D. azteca* | | 1.2% | ---- | 0.1% | ---- | ---- |
| *D. mainlandi* | | ---- | ---- | 0.6% | ---- | ---- |
| *D. melanogaster* | | 0.4% | ---- | 0.2% | ---- | ---- |
| *D. mathisi* | | 0.2% | 0.1% | ---- | ---- | ---- |

References: Markow, T.A., and P.M. O'Grady 2006, Drosophila, *A Guide to Species Identification and Use*. Academic Press.

## ***Drosophila carbonaria:*** **reproductive notes and a new recipe to rearing it in laboratory.**

**Castrezana, Sergio.** Drosophila Tucson Stock Center, University of Arizona, Tucson, AZ 85705, USA; E-mail: castreza@email.arizona.edu

*Drosophila carbonaria* Patterson and Wheeler 1942 is a single species in the carbonaria group (Sturtevant, 1942) within the subgenus *Drosophila*. This species is found in the Sonoran and Chihuahan Deserts of the Southwestern United States and Mexico. In nature, *D. carbonaria* are associated with the sap fluxes of mesquite trees (*Prosopis* spp.), and occasionally, windfall citrus fruits (Patterson, 1943). It is extremely rare to collect *D. carbonaria* on banana baits even when these baits were close to the mesquite trees (Pers. obs.). Recently, *D. carbonaria* has been introduced in the Hawaiian archipelago along with its host (Wagner, *et al*., 1990). Nevertheless, Hawaiian collections of *D. carbonaria* were associated to the sap fluxes of monkeypod trees *Samanea saman* (O'Grady, *et al.,* 2002).

Mesquite fluxes have the lowest nitrogen and phosphorus content of several described *Drosophila* host, including the cactus hosts of Sonoran desert *Drosophila* (Jaenike and Markow 2003). Thus, *D. carbonaria* likely has adopted specialized strategies to survive on its nutrient poor diet. Indeed, of 21 yeast species isolated from both mesquite and *D. carbonaria* flies, three of then were unique to this *Drosophila*-plant association (Ganter *et al.,* 1986). The paucity of research on the

reproductive ecology of *D. carbonaria*, coupled with its unique host environment, has made it difficult to adapt this species to laboratory culture in the past.

Patterson (1943) indicated that *D. carbonaria* does not breed well on laboratory medium unless the food is rather soft. I used wild-caught *D. carbonaria* females and their F1-F3 progeny to examine this result empirically. *Drosophila carbonaria* individuals were collected in Wetmore Park at Tucson, Arizona (32.28°N, 110.97°W) and set in 3-liter jars containing mesquite sap flux for three days. A female was then aspirated into a vial with one of three types of softened laboratory culture media: cornmeal, banana/opuntia, or Wheeler-Clayton food (recipes available at http://stockcenter.arl.arizona.edu). At least four ovipositing females were used for each laboratory culture medium. After 24 hours, the female was removed and the number of eggs was counted. Following larval development, pupal cases and eclosed adults were also quantified. F1 individuals were sorted as virgins and matured for ten days in vials containing the same culture media as their larval environment. F1 *D. carbonaria* pairs were then aspirated into vials with softened laboratory culture media and left in the vial for 24 hours. The pair was then removed and eggs, pupae and adults were counted. This procedure was repeated for F2 and F3 *D. carbonaria* individuals.

Results are presented in Table 1. For all media, almost 90% of *D. carbonaria* hatching larvae died before they reached the pupal stage. Furthermore, after three generations in these laboratory food media, all strains of *D. carbonaria* perished. There was some variation, however, in productivity of *D. carbonaria* on different media. Laboratory cornmeal medium was the most sustainable food type compared with Wheeler & Clayton or Banana/opuntia foods.

Table 1. Testing three different laboratory culture media for *Drosophila carbonaria*: Average number of eggs oviposited in 24 hours, total number of larvae reaching pupae stage, and total adults emerging.

| | Wheeler & Clayton | | | | Cornmeal | | | | Banana/Opuntia | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | eggs | pupae | adults | N | eggs | pupae | adults | N | eggs | pupae | adults |
| *Wild*-♀ | 4 | 23.3±3.8 | 1.0±0.4 | 0.8±0.4 | 4 | 45.3±5.5 | 5.3±1.0 | 4.3±0.5 | 4 | 20.8±6.0 | 3.5±1.0 | 0.8±0.3 |
| F1 | 2 | 11.0±1.0 | 3.5±0.5 | 1.5±1.5 | 4 | 24.2±2.7 | 5.0±0.9 | 3.8±1.5 | 2 | 6.5±1.5 | 2.0±1.0 | 1.0±0.0 |
| F2 | 1 | 13 | 3 | 3 | 4 | 15.8±4.1 | 1.25±0.6 | 0.8±0.5 | - | | | |
| F3 | 1 | 24 | 1 | 1 | 4 | 31.0±6.9 | 3.0±1.3 | 1.8±1.0 | - | | | |

Table 2. Testing new laboratory culture media with different concentrations of mesquite sap infusion for *Drosophila carbonaria*: Average number of eggs oviposited in 24 hours and total adults emerging.

| | 0%* | 25% | 50% | 100% |
|---|---|---|---|---|
| N | 4 | 10 | 10 | 10 |
| eggs | 24.2±2.7 | 24.8±2.1 | 25.5±2.1 | 24.7±1.3 |
| adults | 3.8±1.5 | 11.9±1.4 | 11.6±1.5 | 21.4±1.3 |

*Values corresponded to F1 individuals in cornmeal on table 1.

Due to the inability of laboratory media to sustain *D. carbonaria* in culture, I sought to determine the relationship between these insects and their native host: mesquite sap fluxes. It was, therefore, necessary to harvest mesquite sap flux from nature. While collecting sap flux directly from mesquite trees would be ideal, plant physiology and the arid desert environment made this impractical. Indeed, only five milliliters of sap were obtained from 36 mesquite rotten processes visited in eight hours of collection. Thus, I collected mesquite bark covered with dry fluxes, which it is very abundant. The bark was removed from the trees with a 30-centimeter long screwdriver. In order to resuspend these dried fluxes, 500 grams of harvested bark was boiled for five minutes in one-liter of double distilled water. Three dilutions, 25%, 50% and 100%, of the resultant liquid were produced. Five milliliters of this sap dilution was then combined with five milliliters hot cornmeal media in a vial and allowed to cool at room temperature for 6 hours.

The three types of resultant media were used to test *D. carbonaria* food preference. Mature mate pairs of F1 *D. carbonaria* raised on cornmeal were introduced to the new sap dilution media. After 12 hours, the flies were removed and eggs counted. Vials were retained to quantify pupal cases and eclosed adults (Table 2). After six days, a piece of boiled mesquite bark was added for perching purposes.

Regardless of the concentration of mesquite sap dilution, no significant differences were observed the quantity of oviposited eggs ($F_{3,30}$ =. 058, p = 0.981). Therefore, the presence of mesquite sap does not affect the female oviposition preference. On the other hand, significant differences between treatments were observed for larval performance on different media. Specifically, larval survival to adulthood is significantly increased by the presence of mesquite sap on the food ($F_{3,30}$ = 18.741, p = 0.000). On average, larval survivorship of *Drosophila carbonaria* in laboratory exhibits a 3-fold increase on 25% and 50% dilutions of mesquite infusion, and a greater than a 5-fold increase on undiluted mesquite sap infusion. A *Drosophila carbonaria* stock maintained in laboratory conditions with 100% dilution is available at the Tucson Stock Center (label 15400-0011.00 at http://stockcenter.arl.arizona.edu).

References: Garner, P.F., W.T. Starmer, M.A. Lachance, and H.J. Phaff 1986, Oecologia 70: 386-392; Jaenike, J., and T.A. Markow 2003, Func. Ecology 17: 115-120; O'Grady, P.M., J.W. Beardsley, and W.D. Perreira 2002, Bishop museum ocasional papers 698: 34-35; Patterson, J.T., 1943, Univ. Texas Publs 4313: 7-216; Patterson, J.T., and M.R. Wheeler 1942, Univ. Texas Publs 4213: 67-109; Sturtevant, A.H., 1942, Univ. Texas Publs 4213: 5-51; Wagner, W.L., D.R. Herbst, and S.H. Sohmer 1990, *Manual of the Flowering Plants of Hawai'i*, University of Hawaii Press & Bishop Museum Press.

**Loss of paracentric inversions in laboratory stocks of *Drosophila ananassae*.**

**Singh, P., and B.N. Singh***. Genetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi-221005, India. * E-mail: bnsingh@bhu.ac.in

*Drosophila ananassae* exhibits a high degree of chromosomal polymorphism. It harbors a large number of inversions in its natural populations (Singh, 1998). Out of these reported from various parts of the world, most have restricted distribution while the three cosmopolitan inversions namely, Alpha (AL) in 2L, Delta (DE) in 3L and Eta (ET) in 3R show worldwide distribution (Singh, 1998). Population genetics of chromosomal polymorphism in Indian natural populations of *D. ananassae* has been extensively studied (for references see review by Singh, 1998). The results have clearly shown that there is geographic differentiation of inversion polymorphism.

In the present communication, we report about the fate of two new paracentric inversions namely, theta and iota, (Singh and Singh, 2005a,b) detected from an isofemale line from Bhubneswar (Orissa) and Allahabad (Uttar Pradesh), respectively. *D. ananassae* flies from these places were collected during June 2005 and October 2005, respectively. Two isofemale lines were maintained on the simple culture medium by transferring fifty flies (males and females in equal number). Laboratory stock from Bhubneswar was analysed after 18 generations while laboratory stock from Allahabad was analysed after 14 generations by squashing more than fifty larvae. In both of the stocks the two new inversions namely, theta and iota, were found to be eliminated. Although, a large number of paracentric inversions are known to occur in *D. ananassae*, only three have become coextensive with the species. Most of the inversions have localized distribution and have been detected from the few individuals. This is a feature of the pattern of the chromosomal polymorphism

in *D. ananassae* (Carson, 1965; Singh, 1988). In the present communication, the loss of two paracentric inversions each of which was detected from a single naturally inseminated female is discussed.

At its origin, the unique copy of an inversion will be in a single individual in the heterozygous state. In the following generations, if the inversion escapes elimination, it will be found predominantly in the heterozygous state until (and if) it reaches a substantial frequency. Their evolutionary fate, then, depends upon their gene content (Krimbas and Powell, 1992).

Three stages may be identified in the evolutionary history of an inversion (Nei, *et al.*, 1967). Shortly after its origin as a unique event, the inversion will be lost or survive due largely to stochastic events irrespective of population size (Fisher, 1930; Li, 1955). If the inversion survives this early stage to become present in several copies in the population, selective process may begin to control its fate, along with stochastic ones especially in small populations. In the third stage, a balance may be achieved in establishing a stable polymorphism due to some form of selection (Krimbas and Powell, 1992).

Two types of model based on computer simulations and analytical procedures exploring the fate of newly arisen inversions can be distinguished, the *additive model* and the *interactive model*. According to the first model (Sturtevant and Mather, 1938), there have been several linked genes, each with two alleles, one beneficial and one detrimental. Selection was hypothesized to eliminate unfit phenotypes caused by homozygosity of detrimental alleles in case of dominance, or with added selection against heterozygotes in the case of no dominance. The new inversion was introduced in a single copy into a small genetically variable population. Establishment meant either being maintained as stable polymorphism or going to fixation. Inversions with superior allelic content could be established. Gene action (*i.e.*, dominance, absence of dominance, and occasionally overdominance were considered), allelic content of the new inversion, and the initial frequencies of the advantageous alleles in the population were the most important variables in determining the fate of an inversion. Neither overdominance at the genic level nor epistasis was necessary, although they might render easier the establishment of the inversions (Kojima, 1967). Thus the establishment of a new inversion would be a rare event.

The simplest possible *interactive model* consists of two genes with two alleles each (the two locus system). According to Fisher (1930), in a population where alleles at loci A and B interact such that the combinations AB and ab are selectively advantageous over Ab and aB, any genetic variant reducing recombination between the loci will be favored such that AB/ab heterozygotes would produce fewer unfavorable Ab and aB gametes.

Federer *et al*. (1967) proposed a model in which the expected frequency of generation of an inversion is a linearly decreasing function of its length:

$$h_1(v) = 2\,(c-v)/c^2\,,\ \text{with a mean } c/3 \text{ and a variance } c^2/18$$

where, $h_1(v)$ is the frequency of an inversion of length v occurring in a chromosome of total length c.

Van Valen and Levins (1968) also considered a model where the gene content of inversions was crucial in their establishment and that the probability of retention of an inversion is directly related to the number of overdominant loci captured, the expected distribution of lengths favors longer inversions. In *D. ananassae* AL (alpha) inversion, by virtue of being the longest among the three inversions (AL, DE, ET), has more probability of catching two or more genes with favorable epistatic effect on fitness which increases with size of the inversion, *i.e*. the selective advantage gained by the inversion increases with recombination distance between them (Càceres *et al*.,1999; Schaeffer *et al*., 2003). Olvera *et al*. (1979) examined the distribution of 34 naturally occurring inversions in a single chromosome (the third) of *D. pseudoobscura* and concluded that the wider the distribution and higher the frequency the more successful the inversion. Krimbas and Loukas (1980) examined complex inversion combination in *D. subobscura* and concluded that such combinations

will protect from recombination the chromosome more efficiently than would any single inversion of comparable size. Van Valen (1961) studied a new inversion spontaneously appearing in a population of *D. pseudoobscura*. An increase in frequency was followed by a decrease. This was interpreted as indicating exchange of alleles between the new inversion and the common preexisting gene arrangement leading to shifts in karyotypic fitness. These laboratory experiments reveal that retention of inversion is dependent upon its combining abilities with other chromosomes in the population, that is, its ability to be heterotic with other gene arrangements.

It could be said that most often moderately sized inversions are favored due to trade-off between long and short inversions. Long inversions have a higher probability of capturing favorable sets of alleles solely because they capture more of the genome. However, they may loose their favorable content at a higher rate due to double cross overs. Shorter inversions have a low probability of capturing favorable combinations of alleles, but once they do they retain them more efficiently than do longer inversions (Krimbas and Powell, 1992). Also, the role of gene recombination could be important in two ways, one is the production of such gene combinations that are of rare permanent advantage to the species (three cosmopolitan inversions in *D. ananassae*). If the advantageous gene combinations are preserved due to inversion heterozygosity and become established in its genetic structure, then, the inversion heterozygotes prove to be an asset to the species. Second is the production of disadvantageous gene combinations as an insurance against the period when some of them might be needed for adaptation of the population.

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References: Càceres, M., A. Barbadilla, and A. Ruiz 1999, Genetics 153: 251-259; Carson, H.L., 1965, *The Genetics of Colonizing Species,* Academic Press, London, New York: 503-531; Federer, W.T., R.G.D. Steele, and B. Wallace 1967, Genetics 55: 783; Fisher, R., 1930, *The Genetical Theory of Natural Selection*, Carendon Press, Oxford; Li, C.C., 1955, *Population Genetics*, University of Chicago Press, Chicago; Kojima, K.I., 1967, Cienc. Cult. 19: 67-52; Krimbas, C.B., and J.R. Powell 1992, *Drosophila Inversion Polymorphism*, CRC Press, Boca Raton: 14-20; Krimbas, C.B., and M. Loukas 1980, Evol. Biol. 12: 163-234; Nei, M., 1967, Genetics 57: 625-641; Olvera, O., J.R. Powell, M.E. de la Rosa, V.M. Salceda, M.L. Gaso, J. Guzman, W.W. Anderson, and L. Levine 1979, Evolution 33: 381-395; Schaeffer, S.W., M.P. Goetting-Minesky, M. Kovacevic, J.R. Peoples, and J.L. Graybill *et al*., 2003, PNAS 100: 8319-8324; Sturtevant, A.H., and K. Mather 1938, Am. Nat. 72: 447-452; Singh, B.N., 1988, Ind. Rev. Life Sci. 8: 147-168; Singh, B.N., 1998, Ind. J. Expt. Biol. 36: 739-748; Singh, P., and B.N. Singh 2005a, Dros. Inf. Serv. 88: 10-11; Singh, P., and B.N. Singh 2005b, Dros. Inf. Serv. 88: 15-16; Van Valen, L., 1961, Am. Nat. 95: 113-120; Van Valen, L., and R. Levins 1968, Am. Nat. 102: 5-12.

## A study of P element and hybrid dysgenesis phenomena in some *Drosophila melanogaster* populations from Turkey.

**Atli, Kurtulus**. Hacettepe University, Faculty of Science, Department of Biology, Division of Genetics, 06800, Beytepe, Ankara, Turkey; E-mail: kurtulusatli@gmail.com.

Mobile or transposable elements are DNA sequences that have the ability to integrate into the genome at a new site within their cell of origin (Kazazian, 2004). Over the last two decades a large

number of transposable elements have been identified in a wide variety of evolutionarily distant organisms, where they often represent a large fraction of the genome: 12% in *Drosophila*, 45% in humans, 50% in maize and up to 90% in some plants (Dimitri *et al*., 2003). There are more than 40 mobile elements in *Drosophila* genome (Rasmusson *et al*., 1993). P element is the most studied one among them (Dominguez and Albornoz, 1996; Dimitri *et al*., 2003). The number of P transposable elements in any natural population of *Drosophila melanogaster* can categorically be characterized within the dysgenesis system, P and M (Kidwell, 1983a; Kidwell,1986; Engels, 1996; Ozsoy, 2000). In the P-M system, three classes of strains P, Q, and M, have been described on the basis of their phenotypic properties (Kidwell, 1985). P strains have the P cytotype. These strains have the potential to produce variable degrees of P factor activity. M strains possess M cytotype and are divided into two subcategories, true M and M' (pseudo-M). True M strains completely lack any members of the P element family. M' strains carry a variable number of P elements, many and sometimes all of which may be defective. The M' type is common in strains recently derived from natural populations in Europe and Asia (Kidwell, 1986). Hybrids between P strain males and M strain females how significant frequencies of dysgenic traits, notably gonadal dysgenesis (GD sterility) and singed-weak mutability. Q strains are considered to be a subset of P strains; they do not show gonadal sterility in any strain combination, but can produce low frequencies of other dysgenic traits, such as singed-weak mutability and male recombination, in crosses with M-strain females (Kidwell, 1985). The P element-associated dysgenesis, hybrid dysgenesis, of an individual fly is thought to be determined by the very presence of genomic P elements in that fly, and the categories are constructed by the levels of dysgenesis measured by appropriate genetical tests (Kidwell, 1986; Engels, 1996). Geographical variation in P element numbers of natural *D. melanogaster* populations (corresponding to their P-M status) has been documented. Major continental differences in the genomic distribution of P elements are observed when present-day American and European populations are compared. Almost all American populations are of the P/Q type, but in European populations P strains are rare and M' strains are common. The level of P-element susceptibility varies widely across a gradient from west to east (Anxolabèhère *et al*., 1988).

In this study, 6 geographically different natural populations of *Drosophila melanogaster* from Turkey (Ankara, Kerpe and Giresun) were examined and their P-M statuses were determined by using Gonadal Dysgenesis (GD) Sterility Assay. Four Central Anatolian (Eryaman 1, Eryaman 2, Ayrancı, Beytepe; four local populations in Ankara) and two Northern Anatolian (Kerpe and Giresun) populations were collected during the summer. At each location the number of the flies collected exceeded 50 individuals per population. The GD sterility assay allows one to analyze the results of dysgenic crosses with marker strains, the visible changes in the overall morphology of gonads of the F1 female progenies caused by P mobility at developmental temperature above 25°C (exactly 29°C in this study), to determine P-M categories in an easy way (Ozsoy, 2000). Two types of mating, termed as "Cross A" and "Cross A*", were done to determine the P activities and cytotypes of tested natural populations. Cross A and A* were done as three replicas for every natural population (see Kidwell, 1986, for experimental setup and the marker strains used in these specific crosses). Parental hybrid matings were made in culture bottles that contained a standard *Drosophila* medium and immediately placed at 29°C until the eclosion of $F_1$ progeny. $F_1$ females were aged for three to five days after eclosion in order to allow any oocytes present to undergo full maturation. Ovaries were dissected into water. Observations were made with a stereodissection microscope at magnifications ranging from 10.5 to 45× (see picture in Kidwell, 1986). The frequency of ovarian dysgenesis was calculated as the number of dysgenic ovaries divided by the total number of ovaries examined, and cytotypes were determined according to the standard table given in Kidwell 1986 (Table 1).

Table 2 shows the percentage dysgenesis levels per population for the activity potentials and regulatory abilities. According to GD sterility assay, Ery-2, Beytepe and Giresun populations were defined as M' (pseudo-M); Ery-1, Ayrancı and Kerpe populations were determined as Q (weak-P). The highest P activity (58.3%) was obtained in Giresun population and the smallest (2.5%) in Kerpe population. %GD sterility of natural populations in Cross A and Cross A* is illustrated in Figures 1 and 2.

The populations were sampled from two ecologically distinct regions; Central Anatolian and Northern Anatolian parts of Turkey. The P-M status distribution of the populations determined by using the standard table of Kidwell (1986) resembles Eurasian strains which were mentioned Kidwell (1983b). The P-M category of Kerpe population collected from West Black Sea region was defined as Q strain (2.5% GD sterility), that of Giresun population collected from East Black Sea region was determined as M' strain (58.5% GD sterility). Therefore, it can be said that the strain type and the amount of %GD sterility in Black Sea region may change in an easterly direction. To justify this hypothesis, studies should be extended by raising the number of samplings from west to east, in other following cases. Moreover, it was found that Ery-1 and Ayrancı populations were Q strain; Ery-2 and Beytepe populations were M' strain. The finding of the four populations collected in Ankara is considered as a supporting proposal that fruit flies may have genetical varieties. So it is natural to expect more particular differences between two areas considering the fact that some GD sterility differences were gained even in districts of Ankara.

Table 1. Characteristic values for GD sterility in standard tests of various categories of strains (After Kidwell, 1986).

| Strain type | %GD sterility | |
|---|---|---|
| | **Cross A** | **Cross A*** |
| M (true) | 0 | 100 |
| M' (pseudo – M) | 0 - ? | 0 – 100 |
| Q (weak P) | 0 – 10 | 0 - 10 |
| P (moderate) | 11 – 80 | 0 - 10 |
| P (strong) | 81 - 100 | 0 - 10 |

The P-M status distribution of the different natural populations in Turkey has been investigated by some researchers (Konac and Bozcuk, 1990; Konac *et al*., 1995; Ozsoy and Bozcuk, 2000). But these results are not completely satisfactory to draw a general conclusion for Turkey concerning P-M status. The natural populations collected from different regions can be helpful in revealing a map of Turkey's P-M system.

Table 2. Percentages of gonad dysgenesis in the different natural populations of *Drosophila melanogaster*.

| Natural Populations | **Temperature** | Cross Types | | | | | | % GD sterility | | Strain types |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *2N* | | 1N | | **0N** | | | | |
| | | A | A* | A | A* | A | A* | A | A* | |
| Ery-1 | 29ºC | 60 | 55 | 0 | 1 | 0 | 4 | 0 | 7.5 | Q (weak-P) |
| Ery-2 | 29ºC | 40 | 51 | 0 | 1 | 0 | 11 | 0 | 18.3 | M'(pseudo-M) |
| Ayrancı | 29ºC | 60 | 56 | 0 | 0 | 0 | 6 | 0 | 9.7 | Q (weak-P) |
| Beytepe | 29ºC | 57 | 36 | 3 | 2 | 0 | 22 | 2.5 | 38.3 | M'(pseudo-M) |
| Kerpe | 29ºC | 60 | 57 | 0 | 3 | 0 | 0 | 0 | 2.5 | Q (weak-P) |
| Giresun | 29ºC | 60 | 22 | 0 | 6 | 0 | 32 | 0 | 58.3 | M'(pseudo-M) |

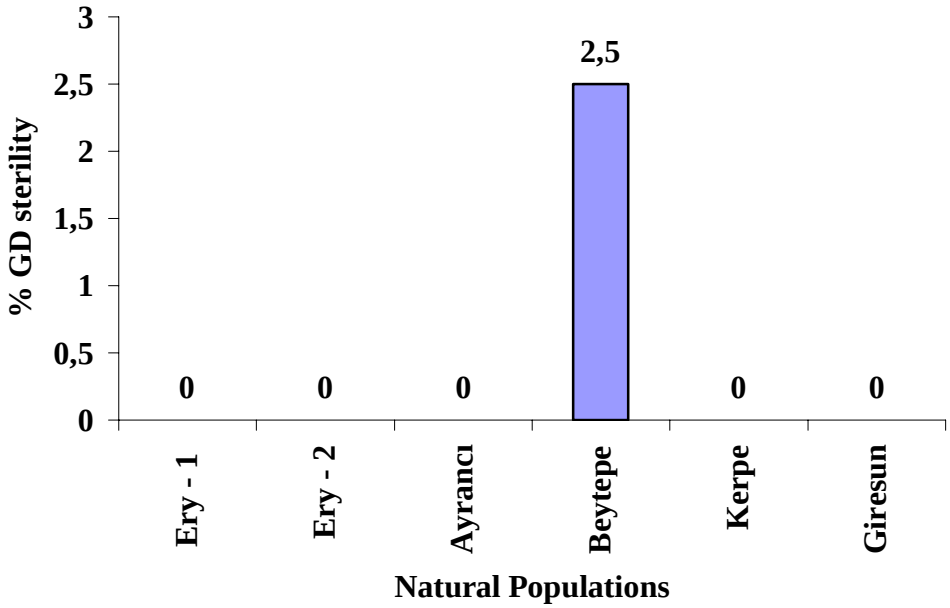


Figure 1. %GD sterility of natural populations of *Drosophila melanogaster* in Cross A.

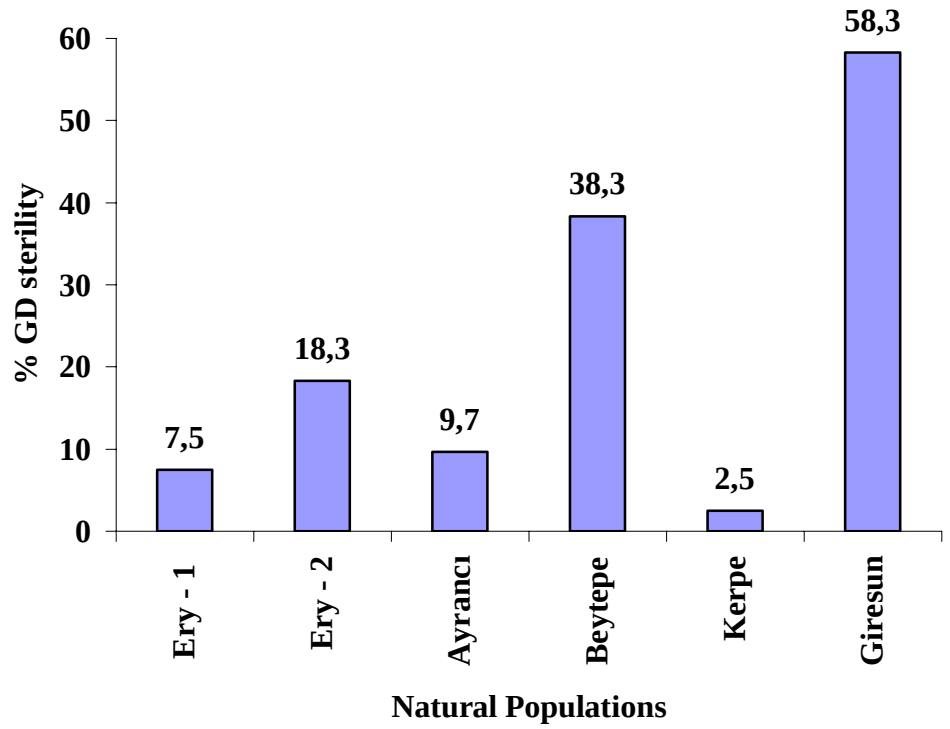


Figure 2. %GD sterility of natural populations of *Drosophila melanogaster* in Cross A*.

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References: Anxolabèhère, D., M.G. Kidwell, and G. Periquet 1988, Mol. Biol. and Evol. 5: 252-269; Dimitri, P., N. Junakovic, and Arca, B., 2003, Mol. Biol. and Evol. 20(4): 503-512; Dominguez, A., and J. Albornoz 1996, Mol. Gen. Genet. 251: 130-138; Engels, W.R.,1996, *In*: *Transposable Elements*, (Saedler, H., and A. Gierl, eds.), 103-123, Springer-Verlag, Berlin Heilderberg; Kazazian, H.H., 2004, Science 303: 1626-1632; Kidwell, M.G., 1983a, Genetics 104: 317-341; Kidwell, M.G., 1983b, Proc. Natl. Acad. Sci. 80: 1655-1659; Kidwell, M.G., 1985, Genetics 111: 337-350; Kidwell, M.G., 1986, *In*: Drosophila*, A Practical Approach* (Roberts, D.B., ed.), 59-81, IRL Press, Oxford; Konac, T., and A.N. Bozcuk 1990, Tr. J. of Biol. 14: 32-41 (in Turkish); Konac, T., A.N. Bozcuk, and A. Kence 1995, Age 18: 19-23; Ozsoy, E.D., and A.N. Bozcuk 2000, Dros. Inf. Serv. 85: 59-62; Rasmusson, K.E., J.D. Raymond, and M.J. Simmons 1993, Genetics 133: 605-622.

## A color and life-history polymorphism in *Drosophila sulfurigaster*.

**van der Linde, Kim.** Department of Biological Science, Florida State University, Tallahassee, FL 32306-1100, USA; E-mail: kim@kimvdlinde.com.

The species of the *nasuta* species subgroup (*immigrans* species group) are highly similar and are best identified using karyotyping (Wilson *et al.*, 1969), although three groups are distinguished based on the coloration of the frons (the area between the eyes). *D. kohkoa*, *D. kepulauana*, *D. nasuta, D. albomicans* and *D. niveifrons* have a white patch on the frons. The various subspecies of the *D. sulfurigaster* complex and *D. pulaua* have white orbits, while the remaining species—*D.* taxon F, *D.* taxon I, *D.* taxon J and *D. pallidifrons*—do not have white on the frons (Wilson *et al.*, 1969; Kitagawa *et al.*, 1982; Kitagawa, 1991; Yu *et al.*, 1999). The coloration is more pronounced in males, but under the right light conditions, this character is also visible in females of *D. sulfurigaster*, though less obvious. Despite many studies, the taxonomy of the group is still not fully known (see Yu *et al.*, 1999; Nagaraja *et al.*, 2004; Bachtrog, 2006), while the assignment of species and subspecies status varies between authors (see Yu *et al.*, 1999).

Three representatives of this group, *D. sulfurigaster* (white orbits), *D. kohkoa* and *D. kepulauana* (both entire white frons), are found in the Philippines; of these, only *D. sulfurigaster* has been recorded from the north of Luzon (Baltazar, 1991; Bächli, 1999-2007; Ruiz-Fiegalan, 2004). In October 1994, I collected *Drosophila* in the Sierra-Madre Mountains, near Cabagan, Isabela province in the northern Philippines. Individuals belonging to the *D. nasuta* subgroup were assigned to two groups based on the absence or presence of the whitish to silvery lines on the frons along the eyes. One group clearly resembles *D. sulfurigaster*, while the second group did not fit the known species of the Philippines ('Type A'). No individuals with a whitish to silvery sheen on the entire frons were found, confirming the absence of *D. kohkoa* and *D. kepulauana* from the north of Luzon (Baltazar, 1991; Bächli, 1999-2007; Ruiz-Fiegalan, 2004).

*D. sulfurigaster* is a generalist species, collected in three habitats (grassland, forest edge and secondary forest), while 'Type A' is only found in the secondary forest. The distance between the collection sides in the secondary forest and in the forest edge was less than one kilometer. Two life-history characteristics, development time and starvation resistance, were measured for both types using standardized methods in a common laboratory environment in the $F_3$ generation (for details, see van der Linde and Sevenster, 2006). The average values for development time and starvation

resistance were for the 'Type A' strain 8.74 days and 3.34 days, respectively. The estimates for the sympatric secondary forest population of *D. sulfurigaster* were 10.08 days and 2.79 days, respectively, a significant difference with 'Type A' for both traits ($p < 0.05$ for each trait). At the same time, the variation between populations of *D. sulfurigaster* is not significant ($P = 0.58$ and $P = 0.19$, respectively), and substantially smaller than the differences between these two types (development time: 9.75 -10.08; starvation resistance: 2.79 - 3.18; van der Linde and Sevenster, 2006).

These obvious differences between the two groups raise the question whether these are separate species or represent a single-gene polymorphism. Individuals of the 'Type A' can be crossed without a problem with individuals of a laboratory strain of *D. sulfurigaster* from the Northern Philippines (personal communication Dr. Y. Fuyama). Unfortunately, various crosses between the different taxa in the *D. nasuta* species subgroup produce $F_1$-offspring, despite that they are morphologically indistinguishable. One example consists of *D. nasuta* and *D. albomicans* that differ only in karyotype (Wilson *et al.*, 1969; Wakahama and Kitagawa, 1972), but they mate freely with each other and produce fertile $F_1$ progeny but their $F_2$ progeny is not. When either *D.* taxon F from Malaysia or *D. pallidifrons* from Ponape (with coloration as the 'Type A' individuals) are crossed with *D. sulfurigaster*, the offspring is completely sterile (Wilson *et al.*, 1969; Kitagawa *et al.*, 1982). The lack of offspring would have been a strong argument in favor of two species, but the reverse is not a conclusive argument that the two types are one and the same species.

To conclude, the unidentified 'Type A' clearly differs in coloration (no white on the frons) and life-history traits (shorter development time and longer starvation resistance) from *D. sulfurigaster*. The same difference in coloration pattern has been observed between species of this subgroup but not within a single species. When crossed, they produce $F_1$-offspring without a problem, which is observed between species of this subgroup. If 'Type A' individuals are variants of *D. sulfurigaster*, the association between life-history traits and coloration suggests a stable single-gene polymorphism with strong pleiotropic effects. Furthermore, this would imply also that the color differences at the frons are not a useful character for the identification of the various species. Further research will be needed to determine whether 'Type A' represents a variant of *D. sulfurigaster* or a separate (and potentially undescribed) species. Regardless whether these two types are color and life-history variants or two different species, the differences between the types provides new clues to the evolution and speciation within this intriguing group of species as well as to the usefulness of color characteristics for the identification of the species.

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References: Bächli, G., 1999-2007, TaxoDros: The Database on Taxonomy of Drosophilidae, http://taxodros.unizh.ch/; Bachtrog, D., 2006, Genet. Res. 88: 13-26; Baltazar, C., 1991, *An inventory of Philippine insects. Part 1: Diptera*, University of the Philippines Los Baños, University Publications Office, Manilla, The Philippines; Kitagawa, O., 1991, *Evolution of Populations (in Japanese)*, Tokyo Univ. Press, Tokyo; Kitagawa, O., K. Wakahama, Y. Fuyama, Y. Shimada, E. Takanashi, M. Hatsumi, M. Uwabo, and Y. Mita 1982, Jpn. J. Genet. 57: 113-141; Nagaraja, J. Nagaraju, and H.A. Ranganath 2004, Genes & Genetic Systems 79: 293-299; Ruiz-Fiegalan, E.C., 2004, Philippine Entomologist 18: 24-43; van der Linde, K., and J.G. Sevenster 2006, Biol. J. Linnean Soc. 87: 115-125; Wakahama, K., and O. Kitagawa 1972, Jpn. J. Genet. 47: 129-131; Wilson, F.D., M.R. Wheeler, M. Harget, and M. Kambysellis 1969, Univ. Texas Publ. 6918: 207-253; Yu, H.J., W. Wang, S. Fang, Y.P. Zhang, F.J. Lin, and Z.C. Geng 1999, Mol. Phylogenet. Evol. 13: 556-565;

**New records of *Zaprionus indianus* Gupta, 1970 (Diptera, Drosophilidae) in North America and a key to identify some *Zaprionus* species deposited in the Drosophila Tucson Stock Center.**

**Castrezana, Sergio.** *Drosophila* Tucson Stock Center, University of Arizona, Tucson, AZ 85705, USA; E-mail: castreza@email.arizona.edu.

The old world native species from subgenus *Zaprionus* and species group *armatus*, *Zaprionus indianus* Gupta, 1970 (Chassagnard, 1988; Chassagnard and Kraaijeveld, 1991; Chassagnard and Tsacas, 1993), was first recorded in the Western Hemisphere in San Paulo, Brazil. Vilela (1999) suggested that the species might have colonized Brazil from the U.S.A. Obviously, no previous records substantiated such an idea. In Brazil, *Z. indianus* was recognized as an active pest mainly in commercial figs where eggs laid at the ostiole gave easy access to the fruit (Vilela *et al.,* 2001; Raga *et al*., 2003). Although *Z. indianus* was reared from several hosts (van der Linde *et al.*, 2006), in all cases the fruit had previous damage. *Zaprionus indianus* spread rapidly through South America where it has established large populations (Castro and Valente, 2001; Goní *et al.,* 2002; Dobbin *et al*., 2004; David *et al.,* 2005). In 2003, a few *Z. indianus* were collected from Isla Contadora, Panama. Then, *Z. indianus* was collected in several Florida counties in 2005 and individuals' numbers are increasing (van der Linde *et al.,* 2006). Apparently, colonization through the Caribbean is the logical explanation for the *Z. indianus* presence in the southeast U.S.A. However, here I present some records from Mexico, a potential alternative route for the incipient *Z. indianus* colonization of the Western U.S.A. Another hypothesis for *Z. indianus* colonization of the western America is through the ports.

University of Arizona *Drosophila* researchers collect continually from Mexico using banana baits in two-liter soda containers as described in O'Grady and Markow (2006). In May 2002, on a *Drosophila* collection trip scheduled in the states of Puebla, Oaxaca and Chiapas, three males and one female of *Z. indianus* were captured three kilometers north of Chiapa de Corzo, Chiapas (16.74$^{o}$N, 92.97$^{o}$W) close to citrus/mango groves. In this sample, 96.4% were *simulans/melanogaster* where *Z. indianus* represented only 0.2% of the sample. The second *Z. indianus* collection took place in January 2004 during a collection trip carried out in the states of Michoacán, Estado de México, Jalisco and Sinaloa. One *Z. indianus* male was captured in El Tuito, Jalisco (20$^{o}$19.1' N, 105$^{o}$19.02' W), in a semi caducifolic rainforest. On this occasion, *Z. indianus* was collected along with indigenous species such as *D. aldrichi*, *D. eremophila, D. nigricruria* and *D. gibberosa.* The latest Mexican collection of *Z. indianus* was made recently, in San Carlos, Sonora (27.97$^{o}$N, 110.99$^{o}$W) when two males were caught in a trash site close to orange/guava/mango groves (December 2006). The species composition in this collection was *D. simulans, D. melanogaster, D. hydei, D. arizonae, D. spenceri, D. repleta, D. pseudoobscura,* and *Z. indianus* (with *Z. indianus* comprising 0.3%).

On the other hand, in 2006 took place the first record of *Z. indianus* in Western United States. In October, one *Z. indianus* male was collected north San Diego bay, California, along with *D. melanogaster* and *D. simulans*. Collection place and the environment was unknown. In the same month, six females and 12 males of *Z. indianus* were collected from banana baits in Tucson, Arizona (32.22$^{o}$N, 110.91$^{o}$W), during the Annual Drosophila Workshop sample demonstrations. Banana bait was located close to citrus and pomegranate (*Punica granatum*) trees. Other species collected in the same bait were *D. simulans, D. melanogaster*, *D. aldrichi, D. longicornis, D. hydei, D. pseudoobscura, D. azteca, D. nigrohydei, D. repleta* and *D. busckii*.

Finally, the Tucson Drosophila Stock Center holds ten species in the *Zaprionus* genus. Jean David from L.E.G.S. in the National Scientific Research Center- (France) kindly donated and identified most of them in 2006. Here is a key to identify these species.

**1**. Fore femur with short stout knob, or tubercle, located near middle of the posteroventral surface.…….. **2**
**1b.** Fore femur unadorned or if knobs present, they are the small base of the setae and no tubercle structure exist.…….……..……... **3**

**2**. Ventral surface of knob with one straight seta…..…….**9**
**2b.** Ventral surface of knob with two strong setae; dorsal surface with a single seta…..……..... *sepsoides*

**3**. Wings strongly shining (reflective), brownish……..…….……...*badyi*
**3b.** Wings hyaline, without shining brownish color…..…… **4**

**4**. Scutellum with a white apical spot…….. *ghesquierei*
**4b.** Scutellum lacking apical white spot……...…….……. **5**

**5**. Frons with a fine, white medial stripe anterior to ocelli…..…….……..… *inermis*
**5b.** Frons without a fine, white medial stripe..…….…….…..... **6**

**6**. Dark species; scutellum black. Mesonotum with two black central bands. Each band has two-dorsocentral setulae rows.…….…….....…. *camerounensis*
**6b.** Yellowish species; scutellum and mesonotum have same color. No black central bands in mesonotum……..…….....…….. **7**

**7**. Tergites without apical band…..……...……..….. *davidi*
**7b.** At least two tergites with visible (or almost) apical bands…….. **8**

**8**. A faint, almost translucent dark band in the apical margin of tergites 2-5. Sub-apical setae in tergites 4-5 arise from dark spot…….....….. *indianus*
**8b.** A dark band in the apical margin of tergites 2-5. No spots on tergites……..… *taronus*

**9**. Flagellomere black. Frons lacking white medial stripe……..…….. *mascariensis*
**9b.** Flagellomere yellow. Frons with a fine, white medial stripe anterior to ocelli.…… *tuberculatus*

References: Castro, F.L., and V.L.S. Valente 2001, Dros. Inf. Serv. 84: 15-17; Chassagnard, M.T., 1988, Naturaliste Canadiénne 115: 305-322; Chassagnard, M.T., and A.R. Kraaijeveld 1991, Ann. Soc. Entomol. (N.S.) 27: 495-496; Chassagnard, M.T., and L. Tsacas 1993, Ann. Soc. Entomol. Fr. (N.S.) 29:173-194; David, J.R., L.O. Araripe, B.C. Bitner-Mathé, P. Capy, B. Goní, L.B. Klaczko, H. Legout, M.B. Martins, J. Voudibio, A. Yassin, and B. Moreteau 2005, Heredity advance online publication; Dobbin, B.F., B.F.D. Leao, and R. Tidon 2004, Ann. Soc. Entomol. Fr. (N.S.) 40: 285-290; Goní, B., M.E. Martínez, G. Techera, and P. Fresia 2002, Dros. Inf. Serv. 85: 75-80; Linde, K.V.D., G.J. Steck, K. Hibbard, J.S. Birdsley, L.M. Alonso, and D. Houle 2006, Florida Entom. 89: 402-404; Markow, T.A., and P.M. O'Grady 2006, Drosophila, *A Guide to Species*

*Identification and Use*, Academic Press; Raga, A., M.F. Souza Filho, and M.E. Sato 2003, Ann. Inst. Biol. San Paulo 70: 287-289; Vilela, C.R., 1999, Dros. Inf. Serv. 82: 37-39; Vilela, C.R., E.P. Teixeira, and C.P. Stein 2001, *In: Historico e impacto das pragras introduzidas no Brasil,* (Vilela, E.F., R.A. Zucchi and F. Cantor, eds.), pp. 48-52, Holos, Ribeirao Preto SP.

**Expression profile analysis of *menin1* mutants.**

**Sturgill, David[a], Aniello Cerrato[a], Sonia Santa Anna[b], Michael Parisi[a], Settara Chandrasekharappa[b], Stephen Marx[a], Francis Collins[b], and Brian Oliver[a].**
[a]National Institute of Diabetes and Digestive and Kidney Diseases, and [b]National Human Genome Research Institute, Bethesda MD 20892.

The absence of the tumor suppressor Menin results in multiple endocrine neoplasia type I (MEN1) in humans (reviewed by Agarwal *et al.*, 2004). *Drosophila* Menin is encoded by *mnn1* (Guru *et al.*, 2001). We assayed the effect of *mnn1* loss-of-function and *mnn1* over expression on adult flies and embryos by microarray experiments and found remarkably little change in expression correlated with *mnn1* genotype, suggesting that *mnn1* has very little effect on gene expression under the tested conditions.

Mammalian Menin is a classic tumor suppression protein, where tumors occur in individuals heterozygous for loss-of-function alleles. Additionally Menin is required for embryonic development, as homozygous embryos die showing hemorrhages and defective neural tube closure (reviewed by Agarwal *et al.*, 2004). In contrast, the *Drosophila Menin1* gene (*mnn1*) is not required for viability, but appears to alter response to stress according to two somewhat incongruent reports (Papaconstantinou *et al.*, 2005; Cerrato *et al.*, 2006). Flies mutant for *mnn1* are also sensitive to mutagens, suggesting that *Drosophila* Menin plays a role in DNA repair (Busygina *et al.*, 2004; Busygina *et al.*, 2006). There is abundant evidence suggesting that Menin interacts with transcription factors (reviewed by Agarwal *et al.*, 2004), including AP1 in both *Drosophila* and mammals (Cerrato *et al.*, 2006). In *Drosophila*, thoracic closure and eye development are particularly susceptible to lowered or raised *mnn1* expression in the context of AP1 mis-expression (Cerrato *et al.*, 2006). In mammals, Menin is a subunit of a Trithorax Complex that methylates Histone H3 at Lysine 4 (Hughes *et al.*, 2004; Yokoyama *et al.*, 2004; Chen *et al.*, 2006; Scacheri *et al.*, 2006). Trithorax complexes typically regulate large batteries of genes, and have especially well studied roles in the regulation of HOX genes (Schuettengruber *et al.*, 2007). Indeed, mammalian Menin is a regulator of at least one HOX gene (Hughes *et al.*, 2004; Yokoyama *et al.*, 2004). Interestingly, even though Menin is bound at many active promoters in mammalian cells, the loss of Menin has very little effect on transcription (Scacheri *et al.*, 2006). To determine if Menin has a general role in transcription in *Drosophila*, we assayed global gene expression in flies lacking or over expressing relative to wild type flies.

We isolated mRNA from *y w; mnn1*$^{\Delta 46}$ and *y w; mnn1*$^{\Delta 79}$ (both are protein null alleles on *mnn1*) adult flies, as well as mRNA from the isogenic *y w; mnn1*$^{+84}$ and *y w; mnn1*$^{+113}$ flies (precise excision lines of the P-element used to generate the null alleles), another *y w* line, and mRNA from heterozygous combinations of *mnn1* alleles. These samples were labeled and hybridized to Affymetrix DrosGenome1 arrays. Gene expression profiles for both females and males were performed, for a total of eleven hybridizations were performed and are available from the Gene

Expression Omnibus (GEO) (Edgar *et al.*, 2002). Replicate hybridizations from different samples showed very good reproducibility ($R^2 \geq 0.98$).

We observed very few significant differences in expression between the *mnn1*$^-$ flies and the remaining *mnn1*$^+$ or *mnn1*$^+$/ *mnn1*$^-$ flies. There was a greater effect of *mnn1*$^-$ loss-of-function on males. Following false discovery rate correction after Benjamini-Hochberg (Hochberg and Benjamini, 1990), two genes are differentially expressed in *mnn1*$^-$ mutants relative to wild type at an adjusted p-value cutoff < 0.01. Both of these genes (*Act88F* and *Acp26Aa*) show lower expression in *mnn1*$^-$ males based on array data. No statistically significant differential expression was found between *mnn1*$^-$ and *mnn1*$^+$ females.

At lower confidence, there are more changes in differential expression. One must be more cautious in using these changes as a basis for further experiments, but several genes might be targets for follow-up work. For example, when we simply observed fold changes, and selected genes at least 2-fold differentially expressed relative to the isogenic control, thirty genes showed 2-fold differences in one of the sexes. Among these genes, Gene Ontology annotation (Harris *et al.*, 2004) for immune response and antibacterial response were significantly enriched ($p << 0.01$). We did not determine the response of *mnn1* mutants on pathogen challenge. Even if all of the genes showing 2-fold differential expression are true direct or indirect targets of Menin, these data suggest that Menin has a limited role in transcriptional regulation in adult flies.

Consistency of the response is also a valuable criterion for follow-up work. Only four genes showed 2-fold differences between *mnn1*$^-$ and *mnn1*$^+$ in both females and males. The *CG11909* gene showed greater expression in *mnn1*$^-$ flies, while the *CG9682*, *Jon99Fi*, and *CG18404* genes showed poorer expression in *mnn1*$^-$ flies. While we have no reason to suspect a sex-biased role for *mnn1*$^+$, sex-biased expression in *Drosophila* is known to be extensive (Zhang *et al.*, 2007). However, these results do not clearly identify a particular non-sex-biased process that responds to Menin.

We over-expressed Menin using the UAS-Gal4 system with the *How24-GAL4* driver (Brand and Perrimon, 1993). This results in defective thoracic closure (Cerrato *et al.*, 2006). An over-expression of Menin in *How24>mnn1*$^+$ was detected in embryos by western blotting (not shown). We therefore collected and aged embryos to assay expression at 2-4, 4-6, 6-8, and 2-16 hrs post egg deposition. As controls, samples from two embryos having only the *How24-GAL4* driver or only the *UAS-mnn1*$^+$ were also collected for each. Expression was assayed using 2-color FlyGEM arrays (Johnston *et al.*, 2004) with dye-flip replicates between each *How24>mnn1*$^+$ and control at each time point. As was observed in adults, there was little differential expression observed that correlated with the *mnn1* genotype in embryos. The only gene showing a time-course change in gene expression consistent with Menin over expression (beginning in 4-6 hr embryos) and the sole significantly differentially expressed gene (FDR adjusted $p < 0.01$) was *white*. Given that Gal4 expression might be expected to increase the expression of the *white*$^+$ marker in the *UAS-mnn1*$^+$ construct, it is quite likely that this is simply a surrogate marker for *mnn1* expression (We did not detect increased expression of *mnn1* transcripts, which we suggest is due to poor performance of the array element for *mnn1*). Briefly, over expression of Menin during embryogenesis does not appear to have a major effect on gene expression.

As previously reported in mammalian cells (Scacheri *et al.*, 2006), our data suggest that *mnn1* has little effect on transcription under standard conditions. This is somewhat enigmatic for a subunit of a major transcriptional regulation complex.

**Materials and Methods**

*Drosophila* mutants, and driver UAS pairs, and standard growth conditions have been previously described (Cerrato *et al.*, 2006). Labeling and hybridization were performed with

standard Affymetrix (Santa Clara, California) protocols by the NIDDK microarray core facility (http://genomics.niddk.nih.gov/array.shtml), or performed as previously described (Johnston *et al.*, 2004).

For Affymetrix experiments, intensities were extracted, normalized, and summarized by robust multi-array average (RMA) (Irizarry, 2003) using the Bioconductor affy package (Irizarry *et al.*, 2003; Gentleman *et al.*, 2004). Differential expression in *mnn1*$^{-}$ mutants compared to wild type was then calculated using Linear modeling using the limma package (Smyth, 2004), with p-values adjusted by the Benjamini and Hochberg method (Hochberg and Benjamini, 1990). Fold-change analyses are from comparisons between mutant strains and isogenic controls.

For FlyGEM experiments, array data were normalized within slides by print-tip loess followed by quantile between-array normalization using the limma package (Smyth, 2004). Fold-change comparisons were performed at each time-point and for each control. Significance measures were calculated using linear modeling as with the Affymetrix arrays, and p-values extracted for mutant vs. control contrasts for each sex separately.

All expression data are available in GEO at these accession numbers: GSM239567, GSM239568, GSM239569, GSM239570, GSM239571, GSM239572, GSM239573, GSM239574, GSM239575, GSM239577, GSM239578, GSM238950, GSM238954, GSM238956, GSM238957, GSM238958, GSM238959, GSM238960, GSM238961, GSM238962, GSM238964, GSM238965, GSM238966, GSM238968, GSM238969, GSM238970, GSM238971, GSM238972, GSM238973.

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References: Agarwal, S.K., A. Lee Burns, K.E. Sukhodolets, P.A. Kennedy, V.H. Obungu, A.B. Hickman, M.E. Mullendore, I. Whitten, M.C. Skarulis, W.F. Simonds, C. Mateo, J.S. Crabtree, P.C. Scacheri, Y. Ji, E.A. Novotny, L. Garrett-Beal, J.M. Ward, S.K. Libutti, H. Richard Alexander, A. Cerrato, M.J. Parisi, A.S. Santa Anna, B Oliver, S.C. Chandrasekharappa, F.S. Collins, A.M. Spiegel, and S.J. Marx 2004, Ann. N. Y. Acad. Sci. 1014: 189-198; Brand, A.H., and N. Perrimon 1993, Development 118(2): 401-415; Busygina, V., M.C. Kottemann, K.L. Scott, S.E. Plon, and A.E. Bale 2006, Cancer Res. 66(17): 8397-8403; Busygina, V., K. Suphapeetiporn, L.R. Marek, R.S. Stowers, T. Xu, and A.E. Bale 2004, Hum. Mol. Genet. 13(20): 2399-2408; Cerrato, A., M. Parisi, S. Santa Anna, F. Missirlis, S. Guru, S. Agarwal, D. Sturgill, T. Talbot, A. Spiegel, F. Collins, S. Chandrasekharappa, S. Marx, and B. Oliver 2006, Dev. Biol. 298(1): 59-70; Chen, Y.X., J. Yan, K. Keeshan, A.T. Tubbs, H. Wang, A. Silva, E.J. Brown, J.L. Hess, W.S. Pear, and X. Hua 2006, Proc. Natl. Acad. Sci. U S A 103(4): 1018-1023; Edgar, R., M. Domrachev, and A.E. Lash 2002, Nucleic Acids Res. 30(1): 207-210; Gentleman, R.C., V.J. Carey, D.M. Bates, B. Bolstad, M. Dettling, S. Dudoit, B. Ellis, L. Gautier, Y. Ge, J. Gentry, K. Hornik, T. Hothorn, W. Huber, S. Iacus, R. Irizarry, F. Leisch, C. Li, M. Maechler, A.J. Rossini, G. Sawitzki, C. Smith, G. Smyth, L. Tierney, J.Y. Yang, and J. Zhang 2004, Genome Biol. 5(10): R80; Guru, S.C., N.B. Prasad, E.J. Shin, K. Hemavathy, J. Lu, Y.T. Ip, S.K. Agarwal, S.J. Marx, A.M. Spiegel, F.S. Collins, B. Oliver, and S.C. Chandrasekharappa 2001, Gene 263(1-2): 31-38; Harris, M.A., J. Clark, A. Ireland, J. Lomax, M. Ashburner, R. Foulger, K. Eilbeck, S. Lewis, B. Marshall, C. Mungall, J. Richter, G.M. Rubin, J.A. Blake, C. Bult, M. Dolan, H. Drabkin, J.T. Eppig, D.P. Hill, L. Ni, M. Ringwald, R. Balakrishnan, J.M. Cherry, K.R. Christie, M.C. Costanzo, S.S. Dwight, S. Engel, D.G. Fisk, J.E. Hirschman, E.L. Hong, R.S. Nash, A. Sethuraman, C.L. Theesfeld, D. Botstein, K. Dolinski, B. Feierbach, T. Berardini, S. Mundodi, S.Y. Rhee, R. Apweiler, D. Barrell, E. Camon, E. Dimmer, V. Lee, R. Chisholm, P. Gaudet, W. Kibbe, R. Kishore, E.M. Schwarz, P. Sternberg, M. Gwinn, L. Hannick, J.

Wortman, M. Berriman, V. Wood, N. de la Cruz, P. Tonellato, P. Jaiswal, T. Seigfried, and R. White 2004, Nucleic Acids Res. 32(Database issue): D258-261; Hochberg, Y., and Y. Benjamini 1990, Stat. Med. 9(7): 811-818; Hughes, C.M., O. Rozenblatt-Rosen, T.A. Milne, T.D. Copeland, S.S. Levine, J.C. Lee, D.N. Hayes, K.S. Shanmugam, A. Bhattacharjee, C.A. Biondi, G.F. Kay, N.K. Hayward, J.L. Hess, and M. Meyerson 2004, Mol. Cell 13(4): 587-597; Irizarry, R.A., B.M. Bolstad, F. Collin, L.M. Cope, B. Hobbs, and T.P. Speed 2003, Nucleic Acids Res. 31(4): e15; Johnston, R., B. Wang, R. Nuttall, M. Doctolero, P. Edwards, J. Lu, M. Vainer, H. Yue, X. Wang, J. Minor, C. Chan, A. Lash, T. Goralski, M. Parisi, B. Oliver, and S. Eastman 2004, Genome Biol. 5(3): R19; Papaconstantinou, M., Y. Wu, H.N. Pretorius, N. Singh, G. Gianfelice, R.M. Tanguay, A.R. Campos, and P.A. Bedard 2005, Mol. Cell Biol. 25(22): 9960-9972; Scacheri, P.C., S. Davis, D.T. Odom, G.E. Crawford, S. Perkins, M.J. Halawi, S.K. Agarwal, S.J. Marx, A.M. Spiegel, P.S. Meltzer, and F.S. Collins 2006, PLoS Genet. 2(4): e51; Schuettengruber, B., D. Chourrout, M. Vervoort, B. Leblanc, and G. Cavalli 2007, Cell 128(4): 735-745; Smyth, G.K., 2004, Stat. Appl. Genet. Mol. Biol. 3: Article3; Yokoyama, A., Z. Wang, J. Wysocka, M. Sanyal, D.J. Aufiero, I. Kitabayashi, W. Herr, and M.L. Cleary 2004, Mol. Cell Biol. 24(13): 5639-5649; Zhang, Y., D. Sturgill, M. Parisi, S. Kumar, and B. Oliver 2007, Nature 450(7167): 233-237.

## Quantitative courtship acts of 3LA inversion homo- and heterokaryotypes of *Drosophila ananassae.*

**Jayaramu, S.C., S.N. Hegde, and S.R. Ramesh.** Department of studies in Zoology, University of Mysore, Manasa Gangothri, Mysore -570006, Karnataka.

The relationship between inversions, morphological traits and fitness characters has been well documented in *Drosophila*. However, the relationship between inversion and behavioural traits has not been studied. Therefore, the present study has been carried out to find out the effect of inversions on courtship acts in *Drosophila ananassae*. Homozygous 3LA inversion stock of *D. ananassae* was established from the females collected at a semi-domestic locality of Mysore, India. This stock was maintained at 22ºC ±1ºC and relative humidity of 70% for ten generations. Before starting the experiments, the inversion heterokaryotypes were generated by crossing males with homokaryotic inversion with normal female or vice versa. When the progeny appeared, the virgin females and bachelor males were isolated, kept separately, aged for five days and used for observation of courtship behaviour. The courtship behaviour of males and females was observed by confining one male and a female of a given type in an Elens-Wattiaux mating chamber. A total of fifteen pairs of the following combinations were studied: a. both male and female heterokaryotypic; b, male homokaryotypic and female heterokaryotypic; c, both male and female homokaryotypic; and d, male heterokaryotypic and female homokaryotypic. The courtship elements were quantified following the procedure of Hegde and Krishna (1997). Following courtship elements such as tapping, scissoring, vibration, circling, licking, ignoring, extruding and decamping were analyzed. The data gathered were subjected to one way ANOVA.

In the present study, it is noticed that, except tapping and wing vibration, courtship acts such as scissoring, circling, licking, ignoring, extruding and decamping were lesser in crosses involving homokaryotypic male and female than in crosses involving heterokaryotypic male and females with 3LA inversion. This suggests that the inversion heterokaryotypes perform greater courtship acts than homokaryotypes. Studies have shown that flies which perform greater courtship acts during courtship

had greater mating success, inseminated more females, and showed greater adaptability than flies with lesser activity during courtship (Hegde and Krishna, 1997). This is in conformity with earlier work of Da Cunha (1955) and Dobzhansky and Wallace (1953). They also found that heterozygote individuals have better adaptability than the homozygotes.

It is also noticed that crosses involving homokaryotypic male and heterokaryotypic female and homokaryotypic female and heterokaryotypic male showed erratic changes in the courtship acts (Table 1). This is because the courtship acts of homo and heterokaryotypes are different.

Therefore, these studies suggest that the heterokaryotype of 3LA inversion has greater courtship activities than homokaryotype.

Table 1. Quantitative courtship acts of 3LA inversion homo and heterokaryotypes of *Drosophila ananassae* (Values are Mean **±** SE).

| Parameters | Both male and female heterokaryotypic | Male homokaryotypic and female heterokaryotypic | Both male and female homokaryotypic | Male heterokaryotypic and female homokaryotypic | F.value |
|---|---|---|---|---|---|
| Taping | 11.7333 ± 1.0931 | 13.0667 ± 1.0577 | 15.2667 ± 1.0711 | 11.2667 ± .9535 | 2.941* |
| Scissoring | 16.2667 ± 1.4124 | 10.6667 ± .8146 | 10.6000 ± .9248 | 15.5333 ± .9704 | 8.382 *** |
| Vibration | 9.6667 ± 1.0764 | 12.4667 ± 1.2979 | 12.6000 ± 1.0226 | 11.0000 ± 1.1297 | 1.481 |
| Circling | 5.8000 ± .5872 | 1.8667 ± .4563 | 2.2667 ± .3446 | 6.6000 ± .6817 | 20.512*** |
| Licking | 12.8000 ± 1.0565 | 3.9333 ± .5973 | 4.2000 ± .5790 | 9.8000 ± .9572 | 27.825*** |
| Ignoring | 6.8000 ± .7051 | 7.2000 ± .5952 | 1.7333 ± .3446 | 4.9333 ± 1.0395 | 12.122*** |
| Extruding | 2.1333 ± .3763 | 1.4000 ± .2895 | .7333 ± .2282 | 2.2000 ± .2960 | 5.239** |
| Decamping | 3.4000 ± .4557 | 3.4000 ± .4557 | 1.0667 ± .3305 | 2.0000 ± .2582 | 8.842*** |

*P<0.05; **P<0.02; ***P< 0.001.

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References: Da Cunha, A.B., 1955, Advances in Genetics 7: 93-138; Dobzhansky, Th.., and B. Wallace 1953, Proc. Natl. Acad. Sci. 39: 16-71; Hedge, S.N., and M.S. Krishna 1997, Anim. Behav. 54: 419-426.

**Erratum**

In J.A. Smith and E.C. Liebl (2005), Identification of the Molecular Lesions in Alleles of the *Drosophila* Abelson Tyrosine Kinase, Dros. Inf. Serv. 88: 20-22, we failed to acknowledge our sources of funding. This material was based upon work supported by the Anderson Summer Research Assistantship Fund of Denison University and the National Science Foundation under Grant No. 0344053.

## Nucleolar Organizer Regions (NORs) in *Drosophila immigrans* Sturtevant and *Drosophila repleta* Wollaston.

**Upadhyay, Kamla, and B.K. Singh.** Cytogenetics Laboratory, Department of Zoology, Kumaon University, Nainital-263002, India, e-mail: kamla_zoo@yahoo.com

In *Drosophila*, as also in various tissues of many eukaryote systems, the nucleolus manifests as a round or oval structure organized by a specific region of the genome commonly known as the nucleolar organizer. In many systems, as in *Drosophila* larval salivary glands, the nucleolus is a very prominent body often attached to the chromocentric region through a bundle of thread-like structures. The thread-like connections seem to penetrate into the nucleolar mass and from different patterns of distribution in the nucleolar matrix.

In Figure 1, the Nucleolar Organizer Region (NOR) in *Drosophila immigrans* Sturtevant is present in X and microchromosomes.

In Figure 2, the Nuclear Organizer Regions (NOR) in *Drosophila repleta* Wollaston as evident from the photograph is present in both X and microchromosomes.

Figure 1. Location of Nucleolar Organizer Region (NOR) in X and 4th chromosomes.

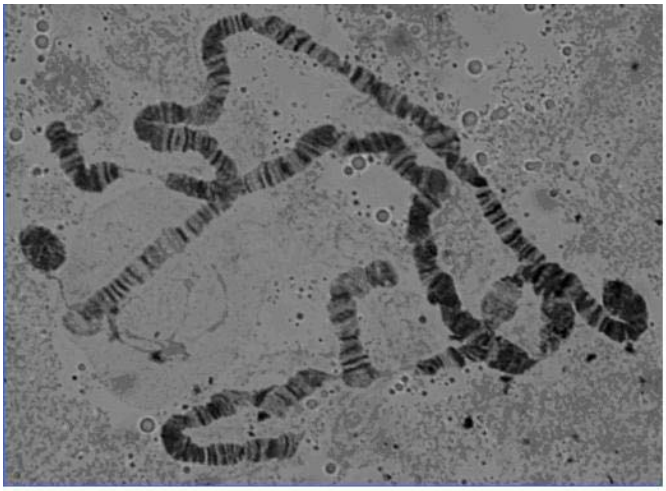


Figure 2. Localization of Nucleolar Organizer Region (NOR) in *Drosophila repleta*.

## Morphology variation between *D. mettleri* collected from different host species.

**Castrezana, Sergio.** Drosophila Tucson Stock Center, University of Arizona, Tucson, AZ 85721, USA; E-mail: castreza@email.arizona.edu.

*Drosophila mettleri* Heed, 1977 is a soil-breeding cactophilic species associated with the large columnar cacti species in the Sonoran Desert, cardon *Pachycereus pringlei*, and saguaro *Carnegiea*

*gigantea*. Nevertheless, *D. mettleri* is considered a generalist, as it can also be collected from other columnar cacti in the Sonoran Desert such as senita *Lophocereus schottii*, echo *P. pecten,* and organ pipe *Stenecereus thurberi.* On the other hand, two *D. mettleri* populations in California switch hosts from columnar cacti. *Drosophila mettleri* uses *Ferrocactus* spp. in the Mojave Desert and *Opuntia* spp. on Catalina Island (Heed, 1978; Heed and Mangan, 1982).

Although host shifts in *Drosophila mettleri* imply behavioral and physiological adaptations, so far no morphological differences between *D. mettleri* populations using different types of cacti as breeding resources have been observed (Castrezana and Markow, *in review*; Castrezana, *personal observation*).

In the present note, I give details of an unexpected observation of morphological differences between *D. mettleri* collected from different host species within a single location. On October 2000, University of Arizona researchers collected *Drosophila* flies at Organ Pipe National Monument (OPNM), in the area known as senita basin (31.93°N, 112.86°W). This area has abundant organ pipe and saguaro cacti but less than one-hundred senita plants. We collected *D. nigrospiracula*, *D. mojavensis, D. pachea,* and *D. mettleri* from both banana baits and natural rots of the mentioned cacti (Table 1).

Table 1. *Drosophila* collections in Organ Pipe National Monument, Arizona (10/2000): Natural rots and banana baits.

| Species | Senita *Lophocereus schotti* | Saguaro *Carnegiae gigantea* | Banana baits | Total |
|---|---|---|---|---|
| *Drosophila nigrospiracula* | - | 229♀ 273♂ | 91♀ 61♂ | 654 |
| *Drosophila pachea* | 31♀ 29♂ | 7♂ | 1♀ 11♂ | 79 |
| *Drosophila mettleri (A)* | 34♀ 89♂ | - | 6♀ 7♂ | 136 |
| *Drosophila mettleri (B)* | - | 3♀ 5♂ | 5♀ 2♂ | 15 |
| *Drosophila mojavensis* | - | - | +80 | N/A |

Table 2. Time to copulation and percent of males unable to detach after copulation in three strains of *Drosophila mettleri.*

| Strain | N | Time to copulation | % pairs stuck after mating |
|---|---|---|---|
| OPNM (A) | 28 | 380.6 ± 5.6 | 21.4% |
| OPNM (B) | 21 | 374.9 ± 8.8 | 0.0% |
| Superstition | 71 | 374.4 ± 6.6 | 0.0% |
| Sup♀ x OPNM(A)♂ | 10 | 407.6 ± 14.0 | 80.0% |
| Sup♀ x OPNM(B)♂ | 8 | 357.8 ± 14.2 | 0.0% |

An anatomical review showed that *D. mettleri* individuals from OPNM could be distinguished in two different morphological types. The *D. mettleri "*type B" was collected from both banana baits and saguaro rots. Individuals of this type exhibited no morphological differences from other *D. mettleri* populations in the species distribution. On the other hand, *D. mettleri "*type A" individuals, which were collected directly from senita rots, were morphologically different from typical *D. mettleri* "B". Compared to "type B", *D. mettleri* "type A" has the following unusual characters: a)ground thorax coloration is reddish; b)Thorax dot-pattern is smaller and perhaps more fused; c) testes are slightly more reddish; d) the ventral margins of the epandrium are more straight and reddish; e) a reduction of 1-2 presisetae teeth (n = 5 males)

Both OPNM *D. mettleri* strains were maintained in potato-saguaro food with pieces of senita cacti for strain "type A" and pieces of saguaro for "type B" to stimulate female oviposition (Castrezana, 1997). Later, in March 2001, *D. mettleri* males from both OPNM strains were used in a no-choice test with *D. mettleri* females from Superstition Mountains, Arizona (33.38°N, 111.37°W), a stock established in March 1997. Data on the time to copulation are presented in Table 2.

The analysis of variance for the time until copulation did not differ between couples ($F_{4,133}$ = 1.5798, P = 0.18). However, a problem following copulation was detected in the OPNM (A) strain. 21.4% of *D. mettleri* OPNM (A) males got stuck after mating with their own females. Nonetheless, in less than two minutes, all pairs were separated. On the other hand, eight of ten pairs (80%) had this problem when the female was from Superstition and the male was from the OPNM (A) strain. Moreover, in six cases the males were unable to break away from the female and both individuals died after three hours. The remaining two pairs produced fertile offspring.

Unfortunately, because of difficulties associated with maintaining *D. mettleri* in the laboratory, the strains used in the present note were lost and access to senita basin area in Organ Pipe National Monument is currently prohibited. Nevertheless, the failure to detach following copulation was also observed in another *D. mettleri* population collected from senita in San Ignacio, Baja (28.03°N, 113.40°W). So, it is possible that a number of important changes occurred in *D. mettleri* flies while using the highly toxic senita cactus. In fact, recently, important molecular differences between OPNM (A) and OPNM (B) were discovered from studies of DNA vouchers. Perhaps in the future, detailed ecological, behavioral, and molecular studies will reveal the extent of differentiation between *D. mettleri* that breed in different host species.

References: Castrezana, S., 1997, Dros. Inf. Serv. 80: 92-93; Heed, W.B., 1977, Proc. Entomol. Soc. Wash. 79: 649-654; Heed, W.B., 1978, In: *Proceedings in the Life Science. Ecological Genetics: The Interface.* (Brussard, ed.), pp.109-126, Springer-Verlag, Berlin; Heed, W.B., and R.L. Mangan 1986, In: *Genetics and Biology of* Drosophila, vol. 3e (Ashburner, M., H.L. Carson, and J.N. Thompson, jr., eds.), pp. 312-345, Academic Press, London.

## Studies on the Nucleolar Chromatin Threads (NCT) of *Drosophila immigrans* Sturtevant and *Drosophila repleta* Wollaston collected from Kumaon region, India.

**Upadhyay, Kamla[1], and B.K. Singh.** Cytogenetics Laboratory, Department of Zoology, Kumaon University, Nainital-263002, India.
e-mail: kamla_zoo@yahoo.com

### Introduction

The nucleolus in giant cells of larval salivary glands of *Drosophila* reveals typical thread like structures which have been shown to be DNA in nature (Barr and Plaut, 1966). Very likely these DNA threads are looped out from the main chromatin and contains the r-DNA sequences in the manner the DNA loops out in the puffs (Chowdhry and Godward, 1979). However, an interesting feature observed about these threads in *Drosophila* is that the pattern of the thread like connection in the matrix of the nucleolus is not constant. The pattern varies not only within the species (Barr and Plaut, 1966), but within the same species there is a considerable degree of variation in the morphological configuration of the threads.

## Materials and Methods

The samples of different strains of *Drosophila immigrans* Sturtevant for the study of chromosomal polymorphism were collected from different parts of Kumaon region *viz.,* Kausani (Bageshwar district), Dunagiri (Almora district) and Nainital. Meanwhile, *D. repleta* Wollaston was also collected from Dwarahat for the study of Nucleolar Chromatin Threads (NCT) in polytene chromosome from salivary gland chromosomes. The flies of this species were collected by exposing fermenting fruits as baits. The flies thus collected were sorted out and single females were transferred in glass vials containing usual laboratory food medium, assuming that it is already inseminated in the nature. The stock culture from single female for each species was thus established in the laboratory.

The slides of salivary gland chromosomes were prepared as suggested by Ashburner (1967). The larvae were grown on the laboratory food medium. The slides were examined with a Wild-Leitz research microscope.

## Types of Nucleolar Chromatin Threads (NCT) in *Drosophila immigrans* Sturtevant

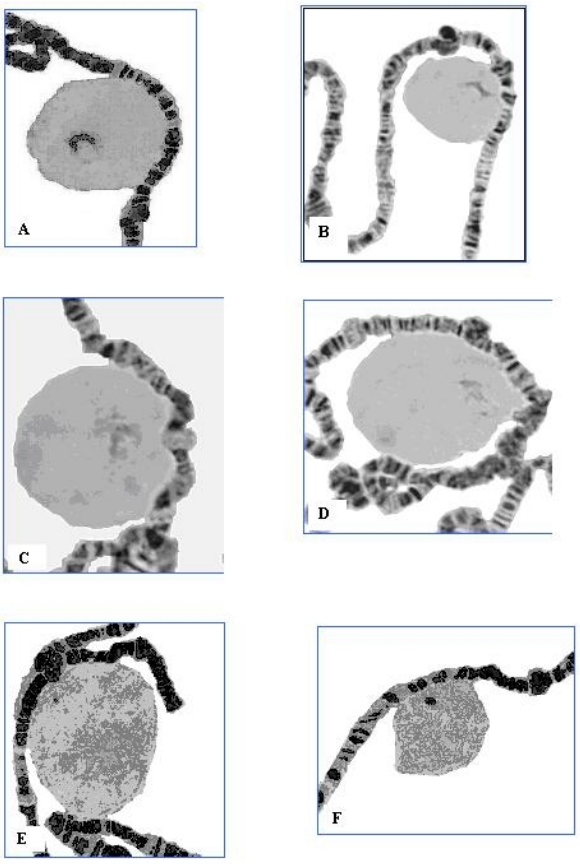



Figure 1. A-F, showing different types of Nucleolar Chromatin Threads.

Six types of Nucleolar Chromatin Threads were observed in *Drosophila immigrans* collected from different geographical localities in Kumaon region are as follows:

**Type I (Figure 1A)**

Thread not visible, branched with light granules and forming a dark semilunar structure.

**Type II (Figure 1B)**

Thread not visible, highly branched and scattered throughout the Nucleolar mass with a large darkly stained body.

**Type III (Figure 1C)**

The thread is condensed and positively stained with a number of darkly stained granules concentrated towards the periphery of the nucleolus.

**Type IV (Figure 1D)**

More than one thread, ramified and with less condensed granules concentrated at the point of origin.

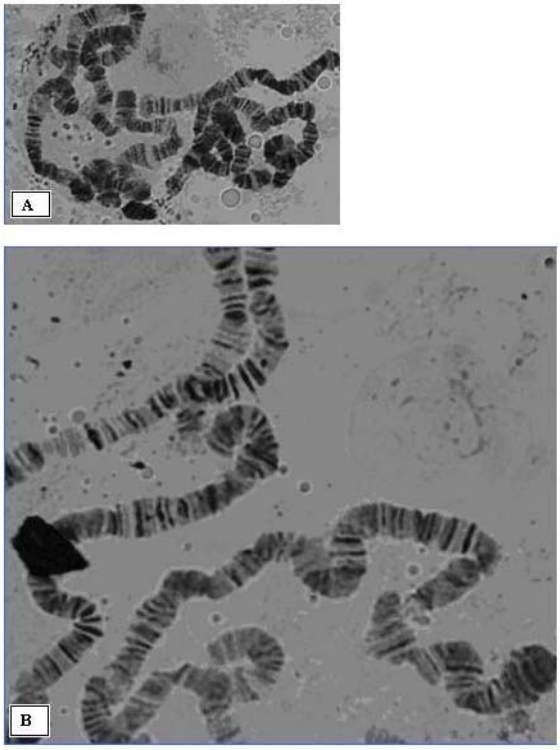
**Type V (Figure 1E)**

Thread not visible, ramified and scattered throughout the nuclear mass but more condensed and prominent in the center.

**Type VI (Figure 1F)**

Thread small, ramified, darkly stained with a round chromatin mass at the point of origin.

## Types of Nucleolar Chromatin Threads (NCT) in *Drosophila repleta* Wollaston




Two types of Nucleolar Chromatin Threads (NCT) were observed in *D. repleta* collected from Dwarahat in Kumaon region which are as follows:

**Type I (Figure 2A)**

Thread not visible, ramified and scattered throughout the nucleolus with granules concentrated towards the periphery of the nucleolus.

**Type II (Figure 2B)**

Thread very thin, ramified and scattered throughout the nucleolus with small light granules.

References: Ashburner, M., 1967, Chromosoma 21**:** 389-428; Barr, H.J., and W. Plaut 1966, J. Cell Biology 31: C17-C22; Chowdhry, A., and M.B.E. Godward 1979. *The Nucleus* 21(3).

Figure 2. A-B, Types of Nucleolar Chromatin Thread (NCT) present in *Drosophila repleta*.

## Genetic ablation of antennae or basiconic sensillia reduces *Drosophila melanogaster's* ability to perceive odors from the fruit of *Morinda citrifolia.*

**Jones, Corbin D.**[1] University of North Carolina, Department of Biology, CB #3280, Chapel Hill, NC 27599, USA; TEL: 919-843-5162; FAX 919-962-1625; Corresponding author email: cdjones@email.unc.edu.

### Abstract

*Drosophila* use a variety of sensory organs to detect odorants in their environment. While recent work in *Drosophila melanogaster* has increased our knowledge of how these organs detect odorants, our understanding of how these organs function in an ecological context is limited. Here, I use several developmental mutations that ablate sensory organs in flies to see which are critical to *D. melanogaster's* ability to perceive odorants from the fruit of *Morinda citrifolia*, which is the primary host of *D. sechellia*. I show that basiconic sensillia on antennae are particularly important to perceiving this fruit, although tarsal receptors may also play a role.

### Introduction

Today we know that odor and taste perception in flies occurs via antennae, maxillary palps, proboscis, and tarsi (reviewed in Hallem, *et al*., 2006). In the antennae, odorants pass through

cuticular pores in sensilla. These odorants are then bound by odorant binding proteins (*Obps*) and delivered to odorant receptors (*Ors*) on the surface of the insect odorant receptor neurons. Taste is perceived in an analogous manner, except that odorants are trafficked to gustatory receptors (*Grs;* Ebbs and Amrein, 2007). Odorant receptor neurons converge to spatially invariant antennal lobe glomeruli. From these glomeruli, neurons project into the mushroom body where higher order processing is believed to occur.

How sensing these odor cues results in ecologically important behaviors, however, is much less well understood. To better understand this problem, considerable work has focused on the host specialist, *Drosophila sechellia* and its generalist sister species, *D. melanogaster*. On its native islands, the Seychelles, *D. sechellia* almost exclusively uses the fruit of *Morinda citrifolia* (Morinda), a plant common around the Indian Ocean and Polynesia (Louis and David, 1986; Jones, 2005). *D. sechellia* has evolved strong preference for, and resistance to, the toxins in Morinda (Louis and David, 1986; R'Kha, *et al.,* 1991; Jones, 1998, 2004, 2005). *D. melanogaster*, on the other hand, is a human commensal that originally arose in Africa (Lachaise and Silvain, 2004). Several compounds found in the Morinda fruit are toxic and noxious to *D. melanogaster* and other *Drosophila*. These include octanoic and hexanoic acid (Jones, 2005). *D. melanogaster,* as a result, typically avoids this plant. In contrast, *D. sechellia* responds positively to Morinda's olfactory cues. When female *D. sechellia* detect Morinda, for instance, they increase egg production and ovipositioning (R'Kha, *et al*., 1991). Field experiments suggest that *D. sechellia* can detect Morinda at distances up to 50m (R'Kha, *et al*., 1991).

Several recent studies have investigated how *D. sechellia* perceives Morinda's odor differently from *D. melanogaster* (Dekker, *et al*., 2006; Matsuo, *et al*., 2007; McBride, 2007). Dekker *et al*. (2006) recently suggested that *D. sechellia* differs from *D. melanogaster* in the numbers and types of sensilla and that this difference in the ability to perceive odors from Morinda leads to the behavioral differences between *D. sechellia* and *D. melanogaster*. Congruent with this observation, McBride (2007) has shown that several *Ors* and *Grs* appear to have become non-functional in *D. sechellia*. In contrast to the Dekker *et al.* and McBride result, Matsuo *et al.* (2007) have recently suggested that a change in *Obp* expression in the tarsi of *D. sechellia* is key to the behavioral difference between *D. sechellia* and other *Drosophila*.

Here I use a suite of mutations that affect sensory organs to determine which are critical for perceiving odors from the fruit of Morinda.

## Material and Methods

*Stocks:* The following *D. melanogaster* stocks were obtained from the *Drosophila* Stock Center in Bloomington, IN: OR-R, *ant, ro, Dfd[3] red e/TM3, Sb[1], kni Dfd[9] e/TM3, Sb[1], th st Ki pb[4] pp/TM3, Sb[1], pb[27]/TM3,] Sb[1], lz[3]* and *lz[77a7]*. *D. sechellia* Line 1 ("Robertson" collected from Seychelles in 1981 by Tsacas and Bächli (1981)), *D. sechellia* Syn A (a wild-type non-isofemale line; courtesy of J. Coyne), and *D. simulans* sim6 (an isofemale line from Winters CA, courtesy D. Begun) were used for most comparisons. Except where noted, all stocks were reared on agar-yeast-cornmeal medium at room temperature.

*Preference assay—oviposition:* Following Jones (2004), oviposition-site preference was scored by presenting inseminated, ovipositing females with a choice of oviposition substrates, one tainted with octanoic acid and one untainted. Media was prepared using *Drosophila* Instant Medium (Carolina Biological Supply Co.). The toxic media was 0.07% octanoic acid by weight (Sigma Chemical Co.). This dose does not kill susceptible flies (Jones, 2001).

Each female was placed in a chamber with the two types of media. She was allowed to oviposit for two days, after which the number of eggs laid on each type of media was counted. The

female was then shifted to a fresh pair of tainted and untainted media. After 2 more days, her preference was scored again. All assays were conducted in a constant temperature room at 20°C with relative humidity between 50-70%.

Egg counts were converted to a preference index by the following formula:

$$\text{Preference Index} = \frac{\left(N_{Eggs\ on\ toxic} - N_{Eggs\ on\ control}\right)}{N_{Both}}$$

Positive values indicate preference for tainted media, whereas negative values indicate avoidance of tainted media. Unless otherwise noted, data were pooled across both days.

High throughput assays of preference were performed in test chambers (2L) containing two standard fly bottles (Genesee Scientific, San Diego CA, USA); either one bottle of control media and one bottle of Morinda toxin media or two bottles of control media. Control media was 44 ml of water with 8.5 of Carolina 4-24 instant media (Carolina Biological Supply, NC, USA). Morinda toxin media was made by combining 44 ml of water with 8.5g of Carolina 4-24 instant media with 90 µl octanoic acid and 30 µl of hexanoic acid (Arcos Organics, NJ, USA). The combination was gently agitated to ensure even distribution of the hydrophobic octanoic and hexanoic acids. Morinda fruit has a 3:1 ratio of octanoic to hexanoic acid (Legal, *et al.*, 1994). The concentration of Morinda toxins in the media was low relative to what is typically observed in nature, but not outside the normal range. This concentration was necessary to minimize mortality in *D. simulans*.

Roughly 90 one-day-old females were collected and allowed to mate *ad libitum* with males of their own species for three days. Females were then separated and allowed to recover for one day. They were then lightly sedated and placed in test chambers, which were then placed in an environmental chamber with constant humidity and temperature (65%; 25°C). They were allowed to roam the test chamber freely and to choose media to oviposit on.

*Genetic ablation of sense organs:* We used developmental mutants to ablate sense organs in order to determine which organs are important for oviposition-site preference. To establish the role of the proboscis, we used the *proboscipedia (pb* ) mutation which transforms the proboscis into a (non-functional) leg. To produce these flies, we crossed *th st Ki pb*[4] *pp*/TM3, *Sb* to *pb*[27]/TM3, *Sb*. we collected and tested the resulting $F_1$ *pb*[4]/*pb*[27] females. To test the effect of maxillary palp ablation, we crossed *Dfd*[3] *red e*/TM3, *Sb* to *kni Dfd*[9] *e*/TM3, *Sb*. A fraction of the $F_1$ *Dfd*[3/ *Dfd*[9] females have greatly reduced maxillary palps. Finally, we tested the preference of *D. melanogaster* flies homozygous for a mutation that renders them antennaless (*ant*). This mutation normally causes the loss of one or both antennae; we used only those flies missing both antennae. *ant* has no known effect on tarsi.

As *ant* had a large effect in the oviposition assay, we wanted to see if ablating the antennae had the same effect in our choice-no choice assay. Unfortunately, the *ant* stock was lost and is no longer available from either the Bloomington or Kyoto Stock Centers. Instead, we used alleles of *lozenge* (*lz[3]* and *lz[77a7]*) that remove the antenneal sensillia thought to be important to detecting fatty acids in Morinda.

## Results

*Ablation of antenna removes preference:* The electrophysiological data of Dekker *et al.* (2006) suggests that olfactory receptors on the antennae are important to a fly's perception of Morinda fruit. In contrast, the genetic data of Matsuo *et al*. (2007) suggest that tarsal taste receptors are playing an important role in oviposition-site preference. To clarify this issue, we used *D. melanogaster* mutants lacking proboscis, maxillary palps or antennae to see if sense organs on the

head are important for preference behavior. If any of these organs are important, flies lacking these structures should be indifferent to the presence *versus* absence of the toxin (Preference Index ≈ 0). Only *antennaless* (*ant/ant*) flies are indifferent (Figure 1; one sample sign tests: *ant versus* no preference: $P = 0.4049$; *Dfd versus* no preference: $P < 0.0001$; *pb versus* no preference: $P = 0.0005$). This result suggests that the antennae play a role in the detection of Morinda.

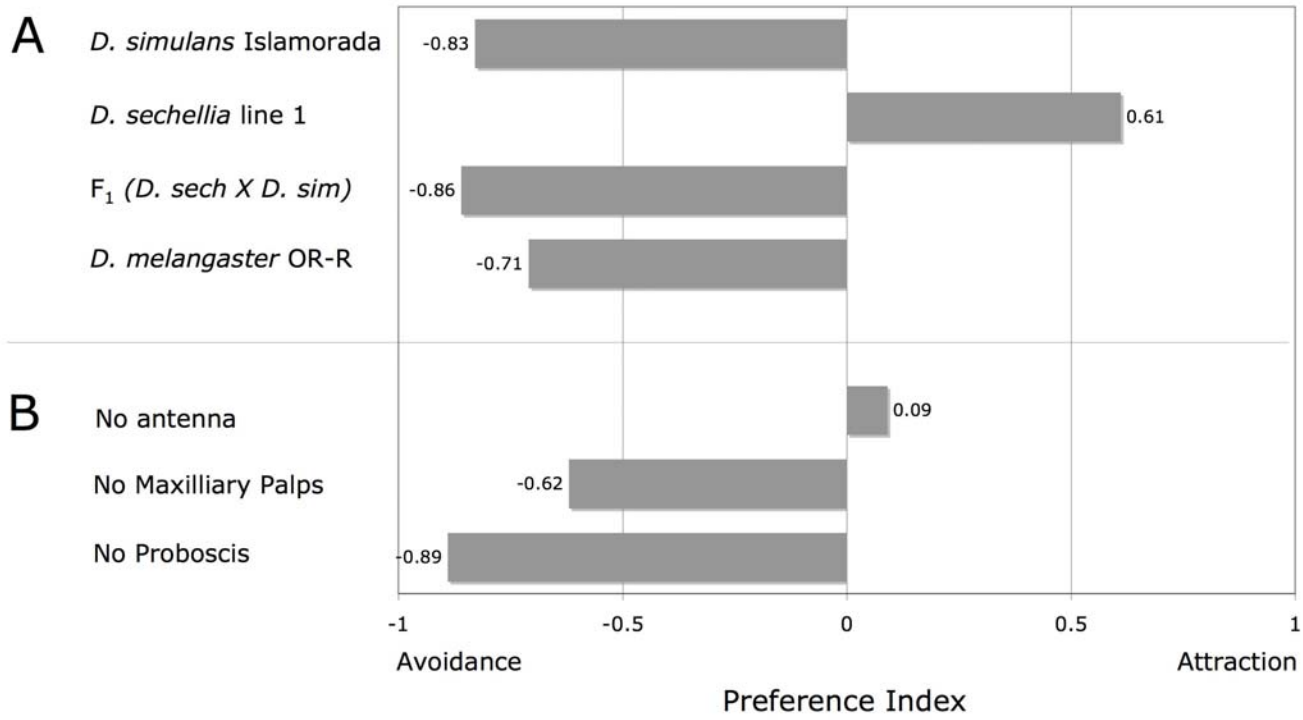



Figure 1. Comparison of oviposition-site preference across species and among sense-organ ablation lines. Oviposition-site preference was scored by presenting inseminated, ovipositing females with a choice of oviposition substrates, one tainted with octanoic acid and one untainted (see Methods). *D. sechellia* shows strong preference for tainted media (A). This behavior is recessive to the avoidance of *D. simulans* and *D. melanogaster*. Only ablation of the antennae causes a significant shift in fly behavior (B).

We confirmed the effect of antenna ablation using our choice-no choice assay. Unfortunately, the *ant* stock was lost and is no longer available. Instead, we used alleles of *lozenge* (*lz[3]* and *lz[77a7]*) to remove some or all of the basiconic sensillia—which are thought to be important to detecting the aromatics in Morinda (Dekker *et al*., 2006)—from the antennae. *lz[3]* antennae lack all basiconic sensillia and show a slight preference for Morinda media (61% of flies on Morinda media; N = 67). *lz[3]* also have tarsal claw defects so it is difficult to wholly exclude an effect of taste receptors on the tarsi. *lz[77a7]* has severely reduced basiconic sensillia and no tarsal defect. These mutants have a tendency to avoid Morinda media more than the *lz[3]* flies ($\chi 2 = 38.0$; $P < 0.0001$), but much less than wild-type (38% of *lz[77a7]* flies on Morinda media; N = 169; $\chi 2 = 384.4$; $P < 0.0001$). At this point, it is unclear whether the difference between the two *lz* alleles results from the presence/absense of the tarsal claw defect, the residual basiconic sensillia in *lz[77a7]* flies, or genetic background effects. It is clear, however, that antennae are important to a fly's ability to detect and respond to odorant from Morinda.

**Discussion**

My data show that antennae are important to *D. melanogaster's* ability to detect and respond to odorants from Morinda fruit. In particular, the removal of the basiconic sensillia clearly reduces avoidance of this fruit, genetically confirming the electrophysiological data of Dekker *et al.* (2006). My data also suggest that tarsal taste receptors (*sensu* Matsuo *et al.,* 2007) may influence behavior, although not as much as antennae. Interestingly, ablation of sensory organs in *D. melanogaster* is not sufficient to engender *D. sechellia*-like behavior (Figure 1). This result suggests that the host specific behavior is not simply a loss of the ability of *D. sechellia* to perceive its host.

Acknowledgments: I thank Ian Dworkin for comments on this note and William Jeck for technical assistance. This work was supported by funds from the National Science Foundation.

References: Dekker, T., I. Ibba, et al., 2006, Curr. Biol. 16(1): 101-9; Ebbs, M.L., and H. Amrein 2007, Pflugers Arch 454: 735-47; Hallem, E.A., A. Dahanukar, et al., 2006, Ann. Rev. Entomol. 51: 113-35; Jones, C.D., 1998, Genetics 149: 1899-908; Jones, C.D., 2001, Genet. Res. 78: 225-33; Jones, C.D., 2004, Heredity 92: 235-41; Jones, C.D., 2005, Genetica 123: 137-45; Lachaise, D., and J.F. Silvain 2004, Genetica 120(1-3): 17-39; Legal, L., B. Chappe, et al., 1994, Journal of Chemical Ecology 20: 1931-1943; Louis, J., and J.R. David 1986, Acta Oecologica 7: 215-229; Matsuo, T., S. Sugaya, et al., 2007. PLoS Biol 5: e118; McBride, C.S., 2007, Proc. Natl. Acad. Sci. USA 104: 4996-5001; R'Kha, S., P. Capy, et al., 1991, Proc. Natl. Acad. Sci. USA 88: 1835-9; Tsacas, L., and G. Bächli 1981, Revue fr. Ent. NS 3: 146-150.

**Chromosomal aberrations in *Drosophila ananassae*.**

**Singh, P., and B.N. Singh***. Genetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi-221005, India. * E-mail: bnsingh@bhu.ac.in.

*Drosophila ananassae* exhibits a high degree of chromosomal polymorphism. It harbors a large number of inversions in its natural populations (Singh, 1998). Out of these reported from various parts of the world, most have restricted distribution while the three cosmopolitan inversions namely, Alpha (AL) in 2L, Delta (DE) in 3L, and Eta (ET) in 3R show worldwide distribution (Singh, 1998). Population genetics of chromosomal polymorphism in Indian natural populations of *D. ananassae* has been extensively studied (for references see the review by Singh, 1998). The results have clearly shown that there is geographic differentiation of inversion polymorphism. Present communication gives the details of chromosomal aberrations detected from natural populations and laboratory stocks of *D. ananassae.* We have tried to include all detected chromosomal aberrations so far in *D. ananassae* in natural populations and laboratory populations to give the holistic picture of chromosomal variability as well as unusual mutational property.

The details of pericentric inversions and translocations detected in *D. ananassae* are given in Tables 1 and 2, respectively. The numbers of pericentric inversions and translocations are twenty one and forty eight, respectively. The occurrence of pericentric inversions (heterozygotes for pericentric inversions produce unbalanced gametes, their appearance, therefore, is opposed by natural selection) and translocations, which are rare in other species of *Drosophila*, reflect unusual mutational properties of *D. ananassae*.

The paracentric inversions are depicted in Figures 1-6 via line diagram. We have followed the improved edition of polytene chromosome reference photomap of *D. ananassae* (Moriwaki and

Table 1. Pericentric inversions in *D. ananassae.*

| S. No. | Pericentric inversion | Locality | Reference |
|---|---|---|---|
| 1. | In (2LR)a | Brazil | Freire-Maia,1955,1961 |
| 2 | In (2LR)A | Niue | Futch,1966; Seecof, 1957; Hinton and Downs,1975 |
| 3. | In (2LR)Lo | # | * |
| 4. | In (2LR) | India | Singh et al., 1971 |
| 5. | In (2LR)9 | India | Reddy and Krishnamurthy,1972b |
| 6. | In (2LR)B | Guam | * |
| 7. | In (2LR)B,Ubx | # | ¶ |
| 8. | In (2LR)C | Noumea | * |
| 9. | In (3LR)a | Brazil | Freire-Maia,1955,1961 |
| 10. | In (3LR)b | Brazil | Freire-Maia,1955,1961 |
| 11. | In (3LR)c | Brazil | Freire-Maia, 1955,1961 |
| 12. | In (3LR)D,Bl$^2$ | # | ¶ |
| 13. | In (3LR)d | Brazil | Freire-Maia,1955,1961 |
| 14. | In (3LR)E,stw | # | ¶ |
| 15. | In (3LR)A | Niue | Seecof,1957;Futch, 1966 |
| 16. | In (3LR)B | Niue | Seecof,1957;Futch, 1966 |
| 17. | In (3LR)C | Chiang Mai | * |
| 18. | In (3LR)F | Wau | * |
| 19. | In (3LR)G | Hyderabad | * |
| 20. | In (3LR)H | Coimbatore | * |
| 21. | In (3LR)I | Coimbatore | * |

Note: * for references see, Tobari, 1993; # detected from various laboratory stocks; ¶ Hinton (unpublished)

Ito, 1969) by Tomimura and Tobari, Tobari (1993). As for naming different inversions, to make it uniform, we have numbered them in chronological order as given in Tobari (1993). It is difficult to know the exact number of paracentric inversions, since different investigators have named inversions independently. Also all the new inversions have not been reported or documented in relevant journals. In the present communication despite these limitations we have tried to include all possible inversions, thus, taking the total tally to seventy eight. The second and third chromosome carried the maximum number of inversions. Among these, 2L carried the maximum of twenty one inversions, followed by 2R (20), 3R (17), 3L (14), XL and XR, three each. With the exception of few cases, there has been no clustering of break points in any particular region in all the arms. So, it could be said that naturally occurring inversions in *D. ananassae* are randomly distributed in X, second, and third chromosomes.

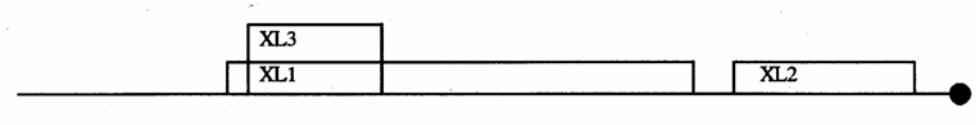



Figure 1. Location of different inversions in XL of *D. ananassae.*

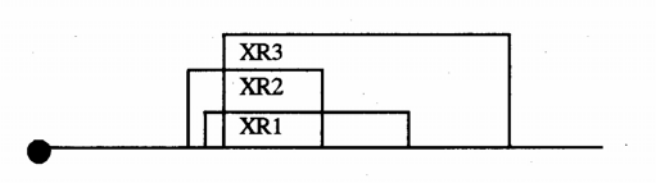



Figure 2. Location of different paracentric inversions in XR of *D. ananassae*.

Although a large number of paracentric inversions are known to occur in *D. ananassae*, only three have become coextensive with the species. Most of the inversions have localized distribution and have been detected from the few individuals. This is a feature of the pattern of the chromosomal polymorphism in *D. ananassae* (Carson, 1965; Singh, 1988). A given arrangement may occur in one region but may be absent in other. An explanation to account for such disjunct distribution of gene arrangements may be that all types of arrangements did not occur simultaneously in the past history of the species. The former types, being older, had greater opportunities for migration and thereby at present they have a distribution throughout the world (Kaufmann, 1936a,b; Kikkawa, 1938; Dobzhansky and Dreyfus, 1943). On the contrary, those rearrangements of relatively recent origin and due to obvious impediments in the

means of migration could not migrate from their respective native place to the localities where they have been found wanting (Ray-Chaudhuri and Jha, 1967).

Table 2. Translocations in *D. ananassae.*

| S.No. | Translocation | Locality | Reference |
|---|---|---|---|
| 1. | T (XL;2R) A | Niue | Seecof,1957;Futch,1966 |
| 2. | T (XR;2R) | India | Sajjan and Krishnmurthy,1970 |
| 3. | T (XR;2L) 8 | India | Reddy and Krishnamurthy,1972b |
| 4. | T (XL;2L) B | Nauru | * |
| 5. | T (XR;2R)A,M Ubx ca | # | Hinton,1979 |
| 6. | T(XL;XR)B,ca Th | # | Hinton,1981 |
| 7. | T (1;3)A, Mo | # | ¶ |
| 8. | T (Y;2L)A, ca | # | Hinton,1979 |
| 9. | T (Y;2L)B, ca | # | Hinton,1979; Hinton and Downs,1975 |
| 10. | T (Y;2L)C, ca | # | Hinton,1979,1980, Hinton and Downs,1975 |
| 11. | T (Y;2;3)A,M ca stw | # | Hinton,1980 |
| 12. | T (Y;3)A.stw | # | Hinton,1980 |
| 13. | T (Y;3R)B | # | Hinton and Downs,1975 |
| 14. | T (Y;3L)C,e se;ru | # | ¶ |
| 15. | T (Y;3L)$pe^{v}$ | # | * |
| 16. | T (2L;3L) | Brazil | Dobzhansky and Dreyfus,1943 |
| 17. | T (2L;3L)66 | Honolulu | * |
| 18. | T (2L;3L) 9 | India | Reddy and Krishnamurthy,1972a |
| 19. | T (2L;3L)10 | India | Sajjan and Krishnamurthy,1972 |
| 20. | T (2L;3L)8 | India | Reddy and Krishnamurthy,1974 |
| 21. | T (2R;3R) | Brazil, India | Freire-Maia,1961; Sajjan and Krishnamurthy,1970 |
| 22. | T (2R;3R)AA,Ly | # | ¶ |
| 23. | T (2R;3R)A,ca stw | # | Hinton,1979; Hinton and Downs,1975 |
| 24. | T (2R;3R)B,M ca stw | # | Hinton,1979; Hinton and Downs,1975 |
| 25. | T (2L;3R)C,M ca stw | # | Hinton,1979; Hinton and Downs,1975 |
| 26. | T (2R;3R)D,ca stw | # | Hinton,1979,Hinton and Downs,1975 |
| 27. | T (2L;3R)E,ca stw | # | Hinton,1979,1980,Hinton and Downs,1975 |
| 28. | T (2L;3L)F,ca stw | # | Hinton,1979 |
| 29. | T (2L;3L)G,ca stw | # | Hinton,1979, Hinton and Downs,1975 |
| 30. | T (2L;3R)H,ca stw | # | Hinton,1979,1980, Hinton and Downs,1975 |
| 31. | T (2R;3R)J, Xa ca stw | # | Hinton,1979,1980, Hinton and Downs,1975 |
| 32. | T (2L;3L)K,ca stw | # | Hinton,1979,1980, Hinton and Downs,1975 |
| 33. | T (2R;3L)L,ca stw | # | Hinton,1979,1980, Hinton and Downs,1975 |
| 34. | T (2L;3L)M,ca stw | # | Hinton,1979,1980, Hinton and Downs,1975 |
| 35. | T (2L;3R)N,ca stw | # | Hinton,1979,1980, Hinton and Downs,1975 |
| 36. | T (2R;3L)O,ca stw | # | Hinton,1979,1980, Hinton and Downs,1975 |
| 37. | T (2L;3R)P,ca stw | # | Hinton,1979,1980, Hinton and Downs, 1975 |
| 38. | T (2R;3R)Q,ca stw | # | Hinton,1979,1980, Hinton and Downs,1975 |
| 39. | T (2;3)R | # | Hinton,1981 |
| 40. | T (2R;3R)S | # | Hinton,1981, Hinton and Downs,1975 |
| 41. | T (2L;3R)T | # | Hinton,1981,Hinton and Downs,1975 |
| 42. | T (2;3)U,ca cy | # | Hinton,1981,Hinton and Downs,1975 |
| 43. | T (2L;3R)V | # | Hinton and Downs,1975 |
| 44. | T (2L;3L)W,$Cy^{EX}$ | # | Hinton and Downs,1975 |
| 45. | T (2;3)Z,Mot | # | ¶ |
| 46. | T (2L;3L)15 | India | Singh,1991 |
| 47. | T (3L;4)Pm | # | Kikkawa,1938 |
| 48. | T (3L;4) | India | Ray-Chaudhuri and Jha,1966 |

Note: #, Detected from various laboratory strains; *, For references see Tobari,1993; ¶, Hinton (unpublished).

Disappearance of new sequences suggests that population, in question, might have developed a kind of resistance to acquire new gene arrangements in its genetic structure, because they could not yield adaptive values or heterotic effects to their carriers. In other words, the resistance might be

against any further increase in the amount of load of chromosomal polymorphism in natural populations of *D. ananassae*. With the same token, the existing load of chromosome polymorphism due to three cosmopolitan inversions in natural populations of *D. ananassae* might be too high to acquire any new gene arrangement as ameans of adaptation of population to the extremes of the environmental conditions (White, 1958).

Freire-Maia's (1961) suggestion that that some special mechanism exists in *D. ananassae* to permit the retention of disadvantageous rearrangements in natural populations deserves exploration. Alternatively, the high incidence of such rearrangements may reflect high mutability in this species, a possibility proposed by Kikkawa (1938).

The geographical distribution of the three cosmopolitan inversions has been shown in Table 3. It is apparent from the table that the three cosmopolitan inversions are of frequent occurrence in natural populations and have become coextensive with the species.

Table 3. Geographical distribution of three cosmopolitan inversions in *D. ananassae*.

| Area | Subterminal (alpha) | Terminal (delta) | Basal (eta) | Source |
|---|---|---|---|---|
| Alabama | + | + | + | Kaufmann,1936 |
| Texas | + | - | + | Shirai and Moriwaki 1952 |
| Hawaii | + | + | + | Shirai and Moriwaki 1952 |
| Majuro | + | + | + | Seecof,1957 |
| Cuba | + | + | + | Futch,1966 |
| Mexico | + | + | + | Shirai and Moriwaki 1952; Futch,1966 |
| Brazil | + | + | + | Dobzhansky and Dreyfus,1943; Shirai and Moriwaki 1952; Freire- Maia,1955 |
| China | + | + | + | Kikkawa,1938 |
| Formosa (Taiwan) | + | + | + | Kaufmann,1936; Kikkawa,1938 |
| Japan | + | + | + | Kaufmann,1936; Kikkawa,1938 |
| India | + | + | + | Ray-Chaudhuri and Jha,1966; Sajjan and Krishnamurthy,1970; Reddy and Krishnamurthy,1972; Singh, B.N.,2001 |
| Africa | + | + | + | Shirai and Moriwaki 1952 |
| Micronesia (Caroline Island, Marshal Island, Mariana Island) | + | + | + | Seecof (Stone et al.1957);Futch,1966 |
| Melanesia (Papua New Guinea,Calodenia Island, Fiji) | + | + | + | Futch, 1966 |
| Polynesia (Samoa, Cook Island) | + | + | + | Futch, 1966 |
| Mauritius | + | + | + | * |
| Sri Lanka | + | + | + | * |
| Myanmer | + | + | + | * |
| Thiland | + | + | + | * |
| Malaysia | + | + | + | Singh,1983a,b; * |
| Borneo | + | + | + | Singh,1983b;* |
| Philippines | + | + | + | * |
| Singapore | + | + | + | * |

* for references see Tobari, 1993; + indicates presence of inversion; - indicates absence of inversion.

Dobzhansky and Dreyfus (1943) pointed out that *D. ananassae* probably originated in some area of eastern and southeastern Asia and has depended on man for its present widespread distribution. *D. ananassae* certainly appears to qualify as a polytypic species. Its widespread circum-tropical distribution, especially through the scattered island groups in the Pacific ocean, has

permitted recognizable genetic differences between parts of species population to become so well developed that geographic races can be distinguished.

Relatively low number of inversions were observed in dark form *ananassae*, thus we suggest that the dark form *ananassae* was distributed around Polynesian Islands before the cosmopolitan form had a chance to expand its territory throughout the entire tropical and subtropical world. Because no reproductive isolation had developed between the two forms, the cosmopolitan form with its cosmopolitan inversions introgressed into Polynesian populations. Then these cosmopolitan inversions were distributed in many places where the dark form *ananassae* had been the precedent inhabitants. These widespread cosmopolitan inversions would be maintained in natural populations by the strong superiority of the inversion heterozygotes (Tobari, 1993).

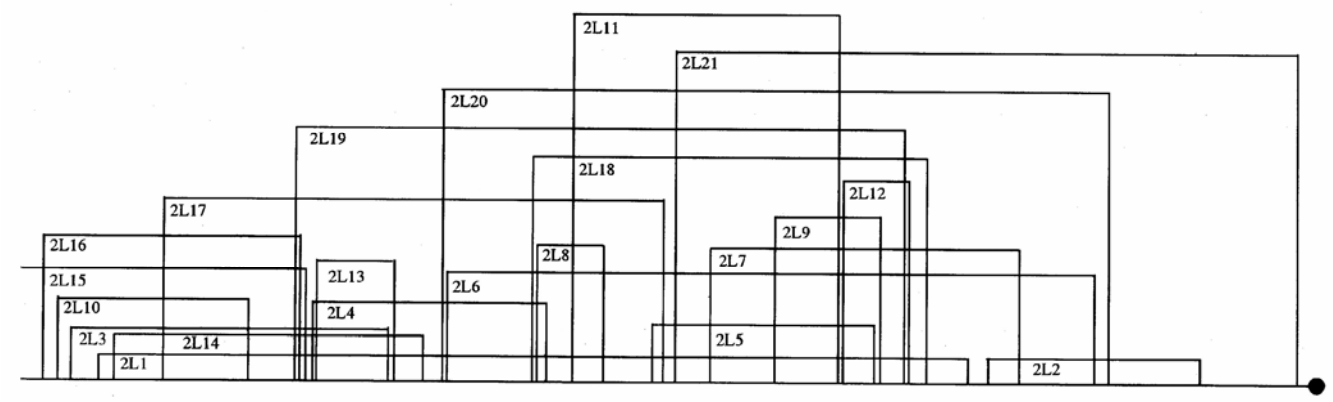



Figure 3. Location of different paracentric inversions in 2L of *D. ananassae*.

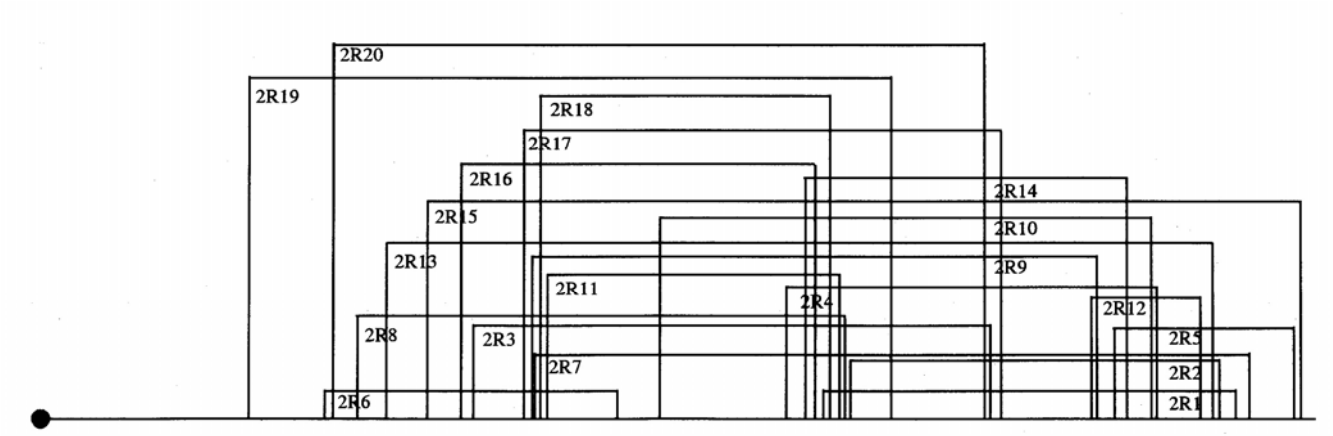



Figure 4. Location of different paracentric inversions in 2R of *D. ananassae*.

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References: Carson, H.L., 1965, *The Genetics of Colonizing Species,* Academic Press, London, New York: 503-531; Dobzhansky, Th., and A. Dreyfus 1943, Proc. Natl. Acad. Sci. USA 29: 301-305; Freire-Maia, N., 1955, Dros. Inf. Serv. 28: 118-119; Freire-Maia, N., 1961, Evolution 15: 486-495; Futch, D.G., 1966, Univ. Texas Publ. 6615: 79-120; Hegde, S.N., and M. Jayashankar 1992, Dros. Inf. Serv. 71: 160; Hinton, C.W., 1979, Genetics 92: 1153-1171; Hinton, C.W., 1980,

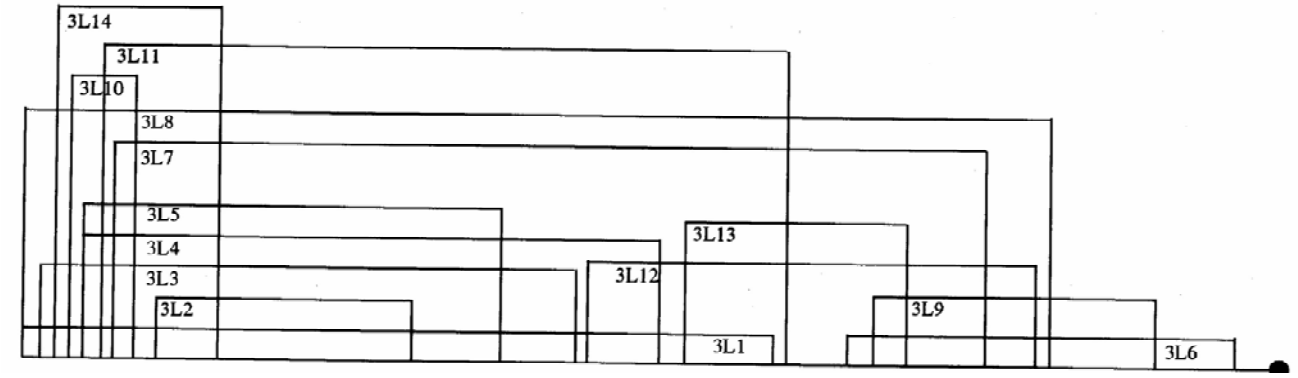


Figure 5. Location of different paracentric inversions in 3L of *D. ananassae*.

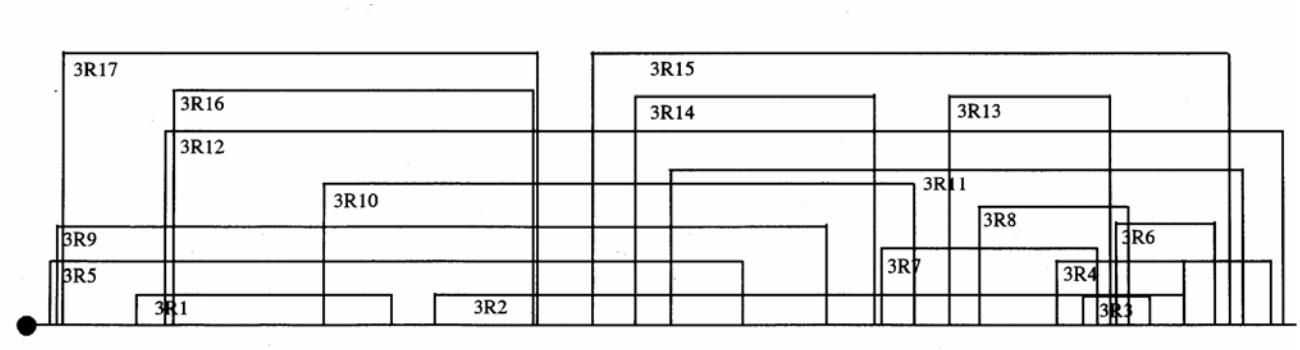


Figure 6. Location of different paracentric inversions in 3R of *D. ananassae*.

Dros. Inf. Serv. 55: 213-217; Hinton, C.W., 1981, Genetics 98: 77-90; Hinton, C.W., and J.E. Downs, 1975, J. Heredity 66: 353-361; Kaufmann, B.P., 1936a, Science 83: 39; Kaufmann, B.P., 1936b, Proc. Natl. Acad. Sci. USA 22: 591-594; Kikkawa, H., 1938, Genetica 20: 458-516; Moriwaki, D., and S. Ito 1969, Jpn. J. Genet. 44: 129-138; Ray-Chaudhuri, S.P., and A.P. Jha 1966, Proc. Int. Cell Biol. Meet. Bombay: 325-383; Ray-Chaudhuri, S.P., and A.P. Jha 1967, Nucleus 10: 81-89; Reddy, G.S., and N.B. Krishnamurthy 1972a, Dros. Inf. Serv. 48: 139-140; Reddy, G.S., and N.B. Krishnamurthy 1972b, Dros. Inf. Serv. 48: 140-142; Sajjan, S.N., and N.B. Krishnamurthy 1970, Dros. Inf. Serv. 45: 166; Sajjan, S.N., and N.B. Krishnamurthy 1972, Dros. Inf. Serv. 48: 103-104; Singh, B.N., 1983a, Experientia 39: 99-100; Singh, B.N., 1988, Ind. Rev. Life Sci. 8: 147-168; Singh, B.N., 1991a, Dros. Inf. Serv. 70: 203; Singh, B.N., 1998, Ind. J. Expt. Biol. 36: 739-748; Singh, B.N., 2001, Ind. J. Expt. Biol. 39: 611-622; Singh, P., and B.N. Singh 2005a, Dros. Inf. Serv. 88: 10-11; Singh, P., and B.N. Singh 2005b, Dros. Inf. Serv. 88: 15-16; Singh, V.K., M. Mishra, and A.P. Jha 1971, Dros. Inf. Serv. 47: 97; Tobari, Y.N. (ed), 1993, *Drosophila ananassae: Genetical and Biological Aspects,* Japan Scientific Societies Press, Tokyo; White, M.J.D., 1958, Cold Spring Harb. Symp. Quant. Biol. 23: 307-310.

**Effect of larval size and weight on pupation site preference in different species of *Drosophila*.**

**Vandal, N.B., and N. Shivanna.** Department of Studies in Zoology, Karnatak University, Dharwad 580003, India; E-mail: drnshivanna@rediffmail.com.

The larval pupation site preference (PSP) is an important event in *Drosophila* preadult development, because the place selected by the larva has decisive influence on the subsequent survival as pupae (Sameoto and Miller, 1968). The larval PSP has been analyzed by two types of phenotypic characters, the pupation height and pupation site preference. The pupation height has been studied by measuring the distance a larva pupated above the surface of the food medium (Sokal *et al.,* 1960; Sokolowski, 1985; Casares and Carracedo, 1987; Schnebel and Grossfield, 1992; Singh and Pandey, 1993). The larval PSP has also been analyzed by measuring the percentage of larva pupated at different sites *viz*; cotton, glass and medium and revealed that most of the *Drosophila* species prefer maximum media and few species prefer glass and cotton for pupation (Barker, 1971; Shirk *et al.,* 1988; Shivanna *et al.,* 1996; Shivanna and Ramesh, 1997; Vandal *et al.,* 2003).

The effect of abiotic and biotic factors on pupation height has been studied in different species of *Drosophila* (de Souza *et al.,* 1970; Sokolowski, 1985; Mueller and Sweet, 1986; Godoy-Herrera, 1986; Casares and Carracedo, 1987; Rodriguez and Sokolowski, 1987; Schnebel and Grossfield, 1992; Pandey and Singh, 1993; Joshi and Mueller, 1993; Hodge *et al.,* 1996; Hodge and Caslaw, 1997; Joshi, 1997) and it reveals that these factors influence the pupation height.

The studies of Shivanna and Ramesh (1995) on larval salivary gland secretions (glue proteins) and the gland size in 15 species of *Drosophila* reveal that the quantity of secretions synthesized is independent of size of the salivary glands. The correlation studies between larval PSP and the quantity of larval salivary gland protein called glue protein revealed that the larvae which secrete a larger quantity of glue protein tend to pupate on media and those that synthesize half of the quantity of glue protein prefer to pupate on glass wall, and very low or negligible quantity of glue protein prefer to pupate on cotton (Shivanna *et al.,* 1996). But the importance of size and weight of the larva in relation to PSP has not been studied. The present study was undertaken to analyze whether there is any relation between the size and weight of larvae and their pupation site preference.

The following *Drosophila* species were used to study the relationship between size and weight of the larva on PSP. *D. melanogaster*, *D. simulans, D. yakuba* and *D. mauritiana* are sibling species belonging to the *melanogaster* subgroup species. *D. ananassae, D. bipectinata, D. malerkotliana* and *D. rajasekari* are closely related sympatric species and belong to *ananassae* subgroup of *melanogaster* species group. *D. virilis* and *D. novamexicana* belong to the *virilis* group and *D. hydei* belongs to *repleta* species group (Bock and Wheeler, 1972; Ehrman, 1978; Ranganath *et al.,* 1985; Ashburner, 1989; Singh and Pandey, 1991).

In order to maintain uniformity with regard to the age of the larvae, the eggs were collected every 6 hours using the modified technique of Delcour as described by Ramachandra and Ranganath (1988) and allowed to hatch. The culture was maintained at 22±1°C with 80% RH. Late third instar larvae (96 hour old) were isolated from the cultures, and the size of the larva was measured from the anterior spiracles to the posterior spiracles (Length and Breadth). Using an electronic balance, the weight of the larva was quantified for each individual (twenty five larvae). Then the larvae were

allowed to pupate in the culture vials. The partial correlation analysis was used to correlate the size and weight of the larva with PSP.

Table 1 shows the mean of larval size (length and breadth) weight and percentage of PSP in different species of *Drosophila*. Among the media-pupating species, *D. simulans, D. yakuba, D. mauritiana, D. bipectinata* and *D. malerkotliana* prefer maximum media for pupation (93.4%, 71.4%, 53.2%, 86.6% and 74%, respectively). The approximate larval size of these species varies between 3 to 3.5 mm and weight varies between 0.6 to 0.7 mg. The larvae of *D. melanogaster, D. ananassae, D. virilis, D. novamexicana,* and *D. hydei* prefer maximum glass for pupation (94.2%, 78%, 95%, 76.6% and 74.25, respectively). The larval size of these species varies between 3.3 to 6 mm and weight varies between 0.6 to 1.7 mg. The larvae of *D. rajasekari* prefer maximum cotton for pupation (61.8%). The larval size is 3.5 mm and weight is 0.6 mg.

Table 1. Size and weight of third instar larvae and their maximum PSP in different species of *Drosophila*.

| Species | Size (LXB) (mm) | Weight (mg) | Percentage of pupation (%) |
|---|---|---|---|
| Media pupating species | | | |
| *D.simulans* | 3.3 | 0.6 | 93.4 |
| *D.mauritiana* | 3.5 | 0.6 | 71.4 |
| *D.yakuba* | 3.4 | 0.6 | 53.2 |
| *D.bipectinata* | 3.5 | 0.7 | 86.6 |
| *D.malerkotliana* | 3.0 | 0.7 | 74.0 |
| Glass pupating species | | | |
| *D. melanogaster* | 3.3 | 0.6 | 94.2 |
| *D.ananassae* | 3.5 | 0.7 | 78.0 |
| *D. virilis* | 5.0 | 1.1 | 95.0 |
| *D. novamexicana* | 6.0 | 1.7 | 76.6 |
| *D.hydei* | 5.5 | 1.7 | 74.2 |
| Cotton pupating species | | | |
| *D.rajasekari* | 3.5 | 0.6 | 61.8 |

Size- Mean length and breadth of larvae includes anterior and posterior spiracles (mm). Weight- Mean weight of late third instars larvae (mg).

The comparison between the larval size and weight with maximum PSP in different species analyzed shows significantly negative correlation (partial correlation) with their maximum PSP; on media *D. simulans* (r = -0.9545 and r = -0.9981), *D. yakuba* (r = -0.9559 and r = -0.9965), *D. mauritiana* (r = -0.9065 and r = -0.9973), *D. bipectinata* (r = -0.9644 and r = -0.9971) and *D. malerkotliana* (r = -0.9979 and r = -0.9458), on glass *D. melanogaster* (r = -0.9605 and r = -0.9984), *D. ananassae* (r = -0.9661 and r = -0.9961), *D. virilis* (r = -0.983 and r = -0.9439), *D. novamexicana* (r = -0.97 and r = -0.9945) and *D. hydei* (r = -0.9769 and r = -0.9924) and on cotton *D. rajasekari* (r = -0.9165 and r = -0.9977).

The larvae of different species show differences in the larval size and weight with their pupation site preference. The glass pupating species *D. virilis*, *D. novamexicana* and *D. hydei* are larger in size and weight than media pupating species, *D. simulans, D. yakuba, D. mauritiana, D. bipectinata,* and *D. malerkotliana*. The larvae of glass pupating species *D. melanogaster* and *D. ananassae* and cotton pupating species *D. rajasekari* are more or less similar in their size and weight. In contrast, *D. virilis, D. novamexicana* and *D. hydei* have bigger size than the larvae of *D. melanogaster*, *D. ananassae,* and *D. rajasekari,* but they prefer to pupate on the glass. The culture conditions were maintained constant for all the species analyzed.

The larval developmental period is determined by the time needed to reach the critical weight, and the time from the critical stage to pupation and final body weight is determined by the critical body weight and the possibilities for additional growth before the onset of pupariation as determined by the availability of resources (Robertson, 1963). The hormonal event leading pupariation are initiated in the third larval instar when critical stage is reached, after which there is a fixed period of post critical feeding growth before pupariation occurs (Riddiford, 1985). In *D. melanogaster*, the critical stage occurs right after the second moult. The size of the larva reaching the critical stage of

commitment to pupariation is referred to as its critical weight. The critical weight is a symptom of the underlying physiology and need not imply a direct relation between size and decision to pupate. Larval critical weight will partly determine the way age and size at maturity respond to environmental variations and is, therefore, important life history evolution (Bernardo, 1993). The studies of larval size, developmental stage, and age on pupation at different temperatures in different populations of *D. melanogaster* reveal that the weight of the larva within the age is significantly correlated with pupation probability in tropical populations, whereas in temperate populations no relation with larval weight, age, and with pupation probability, and most of the larvae succeeded in pupating on/in and then produced small adults (Bochdanovitz and de Jong, 2003). The same larval weight or age might have different meaning for different genotype, and higher probability of pupating was associated with lower adult size once feeding was stopped. Minimal size is needed to pupate, and that might vary between genotypes within populations of *Drosophila* (Bakker, 1961; Bochdanovitz and de Jong, 2003).

Shivanna and Ramesh (1995) studied the larval salivary gland and quantity of glue protein and reported that the secretion of larval glue protein is not associated with size of the salivary gland, and quantity of the secretion is independent of the salivary gland. The larvae which secrete a larger quantity of glue protein tend to pupate on media, and lesser preferred glass for pupation. It reveals that the larval PSP depends on the quantity of glue protein synthesized by the late third instar larvae (Shivanna *et al.,* 1996). The present study reveals that, irrespective of size and weight of the larvae, the species *D. melanogaster, D. ananassae, D. virilis, D. novamexicana* and *D. hydei* prefer to pupate on glass. *D. rajasekari* having similar size and weight with glass and media pupating species prefer to pupate on cotton. *D. virilis, D. novamexicana* and *D. hydei* have more size and weight than other species and prefer to pupate on glass. The *melanogaster* and *ananassae* group species larvae were found to pupate on glass and media though they have similar size and weight. The result shows that the pupation site preference is not taxonomically related. Further it is concluded on the basis of the above result that the size and weight of the larvae has no relationship with their pupation site choice.

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References: Ashburner, M., 1989, Drosophila*, A Laboratory Handbook*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA; Bakker, K., 1961, Arch. Neerland. Zool. 14: 200-281; Barker, J.S.F., 1971, Oecologia 8: 139-156; Bauer, S.J., 1984, J. Heredity 75: 131-134; Bernardo, J., 1993, Trends Ecol. Evol. 8: 166-173; Bock, I.R., and M.R. Wheeler 1972, The *Drosophila melanogaster* species group, Univ. Tex. Publ. No. 7213, 1-102 pp.; Bochdanovits, Z., and G. De Jong 2003, J. Evol. Biol. 16: 1159-1167; Casares, P., and M.C. Carracedo 1987, Behav. Genet. 17: 523-535; De Souza, H.L, A.B. Da Cunha, and E.P. Dos Santos 1970, Amer. Nat. 102**:** 583-586; Ehrman, L., 1978, Sexual behaviour. In *The Genetics and Biology of* Drosophila, Vol 2b, (Ashburner, M., and T.R.F. Wright, eds.). Academic Press, New York, pp. 127-157; Godoy-Herrera, R., 1986, Heredity 56: 3-41; Hodge, S., R. Campbell-Smith, and N. Wilson 1996, Entomologists 115: 129-139; Hodge, S., and P. Caslaw 1997, J. Insect Behav. 11: 47-59; Joshi, A., 1997, Curr. Sci. 72: 555-562; Mueller, L.D., and V.F. Sweet 1986, Evolution 40: 1354-1356; Pandey, M.B., and B.N. Singh 1993, Ind. J. Exp. Biol. 31: 912-917; Ranganath, H.A., L.S. Gowda, and M.R. Rajasekarshetty 1985, Entomon 10: 249-253; Ramachandra, N.B., and H.A. Ranganath 1988, Genome 30: 58; Riddiford, L.M., 1985, Adv. Insect Physiol. 24: 213-274; Rodriguez, L., and M.B. Sokolowski 1987, Behav. Genet. 17: 600-604; Robertson, F.W., 1963, Genet. Res. 4: 74-92; Sameoto, D.D., and R.S. Miller 1968, Ecology 49: 177-180; Schnebel, E.M., and J. Grossfield 1992, J. Insect. Physiol. 38: 727-732; Shirk, P.D., P.A. Roberts, and C.H. Harn 1988, Roux's Arch. Dev.

Biol. 29: 926-929; Shivanna, N., and S.R. Ramesh 1995, Curr. Sci. 68: 1246-1249; Shivanna, N., G.S. Siddalingamurthy, and S.R. Ramesh 1996, Genome 39: 105-111; Shivanna, N., and S.R. Ramesh 1997, Dros. Inf. Serv. 80: 2-4; Singh, B.N., and M.B. Pandey 1991, Ind. J. Exp. Biol. 29: 926-929; Singh, B.N., and M.B. Pandey 1993, Behav. Genet. 23: 239-243; Sokal, R.R., P. Ehrlich, P. Hunter, and G. Schlager 1960, Ann. Ent. Soc. Amer. 53: 174-182; Sokolowski, M.B., 1985, J. Insect Physiol. 31: 857-864; Sokolowski, M.B., and S.J. Bauer 1989, Heredity 62: 177-183; Vandal, N.B., S.A. Modagi, and N. Shivanna 2003, Ind. J. Exp. Biol. 41: 918-920.

## Revised list of drosophilid species so far described and recorded from the Kumaon region, Uttarakhand State, India, with replacement names for two homonyms.

**Fartyal, R.S.,*[1] and B.K. Singh[2].** [1]Department of Zoology & Biotechnology, H.N.B. Garhwal University, Srinagar Garhwal– 246 174 Uttarakhand, India; [2]Department of Zoology, D.S.B. Campus, Kumaon University, Nainital-263 002, Uttarakhand, India. Email:* fartyalrs@rediffmail.com; fartyalrs@yahoo.com

### Introduction

The Cytogenetics Laboratory in the Department of Zoology, Kumaon University, Nainital, India, is actively exploring the Drosophilid fauna of the Kumaon region since 1984, which was completely unknown for its drosophilid fauna. Since then a number of papers have been published on Kumaon Drosophilidae (Singh and Negi, 1989, 1992, 1995; Singh and Dash, 1993, 1998; Singh, Dash and Fartyal, 2000, 2004; Singh and Fartyal, 2002; Fartyal, Singh and Toda, 2005; Joshi, Fartyal and Singh, 2005; Upadhyay and Singh, 2006). The area is characterized by having dense evergreen coniferous forests with medium to very steep slopes and extremely moist condition due to heavy rain fall. This paper deals with the list of drosophilid species so far described and recorded from the Kumaon region, India, with replacement names for two homonyms, *i.e*. *Drosophila* (*Drosophila*) *kulouriensis*; old name e*longata* Singh, Dash and Fartyal, 2004, (Ref. Senck. Biol., 83(2): 173, 2004), and *Drosophila (Drosophila*) *sattalensis*; old name *serrata* Singh, Dash and Fartyal, 2004, (Ref. Senck. Biol., 83(2): 176, 2004).

### Materials and Methods

This list is the result of drosophilid collections undertaken by the students of the Cytogenetics Laboratory, Department of Zoology, Kumaon University, Nainital, India, since 1984. The collections were largely made by net sweeping over wild vegetation and by trap baiting. The collected flies were preserved in 70% alcohol and stored for their head, thorax and abdomen.

### Observations

Out of 82 species collected, a total of 37 species belonging to different genera of the family Drosophilidae were described as new to science, and 18 species were recorded for the first time from India since 1984 from the Kumaon region, India. The genera *Lordiphosa*, Malloch and

*Microdrosophila* Malloch were recorded for the first time from Uttarakhand, and the genus *Dichaetophora* Duda was recorded for the first time from India.

Table 1. List of Drosophilid species and collection localities so far described and recorded from the Kumaon region, India, with replacement names for two homonyms marked with (*).

| Genus / Subgenus | Species | Collection locality |
|---|---|---|
| Genus *Amiota* Loew | 1. *bandes* Singh & Negi, 1992 | Okhalkanda, Sargakhet,Chaubattia garden, Bageshwar, Amsysri,Nainital |
| Subgenus *Phortica* Schiner | 2. *biprotrusa* Chen & Toda, 1998 | Nainital,Gahna |
| | 3. *pseudotau* Toda & Peng, 1990 | Mukteshwar |
| Genus *Dettopsomyia* Lamb | 4. *nigrovittata* (Malloch, 1924) | Latoli, Lariyakanda, Kailakhan, Champawat |
| Genus *Dichaetophora* Duda | 5*. acutissima* Okada, 1956 | Kausani |
| Genus *Drosophila* Fallén | | |
| Subgenus *Dorsilopha* Sturtevant | 6. *busckii* Coquillet,1901 | Nainital, Almora, Dunagiri, Dwarahat, Ranikhet, Chaukhutia, Kausani, Pinath, Amsyari, Pithoragarh |
| Subgenus *Drosophila* Fallén Str. | 7. *analspina* Singh & Negi, 1995 | Nainital,Letibuga, Kausani, Chaubattia Garden |
| | 8. *bageshwarensis* Singh, Dash & Fartyal, 2004 | Pithoragarh, Lohaghat, Ranikhet, Amsyari, Bageshwar |
| | 9. *bishtii* Singh & Negi, 1995 | Nainital, Okhalkanda, Sargkhet, Ranikhet |
| | 10. *bizonata* Kikkawa & Peng, 1938 | Nainital, Mukteshwar, Kausani |
| | 11. *dwarahatensis* Upadhyay & Singh, 2006 | Dwarahat |
| | 12. *hexaspina* Singh, Dash & Fartyal, 2004 | Nainital, Dhari |
| | 13. *hydei* Sturtevant, 1921 | Kausani, Dwarahat, Nainital |
| | 14. *immigrans* Sturtevant, 1921 | Nainital,Sheetla, Ranikhet, Dunagiri, Dwarahat, Chaukhutia, Almora, Pithoragarh, Gangolihat, Kausani, Pinath, Amsyari |
| | 15. *khansuensis* Singh, Dash & Fartyal, 2004 | Nainital, Tanakpur, Chaukhutia, Kausani |
| | 16. *kulouriensis** (old name *elongata* Singh, Dash & Fartyal, 2004; Ref. Senck. Biol. 83(2):163-176, 2004) | Nainital, Dhari |

| | | |
|---|---|---|
| | 17. *lacertosa* Okada, 1956 | Nainital,Dhari ,Ranikhet, Dunagiri, Dwarahat, Chaukhutia, Almora, Pithoragarh, Gangolihat, Kausani, Pinath, Amsyari, Rudarpur |
| | 18. *mukteshwarensis* Joshi, Fartyal & Singh, 2005 | Mukteshwar |
| | 19. *nainitalensis* Singh & Bhatt, 1988 | Ganguachaur, Nainital, Nagpani Chaubattia |
| | 20. *nasuta* Lamb,1914 | Nainital, Haldwani, Rudrapur |
| | 21. *notostriata* Okada, 1966 | Kashialekh, Nainital |
| | 22. *paharpaniensis* Singh, Dash & Fartyal, 2004 Nainital ,Okhalkanda, Ranikhet | |
| | 23. *painaii* Singh & Negi, 1995 | Bhatelia, Nainital, Bhowali, Almora, Dunagiri, Kausani |
| | 24. *paramarginata* Singh, Dash and Fartyal, 2004 | Nainital, Okhalkanda, Champawat (Mayawati Ashram) |
| | 25. *parazonata* Dwivedi & Gupta, 1980 | Nainital |
| | 26. *paunii* Singh & Negi, 1989 | Sargakhet,Nainital, Kausani, Pithoragarh, Almora, Ranikhet |
| | 27. *repleta* Wollaston, 1858 | Latoli,Nainital,Lalkuan, Dunagiri, Dwarahat, Ranikhet, Kausani |
| | 28. *repletoides* Hsu, 1943 | Dwarahat |
| | 29. *sattalensis* ***** (old name *serrata* Singh, Dash & Fartyal, 2004; Ref. Senck. Biol. 83 (2): 163-176, 2004) | Sattal - bhimtal(Nainital), Chaubattia garden, Ranikhet |
| | 30. *sulfurigaster* Duda, 1923 | Letibuga, Nainital, Pithoragarh, Bageshwar |
| | 31. *surangensis* Singh, Dash & Fartyal, 2004 | Nainital |
| | 32. *tetradentata* Singh & Gupta, 1980 | Dunagiri, Kausani |
| | 33. *trizonata* Okada, 1966 | Dhari, Nainital, Bhowali, Dhari, Khansu |
| Subgenus *Sophophora* Sturtevant | 34. *bifasciata* Pomini, 1940 | Sheetla, Nainital,Haldwani, Dunagiri, Kausani,Lohaghat, Tanakpur, Pithoragarh |
| | 35. *hubeiensis* Sperlich & Watabe, 1997 | Sargakhet, Nainital, Dunagiri, Kausani |
| | 36. *jambulina* Parshad and Paika, 1964 | Dhari, Nainital, Dwarahat, Chaubattia Garden, Chaukhutia,Gangolihat. |
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|---|---|---|
| | 37. *kikkawai* Burla, 1954 | Sheetla, Nainital,Ranikhet, Dunagiri, Dwarahat, Chaukhutia, Bageshwar, Kausani, Gangolihat |
| | 38. *malerkotliana malerkotliana* Parshad & Paika, 1964 | Pithoragarh (Ghat), Champawat (Mayawati Ashram), Haldwani, Dunagiri, Kausani, Nainital |
| | 39. *melanogaster* Meigen, 1830 | Gahna, Nainital, Sat Tal, Dwarahat, Chaukhutia, Almora, Kausani, Pithoragarh,Rudrapur, Binsar Mahadev (Almora) |
| | 40. *neobaimai* Singh & Dash, 1998 | Nainital, Dunagiri, Kausani, Kanalichchina(Pithoragarh) |
| | 41. *neokhaoyana* Singh & Dash, 1998 Nainital, Gangolihat, Dhari | |
| | 42. *nepalensis* Okada, 1955 | Nainital, Almora, Ranikhet, Dunagiri, Dwarahat,Chaukhutia, Pithoragarh, Kausani, Pinath, Amsyari |
| | 43. *punjabiensis* Parshad & Paika, 1964 | Nainital, Chaubattia garden |
| | 44. *sargakhetensis* Joshi, Fartyal & Singh, 2005 | Sargakhet |
| | 45. *saraswati* Singh & Dash, 1995 | Kausani,Mukteshwar, Pithoragarh, Bageshwar, Gangolihat. |
| | 46. *suzukii indicus* Parshad & Paika, 1964 | Latoli, Nainital, Almora, Ranikhet, Dwarahat,Chaukhutia, Amsyari, Kausani, Gangolihat |
| | 47. *takahashii* Sturtevant, 1927 | Bhatelia, Nainital, Ratighat, Almora,Ranikhet, Dwarahat, Dunagiri, Gangolihat ,Amsyari, Kausani |
| Genus *Gitona* Meigen | 48. *distigma* Meigen, 1830 | Dhari, Nainital, Lariyakanda |
| Genus *Hirtodrosophila* Duda | 49. *hexaspina* Fartyal & Singh, 2002 | Letibuga, Nainital |
| | 50. *quadrivittata* Okada, 1956 | Sheetla, Nainital |
| *Leucophenga* Mik | 51. *albiceps* de Meijere, 1914 | Ganguachaur, Nainital, Lariyakanda, Chaubattia garden, Dunagiri, Kausani, Gangolihat |
| | 52. *angulata* Singh, Dash & Fartyal, 2000 | Gahna, Nainital, Dunagiri, Kausani, Pinath. Sargakhet |
| | 53. *angusta* Okada, 1956 | Nainital, Pithoragarh |
| | 54. *argentata* de Meijere, 1914 | Dunagiri, Dwarahat, Gangolihat |
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| | | |
|---|---|---|
| | 55. *bellula* (Bergroth, 1894) | Mukteshwar, Nainital, Ratighat, Ranikhet, Dunagiri, Kausani, Pinath, Amsyari |
| | 56. *champawatensis* Fartyal, Singh & Toda, 2005 (= *singhi*, Ref. D.I.S. 83:71, 2000) | Nainital, Kausani, Champawat |
| | 57. *chaubattianensis* Fartyal, Singh & Toda, 2005 | Chaubattia garden, Ratighat, Nainital |
| | 58. *clubiata* Singh, Dash & Fartyal, 2000 | Dhari, Nainital, Bhowali, Dunagiri, Kausani |
| | 59. *kumaonensis* Fartyal, Singh & Toda, 2005 | Nainital |
| | 60. *nainae* Fartyal, Singh & Toda, 2005 | |
| | Nainital, Chaubattia garden | |
| | 61. *neointerrupta* Fartyal, Singh & Toda, 2005 | Nainital,Pilkholi, Dwarahat, Kausani, Pinath |
| | 62. *neolacteusa* Singh & Bhatt, 1988 | Latoli, Nainital, Chaubattia garden. |
| | 63. *okhalkandensis* Singh, Dash & Fartyal, 2000 | Nainital, Ranikhet, Chaubattia garden,Dunagiri, Kausani |
| | 64. *ornata* Wheeler, 1959 | Kashialekh, Nainital, Chaubattia garden, Dunagiri, Kausani |
| | 65. *quadripunctata* (de Meijere, 1908) | Nainital |
| | 66. *subpollinosa* (de Meijere, 1914) | Sheetla, Nainital, Bhowali,Chaubattia Garden |
| | 67. sp. 1, Upadhyay & Singh (In press) | Kausani |
| Genus *Lissocephala* Malloch | 68. *parasiatica* Takada & Momma, 1975 | Mukteshwar, Nainital, Almora, Ranikhet, Pithoragarh |
| Genus *Lordiphosa* Malloch, | 69. *tripartita* Okada 1966 | Dunagiri (Almora) |
| Genus *Microdrosophila* Malloch, Subgenus *Microdrosophila* | 70. sp. 2, Upadhyay & Singh (In press) | Dunagiri (Almora) |
| Genus *Paraleucophenga* Hendel | 71. *neojavanaii* Singh & Negi, 1992 | Dwarahat (Almora) |
| | 72. *todai* Fartyal & Singh, 2004 | |
| Genus *Hirtodrosophila* Duda | 73. *actinia* Okada,1991 | Nainital, Dhari, Pithoragarh, Amsyari |
| Genus *Scaptodrosophila* Duda | 74. *chandraprabhiana* Gupta & Ray-Chaudhuri, 1970 | Nainital, Bageshwar,Kausani, Champawat, Lohaghat |
| | | |

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|---|---|---|
| | 75. *coracina* Kikkawa & Peng, 1938 | Nainital, Bhatelia, Champawat, Almora, Dunagiri, Kausani |
| | 76. *hirsuata* Singh & Dash, 1998 | Sargakhet, Haldwani, Nainital, Bageshwar, Amsyari, Rudrapur, Tanakpur |
| | 77. *subtilis* Kikkawa & Peng, 1938 | Kausani |
| Genus *Scaptomyza* Hardy | 78. *elmoi* Takada, 1970 | Latoli, Nainital, Pithoragarh |
| | 79. *himalayana* Takada, 1970 | Nainital, Almora, Ranikhet, Dwarahat, Dunagiri,Chaukhutia, Pithoragarh, Gangolihat, Kausani, Pinath, Amsyari |
| | 80. *quadruangulata* Singh & Dash, 1993 | Letibuga,Bhimtal, Sat Tal, Ramnagar, Nainital, Ranikhet, Dwarahat, Dunagiri, Chaukhutia, Pithoragarh, Gangolihat, Kausani |
| Genus *Stegana* Meigen | 81. *nainitalensis* Singh & Fartyal, 2002 | Nainital |
| Genus *Zaprionus* Coquillett | 82. *indianus* Gupta, 1970 | Mukteshwar, Nainital, Almora, Ranikhet, Dwarahat, Dunagiri, Chaukhutia, Pithoragarh, Gangolihat, Kausani, Pinath, Amsyari |

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References: Singh, B.K., and N.S. Negi 1989, Proc. Zool. Soc. Cal. 40: 19-26; Singh, B.S., and N.S. Negi 1992, Senckenbergiana Biol. 72: 321-327; Singh, B.S., and N.S. Negi 1995, Entmol. Z. 105(21): 421-440; Singh, B.K., and S. Dash 1993, Proc. Zool. Soc. Cal. 46: 131-140; Singh, B.K., and S. Dash 1998, Proc. Zool. Soc. Cal. 51: 45-56; Singh, B.K., S. Dash, and R.S. Fartyal 2000, Senckenbergiana Biol. 80(1/2): 149-154; Singh, B.K., S. Dash, and R.S. Fartyal 2004, Senckenbergiana Biol. 83(2): 163-176; Singh, B.K., and R.S. Fartyal 2002, Proc. Zool. Soc. Cal. 50 (1): 11-18; R.S. Fartyal, B.K. Singh, and M.J. Toda 2005, Entomol. Sci. 8: 405-417; Joshi, M., R.S. Fartyal, and B.K. Singh 2005, Entomon 30(1): 75-84; Upadhyay, K., and B.K. Singh 2006, Proc. Zool. Soc. Cal. 59(2): 195-202.

**Stochastic and factorial components of phenotypic variation in morphological traits in laboratory populations of *Drosophila ananassae.***

**Vishalakshi, C., and B.N. Singh**.* Genetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi-221005, India; *Corresponding Author; email: **bnsingh@bhu.ac.in**; bashisthsingh2004@rediffmail.com.

## Introduction

Phenotypic variation is a universal requirement for evolution upon which natural selection acts. It has been observed in a wide variety of traits across the populations and species (Woods *et al*., 1999; Belade *et al*., 2005). But how does this variation arise? How do new variants evolve? What constrains variations? These are the central questions of interest to ecologists and evolutionary biologists. Phenotypic variation reflects the genetic differences among individuals and the diversity of the environment (Lajus and Alekseev, 2000). Most of the studies have focused much on the genotypic variation than non-genotypic variation, which provides the basis for population's adaptive response to change in environmental factors by means of selection (Lajus and Alekseev, 2004). But some experiments have shown that even when both genetic and environmental factors have been minimized variation still exists suggesting that there is some "independent variation" which can be neither reduced due to genotypic differences nor due to direct effect of environmental factors (Belade *et al*., 2005; Astaurov, 1930). Later, it has been proposed that this kind of variation results from developmental instability, which reflects the deviations of developmental trajectories from the target determined for a particular genotype and environment (Lajus and Alekseev, 2000). It is measured by fluctuating asymmetry (FA), *i.e*., variance in random deviations from the perfect bilateral symmetry (Van Valen, 1962) and its levels increase due to either genetic or environmental stresses (Leamy and Klingenberg, 2005). There are different ways to partition total phenotypic variation, but most of them require special experimentation and are very difficult to apply to the populations of out-breeding species in natural and laboratory conditions (Lajus and Alekseev, 2000). In non-symmetrical structures, two kinds of the variation, heterogeneity among individuals and within-individual variation, impact total phenotypic variation, but it is impossible to separate them (Lajus, 2001). In symmetrical traits total phenotypic variation can be divided into two components: stochastic and factorial components using a special index of fluctuating asymmetry (Lajus and Alekseev, 2000). The stochastic component reflects developmental instability while the factorial component reflects the heterogeneity of individuals within a population and includes genotypic, macro- and micro-environmental, and ontogenetic variations (Kozhara, 1994; Lajus *et al*., 2003a). Genotypic variations reflect the genetic differences among individuals. Macro-environmental variations are due to environmental variations between distinct environments, whereas micro-environmental variations are due to relatively minor and local environmental variations. The ontogenetic component represents variation among different stages of growth, *e.g*., juveniles and senescent adults differ in their phenotypes (Lajus *et al*., 2003a). However, there is no report on the relative contribution of stochastic and factorial components of phenotypic variance in any bilateral trait in *Drosophila* (D. Lajus, pers. comm.). In view of this, we have made an attempt to analyze the phenotypic variation in *D. ananassae* Doleschall, a cosmopolitan and domestic species, which occupies a unique status among the *Drosophila* species due to certain unusual genetic features (Singh, 2000).

## Materials and Methods

*Fly stocks and Trait measurements*

The details of the eighteen mass culture stocks of *D. ananassae* used in the present study have been described elsewhere (Vishalakshi and Singh, 2006). The stocks were maintained in the laboratory on simple yeast-agar medium at approximately 24$^{o}$C temperature. Virgin flies collected from these mass culture stocks were aged for 4 days in food vials. Different morphological traits *viz*., thorax length, wing length, wing to thorax ratio and sternopleural bristle number, sex comb tooth number, and ovariole number were measured as described earlier (Vishalakshi and Singh, 2006). Except thorax length, all the traits were measured on both left and right sides in a total of 1800 individuals (50 males and 50 females from each population).

*Data analyses*

Variation among populations and sexes was tested by analysis of variance (ANOVA) for each morphological trait. The variability of each population was estimated using the coefficient of variation (CV). Comparisons of phenotypic variability among populations in both sexes were performed using test of homogeneity for coefficient of variation (Zar, 2005).

For the asymmetry analysis, the framework laid by Palmer (1994) was followed. Measurement error (ME) will artificially inflate the estimates of FA. Therefore, for this reason, it is important to have the confidence that there are differences in R-L among individuals and not simply an artifact of ME. For measurement error, 32 flies randomly collected from the cultures and two replicate counts were made for different traits per fly, each on different day. Measurement error was assessed using two-way mixed model ANOVA in which, sides were entered as fixed factor and individuals as a random factor (Palmer, 1994). The tests for FA differences are only justified if interaction (Side × Individual) variance is significant. In all ANOVA (not shown), the interaction between side and individual is highly significant ($P < 0.001$), indicating that the measurement error in all the traits is negligible compared with the variation between sides. In all ANOVA (not shown), the interaction between side and individual is highly significant ($P < 0.001$), indicating that the measurement error in all the traits is negligible compared with the variation between sides. Further, individual asymmetry was measured as D = R - L, where R is the value of the trait on the right side and L is the value of the trait on the left side.

*Partitioning total phenotypic into stochastic and factorial components*

The partitioning of stochastic and factorial components is justifiable only if the asymmetry is fluctuating asymmetry and not directional asymmetry and antisymmetry. Therefore, it is important to check the kind of asymmetry before proceeding with an analysis (Lajus *et al*., 2003a). Thus, one sample t – test on the signed differences (R-L) for each trait was performed to determine whether the mean values differ from zero for directional asymmetry (Palmer, 1994). For antisymmetry, we checked departures from normality of the distribution of the signed differences (R-L) using the Kolmogorov-Smirov test. Fluctuating asymmetry in bilateral traits (wing length, W/T ratio, sternopleural bristle number, sex comb tooth number and ovariole number) in 18 laboratory populations of *D. ananassae* was calculated. Further, phenotypic variance of bilateral traits, *e.g*., wing length, W/T ratio, sternopleural bristle number, sex comb tooth number, and ovariole number was partitioned into stochastic ($\sigma^2_S$) and factorial ($\sigma^2_F$) components (Kozhara, 1989, 1994) $\sigma^2 = \Sigma (Xi - M)^2/ 2 (n-1)$; $\sigma^2_S = \Sigma (R-L)^2/ 2 n$; $\sigma^2_F = \sigma^2 - \sigma^2_S$ where $\sigma^2$ is the total phenotypic variance, $\sigma^2_S$ is the stochastic component of total variance, $\sigma^2_F$ is the factorial component, $X_i$ is both right and

left manifestation of the trait, M is the mean value, R is the right manifestation, and L is the left manifestation of the trait, and n is the number of individuals. Variations among the populations for both stochastic ($\sigma^2_S$) and factorial ($\sigma^2_F$) components in both sexes were tested by test of homogeneity of variance for all the traits separately. To compare the difference in the components (stochastic and factorial) and sexes (males and females), F-test was performed.

Table 1. Comparisons of morphological traits between populations and sexes in laboratory populations of *D.ananassae* by two –way Analysis of Variance.

| Traits | Source of variation | df | MS | F |
|---|---|---|---|---|
| Thorax length | Sex (S) | 1 | 19529.5 | 211.26*** |
| | Populations (P) | 17 | 152.44 | 25.74*** |
| | S x P | 17 | 92.44 | 15.61*** |
| | Error | 1764 | 5.921 | |
| SBN | Sex (S) | 1 | 991.63 | 58.47*** |
| | Populations (P) | 17 | 46.02 | 8.32*** |
| | S x P | 17 | 16.96 | 3.07*** |
| | Error | 1764 | 5.52 | |
| WL | Sex (S) | 1 | 47228 | 235.18*** |
| | Populations (P) | 17 | 250.88 | 21.59*** |
| | S x P | 17 | 200.82 | 17.28*** |
| | Error | 1764 | 11.62 | |
| W/T ratio | Sex (S) | 1 | 0.0346 | 7.208* |
| | Populations (P) | 17 | 0.095 | 256.76*** |
| | S x P | 17 | 0.0048 | 12.98*** |
| | Error | 1764 | 0.00037 | |
| ST | Sex (S) | 1 | 791533.6 | 949.39*** |
| | Populations (P) | 17 | 1447.28 | 4.203*** |
| | S x P | 17 | 833.72 | 2.421** |
| | Error | 1 | 344.32 | |

*$P$<0.05; **$P$<0.01; ***$P$<0.001.
TL-Thorax length; SBN-Sternopleural bristle number; WL-Wing length; W/T– Ratio of wing length and thorax length; ST- sexual traits (in females- Ovariole number and in males - Sex-comb tooth number)

## Results and Discussion

The mean value of different morphological traits *viz*., thorax length, wing length, wing to thorax ratio and sternopleural bristle number, sex comb tooth number, and ovariole number vary significantly among populations (Table 1) providing the evidence for genetic heterogeneity among laboratory populations of *D. ananassae*. There is significant difference between the males and females for all traits (Table 1). Test of homogeneity of coefficient of variation (CV) reveals that there are significant differences among individual variation for all the morphological traits in both

Table 2. Population variance (Var) and test for homogeneity for coefficient of variations ($\chi^2$) among populations for different traits in *D.ananassae*.

| Traits | MALES | | FEMALES | |
|---|---|---|---|---|
| | Var | $\chi^2$ | Var | $\chi^2$ |
| **TL** | 8.59 | 65.51** | 7.63 | 123.57** |
| SBN | 1.89 | 30.99* | 3.781 | 108.15** |
| WL | 24.44 | 69.73** | 14.48 | 1302.66** |
| W/T ratio | 0.004 | 134.79** | 0.0036 | 363.9** |
| ST | 74.16 | 33.00* | 17.47 | 69.04** |

* Significant at $P<0.05$, ** Significant at $P<0.001$

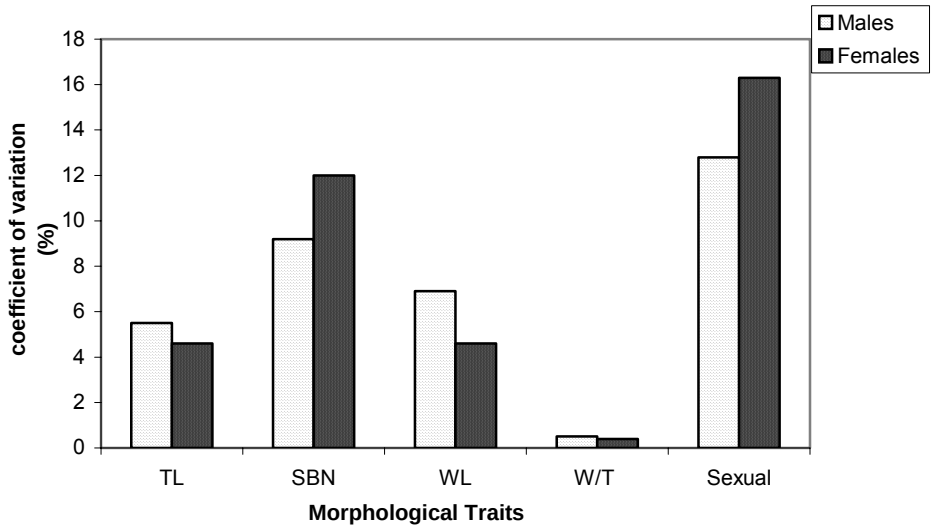


Figure 1. Phenotypic coefficient of variation (CV) for morphological traits in males and females. (The abbreviations used: TL - Thorax length, SBN - Sternopleural bristle number, WL – Wing length, W/T- Ratio of wing length and thorax length, sexual traits (SCTN-Sex comb tooth number and ON- Ovariole number).

sexes (Table 2). The phenotypic variability of sexual traits is more than that of the non-sexual traits (Figure 1). To compare phenotypic variations between traits, we considered trait CVs separately in males and females. Traits differed significantly in their CVs by Kruskal-Wallis tests in both the sexes (males: $F = 50.822$, df = 4, $P < 0.001$; females: $F = 21.33$, df = 4, $P < 0.001$). This difference in CV arises due to enormous diversity of the genetic and environmental factors difficult to disentangle (Houle, 1992) and also depends on the forces acting on them. For example, in the present study the traits that we have selected can be categorized into: meristic traits (SBN, SCTN, and ON) and metric traits (WL, W/T ratio, and TL) and also in non-sexual traits (TL, SBN, WL, and W/T ratio), and sexual traits (SCTN and ON). Across the populations (Figure 1), the CVs of the meristic traits (SBN, SCTN, and ON) are relatively higher than the metric traits (WL, W/T ratio, and TL) suggesting that meristic traits are more prone to phenotypic variability than metric traits (Woods *et al*., 1999). Trait differences in CVs also reflect the forces acting on them, which is evident from the larger CV of sexual traits (SCTN and ON) than non-sexual traits (SBN, WL, W/T ratio, and TL). This suggests that any trait that enhances mating and fertilization opportunities will be favored by sexual selection (Andersson, 1994) and also are sensitive to the effect of environmental perturbations leading to high phenotypic variability (M∅ller and Cuervo, 2003) than the non-sexual traits, which are supposed to be under natural selection. Interestingly, the variability is more in ovariole number (in females) than in sex comb tooth number (in males), suggesting intense sexual selection will reduce variability in males, which supports the theory that strong directional selection reduces underlying genetic and phenotypic variation (Falconer and Mackay, 1996). Moreover, in the non-sexual traits, the lower CVs in the wing traits (WL and W/T ratio) than bristle number in both sexes suggests that the traits that are more closely related to fitness are expected to be better buffered against environmental effects (Lerner, 1954; Woods *et al*., 1999).

*Directional asymmetry and antisymmetry*

One-sample t-test reveals that mean values of each trait did not differ significantly from zero. For example, in males, for SBN (t = -1.273, df = 1798, $P$ = 0.203), WL (t = 0.238, df = 1798, $P$ = 0.814), W/T ratio (t = 0.697, df = 1798, $P$ = 0.486) and SCTN (t = 0.505, df = 1798, $P$ = 0.614) and in females for SBN (t = -0.093, df = 1798, $P$ = 0.926), WL (t = 1.633, df = 1798, $P$ = 0.103), W/T ratio (t = -0.693, df = 1798, $P$ = 0.488) and ON (t = -1.552, df = 1798, $P$ = 0.121). The distribution of the signed differences (R-L) showed normal distribution in the Kolmogorov-Smirov test for normality. Moreover, none of skewness (t = -1.509, df = 8, $P$ = 0.170) and kurtosis (t = 1.752, df = 8, $P$ = 0.118) values differed from zero for all the traits. This indicates that we are observing true FA rather than directional asymmetry and antisymmetry in our data. Fluctuating asymmetry have been calculated as mean of absolute trait asymmetry (|R-L|) for males and females.

*Partitioning of stochastic ($\sigma^2_S$) and factorial ($\sigma^2_F$) components*

Further, we have partitioned out the total phenotypic variance into stochastic ($\sigma^2_S$) and factorial ($\sigma^2_F$) components for all the bilateral traits (sternopleural bristle number, wing length, wing to thorax ratio, ovariole number, and sex comb tooth number). There are significant differences among the populations for stochastic component of variance for all the traits in males (except SCTN) and females (Table 3). The populations also differ significantly for the factorial components of all traits except WL and SCTN (Table 3). The cause of this variability is due to genotypic variation and internal component of environment (Zhang and Hill, 2005). The percentage contribution of factorial component to the total phenotypic variation is more than that of the stochastic component (Table3). Interestingly, the percentage contribution of the stochastic variance for wing traits decreases in comparison to the SCTN, ON, and SBN supporting the view of Lajus *et al.,* (2003a), that the contribution of stochastic variance to the total phenotypic variance decreases as the mean increases. In males, factorial and stochastic component of variance differ significantly for WL and W/T ratio but not for SBN and SCTN (Table 3 A). Similarly, in females the two components differ significantly for WL, W/T ratio, and ON except SBN (Table 3 B).

Table 3. Magnitude of stochastic ($\sigma^2_S$) and factorial ($\sigma^2_F$) components of the variance of different morphological traits in *D. ananassae.*

| Traits | Variance Among populations ($\chi^2$) | | F-test |
|---|---|---|---|
| | $\sigma^2_S$ | $\sigma^2_F$ | |
| a) Males | | | |
| SBN | 91.22* | 918.22* | 0.782 |
| WL | 914.76* | 3.289 | 511.17* |
| W/T | 1055.45* | 1019.20* | 128.50* |
| SCTN | 0.505 | 1.125 | 2.57 |
| b) Females | | | |
| SBN | 112.94* | 862.27* | 1.22 |
| WL | 628.51* | 3.21 | 100.65* |
| W/T | 1050.9* | 443.33* | 72.61* |
| ON | 111.82* | 4.082* | 7.259* |

* Significant at $P$<0.05,

However, the magnitude of factorial component in all the traits is more than that of the stochastic component in all the traits except SBN in both the sexes (Figure 2). In order to test the difference statistically between the two sexes, F –test was employed. Males and females have a similar level of stochastic component of variation for sternopleural bristle number ($F$ = 1.299, $P$ > 0.05), wing length ($F$ = 1.069, $P$ > 0.05) and wing to thorax ratio ($F$ = 2.08, $P$ > 0.05) but not for the sexual traits ($F$ = 6.357, $P$ < 0.001). These results support the previous findings (Vishalakshi and Singh, 2006) in these laboratory populations where the levels of fluctuating asymmetry ( |R-L| ) are similar in non-sexual traits (SBN, WL, and W/T ratio).

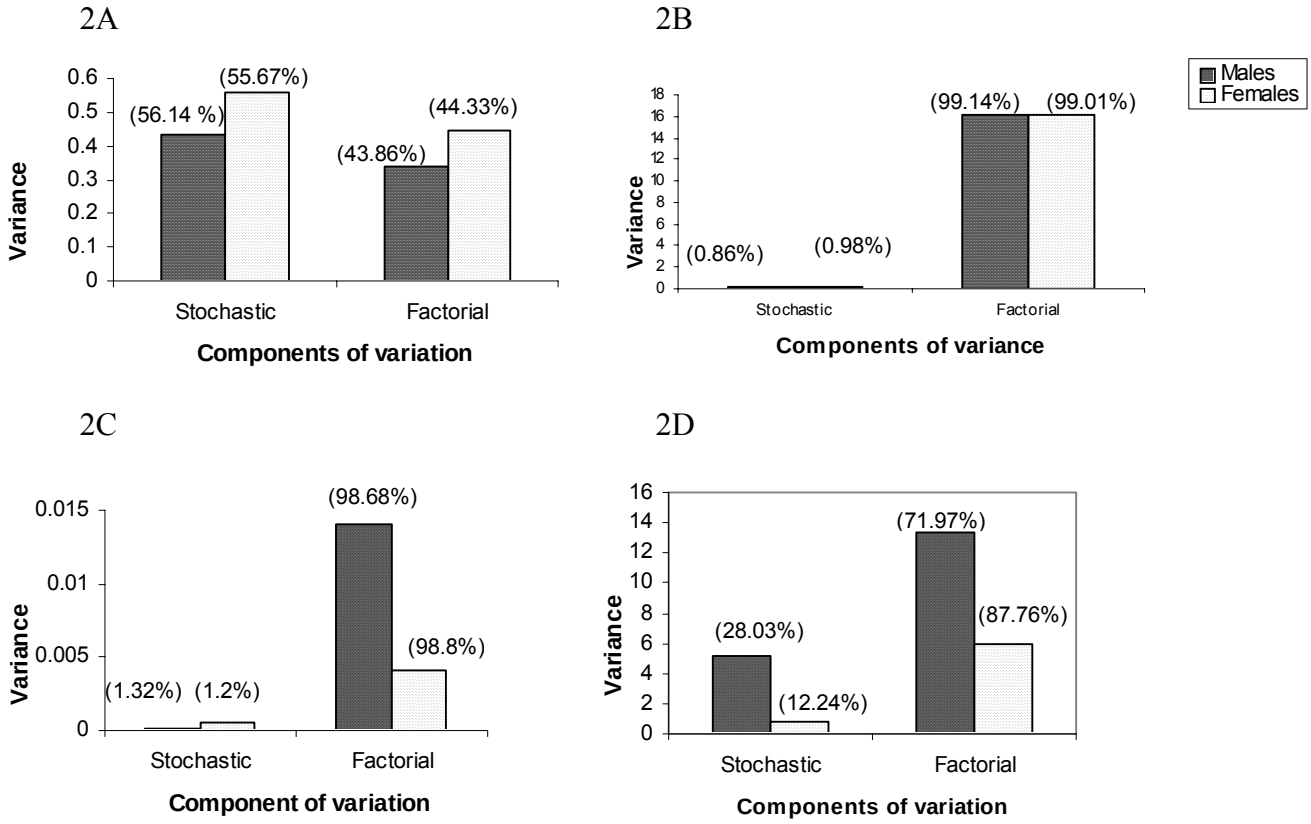


Figure 2. Magnitude of stochastic and factorial components of phenotypic variation in different populations of *D.ananassae* for different morphological traits, A) sternopleural bristle number, B) Wing length, C) Wing to thorax ratio D) Sexual traits. In the parentheses, the percentage values of the contribution of the stochastic and factorial component of the total variance are given).

Similarly, both sexes have the similar level of the factorial component for sternopleural bristle number ($F = 1.368$, $P > 0.05$), wing length ($F = 1.069$, $P > 0.05$), and sexual traits ($F = 2.248$, $P > 0.05$). Moreover, the magnitude of stochastic components is higher in males for SCTN than in female for ON (See Figure 2D) suggesting that sexual traits in males are more prone to developmental instability than in females (Vishalakshi and Singh, 2006).

The relationship between stochastic and factorial components was tested for all the traits in males and females. In males, there is negative correlation between stochastic and factorial components for SBN($r = -0.584$, $P < 0.01$), W/T ratio ($r = -0.305$, $P < 0.219$) and SCTN($r = -0.302$, $P < 0.224$) where as positive correlation for WL ($r = 0.114$, $P = 0.651$). Interestingly, in females there is negative correlation between the two components for WL ($r = -0.394$, $P = 0.106$), W/T ratio ($-0.168$, $P = 0.504$) and ON ($r = -0.433$, $P = 0.072$), whereas as positive correlation for SBN($r = 0.282$, $P < 0.257$). When the data of males and females were pooled for both stochastic and factorial components there is a negative correlation for WL ($r = -0.354$, $P < 0.05$), W/T ratio ($r = -0.156$, $P = 0.585$), whereas there is a positive correlation for SBN ($r = 0.007$, $P = 0.967$) and sexual traits ($r = 0.366$, $P < 0.05$). The association between the stochastic and factorial variance of the same traits is positively correlated (Lajus, 1991; Lajus and Alekseev, 2000). In constrast to this, there is negative correlation for wing traits but positive for SBN and sexual traits. The positive significant association of stochastic and factorial component for sexual traits suggests that the underlying mechanisms are

responsible for buffering the traits development against both external and internal environmental variations are interrelated (Lajus *et al.,* 2003b).

In conclusion, the present study provides the evidence for 1) phenotypic variability among populations and individuals which is caused due to genotypic and micro environmental diversity and within-individual variation is caused due to the developmental instability, 2) trait variability arises due to the evolutionary forces (sexual and natural selection) acting on them at individual level, and 3) the contribution of stochastic variance in comparison to the factorial variance to the total phenotypic variance is small, but is considerable. This is the first report on the relative contribution of stochastic and factorial components of phenotypic variance in any bilateral trait in the genus *Drosophila*. However, present study provides new avenues for further research in order to get more information regarding the contribution of factorial and stochastic variance in other *Drosophila* species with a much wider range of traits.

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References: Andersson, M., 1994, *Sexual Selection*, Princeton University Press, Princeton, N.J.; Astaurov, B.L., 1930, Zeit. in Abs. und Verb. 55**:** 183-263; Belade, P., P.M. Brakefield, and A.D. Long 2005, Evol. Dev. 7: 101-107; Falconer, D.S., and T.F.C. Mackay 1996, *Introduction to Quantitative Genetics*, Longman, Harlow; Houle, D., 1992, Genetics 130: 195-204; Møller, A.P., and J.J. Cuervo 2003, Asymmetry, size and sexual selection: factors affecting heterogeneity in relationships between asymmetry and sexual selection. In: *Developmental Instability: Causes and Consequences* (Polak, M., ed.), pp. 262-275. Oxford University Press, New York; Kozhara, A.V., 1989, Genetika (USSR) 25: 1508-1513; Kozhara, A.V., 1994, J. App. Icht. 10**:** 167-181; Lajus, D.L., 1991, Trudy Zool. Inst. 235**:** 113-122; Lajus, D.L., 2001, Biol. J. Linn. Soc. 74: 237-253; Lajus**,** D.L., and V. Alekseev 2000, Hydrobiologia 417: 25-35; Lajus, D.L., and V. Alekseev 2004, J. Limnol. 63**:** 37-44; Evolution 59: 1237-1244; Lajus, D.L., M. Ciostek, M. Makowiecka, and T. Sywula 2003a, Ann. Zool. Fenn. 40: 411-429; Lajus, D.L., J.H. Graham, and A.V. Kozhara 2003b, Developmental instability and the stochastic component of total phenotypic variance. In: *Developmental Instability: Causes and Consequence.* (Polak, M., ed.), pp. 343-363. Oxford University Press, New York; Leamy, L.L., and C.P. Klingenberg 2005, Ann. Rev. Ecol. Evol. Syst. 36: 1-21; Lerner, I.M., 1954, *Genetic Homeostasis*, John Wiley, New York; Palmer, A.R., 1994, Fluctuating asymmetry analysis: a primer, In: *Developmental Instability: Its Origins and Evolutionary Implications* (Markow, T.A, ed.), pp. 335-364. Kluwer, Dordrecht; Singh, B.N., 2000, Curr. Sci. 78: 391-398; Van Valen, L., 1962, Evolution 16: 125-142; Vishalakshi, C., and B.N. Singh 2006, Genome 49**:** 777-785; Woods, R.E., C.M. Srgo, M.J. Hercus, and A.A. Hoffmann 1999, Evolution 53**:** 493-505; Zar, J.H., 2005, *Biostatistical Analysis*, Pearson Education, Delhi, India; Zhang, Xu- Sheng, and W.G. Hill 2005, Evolution 59: 1237-1244.

**Parallel trend in pigmentation and desiccation tolerance: altitudinal and latitudinal effects in *Drosophila melanogaster*.**

**Rajpurohit, Subhash[1,*], Ravi Parkash[1], Seema Ram Niwas[1], Oldrich Nedved[2,3], and Shama Singh[1]**. [1]Department of Biochemistry and Genetics, Maharshi Dayanand University, Rohtak-124 001, India; [2]Biology Centre, Czech Academy of Sciences;

[3]Faculty of Science, University of South Bohemia, Branisovska 31, CZ-370 05, Ceske Budejovice, Czech Republic. *Corresponding author's present address: Institute of Evolution, The University of Haifa, Haifa-31905, Israel; email: srmailbox@rediffmail.com.

**Abstract**

Body melanization and desiccation tolerance were analyzed in ten *Drosophila melanogaster* populations collected at high and low altitudes from five latitudes along the Indian subcontinent. Significant latitudinal cline (11–31°N) and altitudinal (10–2200 m) differentiation were observed for cuticle pigmentation (8–30 points of maximum 60) and survival time of desiccation (17–25 hours). Both traits were found to be closely associated with average temperature (10–28°C) and relative humidity (40–73%) among study sites. Desiccation tolerance was linearly correlated with pigmentation ($D = 0.31.P+15.5$ hours; $r = 0.94$, $p < 0.0001$). The results from this study suggest that factors linked to temperature are likely selective forces and that genes controlling pigmentation and desiccation tolerance are good candidates for studying climatic adaptation.

**Introduction**

Morphological and life history traits of living organisms may vary gradually along existing environmental clines. Two of the geographical characteristics may be easily identified and correlated with organism traits: latitude (distance from equator) and altitude (height above sea level). Important environmental conditions like ambient temperature are dependent on both these clines, although the rate of change is hardly comparable. Altitudinal temperature changes are generally steep and the climate is less predictable, while latitudinal gradients are smoothly spread over long distances (Heath and Williams, 1979; Baur and Raboud, 1988). For example, mean temperature difference between the foot (200 m) of Sierra Nevada, California, and the peak (3979 m) of Mount Dana at a distance of 97 km is around 24°C. This difference is roughly equivalent to 4500 km in south–north (latitude) direction (Hopkins, 1938). On the Indian sub-continent, both latitudinal and altitudinal ranges are substantial, offering a chance for local adaptation of populations to prevailing climates. The average temperature differs approximately by 2.6°C between south (Kaniya kumari: 8.05 °N / 37 m) and north (Chandigarh: 30.44 °N / 347 m) regions, whereas from lowland Kalka (30.51 °N / 600 m) to mountain Shimla (31.06 °N / 2202 m), the average temperature differs around 10.2°C. The distance between Kaniya kumari to Chandigarh is around 2400 km and between Kalka to Shimla it is around 150 km.

Despite high thermal and other environmental differences between close localities at very different altitudes, genetic adaptation to high-altitude conditions appeared weak in two dung fly species (Blanckenhorn, 1997). The phenotypic plasticity of the populations was extensive, while genotypic differences were probably reduced by substantial gene flow across short distance over an altitudinal cline. Similarly, water loss rate showed high variation (3.7×) among populations, seasons, and acclimation treatments in *Glossina pallidipes* (Terblanche *et al.*, 2006), but most of the intraspecific variation was accounted for by within-generation phenotypic plasticity in response to temperature acclimation. Limitations of spreading and distribution of organisms in the tropics caused by high mountain ranges were reviewed by Ghalambor *et al.* (2006).

Genetics as well as ecophysiology of fruit flies have been thoroughly studied previously. Latitudinal clines in physiological tolerance to environmental stress have been documented in wild populations of *Drosophila melanogaster* in several continents (James *et al.*, 1995; James and

Partridge, 1995; Azevedo *et al*., 1996; Hoffmann *et al.*, 2002; Watada *et al.*, 1986; Capy *et al*., 1993; Hallas *et al.*, 2002). The wide geographical distribution range of *D. melanogaster* is exceptional, providing large opportunity for selection of strains adapted to local climate. However, fruit-flies generally tend to have wide distribution with low beta diversity among sites (Novotný *et al.*, 2007). The widespread existence of clinal variability suggests that the climate plays a key role in life history and physiology of the flies. On the other hand, altitudinal gradients in traits of *Drosophila* have been less well studied (Etges and Levitan, 2004; Sorensen *et al.*, 2001, 2005; Parkash *et al.*, 2005; Collinge *et al.*, 2006).

Heritable clines along latitude in eastern Australia have been established for quantitative traits of *D. melanogaster* including wing length/area, thorax length and cold and heat resistance. Expected clinal pattern for resistance to desiccation has not been supported (Hoffmann and Weeks, 2007). Desiccation resistance was higher under summer compared with winter simulating laboratory conditions, but this trait did not exhibit clinal variation across a latitudinal range of 27 degrees in eastern Australia (Hoffmann *et al.*, 2005). In cool, high latitude regions, *Drosophila* species are often active throughout the day, whereas in lower hot latitudes, they are most active early and late during the daylight hours. Indeed, a heritable latitudinal cline in circadian activity has been found suggesting avoidance of potential desiccation and/or thermal stress (Simunovic and Jaenike, 2006).

In this study, we examine a morphological (pigmentation) and an ecophysiological (desiccation resistance) trait in *D. melanogaster* collected from high and low altitude sites at five locations along the Indian subcontinent. We directly assess the relative importance of altitudinal and latitudinal clinal differences for the adaptations of local populations. We, therefore, test the hypothesis that populations of *D. melanogaster* are adapted to local conditions for morphological and physiological traits. In addition, we evaluate potential correlations between these traits among populations. Pigmentation was studied because previous studies (Munjal *et al.*, 2007) found it to be positively correlated with both the latitude and altitude of original populations (not original populations). Although a clinal variation of desiccation tolerance has not been previously found in other continents (Hoffmann and Weeks, 2007), our preliminary data suggested adaptive pleiotropic effects of melanisation in coping with problems related to thermal balance as well as water balance in *D. melanogaster*.

## Material and Methods

From each collection site, about 80–90 wild caught individuals were obtained using banana bait traps. Isogroups were set up from field collected individuals. All collections were done in winter months (Nov–Dec, 2004) when flies are commonly available throughout India. The flies were collected from five pairs of low and high altitudinal sites at five different latitudes (Calicut/ 11°15’ and Coonoor/ 11°21’; Ratnagiri/ 16°59’ and Mahabaleshwar/ 17°56’; Deesa/ 24°12’ and Mt. Abu/ 24°36’; Bareilly/ 28°22’ and Nainital/ 29°24’; Chandigarh/ 30°44’ and Shimla/ 31°06') along the Indian sub-continent (Figure 1). Except for the Calicut (coastal region) site, the rest of the collection sites were inland. The sampling sites were characterised by their latitude, altitude, longitude, average temperature and relative humidity. The climatic data for the geographical sites were obtained from climatological tables of Meteorological Department of India which represent last 30 years averages.

Flies of each population were reared on cornmeal sugar medium inoculated with live yeast. All experiments were performed with F1 generation adults. For each population, ten randomly chosen individuals (females only) from each replicate (a total of four replicates for each isogroup) of each isogroup (a total of four isogroup) were simultaneously analyzed for pigmentation scores and desiccation tolerance.

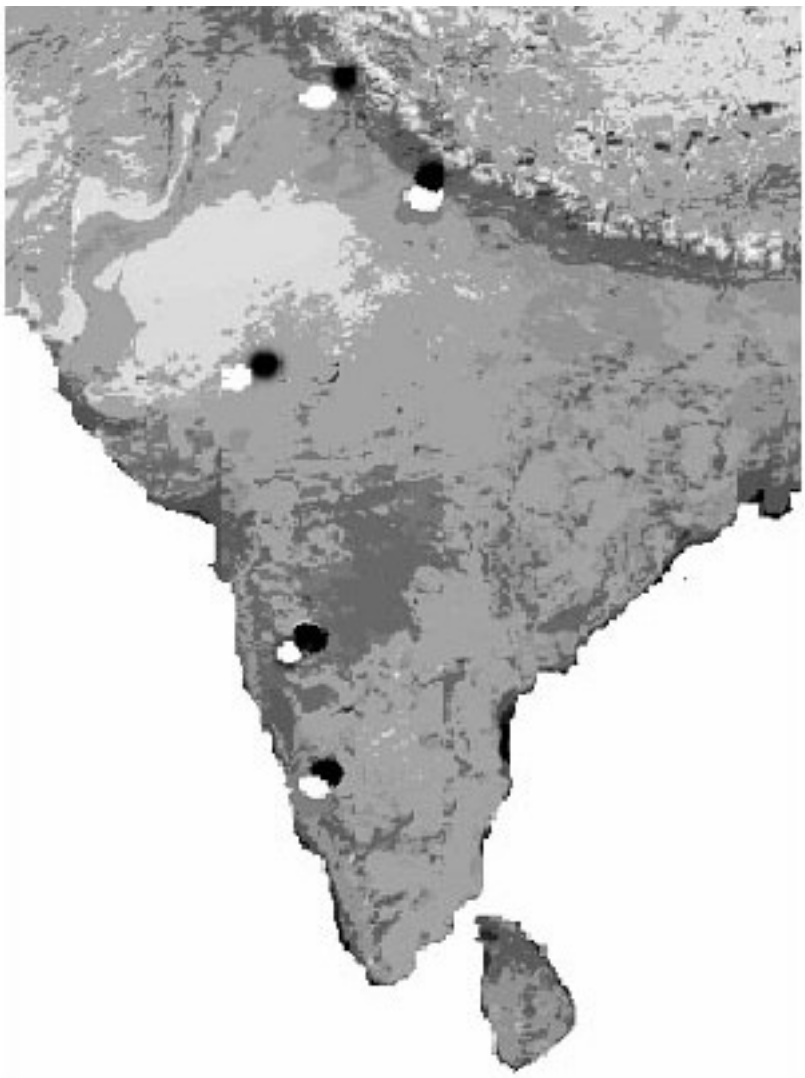

Figure 1. Map of India showing origin of populations of *Drosophila melanogaster*. Locations with black, and white circles correspond to highland and lowland sites.

For abdominal pigmentation and desiccation tolerance the methods of David *et al*. (1990) and Hoffmann and Parsons (1989) were followed. The degree of pigmentation was estimated from a lateral view of the female abdomens giving values ranging from 0 (no pigment) up to 10 (complete darkness) for each of the six visible segments. To improve the accuracy of the measurements, double blinded study (*i.e*., two persons did the pigmentation scoring and they did not know the origin of the population) was done and good repeatability observed.

For measuring desiccation tolerance (as survival time under low humidity conditions), ten individuals of each line were isolated in a dry plastic vial. These vials contained 2 g of silica gel at the bottom of each vial and were covered with a disc of plastic foam to create a <10% relative humidity environment. Four replicates were run for each isogroup to yield a total sample of n = 40. The vials were inspected hourly and the number of dead flies (completely immobile) was recorded (in order to confirm this vials was shaken twice to check the fly response). When the numbers of dead individuals approached 50%, vials were inspected every 30 minutes until all the flies had died.

ANOVA was undertaken to examine the effects of the latitude and altitude on the traits of the populations as well as the interactions between these effects. These factors were treated as fixed effects, because we deliberately selected latitude points along the Indian sub-continent and high/low altitude sites. Multiple regression analyses (Statistica 7) were carried out to examine associations between population trait means and the two macroclimatic variables: mean annual temperature and mean annual humidity and the influence of altitude and latitude. Under strong climatic selection, we might expect association between trait means and climatic variables regardless of the distance between populations.

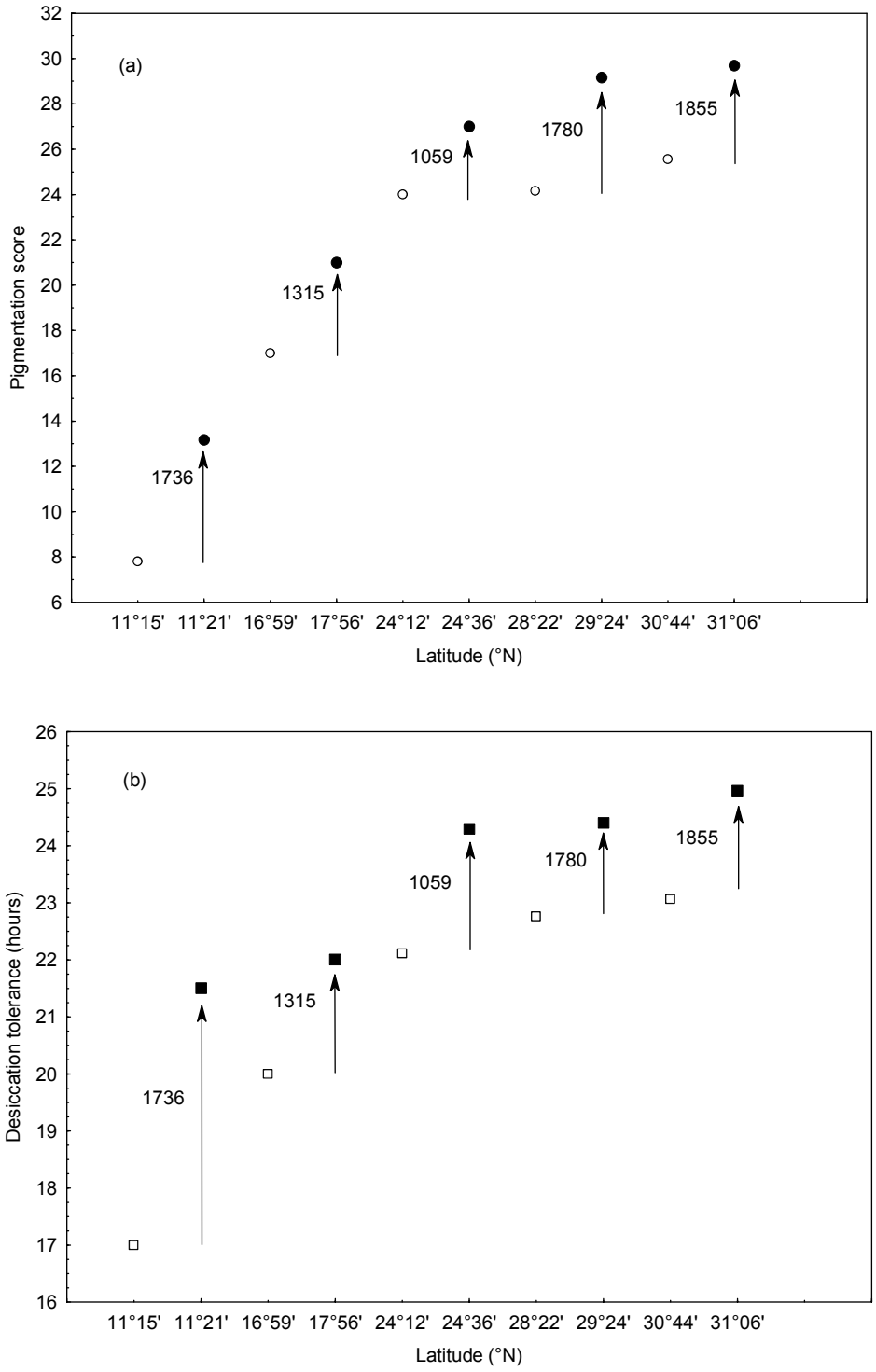


Figure 2. Trait variability along latitude for pigmentation sum (a) and desiccation tolerance (b). Numeric figures given besides arrows indicate altitudinal difference in meters between lowland and highland sites. All the lowland populations are represented in black circles or blank rectangles and highland populations in filled circles or filled rectangles.

## Results

The degree of pigmentation was dependent on latitude (one-way ANOVA: $F_{x,y} = 1877$, $p < 0.001$), altitude ($F_{x,y} = 864$, $p < 0.001$), and also latitude by altitude interaction ($F_{x,y} = 8.6$, $p < 0.01$). Flies from the northernmost regions showed highest pigmentation scores (Shimla: 29.70 ± 0.86 and Chandigarh: 25.70 ± 0.71). All highland populations showed stronger pigmentation than the corresponding lowland populations (Figure 2a).

Populations from the northern India had greater desiccation tolerance (Shimla: 24.97 ± 1.21 h and Chandigarh: 23.06 ± 1.31 h) than those from southern parts (Figure 2b) of India (Calicut: 17.00 ± 0.90 h and Coonoor: 21.50 ± 0.87 h, one-way ANOVA: $F_{x,y} = 246$, $p < 0.001$). All highland populations showed better desiccation tolerance compared to lowland populations localized nearby ($F_{x,y} = 406$, $p < 0.001$, interaction between latitude and altitude: $F_{x,y} = 15.7$, $p < 0.01$).

Significant correlation coefficients were obtained when the two traits of flies were pairwise correlated with geographic ($r = 0.41$ for Pigmentation vs. Altitude; $r = 0.61$ for Desiccation vs. altitude; $r = 0.93$ for pigmentation vs. latitude; $r = 0.81$ for desiccation vs. latitude) and climatic factors ($r = -0.65$ for pigmentation vs. temperature; $r = -0.82$ for desiccation vs. temperature; $r = -0.78$ for pigmentation vs. relative humidity; $r = -0.75$ for desiccation vs. relative humidity), except for longitude ('r' less than 0.26 for both the traits). Pigmentation and desiccation tolerance increased with altitude and latitude, and in the same time decreased with average temperature and relative humidity of the site of origin. Both temperature and humidity decreased with both altitude and latitude. Both traits were strongly correlated ($R^2 = 0.88$, $p < 0.0001$).

The correlation parameters further increased when all four significant geographic and climatic parameters were used in a common multiple regression ($R^2 = 0.95$, $p < 0.002$ for pigmentation, $R^2 = 0.89$, $p < 0.003$ for desiccation). However, latitude was a single significant factor among the four ones ($r = 0.74$) predictive for pigmentation, the other (including altitude) contributed only slightly for the correlation (Figure 3a). For desiccation, no single factor had a significant predictive contribution to the correlation. For this reason, we used forward stepwise method of regression, resulting in two significant predictive factors for desiccation – temperature ($r = –0.53$) and humidity ($r = –0.38$) (Figure 3b).

Genetic basis of trait variability was analyzed on the basis of repeatability across generations as well as by subjecting the isofemale line data to ANOVA. For the two traits (pigmentation and desiccation), repeatability was further analyzed in two more generations (F3 and F7 added to F1). Correlation across generations (F1-F3, F3-F7 and F1-F7) showed highly significant values (0.91, 0.89, and 0.86 for pigmentation and 0.86, 0.80, and 0.83 for desiccation).

In order to explore potential associations between pigmentation and desiccation resistance, preliminary experiments were set up with wild female flies from a single mid–altitude site (Barog/1680m a.s.l.; 30°44'N, 77°01'E). The flies were separated into two groups of high and low pigmentation. The two groups differed in their desiccation tolerance (data not shown). This was further confirmed on wild females (n = 60+65) collected in January. Within this sampling location, there was, therefore, a significant positive correlation between darker flies and longer desiccation tolerance and paler flies and shorter desiccation tolerance ($r = 0.79$, $p < 0.003$).

## Discussion

In the present study, populations of *D. melanogaster* sampled in a range of latitudes (11–31°N) and altitudes (0–2200 m a.s.l.) within the Indian sub-continent showed significant phenotypic differences in the degree of abdominal melanic pigmentation and desiccation tolerance (survival

time). Although a clinal trend of desiccation tolerance with latitude has not been previously found in other continents (Hoffmann and Weeks, 2007), the latitudinal pattern described here (strong increase of values of both traits with latitude) is consistent with previous studies done by Parkash and Munjal (1999) and Das (1995). High latitude sites were cooler and in the same time drier – an opposite situation to subtropical–temperate climatic regions.

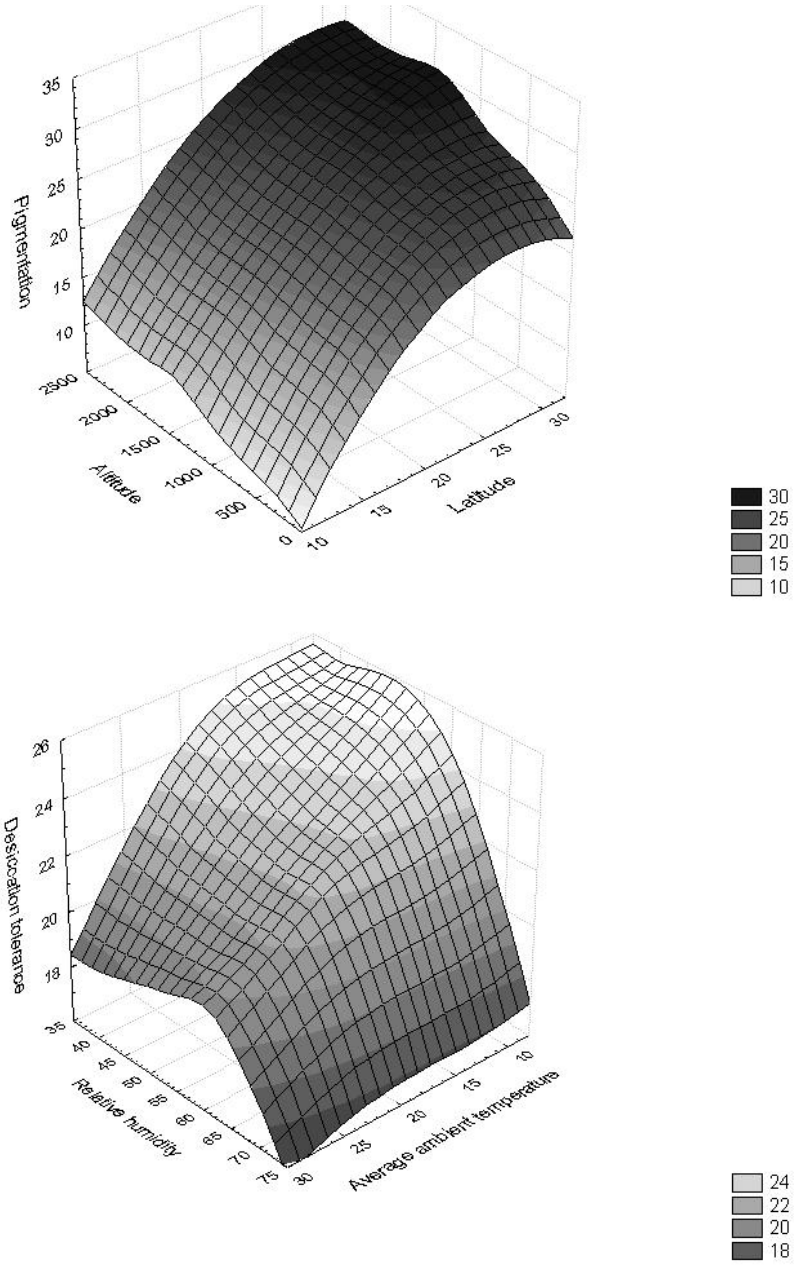


Figure 3. General trends of (a) increase of melanic pigmentation score of abdomen with latitude (°N) and altitude (m a.s.l.), and (b) increase of desiccation tolerance (hours of survival) with average ambient temperature (°C) and relative humidity (%) through Indian subcontinent in female *Drosophila melanogaster*. Surface = Distance Weighted Least Squares.

Higher altitude also hosted flies that were more resistant to desiccation and darker. This correlation of the traits with altitude was not so strong over the entire scale and when analysed by multivariate regression, but constant in pairwise comparison. Mountain sites were always cooler and also drier than correspondent lowland sites.

Because mean ambient temperature and relative air humidity are inversely correlated with both latitude and altitude, it might be possible to explain the observed variation as the result of adaptation to local environments. We tried to decide between the roles of either geographical coordinates or climatic factors by forward stepwise method of multiple regression. While the desiccation tolerance (survival time) increased with decreasing ambient temperature and air humidity, *i.e*., with climatic factors (Figure 3b), degree of pigmentation increased significantly only with latitude and slightly with altitude of the site of origin (geographical factors). The strong correlation of desiccation tolerance with climatic variables supports a direct role for climatic selection in generating altitudinal as well as latitudinal patterns in this trait. The selection effect on pigmentation may be non-direct or more complicated. Gene flow between sites with different climate may be more effective for pigmentation and penalized by selection for desiccation tolerance. Although proximate selection factors for the two traits may be different, they were repeatedly found to be strongly correlated to each other.

The role of body melanization for thermoregulation has been explored in several ectothermic animals, *e.g*., in ladybird beetles (*Adalia bipunctata*) and in *Colias* butterflies (de Jong and Brakefield, 1998; Ellers and Boggs, 2002, 2004). Clusella-Trullas *et al*. (2007) and Majerus (1998) have reviewed all such evidence in favor of thermal melanism. Heritable increase in melanization of abdominal segments with latitude and altitude in *D. melanogaster* agree with the hypothesis that black body surfaces better absorb solar radiation in order to maintain optimum body temperature under colder ambient temperatures. A disadvantage of being darker is that the animal may overheat more easily, but this is often compensated by behavioural mechanisms, as in the firebug (Honěk, 1986). The assumption that colour, thermal physiology and behaviour are coadapted has been supported (Clusella-Trullas *et al.*, 2007).

Direct evidence of different effects of solar radiation and thermal properties on melanics versus typical pale forms has been demonstrated in butterflies and in beetles (Roland, 1982; Guppy, 1986; Brakefield and Willmer, 1986). Significant differences in behavioural thermo regulation between dark and pale individuals appeared in females, but not in males of the grasshopper *Tetrix undulata* (Forsman *et al.*, 2002). Surprisingly, behavioural differences between individuals belonging to different colour morphs were genetically determined, rather than simply reflecting a response to different heating rates according to the actual body coloration.

In montane habitats, organisms have to cope with colder and usually wetter conditions. In all our sites throughout Indian subcontinent, the conditions at high altitudes were slightly drier. At low ambient temperature, the *absolute* water content of the ambient air is reduced even at the same *relative* humidity. Several investigations have considered interspecific differences in desiccation tolerance with mechanistic link to the problems of water balance (Zachariaseen, 1996; Gibbs *et al.*, 1997; Hoffmann and Harshman, 1999; Addo-Bediako *et al.*, 2001). However, similar studies on intraspecific level are limited (Eckstrand and Richardson, 1981). Amount of cuticular lipids varied among populations of *Glossina pallidipes* and was not correlated with prevailing temperatures, humidities, and vegetation density (Jurenka *et al.*, 2007).

The present study suggests that *D. melanogaster* females from high altitude locations survive desiccating conditions significantly longer than lowland populations, at least when geographically close pairs of localities are considered. This could be because of selection to reduced rates of water loss and an increased ability to tolerate dehydration. Numerous studies have considered the mechanistic basis of water balance under hot and dry environmental conditions in diverse taxa of

insects (Gibbs *et al.*, 1997). Neither a reduction in body size (which might be selected to reduce chances of freezing at high altitudes) nor corresponding increase of water loss (and thus probable reduction of survival time in desiccating conditions) were observed. Only the Nainital locality periodically experiences subzero temperatures.

The water loss rate can be strongly influenced by body size variation, while little direct support could be found for the assumption that there is a consistent melanism-body size tradeoff (Clusella-Trullas *et al.*, 2007). This problem is reviewed by Chown and Terblanche (2007). We did not observe a consistent pattern of body size in our material.

Moreover, desiccation tolerance was not linearly dependent on the ambient relative humidity, but rather steeply increased with decreasing humidity between 75–60%, and then remained unchanged (Figure 3b). Dependence of desiccation tolerance on average temperature was gradual.

Pleiotropic effect of genes on several phenotypic traits was found in some animals. In flour moth (*Ephestia kuehniella*) the effects of genes controlling melanism resulted in significant higher flight as well as walking activities in melanics than non-melanic genotype (Verhoog *et al.*, 1998). Since our finding of strong intercorrelation and heritability of pigmentation and desiccation tolerance has no direct explanation, we consider it as a hidden pleiotropic effect of genes. These findings are consistent with the hypothesis of multiple-trait coevolution demonstrated in females of a grasshopper (Forsman *et al.*, 2002), and suggest that the different colour morphs represent alternative evolutionary strategies. Since filial (F1) generation of wild-caught flies was studied in the laboratory, the revealed differences in measured traits among populations may be mostly related to heritable (genotypic) differences, while the role of phenotypic plasticity in processes of local adaptation (Driessen *et al.*, 2007) remains relatively unclear (see Hoffmann *et al.*, 2005; Terblanche *et al.*, 2006, for discussion).

In conclusion, analysis of populations of *D. melanogaster* demonstrates that body pigmentation is genetically variable and subjected to natural selection pressure under colder and drier conditions. Parallel and correlated to pigmentation was desiccation tolerance, while selection responses might slightly differ. Melanisation is a likely candidate for cuticular impermeability for reducing water loss under increasing dehydrating conditions along longitudinal and altitudinal transects. In almost all cases, altitudinal differences match patterns evident at the latitudinal level. The analysis of climatic factors has shown that temperature average and humidity can be responsible for maintaining genetic heterogeneity in the traits related to thermal and water balance.

References: Alpert, P., 2006, J. Exp. Biol. 209 (9): 1575–1584; Azevedo, R.B.R., V. French, and L. Partridge 1996, Evolution 50: 2338–2345; Baur, B., and C. Raboud 1988, J. Anim. Ecol. 57: 71–87; Blanckenhorn, W.U., 1997, Oecologia 109: 342–352; Capy, P., E. Pla, and J.R. David 1993, Genet. Sel. Evol. 25: 517–536; Chown, S.L., and J.S. Terblanche 2007, Adv. Insect Physiol. 33: 50–152; Clusella-Trullas, S., J.H. van Wyk, and J.R. Spotila 2007, J. Theor. Biol. 32: 235–245; Collinge, J.E., A.A. Hoffmann, and S.W. Mckenchnie 2006, J. Evol. Biol. 19: 473–482; Das, A., 1995, J. Zool. Syst. Evol. Research. 33: 84–87; Driessen, G., J. Ellers, and N.M.V. Straalen 2007, Eur. J. Entomol. 104(1): 39–46; Etges, W.J., and M. Levitan 2004, Biol. J. Linn. Soc. 81: 395–411; Forsman, A., K. Ringblom, E. Civantos, and J. Ahnesjo 2002, Evolution 56 (2): 349–360; Ghalambor, C.K., R.B. Huey, P.R. Martin, J.J. Tewksbury, and G. Wang 2006, Integrative Compar. Biol. 46 (1): 5–17; Hallas, R., M. Schiffer, and A.A. Hoffmann 2002, Genet. Res. 79: 141–148; Heath, D., and D.R. Williams 1979, *Life at High Altitude*. Edward Arnold, London; Hoffmann, A.A., and A.R. Weeks 2007, Genetica 129(2): 133–147; Hoffmann, A.A., A. Anderson, and R. Hallas 2002, Ecol. Lett. 5: 614–618; Hoffmann, A.A., J. Shirriffs, and M. Scott 2005, Func. Ecol. 19(2): 222–227; Honěk, A., 1986, Acta Entomologica Bohemoslovaca 83 (6): 411–417; James, A.C., and L. Partridge 1995, J. Evol. Biol. 8: 315–330; James, A.C., R.B.R. Azevedo, and L. Partridge 1995, Genetics 140: 695–666; Jurenka, R., J.S. Terblanche, C.J. Klok, S.L. Chown, and E.S. Krafsur 2007, Physiol. Entomol. 32 (3): 287–293; Majerus, M., 1998, *Melanism: Evolution in*

*Action*. Oxford University Press, Oxford, U.K.; Munja, A.K., D. Karan, P. Gibert, B. Moreteau, R. Parkash, and J.R. David 1997, Genet. Sel. Evol. 29(6): 601–610; Novotný, V., S.E. Miller, J. Hulcr, R.A.I. Drew, Y. Basset, M. Janda, G.P. Setliff, K. Darrow, A.J.A. Stewart, J. Auga, B. Isua, K. Molem, M. Manumbor, E. Tamtiai, M. Mogia, and G.D. Weiblen 2007, Nature 448(7154): 692–U8; Parkash, R., and A.K. Munjal 1999, J. Zool. Syst. Evol. Res. 37: 195–202; Parkash, R., P.K. Tyagi, I. Shrama, and S. Rajpurohit 2005, Physiol. Entomol. 30: 353–361; Simunovic, A., and J. Jaenike 2006, Evol. Ecol. Res. 8(5): 803–811; Sorensen, J.G., J. Dahlgaard, and V. Loeschcke 2001, Funct. Ecol. 15: 289–296; Sørensen, J.G., F.M. Norry, A.C. Scannapieco, and V. Loeschcke 2005, J. Evol. Biol. 18: 829–837; Starmer, W.T., and L.L. Wolf 1997, Biol. J. Linn. Soc. 62: 459–473; Terblanche, J.S., C.J. Klok, E.S. Krafsur, and S.L. Chown 2006, Am. J. Trop. Med. Hyg. 74(5): 786–794; Van't Land, J., P. Van Putten, B. Zwaan, A. Kamping, and W. Van Delden 1999, J. Evol. Biol. 12: 222–232; Watada, M., S. Ohba, and Y.N. Tobari 1986, Jpn. J. Genet. 61: 469–480.

## Analysis of the drosophilid *Zaprionus indianus* introduction in Brazil: contribution of esterase loci polymorphisms.

**Galego, Luís Gustavo C., and Claudia Márcia A. Carareto**. UNESP – São Paulo State University, Rua Cristóvão Colombo, São José do Rio Preto - São Paulo – Brasil; e-mail: carareto@ibilce.unesp.br.

### Introduction

The colonization of environmental niches by exotic species constitutes an unplanned experiment that provides historical information on ecology and evolution (Carroll and Boyd, 1992; Cox, 2004; Strauss *et al*., 2006). Such invasions allow comparing the biological responses to the new niche with those to the ancestral environments, by direct observation rather than by inference as it is usually done, as observed by Carroll (2007).

Recently, a drosophilid - *Zaprionus indianus* - was introduced in Brazil (Vilela, 1999). This is an African species (Tsacas, 1985) that had already spread over Asia several decades ago (Gupta, 1970; Amoudi, 1993; Parkash and Yadav, 1993a,b; Karan *et al*., 2000). Since then, a number of reports have indicated its occurrence in several regions of Brazil (Galinkin and Tidon, 2000; Moraes *et al*., 2000; Vilela *et al*., 2001; Toni *et al*., 2001; Tidon *et al*., 2003; Machado *et al*., 2005; Galego *et al*., 2006), in other South American countries (Goñi *et al*., 2001, 2002) and recently in the United States of America (Linde *et al*., 2006). In Brazil, *Z. indianus* was known as fig fly because of the loss of 50% of the fig crop in 1999 due to larvae infestation. The potential status of pest during the early period of its introduction stimulated Brazilian researchers to study *Z. indianus* aiming at understanding the biology, ecology, genetics, and population structure of this well succeeded invader. An adequate tool to investigate the genetic structure and the relationships between populations are the alloenzyme markers as, for instance, esterases, a multi-functional and heterogeneous group of enzymes that frequently show polymorphic loci in Drosophilidae (Brady and Richmond, 1990; Parkash and Yadav, 1993; Russell *et al.*, 1996; Dumancic *et al.*, 1997; Nascimento and Bicudo, 2002; Campbell *et al.*, 2003; Machado *et al*., 2005; Galego *et al.,* 2004, 2006).

In this study, populations from 22 regions of Brazil were sampled, so as to propose a model of *Z. indianus* introduction in the country, using as a reference the genetic structure of these populations by means of data on esterase polymorphisms. The polymorphic Est3 locus was used as marker,

which present four alleles ($Est3^1$, $Est3^2$, $Est3^3$, $Est3^4$) in most Brazilian populations (Galego *et al.,* 2006).

Table 1. Populations of *Z. indianus* sampled for this study, with their regions, geographic location and sample collector.

| Region | Locality | Geographic location | Collector |
|---|---|---|---|
| *State of São Paulo* (SP) | Mirassol | 49º30´W/20º47´S | Galego, LGC |
| | Onda Verde | 49º30´W/20º62´S | Granzotto, A |
| | São José do Rio Preto | 49º22´W/20º49´S | Galego, LGC |
| | Itatiba | 46º50´W/23º00´S | Galego, LGC |
| | Ilhabela | 45º21´W/23º46´S | Fazza, AC |
| | Paulo de Faria | 49º30´W/20º62´S | Granzotto, A |
| | São Paulo | 46º50´W/23º31´S | Setta, N |
| | Paraibuna | 45º41´W/23º26´S | Galego, LGC |
| | Maresias | 45º21´W/23º21´S | Galego, LGC |
| | Rio Claro | 44º08´W/22º43´S | Carareto, CMA |
| | Ibirá | 49º14´W/21º04´S | Galego, LGC |
| | Olímpia | 48º54´W/20º44´S | Galego, LGC |
| | Sud Menucci | 50º55´W/20º41´S | Lofego, AC |
| *Southeast (Except to SP) and Centerwest Regions* (SE+MW) | Alfenas (MG) | 46º10´W/21º20´S | Galego, LGC |
| | Belo Horizonte (MG) | 43º56´W/19º55´S | Galego, LGC |
| | Córrego Danta (MG) | 45º55´W/19º24´S | Galego, LGC |
| | Poços de Caldas (MG) | 46º33W/21º47´´S | Machado et al. (2005) |
| | Rio de Janeiro (RJ) | 43º12´W/22º54´S | Bitner-Mathé, B |
| | Brasília (DF) | 47º55´W/15º46´S | Tidon, R |
| *South Region* (S) | Porto Alegre (RS) | 51º13´W/30º01´S | Valente, V |
| | Santa Maria (RS) | 53º48´W/29º41´S | Valente, V |
| | Florianópolis (SC) | 48º32´W/27º35´S | Hoffman, P |
| *Northeast Region* (NE) | Jequié (BA) | 40º04´W/13º51´S | Luizon, MR |
| | Lençóis (BA) | 41º23´W/12º33´S | Machado et al. (2005) |
| | Beberibe (CE) | 38º53´W/04º10´S | Machado et al. (2005) |

SP: São Paulo state; SE: Southeast; MW: Mid-West; S: South; NE: Northeast.

## Material and Methods

The collections of *Z. indianus* were performed with traps containing attractive baits made of banana and biological yeast, as described by Galego *et al*. (2006). The populations collected were joined in geographic groups (Table 1), as the Brazilian state of collection localities: Northeast, Mid-West, Southeast and South, with exception of the São Paulo state that was considered as a block out of Southwest region, on account of the great number of collections performed in this state. The Asia and Africa data used in the analyses were those published by Machado *et al*. (2005). The esterases were detected as described by Galego *et al.* (2006). The alloenzyme data were analyzed using the computer softwares TFPGA version 1.3 (Miller, 1997) and Genetix version 4.05.2 (Belkier *et al*., 1996). The allele and genotype frequencies of the polymorphic loci and the observed ($H_O$) and expected ($H_E$) heterozygosity were determined with TFPGA. UPGMA analysis (Swofford and Olsen, 1990) was performed using the same software. Genetic distances (Nei, 1978) were

determined by Genetix. Excel and Minitab softwares were used for the multivariate analyses of genetic data.

**Results and Discussion**

Allele frequencies of Est3 locus (Figure 1) ranged between 0.471 (South) and 0.150 (Northeast) for $Est3^1$, 0.362 (Northeast) and 0.183 (São Paulo State) for $Est3^2$, 0.380 (Northeast) and 0.127 (Southeast + Mid-west) for $Est3^3$ and 0.345 (São Paulo State) to 0.108 (Northeast) for $Est3^4$. In Asia and Africa, the frequency of these alleles ranged from, respectively, 0.361 to 0.403 for $Est3^1$, 0.472 to 0.238 ($Est3^2$), 0.167 to 0.285 ($Est3^3$) and 0 to 0.074 ($Est3^4$). The absence of allele $Est3^4$ in the Asian populations suggests that the propagule of *Z. indianus* that invaded Brazil may have come from Africa rather than from Asia, since the African lineages present this allele, even if at a low frequency (0.074). Reinforcing this hypothesis, David *et al*. (2006b), who studied quantitative traits related to body size, suggested that the propagule that colonized Brazil might have come from high-latitude African regions, due to similarities between the Brazilian populations and those of those latitudes.

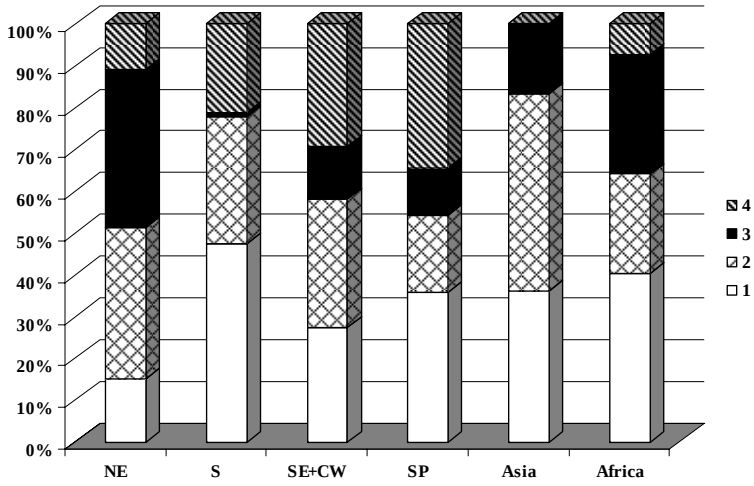


Figure 1. Allele distribution in the Est3 locus ($Est3^1$: 1, $Est3^2$: 2, $Est3^3$: 3, $Est3^4$: 4) from 21 Brazilian populations of *Z. indianus* clustered in four regions and Africa and Asia populations studied by Machado *et al*. (2005) (*). SP: São Paulo state; SE: Southeast; CW: Mid-west; NE: Northeast; S: South.

Multivariate analysis with genetic variables (Est3 allele frequencies, observed and expected heterozygosity) grouped samples of São Paulo, other Southeastern, Mid-western and Southern populations of *Z. indianus* together the African populations (Figure 2). The Northeastern and Asian populations showed less similarity to this cluster, and Asian is an outgroup of Brazilian and African populations. UPGMA analysis using the genetic distances (Figure 3) grouped the populations from Southeast together with those from Asia and Africa (ancestral populations). São Paulo city and Itatiba populations were closely related to African populations. The similarity showed by both analyses is congruent with the chronological reports of invasion along the Brazil: 1999, in São Paulo state, Southwestern and Mid-western regions (Vilela, 1999; Vilela *et al.*, 2000; Tidon *et al*., 2003), 1999-2000 in Southern (De Toni *et al*., 2001; Castro and Valente, 2001), and 2001 in Northeastern (Santos *et al.*, 2003; Machado *et al*., 2006) regions.

The data obtained in the present study indicate that the invasion of the Brazilian territory by *Z. indianus* is according to the theoretical invasion scenario 1 proposed by Facon *et al*. (2006) in which a change in the migration regime, possibly as a result of human activity, is responsible for the process of invasion. Our data corroborate, too, the scenario suggested by Machado *et al*. (2005) and David *et al*. (2006b) that a single invasion occurred by a unique and numerous propagule, followed by rapid expansion. Their idea is that the propagule probably came from tropical Africa and, once in Brazil,

spread from one locality to another by jumps, thanks to human transportation (David *et al*., 2006b). The African origin of Brazilian populations of *Z. indianus* was reinforced by analysis of chromosome inversion polymorphism (Ananina *et al*., 2006) and quantitative traits (David *et al*., 2006a,b).

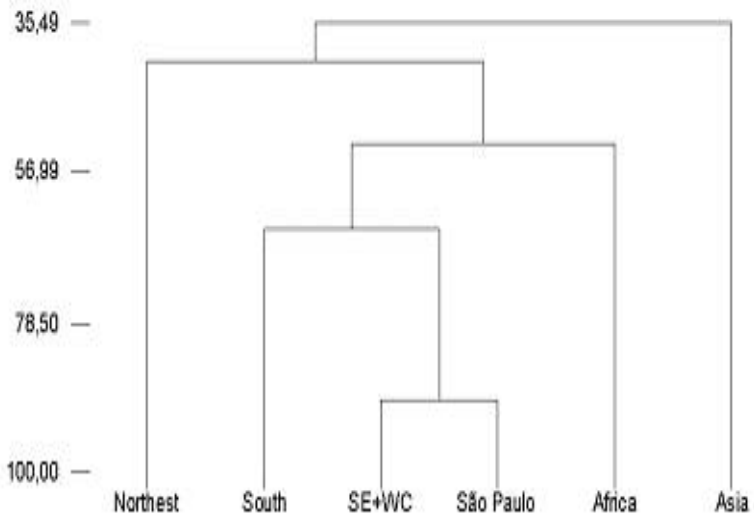


Figure 2. Multivariate analysis of *Z. indianus* populations using genetic variants regarding to Est3 locus (Est3 allele frequencies, observed and expected heterozygosity). Asia and Africa data from Machado *et al*. (2005). SE: Southest; CW: Mid-west.

The UPGMA and multivariate analyses here reported and particularly the common occurrence of allele Est3[4] in a population of African origin and in Brazilian populations corroborate with genetic data the hypothesis of African origin of *Z. indianus* that colonized Brazil. These analyses further indicate that the arrival of the invaders in Brazil occurred in the State of São Paulo, spreading over the country later on. Vilela *et al*. (2000) suggested that *Z. indianus* introduction occurred by plane, possibly due to presence of eggs and larvae in meals, but the conclusions of Machado *et al*. (2005), David *et al*. (2006a,b), and Ananina *et al*. (2006) indicate that the founder propagule was numerous. Accordingly, this hypothesis makes more probable the introduction by ships, considering that a numerous propagule demands a big reservoir as a fruit charges and the fruits international commercialization and transportation was mainly made by maritime waterway (França and Gondin, 1999, in http://www.bnb.gov.br/content/Aplicacao/ETENE/Rede_Irrigacao/ Docs/Fruticultura%20I-%20Uma%20visao%20geral%20do%20mercado.PDF). Taking also into consideration that Brazil imports fruit from Africa (http://www.mre.gov.br/index.php?option=comcontentandtask= categoryandsectionid=5andid=11andItemid=557), it is rather likely that the entrance has occurred by the Port of Santos, a port that accounts yearly for one fourth of all products traded by the country on the international market (Available at <http:// http://www.vivasantos.com.br/ 04/04a.htm>, accessed on 11/03/2007), which shows the importance of this port for the entrance of merchandise from other countries. Furthermore, a great part of the goods that arrive in Santos is distributed throughout the State of São Paulo and from there to the rest of the country.

It is probable that after the arrival, *Z. indianus* has spread over the State of São Paulo by highway transportation and from there to the whole country, mainly as a result of fruit commerce, as suggested by Tidon *et al*. (2003). The UPGMA and multivariate analyses support this scenario. In both, large blocks of populations from the State of São Paulo were grouped together with the ancestral populations from Africa. After its introduction, *Z. indianus* rapidly spread over the southeastern, southern and mid-western regions, as shown by the publication records (Vilela, 1999; Galinkin and Tidon, 2000; Moraes *et al*., 2000; Toni *et al*., 2001; Tidon *et al*., 2003), only reaching the northern and north-eastern regions later on. The structure of the Brazilian *Z. indianus* populations studied here does not suggest the occurrence of spontaneous and progressive spreading of the marginal populations. The UPGMA analyses clustered populations far from each other, which

suggests that the main way of spreading of this species in Brazil continues to be the commercial food transportation, which is one of the two possibilities raised by David *et al*. (2006b). The analyses also show that the colonization was more pronounced and early in areas with heavier highway traffic. Genetic characterization of populations of a colonizer species soon after its introduction, as it is the case of *Z. indianus* in South America, besides its relevance in offering a possibility to determine the source of the invasion and demographic parameters of the species, also represents a unique opportunity to follow-up the evolutionary dynamics of the invader species over time.

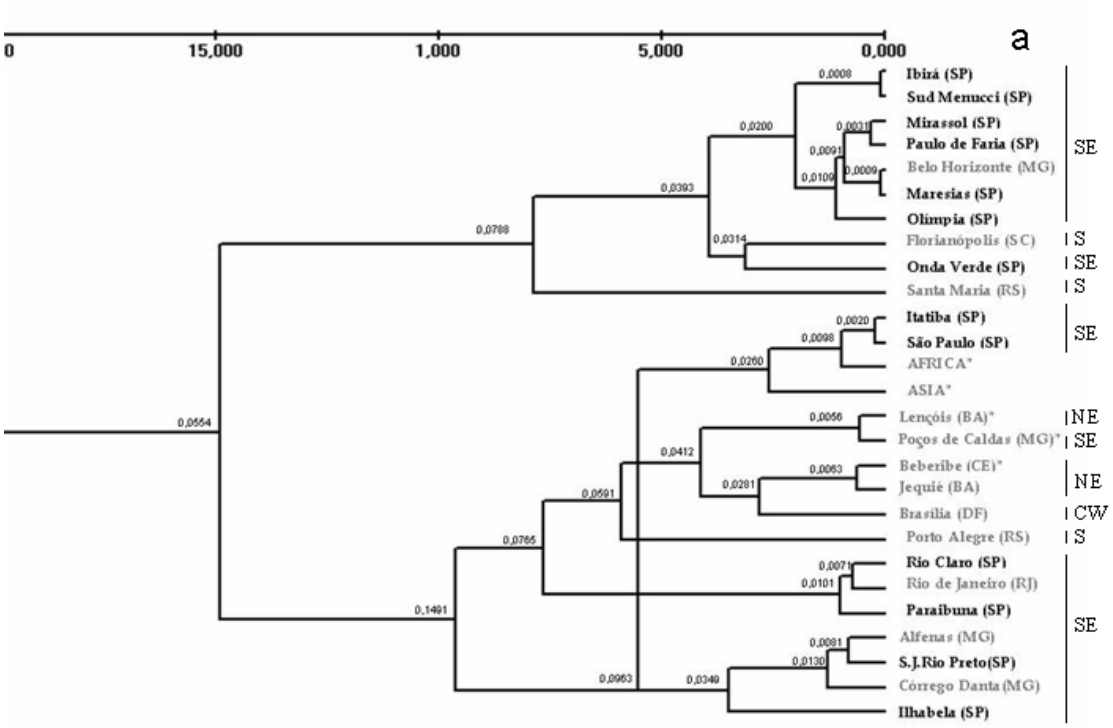


Figure 3. UPGMA analyses using the Nei´s genetic distances (1978) between 21 Brazilian populations and Asia and Africa populations of *Z. indianus*. The * indicate the populations studied by Machado *et al*. (2005). Localities in grey scale are outside São Paulo state. SP: São Paulo state; SE: Southest; CW: Mid-west; NE: Northest; S: South.

References: Amoudi, M.A., F.M. Diab, and S.S.M. Abou-Fannah 1993, Pakistan J. Zool. 25: 37-40; Ananina, G., C. Rohde, J. David, V.L. Valente, and L.B. Klaczko 2006, Genetica 131: 117-125; Belkhier, J., C. Segarra, A. Prevosti, and L. Serra 1996, GENETIX 4.04, Logiciel sous Windows pour la génétique dês populations.Montpellier, France; Brady, J.P., and R.C. Richmond 1990, Proc. Nat. Acad. Sci. USA 87: 8217-8221; Campbell, P.M., G.C.Q. Robi, L.N. Court, S.J. Dorrian, R.J. Russell, and J.G. Oakeshott 2003, Insect Molecular Biology 12: 459-471; Carroll, S.P., 2007, 129: 193-204; Carrol, S.P., and C. Boyd 1992, Evolution 46: 1052-1069; Castro, F.L., and V.L.S. Valente 2001, Dros. Inf. Serv. 84: 15-17; Cox, G.W., 2004, *Alien Species and Evolution*. Island Press, Washington; David, J.R., L.O. Araripe, B.C. Bitner-Mathe, P. Capy, B. Goni, L.B. Klaczo, H. Legout, M.B. Martins, J. Vouidibio, A. Yassin, and B. Moreteau 2006a, Genetica 128(1-3): 109-122; David, J.R., L.O. Araripe, B.C. Bitner-Mathe, P. Capy, B. Goni, L.B. Klaczo, H. Legout, M.B. Martins, J. Vouidibio, A. Yassin, and B. Moreteau 2006b, Heredity 96: 53-62;

Dumancic, M.M., J.G. Oakeshott, R.J. Russell, and M.J. Healy 1997, Genetics 35: 251-270; Facon, B., B.J. Genton, J. Shykoff, P. Jarbe, A. Estoup, P. David 2006, Trends in Ecology and Evolution 21: 130-135; Galego, L.G.C., C.R. Ceron, and C.M.A. Carareto 2004, Revista UNORP: Saúde 3: 97-114; Galego, L.G.C., C.R. Ceron, and C.M.A. Carareto 2006, Genetica 126: 89-99; Galinkin, J., and R. Tidon 2000, Resumos do XXIII Congresso Brasileiro de Zoologia, 273-274; Gõni, B., P. Fresia, M. Calviño, M.J. Ferreiro, V.L.S. Valente, and L. Basso da Silva 2001, Dros. Inf. Serv. 84: 61-65; Gupta, J.P., 1970, Proceedings of the Indian Science Academy 36B: 62-70; Karan, D., S. Dubey, B. Moreteau, R. Parkash, and J.R. 2000, Genetica 108: 91-100; Linde, K., G.J. Steck, K. Hibbard, J.S. Birdsley, L.M. Alonso, and D. Houle 2006, Florida Entomologist 89(3): 402-403; Machado, T., A.M. Solé-Cava, J. David, and B.C. Bitner-Mathé 2005, Ann. Soc. Fr. 41: 7-13; Miller, M.P., 1997, Tool for population genetic analyses – TFPGA – 1.3: A Windows program for the analysis of allozyme and molecular population genetic data. Computer software distributed by author; Moraes, E.M., R. Mateus, and F.M. Sene 2000, Abstract book – XXI International Congress of Entomology, 271; Nascimento, A.P., and H.E.M. Campos Bicudo 2002, Genetica 114: 41-51; Nei, M., 1978, Genetics 89: 583-590; Parkash, R., and J.P. Yadav 1993a, Hereditas 119: 161-173; Parkash, R., and J.P. Yadav 1993b, Dros. Inf. Serv. 72: 150-152; Russell, R.J., G.C. Robin, P. Kostakos, R.D. Newcomb, T.M. Boyce, K.M. Medveczky, and J.G. Oakeshott 1996, Insect Biochem. Mol. Biol. 26: 235-247; Santos, J.F., T.T. Rieger, S.R.C. Campos, A.C.C. Nascimento, P.T. Felix, S.V.O. Silva, and F.M.R. Freitas 2003, Dros. Inf. Serv. 86: 92-95; Strauss, S.Y., J.A. Lau, and S.P. Carroll 2006, Ecol. Lett. 9: 357-374; Swofford, D.L., and G.J. Olsen 1990, Phylogeny reconstruction. In: *Molecular Systematics* (Hillis, D.M., and C. Moritz, eds.). Sinauer Associates, Inc., Sunderland, MA; Tidon, R., D.F. Leite, and B.F.D. Leão 2003, Biological Conservation 112: 299-305; Toni, D.C., P.R.P. Hofmann, and V.L.S. Valente 2001, Biotemas 14: 71-85; Tsacas, L. 1985, Annls. Soc. Ent. Fr. 21: 343-344; Vilela, C.R., 1999, Dros. Inf. Serv. 82: 37-39; Vilela, C.R., E.P. Teixeira, and C.P. Stein 2000, Mosca-africana-do-figo, Zaprionus indianus (Diptera: Drosophilidae). In: *Histórico e impacto das pragas introduzidas no Brasil. Editora Holos, São Paulo.* (Vilela, E., R.A. Zucchi, and F. Cantor, eds.), 48-52.

## Toxicological tests of tibolone in *Drosophila melanogaster* wild type and Oregon-*flare* strains.

**Castañeda-Partida, L.,[1] A. Silva-Galicia[1], O.F. Jiménez[1], C.F. Román[1], A. Silíceo[1], A. Durán-Díaz[2], I.E. Dueñas-García[1] and M.E. Heres-Pulido[1].** [1]Lab. Genética Toxicológica, [2]Matemáticas. Dept. Biología. FES Iztacala-UNAM. Av. de los Barrios 1. Los Reyes Iztacala, Tlalnepantla. Edo. de México, México. E-mail*: lauracp@campus.iztacala.unam.mx*

### Introduction

For many years estrogen replacement has been used effectively to prevent osteoporosis and other climacteric complains such as vaginal dryness, hot flushes and mood symptoms that are related to the marked decline in plasma estradiol levels in postmenopausal women. Estrogen replacement, however, may lead to cell proliferation in the uterus and breast. In particular in the uterus, unopposed estrogen replacement treatment leads to an increased risk of developing uterine cancers. Therefore, an ideal therapy would be one that acts as an estrogen on the bone and the urogenital system, but not on the uterus or the breast (Bloom, 2006).

Tibolone (Livial, Org OD 14), produced by Organon (West Orange, NJ), is a synthetic steroid that has estrogenic, androgenic and progestagenic properties. It has been used in many countries for almost two decades, primarily for the prevention of postmenopausal osteoporosis and beneficial effects on menopausal and postmenopausal vasomotor, bone, vaginal and mood symptoms without affecting the endometrial, breast or cardiovascular systems (Falany *et al.,* 2004). Tibolone itself has no biological activity; its estrogenic, progestagenic and androgenic properties are the result of the activity of its metabolites on various tissues. After administration, tibolone is quickly metabolized into 3α-hydroxytibolone (3α-OH-tibolone) and 3β-OH-tibolone compounds, which are also present in an inactive, sulfated form (Modelska and Cummings, 2002). Sulfation is the major conjugation pathway involved in tibolone metabolism and may be significant in determining the tissue-specific effects of tibolone and its metabolites by modulating activity in situ. In general, sulfation inhibits the biological activity of steroidal compounds by prevention of binding to hormone receptors. Sulfation of tibolone and its metabolites is proposed to have an important role in regulating their tissue-specific effects. Selective inhibition of sulfatase activity by tibolone and its metabolites has been proposed as a mechanism for the specific effects of tibolone in breast and bone cells. Inhibition of sulfatase activity would decrease the conversion of the sulfates of tibolone and its metabolites to their unconjugated active forms. Also, the presence of specific sulfotransferase (SULT) isoforms in different human tissues may be involved in regulating tibolone activity in a tissue-specific manner (Falany *et al.,* 2004). A third compound, the Δ4-isomer, is formed from tibolone directly or from the 3β-OH-metabolites. The 3α- and 3β-OH-metabolites bind solely to the estrogen receptor (ER), whereas the Δ4-isomer has affinity for progesterone receptor (PR) and androgenic receptor (AR), but not ER (Modelska and Cummings, 2002). Since tibolone causes an increase in bone mineral density and is effective in the reduction of climacteric complaints but has no estrogenic effect on the endometrium, it is, therefore, considered to be the first member of a unique class of compounds described as selective, tissue estrogenic activity regulators or STEARs (Kloosterboer *et al.,* 2003). The concentrations of tibolone metabolites and the metabolic regulation of hormonal activities vary depending on tissue type. Tibolone given orally (2.5 mg) is rapidly absorbed, appearing in the plasma within 30 min and peaking in 4 h. Tibolone is metabolized mainly in the liver and is excreted in the urine and feces. The elimination half-life is approximately 45 h (De Gooyer *et al.,* 2001; Modelska and Cummings, 2002).

From 1996 to 2001 Beral *et al.* (2003) studied a million women between 50 and 64 years old confirming that 184 out of 18,186 ingesting tibolone developed breast cancer; besides that, the million women study revealed that this synthetic estrogen increased cancer risk in 1.45%. Other effects of tibolone in postmenopausal women, such as its influence on lipid metabolism, hemostasis, and sexual function, are less certain. In addition, the long-term effects of tibolone, particularly in reducing fractures, breast cancer, and cardiovascular disease, are still unknown (Modelska and Cummings, 2002).

The fruit fly *Drosophila melanogaster* is a multicellular eukaryote widely used in scientific research. It requires simple facilities, inexpensive culture media, it has a short generation time (about 10 days at 25ºC), it breeds a large number of individuals per generation, and *in vivo* assays can be done easily. *Drosophila* has also gained importance as a biological model in short term tests for toxicity screening of natural and synthetic compounds (Lewis *et al.,* 1998; Heres *et al.,* 2005; Dueñas *et al.,* 2005; Castañeda *et al.,* 2001). On the other hand, approximately 80% of human genes have a genetic homologue in *Drosophila melanogaster*. Most human genes are duplications and elaborations of their insect equivalents. In fact, not only individual domains and proteins but entire complexes and metabolic pathways are conserved between *Drosophila* and *Homo sapiens.* The knowledge from studying these *Drosophila* genes and the biological processes in which they

participate contributes to our understanding of the mechanisms of action of their human counterparts (Mackay; 2006; St. John and Xu, 1997).

With the concerns involving the use of estrogens in hormonal replacement therapy, there is a renewed interest in evaluating their potential side effects due to long-term use in postmenopausal women. In order to provide more information on tibolone toxic effects, we conducted toxicological tests in third instar larvae of *Drosophila melanogaster* wild type and Oregon-*flare* strains with regulated and highly constitutive CYP450 levels, respectively. This difference would indirectly indicate whether CYP450s are involved in tibolone metabolism in these *Drosophila* strains or not.

## Materials and Methods

*Chemical compounds*

Tibolone (Livial, Organon), (Figure 1).

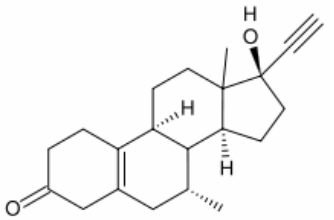


Figure 1. Molecular structure of tibolone.

*Strains*

Wild type (+/ +) *Drosophila melanogaster:* with regulated cytochrome P450 (CYP450) levels. Oregon-*flare* (ORR(1)/ ORR(2); *flr3/In(3LR)TM3, BdS)*: with highly constitutive CYP450 levels.

*Toxicological tests*

Adult flies were raised at 25ºC and aged in culture bottles containing mashed potato flakes medium and a conservative solution (Dueñas *et al*., 2001). Eggs from both strains were collected separately by shaking the flies into bottles (250 ml) containing an approximately 5 cm layer of fermenting fresh baker's yeast supplemented with sucrose (Graf and van Schaik, 1992). The egg collection bottles were then kept undisturbed in the dark for 8 h at 25ºC and a relative humidity of 65%. After removing the parental flies, the egg collection bottles were taken back to the incubator with the same conditions. Three days later, the 72 h ± 4 h, third instar larvae were washed out of the bottles with tap water at room temperature through a fine-meshed stainless steel strainer and thoroughly washed free of yeast while still in the strainer. Ten larvae were transferred to vials containing 0.5 g of mashed potato flakes prepared with 2 ml of Tibolone (Livial, Organon. CAS Nº 5630-53-5, 99% purity) at 0, 0.07, 0.156, 0.312, 0.625, 1.25 and 2.5 mg (therapeutic dose) in distillated water. Three replicates were made for each concentration in three independent chronic experiments for each strain. The treatment vials were kept at 25ºC and a relative humidity of 65% until pupation. The surviving flies were collected in alcohol 70% from the vials on days 10 to 12 after egg laying to quantify mortality. The mortality rates were plotted against tibolone concentration in order to calculate fitting by regression. Results were analyzed with one-way analysis of variance (ANOVA) where F test was calculated for statistically significant differences between concentrations and strains.

## Results

Figures 2 and 3 show the mortality rates of Oregon-*flare* and wild type (+/ +) third instar larvae exposed to tibolone. There was not a dose-response effect in any of the strains and mortality rates were not higher than 15-20%.

The ANOVA results for the Oregon-*flare* strain showed there was no interaction between control and experimental treatments ($P = 0.2487$). In contrast, the ANOVA results for the wild (+/+) strain showed statistically significant differences between experimental treatments ($P = 0.037$); however, it is not possible to assure there is a biological explanation to this because of the standard

deviations. Finally, the ANOVA results between strains did not yield significant differences (P = 0.1315).

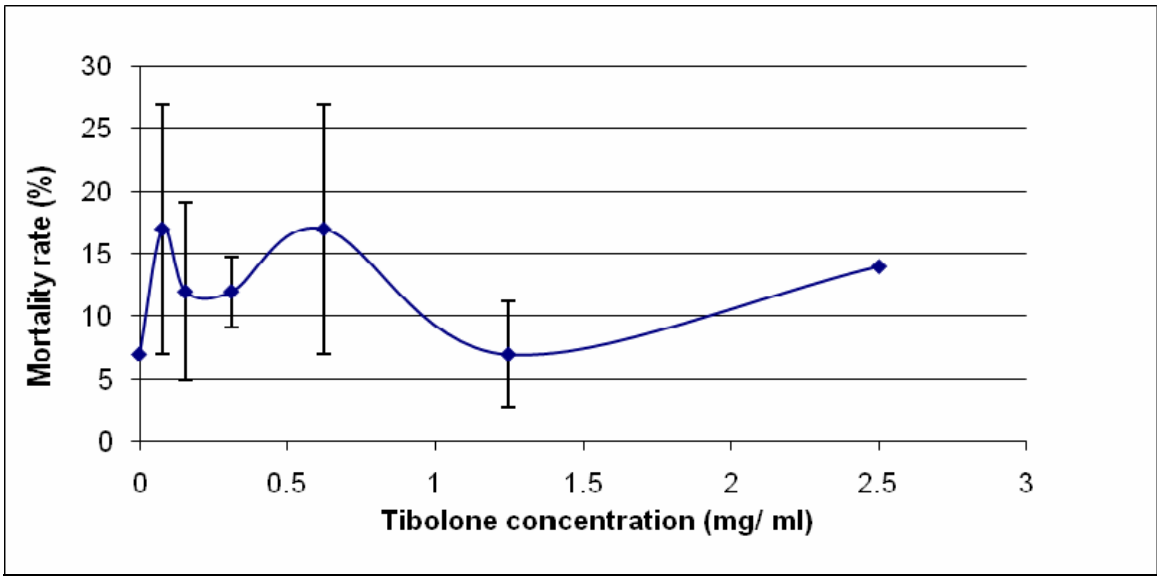


Figure 2. Mortality rate in Oregon-*flare* third instar larvae exposed to tibolone.

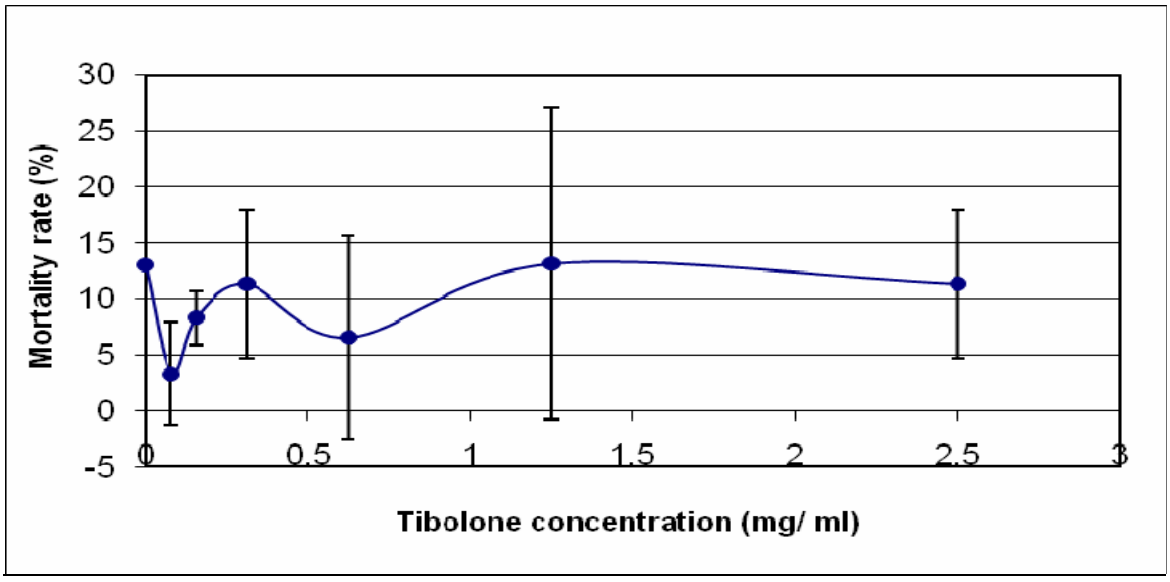


Figure 3. Mortality rate in wild type (+/+) third instar larvae exposed to tibolone.

Figure 4 shows the mortality curves for both *Drosophila* strains displaying a "mirror" image.

The main explanation to this would be the regulated CYP450 levels in the wild type (+/+) strain and the highly constitutive levels of these drug metabolizing enzymes in the Oregon-*flare* strain. All along the tested concentrations of tibolone the strains seem to respond in a different manner with respect to each other in three different moments and not showing a dose-response effect. We did not conduct experiments with tibolone concentrations higher than 2.5 mg, the therapeutic

dose, in order to keep our objectives in an actual toxicological perspective. Unfortunately, the ANOVA results and the standard deviations do not allow to assure this "mirror" image represents a biological process in answer to tibolone exposure in these different *Drosophila* strains.

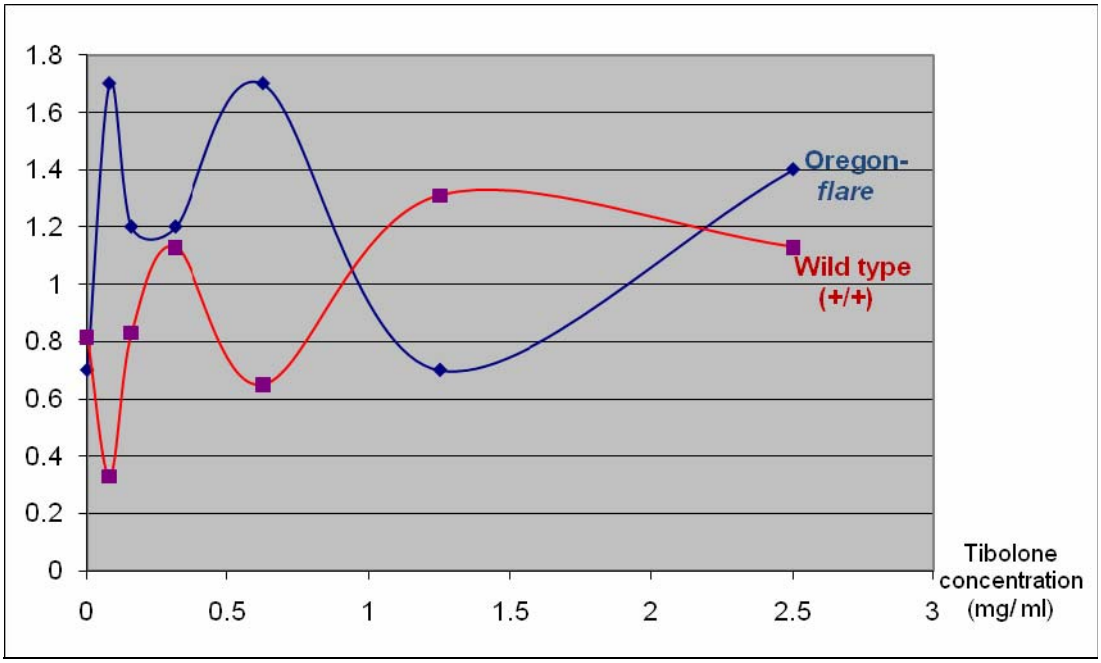


Figure 4. Mortality curves for Oregon-*flare* and wild type (+/+) third instar larvae exposed to tibolone.

**Discussion**

Toxicological tests were performed for toxicity screening of tibolone, a synthetic estrogen widely used in hormonal replacement therapy. Third instar larvae of the *Drosophila* Oregon-*flare* and wild type (+/+) strains were chronically exposed to the compound until pupation. The results seemed to show differences that could be accounted for by the CYP450 levels in these strains. From this point of view, the lowest concentration would have been biotransformed into a toxic metabolite by the constitutive CYP450s in Oregon-*flare* causing a relative mortality. In the case of the wild type (+/+) strain with regulated CYP450 levels, the lowest concentration of tibolone would have not been enough to trigger a cellular response (*i.e.*, CYP450s synthesis). Should this be true, the CYP450 enzymes would be involved in tibolone metabolism in *Drosophila* and not Phase II sulfotransferases (SULTs) that regulate tibolone in humans (Falany *et al.*, 2004). However, the standard deviations do not validate this idea, proving there would be another metabolic pathway in *Drosophila* common to both strains and without a dose-response effect which is very interesting.

There also exists the question whether tibolone was actually ingested by the larvae and absorbed as a xenobiotic or just as one more molecule in the diet. It is well known estreoids do not exist in invertebrates; in turn, they present ecdysteroids that perform hormonal functions: metamorphosis modulation (Terashima *et al*., 2005; Kozlova and Thummel, 2002), embryonic development and oogenesis (Terashima *et al.,* 2005). Ecdysteroids are produced by the prothoracic glands or ring glands located in the brain (Terashima *et al*., 2005; Kozlova and Thummel, 2002).

One of the main ecdysteroids is ecdysone, the metamorphosis hormone that regulates embryonic development.

Anyhow, if tibolone was ingested and/or absorbed by the larvae, we do not know its metabolism in *Drosophila*. Therefore, we can only suggest it was possibly processed in the fat body and the Malpighian tubules, the insect organs associated with metabolism, the ability to respond to xenobiotics and excretion (King-Jones *et al*., 2006; Yang *et al.,* 2007). Many of the response genes to xenobiotic compounds are highly expressed in these organs, leading to the opinion that they play similar roles to those of the liver and kidney in mammals (McGettigan *et al.,* 2005; Yang *et al*., 2007). It is precisely in these organs where gen *drh96* expresses, belonging to a superfamily of hormone nuclear receptors (Fisk and Thummel, 1995); the gen codifies receptor DRH96, a molecule exclusively found in the nucleus, related to transcriptional response to xenobiotics; mutant strains for this gene show certain resistance to phenobarbital (King- Jones *et al.,* 2006). Even more, many of the genes that regulate DRH96 codify diverse members of detoxifying enzymes that are conserved from insects to humans: cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs), carboxylesterases, and UDP-glucuronosyl transferases (UGTs) (King-Jones *et al*., 2006).

In humans are present the *Steroid and Xenobiotic Receptor* (SXR*)* and the *Constitutive Androstane Receptor* (CAR) ortholog transcription factors to DHR96. These bind directly to endogen as well as to exogen lipophilic compounds to promote transcription of genes that codify Phase I and II enzymes, such as CYP450s, GSTs, UGTs, and SULTs (King- Jones *et al.,* 2006).

In view of these physiological similarities between organs and other molecular structures of insects and mammals, it is possible that tibolone had been absorbed and processed by similar metabolic pathways.

In women, tibolone activation is attributed to sulfation carried out by certain SULTs of Phase II (Falany *et al*., 2004). The parental molecule is sulfated (inactive form) mainly in the liver and distributed through the blood vessels to all the body (De Gooyer *et al.,* 2001); and since sulfation inhibits the biological activity of chemical compounds by preventing their binding to their receptors (Falany *et al.,* 2004), the titers of SULTs in the cells of a given tissue are decisive for the activation, explaining tibolone´s tissue specificity (De Gooyer *et al.,* 2001). It is thought tibolone has no activity on mammary tissue, because sulfatase activity is inhibited there (Falany *et al*., 2004; Modelska and Cummings, 2002).

So there is a minimal probability that tibolone has a toxic/genotoxic effect in mammary tissue cells which could lead to cancer. However, tibolone ingestion may extend at least a decade and, therefore, is more likely to cause some renal pathology.

Every day, each kidney filters approximately 1,700 L of blood, concentrating refuse residues in almost 1 L of urine with constant exposure to toxic substances that make them susceptible to lesions. Actually, nephropathy or nephritis can derive from excessive consumption of drugs such as analgesics (MedlinePlus, 2007).

## Conclusion

Tibolone, at therapeutic doses, did not result toxic to *Drosophila* Oregon-*flare* and wild type third instar larvae, producing low mortality rates between 15-20% without a dose-response relation. This is good news for premenopausal and postmenopausal women undergoing chronical hormonal replacement therapy; however, special attention still must be paid to its effects on uterus, mammary glands, liver, and kidneys among other organs and systems.

Finally, it can not be affirmed from our data that the CYP450 enzymes are involved in tibolone metabolism, because no statistically significant differences were observed between

*Drosophila* Oregon-*flare* and wild type strains; the resulting standard deviations do not allow to clearly establish the biological effect of this synthetic estrogen on the larvae.

References: Beral, V., (Million Women Study Collaborators) 2003, The Lancet 362: 419-27; Blom, M.J., M. Groot Wassink, M.E. De Gooyer, A.G.H. Ederveen, H.J. Kloosterboer, J. Lange, J.G.D. Lambert, and H.J.Th. Goos 2006, J. of Ster. Bio. and Mol. Biol. 101: 42–49; Castañeda, P.L., G.L.E. Muñoz, D.A. Durán, P.M.E. Heres, and G.I.E y Dueñas 2001, Dros. Inf. Serv. 84: 44-47; De Gooyer, M.E., G.T. Overklift Vaupel Kleyn, K.C. Smits, A.G.H. Ederveen, H.A.M. Verheul, and H.J. Kloosterboer 2001, Mol. and Cel. Endocr. 183: 55-62; Dueñas, I.E.; M.E. Heres, P.L. Castañeda, and U. Graf 2002, Dros. Inf. Serv. 84: 166; Dueñas-García, I.E., V. Vega-Contreras, J.C. Gómez-Luna, L.F. Santos-Cruz, L. Castañeda-Partida, A. Durán-Díaz, and M.E. Heres-Pulido 2005, Dros. Inf. Serv. 88: 83-89; Falany, J.L., N. Macrina, and C.N. Falany 2004, J. of Ster. Bio. and Mol. Biol. 88: 383-391; Fisk, G.J., and C.S. Thummel 1995, Proc. Natl. Acad Sci., 92: 10604-10608; Heres-Pulido, M.E., G. Ávila-Acevedo, H. Dionicio-López, G.L.B. Hernández-Portilla, L. Castañeda-Partida, A.M. García-Bores, G. Martínez-Cortés, S.L. Cárdenas, G. Cerón, K. Tavera-Briseño, A. Vargas-Vera, and I.E. Dueñas-García 2005, Dros. Inf. Serv. 88: 103-107; Graf, U., and N. van Schaik 1992, Mutat. Res. 271: 59-67; King-Jones, K., M.A. Horner, G. Lam, and C.S. Thummel 2006, Cell Metabolism 4: 37-48; Kloosterboer, H.J., and A.G.H. Ederveen 2003, J. Steroid Biochem. Mol. Biol. 83: 157–165; Kozlova, T., and C.S. Thummel 2002, Development 129: 1739-1750; Lewis, D.F.V., C. Loannides, and D.V. Parke 1998, Environ. Health Pespect. 106: 633-641; Mackay, T.F.C., and R.R.H. Anholt 2006, Annu. Rev. Genomics Hum. Genet. 7: 339–67; McGettigan, J., R.K.J. McLennan, K.E. Broderick, L. Kean, A.K. Allan, P. Cabrero, M.R. Regulski, V.P. Pollock, G.W. Gould, S.-A. Davies, and J.A.T. Dow 2005, Insect Biochem. Mol. Biol. 35: 741–754; MedlinePlus 2007, http://www.nlm.nih.gov/medlineplus/spanish/ency/article/000464.htm; Modelska, K., and S. Cummings 2002, Clin. Endocrinol. Metab. 87: 6–23; St. John, M.A.R., and T. Xu 1997, Am. J. Hum. Gen. 61: 1006-1010; Terashima, J., K. Takaki, S. Sakurai, and M. Bownes 2005, J. of Endocrinology 187: 69-79; Yang, J., C. McCart, D.J. Woods, S. Terhzaz, K.G. Greenwood, R.H. Ffrench-Constant, and J.A.T. Dow 2007, Physiol. Genomics 30: 223-231.

## A novel record of Drosophilidae species in the Cerrado biome of the state of Mato Grosso, west-central Brazil.

**Blauth, Monica L.,[1] and Marco S. Gottschalk[2]**. [1]Universidade do Estado de Mato Grosso (UNEMAT), *Campus* de Tangará da Serra, Departamento de Ciências Biológicas; [2]Universidade Federal do Rio Grande do Sul (UFRGS), Departamento de Genética, Laboratório de *Drosophila.*

### Introduction

Systematic studies on the ecology and distribution of Drosophilidae species in Brazil started in the 1940's (Dobzhansky and Pavan, 1943, 1950; Pavan, 1959). From then on, research groups were formed to investigate Drosophilidae species in different localities in Brazil, especially in the south-east and Atlantic Forest. More recently, by the end of the 1990's, systematic studies were conducted also in west-central Brazil, more specifically in the Cerrado biome (Tidon *et al.*, 2003, 2005; Tidon, 2006), in southern Brazil, in the Atlantic Forest biome (De Toni and Hofmann, 1995; Schmitz *et al.*, 2007; Gottschalk *et al.*, in press), and in the north, in the Amazonian biome (Martins,

1987, 2001).

Of the 3,952 Drosophilidae species described (Bächli, 2007), 308 have been recorded in Brazil (M.S. Gottschalk, personal communication), the majority of which are in south-eastern Brazil (Tidon-Sklorz and Sene, 1999; Medeiros and Klaczko, 2004).

Table 1. List of species with records in the state of Mato Grosso and respective references.

| Species reccorded in Mato Grosso, Brazil | References |
|---|---|
| *Chymomyza procnemis* (Williston, 1896) | Kahl (1917) |
| *Drosophila aguape* Val & Marques, 1996 | Val & Marques (1996) |
| *D. bocainensis* Pavan & Cunha, 1947 | Pavan (1959) |
| *D. capricorni* Dobzhansky & Pavan, 1943 | Pavan (1959), Dobzhansky & Pavan (1950) |
| *D. eleonorae* Tosi, Martins, Vilela & Pereira, 1990 | Tosi *et al.* (1990) |
| *D. equinoxialis* Dobzhansky, 1946 | De Toni *et al*. (2005) |
| *D. fumipennis* Duda, 1925 | Pavan (1959), Dobzhansky & Pavan (1950) |
| *D. immigrans* Sturtevant, 1921 | Dobzhansky & Pavan (1950) |
| *D. maculifrons* Duda, 1927 | Pavan (1959), Dobzhansky & Pavan (1950) |
| *D. malerkotliana* Parshad & Paika, 1964 | Val & Marques (1996), De Toni *et al*. (2005) |
| *D. mediostriata* Duda, 1925 | Pavan (1959), Dobzhansky & Pavan (1950) |
| *D. nebulosa* Sturtevant, 1916 | Pavan (1959), Dobzhansky & Pavan (1950), Val & Marques (1996) |
| *D. neomorpha* Heed & Wheeler, 1957 | De Toni *et al*. (2005) |
| *D. pallidipennis* Dobzhansky & Pavan, 1943 | Dobzhansky & Pavan (1950) |
| *D. paranaensis* Barros, 1950 | Vilela (1983) |
| *D. parthenogenetica* Stalker, 1953 | De Toni *et al*. (2005) |
| *D. paulistorum* Dobzhansky & Pavan *in* Burla *et al.*, 1949 | De Toni *et al*. (2005) |
| *D. polymorpha* Dobzhansky & Pavan, 1943 | Pavan (1959) |
| *D. repleta* Wollaston, 1858 | Vilela (1983), Val & Marques (1996) |
| *D. saltans* Sturtevant, 1916 | De Toni *et al*. (2005) |
| *D. simulans* Sturtevant, 1919 | Dobzhansky & Pavan (1950), Val & Marques (1996), De Toni *et al*. (2005) |
| *D. sturtevanti* Duda, 1927 | Dobzhansky & Pavan (1950), Pavan (1959), Val & Marques (1996), De Toni *et al*. (2005) |
| *D. tropicalis* Burla & Cunha *in* Burla *et al.*, 1949 | De Toni *et al*. (2005) |
| *D. willistoni* Sturtevant, 1916 | De Toni *et al*. (2005) |
| *Rhinoleucophenga obesa* (Loew, 1872) | Malogolowkin (1946) |
| *Scaptodrosophila latifasciaeformis* (Duda, 1940) | Pavan (1959), Dobzhansky & Pavan (1950), Val & Marques (1996) |
| *Zaprionus indianus* Gupta, 1970 | David *et al*. (2006) |
| *Zygothrica bilineata* (Williston, 1896) | Grimaldi (1990) |
| *Z. microeristes* Grimaldi, 1987 | Grimaldi (1987) |
| *Z. prodispar* Duda, 1925 | Grimaldi (1987) |

In the state of Mato Grosso, few studies on Drosophilidae species have been conducted, and only 30 species have been recorded (Table 1). Three biomes are observed in the state: Amazon, Pantanal and Cerrado, which covers most of the state's area. Cerrado is the second Brazilian biome in size. The prevailing vegetal physiognomy is the savannah, though forest patches are observed, in which vegetation aspect and physiognomy are varied (Valente, 2006). Estimates say that the biome comes second in species richness in the world. Yet, the Cerrado is endangered by the increase in plantation and pasture areas. Nowadays the Cerrado is considered a global conservation hotspot (Myers *et al.*, 2000).

In an effort to add to the current knowledge of drosophilid distribution in the Cerrado biome, our study lists 18 records of Drosophilidae species in the Tangará da Serra region, state of Mato Grosso, west-central Brazil, new to the region.


Figure 1. South America map indicating the municipality of Tangará da Serra, state of Mato Grosso (MT), Brazil.

**Material and Methods**

Sample collections were carried out in the municipality of Tangará da Serra, state of Mato Grosso (14°04'38"S; 57°03' 45"W) (Figure 1), located between Parecis and Tapirapuã mountain ranges. The prevailing vegetation class is the Seasonal Semi-deciduous Forest. Nowadays, the patches of intact original vegetation are rare in the region. This area of the Cerrado biome undergoes the influence of the Amazonian biome, receiving an expressive number of animal and plant species native to the latter. Adult fly specimens were collected (i) on specially prepared banana baits (Tidon and Sene, 1988) and (ii) flying over fruit lying in the collection site, using an entomological net, whereas larvae were collected with fruit and flowers and reared in the laboratory upon emergence of adult flies. Plant species on which individuals were collected (adult or as immature) were also recorded. As for immature individuals, the material was sent to the laboratory and stored in a controlled temperature environment (21 ± 1°C) for one month. Emerging flies were retrieved daily. Table 2

shows the dates, locations and collection approaches adopted.

The characterization of the drosophilids collected was based on external morphologic traits and male genitalia, according to Wheeler and Kambysellis (1966).

Table 2 – Date, location, collection method, and plant species collected and used as feeding or breeding sites.

| | Date | Location | Collection method / resource collected |
|---|---|---|---|
| 01 | March 22nd 2007 | Garden of UNEMAT *Campus* | Flying over fruits of *Artocarpus heterophyllus* (Thunb.) (Moraceae) – jackfruit |
| 02 | March 22nd 2007 | Garden of UNEMAT *Campus* | Flying over fruits of *Persea americana* Mill. (Lauraceae) – avocado |
| 03 | March 25th 2007 | Garden of UNEMAT *Campus* | Flying over fruits of *Artocarpus heterophyllus* (Thunb.) (Moraceae) – jackfruit |
| 04 | March 31st 2007 | Forest patch near Agricultural School | Flying over fruits of *Bactris coccinea* Barb. Rodr. (Palmae) – red club palm |
| 05 | April 13th 2007 | Garden of UNEMAT *Campus* | Flying over fruits of *Malpighia glabra* L. (Malpighiaceae) – acerola fruit |
| 06 | 23rd to 26th April 2007 | Garden of UNEMAT *Campus* | Flying over fruits of *Persea americana* Mill. (Lauraceae) – avocado |
| 07 | 23rd to 26th April 2007 | Garden of UNEMAT *Campus* | Flying over fruits of *Pyrus* sp. (Rosaceae) – common pear |
| 08 | 24th April 2007 | Park in town center | Emerged from flowers of Convolvulaceae |
| 09 | 13th May 2007 | Vacant lot in downtown | Emerged from flowers of Convolvulaceae |
| 10 | 13th May 2007 | Park in town center | Emerged from *Buchenavia tomentosa* Eichler (Combretaceae) – mirindiba fruit |
| 11 | 14th May 2007 | Garden of UNEMAT *Campus* | Flying over fruits of *Artocarpus heterophyllus* (Thunb.) (Moraceae) – jackfruit |
| 12 | 18th May 2007 | Plantation near UNEMAT *Campus* | Inside flowers of *Cucurbita pepo* L. (Cucurbitaceae) – zucchini |
| 13 | 18th to 21st May 2007 | Forest patch near UNEMAT *Campus* | Collection using traps according to Tidon & Sene (1988) |
| 14 | 18th to 21st May 2007 | Pasture area near UNEMAT *Campus* | Collection using traps according to Tidon & Sene (1988) |

## Results and Discussion

A total of 1,187 individuals were collected. Specimens belonged to 35 species and four genera of Drosophilidae, the majority of which associated to fruit (Table 3). The only species associated to flowers were *D. denieri* and one *Drosophila* species belonging to the *bromeliae* group, not yet described — *Drosophila* sp.1 (H.J. Schmitz, personal communication). Schmitz and Hofmann (2005) state that *D. denieri* was recorded in Argentina, Uruguay, and in the Brazilian states of Rio Grande do Sul and Santa Catarina. The authors draw attention to the hypothesis that the apparently restricted distribution of *D. denieri* is due to inappropriate collection approaches. The first record of the species in the Mato Grosso section of the Cerrado biome confirms the notion.

Table 3. Species collected in the region of Tangará da Serra, state of Mato Grosso, Brazil. Numbers indicate collections mentioned in Table 2.

| | 01 | 02 | 03 | 04 | 05 | 06 | 07 | 08 | 09 | 10 | 11 | 12 | 13 | 14 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Drosophila* | | | | | | | | | | | | | | | |
| *atrata* group | | | | | | | | | | | | | | | |
| *D. calloptera* Schiner, 1868* | - | 1 | - | 5 | - | - | - | - | - | - | - | - | - | - | 6 |
| *bromeliae* group | | | | | | | | | | | | | | | |
| *Drosophila* sp.1 | - | - | - | - | - | - | - | 8 | - | - | - | - | - | - | 8 |
| *canalinea* group | | | | | | | | | | | | | | | |
| *D. canalinea* Patterson & Mainland, 1944* | 1 | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 2 |
| *cardini* group | | | | | | | | | | | | | | | |
| *D. cardini* Sturtevant, 1916* | 1 | 3 | 3 | - | - | - | - | - | - | 1 | - | - | 55 | 78 | 141 |
| *D. cardinoides* Dobzhansky & Pavan, 1943* | - | - | - | - | - | - | - | - | - | 52 | - | - | - | - | 52 |
| *D. polymorpha* Dobzhansky & Pavan, 1943 | - | - | 1 | - | - | 1 | - | - | - | - | - | - | - | - | 2 |
| *melanogaster* group | | | | | | | | | | | | | | | |
| *D. ananassae* Doleschall, 1858* | 1 | - | 1 | - | - | - | - | - | - | 1 | 3 | - | - | - | 6 |
| *D. kikkawai* Burla, 1954* | - | - | - | - | - | - | - | - | - | 1 | - | - | - | - | 1 |
| *D. malerkotliana* Parshad & Paika, 1964 | 23 | - | - | - | 1 | 1 | 1 | - | - | 12 | - | - | 83 | 11 | 132 |
| *D. melanogaster* Meigen, 1830* | - | - | - | - | - | - | - | - | - | - | 2 | - | - | 4 | 6 |
| *D. simulans* Sturtevant, 1919 | - | - | - | - | 5 | - | - | - | - | - | - | - | 2 | 5 | 12 |
| *repleta* group | | | | | | | | | | | | | | | |
| *D. hydei* Sturtevant, 1921* | 37 | - | 1 | - | - | - | - | - | - | - | - | - | - | 7 | 45 |
| *D. mercatorum* Patterson & Wheeler, 1942* | - | - | - | - | - | - | - | - | - | - | - | - | - | 3 | 3 |
| *D. moju* Pavan, 1950* | - | - | - | - | - | - | - | - | - | - | - | - | 2 | - | 2 |
| *D. paranaensis* (Barros, 1950) | - | - | - | - | - | - | - | - | - | - | - | - | 4 | 1 | 5 |
| *D. zottii* Vilela, 1983* | - | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 |
| *saltans* group | | | | | | | | | | | | | | | |
| *D. prosaltans* Duda, 1927* | - | - | 1 | - | - | - | - | - | - | - | 1 | - | 9 | 2 | 13 |
| *D. sturtevanti* Duda, 1927 | - | - | 1 | - | - | - | - | - | - | - | 3 | - | 25 | 3 | 32 |
| *Drosophila* sp.2 | - | - | - | - | - | - | - | - | - | - | 2 | - | 2 | - | 4 |
| *tripunctata* group | | | | | | | | | | | | | | | |
| *D. cuaso* Bächli, Vilela & Ratcov, 2000* | - | - | - | - | - | - | - | - | - | 25 | - | - | - | - | 25 |
| *D. mediopunctata* Dobzhansky & Pavan, 1943* | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - | 1 |
| *D. mediostriata* Duda, 1925 | - | - | - | 3 | - | - | - | - | - | 18 | - | - | 2 | 2 | 25 |
| *D. neoguaramunu* Frydenberg, 1956* | - | - | - | 1 | - | - | - | - | - | - | - | - | 1 | - | 2 |
| *D. paramediostriata* Townsend & Wheeler, 1955* | - | 7 | 1 | - | - | - | - | - | - | - | - | - | - | - | 8 |
| *D. trapeza* Heed & Wheeler, 1957* | - | - | 3 | - | - | - | - | - | - | - | - | - | 2 | - | 5 |
| *willistoni* group | | | | | | | | | | | | | | | |
| *D. nebulosa* Sturtevant, 1916 | - | - | - | - | 2 | - | 2 | - | - | 48 | - | - | 2 | 1 | 55 |
| *willistoni* subgroup | 4 | - | - | - | - | - | - | - | - | 7 | - | - | 4 | 1 | 16 |
| *Drosophila* sp.3 | - | - | - | - | - | - | 1 | - | - | - | - | - | - | - | 1 |
| not grouped | | | | | | | | | | | | | | | |
| *D. denieri* Blanchard, 1938* | - | - | - | - | - | - | - | - | 1 | - | - | 24 | - | - | 25 |
| *Drosophila* sp.4[†] | - | - | - | - | - | - | - | - | - | - | - | - | 2 | - | 2 |
| ***Rhinoleucophenga*** | | | | | | | | | | | | | | | |
| *R. obesa* (Loew, 1872) | - | - | - | - | - | - | - | - | - | - | - | - | 1 | - | 1 |
| *Rhinoleucophenga* sp.1 | - | 1 | - | - | - | - | - | - | - | - | - | - | - | - | 1 |
| *Rhinoleucophenga* sp.2 | - | - | - | - | - | - | - | - | - | - | - | - | - | 3 | 3 |
| ***Scaptodrosophila*** | | | | | | | | | | | | | | | |
| *latifasciaeformis* group | | | | | | | | | | | | | | | |
| *S. latifasciaeformis* (Duda, 1940) | 116 | 8 | 36 | - | 2 | 4 | 1 | - | - | - | 5 | - | 27 | 168 | 367 |
| ***Zaprionus*** | | | | | | | | | | | | | | | |
| *vittiger* group | | | | | | | | | | | | | | | |
| *Z. indianus* Gupta, 1970 | - | - | 1 | - | 14 | - | - | - | - | 33 | 1 | - | 23 | 105 | 177 |
| Total | 183 | 20 | 49 | 10 | 24 | 6 | 5 | 8 | 1 | 198 | 17 | 24 | 248 | 394 | 1187 |

species with first record in the state of Mato Grosso

[†]same *Drosophila* sp.4 mentioned in Gottschalk *et al*. (in press*)

The lack of collections in the region also explains why *D. calloptera* and *D. moju*, which distribute in the Amazon and in the Atlantic Forest (Val *et al*., 1981; De Toni *et al*., 2007; Gottschalk *et al*., in press), had not been recorded in the Cerrado before. In turn, *D. cuaso* distribution was confined to the Atlantic Forest, and the present record being the first outside that biome (Bächli *et al*., 2000).

The other species presenting novel records in the state of Mato Grosso are: *D. canalinea*, *D. cardini*, *D. cardinoides*, *D. hydei*, *D. mediopunctata*, *D. mercatorum*, *D. neoguaramunu*, *D. paramediostriata*, *D. prosaltans*, *D. trapeza* and *D. zottii*. However, these species had previously been described in other Cerrado regions (Sene *et al*., 1980; Martins, 2001; De Toni *et al*., 2007). For the first time in the state the following species of the *melanogaster* groups were recorded: *D. ananassae*, *D. kikkawai,* and *D. melanogaster*. These species are cosmopolitan, exotic to the Neotropical region, and are very common in open areas of all South American continent (Ferreira and Tidon, 2005; Gottschalk *et al*., in press).

In spite of the efforts by Brazilian researchers to obtain representative samples of Drosophilidae in the country's territory as a whole, the biogeographic and ecological studies conducted have failed to cover an expressive extension of the territory, leaving several areas totally unexplored or poorly sampled. Considering the current stage of degradation of most biomes, a considerable body of evidence on species distribution might now be irremediably lost, let alone the fact that the extent of endemism areas that have vanished is unknown.

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References: Bächli, G., C.R. Vilela, and V. Ratcov 2000, Mitt. schweiz. ent. Ges. 73: 67-92; Bächli, G., 2007, TaxoDros: The database on Taxonomy of Drosophilidae. Electronic Database accessible at http://www.taxodros.unizh.ch. Captured on 01 December 2007; Chaves, N.B., and R. Tidon 2005, Dros. Inf. Serv. 88: 25-27; David, J.R., L.O. Araripe, B.C. Bitner-Mathé, P. Capy, B. Goñi, L.B. Klaczko, H. Legout, M. Martins, J. Vouidibio, A. Yassin, and B. Moreteau 2006, Heredity 96: 53-62; De Toni, D.C., and P.R.P. Hofmann 1995, Rev. bras. Biol. 55: 347-350; De Toni, D.C., J.A. Brisson, P.R.P. Hofmann, M. Martins, and H. Hollocher 2005, Dros. Inf. Serv. 88: 33-38; De Toni, D.C., M.S. Gottschalk, J. Cordeiro, P.R.P. Hofmann, and V.L.S. Valente 2007, Neotropical Ent. 36: 356-375; Dobzhansky, T., and C. Pavan 1943, Bolm Fac. Filos. Ciênc. S. Paulo 46: 7-72; Dobzhansky, T., and C. Pavan 1950, J. Anim. Ecol. 19: 1-14; Ferreira, L., and R. Tidon 2005, Biod. Conserv. 14: 1809-1821; Gottschalk, M.S., D.C. De Toni, P.R.P. Hofmann, and V.L.S. Valente 2007, Neotropical Ent. 36: in press; Grimaldi, D.A, 1987, Bull. Am. Mus. Nat. Hist. 186: 103-268; Grimaldi, D.A., 1990, Am. Mus. Novit. 2964: 1-31; Kahl, H., 1917, Annals of Carnegie Museum 11: 364-393; Malogolowkin, C., 1946, Rev. bras. Biol. 6: 415-426; Martins, M., 1987, Bolm Mus. Para. Emilio Goeldi 3: 195-218; Martins, M., 2001, In: *Lessons from Amazonia: The Ecology and Conservation of a Fragmented Forest* (Bierregaard, R.O., C. Gascon, T.E. Lovejoy, and R. Mesquita, eds.), pp. 175-186; Medeiros, H.F., and L.B. Klaczko 2004, Biota Neot. 4: http://www.biotaneotropica.org.br/v4n1/pt/abstract?article+BN01604012004; Myers, N., R.A. Mittermeier, C.G. Mittermeier, G.A.B. Fonseca, and J. Kent 2000, Nature 403: 853-858; Pavan, C., 1959, Bolm Fac. Filos. Ciênc. S. Paulo 11: 1-81; Schmitz, H.J., and P.R.P. Hofmann 2005, Dros. Inf. Serv. 88: 97-101; Schmitz, H.J., V.L.S. Valente, and P.R.P. Hofmann 2007, Neotropical Ent. 36: 53-64; Sene, F.M., F.C. Val, C.R. Vilela, and M.A.Q.R. Pereira 1980, Pap. Avul. Zool. 33: 315-326; Tidon, R., and F.M. Sene 1988, Dros. Inf. Serv. 67: 90; Tidon, R., D.F. Leite, and B.F.D. Leão 2003, Biol. Cons. 112: 299-305; Tidon, R., D.F. Leite, L.B. Ferreira, and B.F.D. Leão 2005, In: *Ecologia e biodiversidade do Cerrado* (Scariot, A., J.M. Felfili, and J.C. Souza-Silva, eds.), pp. 337-352; Tidon, R., 2006, Biol. J. Linn. Soc. 87: 233-247; Tidon-Sklorz, R., and F.M. Sene 1999, In: *Biodiversidade do Estado de São Paulo, Brasil, síntese do conhecimento ao final do século XX. Invertebrados*

*terrestres* (Brandão, C.R., and E.M. Cancello, eds.), pp. 245-261; Tosi, D., M. Martins, C.R. Vilela, and M.A.Q.R. Pereira 1990, Braz. J. Genet. 13: 19-31; Val, F.C., and M.D. Marques 1996, Pap. Avul. Zool. 39: 223-230; Val, F.C., C.R. Vilela, and M.D. Marques 1981, In: *The Genetics and Biology of* Drosophila (Ashburner, M., H.L. Carson, and J.N. Thompson, Jr., eds.), pp. 123-168; Valente, C.R., 2006, In: *Natureza viva cerrado: caracterização e conservação* (Guimarães, L.D., M.A.D. Da Silva, and T.C. Anacleto, eds.) pp. 20-44; Vilela, C.R., 1983, Rev. bras. Ent. 27: 1-114; Wheeler, M.R., and M.P. Kambysellis 1966, Univ. Texas Publs. 6615: 533-565.

**Screening for transposable elements in South America invasive species *Zaprionus indianus* and *Drosophila malerkotliana*.**

**de Setta, N. and C.M.A. Carareto.** UNESP – Universidade Estadual Paulista, São José do Rio Preto, SP, Brazil; e-mail: carareto@ibilce.unesp.br

Transposable elements (TEs) have usually been studied focusing on distribution, structure, activity and evolution. Such analyses primarily aim at understanding the impact of TEs in the genetic dynamics and evolution of the host genomes. A kind of impact analysis that has not been enough explored is to look at genomic dynamics of TEs under stressful conditions, like environment invasion and colonization. According to the "wake-up" hypothesis (Vieira *et al.*, 1999), there is a tendency of mobilization intensification in colonizing populations resulting in an increasing of copy number. An example is given by the comparison between *D. melanogaster* and *D. simulans*, two cosmopolitan sibling species. *D. melanogaster* has dispersed around the world some centuries before *D. simulans* (Capy and Gilbert, 2004) and among 34 transposable elements analyzed, *D. melanogaster* appears to have higher insertion site numbers than *D. simulans* for 29 TEs when populations of America, Asia, Europe, Australia and Africa, the last one being the native place of both species, are compared (Vieira *et al.*, 1999).

Besides *D. melanogaster* and *D. simulans*, numerous other Drosophilidae were able to invade new habitats and had recently colonized the Brazilian territory mainly by antropic activity; among them, *Zaprionus indianus* (from Africa) and *D. malerkotliana* (from Asia). *Zaprionus indianus* was first collected in 1998, in a fig culture at Valinhos city, Sao Paulo (Vilela, 1999), and now it can be collected in several regions of Brazil (Vilela, 1999; Castro and Valente, 2001; Kato *et al.*, 2004), Uruguay (Gõni *et al.*, 2002), and southern North America (van der Linde *et al.*, 2006). *D. malerkotliana* has invaded South America in the 1970's decade (Val and Sene, 1980) and nowadays it is frequently collected in southern Brazil. The recent invasion of a continent by a species provides a useful tool for studying the dynamics of TEs during colonizing stress, because we can monitor populations since the first steps of introduction. To do that, it is necessary to accumulate information about distribution, history, and dynamics of its transposable elements. The goal of this study was then to make an initial search for transposable elements in *Z. indianus* and *D. malerkotliana* genomes to guide further studies on these invasive species.

Seven retrotransposons were searched (*copia*, *mdg-1*, *412*, *gypsy*, *297*, *micropia,* and *roo/B104*), two non-LTR retrotransposons (*jockey* and *doc*), and one transposon (*bari-1*) in a Brazilian population of *Z. indianus* (Mirassol, SP) and *D. malerkotliana* (Onda Verde, SP) by the Dot blotting method. The probes were prepared by two different methods: (1) PCR reactions were performed in 25 μl using approximately 50 ng of each TE plasmid, 0.4 μM of each specific primer

(Table 1), 200 mM of dNTPs, 1.5 mM of $MgCl_2$ and 1U of Taq Platinum Polymerase (Invitrogen) in 1× PCR buffer; for amplification we used an initial denaturation step of 3 min at 94°C, 30 cycles consisting of 30 sec at 94°C, 30 sec at 56°C and 1 min at 72°C, finally a additional extension step of 10 min at 72°C were performed; (2) 5 μg of each plasmid was digested with appropriate restriction enzymes (Table 1). The fragments were isolated from agarose 1% gels, purified using GFX PCR DNA and Gel Band Purification (GE Healthcare), and probed using Gene Images Random Prime Labelling Module (GE Healthcare) according to the manufacturer's instructions. For screening the TEs, 8 mg of genomic DNA from each strain was dropped in the Hybond N+ nylon membranes (GE Healthcare). For hybridization and detection we used the chemioluminescent hybridization system Gene Images (GE Healthcare) at high stringency (60°C) according to the manufacturer's instructions. Ultra pure water and 1 mg of each plasmid were used as negative and positive controls, respectively.

Table 1. Plasmids, primers and restriction enzymes used to prepare probes for detection of transposable elements in *Z. indianus* and *D. malerkotliana*.

| Element Plasmid [1] | Probe type | Primers / Restriction enzymes | Probe extension[2] |
|---|---|---|---|
| *copia* - p77E4 | PCR | LTR-5´CTATTCAACCTACAA AAATAACG3´<br>PCS-5´ATTACGTTTAGCCTTGTCCAT3´ | 439 bp |
| *micropia* - dhMiF$_2$ | PCR | 2813-5´TTAACTCCTAGAGTTCATCGCTGG3´<br>2814-5´CATGTACCTGGTTAACTACTGACC3´ | 387 bp |
| *gypsy* - pGGHS | PCR | GM003-5´GTACTGAACATTATCAGAATC3´<br>GM004-5´TCTAAGGAGTCCTCTGCAAGG3´ | 542 bp |
| *412* - *pBR322* | Restriction fragment | Hind III | ~850 bp |
| *297* - *pBR322* | Restriction fragment | Eco RI | ~2,3 kb |
| *mdg-1* - *pBR322* | Restriction fragment | Eco RI / Hind III | ~1,2 kb |
| *roo/ B104* - *pBR322* | Restriction fragment | Eco RI / Hind III | ~2,3 kb |
| doc - *pBspt Kst* | Restriction fragment | Eco RI / Hind III | ~800 kb |
| *jockey* - *puC19* | Restriction fragment | Eco RI / Hind III | ~1,5 kb |
| *bari-1* - *puC8* | PCR | Br1 – 5' ATTCGTCGCAGGCTAAAAGA 3'<br>Br2 – 5' TTGTAACACCACCTTTGGCA 3' | 703 bp |

[1] Plasmid source: *copia* and *412* – E. Loreto (UFSM, Santa Maria, RS, Brazil); *gypsy* - D. Dorsett (Memorial Sloan-Kettering Cancer Center, USA); *micropia* – D.H Lankenau (University of Heidelberg, Heidelberg, Germany); *297*, *mdg-1*, *roo/B104* and *doc* – C. Vieira (Université Lyon I, Lyon, France); *jockey* – D.J. Begun (University of California, Davis, USA); *bari-1* – R. Caizzi (Universidade de Bari, Bari, Itália). [2] TE sequences inserted into plasmids were derived from:*D. melanogaster* (p77E4, pGGHS, *pBR322*, *pBspt Kst*, *puC19* and *puC8*) and *D. hydei* (dhMiF$_2$).

The 10 TEs investigated were identified in both *Z. indianus* and *D. malerkotliana*; however, the distribution of sequences homologous to these elements showed different hybridization signals, varying from very strong to weak signals (Figure 1).

The distribution of transposable elements of *Z. indianus* and *D. malerkotliana* is in agreement with the few data previously reported. Among the TEs here analyzed, only *gypsy* had been searched in *Z. indianus* (Heredia *et al.*, 2004), and the authors proposed that this retrotransposon was received

from *D. simulans* through an event of horizontal transfer. Likewise, our result showed a strong homology between *gypsy* probe from *D. melanogaster* and *Z. indianus* sequences, reinforcing the previous data, since *D. melanogaster* and *D. simulans* are sibling species and their *gypsy* sequences are highly similar (Heredia *et al.*, 2004). *D. malerkotliana* had just been studied for *copia* and *412* occurrences. *Copia* had been identified by weak *in situ* hybridization signal on the chromocenter (Biémont and Cizeron, 1999), and *412* had also been shown on the chromocenter both by *in situ* hybridization and Southern blotting (Cizeron *et al.*, 1998). Our analysis showed that *copia* and *412* are components of *D. malerkotliana* genome, but the hybridization signals obtained are also very weak, suggesting that both TEs of *D. malerkotliana* have low homology with those of *D. melanogaster*.

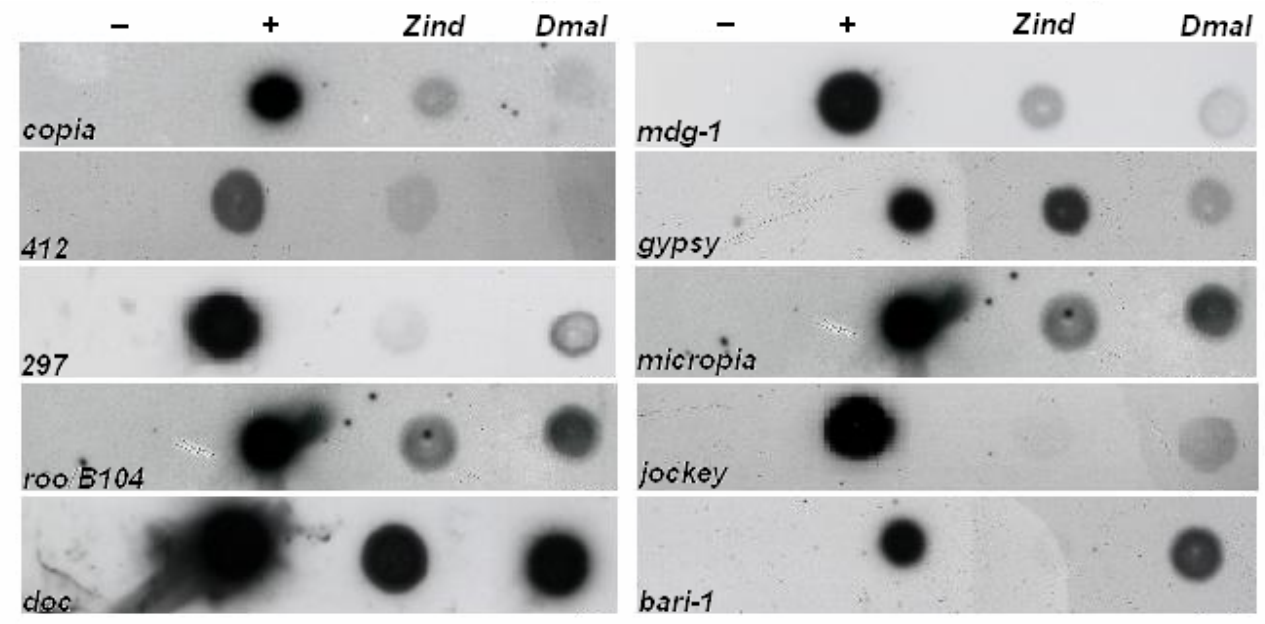



Figure 1. Dot blotting analysis of the transposable elements *copia*, *mdg-1*, *412*, *gypsy*, *297*, *micropia*, *roo/B104*, *jockey*, *doc* and *bari-1* in *Z. indianus* (*Zind*) and *D. malerkotliana* (*Dmal*). (–, negative control; +, positive control).

The results here presented show for the first time the occurrence of several transposable elements in two species scarcely studied in this respect. Except for *doc* and *gypsy* in *Z. indianus* and *doc*, *micropia* and *bari-1* in *D. malerkotliana*, all the analyses produced weak hybridization signals (Figure 1), which suggest that these TEs are divergent from those of *D. melanogaster* and *D. hydei* (for *micropia*) and they should have evolved vertically, accumulating nucleotide variation since the last common ancestor. On the other hand, *doc* and *gypsy* of *Z. indianus* and, *doc*, *micropia* and *bari-1* of *D. malerkotliana* have shown remarkably strong hybridization signals (Figure 1). Further analysis could test which is the most parsimonious hypothesis to explain that high homology between *Z. indianus* / *D. malerkotliana* and *D. melanogaster* / *D. hydei* sequences, if horizontal transfer of these elements between these species or any other alternative factor that can explain high similarity between elements of distantly related species (for example, Almeida and Carareto, 2005; Setta *et al.*, 2007). This report is the first step of a study regarding the dynamics of transposable elements in the

invasive species *Z. indianus* and *D. malerkotliana* and will be used for selecting candidate TEs for more detailed studies.

Acknowledgments: The authors are grateful to CNPq and COFECUB-CAPES for fellowships to NS and to CNPq by fellowship to CMAC.

References: Biémont, C., and G. Cizeron 1999, Genetica 105: 43-62; Capy, P., and P. Gilbert 2004, 120: 5-16; Castro, F.L., and V.L.S. Valente 2001, Dros. Inf. Serv. 84: 15-17; Cizeron, G., F. Lemeunier, C. Loevenbruck, A. Brehm, and C. Biémont 1998, 15: 1589-1599; de Almeida, L.M., and C.M.A. Carareto 2005, Mol. Phylogenet. Evol. 35: 583-594; de Setta, N., E.L. Loreto, and C.M.A. Carareto 2007, J. Mol. Evol. doi: 10.1007/s00239-007-9051-7; García Guerreiro, M.P., and A. Fontdevila 2001, Genet. Res. 77: 227-238; Gõni, B., M.E. Martinez, G. Techera, and P. Fresia 2002, Dros. Inf. Serv. 85: 75-80; Heredia, F., E.L. Loreto, and V.L. Valente 2004, Mol. Biol. Evol. 21: 1831-1842; Kato, C.M., L.V. Foureaux, R.A. Cesar, and M.P. Torres 2004, Ciênc. Agrotec. 28: 454-455; Labrador, M., M.C. Seleme, and A. Fontdevila 1998, Mol. Biol. Evol. 15: 1532-1547; Val, F.C., and F.M. Sene 1980, Papéis Avulsos de Zoologia 33: 293-298; van der Linde, K., G.J. Steck, K. Hibbard, J.S. Birdsley, L.M. Alonso, and D. Houle 2006, Fla. Entomol. 89: 402-404; Vieira, C., D. Lepetit, S. Dumont, and C. Biémont 1999, Mol. Biol. Evol. 16: 1251-1255; Vilela, C.R., 1999, Dros. Inf. Serv. 82: 37-39.

## Effects of Phloxine B and Hematoporphyrin IX on immature stages of *Drosophila melanogaster.*

**Massaldi-Fuchs, A.M.M.[1*], L.M. Pujol-Lereis[1*], A. Rabossi[1], A. Filiberti[2], C.E. Argaraña[2], and L.A. Quesada-Allué[1**].** [1]FCEyN- University of Buenos Aires, CONICET and Fundación Instituto Leloir, Patricias Argentinas 435, (1405) Buenos Aires, Argentina; [2]CIQUIBIC- University of Córdoba, CONICET. Ciudad Universitaria, Córdoba, Argentina; * These authors equally contributed to this work; ** corresponding author (E-mail: lualque@iib.uba.ar).

### Introduction

Photosensitizers such as xanthene derivatives (*e.g*., phloxine B) and porphyrins (*e.g*., hematoporphyrin IX) are endowed with photoinsecticidal properties. Xanthene derivatives showed acute photo-toxicity against several dipterans (Ben Amor and Jori, 2000, Berni *et al*., 2002, 2003). Pujol-Lereis (2006) compared the photo-toxic effect of phloxine B (PhB) during the postembryonic development of *D. melanogaster* (*Dm*), *Haematobia irritans*, and *Ceratitis capitata*, and determined that *Dm* is affected during larval development. Low concentrations of hematoporphyrin (HP) rapidly decrease adult survival rates of *Ceratitis capitata*, *Bactrocera (Dacus) oleae*, and *Stomoxys calcitrans* (Ben Amor *et al.,* 1998, 2000).

Photosensitizer molecules react upon absorption of visible radiation with the subsequent formation of reactive oxygen species, mediating signaling cascades which either fortify antioxidant defenses of cells or switch to apoptotic death if oxidative pressure is too great (Girotti, 1998). There are two mechanisms by which the photosensitizer can react with biomolecules: type I reactions produce highly reactive oxygen species (*e.g*., the superoxide and the peroxide anions) which usually activate enzymatic antioxidant defense, and type II reactions result in the formation of singlet oxygen, leading mainly to lipid peroxidation. Studies of the effects in immature stages were carried

out in order to understand which phototoxic pathway is triggered and which main cellular and sub-cellular targets are affected.

## Methods

Wild type (strain Oregon-R-C) and antioxidant enzyme mutant strains of *D. melanogaster* were obtained from Bloomington *Drosophila* Stock Center (Bloomington, IN). Larvae were reared in Formula 4.24 Instant *Drosophila* Medium (Carolina Biological Supply, Ca). Batches of 30 newly hatched larvae I of *Drosophila* were placed on the surface of the larval media with or without 0.1 mM PhB disodium salt (D&C Red Nº 28, Warner Jenkinson, St Louis, MO) and 0.1 mM hematoporphyrin IX (Sigma-Aldrich Inc., St Louis, MO). Cultures were maintained in the dark in a Conviron chamber CMP 3244 (Canadá), at 23°C, 50-60% RH, and exposed to light (5000 lux) during the wandering period. After pupariation, insects were maintained in the dark until imago ecdysis. Separate cultures of wt *Drosophila* were maintained in the dark throughout the entire experiment.

For semi-quantitative PCR analysis of antioxidant enzymes, stage III wandering larvae were transferred to 1% agar plaques and exposed to 1 hour of light. RNA was extracted with TRIzol Reagent (Life Technologies, GIBCO-BRL). cDNA was synthesized following the SuperScript Pre-amplification System for First Strand cDNA Synthesis (BRL).

Pairs of oligonucleotide primers were designed for amplification of *Actin* (5′ AAGCGTGGTATCCTCACCC 3′ and 5′ TCCTCCTCCTCCTCCAGC 3′), *Catalase* (*Cat*) (5′ CATGTTCTGGGACTTCCTC 3′ and 5′ GTC TGATCCGTCGAATCC 3′), *Glutathione S-transferase* (*Gst*) (5′GTATTTGGTGGAGAAGTACG 3′ and 5′ ATTGTCTTTGGAGACAGGC 3′), Mn *Superoxide Dismutase* (Mn*Sod*) (5′ CGACACCACCAAGCTGATTCAG 3′) and Cu, Zn *Superoxide dismutase* (Zn/Cu*Sod*) (5′ CCAAGATCATCGGCATCGG 3′ and 5′ TGACAACACCAATGGCTGC 3′). PCR reactions (50 µl) were carried out with 1 µl of each primer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 2 units Taq polymerase (Promega, WI). Amplification conditions were: 94ºC for 5 min, 25 cycles of 94ºC × 30 sec, annealing temperature × 30 sec, and 72ºC × 1 min, followed by a final extension at 72ºC for 5 min. Annealing temperatures were: 47ºC for *Gst,* 52ºC for *Actin*, *Cat* and Zn/Cu*Sod*, and 54ºC for Mn*Sod*. All PCR reactions were performed with a PCR*Express* Thermo Hybaid thermal cycler. PCR products were run through a 1.2 % agarose gel and band intensities relative to *Actin* were analyzed with ImageJ program.

## Results and Discussion

The survival data of wt *Drosophila* when HP was added to the larval media showed that the photosensitizer was not toxic when the insects were maintained in the dark throughout development (Table 1). Instead, wt *Drosophila* treated with HP and exposed to light reduced the survival from 80% to 47.5%. Further mortality was not registered in wt after pupariation (not shown). We determined that the 50% lethal concentration ($LC_{50}$) of HP of wt *Drosophila* from egg eclosion to imago ecdysis was 0.11 mM.

Surprisingly, Catalase (*Cat*) and Glutathione S-transferase (*Gst*) mutant strains were not affected by the addition of HP to the larval media (Table 1). Survival of Zn/Cu Superoxide dismutase (Zn/Cu*Sod*) mutant strain showed an important survival reduction from larvae I to white pre-pupa when treated with HP and exposed to light (65% of the larvae could pupariate without HP whereas only 15% with HP). The percent of adult ecdysis of Zn/Cu*Sod* mutant strain was 7.5%, indicating that additional mortality occurred during the stages within the puparium (not shown).

Table 1. Percentage survival of wt and antioxidant enzyme mutant strains of *D. melanogaster* reared with 0.1mM HP. Each value represents the average of 3 replicas.

| *D. melanogaster* mutant strains | LI to white pre-pupa survival (mean % ± sem) | | |
|---|---|---|---|
| | 0 mM HP | 0.1 mM HP | Δ |
| Dark conditions | | | |
| wt Oregon-R-C | 74.4 ± 17.1 | 72.5 ± 8.7 | -1.9 |
| Light during wandering | | | |
| wt Oregon-R-C | 80 ± 10 | 47.5 ± 2.5 | - 32.5 |
| *Sod* 4018 | 65 ±10 | 15.0 ± 3 | - 50.0 |
| *Cat* 17939 | 70 ± 2 | 75.0 ± 5 | + 5.0 |
| *Gst* 14114 | 40 ± 5 | 25.0 ± 10 | - 15.0 |

Table 2. Survival decrease of wt and antioxidant enzyme mutant strains of *D. melanogaster* reared with 0.1 mM PhB. Each value represents the average of 3 replicas.

| *D. melanogaster* mutant strains | Decrease in survival (mean %)* | |
|---|---|---|
| | LI to white pre-pupa | LI to adult ecdysis |
| Light during wandering | | |
| wt oregon-R-C | 26.2 | 65.3 |
| *Sod* 4018 | 0.0 | 32.3 |
| *Cat* 17939 | 11.0 | 43.8 |
| *Gst* 14114 | 8.0 | 20.7 |

(*)Maximum standard deviation in the experiments was ± 12.0 %

Table 3. Transcription levels of antioxidant enzymes of wt *Drosophila* reared with PhB or HP.

| | Intensity (mean ± se) | | |
|---|---|---|---|
| | 0mM | 0.1mM PhB | 0.1mM HP |
| Zn, Cu Sod | 0.88 ± 0.35 | 0.84 ± 0.34 | 1.29 ± 0.28 |
| MnSod | 1.03 ± 0.32 | 0.86 ± 0.7 | 0.68 ± 0.14 |
| Cat | 1.16 ± 0.04 | 1.08 ± 0.13 | 1.01 ± 0.3 |
| Gst | 0.98 ± 0.1 | 0.90 ± 0.84 | 0.80 ± 0.10 |

Intensity: arbitrary units (Image J program) determined as relative to actin levels.

As shown with HP, 0.1 mM PhB was not toxic to wt *Drosophila* maintained in the dark throughout development (73.3% survival). Significant light-dependent toxicity was observed in wt both at pupariation (26.2% reduction) and at adult ecdysis (65.3%, Table 2). The 50% lethal concentration ($LC_{50}$) for PhB during the postembryonic development of wt *Drosophila* was 0.06 mM. No additional mortality due to PhB activation was observed in antioxidant enzyme mutant strains (Table 2).

The transcription levels of the four antioxidant enzymes corresponding to the above tested mutants (Zn/Cu*Sod*, *Cat*, *Gst*, and Mn*Sod*) were estimated by semi-quantitative PCR, followed by gel electrophoresis, for larvae reared with or without 0.1 mM PhB or HP (Table 3). In agreement with the above results no significant increase in transcription of Mn*Sod*, *Cat*, and *Gst* antioxidant enzymes was registered with HP nor with PhB (Table 3). Therefore, Type I reactions do not seem to prevail in our experimental model with photosensitizers.

However, survival was significantly reduced in mutant strain 4018 when HP was added to the diet. This strain presents mutation of Zn/Cu*Sod* enzyme. Moreover, although not statistically significant, the transcription level of Zn/Cu*Sod* seems to be enhanced when wt *Drosophila* was reared with HP. These experiments must be repeated in order to attain statistical significance.

From these results we can conclude that in our experimental conditions, Type II reactions are likely implied in photo-toxicity. Preliminary studies in the medfly *Ceratitis capitata* showed 2-fold increase in the level of weak chemoluminiscence associated to lipid peroxidation, when treated with comparable concentrations of PhB and HP and exposed to light (data not shown). Further studies are on the way to confirm the existence of rapid lipid peroxidation (type II mechanism).

References: Ben Amor, T., L. Bartolotto, and G. Jori 1998, Photochemistry and Photobiology 68: 314-318; Ben Amor, T., and G. Jori 2000, Insect Biochem. Mol. Biol. 30: 915-925**;** Berni, J., 2002, PhD Dissertation, University of Buenos Aires; Berni, J., A. Rabossi, and L.A. Quesada-Allué 2003, J. Econ. Entomol. 96: 662-668**;** Girotti, A.W., 1998, J. of Lipid Research. 39: 1529-1542; Pujol-Lereis, L.M., A. Rabossi, A. Filiberti, C.E. Argaraña, and L.A. Quesada-Allué 2006, Dros. Inf. Serv. 89: 82-84.

## The *white*$^{-}$ mutation provides a growth advantage for mosaic clones in *D. melanogaster* and *D. simulans*.

**Siderov, Roman[1], Elena Ugnivenko[2], Kirill Kirsanov[1], Gennady A. Belitsky[1], and Elizabeth M. Khovanova[2]**. [1]Inst. Carcinogenesis, N.N. Blokhin Cancer Research Center RAMS, Moscow, Russian Federation; [2]N.K. Koltzov Institute of Developmental Biology RAS, Moscow, Russian Federation.

In this short communication we demonstrate that a *white* mutation can provide a positive bias to a mosaic clone homozygous for a recessive marker linked with *white*$^{-}$ compared to its twin clone homozygous for another marker linked with *white*$^{+}$. The effect is not very strong and requires for its observation a large sample or a strong mutagenic treatment generating enough clones. At the same time, the effect remains persistent and statistically significant in two fly species (*D. simulans* and *D. melanogaster*), with different alleles of *white* and mosaic clone markers and with different mutagenic agents applied. The protein White is a part of ATP-dependent nucleotide and amino acid transporters, particularly for guanine and tryptophan, the precursors for eye pigments (Evart *et al*., 1994). The gene *white* is also known to affect courtship behavior and response to anesthesia (Svetec *et al*., 2005; Campbell and Nash, 2001). No function relevant to cell growth has been reported for this gene by now.

Genes. $y^2$ – *D. simulans yellow*$^2$, *yellow* chaetae, normal wings, the mutation of spontaneous origin in the *vermilion* strain; *sn* – *D. simulans singed*, "singed" chaetae, homozygous females are sterile, radiation-induced mutation in the *vermilion* strain; *v* – *D. simulans vermilion*, vermillion, brilliant red eye color; n$H^{+}$ – an autonomous *D. simulans* transposable element located in X-chromosome, the genetic instability $H^{+}$-factor, n represents the number of $H^{+}$ copies in the X chromosome; *w* – *D. simulans white*, white eye color; *wy* – unspecified *D. simulans wavy* allele, grey wavy wings, the mutation was originated spontaneously in the *sn v* strain; *y* – *D. melanogaster* unspecified *yellow* allele; *w* – *D. melanogaster* unspecified loss-of-function *white* allele; $w^{1118}$ – *D. melanogaster* loss-of-function *white* allele, homozygotes have white eyes with only 1% red pigment (compared to 100% pigment in wild type); $sn^3$ – *D. melanogaster singed*$^3$ allele, homozygous viable and fertile.

Stocks and Crosses. *Drosophila simulans*: (1) $y^2$, $H^{+}$, (2) *sn v*, $H^{+}$ / $\overset{\wedge}{XX}$ , (3) $y^2$ *w*, (4) *sn v wy*, $2H^{+}$/ $\overset{\wedge}{XX}$ . *Drosophila melanogaster*: (1a) *y*, (2a) *w* $sn^3$, (3a) *y* $w^{1118}$; (4a) $sn^{MR2}$. The following crosses were performed to obtain *y* + +/+ + *sn*, *y w* +/+ + *sn* and *y* + +/+ *w sn* heterozygotes: (I) ♀ $y^2$, $H^{+}$ × ♂ *sn v*, $H^{+}$ /*Y* ; (II) ♀ $y^2$ *w* × ♂ *sn v wy*, $2H^{+}$ /*Y* (*D. simulans*); (III) ♀ *y* × ♂ *w* $sn^3$ ; (IV) ♀ *y* $w^{1118}$ × ♂ $sn^{MR2}$ (*D. melanogaster*).

Mutagenesis. To generate mosaic clones by somatic recombination, we used the following procedure. The $F_1$ 48-hrs old larvae of the cross (III) were treated with 0.3 ml per vial 0.5% water solution of phosphemide (CAS # 882-58-6). Analogously, the larvae from the cross (IV) were treated

with 2 mg/ml water solution of oxoplatin (CAS # 53261-25-9). $F_1$ larvae from the cross (III) were treated by γ-rays (600, 900 or 1200r) after 72, 96, or 120 hrs of development. Untreated $F_1$ cross (III, IV) larvae were used for a control. $H^+$-dependent mutagenesis in the offspring of the crosses (I) and (II) occurred spontaneously.

Observation

Within the $F_1$ of all the crosses the y + +/+ + *sn*, *y w* +/+ + *sn* and *y* + +/+ *w sn* heterozygous females were examined for *yellow* and *singed* clones on head, notum, and humeri with MBS-10 stereomicroscope. The clone number and frequencies were calculated. The clones were ranked according their size in terms of the macrochaetae number (clone number / flies population) × 100%. In *D. simulans* individuals heterozygous for the $H^+$-factor the clone frequency on the humeri was registered separately as a result of the humeri-specific *H*-factor mutagenesis.

*Statistics*. Hypotheses regarding the yellow and singed clone proportion were estimated in a standard χ2 – test. All the biased *y*:*sn* clone proportions given in the figures differ from 1:1 at least at $P < 0.05$.

Results and Conclusions

1. In flies heterozygous for *white* and other X-linked markers, the marker linked to the *white* mutation in *cis*- possesses higher mosaic clone frequency than the marker in *trans*-. For example, in *y* + + / + *w sn* heterozygotes the *singed* clone frequency is higher than the frequency of the twin *yellow* clones, and *vice versa*, in *y w* + / + + *sn* heterozygotes *yellow* clones are more frequent than *singed* ones. In absence of the *white* mutation (in *y* +/+ *sn* flies), frequencies of *yellow* and *singed* clones are equal. Thus, the mosaic clones homozygous for a *white* mutation have a growth advantage on a *white*/+ background compared to twin clones homozygous for $white^+$.

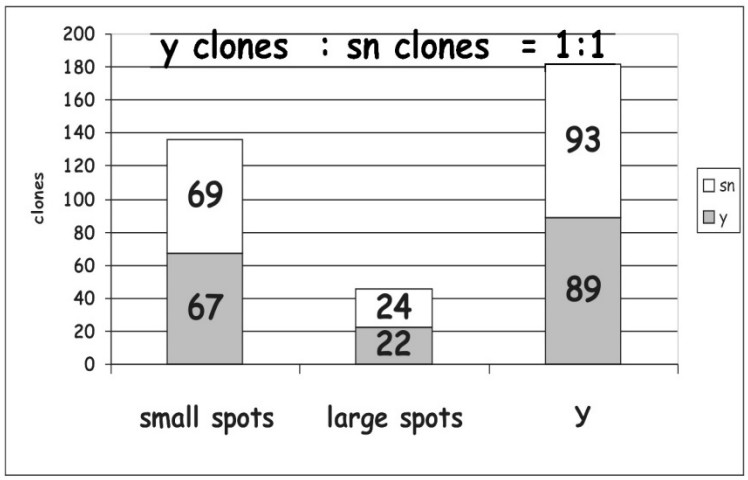



Figure 1. In the absence of the *white* mutation, (*y*) to (*sn*) clone ratio is 1:1.

Flies: *y* + / + *sn*, 8819 *D. simulans* females, spontaneous mosaicism.

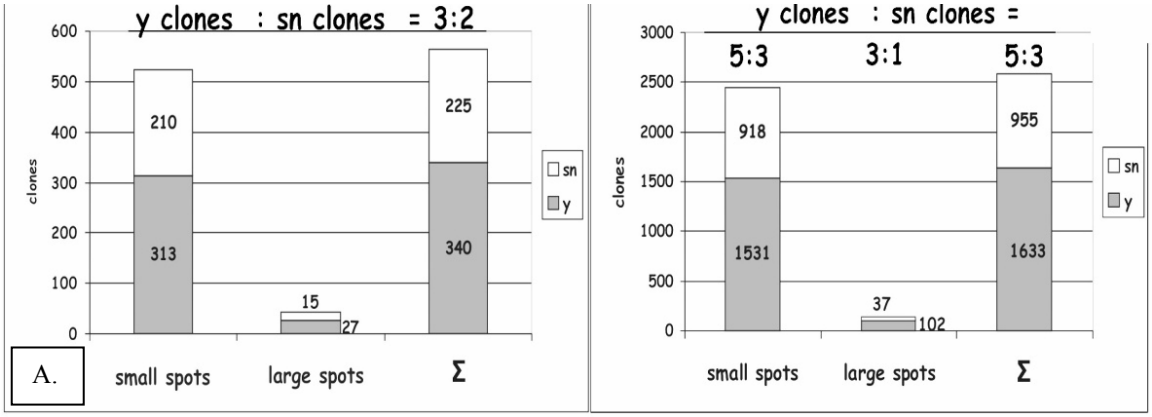
2. This effect was observed in two *Drosophila* species, *D. melanogaster* and *D. simulans*, and within a species it is true for different alleles of *white*, *yellow*, and *singed*.

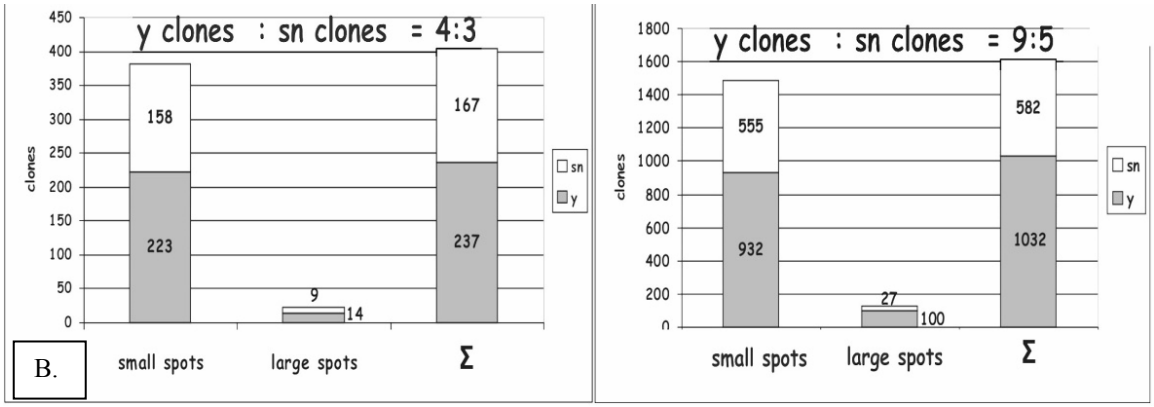
3. The effect is true for different types of mutagenesis – spontaneous, chemical, γ-ray, and genetic instability factor-induced.

4. The manifestation of the effect depends on the developmental stage at which the clones were induced. The effect is clear for later induced (smaller) clones, in this case the proportion of

white-homozygous to *white*$^{+}$-homozygous clones (both marked with either *yellow* or *singed*) is far from 1:1 (up to 4:1). However, for early (large) clones, the proportion of *y* and *sn* clones is near 1:1. As the large clones typically originate early in the development, and most of the small clones originate later, the same regularity is applicable for large and small clones as well.

5. The effect extent depends on the applied dose of mutagen. The stronger was the mutagen, the higher preponderance of white homozygous clones over *white*$^{+}$ homozygous twins we observed.



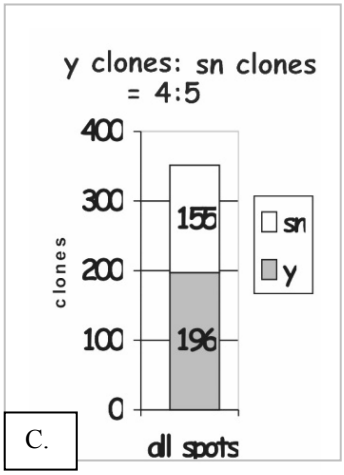




Figure 2. (*y w*) clones dominate over their (+ *sn*) twins. 2A. Spontaneous mosaicism (left panel) and *H*-factor-induced mosaicism (right panel) in the same *D. simulans y w* + / + + *sn*, *1H*$^{+}$ flies. Sample size is 60796 females. 2B. Spontaneous mosaicism (left panel) and *H*-factor-induced mosaicism (right panel) in the same *D. simulans y w* + / + + *sn*, *2H*$^{+}$ flies. Sample size is 20045 females. 2C. Oxoplatin-induced mosaicism in *D. melanogaster y w* + / + + *sn* flies. Sample size is 1250 females.

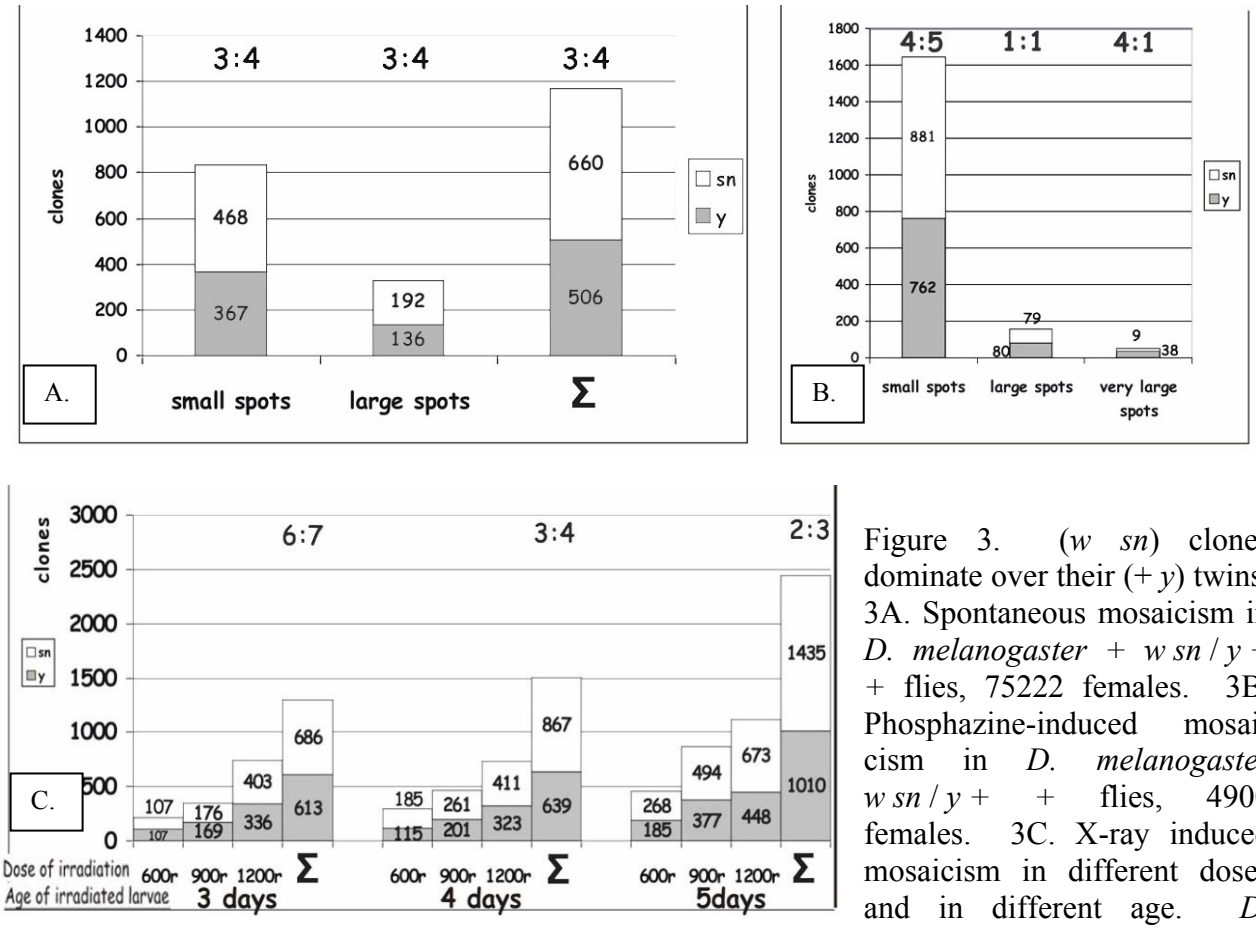



Figure 3. (*w sn*) clones dominate over their (+ *y*) twins. 3A. Spontaneous mosaicism in *D. melanogaster* + *w sn* / *y* + + flies, 75222 females. 3B. Phosphazine-induced mosaicism in *D. melanogaster* *w sn* / *y* + + flies, 4900 females. 3C. X-ray induced mosaicism in different doses and in different age. *D. melanogaster*, 15600 females

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References: Evart, G.D., D. Cannell, G.B. Cox, and A.J. Howells 1994, J. Biol. Chem. 269(14): 10370-10377; Svetec, N., B. Houot, and J.F. Ferveur 2005, Genet. Res. 85(3): 183-193; Campbell, J.L., and H.A. Nash 2001, J. Neurobiol. 49(4): 339-349.

## Guide to Authors

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# Signs of photoreceptor neurodegeneration in a wild type Canton-S strain of *Drosophila melanogaster* as revealed by electron microscopy characterization of ommatidia morphology.

**Koles, Kate, and Vladislav M. Panin.** Texas A&M University, Biochemistry and Biophysics Department, College Station, TX 77843. Email: panin@tamu.edu.

## Abstract

*Drosophila* has a long and fruitful history in the use of photoreceptor morphology to study neuronal degeneration. Even subtle early changes in the cytoplasm can be easily noticed by transmission electron microscopy of photoreceptor sections. In the course of characterizing the mutant phenotype of our gene of interest, we have unexpectedly noted major ultrastructural abnormalities in the wild type Canton-S photoreceptors. These flies were obtained from the Bloomington stock center and were not contaminated by Wolbachia or any other micro-organism sensitive to tetracycline treatment. The observed "phenotype" disappeared after crossing the flies to different stocks indicating that the causative agent is not contagious and that it has a genetic basis. Our results demonstrate that caution should be practiced by scientists who are about to embark on the labor-intensive electron microscopic characterization of their mutant phenotypes to first ascertain the normal morphology of a control genotype, especially if it is related to the Canton-S genotype recently obtained from the Bloomington stock center.

## Introduction

The optic lobe of the fruit-fly is ideally suited for histological analysis both by light and transmission electron microscopy as its symmetric and patterned structure yields itself to easy identification and sample preparation. EM analysis of numerous genes involved in cell polarity (Pellikka *et al.*, 2002), phototransduction (Harris and Stark, 1977), polyglutamine expansion induced neurodegeneration (Jackson *et al.*, 1998), etc. have revealed different aspects of abnormal photoreceptor subcellular morphology and provided significant insight into the pathology and molecular pathways affected in these mutants. Many aspects of neurodegeneration can be readily discerned in the eyes of mutant flies or in mutant eye clones, and in this way EM is well suited for the characterization of mutations in novel genes that are expressed in the optic lobes.

This was our rationale to study the morphology and subcellular appearance of a mutant that we have been working with during the past few years. However, in the course of our experiments we have noted that our control Canton-S fly eyes did not have a healthy appearance. Here we briefly report the electron microscopic appearance of the #1 Bloomington stock, Canton-S wild-type fly ommatidia.

## Materials and Methods

*Fly Stocks*

Canton-S flies were from Bloomington (stock number 1), unless specified otherwise. All flies were maintained at 25$^{o}$C on a 12 hr dark : 12 light cycle.

*Transmission Electron Microscopy*

Appropriately aged eyes from adult flies were dissected using razor blades and fixed in 2% paraformaldehyde, 2% glutaraldehyde, 0.1 M Na-cacodylate (pH 7.2) overnight at 4°C and postfixed in 2% $OsO_4$ for 2 hr at ambient temperature. After dehydration and embedding, 50 nm thin sections were cut. Sections were stained with 4% uranyl acetate and 2.5% lead nitrate. TEM was performed on JEOL 1200 EX electron microscope; negatives were scanned and processed in Adobe Photoshop.

*Wolbachia PCR*

Primers specific for the 16S rDNA were designed according to (O'Neill *et al.*, 1992), namely Wolbachia_99F: 5'-TTGTAGCCTGCTATGGTATAACT-3' and Wolbachia_994R: 5'-GAATAGGTATGATTTTCATGT-3'. PCR was performed as described in (O'Neill *et al.*, 1992).

*Tetracycline treatment*

Tetracycline was added to regular fly food to yield 0.25 mg/ml final concentration. Flies were passed through two generations of tetracycline treatment.

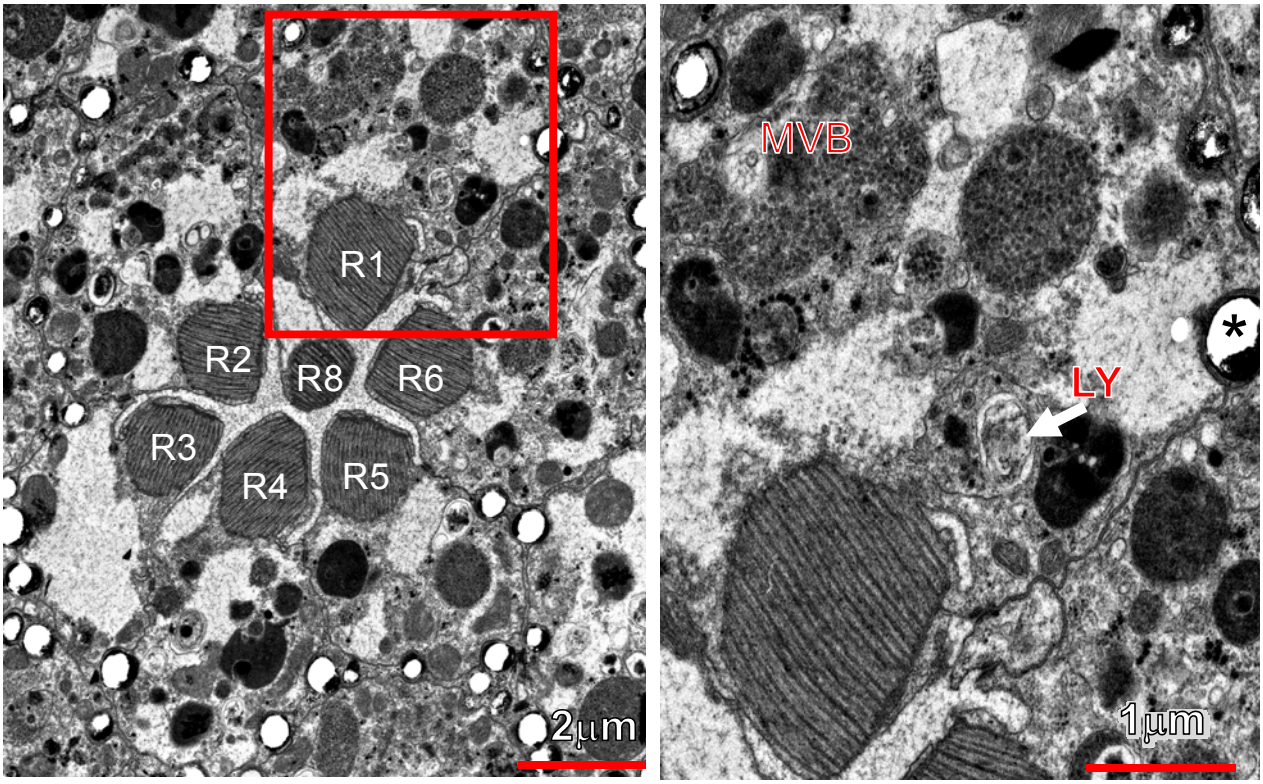


Figure 1. Bloomington #1 Canton-S fly ommatidium at 2000 × magnification. On the right, inset magnified to show the cytoplasm in more detail, characterized by the absence of the characteristic clean cytoplasm, and instead cluttered with lysosomes (white arrow), large multivesicular bodies (MVB) and dark aggregates. The rhabdomeres (dark stacks of cisternae in each individual photoreceptor cell) do not appear to be affected at this stage, although in some cases degenerative changes can also be noted in these structures. * indicates the pigment granules.

**Results and Discussion**

Since our gene of interest (CG4871) was shown to be expressed in the optic lobes and specifically in the photoreceptor cell by *in situ* hybridization, we were curious to examine the photoreceptor cells in the mutant background of CG4871. Wild type (Canton-S from Bloomington, stock number 1) and mutant flies in the same Canton-S background were aged for 20 days and then processed for transmission electron microscopy (see Methods). To our surprise, wild-type flies had indications of quite severe pathological changes in the cytoplasm of all the photoreceptors (R1-R8) examined (Figure 1).

We have subsequently re-ordered the same Canton-S stock from Bloomington (approximately 2 years after the ordering of the first batch of Canton-S flies), and to our surprise we found the very same morphological changes (data not shown). It is known that Wolbachia infection can result in the accumulation of bacteria inside the photoreceptor cells and lead to significantly abnormal ommatidium morphology somewhat similar to neurodegeneration phenotype detected in our experiments (Min and Benzer, 1997). Thus, we have tested for the presence of Wolbachia infection using a PCR protocol (O'Neill *et al.*, 1992), but found no evidence of Wolbachia contamination (Figure 2).

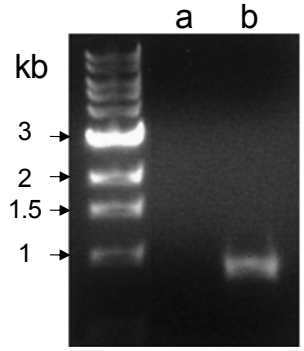


Figure 2. PCR screen of a) CS wild-type flies from Bloomington, and b) FTF1 control flies (from David Rand, Brown University) for Wolbachia infection. Absence of the PCR product from 16S rDNA in wild type Canton-S flies suggests the absence of Wolbachia infection.

Repeated tetracycline treatment did not improve the photoreceptor morphology of Canton-S flies (data not shown). Outcrossing to different genotypes did not lead to the "infection" of the progeny and the eye phenotype became indistinguishable from the one of the Canton-S flies received from an alternative source and used as "healthy" control (Figure 3). These observations suggest that a certain combination of genetic factors has accumulated in the Bloomington Canton-S fly stock that results in the abnormal appearance of the photoreceptors.

We have not examined in further detail the nature of detected defects and whether the putative genetic factors causing the phenotype are linked to particular genomic locations. Similar unexpected defects have not been unnoticed in the past, *e.g*,. a *Drosophila virilis* stock that did not have R7s (also from Bloomington) have previously been reported in DIS (William Stark, personal communication). We wanted to raise awareness among members of the *Drosophila* community about this issue, especially if their near-future research plan includes the ultrastructural characterization of photoreceptors. The morphological abnormalities described in this work (Figure 1) could be used for comparison in electron microscopy characterization of photoreceptor morphology of various genetic backgrounds. The sensitivity of ultrastructural eye morphology to a genetic background (even in the case of not isogenized "wild type" strain, as it was demonstrated in our experiments) underlines the importance of generating control strains with the genetic background as close as possible to the genetic background of the flies under investigation.

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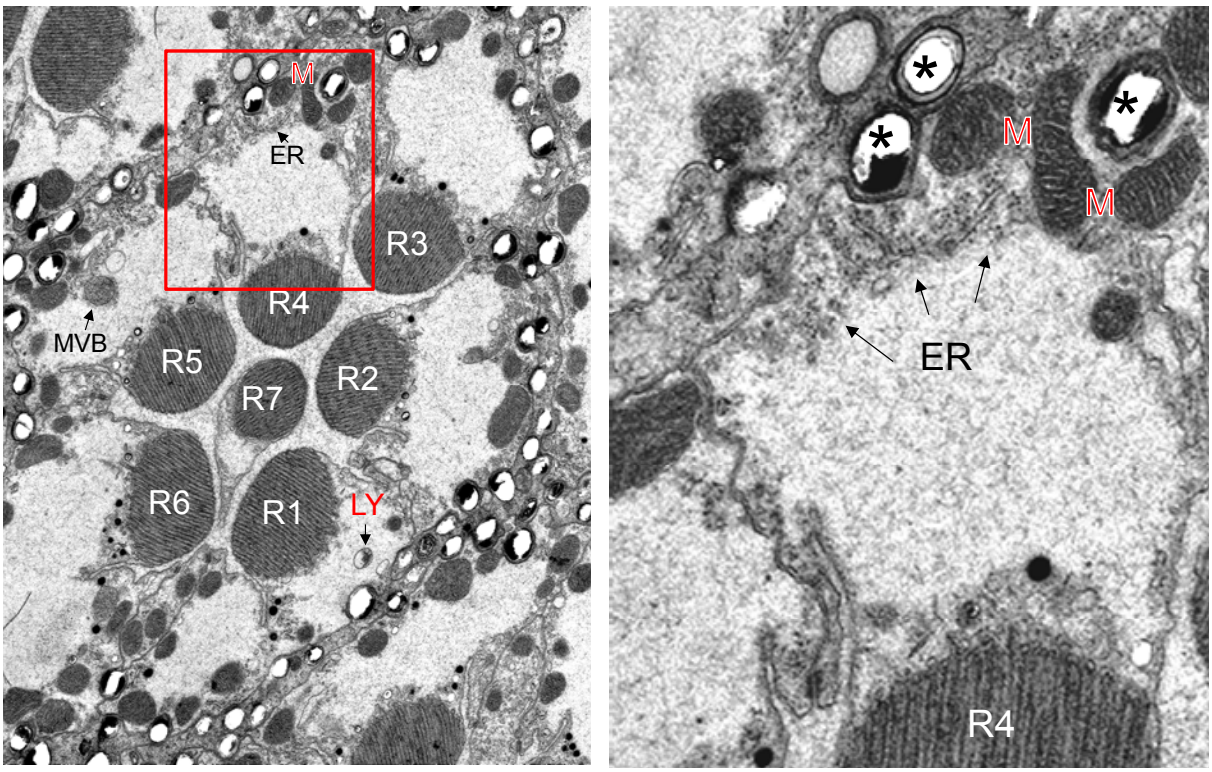


Figure 3. A healthy ommatidium with normal morphology from an alternative source of wild-type Canton-S flies (obtained from Howard Nash, NIMH, Bethesda, MD). On the right side panel the cytoplasm of R4 photoreceptor cell is shown at higher magnification. Note the relatively clean, uncluttered appearance of the cytoplasm, with the endoplasmic reticulum (ER, arrows) and a few mitochondria (M) near the cell border. * indicates the pigment granules; these often fall out during thin sectioning, hence the empty appearance of these structures.

References: Pellikka, M., G. Tanentzapf, M. Pinto, C. Smith, C.J. McGlade, D.F. Ready, and U. Tepass 2002, Nature 416: 143-149; Harris, W.A., and W.S. Stark 1977, J. Gen. Physiol. 69: 261-291; Jackson, G.R., I. Salecker, X. Dong, X. Yao, N. Arnheim, P.W. Faber, M.E. MacDonald, and S.L. Zipursky 1998, Neuron 21: 633-642; O'Neill, S.L., R. Giordano, A.M. Colbert, T.L. Karr, and H.M. Robertson 1992, Proc. Natl. Acad. Sci. U S A 89: 2699-2702; Min, K.T., and S. Benzer 1997, Proc. Natl. Acad. Sci. U S A 94: 10792-10796.

**Growth temperature, duration of development, preadult viability, and body size in *Drosophila melanogaster*.**

**Obradović, Tanja, Sofija Pavković-Lučić, and Vladimir Kekić.** Institute of Zoology, Faculty of Biology, University of Belgrade, Studentski trg 16, Belgrade, Serbia.

Temperature is one of the most important environmental factors that influences all biological processes in *Drosophila*, from molecular level up to behavior (David *et al.*, 1983). Beside immediate influences, temperature may have delayed effects, which can express in later stages during life. For instance, many quantitative morphological traits of *Drosophila* adults depend on temperature at which they were exposed during the larval and/or pupal stage (David and Clavel, 1967), *e.g.*, low temperature, as a rule, influences slower developmental time and larger adult body size (Atkinson, 1994; French *et al.*, 1998; Gibert *et al.*, 2004).

The present study examined the effects of two growth temperatures (18°C and 25°C) on duration of development, preadult viability, and body size in *D. melanogaster*.

Table 1. Mean preadult viability of *D. melanogaster* flies reared at two growth temperatures, 18°C and 25°C.

| Temperature | N | $\overline{X} \pm SE$ |
|---|---|---|
| 18°C | 42 | $0.89 \pm 0.02$ |
| 25°C | 42 | $0.91 \pm 0.02$ |

Table 2. Mean duration of development (males and females) of *D. melanogaster* at two growth temperatures, 18°C and 25°C.

| Temperature | N | $\overline{X} \pm SE$ |
|---|---|---|
| 18°C | 992 | $18.22 \pm 0.06$ |
| 25°C | 1062 | $10.39 \pm 0.04$ |

Table 3. Mean wing length of males and females reared at different growth temperatures, 18°C and 25°C ; 1 mm ≈ 71 measurement units.

| Temperature | N | $\overline{X} \pm SE$ | t | df | P |
|---|---|---|---|---|---|
| 18°C males | 210 | $115.53 \pm 0.18$ | 49.63 | 418 | << 0.001 |
| 25°C males | 210 | $103.62 \pm 0.18$ | | | |
| 18°C females | 210 | $130.34 \pm 0.24$ | 44.93 | 418 | << 0.001 |
| 25°C females | 210 | $118.81 \pm 0.22$ | | | |

Flies used in this experiment belonged to $F_{59}$ laboratory generation of *D. melanogaster* flies from *BGSK* strain. This strain was originated by mixing samples from natural populations collected in Belgrade, Sremska Kamenica and Kragujevac, Serbia. Flies were maintained in mass culture, in 250 ccm glass bottles, without competition, at 25°C, relative humidity of 60%, and light conditions 12h L: 12h D, light from 8 a.m. to 8 p.m. During first 40 generations, flies were maintained on standard cornmeal-agar-yeast medium; later, their rearing was continued on yeast's substrate (dry baker's yeast -sucrose - water - agar - nipagin).

From $F_{58}$ generation, virgin males and females were randomly taken. When they were six days old, males and females were crossed: every male stayed with two females for 24 hours; later, every female was transferred into separate flacon with fresh yeast's substrate, at 25°C. When female laid between 20 and 30 eggs (which was controlled several times during the day), she was transferred into new flacon with fresh medium. In total, there were 42 females: every female laid eggs in two flacons, one was put at 25°C, and the other one at 18°C (those flacons stayed during the first 24 hours also at 25°C during egg laying).

Viability is a major component of preadult fitness, and it is usually defined as the proportion of laid eggs which reach the adult stage. It is itself a combination of three components: egg hatchability, larval viability, and pupal viability (David *et al*., 2004). At both temperatures, preadult viability was about 90% (Table 1).

So high viability is not unexpected, as both temperatures are very close to the species optimum (David *et al.*, 1983).

Duration of development (time from egg laying up to adult eclosion) in *D. melanogaster* highly depends on temperature (Pétavy *et al*., 2001). As in our experiment differences in age of the laid eggs in the same flacon might be from several up to almost 24 hours, estimated duration of development (in days), presented on Table 2, are not precise, but it is obvious that development at 25°C is almost doubly faster.

Adult body size was estimated as right wing length (Table 3), as lenght of the third longitudinal vein, from the anterior cross vein to the distal edge (Partridge *et al.*, 1987).

Flies reared at 18°C were about 10% larger than flies of the corresponding sex reared at 25°C, and this diffrence, estimated by t-test, was highly statistically significant.

Effects of growth temperature on traits that we have observed are consistent with literature data, while some deviations, always present when comparing different populations and/or different samples are consequences of the genetic differences between those populations (see *e.g.*, Trotta *et al.*, 2006), as well as specific interactions among environmental conditions, genetic basis and experimental design. Using the *BGSK* strain, we intend to observe effect of growth temperature also on some other, less investigated phenotypic characteristics, especially on some behavioral traits.

References: Atkinson, D., 1994, Adv. Ecol. Res. 25: 1-58; David, J.R., and M.F. Clavel 1967, J. Insect Physiol. 13: 717-729; David, J.R., R. Allemand, J. Van Herewege, and Y. Cohet 1983, Ecophysiology: abiotic factors. In: *The Genetics and Biology of* Drosophila (Ashburner, M., H.L. Carson, and J.N. Thompson, jr., eds.). Vol. 3d, pp. 105-170, Academic Press, London; David, J.R., R. Allemand., P. Capy, M. Chakir, P. Gibert., G. Pétavy, and B. Moreteau 2004, Genetica 120: 151-163; French, V., M. Feast, and L. Partridge 1998, J. Insect Physiol. 44: 1081-1089; Gibert, P., P. Capy, A. Imasheva, B. Moreteau, J.P. Morin, G. Pétavy and J.R. David 2004, Genetica 120: 165-179; Partridge, L., A. Hoffmann, and J.S. Jones 1987, Anim. Behav. 35: 468-476; Pétavy, G., J.R. David, P. Gilbert, and B. Moreteau 2001, J. Therm. Biol. 26: 29-39; Trotta, V.F., C.F. Calboli, M. Ziosi, D. Guerra, M.C. Pezzoli, J.R. David, and S. Cavicchi 2006, BMC Evol. Biol. 6: 67.

**Growth temperature, mating latency, and duration of copulation in *Drosophila melanogaster*.**

**Kekić, Vladimir, Tanja Obradović, and Sofija Pavković-Lučić**. Institute of Zoology, Faculty of Biology, University of Belgrade, Studentski trg 16, Belgrade, Serbia.

For ectotherm species, such as *Drosophila*, temperature is one of the crucial environmental factors (David *et al.*, 1983; Precht *et al.*, 1955). Many papers considering influence of temperature on *Drosophila* geographic distribution, their adaptation on local thermal regimes, on phenotypic plasticity, as well as on different processes in all developmental stages were published (see *e.g.*, Anderson, 1973; James and Partridge, 1995; Hoffmann *et al.*, 2003; David *et al.*, 2004; Ayrinhac *et al.*, 2004; Trotta *et al.*, 2006). Delayed effects of growth temperature (temperature at which development occurs, from egg laying up to eclosion) on different phenotypic traits of adults are especially interesting (David *et al.*, 1983; Atkinson, 1994; French *et al.*, 1998). However, very little is known about delayed effect of growth temperature on behavior of *Drosophila* adults.

The present study examined the effect of two different growth temperatures (18°C and 25°C) on two components of mating behavior: *mating latency* (time between introduction of females and males into mating vial until inception of copulation) and *duration of copulation* (time from inception to the termination of copulation). Mating latency is an important component of fitness in *Drosophila* (Prakash, 1967) and is correlated with different fitness components, like fecundity, fertility and longevity (Hegde and Krishna, 1999; see also Rose *et al.*, 2004). Duration of copulation is primarily under genetic control, but may be affected by different factors, like previous mating experience (Singh and Singh, 2004; Pavković-Lučić and Kekić, 2006).

*D. melanogaster* flies used in this experiment belonged to the *BGSK* laboratory strain (see Obradović *et al.*, 2007). Flies were maintained on yeast's substrate (dry baker's yeast - sucrose - water - agar - nipagin), in mass culture, in 250 ccm glass bottles, without competition, at 25°C, relative humidity of 60%, and light conditions 12h L: 12h D, light from 8 a.m. to 8 p.m. From $F_{67}$ generation, 12 males and 12 females were taken and 12 families were formed. Part of the eggs collected from each pair was put into a climate room at 18°C, while the other part was maintained at 25°C. After eclosion, males that had developed on both temperatures were aspirated and kept into separate flacons during acclimatization at room temperature (about 23°C) until experiment started. Of course, as duration of development differed at different temperatures (see Obradović *et al.,* 2007), it was necessary to synchronize eclosion, as it was important to use males of similar age (4 to 8 days). In experiments, virgin females of the same age from basic laboratory population, reared at 25°C were used.

Mating behavior was tested during morning hours, at room temperature, in *female choice* experimental design: in the first place, males (10 males that developed at 25°C + 10 males developed at 18°C) were introduced into mating vials (250 ccm glass bottles), followed by 10 virgin females. All males introduced into one mating vial were full-sibs. Two replicates *per* family were made, or, in total, 24 replicates. Males that had developed at different temperatures were marked 24 hours before experiment started with fluorescent UV dust, as it does not influence their activity (Crumpacker, 1974; Terzić *et al.*, 1994). Observation lasted one hour *per* replicate; mating pairs were gently aspirated from mating vial and put into separate flacons.

Mean values of observed components of mating behavior were pooled for all 12 families and presented in Table 1. No significant differences in mating latency, as well as in duration of copulation between full-sib males which had developed on different temperatures were observed.

Table 1. Mean mating latency (*log* transformed) and duration of copulation (in *minutes*) in mating pairs in which males differed in growth temperatures.

| Males growth temperature | Mating latency | | | | | Duration of copulation | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | N | $\overline{X} \pm SE$ | t | df | P | N | $\overline{X} \pm SE$ | t | df | P |
| 18°C | 112 | 2.761 ± 0.044 | 1.03 | 216 | >0.05 | 103 | 18.37 ± 0.43 | 1.65 | 194 | >0.05 |
| 25°C | 106 | 2.695 ± 0.046 | | | | 93 | 19.34 ± 0.38 | | | |

Males that had developed at 18°C were significantly larger than those reared at 25°C. Adult body size was approximated as right wing length, as length of the third longitudinal vein, from the anterior cross vein to the distal edge (Partridge *et al.,* 1987). Mean wing length of males developed at 18°C was: $\overline{X} \pm SE = 114.14 \pm 0.26$; N = 107; at 25°C it was $\overline{X} \pm SE = 102.63 \pm 0.28$; N = 94; this difference, estimated by t-test, was highly statistically significant ($t = 29.51$; $P < 0.01$; $df = 199$).

On the other side, when body size variation was created by varying the degree of crowding among larvae from an inbred strain of *D. melanogaster,* copulation duration was shown to depend on female body size, but either not or much less on male body size (Lefranc and Bundgaard, 2000). Unfortunately, in our experiment, we did not use females that had developed at 18°C; it is possible that variation in female body size created by growth temperature has more influence on copulation duration than variation in male body size. Our previous results also suggested much more active and more important role of females in control of duration of copulation (Pavković-Lučić and Kekić, 2006).

References: Anderson, W.W., 1973, Evolution 27: 278-284; Atkinson, D., 1994, Adv. Ecol. Res. 25: 1-58; Ayrinhac, A., V. Debat, P. Gibert, A.G. Kister, H. Legout, B. Moreteau, R. Vergilino,

and J.R. David 2004, Funct. Ecol. 18: 700-706; Crumparker, D.W., 1974, The American Midland Naturalist 91: 118-129; David, J.R., R. Allemand, J. Van Herewege, and Y. Cohet 1983, Ecophysiology: abiotic factors. In: *The Genetics and Biology of* Drosophila (Ashburner, M., H.L. Carson, and J.N. Thompson, jr., eds.). Vol. 3d, pp. 105-170, Academic Press, London; David, J.R., P. Gibert, and B. Moreteau 2004, Evolution of reaction norms. *Phenotypic Plasticity: Functional and Conceptual Approaches* (De Witt, T.J., and S.M. Scheiner, eds.), pp. 50-63. Oxford University Press, Cary, NC; French, V., M. Feast, and L. Partridge 1998, J. Insect Physiol. 44: 1081-1089; Hegde, S.N., and M.S. Krishna 1999, Curr. Sci. 77: 178-179; Hoffmann, A.A., J.G. Sorensen, and V. Loeschcke 2003, J. Therm. Biol. 28: 175-216; James, A.C., and L. Partridge 1995, J. Evol. Biol. 8: 315-330; Lefranc, A., and J. Bundgaard 2000, Hereditas 132: 243-247; Obradović, T., S. Pavković-Lučić, and V. Kekić 2007, Growth temperature, duration of development, preadult viability and body size in *Drosophila melanogaster*. Dros. Inf. Serv. 90: *in press;* Partridge, L., A. Hoffmann, and J.S. Jones 1987, Anim. Behav. 35: 468-476; Pavković-Lučić, S., and V. Kekić 2006, Period. Biol. 108: 81-84; Prakash, S., 1967, Genetics 57: 655-663; Precht, H., J. Christophersen, and H. Hensel 1955, Temperatur und Leben. Springer. Berlin; Rose, M.R., H.B. Passananti, and M. Matos (eds.) 2004, *Methuselah Flies: A Case Study in the Evolution of Aging*. Word Scientific Publishing, Singapore; Singh, S.R., and B.N. Singh 2004, Curr. Sci. 86: 465-470; Terzić, T., M. Andjelković, and M. Stamenković-Radak 1994, Dros. Inf. Serv. 75: 164; Trotta, V., F.C.F. Calboli, M. Ziosi, D. Guerra, M.C. Pezzoli, J.R. David and S. Cavicchi 2006, BMC Evol. Biol. 6: 67.

**Amplifications of orthologous DNA fragments in three *Drosophila* species endemic to India.**

**Mohanty, Sujata*, and Deepshikha Pandey.** Department of Biotechnology, Jaypee Institute of Information Technology University, A-10, Sector 62, Noida – 201307, Uttar Pradesh, India**;** *Author for correspondence (e-mail: sujata.mohanty@jiit.ac.in).

The footprint of evolution is very often reflected at the molecular organisation in the closely related taxa (Takano, 1998). Such footprints can easily be traced by the identification of conserved DNA sequences across different taxa (otherwise known as orthologous DNA fragments). These conserved DNA sequences can then be utilized to reconstruct phylogenetic positions of these taxa (Goodstadt and Ponting, 2006) and to infer detailed evolutionary history of a group of closely related taxa. Thus, much attention has been paid in recent years to use of single-locus homologous DNA markers for these kinds of study. However, it has been shown that evolutionary history reconstruction through the use of multilocus DNA fragments provides robust inference to this aspect (Kopp and Barmina, 2005).

To this respect, the model organism *Drosophila* has been used to test this hypothesis in great detail. Since wide varieties of *Drosophila* species are sympatric to many tropical habitats of the globe, it is interesting to understand evolutionary history of these species and thus to correlate with ecological adaptations. Evolutionary history inferred from multilocus DNA fragments, that too using putatively neutral markers are especially important, as demographic events related to species differentiation could be inferred baring the effect of natural selection. *Drosophila* species, due to manifold advantages for use in both classical and molecular genetic work, are organisms of choice to test the differential evolutionary hypotheses and also the role of different evolutionary forces in speciation.

India harbours a wide range of *Drosophila* species. Species that are found in domestic or semi-domestic areas are often common. Among these, three different species of *Drosophila* are of fairly common in occurrence in the wild; *Drosophila ananassae*, *D. malerkotliana* and *D. melanogaster*. While the first species is fairly common in almost all seasons, *D. malerkotliana* is widely available for collection during rainy season of the year and *D. melanogaster* during winter. However, the latter species is becoming rare in the wild in India. Ecologically, *D. melanogaster* and *D. ananassae* almost utilize a similar food resource and both are found to be strictly domestic, while *D. malerkotliana* is semi-domestic and mostly seen in orchards, gardens, and places just close to human habitation, but rarely inside houses. Thus, while the almost-common ecological habitat of all the three species in India provides an opportunity to understand similarities at the DNA level, it is of high interest, on the other hand, to look for genetic dissimilarities as well, by which each have adapted to different ecological, seasonal conditions in India.

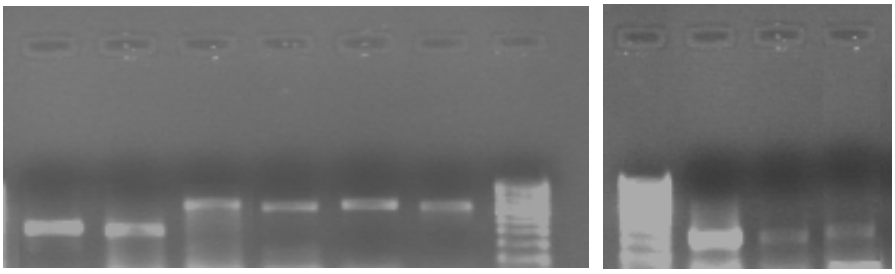


Figure 1. Agarose-gel electrophoretic pictures showing three PCR-amplified nuclear DNA fragments in the genomic DNA of three species of *Drosophila* endemic to India. L = 100 base pair DNA ladder; M1 to M 3 = *D. malerkotliana* DNA fragment no. 1 to 3; A1 to A3 = *D. ananassae* DNA fragment no. 1 to 3; ME1 to ME3 = *D. melanogaster* DNA fragment no. 1 to 3.

The present study, a part of the long-term genomic understanding of speciation history of common *Drosophila* species in India focuses on identifying common nuclear DNA fragments and utilizes these markers to understand evolutionary history of these species in India. To initiate such a kind of study, we have utilized the whole genome sequence information (Adams *et al.*, 2000) of *D. melanogaster* to design primers in the coding regions of different genes (exon parts) to amplify the flanking non-coding introns. As stated above, such types of DNA fragments serve as putatively neutral nuclear DNA markers (Das *et al.*, 2004). We have designed as many as 20 primer pairs based on the *D. melanogaster* whole genome sequence information of the X-chromosome and could successfully amplify only three fragments in all the three species so far (Figure 1). Considering a large reported divergence time among these three species (Kopp and Barmina, 2005), the data on the low amplification rate of DNA fragments across three different species is not surprising, even though the primers are designed in the coding regions of the genome. These fragments will further be sequenced followed by sequence alignments and phylogenetic tree construction which is in process in this laboratory.

Acknowledgments: We thank the Department of Science and Technology, Government of India for an extramural research project to SM. We also thank the Vice Chancellor, JIITU for extending facilities in the department for carrying out research on *Drosophila*.

Reference: Adams, M.D. *et al.*, 2000, Science 287: 2185-2195; Das, A., S. Mohanty, and W. Stephan 2004, Genetics 168: 1975-1985; Goodstadt, L., and C.P. Ponting 2006, PLoS Comp. Biol. 2(9) e133; Kopp, A., and O. Barmina 2005, Genet. Res. 85: 23-46; Takano, T.S., 1998, Genetics 149: 959-970.

**Multilocus nuclear DNA markers for population genetic study in *Drosophila malerkotliana.***

**Mohanty, Sujata*, and Deepshikha Pandey.** Department of Biotechnology, Jaypee Institute of Information Technology University, A-10, Sector 62, Noida – 201307, Uttar Pradesh, India**;** *Author for correspondence (e-mail: sujata.mohanty@jiit.ac.in).

In recent years, major emphasis has been given in the use of multilocus nuclear DNA markers for inference of population evolutionary history of species (Das *et al*., 2004). Such markers provide stable inferences on population history of *Drosophila ananassae* and could ascertain both genetic drift and natural selection in different genomic regions of this species (Baines *et al.*, 2004; Das *et al*., 2004). These approaches have also been followed in population genetic studies of other different taxa out of the *Drosophila* model.

*D. malerkotliana* is a member of the *ananassae* subgroup of the *D. melanogaster* species group (Bock, 1980). This species belongs to the *bipectinata* species complex that consists of four closely related species - *D. bipectinata*, *D. parabipectinata*, *D. malerkotliana* and *D. pseudoananassae* (Singh and Singh, 2001). Evolutionary history of this species complex has been inferred recently using molecular markers (Kopp and Barmina, 2005) with both mitochondrial and nuclear loci. This study revealed that the four members of this species complex have diverged only 283,000 to 385,000 years ago. The later study had made the member of this species complex very interesting. India is presumed to be the homeland to *D. malerkotliana* (species name synonym to a place in Eastern Punjab, Malerkotla) (Tsacas *et al.*, 1981). Interestingly, this species has been introduced to the rest of the world relatively recently; to the Afro-tropical regions and the Seychelles during the 20$^{th}$ century (Louis and David, 1986), to Brazil in 1970s (Val and Sene, 1980; Santos *et al.*, 2003) and to Mexico and Cuba probably during 1980s (Chassagnard *et al.*, 1989). Recent reports confirm its invasion into North America, plausibly through range expansions from the South (Birdsley, 2003; Medeiros *et al.*, 2003). Parts of these hypotheses are substantiated by a recent study on correlation of genome size variation to colonization events (Nardon *et al.*, 2005). Thus, *D. malerkotliana* appears to be an interesting species to understand genetic mechanisms of adaptation to new environments.

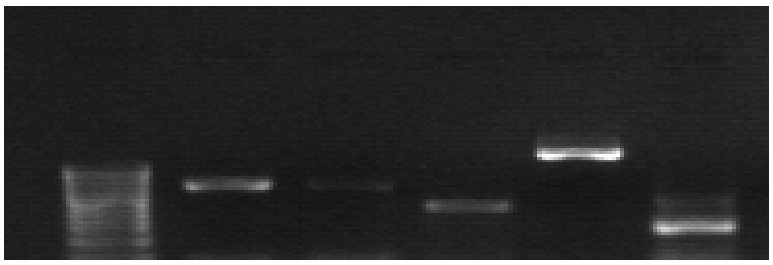


Figure 1. Agarose-gel electrophoretic pictures showing five PCR-amplified nuclear DNA fragments in the genomic DNA of *Drosophila malerkotliana*. L = 100 basepair DNA ladder; M1 = Marker 1; M2 = Marker 2; M3 = Marker 3; M4 = Marker 4; M5 = Marker 5.

In order to initiate population genetic study in India with multilocus nuclear DNA markers, we have utilized the published whole genome sequence information of *D. melanogaster* and designed EPIC (exon priming intron crossing) primers in both the forward and reverse directions. Nuclear DNA of *D. malerkotliana* from an Indian natural population sample was isolated, and amplifications of different fragments were tested. Out of 20 sets of primers tested we have obtained amplifications for five fragments (Figure 1). The extreme

left of the picture contains the 100 base pair ladder for comparison. The first, second and fourth fragment are the largest ones (about 800-900 nucleotide base pair length), while the third and fifth fragments are of intermediate size (about 400 to 600 bp). The sizes of these fragments are within the limit of such fragments used for population genetics studies (Das *et al.*, 2004). However, detail sequencing of all these five fragments and further detection of single nucleotide polymorphisms (SNPs) through comparison of about 10 individuals with DNA sequence assembly and alignments will further confirm their utilization in population genetic analyses. This preliminary work is currently under process in our laboratory.

Acknowledgments: We thank the Department of Science and Technology, Government of India for an extramural research project to SM. We also thank the Vice Chancellor, JIITU for extending facilities in the department for carrying out research on *Drosophila*.

References: Bock, I.R., 1971, Univ. Tex. Publ. 7103: 273- 280; Baines, J.F., A. Das, S. Mousset, and W. Stephan 2004, Genetics 168: 1987-1998; Birdsley, J.S., 2003, Dros. Inf. Serv. 86: 112-113; Chassagnard M.T., M. Solignac, and L. Tsacas 1989, Annls. Soc. Ent. Fr. 25: 247-248; Das, A., S. Mohanty, and W. Stephan 2004, Genetics 168: 1975-1985; Kopp, A., and O. Barmina 2005, Genet. Res. 85: 23-46; Louis, J., and J.R. David 1986, Acta Oecol. Oecol. Gen. 7: 215-229; Medeiros, H.F., B.M. Marlucia, and J.R. David 2003, Dros. Inf. Serv. 86: 89-92; Nardon, C., G. Deceliere, C. Loevenbruck, M. Weiss, C. Vieira, and C. Biemont 2005, Mol. Ecology 14: 869-878; Santos, J.F., T.T. Rieger, S.R.C. Campos *et al.*, 2003, Dros. Inf. Serv. 86: 92-95; Singh, S., and B.N. Singh 2001, Indian J. Exp. Biol. 39: 835-844; Tsacas, L., D. Lachaise, and J.R. David 1981, In: *Genetics and Biology of* Drosophila, (Ashburner, M., H.L. Carson and J.N. Thompson, jr., eds.), Vol 3a: 197-259, Academic Press, London; Val, F.C., and F.M. de Sene 1980, Papeis Avulsos Do Depart. de Zool. da Soc. Agric. 33: 293-298.

**Endemic inversions in Brazilian populations of *Drosophila melanogaster*.**

**Ananina, Galina, and Louis B. Klaczko.** Departamento de Genética e Evolução, Instituto de Biologia, Universidade Estadual de Campinas, UNICAMP, Cx. Postal 6109, Campinas 13083-070 SP, Brazil; Author for correspondence: G. Ananina; e-mail: g.ananina@gmail.com.

## Introduction

*Drosophila melanogaster* is a cosmopolitan species with a very large area of distribution (Keller, 2007). It is natural that, exploiting a wide range of climatic conditions, *D. melanogaster* exhibits a large variability in morphologic and genetic characters. One such character is chromosomal inversion polymorphism (Krimbas and Powell, 1992; Lemeunier and Aulard, 1992; Powell, 1997). *D. melanogaster* chromosomal inversions can be categorized as cosmopolitan (common and rare) and endemic (recurrent and unique), depending on the geographical distribution and frequency (Mettler *et al*., 1977). Cosmopolitan inversions are those that have been observed in populations from all parts of species' geographical range. Endemic inversions are geographically restricted. Recurrent endemic inversions are observed more than once in a given population or may be observed in adjacent populations, while unique endemic inversions are observed only in a single individual from a single population (Mettler *et al*., 1977). Inoue and Igarashi (1994) also described recurrent inversions as `polymorphic endemics'. Comparisons of the endemic inversions found by

different researchers are sometimes very hard due to the absence of microphotographs. Here we report and document endemic inversions found in Brazilian populations of *D. melanogaster*.

## Materials and Methods

Adult flies were collected in urban and suburban areas using traps containing fermented banana baits. We examined samples of *n* individuals from the following locations: Fortaleza, CE (2005; n = 21); Recife, PE (2004, 2005; n = 178); Porto Seguro, BA (2006; n = 18); Rio de Janeiro, RJ (2004, 2005; n = 180); Campinas, SP (2004, 2006; n = 131); Florianópolis, SC (2006; n = 21); Santa Maria, RS (2006; n = 25); Porto Alegre, RS (2006; n = 45).

Males collected from the wild were individually crossed to virgin *Canton-S* females, and male genotypes were determined by examining the salivary gland chromosomes of the F1 larvae. Polytene chromosomes were prepared by 1N HCl treatment and subsequent lacto-acetic-orcein staining of salivary gland cells from 3rd instar larvae (Ashburner, 1989, p 31). Slides were examined using a Nikon Eclipse E800 microscope with 100× objective magnification. Photomicrographs were captured using the digital camera Cool SNAP-Pro (Color). Then, they were digitized with the software Image Pro-Plus v. 4.1., and finally edited using Adobe Photoshop (Adobe 2002 7.0, v 701, San Jose, California, Adobe Systems Inc).

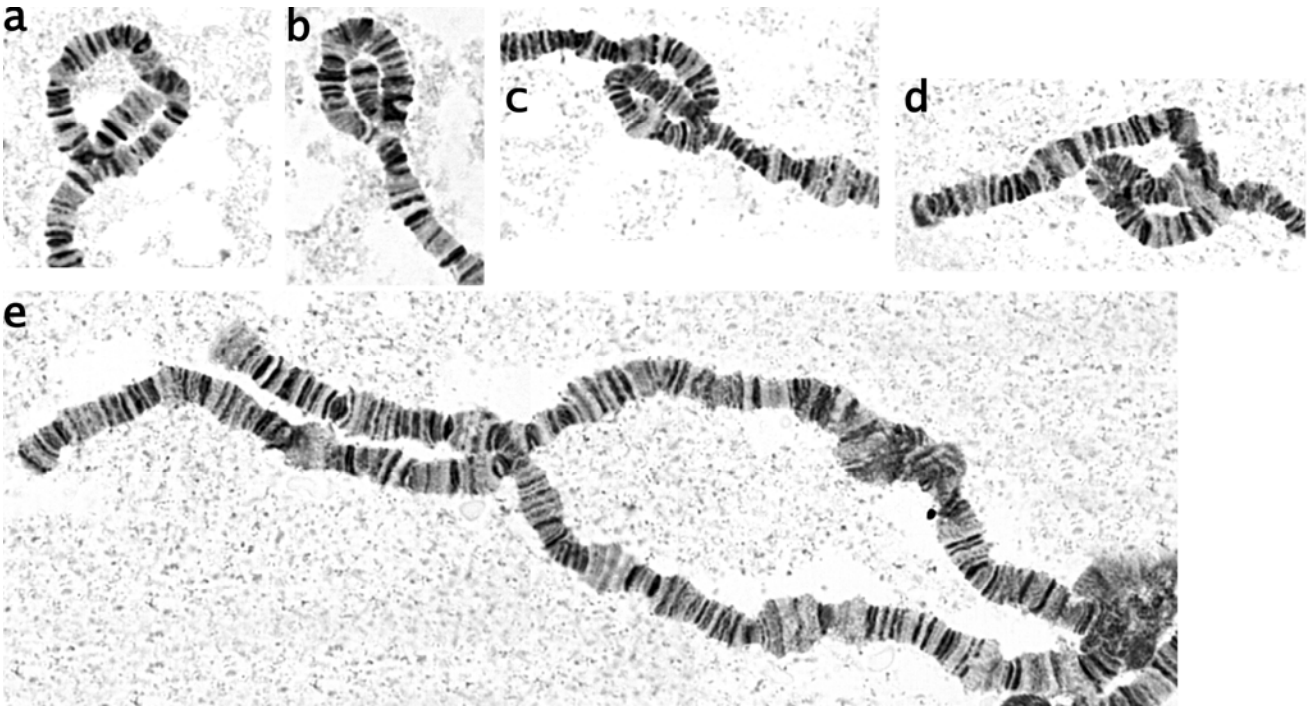


Figure 1. Endemic inversions found in Brazilian populations of *D. melanogaster*, chromosome 2: **a** – *In(2L) 22A;28B* ; **b** – *In(2R)56D;60C*; **c** – *In(2R)49D;52F*; **d** – *In(2R)50AB;55E + In(2R)51D;52F*; **e** – *In(2LR)26A;52F*.

## Results

Endemic rearrangements identified in Brazilian populations of *D. melanogaster* (number of individuals found with inversion):

Chromosome 2:

1) *In(2L) 22A;28B*, Figure 1-a, unique endemic: Rio de Janeiro, RJ, 2004. (1)
2) *In(2LR)26A;52F*, Figure 1-e, pericentric inversion, unique endemic: Campinas, 2004 (1).
3) *In(2R)42A;52F*, Figure 2-c, unique endemic: found in two flies from Rio de Janeiro, RJ, 2004. (2)
4) *In(2R)42C;57E*, Figure 2- b, recurrent endemic: Recife, PE, 2002, 2004; Rio de Janeiro, RJ, 2004. (4)
5) *In(2R)42C;56E*, Figure 2- a, unique endemic: found in three flies from Porto Alegre, RS, 2005. (3)
6) *In(2R)44E;47E*, Figure 2-h, unique endemic: Campinas, SP, 2006. (1)
7) *In(2R)44DE;48F*, Figure 2-e, recurrent endemic: Campinas, SP, 2005, Florianópolis, SC, 2006. (3)
8) *In(2R)45F;59B + In(2R)51E;57A*, Figure 2-j, unique endemic: Rio de Janeiro, RJ, 2004. (4)
9) *In(2R)46F-47A;59B*, Figure 2-f, unique endemic: found in three flies from Campinas, SP, 2004. (3)
10) *In(2R)47B;51C*, unique endemic: Florianópolis, SC, 2006. (1)
11) *In(2R)47E;51F-52A*, Figure 2-k, unique endemic: Rio de Janeiro, RJ, 2004. (1)
12) *In(2R)47F;50E*, Figure 2-d, unique endemic: Campinas, SP, 2006. (1)
13) *In(2R)49E;53F*, Figure 2-i, unique endemic: Santa Maria, RS, 2006. (2)
14) *In(2R)49D;52F*, Figure 1-c, unique endemic: Florianópolis, SC, 2006. (1)
15) *In(2R)50A;55E*, Figure 2-l, recurrent endemic: Campinas, SP, 2004, 2005. (4)
16) *In(2R)50AB;55E + In(2R)51D;52F*, Figure 1-d, recurrent endemic: Campinas, SP, 2004, 2006. (2)
17) In*(2R)51A;55E*, recurrent endemic: Rio de Janeiro, RJ, 2004; Campinas, SP, 2004. (2)
18) *In(2R)51B;59B*, Figure 2-g, unique endemic: Campinas, SP, 2005. (1)
19) In*(2R)53EF;55C*, unique endemic: Rio de Janeiro, RJ, 2004. (1)
20) *In(2R)55DE;59A*, unique endemic: Rio de Janeiro, RJ, 2004. (1)
21) *In(2R)56D;60C*, Figure 1-b, recurrent endemic: Rio de Janeiro, RJ, 2004, 2005. (3)

Chromosome 3

1) *In(3L)63F;64A*, unique endemic: Campinas, SP, 2004. (1)
2) *In(3L)64A;66B*, Figure 3-a, recurrent endemic: Recife, PE, 2004, 2005; Rio de Janeiro, RJ, 2004, 2005; Campinas, SP, 2004, 2005, 2006. (20)
3) *In(3L)64A;67B*, Figure 3-b, recurrent endemic: Rio de Janeiro, RJ, 2004, 2005; Campinas, SP, 2004, 2005, 2006. (22)
4) *In(3L)65DE;67C*, Figure 3-c, unique endemic: Rio de Janeiro, RJ, 2004. (1)
5) *In(3L)66D;72EF*, unique endemic: Campinas, SP, 2005. (1)
6) *In(3L)67A;71B*, Figure 3-h, recurrent endemic: Fortaleza, CE, 2005; Rio de Janeiro, RJ, 2004, 2005; Campinas, SP, 2005. (8)
7) *In(3L)67B;69E*, Figure 3-d, recurrent endemic: Rio de Janeiro, RJ, 2004; Campinas, SP, 2004, 2005; Florianópolis, SC, 2006. (7)

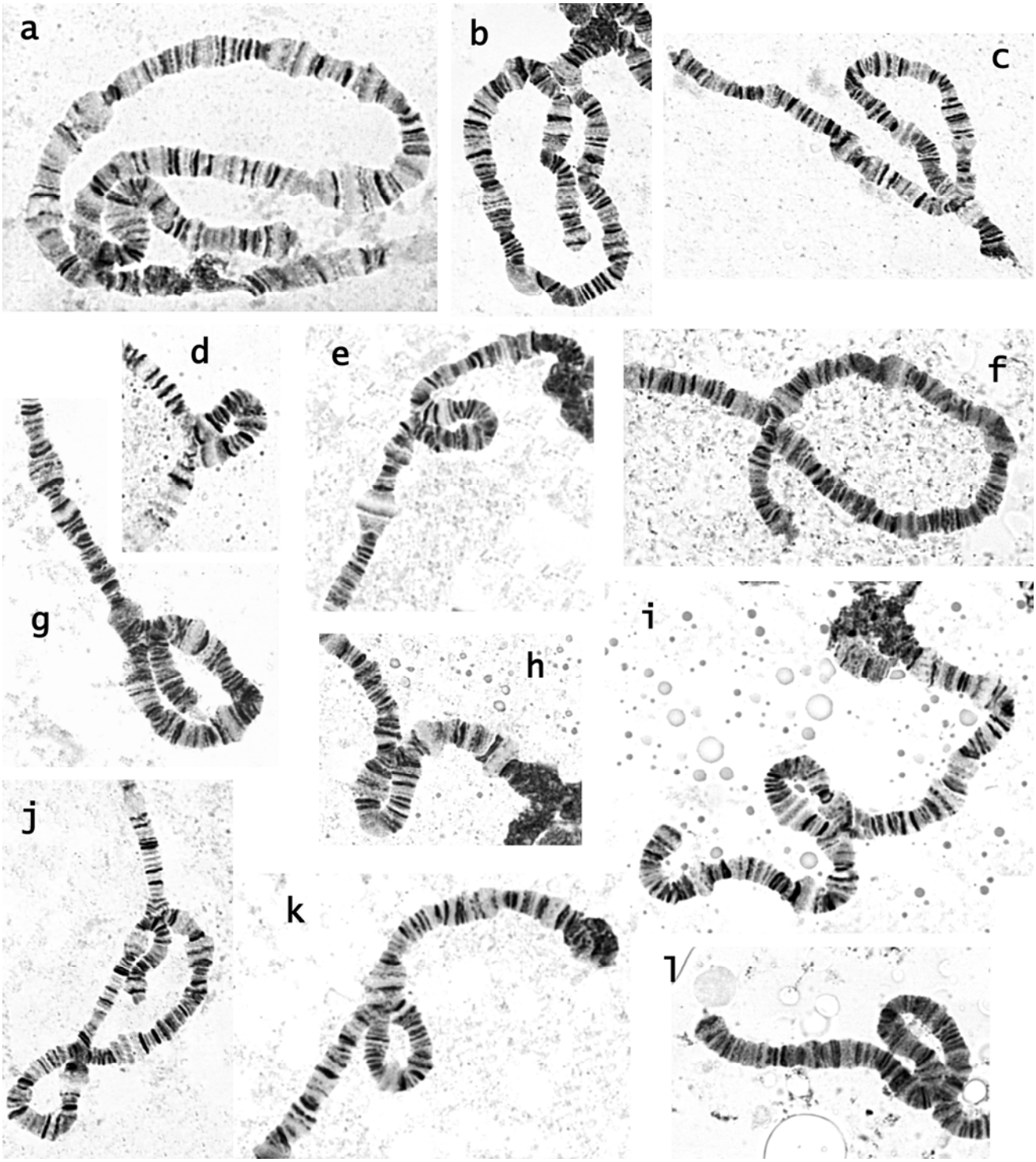


Figure 2. Endemic inversions found in Brazilian populations of *D. melanogaster*, chromosome 2, *2R* arm: **a** – *In(2R)42C;56E*; **b** – *In(2R)42C;57E*; **c** – *In(2R)42A;52F*; **d** – *In(2R)47F;50E*, **e** – *In(2R)44DE;48F*; **f** – *In(2R)46F-47A;59B*; **g** – *In(2R)51B;59B*; **h** – *In(2R)44E;47E*; **i** – *In(2R)49E;53F*; **j** – *In(2R)45F;59B* + *In(2R)51E;57A*; **k** – *In(2R)47E;51F-52A*; **l** – *In(2R)50A;55E*.

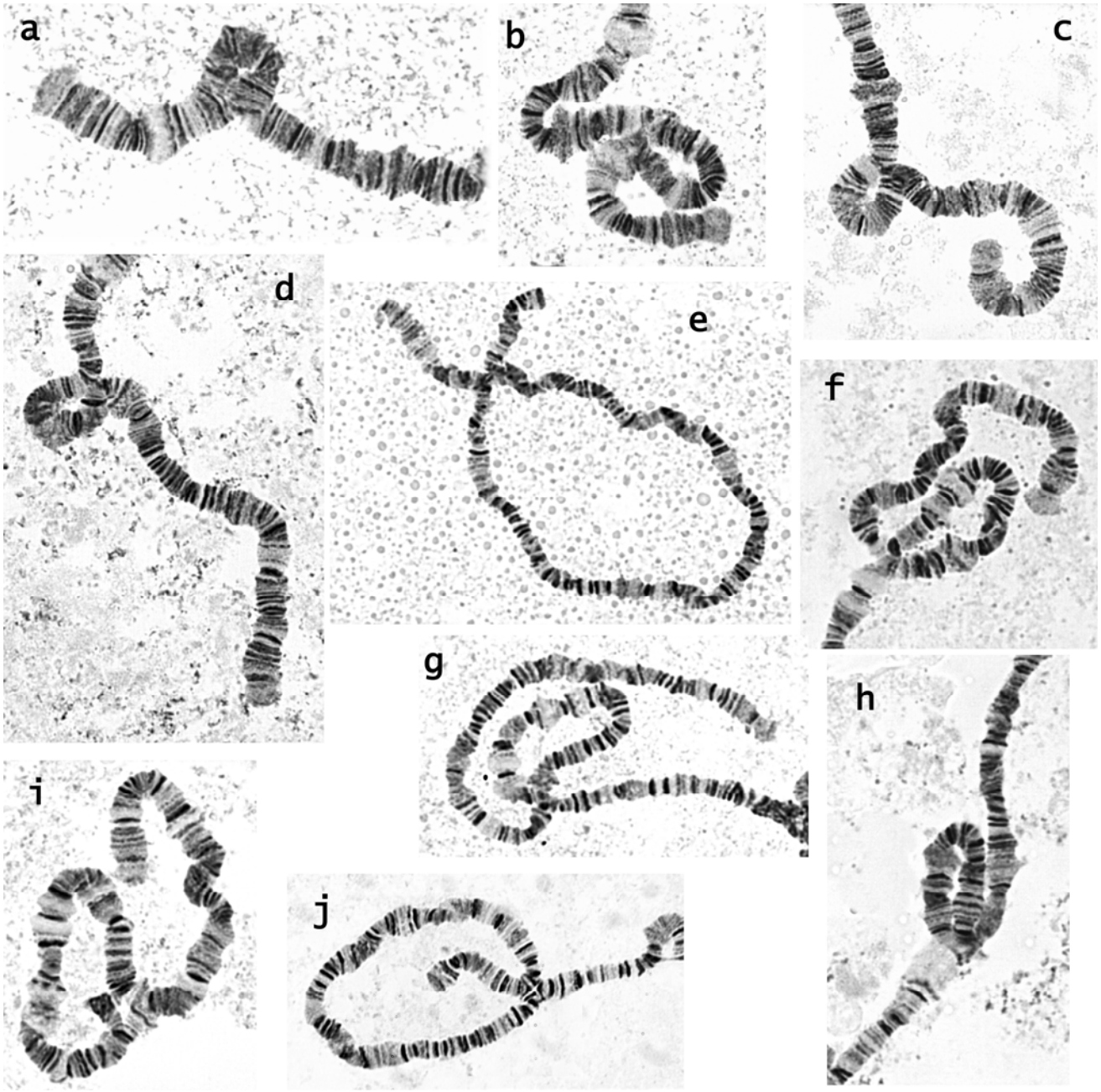


Figure 3**.** Endemic inversions found in Brazilian populations of *D. melanogaster*, chromosome 3: **a** – *In(3L)64A;66B*; **b** – *In(3L)64A;67B*; **c** – *In(3L)65DE;67C*; **d** – *In(3L)67B;69E*; **e** – *In(3R)84D;98F*; **f** – *In(3L)67B;74C*; **g** – *In(3L)68F;75C*; **h** – *In(3L)67A;71B*; **i** – *In(3L)68CD;79F*; **j** – *In(3R)87E;98C*.

8) *In(3L)67B;74C*, Figure 3-f, unique endemic: Florianópolis, SC, 2006. (1)

9) *In(3L)68CD;79F*, Figure 3-i, recurrent endemic: Recife, PE, 2002, 2005; Porto Seguro, BA, 2006; Rio de Janeiro, RJ, 2004, 2005; Campinas, SP, 2004, 2005, 2006; Florianópolis, SC, 2006. (19)

10) *In(3L)68F;75C*, Figure 3-g, recurrent endemic: Rio de Janeiro, RJ, 2004; Campinas, SP, 2004, 2005. (7)

11) *In(3L)68F;70C*, unique endemic: Rio de Janeiro, RJ, 2004. (1)
12) *In(3R)84D;98F*, Figure 3-e, unique endemic: Rio de Janeiro, RJ, 2005. (1)
13) *In(3R)87F;92F*, recurrent endemic: Rio de Janeiro, RJ, 2004; Florianópolis, SC, 2006. (2)
14) *In(3R)87E;98C*, Figure 3-j, recurrent endemic: Rio de Janeiro, RJ, 2004; Campinas, SP, 2004. (4)

References**:** Inoue, Y., and Y. Igarashi 1994, Jpn. J. Genet. 69: 105-118; Keller, A., 2007, Curr. Biol. 17: 77-81; Krimbas, C.B., and J.R. Powell 1992, *Drosophila Inversion Polymorphism* (Krimbas, C.B., and J.R. Powell, eds.), pp. 1–52, Boca Raton, CRC Press; Lemeunier, F., and S. Aulard 1992, *Drosophila Inversion Polymorphism* (Krimbas, C.B., and J.R. Powell, eds.), pp. 339–405, Boca Raton, CRC Press; Powell, J.R., 1997, *Progress and Prospects in Evolutionary Biology, The Drosophila Model* (Powell, J.R., ed.), New York, Oxford University Press; Mettler, L.E., R.A. Voelker, and T. Mukai 1977, Genetics 87: 169-176.

**Hearing defects in Johnston's Organ Gal4 lines**.

**Kotla, Sumankrishna[1], Maura Cosetti[2], Peter F. O'Brien[1], and Frances Hannan[1]**.
[1]Departments of Cell Biology & Anatomy, and Otolaryngology, New York Medical College, Valhalla, NY 10598; [2]Department of Otolaryngology, New York Eye & Ear Infirmary, New York, NY 10003; Email: frances_hannan@nymc.edu.

## Introduction

The *Drosophila* auditory organ, Johnston's Organ (JO), is housed in the second antennal segment (a2; Caldwell and Eberl, 2002). It consists of an array of more than 200 mechanosensory chordotonal organs termed scolopidia. Each scolopidium contains two to three bipolar neurons, and a number of support cells including the scolopale cell which ensheaths the ciliated dendritic processes of the neurons. The basal ends of the scolopidia are attached to the inner surface of a2, while the apical ends are attached to the joint between the a2 and a3 segments by a dendritic cap that is secreted by the scolopale cell and possibly other support cells. The vibrating air particles of a near-field sound, typically the flies' courtship song, cause deflection of the arista and rotation of the third antennal segment (a3) about the a2/a3 joint. This stretches the entire array of scolopidia, initiating transmission of a signal to the central brain via the antennal nerve.

A number of Gal4 lines that label specific subsets of neurons within the JO have been identified (Sharma *et al*., 2002; Kamikouchi *et al*., 2006). The JO1 line labels most neurons in the JO (94%), while the JO3 line labels 67% of JO neurons. Three other lines label 22-38% of the JO neurons (JO2, JO4, JO15). An additional twelve lines label JO neurons as well as other cells within the antenna and forehead region (JO21-JO32). All of the lines also label cells elsewhere in the fly brain, except JO15, which expresses Gal4 only in the JO (Sharma *et al*., 2002). The JO15 line expresses Gal4 under control of a JO specific enhancer fragment, originally identified and cloned from a *hobo* enhancer trap line that specifically stains the JO neurons (Sharma *et al*., 2002).

The spatial organization of the JO neurons expressing Gal4 in each line has been determined, as well as their projection patterns to the antennal mechanosensory and motor center, the AMMC (Kamikouchi *et al*., 2006). Within the JO, the cell bodies of the JO neurons form a bottomless bowl shape. The JO3 line labels cells throughout the entire bowl region, while the JO2 line labels a middle

ring of cells, and the JO4 and JO15 lines each label clusters of cells in the anterior and posterior regions. Five distinct zones within the central brain receive projections from the JO neurons, four of which are within the AMMC, while the other extends over the ventrolateral protocerebrum and the subesophageal ganglion.

We are interested in using these lines to drive the expression of genes of interest and RNAi constructs in the JO to assess the effect of those constructs on hearing. Prior to using the JO lines for this purpose, however, we needed to determine whether any of the JO-Gal4 insertions disrupt hearing. We present data here showing that many of these JO-Gal4 lines do indeed have defective hearing, even after extensive outcrossing to wild type flies for five generations. Both dominant and recessive effects on hearing are observed, potentially identifying novel hearing genes. We also detected several JO-Gal4 lines that express strongly in the JO but do not affect hearing. These will be useful for our future studies and for other *Drosophila* hearing researchers.

**Materials and Methods**

Fly Stocks:

JO15/TM3Sb contains a pPTGAL element with a JO specific enhancer (Sharma *et al*., 2002). The remaining JO-Gal4 lines; JO1, JO2, JO3/CyO, JO4, JO21, JO22, JO23, JO24, JO25/+, JO26/CyO, JO27/CyO, JO28, JO29, JO30, and JO31, were identified by Kamikouchi *et al*. (2006) from the NP series of lines produced by mobilization of pGawB in a *y w (iso)* background (Yoshihara and Ito, 2000). The isogenic control line used in this study is $w^{118isoCJ}$ (Yin *et al*., 1994). Flies were raised on a cornmeal medium at 25°C, and females were collected from 2-4 days post-eclosion for electrophysiological recording of Sound Evoked Potentials (SEPs).

Electrophysiology:

Unanesthetized flies were mounted into a 200 μl micropipette tip with the protruding head immobilized by modeling wax, leaving the antennae free to vibrate. A sharp tungsten electrode was placed in the joint between the first and second antennal segments (recording electrode), and a second (reference) electrode was placed in the brain in the dorsal medial region. The electrodes were connected to a DP-301 differential amplifier (Warner Instruments, CT) with gain set at 1000, low pass filter at 10kHz and high pass filter at 10Hz. The sound stimulus consisting of 5 pulses with 35ms interpulse interval (mimicking the pulse component of *Drosophila* courtship song) was delivered from a speaker via a 0.25 inch i.d. tubing to ensure near field acoustic conditions. Signals were acquired and digitized using a PCI-6023E data acquisition board, and Lab View software, Version 8.0 (National Instruments, Austin, TX). The entire apparatus was set up in a Faraday cage to eliminate electrical disturbances. Responses to 10 presentations of the stimulus were averaged in each trial, and the maximum amplitude of the peak responses was calculated. Statistical analyses were performed using Prism software, Versions 4.0c/5.00 (Graph Pad, San Diego, CA).

**Results and Discussion**

Previous studies in our lab have shown that hearing in female flies is more sensitive to disruption than male hearing (Cosetti *et al*., submitted); therefore, we restricted this study to female flies. We first recorded SEPs from all the JO-Gal4 lines, and compared them with recordings from the $w^{118isoCJ}$ isogenic control line that is wild type for hearing, and is hereafter referred to as WT. This line is commonly used for behavioral studies (Yin *et al*., 1994) and is similar to the lines used to generate the transgenic JO-Gal4 insertions (see Methods). The peak amplitudes of the SEPs in

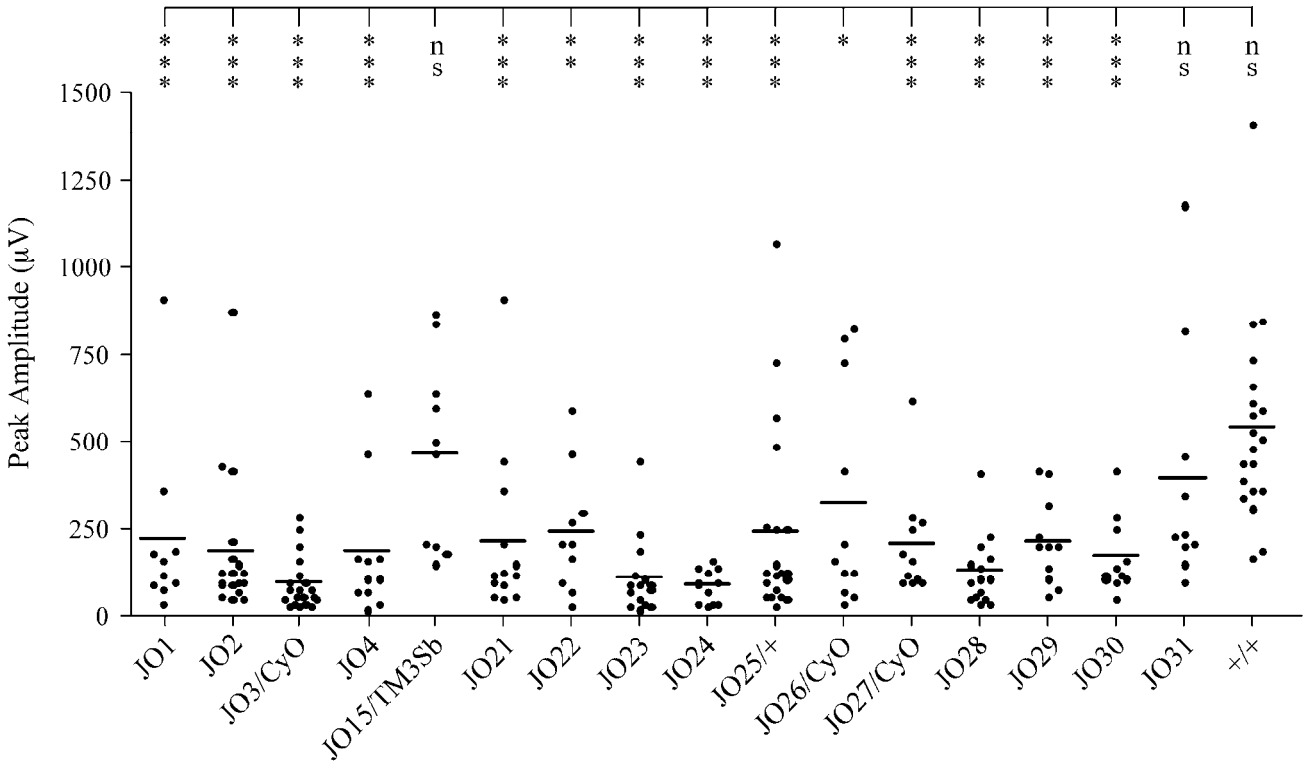


Figure 1. Peak amplitudes of SEPs in JO-Gal4 lines are reduced in comparison to wild type controls. The majority of flies with JO-Gal4 insertions exhibit SEP responses ranging from ~20-500 µV, that are much lower than wild type SEPs. Representative responses of wild type (+/+) flies range from ~100-1500 µV. Mean values for each experimental group are plotted as horizontal bars. The mean value of an expanded wild type (WT) dataset (n = 80) is shown as a horizontal dashed line. Statistically significant reductions in median SEPs are observed for most JO-Gal4 lines compared to the WT dataset (* $p < 0.05$; ** $p < 0.005$; ***$p < 0.005$; Mann-Whitney). See Table 1 for detailed statistical analyses.

individual flies of each genotype, including a representative group of contemporaneously recorded WT flies (+/+), are plotted graphically in Figure 1. The peak amplitude of the response in wild type females ranges from 100 µV up to 1500 µV, while many of the JO lines exhibited responses below 100 µV, especially JO3, JO23, JO24 and JO28. The JO3, JO15, JO26 and JO27 lines were supplied as balanced stocks; however, the presence of the CyO or TM3 balancer chromosomes had no effect on SEPs (Figure 1; data not shown). The mean response for each genotype is shown as a horizontal bar, while the mean response of a large WT dataset (n = 80), collected over several months of recordings, is shown as a horizontal dashed line (Figure 1). Statistical analyses of the data, including minimum, maximum, median, mean and standard error of the mean (SEM) are presented in Table 1. P values were calculated by non-parametric Mann-Whitney analysis of median values for each group in comparison to the large WT dataset (Table 1). Significant reductions in median peak response amplitude were observed for most of the JO-Gal4 lines when compared to WT flies (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$; Figure 1), except for the JO15/TM3Sb and JO31lines (ns $p > 0.05$; Figure 1).

Table 1. Statistical evaluation of SEPs in JO-Gal4 lines.

| Genotype | N | Minimum (mV) | Maximum (mV) | Median (mV) | Mean (mV) | SEM (mV) | P value vs WT |
|---|---|---|---|---|---|---|---|
| WT | 80 | 110 | 1410 | 525 | 509 | 30 | 1 |
| JO1 | 10 | 40 | 910 | 140 | 223 | 81 | 0.0005 |
| JO2 | 18 | 50 | 880 | 115 | 187 | 48 | <0.0001 |
| JO3/CyO | 20 | 30 | 290 | 70 | 96 | 17 | <0.0001 |
| JO4 | 12 | 20 | 640 | 135 | 183 | 53 | <0.0001 |
| JO15/TM3Sb | 10 | 150 | 870 | 485 | 466 | 86 | 0.7531 |
| JO21 | 13 | 50 | 910 | 120 | 216 | 67 | <0.0001 |
| JO22 | 10 | 30 | 590 | 210 | 242 | 56 | 0.0016 |
| JO23 | 15 | 20 | 450 | 90 | 113 | 29 | <0.0001 |
| JO24 | 12 | 30 | 160 | 95 | 90 | 13 | <0.0001 |
| JO25/+ | 20 | 30 | 1070 | 130 | 242 | 61 | <0.0001 |
| JO26/CyO | 11 | 40 | 830 | 160 | 326 | 95 | 0.0440 |
| JO27/CyO | 11 | 100 | 620 | 160 | 209 | 46 | 0.0001 |
| JO28 | 15 | 40 | 410 | 110 | 129 | 25 | <0.0001 |
| JO29 | 11 | 60 | 420 | 200 | 216 | 37 | 0.0002 |
| JO30 | 11 | 50 | 420 | 120 | 170 | 32 | <0.0001 |
| JO31 | 10 | 100 | 1180 | 235 | 394 | 109 | 0.0744 |
| +/+ | 20 | 170 | 1410 | 495 | 540 | 62 | 0.7271 |

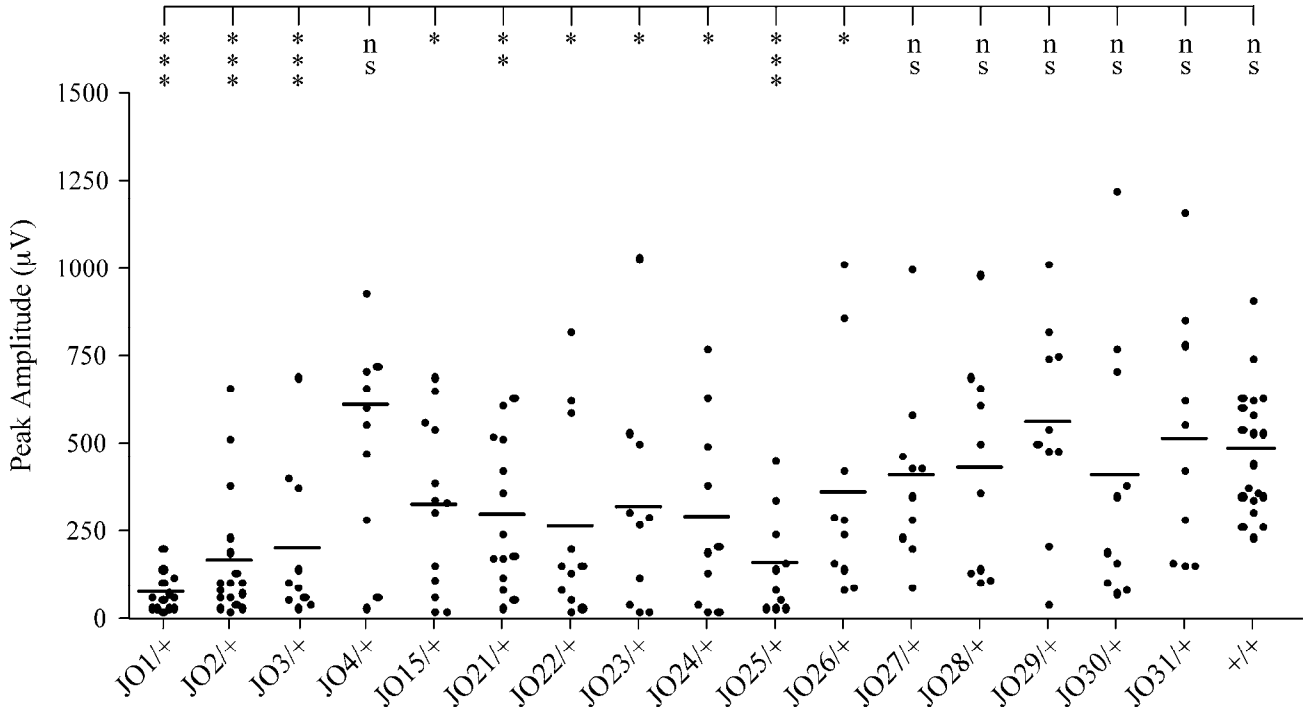


Figure 2. Peak amplitudes of SEPs are improved in JO-Gal4 heterozygotes. Many JO-Gal4 heterozygotes exhibit SEP responses that are statistically similar to wild type values (ns $p > 0.05$), ranging from ~50-1200 µV. Representative responses of wild type (+/+) flies range from ~200-950 µV. Mean values for each experimental group are plotted as horizontal bars. The mean value of an expanded WT dataset (n = 80) is shown as a horizontal dashed line. Statistically significant

reductions in median SEPs are still observed for some JO-Gal4 lines compared to the WT dataset (* $p < 0.05$; ** $p < 0.005$; ***$p < 0.005$; Mann-Whitney). See Table 2 for detailed statistical analyses.

Table 2. Statistical evaluation of SEPs in JO-Gal4 heterozygotes.

| Genotype | N | Minimum (mV) | Maximum (mV) | Median (mV) | Mean (mV) | SEM (mV) | P value vs WT |
|---|---|---|---|---|---|---|---|
| JO1/+ | 14 | 20 | 200 | 60 | 71 | 14 | <0.0001 |
| JO2/+ | 17 | 20 | 660 | 100 | 164 | 45 | <0.0001 |
| JO3/+ | 10 | 30 | 690 | 95 | 197 | 69 | 0.0005 |
| JO4/+ | 11 | 30 | 1650 | 600 | 606 | 134 | 0.4252 |
| JO15/+ | 13 | 20 | 690 | 330 | 320 | 66 | 0.0212 |
| JO21/+ | 14 | 30 | 630 | 210 | 292 | 57 | 0.0048 |
| JO22/+ | 11 | 20 | 820 | 150 | 258 | 84 | 0.0060 |
| JO23/+ | 10 | 20 | 1030 | 280 | 312 | 99 | 0.0145 |
| JO24/+ | 10 | 20 | 770 | 200 | 288 | 85 | 0.0251 |
| JO25/+ | 10 | 30 | 450 | 110 | 155 | 46 | <0.0001 |
| JO26/+ | 10 | 80 | 1010 | 260 | 357 | 102 | 0.0419 |
| JO27/+ | 10 | 90 | 1000 | 390 | 405 | 80 | 0.1542 |
| JO28/+ | 10 | 100 | 980 | 430 | 428 | 97 | 0.4411 |
| JO29/+ | 10 | 40 | 1010 | 520 | 557 | 91 | 0.4526 |
| JO30/+ | 10 | 70 | 1220 | 270 | 403 | 121 | 0.1382 |
| JO31/+ | 10 | 150 | 1160 | 485 | 512 | 109 | 0.9590 |
| +/+ | 20 | 230 | 910 | 485 | 479 | 41 | 0.7728 |

Since the JO-Gal4 lines would be used as heterozygotes when driving expression of transgenic constructs, we next analyzed the responses of heterozygous JO-Gal4 flies, after crossing the JO-Gal4 lines to the WT line, as shown in Figure 2. Statistical analyses of the data are presented in Table 2. Hearing was not significantly different from wild type flies in JO4/+, JO27/+, JO28/+, JO29/+, JO30/+ and J031/+ flies (ns $p > 0.05$; Figure 2). There also appeared to be an improvement of SEPs in JO21/+, JO22/+, JO23/+, and JO24/+ flies (* $p < 0.05$; **$p < 0.005$; Figure 2) as compared to JO21, JO22, JO23, and JO24 homozygotes (**$p < 0.005$; ***$p < 0.0005$; Figure 1); however, this "improvement" was not statistically significant ($p > 0.05$; not shown). Several lines, however, were still significantly unresponsive to the stimulus, including JO1/+, JO2/+, JO3/+ and JO25/+ (***$p < 0.005$, Figure 2). Unfortunately three of these lines are among those that express strongly in the JO region of the antenna alone (Kamikouchi *et al*., 2006), and would, therefore, be particularly useful for expressing transgenic RNAi constructs in the JO.

Encouraged by the restoration of normal hearing in some of the JO-Gal4 heterozygotes, we continued to outcross to the WT line for an additional four generations, which should be sufficient to remove any effect of genetic background differences on hearing. The outcrossed JO-Gal4 lines are hereafter distinguished from the original JO-Gal4 lines by a $^{\#}$ superscript. Lines were then made homozygous (except JO15$^{\#}$/TM3 Ser, and JO27$^{\#}$/CyO, which are homozygous lethal/semi-lethal) and SEPs were again recorded and compared to wild type flies, as shown in Figure 3 and Table 3. Significant hearing defects were still observed in the majority of homozygous lines, including JO1$^{\#}$, JO2$^{\#}$, JO3$^{\#}$, JO22$^{\#}$, JO23$^{\#}$, JO24$^{\#}$, JO25$^{\#}$, JO26$^{\#}$, JO28$^{\#}$, and JO31$^{\#}$ (**$p < 0.005$; ***$p < 0.0005$; Figure 3). The SEPs of JO4$^{\#}$, JO15$^{\#}$/TM3 Ser, JO27$^{\#}$/CyO, and JO30$^{\#}$ lines were, however, indistinguishable from wild type flies (ns $p > 0.05$; Figure 3). The apparent improvement of SEPs in JO21$^{\#}$ and JO29$^{\#}$ homozygotes (* $p < 0.05$; Figure 3) compared to the original JO21 and JO29 flies (*** $p < 0.0005$; see Figure 1) was not statistically significant ($p > 0.05$; not shown).

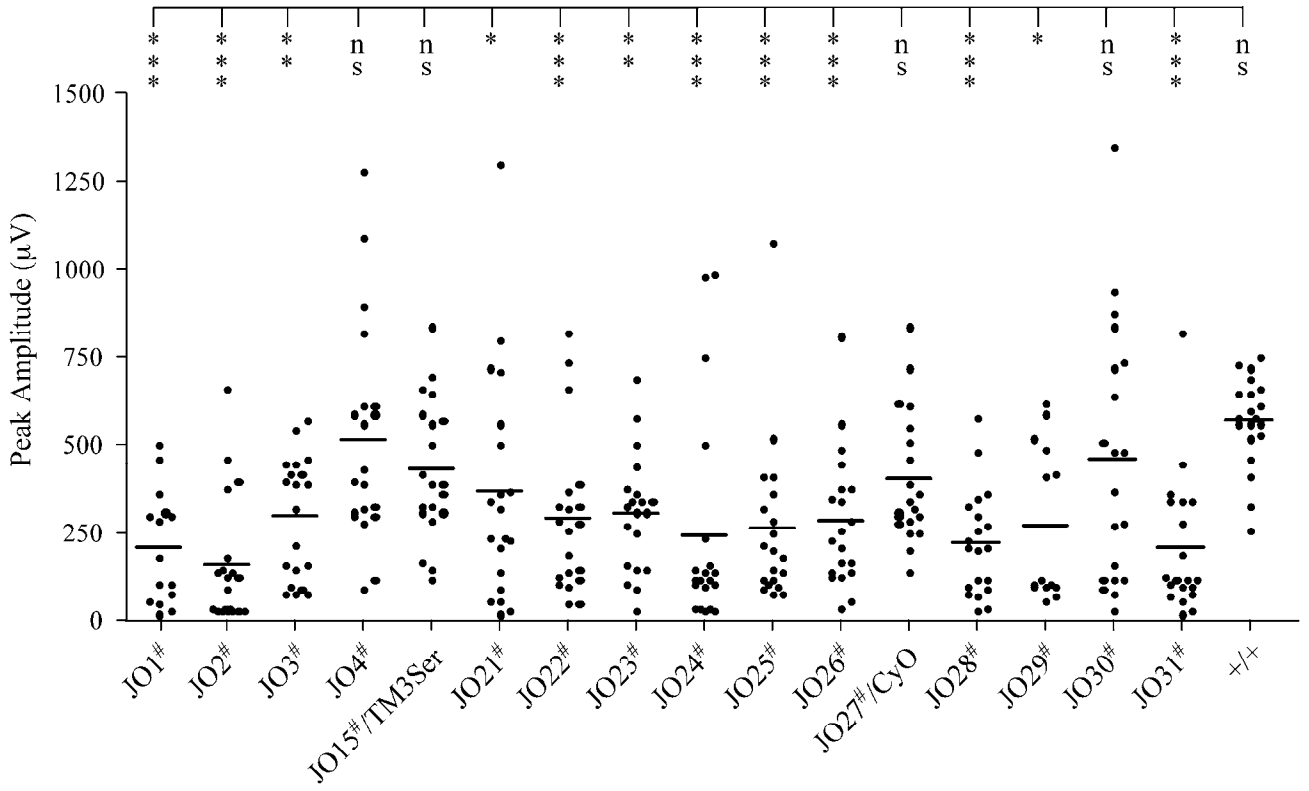


Figure 3. Peak amplitudes of SEPs in most JO-Gal4 lines remain reduced after extensive outcrossing to wild type flies. The majority of flies with JO-Gal4 insertions exhibit reduced SEP responses, ranging from ~20-800 μV, despite extensive outcrossing to wild type controls. Representative responses of wild type (+/+) flies range from ~250-750 μV. Mean values for each experimental group are plotted as horizontal bars. The mean value of an expanded wild type (WT) dataset (n = 80) is shown as a horizontal dashed line. Statistically significant reductions in median SEPs are observed for many JO-Gal4 lines compared to the WT dataset (* $p < 0.05$; ** $p < 0.005$; ***$p < 0.005$; Mann-Whitney). See Table 3 for detailed statistical analyses.

We then analyzed the response of heterozygous outcrossed JO-Gal4 lines, after crossing to the WT line, as shown in Figure 4 and Table 4. The majority of the outcrossed JO-Gal4 heterozygotes exhibit SEPs that are statistically indistinguishable in comparison to wild type flies (ns $p > 0.05$; Figure 4). A few of these lines still show significantly reduced SEPs compared to WT flies, however, suggesting a dominant effect of the Gal4 insertion on hearing in these flies, including JO3$^{\#}$/+, JO26$^{\#}$/+, JO27$^{\#}$/+ and JO31$^{\#}$/+ flies (*$p < 0.05$, Figure 4). The SEP defects in these four JO-Gal4 lines may improve further after additional outcrossing; however, we have decided not to use these lines to drive expression of genes in the JO for the analysis of hearing. Interestingly, the JO27/+ and JO27$^{\#}$/CyO flies appear to have normal hearing (Figures 2 and 3), while the JO27$^{\#}$/+ flies do not (Figure 4). Perhaps there is an interaction with another gene or genes on the WT or CyO second chromosomes.

The dominant defect in SEPs in the JO3, JO26, JO27 and JO31 lines (Figure 4), suggests that the genes disrupted by these JO-Gal4 insertions may be critical for hearing, although this needs to be confirmed by additional experiments. The JO3 line is expressed strongly in the JO alone and has an insertion in the CG13795 gene, encoding a putative extracellular amino acid transporter protein,

Table 3. Statistical evaluation of SEPs in outcrossed JO-Gal4 lines.

| Genotype | N | Minimum (mV) | Maximum (mV) | Median (mV) | Mean (mV) | SEM (mV) | P value vs WT |
|---|---|---|---|---|---|---|---|
| JO1$^{\#}$ | 15 | 20 | 500 | 180 | 211 | 41 | <0.0001 |
| JO2$^{\#}$ | 20 | 30 | 660 | 110 | 158 | 40 | <0.0001 |
| JO3$^{\#}$ | 20 | 80 | 570 | 355 | 297 | 38 | 0.0007 |
| JO4$^{\#}$ | 20 | 90 | 1280 | 415 | 516 | 69 | 0.7630 |
| JO15$^{\#}$/TM3Ser | 20 | 120 | 840 | 390 | 432 | 44 | 0.2776 |
| JO21$^{\#}$ | 20 | 20 | 1300 | 280 | 365 | 72 | 0.0133 |
| JO22$^{\#}$ | 20 | 50 | 820 | 270 | 292 | 50 | 0.0005 |
| JO23$^{\#}$ | 20 | 30 | 690 | 320 | 307 | 37 | 0.0007 |
| JO24$^{\#}$ | 20 | 30 | 990 | 120 | 246 | 68 | <0.0001 |
| JO25$^{\#}$ | 20 | 80 | 1080 | 190 | 262 | 52 | <0.0001 |
| JO26$^{\#}$ | 20 | 40 | 810 | 245 | 287 | 42 | 0.0004 |
| JO27$^{\#}$/CyO | 20 | 140 | 840 | 330 | 402 | 41 | 0.0738 |
| JO28$^{\#}$ | 20 | 30 | 580 | 210 | 222 | 33 | <0.0001 |
| JO29$^{\#}$ | 14 | 60 | 620 | 115 | 273 | 58 | 0.0013 |
| JO30$^{\#}$ | 20 | 30 | 1350 | 425 | 461 | 81 | 0.3476 |
| JO31$^{\#}$ | 20 | 20 | 820 | 120 | 209 | 43 | <0.0001 |
| +/+ | 20 | 260 | 750 | 580 | 571 | 29 | 0.0963 |

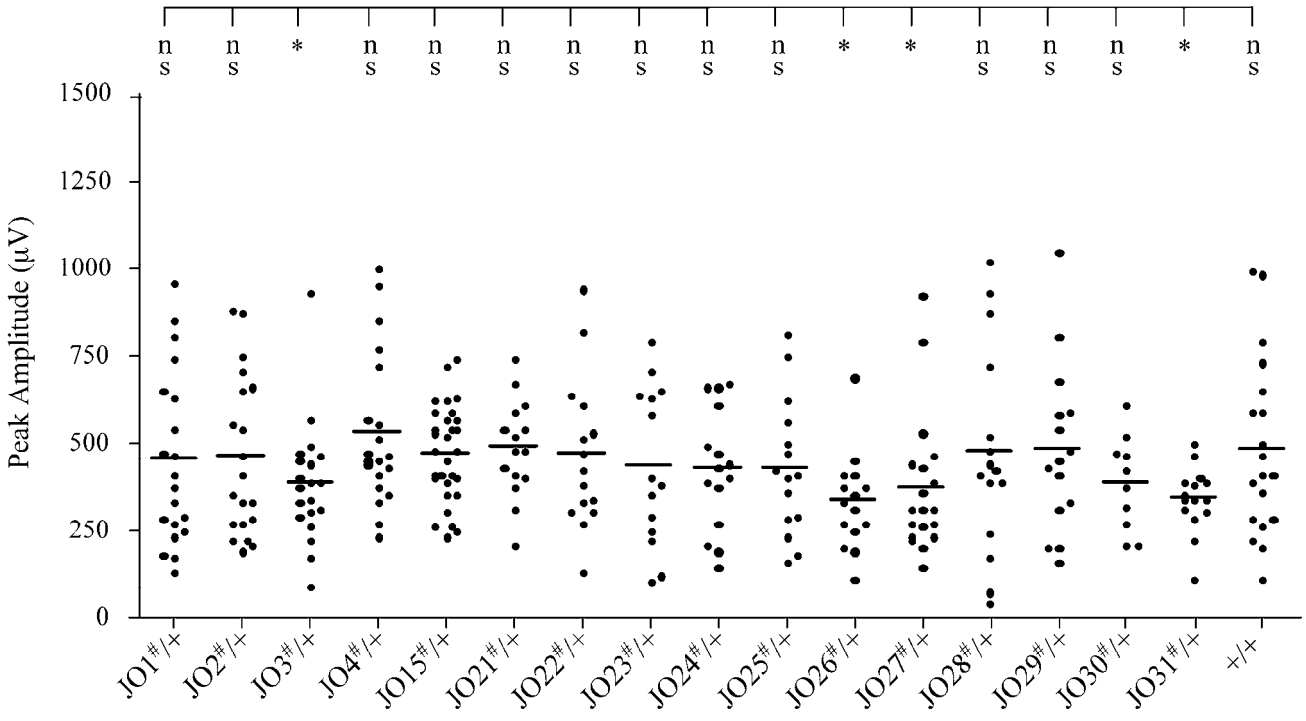


Figure 4. Peak amplitudes of SEPs in outcrossed JO-Gal4 heterozygotes are restored to wild type values. The majority of outcrossed JO-Gal4 heterozygotes exhibit SEP responses that are statistically similar to wild type values (ns $p > 0.05$), ranging from ~100-1000 µV. Representative responses of wild type (+/+) flies also range from ~100-1000 µV. Mean values for each experimental group are plotted as horizontal bars. The mean value of an expanded wild type (WT) dataset (n = 80) is shown as a horizontal dashed line. Statistically significant reductions in median SEPs are observed for only

a few JO-Gal4 lines compared to the WT dataset (* $p < 0.05$; Mann-Whitney). See Table 4 for detailed statistical analyses.

Table 4. Statistical evaluation of SEPs in outcrossed JO-Gal4 heterozygotes.

| Genotype | N | Minimum (mV) | Maximum (mV) | Median (mV) | Mean (mV) | SEM (mV) | P value vs WT |
|---|---|---|---|---|---|---|---|
| JO1#/+ | 20 | 130 | 960 | 390 | 451 | 55 | 0.4081 |
| JO2#/+ | 20 | 190 | 880 | 380 | 458 | 51 | 0.4879 |
| JO3#/+ | 20 | 90 | 930 | 380 | 384 | 39 | 0.0277 |
| JO4#/+ | 20 | 230 | 1000 | 455 | 529 | 49 | 0.7662 |
| JO15#/+ | 30 | 230 | 740 | 480 | 472 | 25 | 0.6798 |
| JO21#/+ | 15 | 210 | 740 | 480 | 487 | 36 | 0.9878 |
| JO22#/+ | 15 | 130 | 940 | 420 | 466 | 56 | 0.5302 |
| JO23#/+ | 14 | 100 | 790 | 390 | 436 | 61 | 0.4967 |
| JO24#/+ | 15 | 140 | 670 | 430 | 427 | 45 | 0.4230 |
| JO25#/+ | 15 | 160 | 810 | 410 | 429 | 50 | 0.3197 |
| JO26#/+ | 15 | 110 | 690 | 330 | 332 | 35 | 0.0089 |
| JO27#/+ | 19 | 140 | 920 | 310 | 372 | 46 | 0.0193 |
| JO28#/+ | 15 | 40 | 1020 | 420 | 474 | 77 | 0.5921 |
| JO29#/+ | 15 | 160 | 1050 | 450 | 481 | 62 | 0.7019 |
| JO30#/+ | 10 | 210 | 610 | 395 | 386 | 42 | 0.1451 |
| JO31#/+ | 15 | 110 | 500 | 340 | 341 | 24 | 0.0079 |
| +/+ | 19 | 110 | 990 | 410 | 484 | 58 | 0.7390 |

while JO26 is downstream of the Hr38 hormone receptor-like gene, JO27 is upstream of the CG17834 gene, encoding a hypothetical protein of unknown function, and JO31 disrupts a glucuronosyltransferase transcript encoded by CG17323 (Kamikouchi *et al*., 2006). The Gal4 insertions in the JO1 and JO2 lines are expressed strongly in the JO alone, and also appear to affect genes that are required for hearing, since our manipulations failed to restore SEPs in JO1 or JO2 homozygous flies (Figure 3). The insertion site in the JO1 line is unknown; however, the JO2 insertion is located in the first intron of the polyhomeotic proximal (ph-p) gene (Kamikouchi *et al*., 2006), that is involved in regulation of the bithorax complex and is required for CNS development, among other things. Conversely, while the Gal4 insertions in the JO4 and JO15 lines are also expressed strongly in the JO, these insertions do not appear to affect hearing. The JO4 insertion is reported to be downstream of a putative gene of unknown function (CG40138; Kamikouchi *et al*., 2006). The JO15 insertion causes a homozygous lethal phenotype; however, the Gal4 expression pattern should be independent of the insertion site, since it is determined by an enhancer element within the pPTGAL construct (Sharma *et al*., 2002).

It is tempting to speculate that the subset of JO neurons labeled by JO2 and JO3 are somehow specialized for hearing, while those labeled by JO4, and perhaps JO15, have some other function such as hygrosensation, or detection of gravity or acceleration. For our future studies we will be able to utilize the JO1#, JO2#, JO4# and JO15# lines, which exhibit no hearing defects in heterozygous flies, to drive expression of our UAS linked constructs in substantial numbers of JO neurons, while not affecting other types of neurons in the antenna. It is also possible that the JO1 and JO2 heterozygotes will be sensitized for hearing defects, fostering identification of subtle hearing defects caused by expression of our constructs. Many of the other JO lines that do not affect hearing in heterozygotes, including JO21#, JO22#, JO23#, JO24#, JO25#, JO28#, JO29# and JO30#, may also prove useful for expressing constructs in the JO, depending on their expression in other brain areas.

Acknowledgments: We thank Dan Eberl for the JO15 line and helpful advice regarding our electrophysiology setup, and Asuza Kamikouchi for providing the remaining JO lines. We also thank Dr. Guopei Yu for advice regarding the statistical analysis of data, and Dr. Steven D. Schaefer MD, FACS for his enthusiastic support and encouragement. This work was supported by funds from the New York Eye & Ear Infirmary, New York, NY; and the Children's Hearing Foundation, New York, NY.

References: Caldwell, J.C., and D.F. Eberl 2002, J. Neurobiol. 53: 172-189; Cosetti, M.K., D. Culang, S. Kotla, P.F. O'Brien, D.F. Eberl, and F. Hannan A unique transgenic model for hearing loss (submitted); Kamikouchi, A., T. Shimada, and K. Ito 2006, J. Comp. Neurol. 499: 317-356; Sharma, Y., U. Cheung, E.W. Larson, and D.F. Eberl 2002, Genesis 34: 115-118; Yin, J.C., J.S. Wallach, M. Del Vecchio, E.L. Wilder, H. Zhou, W.G. Quinn, and T. Tully 1994, Cell 79: 49-58; Yoshihara, M., and K. Ito 2000, Dros. Inf. Serv. 83: 199-202.

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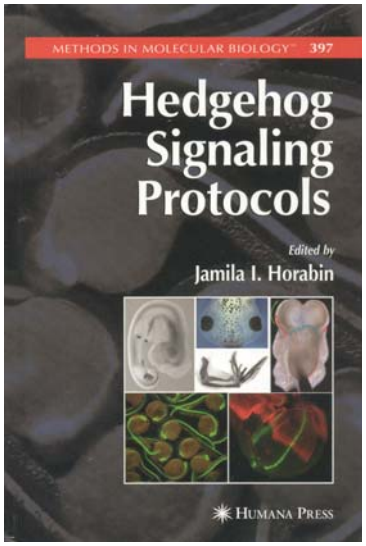
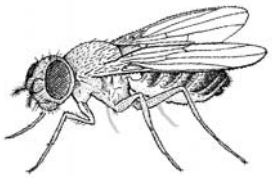



**Hedgehog Signaling Protocols**.

**Horabin, Jamila I**. (editor). 2007. *Hedgehog Signaling Protocols*. Methods in Molecular Biology #397. Humana Press, Totowa, NJ. 256 pp. ISBN: 978-1-58829-692-4.

The Hedgehog signaling pathway is important to understanding the regulation of development in both invertebrates and vertebrates. Its misregulation is associated with many human diseases. This well-organized book presents several different experimental approaches toward understanding its molecular events in a variety of model systems. Protocols are presented in a clear manner with excellent illustrations of techniques, experimental resources, and representative results. In 16 chapters, the 38 contributors provide a valuable resource for geneticists, biochemists, and molecular biologists studying this important pathway. Following a description of Hedgehog protein purification, applications in several model systems are discussed, including the chick limb, *Xenopus*, rats, zebrafish, and *Drosophila*. Specific techniques include manipulating Hedgehog signaling with retroviral expression systems, cell cycle analysis using flow cytometry, detecting tagged Hedgehog with immunocytochemistry, confocal analysis, RNAi, germline clone analysis, clonal analysis in somatic tissues, GAL4/UAS targeted gene expression, biochemical fractionation of cells, immunoprecipitation to study protein-protein interactions, and gene sequence analyses. Each chapter is accompanied by a useful and up-to-date bibliography of cited references. Price: $99.50. For more information, email humana@humanapr.com.



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Technique Notes

**Methods and rationale for high-resolution magnetic resonance imaging (MRI) of *Drosophila*, using an 18.8 Tesla NMR spectrometer.**

**Null, Brian[1*], Corey W. Liu[2], Maj Hedehus[3], Steven Conolly[4], and Ronald W. Davis[1]**. [1]Stanford Genome Technology Ctr/Bio-X Program; [2]Stanford Magnetic Resonance Laboratory; [3]Varian Inc, NMR Instruments; [4]U.C. Berkeley, Dept. of Bioengineering; *Corresponding author

## Introduction

Magnetic resonance imaging (MRI) is proven as an important tool for the study of thick or opaque tissues in living organisms, and its application to the study of development and biomedicine in the smallest model organisms is an exciting frontier. NMR spectrometers, while not typically used for imaging, are capable of generating extremely high magnetic fields, up to approximately 20 Tesla, whereas the more familiar MRI devices used to image humans in the clinical setting operate at only about one Tesla. High field strength is especially important for tiny specimens like the fruit fly, to increase signal to noise ratio and NMR spectral resolution for quantitation of metabolites *in vivo*. The small sample dimensions of the NMR spectrometer are ideal for the study of *Drosophila* and other small model organisms. Further, the ongoing innovation of MR contrast agents which can act as *in vivo* indicators of physiological status such as calcium ion concentration and gene expression, combined with the robustness of *Drosophila* as a model organism with a diverse array of genetic tools and genomic data, would make *in vivo* imaging and spectroscopy a highly desirable technique for the study of *Drosophila*. Over the past several decades, tremendous advances have been made in the capabilities of magnetic resonance methods for imaging and spectroscopic measurement in human subjects. Such methods are increasingly being developed and utilized for small mammal studies; however, while adapting the methods and instrumentation used for human patients to the rodent models has been highly beneficial, it will always face limitations which can be easily overcome by working in the fly. And recent developments in magnetic resonance instrumentation and methods have greatly increased the feasibility of detailed *in-vivo* spectroscopic study of *Drosophila*. The methods described here are part of an ongoing effort to expand the capabilities of magnetic resonance methods and build on our prior genomic studies to aid the future development of new human disease models.

MRS, or magnetic resonance spectroscopy, is a special case of MRI, in which a sample can be analyzed to acquire NMR spectral data for discrete regions of tissue within the magnetic resonance image. MRS uses the excellent chemical specificity of the nuclear magnetic resonance technique to allow the non-invasive quantitation of certain metabolites, which can be correlated with structural and functional data. For example, MRS can easily identify the three phosphate groups of ATP, the energy buffer phosphocreatine, and inorganic phosphate, from which intracellular pH can be derived, allowing the bioenergetic status of tissues to be accurately assessed. When MRS is applied to an intact biological specimen, it begins to open the door to direct detection and measurement of the molecular physiology encoded in the biochemistry of a living organism.

For example, if spectroscopy of neurotransmitters can be accomplished in the living, developing fly, as it has been in the rat, and these data are coupled with the expression data of the

genes involved in neurotransmitter-mediated signal transduction and feedback systems, it will be a powerful tool. A synergistic combination of imaging and genomic tools stands to greatly accelerate research in *Drosophila* disease models and thus our understanding of the molecular-genetic nature of human diseases.

The ideal specimen for high resolution *in vivo* imaging and spectroscopic study must be hardy and unmoving, capable of remaining stationary for long periods of time while immersed in an oxygen-rich perfluorocarbon oil. Additionally, *in vivo* spectroscopy is most effective if the instrumentation is closely matched to the size of the specimen, and generates the highest possible magnetic field. The *Drosophila* pupa has particularly excellent properties as an MRI subject, in that it is immotile and also naturally capable of gluing itself to glass surfaces, where it remains for ~100 hrs (at 25˚C) while undergoing massive developmental reorganization. The opacity of the pupa and adult phases have limited the study of these periods of the life cycle, as investigators have primarily used the light microscope as the instrument of choice.

Of course, light microscopy is indispensable, and in comparison to MRI, conventional imaging using light microscopy has great advantage in the ease of achieving real-time temporal resolution. However, there are obviously limits to the techniques of light microscopy, and there is great opportunity for MRI to be used as a highly complementary tool to existing methods. Light microscopy is limited to tissues that transmit light, and as resolution is increased, the field of view decreases, and the light intensity must be increased dramatically, so that as higher spatial resolutions are approached using methods like confocal microscopy, the intensity of light radiation can easily damage or kill a live specimen. Generally, research which requires detailed light microscopic studies of insect and vertebrate models is limited to dead or damaged specimens, requiring special dissection, chemical fixation, or other deleterious preparation, which can include partial dissection of a live specimen, to afford access of the light microscope to a tiny field of view within the specimen. Although the resolution of light microscopy is fundamentally only limited by the wavelength of light, in practice high resolution *in vivo* studies are severely limited by light requirements and diminishing field of view with increasing resolution, as well as deleterious and logistical challenges. While it may be straightforward to optically image a thin slice of tissue with 10 micron resolution or better, it is hard to imagine any practical method other than MRI for imaging the entire surface of a live animal, in addition to the full volume of its interior, at this resolution. This report describes methods we have employed to this end, imaging *Drosophila* in an NMR spectrometer, in an effort to ascertain the feasibility of MRI and MRS on live *Drosophila* at 18.8 T, with a field of view less than 5 mm. The coming challenge for this technique will be to improve methods and instrumentation for greater spatial and temporal resolution of imaging and spectroscopy *in vivo*.

**Instrumentation, Methods, and Findings**

Proton resonance imaging was performed at the Stanford Magnetic Resonance Laboratory on a Varian 800MHz NMR instrument with vertical bore magnet (Oxford Instruments), the Varian INOVA console and 5 mm inner diameter triple-resonance H {CN} triple-axis gradient NMR liquids probe. Specimens were immersed in a gas-permeable fluorocarbon oil, either “Halo 700” or “Halo 27” (Halocarbon, Inc), which are high molecular weight polymers of chlorotrifluoroethylene [PCTFE] used to prevent desiccation, improve magnetic susceptibility matching between the specimen and the surrounding medium, and decrease background hydrogen signal. Viability testing demonstrated flies were able to survive to adulthood (eclosion) in this oil while subjected to prolonged imaging protocols.

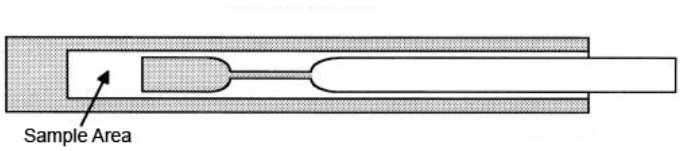
At Left: The 800MHz (18.8 Tesla) Varian NMR Spectrometer. Samples are loaded from the top, with the aid of a stepladder.

Imaging sequences utilized include spin-echo multi-slice, 3D spin echo, and 3D gradient echo. Experimental parameters have varied among experiments and are generally in the following ranges FOV: 2-5 mm, TR: 0.1-2.0 s, TE: 2-25 ms, matrix: 64x64x64 or 128x128x128, scan time ranges greatly, depending on the experiment, from 15 minutes to 12 hours. Pupal flies subjected to an intensive imaging regimen remain viable in this medium and develop to adulthood. Specimen mounting is fairly straightforward, without special oxygenation or anesthesia. Notably, internal movement and developmental speed can be slowed or accelerated by changing the temperature of the specimen using the variable temperature controller of the INOVA instrument console.

Specimen Preparation in NMR Sample Tubes:

Individual adult and pupal *Drosophila melanogaster*, as well as *D. bifurca*, and *D. aracatacas* (Tucson Stock Center #15085-1621.0 and #15040-1171.0) were imaged in 5 mm diameter NMR tubes, either in air or immersed in halocarbon oil. Numerous arrangements were devised to hold the specimen, the most streamlined of which as follows: Samples are placed in 5-10 mm long sections of glass capillary tube that have been flame-sealed at one end. Prepupae and pupae can be gently collected from culture vials and placed by hand, or, for minimal invasiveness, the 'wandering 3rd instar' larvae can be allowed to crawl inside these tubes and glue themselves to the inside as they form white prepupae. Prior to imaging, the tube is filled with halocarbon oil, just covering the pupa. These miniature sample tubes containing the specimen are then placed inside a 5 mm NMR tube and loaded into the spectrometer for imaging. For vertical alignment of the sample and chemical calibration, a special NMR tube was used which contains another smaller diameter tube within it to form two separate sections, concentrically aligned. With the sample placed in the inner section, it is vertically aligned along the long axis of the tube, and the outer section can be filled with 100% $D_2O$ or similar reagent to allow for calibration of the NMR instrument. A benefit of vertical mounting of the specimen is that several samples can be vertically stacked in one tube and then imaged individually, by either moving the field of view via the software interface, or by physically moving the sample tube up or down within the instrument.

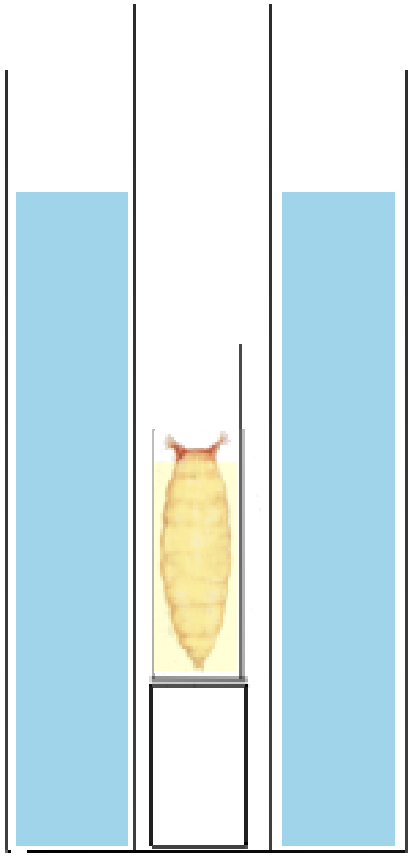
This concentric tube method was used for most imaging experiments; however, with the INOVA 800 system and the particular probe hardware used in this case, for higher resolution scans, it was sometimes preferable to mount the specimen in a transverse (horizontal) configuration. To achieve horizontal alignment of the sample, it can be placed in a 'shigemi' type NMR tube, which has a flat horizontal surface at its bottom, and a glass plunger which fits within the tube and holds the sample in place (Shigemi, Inc., sigma #Z54,334-9).




Shigemi tube diagram

Alternatively, a twisted pair of long glass fibers, monofilament fishing line, or similar mechanical support can be mounted inside a normal 5 mm diameter sample tube (Norell, Inc. #509-UP-8), holding the specimen very lightly like a pincer between the fibers (fabricated in-house). Similarly a glass capillary tube wrapped in spacers made from wrapped strips of Parafilm M (VWR #52858-000) may be mounted concentrically inside the NMR sample tube, the specimen attached at its terminus with double-stick tape, and immersed in halocarbon oil at the bottom of the tube; however, this method can create a syringe-like pressurizing effect, potentially causing issues with air bubbles and hydraulic complications which can be quite challenging, and so is best used as a 'quick and dirty' approach for machine calibration and so forth rather than real biological measurements.

Mounting of adult flies can be accomplished by similar mounting methods as used for pupae, though additional care must be taken due to their greater fragility. When immersed in the halocarbon oil, the adult flies react similarly to anesthetization with $CO_2$ or ether: all movement ceases, and the fly remains in a characteristic life-like position, which is generally not the case for treatments that kill the fly directly. Larvae, when placed in halocarbon oil, appear unfazed and continue to move normally. Thus larvae will require further refinement of the specimen mounting technique for imaging. For larvae it might suffice to cool the sample enough to still the larva's movement (controllable from the INOVA interface), or administer a chemical anesthetic.



Schematic cross section of lowermost portion of the concentric two-chamber NMR tube with pupa mounted vertically and the outer chamber filled with 10% $D_2O$ (dimensions and tolerances not drawn to scale). Exact tube used in no longer available, but a suggested replacement is WILMAD-LABGLASS products #517, 518, or 519-"COMPLETE".

Administration of MR Contrast Reagents (CR) to *Drosophila*:

A gadolinium-based contrast agent ('Magnevist', Berlex Inc.) and a solution of manganese chloride were separately administered and demonstrated effective both by direct feeding and microinjection during larval, pupal, and adult phases of the life cycle, while retaining viability.

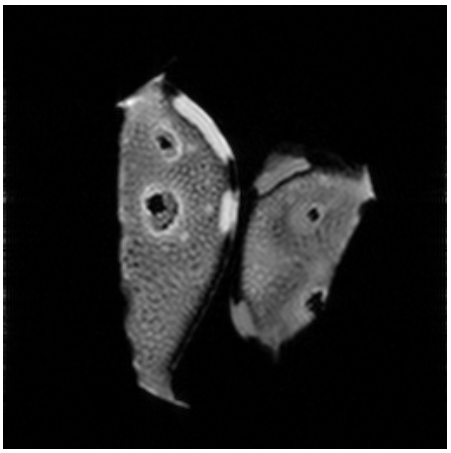
$MnCl_2$ in food:

| Final Concentration | Effect |
|---|---|
| 100 mM | Adults die |
| 50 mM | Adults persist, but no offspring |
| 20 mM | Adults persist, but no offspring |

$MnCl_2$ Preparation: Starting with 1 Molar $MnCl_2$ solution (Sigma #M1787), a 100mM $MnCl_2$ food mixture was prepared by adding 1M solution to heated, molten *Drosophila* food medium and mixing well. 50 and 20 mM food was prepared by further dilution of the 100 mM medium.

Gadopentate Dimeglumine:

Magnevist Injection (Berlex Inc.) is a 0.5 mol/L solution of 1-deoxy-1-(methylamino)-D-glucitol dihydrogen [N, N-bis[2-[bis(carboxymethyl)amino]ethyl]-glycinato-(5-)-] gadolinate(2-) (2:1), a paramagnetic contrast medium used diagnostically for clinical (human) MRI. Each mL of Magnevist Injection contains 469.01 mg gadopentetate dimeglumine, 0.99 mg meglumine, 0.40 mg diethylenetriamine pentaacetic acid and water for injection. The contrast enhancing effect of Magnevist is caused by the di-N-methylglucamine salt of gadopentetate (Gd-DTPA), the gadolinium complex of diethylenetriamine pentaacetic acid. With the appropriate imaging sequence (*e.g*. with $T_1$-weighted spin echo), the resultant shortening of the spin-lattice relaxation time of excited nuclei upon exposure to gadolinium allows an enhancement of signal intensity while reducing acquisition time. **As a preliminary control test of Magnevist in our system, we placed freshly severed sections of plant stem (*Apium graveolens*) into a solution of Magnevist with food dye, and a control solution without Magnevist, overnight. The food dye was observed to travel up the vascular tissues of the stem, and then excised slices of each were imaged side by side, across a series of relaxation times. The results show nicely how Magnevist enhances the signal in tissues:



Plant tissue demonstration of contrast agent: Magnevist-treated sample, on left side, shows brighter signal surrounding vascular tissues than in the non-treated sample, on the right. Spin-echo multi slice (SEMS) method, 256x256 grid, 19.5 micron in-plane resolution, 250 micron z-axis slice thickness.

Administration of Magnevist In *Drosophila* Food Medium:

500 µl Magnevist was prepared in 5ml total volume with water, and mixed with dry food medium until proper consistency. Approximately 3 mL was removed from an existing culture in the form of a slurry of larvae and food, which was then mixed into the new Magnevist CR medium.

Flies reared in this medium showed no obvious effect on viability, and were found to live on the medium until the food supply was depleted.

Administration of Contrast Reagents by Direct Injection:

Gadolinium contrast agent or manganese chloride solution was injected into larvae, pupae, and adults. Gadolinium contrast agent, but not manganese chloride solution, was injected into syncitial (early multinucleate stage) *Drosophila* embryos. Injection was accomplished using a drawn glass capillary needle, like those commonly used for injection of DNA into embryos. Results are as follows:

Embryos: Injection of 60-110 picoliters Magnevist solution (standard human preparation) into syncitial embryos produced no hatching larvae and appeared to have arrested development early. Manganese chloride was not tested.
Larvae and pupae: Green food coloring dye was diluted 1:12, and then mixed 1:1 with the CR for visualization of injected volume with a light microscope during the injection process. This solution was injected within the cuticle of 3rd instar larvae. In pupae, the above solution was injected both internally, into the abdomen of pupae, as well as into the interstitial air space which forms between the body of the developing fly and the pupal case itself which forms from the cuticle of the larva.
Results: When the interstitial space was filled, the fly did not survive and eclose from the pupal case, and development appeared arrested. A small volume injection, such as filling just the empty space anterior to the head, is less lethal, but also allows little access of the CR to the tissues of the developing fly.
When the mix was successfully administered internally, the fly did survive and appeared normal. Volumetric measurements of administered doses cannot be precisely measured with the current setup, and are between 100 pl & 1 µl; the volume was enough to easily visualize the green dye in concentrated drops which rapidly diluted as they were taken up by the fly's open circulatory system.
Adults: the same mix and quantity as above was injected into the abdomen. Flies survive and appear normal.

These results are summarized in the following table:

| | Embryos | Larvae | Pupae | Adults |
|---|---|---|---|---|
| Gd Injection | Further Refinement Needed | Successful | Successful | Successful |
| Gd Food | Not Applicable | Successful | Not Applicable | Successful |
| $MnCl_2$ Injection | Not Tested | Further Refinement Needed | Successful | Successful |
| $MnCl_2$ in Food | Not Applicable | Further Refinement Needed | Not Applicable | Successful |

## Conclusion

Especially given the nature of the hardware used for these experiments, which was not designed for imaging, we were startlingly successful in our goal of producing high resolution virtually-dissectable 3D images of live *Drosophila* specimens, utilizing multiple imaging techniques. Based on these experiments we feel that the application of high-field MRI/MRS to *Drosophila* could represent an important direction for *in vivo* imaging and genetic modeling of human disease, that is worthy of further pursuit and technological development.

## Additional Notes and Discussion

A possible lead for species and biomaterials studies is worth mention. *Drosophila* aracatacas generally yielded poorer imaging results in which the cuticle was less well defined than either *D. melanogaster* or *D. bifurca*. It may be noteworthy that *aracatacas* are an exceptionally sluggish, slow moving species, while *bifurca* is such an exceptionally quick species that is quite difficult even

to transfer flies from one vial to another without losing some of them. This suggests a question as to whether the material properties of the cuticle in these species that are necessary to support their physical dynamism produces an effect in imaging contrast, and that it may be interesting to study insect biomechanics with MRI.

Successful injection of embryos with contrast agent would be an exciting step forward. The possibility of injection of embryos with MRI contrast agents was not fully explored here, and offers fertile ground for future experiments. In general, the primary difficulty facing contrast agent experiments in model organisms is the challenge of how to actually get the contrast reagent to the interior of cells so they can be used as indicators of physiological states such as pH, calcium ion measurement and gene expression can be studied *in vivo*. *Drosophila*'s syncitial development has long been utilized for transgenic and RNAi methods, and could present a special opportunity for use of MRI contrast agents in a genetic model organism, due to the potential to load cells with contrast agent prior to cellularization. If an adequate concentration of contrast reagent is retained in the cells following subsequent divisions, the fly will have intracellular contrast agent in all its cells or only within specific cell lineages. In our preliminary attempt to inject embryos, no optimization was done to the formulation of the solution, and it was beyond the focus of our current effort to pursue the method further. Improvement of the buffer solution used for injection could lead to the development of an intracellular *in vivo* contrast agent system for the fly, a very exciting possibility. Such a method would provide an excellent opportunity for utilizing existing MRI contrast agents and encouraging the development of new varieties of contrast agents for molecular genetic studies.

Imaging with other nuclei:

Carbon imaging (C-13) on an untreated sample was attempted, but did not produce a successful image. Fluorine presents a stronger signal than carbon, and is not normally found in organisms naturally, so could be an interesting direction for imaging labeled molecules. Notably, bacteria can produce fluorinated proteins from fluorine-tagged amino acids, and thus might be used to synthesize *in vivo* labeling agents for use in fluorine imaging.

Acknowledgments: This work was enabled by cooperation and support from Professors Joseph Puglisi, Structural Biology Chair and SMRL Director, and Michael Moseley, Lucas Center Radiology Science Laboratory, in the Stanford School of Medicine. Key assistance was provided by Matthew Fish (embryo injection), and John Sack & Richard Aldrich, and immeasurable support was provided by Varian Inc. to promote and support the advancement of this exciting technology.

## Economical high-throughput DNA extraction procedure in 96-well format for *Drosophila* tissue.

**Holleley, Clare E.** School of Biological, Earth and Environmental Sciences, University of New South Wales, Sydney, NSW 2052, Australia. E-mail: c.holleley@unsw.edu.au.

One of the major benefits of working with *Drosophila* is the ease of achieving highly replicated and statistically powerful experiments. However in molecular studies, the time consuming process of single-tube DNA extraction can create a work-flow bottle-neck. We have modified the standard salting-out DNA extraction method so that it can be conducted in 96-well PCR plates, dramatically increasing the number of extractions that can be performed. The salting-out method is

often preferable to column based extraction methods, because you can manipulate the final volume in which you re-hydrate the purified DNA. Thus it is ideal for species with low expected DNA yields. Also, salting-out DNA extraction methods are usually much less expensive than column based extraction methods. We modified the manufacturer's instructions provided in the Gentra PUREGENE® DNA purification kit (Progenz Ltd, Australia) for use in 96-well plates. However the methods described here can also be applied non-commercial salting-out procedures (Aljanabi and Martinez, 1997; Sunnucks and Hales, 1996).

We first overcame the time consuming process of individually macerating each *Drosophila* tissue sample using a stainless steel 96-well phage colony replicator tool with 1.6 mm round-ended Phage Picker+ pins (Genetix, Millennium Science, Australia). The pins of this commercially available instrument fit exactly into the wells of a standard 96-well PCR plate. Using this instrument, we could macerate 96 tissue samples simultaneously in the chilled cell lysis solution (Tris, EDTA, SDS) by grinding for a minimum of 10 minutes. After macerating each *Drosophila* in 100µl of cell lysis solution, we added 0.5µl of Proteinase K (20µg/ml) to each well. We then sealed the plate, mixed the samples and incubated the samples overnight at 55˚C in a PCR machine.

The following morning, the samples were cooled to room temperature and 50µl protein precipitation solution (ammonium acetate) was added to the cell lysate and vortexed vigorously for at high speed for 20 seconds. Samples were then centrifuged at 4100 rpm for 12 minutes to pellet cell debris. Immediately after centrifugation we *carefully* removed approximately 100µl of supernatant, without disturbing or sucking up any of the pellet using multi-channel pipette. This step required careful observation of marker lines on the pipette tips to gauge depth, and we found that elevating the plate aided visibility. If the pellet is disturbed, re-centrifuge the samples. We then added the supernatant to 100µl of room temperature 100% isopropanol in a new 96-well PCR plate. We sealed the plate, inverted the plate 50 times to mix and then centrifuged at 4100rpm for 12 minutes to pellet the DNA. We then removed the supernatant and washed the DNA pellet by adding 150µl of 70% undenatured ethanol and inverting 10 times. The plate was then centrifuged again for 12 minutes. It was essential to remove all ethanol from the DNA pellets prior to rehydration. Thus if ethanol was still visible in the wells after draining, we inverted the plate on several kim-wipes and centrifuged upside down at 200 rpm for 30 seconds. We then let the plate air-dry for 10-15 minutes. After all visible ethanol had been removed from the wells, we re-hydrated the DNA in the desired volume of hydration solution (TE Buffer: 10mM Tris-HCl, 1mM EDTA, pH 7.0-8.0) or RNase-free water. Finally we re-hydrated the DNA pellet by incubating sample at 65˚C for 1 hour or incubating at room temperature overnight, tapping periodically to disperse DNA. For a single fly, the observed DNA yield ranges from 35ng – 10µg with a mean concentration of 172.4 ng/µl ± 21.3 SE (N = 189) when re-hydrated in 20µl. The failure rate is low (2.1%) and is usually due to a cracked well during excessively vigorous maceration.

References: Aljanabi, S.M., and I. Martinez 1997, Nucleic Acids Res. 25: 4692-4693; Sunnucks, P., and D.F. Hales 1996, Molec. Biol. Evol. 13: 510-524.

## 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 15 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.

**Methods to measure circadian pattern in isolated adults.**

**Cooper, Ann S.** Dept. of Biology, University of KY, Lexington, KY. 40506-0225 USA. Email: asc1029@hotmail.com.

Measuring circadian patterns in a pool of adults can have compounding variables to control, whereas isolated adults reduce social variables when addressing mechanisms driving a circadian pattern. A common approach to index circadian patterns is to monitor locomotor activity (Klarsfeld, 2003). However, monitoring single flies as compared to a population presents challenges. Here I report on two relatively inexpensive and simple approaches to index circadian patterns in single flies with readily accessible instrumentation.

After entrainment of a circadian pattern, one examines if the rhythm remains in the absence of the light cycle (Pittendrigh and Daan, 1976). This can be accomplished in total darkness or with continuous light. In order to detect activity of entrained adult flies a narrow glass tube was used. It was wide enough for the adult to turn around, but does not allow the adult to fly. The glass tube is Pyrex Disposable Pipette (serial number CGW 3597). I used a glass tube that is 5.1 cm in length for these studies.

At one end of the glass tube a clear rubber tube, ½ cm in length, was placed. This made it easy to remove and replace as needed. One end of the rubber tubing was for the foam stopper and the other end was for the standard *Drosophila* corn meal food (Figure 1). At the other end of the glass tube, foam was placed to allow ventilation.

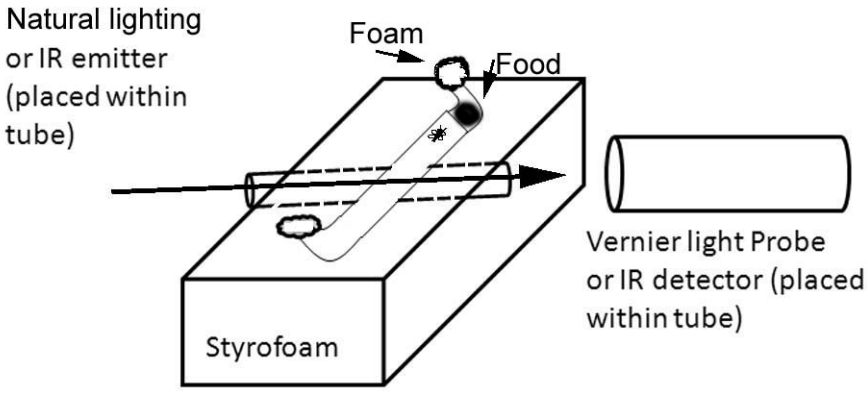



Figure 1.

For measuring the circadian pattern in continuous light I used a light probe (TI Light Probe, TILT-BTA) from Vernier (Vernier Software & Technology, Beaverton, OR, USA). The probe interfaces with a Vernier LabPro (commonly used in many high schools and introductory college science classes). The data are downloaded to a TI-83 hand calculator or directly stored on a computer. This probe is placed on a cut out of Styrofoam to hold it as well as the glass ally way tube (Figure 1). The light probe is placed directly against the glass tube and will detect each crossing of the light path. Normal room lighting may be used for the light source or any standard lighting within a temperature controlled incubator.

For measuring activity in constant darkness, an infrared (IR) light emitter (model 276-142, Radio Shack) was used in conjunction with the light probe (TI Light Probe, TILT-BTA) from Vernier as it is sensitive to IR.

An alternative approach to monitoring activity using different instrumentation was also examined for its feasibility. Activity in constant darkness or with a room well lit, the IR emitter can be used to monitor a fly when crossing an IR beam. The detector (model 276-142, Radio Shack) was

used instead of the Vernier light probe. The IR detector was connected to an impedance amplifier (UFI, model 2991, Morro Bay, CA, USA) in which the output is then passed through an AD board (MacLab 4s interface, ADInstruments, INC; Colorado Springs, CO, USA ) before being collected on a computer with the MacLab Chart (version 5) software. As with the earlier method, Styrofoam is used to cut out holders for aligning the infrared light path and glass alley way tube. The emitter and detector were aligned by use of a larger plastic pipette that had a cut away for placing the glass fly tube (Figure 1).

With either approach, Vernier or impedance detector, the IR emitter (model 276-142, Radio Shack) can be powered by a 9V battery for about 24 hrs. I found the battery strength runs down quickly. Alternatively, use of a DC power source from a 120V AC to a 9V DC transformer is suggested to be used. Each emitter requires a 66 Ohm resistance on one lead. With the transformer as a power source a number of emitters can be used. I used 8 in parallel with 1 resistor in series from the transformer the 8 emitters in parallel. The rate of acquisition, not to over collect but to catch quick movements, is best about 25,000 samples/hour for the Vernier software. Collecting data for 4 hours or less in each set keeps files small enough as to readily open and analyze. The impedance amplifier with the MacLab 4s interface one can collect for 8 hours at 1 KHz to sufficiently detect movements and keep files small enough to manage.

The responses from either method are then plotted as beam breaks over time for determining the activity of individual flies. These methods allow for various experimentations to be utilized, such as readily altering food sources, effects of compounds mixed with food and changes in environmental lighting (Sheward *et al*., 2007; Yoshii *et al*., 2007). Light conditions can be readily altered to total darkness or with visible light while still monitoring the adult locomotor activity if the IR emitter is used as a light source for the detector. Background level of absolute intensity on the Vernier detector will vary when using the IR emitter while the white light is turned on or off as the detectors pick up some of the white light signal.

I found the Vernier LabPro connected directly to a computer to be the easiest approach to set up and monitor activity. The impedance amplifier can saturate and requires monitoring often. Also the LabPro allows 4 probes to be connected simultaneously while the impedance amplifier monitors a signal detector. The net cost is also cheaper with the Vernier hardware as 4 detectors and one Vernier LabPro costs about $500 (USD) while each impedance amplifier costs about $350 (USD).

Acknowledgments: I thank Dr. R.L. Cooper at the University of KY for his advice and help with this study.

References: Klarsfeld, A., J.C. Leloup, and F. Rouyer 2003, Behavioural Processes 64: 161-175; Pittendrigh, C.S., and S. Daan 1976, J. Comp. Physiol. [A] 106: 223–252; Sheward, W.J., E.S. Maywood, K.L. French, J.M. Horn, M.H. Hastings, J.R. Seckl, M.C. Holmes, and A.J. Harmar 2007, J. Neurosci. 27(16): 4351-4358; Yoshii, T., K. Fujii, and K. Tomioka 2007, J. Biol. Rhythms 22(2): 103-114.

**Two robust multiplex PCR reactions for high-throughput microsatellite genotyping in *Drosophila melanogaster*.**

**Holleley, C.E., and W.B. Sherwin.** School of Biological, Earth and Environmental Sciences, University of New South Wales, Sydney, NSW 2052, Australia. E-mail: c.holleley@unsw.edu.au

Microsatellites are currently among the most commonly used genetic markers for population genetic studies, because they allow the inference of several important population parameters, such as

population genetic structure, effective population size, and dispersal rate. Microsatellites are also commonly used for a range of other applications including paternity assignment, population of origin assignment, detection of population bottlenecks, and inbreeding assessment. Single amplification of microsatellite loci is time-consuming, expensive and can require large quantities of template DNA. An alternative to single amplification is multiplex PCR, where multiple microsatellite loci are amplified simultaneously in a single reaction. Despite all of the advantages of multiplex PCR and the common use of *D. melanogaster* in population genetics, there are very few published examples of successfully multiplexed microsatellite loci (England *et al.*, 1996; Schlötterer *et al.*, 1997). We have designed two robust PCR reactions that successfully amplified ten microsatellite loci, and we extensively tested these reactions in 176 *Drosophila melanogaster* populations. We present the basic population genetic data and PCR failure rates obtained from the 4,224 genotyped individuals.

We initially chose twelve microsatellite loci that provided whole genome coverage and minimised physical linkage of loci (Table 1). These twelve loci were organised into two multiplex reactions (MP1 and MP2) (Table 1). Loci with overlapping allele size ranges were labelled using different 5'-fluorescent labels on the forward primer (6-Fam, VIC, PET, NED) (Table 1). All unlabelled reverse primers were tailed with a commercially available seven base sequence (tail) that promotes +A DNA polymerase activity and thus reduces stutter-bands that are associated with poly-A tailing (Applied Biosystems, Foster City, California). Primers in a single multiplex reaction were checked for complementarity and hairpin structures using the programs MULTIPLEX MANAGER (Holleley and Geerts, in prep.) and AUTODIMER (Vallone and Butler, 2004).

Table 1.

| | Reaction Conditions | | | General Microsatellite Information | | | | | Primer Sequences | |
|---|---|---|---|---|---|---|---|---|---|---|
| Short Name | Mplex | Fluro Dye | Primer Conc. Final Reaction | Published Name | Repeat | Chrom. | Cytological Location | Genetic Location (cM) | Forward/ Reverse (5' - 3') | References |
| Msat 1 | MP1 | 6-FAM | 0.2 µM | *DMZW3K25* | $(AT)_{14}$ | X | 3A | 1 | ATTGTCATTTTATTGCTGCCG | a, b |
| | | | | | | | | | TAACGAAGAGAGTTGCGGAGA | |
| Msat 2 | MP1 | 6-FAM | 0.2 µM | *su.var* | $(TG)_{12}$ | II | 29A5-B4 | 31 | GGTTGCTGGGAGAAAGAC | c, d |
| | | | | | | | | | GCCACACATTCGCATCTC | |
| Msat 3 | MP1 | VIC | 0.2 µM | *Cad-GA* | $(GA)_{11}$ | II | 38D4-E1 | 54 | AGGCACTCTCTGGGCGAAAC | d, e |
| | | | | | | | | | CGTCACTAGGTCGGGGTATC | |
| Msat 4 | MP1 | NED | 0.2 µM | *DROMHC* | $(CA)_{13}$ | II | 60 E9 | 107 | AAACCCACACAACAACTGCA | a, b |
| | | | | | | | | | GACATTACCGATATTGGATGCA | |
| Msat 5 | MP1 | NED | 0.2 µM | *DROGPAD* | $(GT)_{9}$ | II | 47A | 62 | GAAATAGGAATCATTTTGAATGGC | a, f |
| | | | | | | | | | AATTAAAAACAAAAAACCTGAGCG | |
| Msat 6 | MP1 | PET | 0.2 µM | *3L8939767ct* | $(CT)_{10}$ | III | 66F | 28 | CCGTCCCGCTCTGGTTTGG | b, g |
| | | | | | | | | | GTTGCTGCTCCTCCGCTGA | |
| Msat 7 | MP2 | 6-FAM | 0.1 µM | *DRO17DC2Z* | $(CT)_{9}$ | III | 84 | 48 | TTCGTGCAAAGGTGTTTTCC | a |
| | | | | | | | | | ATGCAGATACCAGAAACCGC | |
| Msat 8 | MP2 | 6-FAM | 0.4 µM | *DMU1951* | $(TA)_{16}$ | III | 93C | 71 | GGGTCTTTCTGCTTCAGTTACC | a, b |
| | | | | | | | | | GGAATACACGAATCCCCCTT | |
| Msat 9 | MP2 | VIC | 0.1 µM | *Adh-TC* | $(TC)_{11}$ | II | 35B2-B4 | 50 | CAGCACCAGCATCCAAGTAC | d, e |
| | | | | | | | | | AGTCTCTGTGGCAGTGTGAG | |
| Msat 10 | MP2 | NED | 0.1 µM | *DMTENA* | $(TA)_4CC(AT)_{14}$ | X | 11A6 | 38 | CTCTTAGTGCGCAGGGATTC | a, b |
| | | | | | | | | | GAGTCGCTCAATGGCAGG | |
| Msat 11 | MP2 | NED | 0.1 µM | *G410* | $(TC)_{11}(TG)_4$ | II | 33E9-E10 | 46 | TTCGGCTCTTTGTTTGCTTG | d, h |
| | | | | | | | | | AAGCTTAAACCGATCGAAAAC | |
| Msat 12 | MP2 | PET | 0.1 µM | *DMTROPONI* | $(CA)_{11}$ | X | 16F3-6 | 59 | CAAGAGATCCCGAGAGAGAGA | a, b |
| | | | | | | | | | ACGTGTGCGTGTTGTTTCTC | |

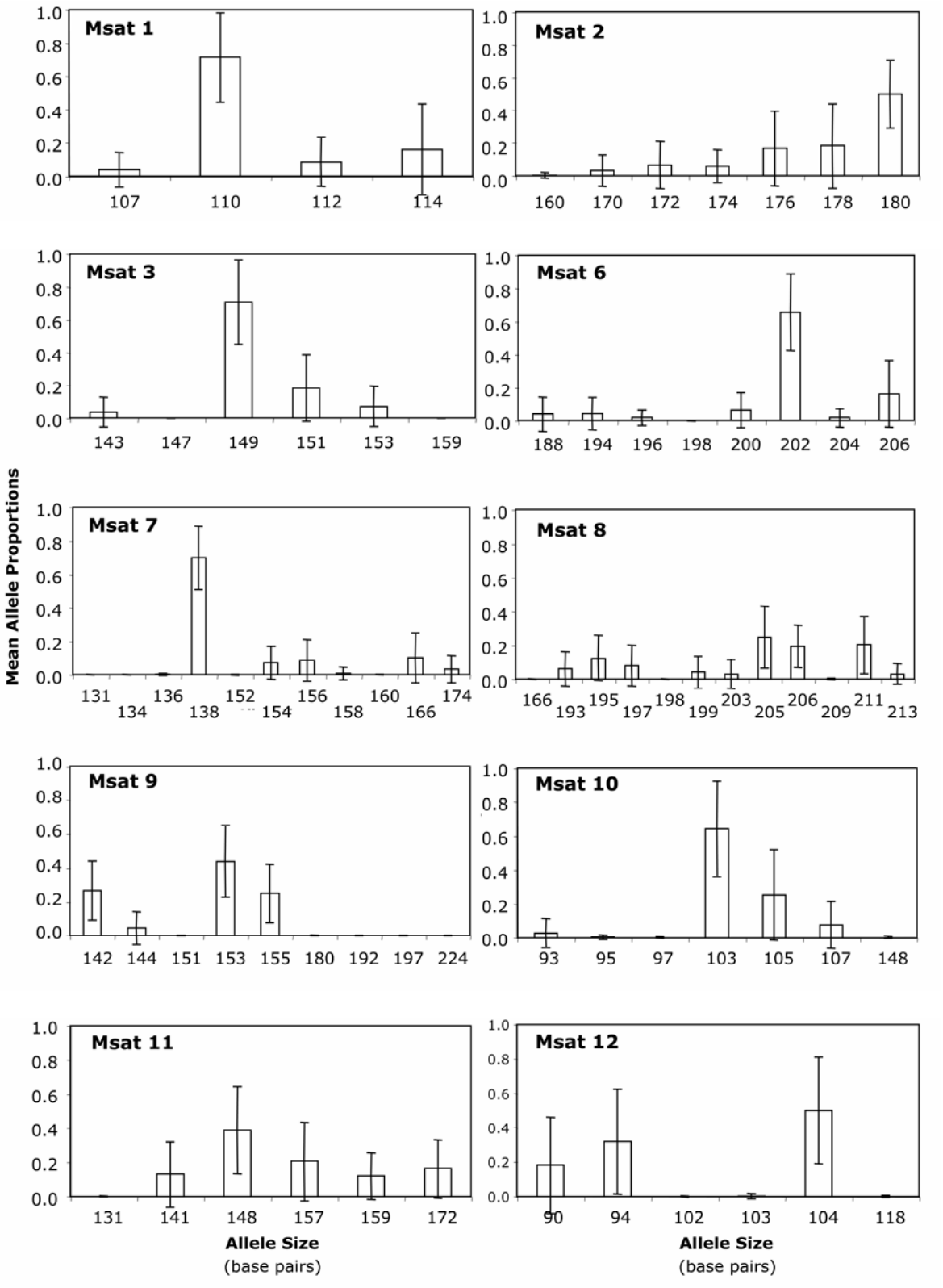



Figure 1. Mean allele proportions of the ten microsatellites amplified in multiplex reactions MP1 and MP2 ($N$ = 176 populations). Bars indicate the standard deviation of the mean.

The PCR reactions consisted of QIAGEN® Multiplex Master Mix [1x], RNase-free water, approximately 20 ng of template DNA (Holleley, 2007) and an equimolar concentration of primers Msat 1 – Msat 6 [0.2μM] for multiplex one (MP1) (Table 1). In multiplex two (MP2), the concentration of Msat 8 was increased [0.4μM] relative to the equimolar concentration of the remaining primers Msat 7 – Msat 12 [0.1μM] to compensate for differential amplification efficiency among loci (Bercovich *et al.*, 1999) (Table 1). Both multiplex reactions were amplified using a step-down PCR protocol. The initial denaturing step of 15 minutes at 95˚C was followed by 10 cycles of 94˚C for 30 seconds, 64˚C for 90 seconds, 72˚C for 90 seconds. After this high stringency cycling, the annealing temperature was reduced in a step-wise fashion from the initial 64˚C to 60˚C in the next 10 cycles, then 56˚C in the next 10 cycles, and 50˚C in the final set of 10 cycles. After the 40 cycle step-down protocol was complete, there was a final extension period of 72˚C for 10 minutes. We found that MP1 amplified best if the above conditions were modified slightly, such that the extension temperature of the 10 × 53˚C annealing temperature cycles was 70˚C instead of 72˚C. This step-down PCR approach allowed co-amplification of primers with different annealing temperatures. Additionally the gradient of high stringency to relaxed stringency cycling increased specificity and yield (Henegariu *et al.*, 1997). This approach can result in preferential amplification of primers with higher annealing temperatures, thus we designed both multiplexes to minimise the range of annealing temperatures (MP1 55˚C – 57.3˚C; MP2 53˚C – 55˚C). PCR products were run on a 48-Capillary 3730 DNA Analyser (Applied Biosystems, Foster City, California), and fragment size analysis was conducted using GENEMAPPER® SOFTWARE 3.7 (Applied Biosystems 2004).

Table 2. Basic population genetic data for twelve microsatellite loci from 176 *Drosophila melanogaster* populations (*N* = 24 individuals per population). For each locus we present the size range of amplified fragments in base pairs, the total number of alleles observed over all populations (Total *Na*), the mean number of alleles per population (Mean *Na*), mean observed heterozygosity per population ($H_o$), mean expected heterozygosity ($H_e$), the percentage of populations that significantly deviated from Hardy-Weinberg equilibrium (HWE) expectations using Fisher's Exact test with Bonferroni correction and the percentage of PCR reactions that failed to amplify. Means are presented ± the standard deviation. Dash (–) indicates that information was not available.

| Short Name | Published Name | Allele size range | Total *Na* | Mean *Na* | Mean $H_o$ | Mean $H_e$ | % HWE deviation | % Failure |
|---|---|---|---|---|---|---|---|---|
| Msat 1 | *DMZW3K25* | 106-114 | 4 | 2.1 ± 0.2 | 0.297 ± 0.2 | 0.279 ± 0.2 | 0 | 0.21 |
| Msat 2 | *su.var* | 160-180 | 7 | 2.9 ± 0.1 | 0.503 ± 0.2 | 0.487 ± 0.1 | 1.1 | 0.14 |
| Msat 3 | *Cad-GA* | 143-159 | 6 | 2.3 ± 0.2 | 0.350 ± 0.3 | 0.331 ± 0.2 | 1.1 | 0.05 |
| Msat 4 | *DROMHC* | - | - | - | - | - | - | - |
| Msat 5 | *DROGPAD* | 168-212 | 9 | 1.7 ± 0.2 | 0.071 ± 0.1 | 0.193 ± 0.2 | 29 | 1.35 |
| Msat 6 | *3L8939767ct* | 188-206 | 8 | 2.8 ± 0.2 | 0.402 ± 0.2 | 0.403 ± 0.2 | 3.4 | 0.14 |
| Msat 7 | *DRO17DC2Z* | 131-174 | 11 | 2.8 ± 0.2 | 0.392 ± 0.2 | 0.397 ± 0.2 | 2.3 | 0.36 |
| Msat 8 | *DMU1951* | 166-213 | 12 | 4.9 ± 0.1 | 0.738 ± 0.1 | 0.695 ± 0.1 | 2.8 | 0.97 |
| Msat 9 | *Adh-TC* | 142-224 | 9 | 3.2 ± 0.1 | 0.598 ± 0.2 | 0.558 ± 0.1 | 1.7 | 0.05 |
| Msat 10 | *DMTENA* | 93-148 | 7 | 2.3 ± 0.2 | 0.359 ± 0.3 | 0.342 ± 0.2 | 0.6 | 0.71 |
| Msat 11 | *G410* | 131-172 | 6 | 3.5 ± 0.2 | 0.584 ± 0.2 | 0.552 ± 0.2 | 3.4 | 0.21 |
| Msat 12 | *DMTROPONI* | 90-118 | 6 | 2.2 ± 0.2 | 0.366 ± 0.2 | 0.351 ± 0.2 | 0 | 0.07 |

Both multiplexes had a low rate of PCR failure (0.07% – 1.4%) and a low mis-scoring error rate of 3.9% (Table 2). Microsatellite 4 (*DROMHC*) showed extreme allelic drop-out under these conditions and thus was excluded from analysis and not scored. Microsatellite 5 (*DROGPAD*) also exhibited evidence of allelic drop-out or null alleles as it had an excess of homozygotes ($H_o$ = 0.071) and 29% of the populations significantly deviated from Hardy-Weinberg expectations (Table 2). Thus microsatellite 5 should be excluded from population genetic analyses. After excluding these two potentially compromised loci, MP1 and MP2 comprise ten microsatellite loci that amplify robustly and provide good but not complete genome coverage. The total number of alleles for the remaining ten loci ranges from 4 – 12 and the mean observed heterozygosity ranged from 0.3 – 0.7 (Table 2). The remaining ten loci adhered to Hardy-Weinberg expectations in 96.6 – 100% of populations, with the mean allele proportions displayed in Figure 1.

We have described a set of optimised PCR reaction conditions that, in conjunction with step-down PCR cycling conditions, result in robust, repeatable and non-preferential amplification of a suite of ten microsatellite markers. Thus this paper provides *Drosophila* researchers with an efficient and cost effective alternative to multiple single PCR reactions.

References: Bercovich, D., Z. Regrev, T. Ratz, A. Luder, Y. Plotsky, and Y. Gruenbaum 1999, BioTechniques 27: 762-770; Caracristi, G., and C. Schlötterer 2003, Molec. Biol. Evol. 20: 792-799; England, P.R., D.A. Briscoe, and R. Frankham 1996, Genet. Res. 67: 285-290; Goldstein, D.B., and A.G. Clark 1995, Nucleic Acids Res. 23: 3382-3386; Harr, B., 2000, Ph.D. Thesis, University of Konstanz Germany; Harr, B., and C. Schlötterer 2000, Genetics 155: 1213-1220; Henegariu, O., N.A. Heerema, S.R. Dlouhy, G.H. Vance, and P.H. Vogt 1997, BioTechniques 23: 504-511; Holleley, C.E., 2007, Dros. Inf. Serv. 90: 137-138; Schlötterer, C., C. Vogl, and D. Tautz 1997, Genetics 146: 309-320; Schug, M.D., T.F.C. Mackay, and C.F. Aquadro 1997, Nat. Genet. 15: 99-102; Schug, M.D., K.A. Wetterstrand, M.S. Gaudette, R.H. Lim, C.M. Hutter, and C.F. Aquadro 1998, Mol. Ecol. 7: 57-70; Swindell, W.R., and J.L. Bouzat 2006, Evolution 60: 1014-1022; Vallone, P.M., and J.M. Butler 2004, BioTechniques 37: 226-231.

## An inexpensive and efficient method for obtaining *Drosophila* heads.

**Pu, Rongsun,[#] Ailleen Manansala,* Matthew Sorensen,* and Opeyemi Tayo-Balogun*.** 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.

During our investigation of calcium/calmodulin-dependent protein kinase II which is highly concentrated in the heads of *Drosophila melanogaster*, we have devised a method to quickly separate the heads of the flies from the bodies.

The flies are moved into a clear, empty vial with a foam cap. Then small chunks of dry ice are put into the vial. The carbon dioxide immobilizes flies within a few seconds, while the low temperature minimizes protein degradation. The vial is manually shaken for a few minutes until the wing tissue comes off, as evident by the clear specks on the sides of the vial. By this time most heads have snapped off. The contents of the vial (including remaining pieces of dry ice) are then examined under a dissecting microscope and the heads could be easily separated from the bodies using a fine brush or dissecting needle.

Using this method, we were able to obtain about 200 fly heads under a dissecting microscope in less than 5 minutes. This method works well when the air is dry. Otherwise, the moisture from the air would keep the flies mushy and inseparable.

**A method to measure associative learning for different size larvae.**

**Cooper, Richard M., and Robin L. Cooper**. Dept. of Biology, University of KY, Lexington, KY. 40506-0225 USA; Email: cooper_richie@yahoo.com

Since the time of Alpatov (1929) people have been interested in studying environmental impacts on the developmental rate of larval fruit flies. Kaznowski *et al.* (1985) demonstrated that the thickness of the cuticle is gradual throughout the instar stages and does not jump with every molt cycle. This study was important since it explained why a gradual development was observed in these molting instars. This also suggests that diet could have an effect on development within an instar stage. Biochemical studies on larval fruit flies showed how the amount of proteins increases with development (Church and Robinson, 1966) and that the nitrogen and phosphate content increased with each molt stage (Watts *et al.,* 2006). These studies suggest that fruit flies are dependent on particular elements. Some factors may be developmentally rate limiting for the whole body. Despite whole larval developmental interest over the years, little attention has been given to dietary regulation on neuronal development of the larval central nervous system (CNS).

The developmental need of the CNS may be more restrictive than the whole body development due to the complexity of factors in formation and maintenance the central nervous system. There are many synapses for the refined communication. So even though whole body size might appear to be normal, one cannot assume the neural functions are fully intact. One approach to examine the effects of diet on proper development of neuronal circuits would be particular behavioral assays. It is apparent that most of the attention given to *Drosophila* behaviors has concentrated on adults, but there are a number of larval behavioral assays established. Eating and locomotion are commonly used larval behaviors (Sewell *et al.* 1975; Neckameyer, 1996; Li *et al.,* 2001). In the last few years, revolutionary studies have shown that larvae have the ability to demonstrate associative learning (Scherer *et al.,* 2003; Gerber *et al.,* 2004; Hendel *et al.,* 2005). However, the assays used are very dependent on the experimental design and might not be best suited for larvae of all developmental stages or sizes that could be altered from developmental retardation.

More direct effects on a neural circuit can also be examined. Recently, neuromodulators, such as serotonin (5-HT) and dopamine, have been shown to have a role on activity of a sensory-CNS-motor circuit in *Drosophila* larvae (Dasari and Cooper, 2004; Dasari *et al.,* 2007) as well as direct effects on the larval heart (Dasari and Cooper, 2006). So one might assume a diet restrictive in essential amino acids that are precursors to 5-HT and dopamine might impact the development in the wiring of the CNS.

In our current studies we used a simple restrictive diet of glucose and water (1 gram for 10 mls of water) and placed newly hatched embryos (1$^{st}$ instar) in this solution and maintained them at room temperature (21$^{o}$C). With this diet larvae develop very slowly compared to ones fed a standard cornmeal-agar-dextrose-yeast medium diet commonly used for culturing. The larvae would remain within each instar for a longer time and some would take 14 days until beginning to form a pupa. The size of the larvae at each stage is drastically reduced compared to an equivalent instar stage in the controls fed an enriched diet. We determined instar stage based on the numbers of teeth present on the mouth hooks (Strasburger, 1932; Demerec, 1994).

To examine the possibility that larvae fed this restrictive diet are impaired in neuronal function we used the relatively recently described associative learning assays. We used a gustatory-visual relationship (Gerber *et al.*, 2004; Hendel *et al.*, 2005). We found that originally described procedures of having the agar dish divided into alternating quarters of light and dark for the initial and final testing for associative learning was biased toward larger larvae since they would cross the boundaries of the dark/light edges more often than smaller larvae over a given time period. If the larvae are in the middle of the dark or light pie shaped quarter then it is a relatively long way to reach a boarder transition (Figure 1A). In order to standardize the experimental design for various size larvae we used a stripped pattern (Figure 1B) and varied the strip distances as well as stripe thickness to equal two body lengths. The restrictive diet never allowed the larvae to reach the full body length of control larvae. However, each experimental group was consistent for body length within a group; we only needed to use a few sets of grids to carry out the testing on developmental time points. We feel that the new experimental design provides more opportunities for the larvae to choose their side of preference before and after associate conditioning.

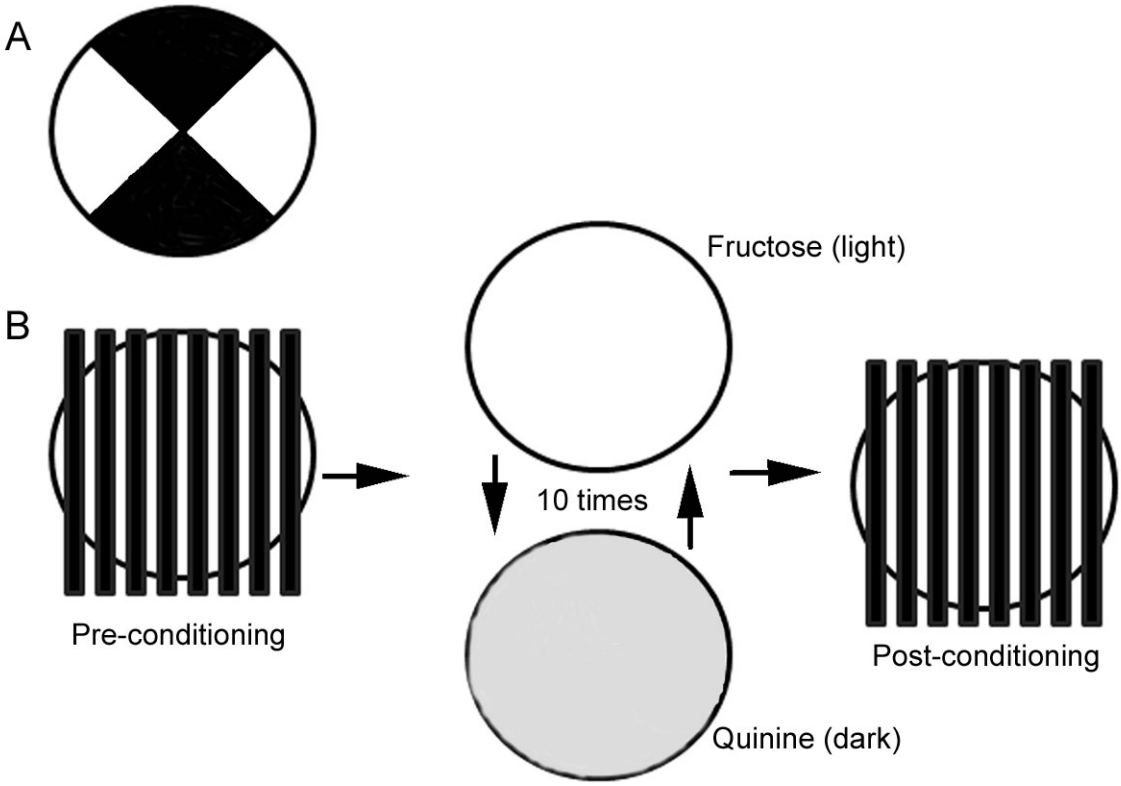



Figure 1.

The 1% agarose plates were used for the initial and final visual (*i.e.*, light/dark) preference test. For the learning assay, two other dishes were used: one for the positive gustatory reinforcement dishes with 1% agarose and 1M fructose (FRU, purity: 99%); and one for the negative reinforcement with 1% agarose and 0.2% quinine hemisulfate (QUI, purity 93%). The gustatory reinforcers were added after the agarose was dissolved and slightly cooled just prior to pouring the agar for the plates. Glass Petri dishes were used as the agarose plates in these studies (90-mm diameter). All experiments were done in a dark room at room temperature (21°C). The dishes were placed on a light box that only allowed light to go through the grids of the dishes. The learning regime consisted of 1 min in the light with a positive gustatory reinforcement, followed by 1 min in the dark with a

negative gustatory reinforcement. This procedure was repeated 10 times. The learning takes longer than 20 min since the 1 min conditioning does not include the time for transferring the larvae. Transferring the larvae occurred as rapidly as possible (~10 seconds). For the visual preference test, the larvae are recorded every 30 sec as being either on a dark side or on a light side for a total of 5 min. The % of larvae before and after training on the various dark/light locations are then compared for significant difference in learning.

Acknowledgments: I thank my sister Ann Cooper for help on this project and Hye Won Cooper for editorial assistance.

References: Alpatov, W., 1929, J. Exp. Zool. 52: 407-437; Church, B., and F.W. Robertson 1966, J. Exp. Zool. 162: 337-352; Dasari, S., and R.L. Cooper 2004, Neurosci. Res. 48: 221–227; Dasari, S., and R.L. Cooper 2006, J. Comp. Physiol. B 176: 349–357; Dasari, S., K. Viele, A.C. Turner, and R.L. Cooper 2007, European J. Neurosci. 26: 424–438; Demerec, M., 1994, *Biology of* Drosophila, CSHL Press, p. 285; Gerber, B., S. Scherer, K. Neuser, B. Michels, T. Hendel, R.F. Stocker, and M. Heisenberg 2004, J. Exp. Biol. 207: 179-188; Hendel, T., B. Michels, K. Neuser, A. Schipanski, K. Kaun, M.B. Sokolowski, F. Marohn, R. Michel, M. Heisenberg, and B. Gerber 2005, J. Comp. Physiol. A. 191: 265–279; Kaznowski, E.C., H.A. Schneiderman, and J.P. Bryant 1985, J. Insect Physiol. 31: 801-813; Li, H., D. Harrison, G. Jones, D. Jones, and R.L Cooper 2001, J. Neurophysiol. 85: 98-104; Neckameyer, W.S., 1996, Dev. Biol. 176: 209-219; Sewell, D., B. Burnet, and K. Connolly 1975, Genet Res. Camb. 24: 163–173; Scherer, S., R.F. Stocker, and B. Gerber 2003, Learn Mem. 10: 217–225; Strasburger, M., 1932, Meig. Zeit. wiss. Zool. 140: 539-649; Watts, T., A. Woods, S. Hargand, J. Elser, and T. Markow 2006, J. Insect Physiol. 52: 187-193.

## Iontophoretic dye injections into *Drosophila* cells.

**Tarasov, Peter[2,3], Alexey Zharinov[3], Jaakko Mattila[4], Leonid Omelyanchuk[1,3], and Valery Maltsev[2,3].** [1]Institute of Cytology and Genetics, [2]Institute of Chemical Kinetics and Combustion, [3]Novisibirsk University; [4]University of Turku, Finland.

### Abstract

Computer managed iontophoretic device was constructed and successfully applied in regeneration study. General scheme of the device, the method of dye injection, and associated software is discussed.

### The Device and Results

Iontophoresis can be used as a method to mark a living cell and its progeny cells with a vital charged dye or to deliver active substances to a cell. Of particular interest are the injections of antisence oligonucleotide probes, giving an opportunity to make a single gene silent (Aramaki *et al.*, 2003). In *Drosophila* research this method was used for marking cells from the wound edges of imaginal discs in regeneration study and to test the cell communications through the newly formed cell contacts (Bryant and Fraser, 1988). The aim of the present communication is to decribe the iontophoretic device according to current technology and to show its use.

As it was mentioned in Bryant and Fraser (1988), the positioning of the iontophoretic capillary within a cell is a critically important factor for successful injection. The correct position can be determined by measuring the membrane potential of the cell. Special scheme was constructed to

register a membrane potential of the cell, as well as to inject a fluorescent dye into this cell. The scheme is presented in Figure 1. The scheme contains the microelectrode, preamplifier, ADC/DAC board L-761, voltage controlled depended current source and reed relay. Physically the preamplifier, relay and current source are positioned onto the same board and shielded. While membrane potential polarizes the microelectrode, voltage produced is delivered to the input terminal of the preamplifier. Preamplifier is assembled on operational amplifier chip LPC-662 manufactured by National Semiconductor Corp. It has a high input resistance >1 Tera Ω and ultra low leakage current 2 fA. The preamplifier is intended to transfer signals from the microelectrode to ADC without any distortion as well as to hamper leakage current occurrence. To reduce the leakage current value, a special chip assembling on the printed-circuit board surface was employed. The output signal is digitized by PCI ADC/DAC board L-761 (L-Card Company). This board also supplied with double-channel DAC. The first channel is employed to control the relay, commutating the microelectrode to the preamplifier input and to the current source output. The second channel controls the current source. The current source is intended to deliver alternate current to microelectrode. Typically current represents a sequence of 200 ms pulses with amplitude of 4 nA and porosity of 2. Such current causes fluorescent dye injection into cell.

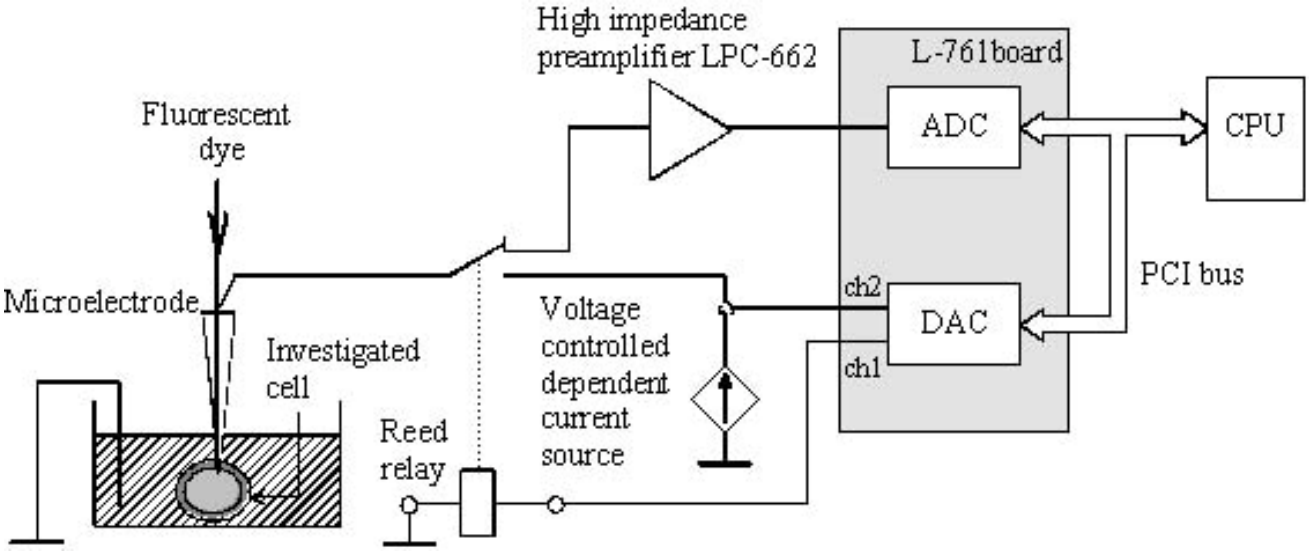



Figure 1. Connection layout of the iontophoretic device. Microelectrode is denoted as a triangle, the cell as a grey oval.

With use of LabVIEW 6.1 package the program to control L-761 board was developed. This program realizes virtual oscilloscope and voltmeter to register a membrane potential that depends on microelectrode penetration depth into cell. This program has the following features.

- Microelectrode signal registration and its shape examination on screen.
- Relay commutation control.
- Delivering of current signal of any given shape to the microelectrode to inject dye into cell.

The iontophoretic capillaries with the tip diameter less than 1 micrometer were filled first by the 200mg/ml rhodamine-conjugated dextran 40S (Sigma) and second by 1.2M LiCl. The fluorescent dye used is positively charged and was injected by pulses of the positive polarity. The capillary was

mounted on Narishige micromanipullator MWO-202 attached to Axiovert-200 Carl Zeiss inverted microscope. Transmitted and fluorescent light images (10× and 20× objectives) were taken by Axiocam MBC digital camera attached to the microscope. Injections of imaginal discs into adult female abdomen were done similarly to those already described (Bryant and Fraser, 1988).

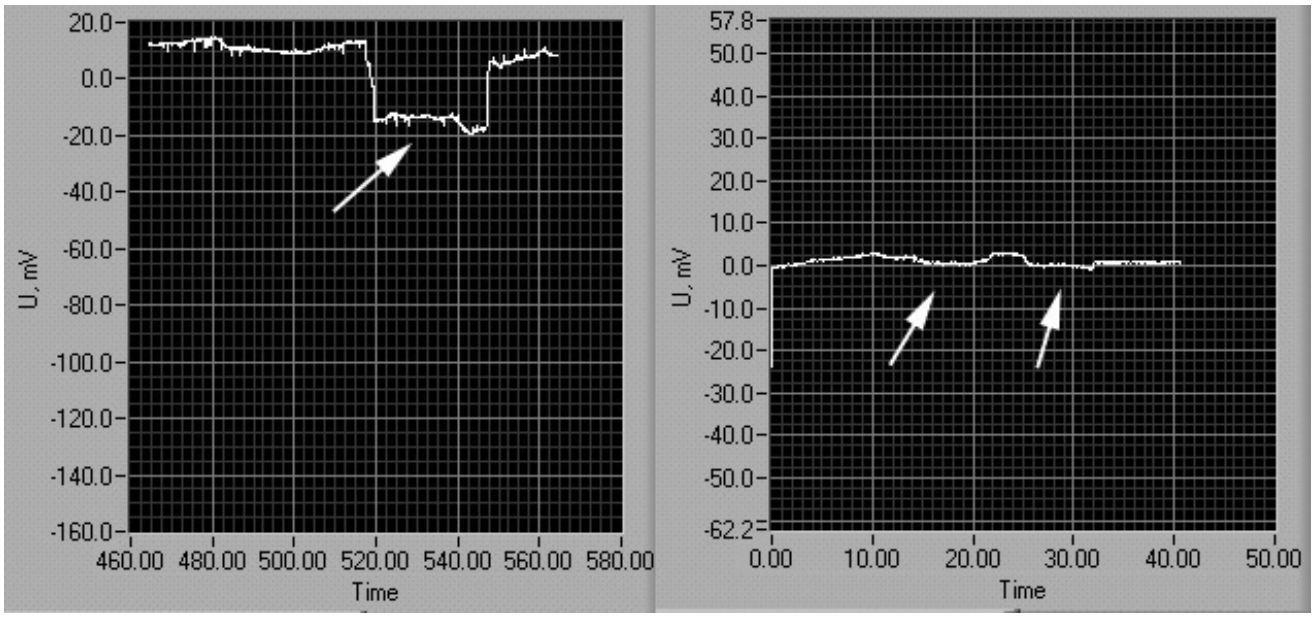



Figure 2. Membrane potential measurement. Oscillogram showing a polarization of microelectrode during introduction and moving off the capillary into the salivary gland polytene cell (right, one rectangular impulse marked with white arrow) and into the diploid imaginal disc cells (left, two negative impulses marked with white arrows). A window of LabVIEW software is shown.

Figure 2 illustrates the oscillogram after introduction and moving off the capillary into the salivary gland polytene cell (right, one rectangular impulse marked with white arrow) and into the imaginal disc cells (left, two negative impulses marked with white arrows). Figure 3 shows the phase contrast image of fragment of salivary gland containing target polytene cell (left, white arrow) and fluorescent image of inotophoretically injected cell (right, position where the capillary was introduced is marked by yellow by the remainder of capillary staying in the cell wall). It can be seen that the rhodamine label does not penetrate into nuclei (denoted by white arrow), but is localized in the cytoplasm. The example of iontophoretic injections into diploid *Drosophila* cells of the wing imaginal disc are given in Figure 4 (merged fluorescent and phase-contarast images). The most left photo - freshly injected cells of imaginal disc. At the next photo the same disc after culturing for 1 day in adult female abdomen is represented. The right photo – the same disc after 2 days of cultivation, note the right rhodamine spot was subdivided into two parts as a result of intercalary proliferation of nearby non-labeled cells or by allocation and proliferation of marked cells. Therefore, the injections made are stable through several cell generations. The application of the system for the study of *D. melanogaster* imaginal discs regeneration was published earlier (Mattila *et al*., 2004).

References: Aramaki, Y., H. Arima, M. Takahashi, E. Miyazaki, T. Sakamoto, and S. Tsuchiya 2003, Intradermal delivery of antisense oligonucleotides by the pulse depolarization iontophoretic system. Biol. Pharm. Bull. 10: 1461-1466; Bryant, P., and S. Fraser 1988, Wound

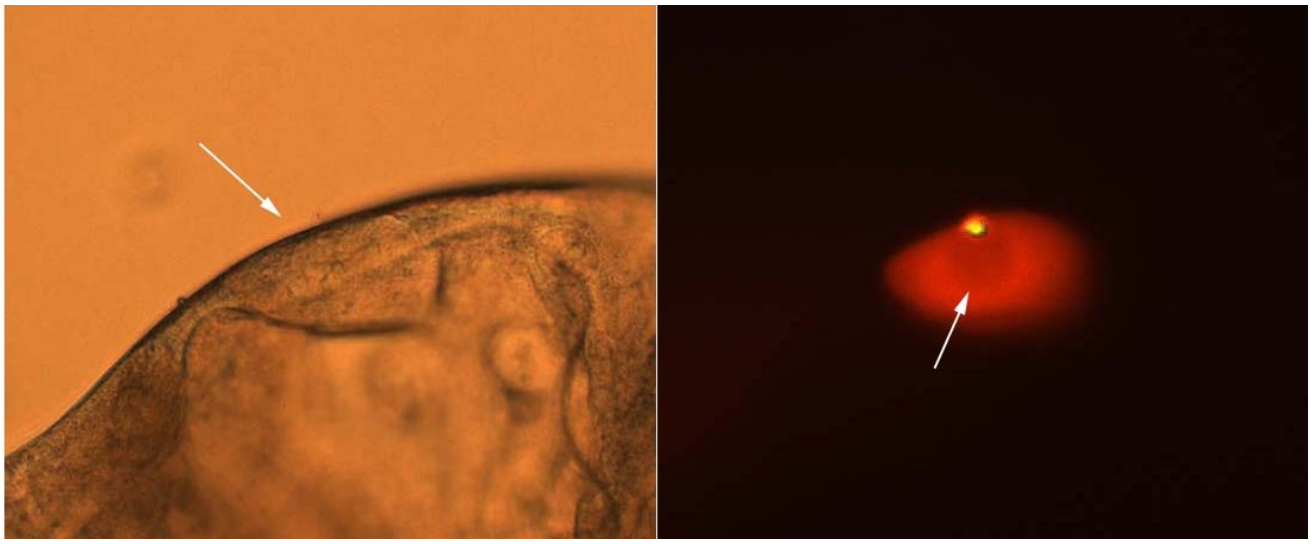


Figure 3. Injections into polytene cell of the larval salivary gland. Left: phase contrast image of fragment of salivary gland, containing target polytene cell (white arrow). Right: fluorescent image (red) of iontophoretically injected target cell (position where the capillary was introduced is marked by yellow by the remainder of capillary staying in the cell wall).

Figure 4. Diploid cells of the wing imaginal disc injections. Merged fluorescent (red) and phase-contrast images are shown. Left: freshly injected cells of imaginal disc. Middle: the same disc after culturing for 1 day in adult female abdomen is represented. Right: the same disc after 2 days of cultivation.

healing, cell communication, and DNA synthesis during imaginal disc regeneration in *Drosophila*. Devel. Biol. 127: 197-208; Mattila, J., L. Omelyanchuk, and S. Nokkala 2004, Dynamics of

decapentaplegic expression during regeneration of the Drosophila melanogaster wing imaginal disc. Int. J. Dev. Biol. 48(4): 343-347.

**Comparison of somatic clones of the eye in the analysis of cell growth.**

**Slade, Jennifer D., and Brian E. Staveley.** Department of Biology, Memorial University of Newfoundland, St. John's, Newfoundland & Labrador, Canada, A1B 3X9; telephone (709) 737-4317; telefax (709) 737-3018; Corresponding author: Dr. Brian E. Staveley; e-mail address: bestave@mun.ca.

## Introduction

Cell proliferation and increases in cell size are the processes that contribute to cell growth. Organs or tissues have a tendency to develop within the constraints of a normal final size and vary from a large number of small cells to a small number of large cells (Day and Lawrence, 2000). One important pathway in the control of cellular growth is the insulin receptor signaling pathway, which is highly conserved among invertebrates and mammals (Brogiolo *et al.*, 2001). When manipulated, several components of this pathway can alter growth rates of *Drosophila melanogaster*. The *Drosophila* eye normally consists of 700 to 800 ommatidia that develop in a highly regulated manner (Baker, 2001) and is ideal for the study of cell growth and cell survival. Mosaic eye clones can be created via the yeast site-specific recombination FLP/FRT system (Theodosiou and Xu, 1998), which depends upon the presence of *FRT* sites and expression of the enzyme FLP directed in the developing eye tissue. In the presence of FLP, homologous chromosomes undergo mitotic recombination between the *FRT* sites located on chromosome pairs. Heterozygous parent cells produce homozygous tissue within a heterozygous organism. In this paper, two methods of generating somatic clones of the eye are compared in the study of cell growth.

## *Drosophila* Strains and Culture

Experiments were carried out on standard media containing cornmeal, molasses, yeast, agar, and water at 25°C. The control line *w; +/+; P[FRT ;w$^{+}$]$^{2A}$ P[ry$^{+}$ neo$^{R}$ FRT]$^{82B}$ akt1$^{+}$* (*w; FRT$^{2A}$ FRT$^{82B}$ akt1$^{+}$*) was obtained from Dr. Norbert Perrimon, Harvard University (Perrimon *et al.*, 1996). The P-element insertion line *akt1$^{04226}$* was obtained from the Bloomington *Drosophila* Stock Center. This line contains a P-element inserted within the 5' untranslated region of the *akt1* gene on the third chromosome (Perrimon *et al.*, 1996; Spradling *et al.*, 1999). The novel derivative of *akt1$^{0422}$*, *akt$^{PR52}$*, was generated by imprecise excision. Both *akt1$^{04226}$* and *akt1$^{PR52}$* were then recombined with the third chromosome *FRT*'s by standard means, and, therefore, have the genotype of *w; +/+; P[FRT ; w$^{+}$]$^{2A}$ P[ry$^{+}$ neo$^{R}$ FRT]$^{82B}$ akt1$^{04226}$(or$^{PR52}$)/TM6B* (*w; FRT$^{2A}$ FRT$^{82B}$ akt1$^{04226}$ (or$^{PR52}$)*). The stocks required for creation of somatic clones of the eye were received from the Bloomington *Drosophila* Stock Center. The full genotype of *Drosophila* stock containing *eyeless-FLP* is *y$^{d2}$ w$^{1118}$; P{ry$^{+t7.2}$=ey-FLP.N}$^{2}$, P{GMR-lacZ.C(38.1)}$^{TPN1}$; P{ry$^{+t7.2}$=neoFRT}$^{82B}$, P{w$^{+t*}$ ry$^{+t*}$=white-un1}$^{90E}$, l(3)cl-R3$^{1}$/TM6B, P{y$^{+t7.7}$ ry $^{+t7.2}$=Car20y}$^{TPN1}$, Tb$^{1}$* for method one (Newsome *et al.*, 2000), hereby termed the *ey-FLP* method. The genotype *y w; P{w$^{+m}$=GAL4-ey.H}$^{3-8}$, P{w$^{+mC}$=UAS-FLP1.D}$^{JD1}$;*

*P{ry$^{+t7.2}$=neoFRT}$^{82B}$*, *P{w$^{+mC}$=GMR-hid}$^{SS4}$*, *l(3)CL-R$^{1}$/TM2* (Stowers and Schwarz, 1999) is the line used for method two, hereby termed the *ey-GAL4/UAS-FLP* method.

## Assay Design

Males of *w; FRT$^{2A}$ FRT$^{82B}$ akt1$^{+}$*, *w; FRT$^{2A}$ FRT$^{82B}$ akt1$^{04226}$* and *w; FRT$^{2A}$ FRT$^{82B}$ akt1$^{PR52}$* lines are crossed to females possessing either *eyeless-FLP* or *eyeless-GAL4* and *UAS-FLP* plus the proximal 3R recombination site (*FRT$^{82B}$*) and a wild-type copy of *akt1*. To obtain the desired individuals, flies are collected upon eclosion based upon phenotypic markers (the absence of *Hu* or *Ubx*). For comparison, homozygous adult males have been analyzed. All collected flies are aged for three to five days, and then flash frozen at –70°C before preparation for Scanning Electron Microscopy (SEM). Preparation included mounting upon aluminum SEM studs, dessication, and sputter coating in gold. Three to five images of each genotype are taken by SEM (Hitachi S-570 SEM) at 150× magnification.

## Statistical Analysis

Images of each genotype are analyzed using NIH Image J software. Ommatidia and bristles are counted from three images. Ommatidia area is determined by three independent measurements of the area of a cluster of seven ommatidia per picture, for three images. Results are graphed using GraphPad Prism (version 4.02). This program calculates the standard error of the mean to statistically compare the averages of ommatidia number, size, and bristle number between genotypes.

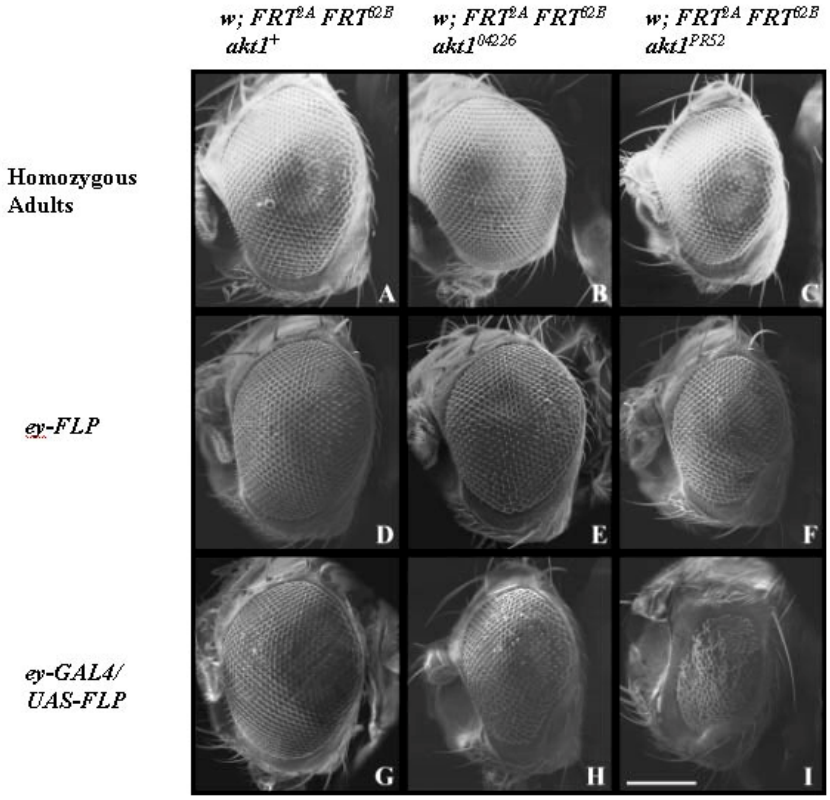



Figure 1. Eyes of *w; FRT$^{2A}$ FRT$^{82B}$ akt1$^{+}$*, *w; FRT$^{2A}$ FRT$^{82B}$ akt1$^{04226}$* and *w; FRT$^{2A}$ FRT$^{82B}$ akt1$^{PR52}$* homozygotes, *ey-FLP*-generated clones, and *ey-GAL4/UAS-FLP*-generated clones. Both methods of creating eye clones counteract the effects of developmental delay, as compared to the homozygous individuals. A-C are from homozygous adult males, D-F are somatic clones of the eye generated by the *ey-FLP* method, and G-I are somatic clones of the eye generated by the *ey-GAL4/UAS-FLP* method. Genotypes are: A, D, and G: *w; FRT$^{2A}$ FRT$^{82B}$ akt1$^{+}$*, B, E, and H: *w; FRT$^{2A}$ FRT$^{82B}$ akt1$^{04226}$*, C, F, and I: *w; FRT$^{2A}$ FRT$^{82B}$ akt1$^{PR52}$*. Scale bar represents 160 um.

## Results

Homozygous *akt1* mutant males have phenotypically smaller eyes than wild-type flies (Figure 1). With the *ey-FLP* method, control somatic eye clones developed 711 ± 15 ommatidia, with an area of 223 ± 5 um$^2$ and 540 ± 14 bristles (Figure 2). The *w; FRT$^{2A}$ FRT$^{82B}$ akt1$^{04226}$* eye was smaller in size compared to the *w; FRT$^{2A}$ FRT$^{82B}$ akt1$^{+}$* control, with 558 ± 14 ommatidia, with an area of 196 ± 4 um$^2$ and a bristle count of 452 ± 15. This weak allele produced a reduced number of ommatidia and exhibited a small amount of abnormal patterning, as well as misshaped ommatidia, not observed for the control clones. The P element derivative allele *w; FRT $^{2A}$ FRT$^{82B}$ akt1$^{PR52}$* developed smaller and fewer ommatidia than either *w; FRT$^{2A}$ FRT$^{82B}$ akt1$^{+}$* or *w; FRT$^{2A}$ FRT$^{82B}$ akt1$^{04226}$*, with 482 ± 32 ommatidia of an area of 182 ± 3 um$^2$ and bristle count of 371 ± 33 that had irregular ommatidial pattern. In the *ey-GAL4/UAS-FLP* method, the *w; FRT$^{2A}$ FRT$^{82B}$ akt1$^{+}$* control eye had an average of 704 ± 3 ommatidia with an area of 212 ± 2 um$^2$ and 563 ± 8 bristles. The *w; FRT$^{2A}$ FRT$^{82B}$ akt1$^{04226}$* eye clones have 508 ± 21 ommatidia of an area of 179 ± 3 um$^2$ with 377 ± 11 bristles. The derivative *w; FRT$^{2A}$ FRT$^{82B}$ akt1$^{PR52}$* in this method produced 261 ± 8 ommatidia with an area of 149 ± 11 um$^2$ and 115 ± 11 bristles. All genotypes had some abnormal ommatidial patterning and shape in the *ey-GAL4/UAS-FLP* method. In both methods, the more severe allele, *w; FRT$^{2A}$ FRT$^{82B}$ akt1$^{PR52}$*, was found to be significantly reduced in ommatidia number and size, and in bristle number. However, it is only in the *ey-GAL4/UAS-FLP* method that a significant difference between the control and the weak allele, *w; FRT$^{2A}$ FRT$^{82B}$ akt1$^{04226}$*, was observed.

## Discussion

The akt protein kinase is a central component in insulin receptor signaling, and acts through many downstream targets in cell survival, growth, proliferation, metabolism, and migration (recently reviewed in Manning and Cantley, 2007). Homozygous *akt1$^{1}$* mutant eyes are small, but co-expression of *akt* in these eyes was able to significantly suppress the reduction in cell size (Staveley *et al.*, 1998; Scanga *et al.,* 2000). Overexpression of *akt* in the developing eye was reported to produce enlarged eyes due to an increase in cell size but not in cell number (Verdu *et al.*, 1999). The akt kinase may exert its influence on cell growth through the activation of the target of rapamycin (TOR) complex, and downstream targets S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein (4EBP), which stimulate the initiation of protein synthesis (Manning and Cantley, 2007). In general, the akt kinase can alter cell number through its influence upon cell survival and proliferation, by blocking pro-apoptotic downstream targets BAD (and homologues) and the transcription factor foxo. The akt kinase can influence the overall size of tissues via cell proliferation through downstream targets, which function within cell cycle regulation. Regardless of the relative contributions of these molecular mechanisms, *akt* activity controls cell growth and survival in the developing *Drosophila* eye.

*Comparison of the Two Methods of Creating Somatic Clones of the Eye*

Both methods use *eyeless* enhancers to induce expression of the *FLP recombinase* gene. In the *ey-FLP* method, the *FLP* gene is under the control of four tandem repeats of a specific enhancer from the *eyeless* gene and a basal *hsp70* promoter. This allows for a direct expression of *FLP*. In the *ey-GAL4/UAS-FLP* method, the expression of *FLP* is indirect by the use of the UAS/GAL4 system. The *ey-GAL4* was constructed by cloning a 3.6 kb *Eco*RI fragment containing the eye-specific enhancer of the *eyeless* gene into a vector (Hazelett *et al.*, 1998). The expression of *eyeless* begins in the 6 to 23 cell-containing eye disc in embryogenesis and lasts until the last cell divisions required to

complete the ~15,000 cell-containing eye disc are carried out during the late third instar (Newsome *et al*., 2000). *FLP* is expressed during this time period and leads to the induction of recombination. There are roughly 10 to 12 rounds of post-embryonic cell divisions necessary to generate the number of cells in the eye disc (Newsome *et al.*, 2000). Heterozygous cells can give rise to homozygous cells during subsequent rounds of cell division, and this is increased with the sustained expression of *FLP* and mitotic recombination.

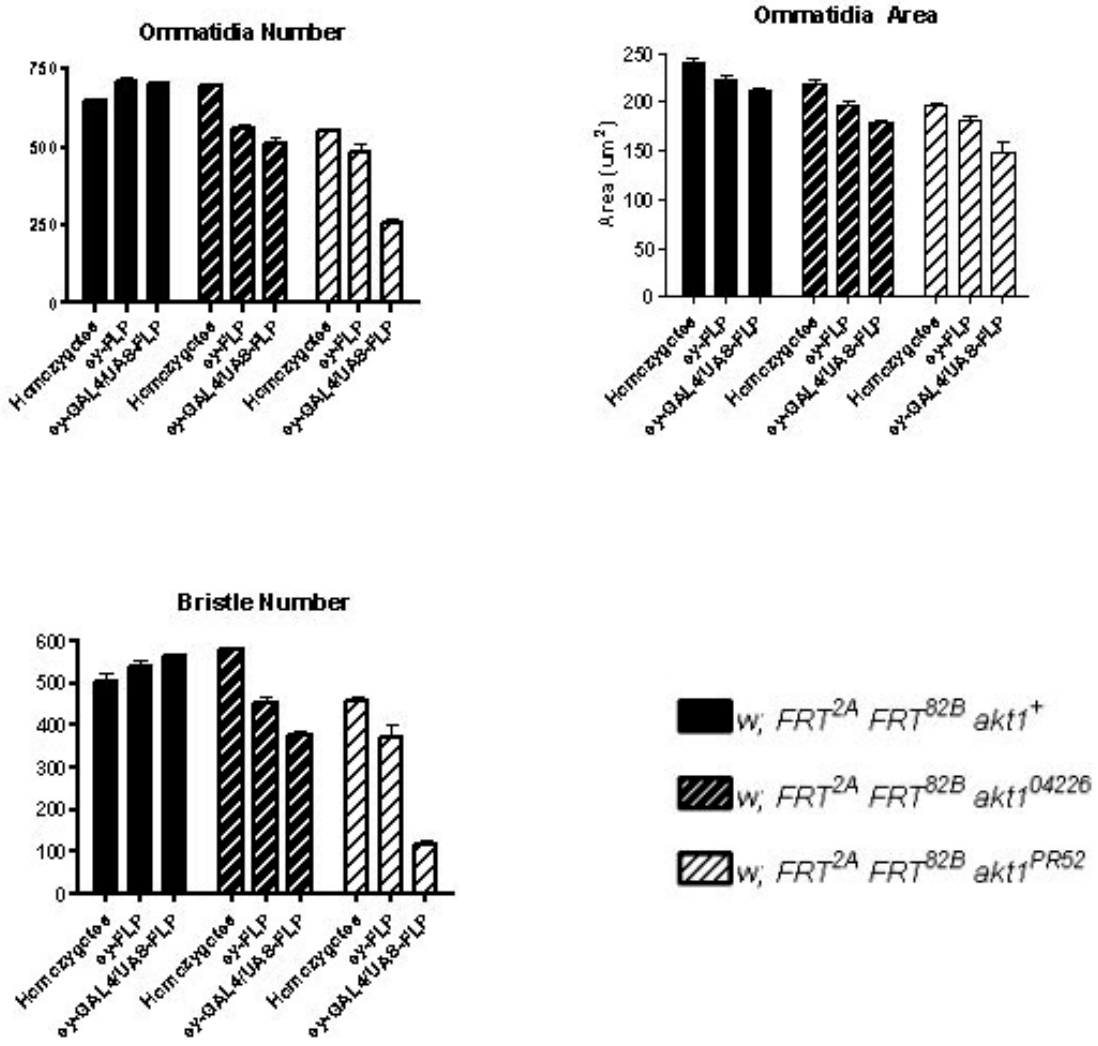



Figure 2. Comparison of ommatidia number, ommatidia size, and bristle number of *akt1* mutant homozygotes and somatic eye clones. The control line exhibits a small increase in both ommatidia and bristle number, and a slight decrease in ommatidia area in somatic clones when compared to homozygous individuals. The P-element insertion line *w; $FRT^{2A}$ $FRT^{82B}$ $akt1^{04226}$* has a larger number of smaller ommatidia and bristles compared to the control as a homozygote, but exhibits a decrease in all three phenotypic categories as a clone. The derivative *w; $FRT^{2A}$ $FRT^{82B}$ $akt1^{PR52}$* has fewer ommatidia and bristles, as well as smaller ommatidia, compared to both the control and *w; $FRT^{2A}$ $FRT^{82B}$ $akt1^{04226}$* in all three analyses. This decrease in size and number of cells is the most severe with the *ey-GAL4/UAS-FLP* method.

The *ey-FLP* method has been used both with and without the presence of a cell lethal. Without a cell lethal, the *ey-FLP* method results in 20-30% homozygous cells for the mutant allele of interest, and 70-80% of either homozygous or heterozygous wild-type cells (Newsome *et al.*, 2000). In the presence of a cell lethal, eye clones produce eyes that consist of 90-100% homozygous cells, although up to 10% of the eye may arise from heterozygous cells (Newsome *et al.*, 2000). The cell lethal is, therefore, able to enhance the generation of clone tissue and, therefore, allow for a more complete analysis of homozygous mutant tissue in an otherwise heterozygous animal.

The *ey-GAL4/UAS-FLP* method expresses *FLP* indirectly, so that an amplification of the recombinase is possible. This works through the *eyeless* control sequences enhancing the expression of *GAL4*, and then many copies of the GAL4 protein binding to *UAS* sites to lead to an excess of *FLP* expression. In addition, there is a *GMR-hid* transgene insertion distal to the *FRT* and wild-type copy of the gene of interest (Stowers and Schwarz, 1999). This gene will lead to the death of cells that carry it, including heterozygous cells. The *GMR* promoter leads to the expression of *hid* in late development, during metamorphosis (Stowers and Schwarz, 1999). While this is sufficient to remove heterozygous and homozygous wild-type cells, it does not leave sufficient time for the developing eye to compensate. The presence of both *GMR-hid* and a cell lethal allows this method to be effective in eliminating the non-mutant cells, and to produce an almost completely homozygous eye. Calculations determine that the percentage of heterozygous cells will decrease by 0.75 fold to result in the formation of 3 to 5% of the eye (Stowers and Schwarz, 1999). This represents a considerably smaller proportion of the eye when compared to the *ey-FLP* method.

*Evaluation of Assay*

The creation of homozygous clones of the eyes is effective in the study of a mutant phenotype in a heterozygous animal. When the homozygote is developmentally delayed, as is the case with *akt1* mutants (Slade and Staveley unpublished), clones are very informative. In homozygous individuals, a delay in development may allow for compensatory growth that could suppress the homozygous phenotype. The two methods for creating somatic clones of the eye, evaluated here, are more effective in revealing the mutant phenotype when compared to the analysis of the homozygous mutant adults. In the *ey-FLP* method, the *FLP recombinase* gene is expressed directly via the *eyeless* enhancer, and surviving heterozygous cells make up part of the eye. The *ey-GAL4/UAS-FLP* method has the *FLP recombinase* gene expressed in an indirect manner through the UAS-GAL4 system. This allows the amount of the FLP recombinase enzyme to be amplified that may lead to a greater effectiveness in the generation of clones and thus be more sensitive to alterations in growth as observed with *akt1* mutants. In addition, the presence of *GMR-hid* allows for heterozygous cells to be removed from the eye, to produce more clone tissue. These elements suggest that the *ey-GAL4/UAS-FLP* method is a sensitive method for analyzing the growth-dependent mutant phenotype in developmentally delayed adult organisms.

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References: Baker N.E., 2001, Semin. Cell Dev. Biol. 12: 499-507; Brogiolo W., H. Stocker, T. Ikeya, F. Rintelen, R. Fernandez, and E. Hafen 2001, Curr. Biol. 11: 213-21; Day, S.J., and P.A. Lawrence 2000, Development 127: 2977-87; Hazelett, D.J., M. Bourouis, U. Walldorf, and J.E. Treisman 1998, Development 125: 3741-51; Manning, B.D., and L.C. Cantley 2007, Cell 129: 1261-74; Newsome, T.P., B. Asling, and B.J. Dickson 2000, Development 127: 851-60; Perrimon,

N., A. Lanjuin, C. Arnold, and E. Noll 1996, Genetics 144: 1681-92; Scanga, S.E., L. Ruel, R.C. Binari, B. Snow, V. Stambolic, D. Bouchard, M. Peters, B. Calvieri, T.W. Mak, J.R. Woodgett, and A.S. Manoukian 2000, Oncogene 19: 3971-7; Spradling, A.C., D. Stern, A. Beaton, E.J. Rhem, T. Laverty, N. Mozden, S. Misra, and G.M. Rubin 1999, Genetics 153: 135-77; Staveley, B.E., L. Ruel, J. Jin, V. Stambolic, F.G. Mastronardi, P. Heitzler, J.R. Woodgett, and A.S. Manoukian 1998, Curr. Biol. 8: 599-602; Stowers, R.S., and T.L. Schwarz 1999, Genetics 152: 1631-9; Theodosiou, N.A., and T. Xu 1998, Methods 14: 355-65; Verdu, J., M.A. Buratovich, E.L. Wilder, and M.J. Birnbaum 1999, Nat. Cell Biol. 1: 500-6.

## Amplification of DNA from 30-year-old aceto-orcein stained salivary gland squash slides.

**Johnson, David A**. Seinan Gakuin University, 6-2-92 Nishijin, Sawara-ku, Fukuoka-shi 814-8511 JAPAN.

Salivary gland squash slides have been used since the early days of genetics to visualize the fruit fly's polytene chromosomes. These slides represent an immense archive of genetic material for *Drosophila* and other genera. Aceto-orcein is commonly used to stain salivary gland preparation, as well as other cytological specimens, especially when trying to visualize chromosomal material. However, aceto-orcein stained tissue proves recalcitrant to yielding DNA for amplification. I describe here a simple procedural modification of a basic DNA extraction protocol that yields PCR-amplifiable DNA from aceto-orcein stained salivary glands.

The slides in question were made over 30 years ago from *Drosophila melanogaster* strains with presumptive tandem duplications of the maroon-like (*mal*) locus. These were apparently the result of spontaneous non-homologous unequal crossing over (Johnson and Smith, 1976; Johnson, 1977). These slides were prepared using a standard protocol involving 1) coating one slide with a gelatin subbing solution, 2) squashing salivary glands in 45% acetic acid between two slides using a bench vice, 3) freezing the slides on dry ice then popping them apart, 4) staining with aceto-orcein, 5) dehydration in alcohol, serial toluene treatment, and mounting a coverslip with CoverBond (Harelco). Various protocols to recover DNA were attempted repeatedly, including using the Pinpoint Slide DNA Isolation System™ (Zymo Research), all yielding no detectable DNA after PCR.

The key that made DNA isolation possible was a brief acidification step, presumably to remove the DNA-bound aceto-orcein. The successful protocol below is a modification of the Wizard® Genomic DNA Purification Kit procedure (Promega). The use of toluene here is to remove CoverBond which is toluene soluble.

### Protocol

1) Carefully scrape salivary gland tissue off the slide with a new razor blade then soak the tissue in toluene for 5 minutes.
2) Add an excess of 95% ethanol and mix gently.
3) Centrifuge briefly to pellet the tissue flake then discard the supernatant.
4) Wash three more times with 95% ethanol (repeat steps 2 and 3).
5) Remove remaining ethanol in a vacuum centrifuge.
6) Dissolve the flake (as much as possible) in 600 µL Wizard Nuclei Lysis Solution.
7) Incubate at 65ºC for 30 minutes.

```
8A21    1     TAGATGTGAAAACGAATTTAATGTTCTGTAGTTAAAAAACATTTTATAAATGTTTTAAAT  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AE014298.4    TAGATGTGAAAACGAATTTAATGTTCTGTAGTTAAAAAACATTTTATAAATGTTTTAAAT

8A21    61    AATAAGATTGTAGTTTCCAAAAATTATTGTCATACAATTGGCCAATTAGCCATTAGCAAC  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AE014298.4    AATAAGATTGTAGTTTCCAAAAATTATTGTCATACAATTGGCCAATTAGCCATTAGCAAC

8A21    121   CATTTTCCTAACACCAATTGTTTGCCAACACTCAGAACATATGTTTGAATAGCCATGCAA  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AE014298.4    CATTTTCCTAACACCAATTGTTTGCCAACACTCAGAACATATGTTTGAATAGCCATGCAA

8A21    181   AGTGCACAACGACTAGTTAATACCGTACAATTTGAGTTTAAAATTCTATGCAATTCAGAG  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AE014298.4    AGTGCACAACGACTAGTTAATACCGTACAATTTGAGTTTAAAATTCTATGCAATTCAGAG

8A21    241   TTAATAATTGGAAAAGATTTGGTAAAATGCTACATCTATAATATTGGTGAGCAGTGTGAG  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AE014298.4    TTAATAATTGGAAAAGATTTGGTAAAATGCTACATCTATAATATTGGTGAGCAGTGTGAG

8A21    301   AATATGATATATATATTACAATAAGCAAACATAATGCACAAGAAGCTTAATAAATTGAGC  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AE014298.4    AATATGATATATATATTACAATAAGCAAACATAATGCACAAGAAGCTTAATAAATTGAGC

8A21    361   TTCGTAAAATGTGTAGGGTGGAGTAAGTTCAAAGAGAAGTCAAAGCAAATGCTCAACTGA  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AE014298.4    TTCGTAAAATGTGTAGGGTGGAGTAAGTTCAAAGAGAAGTCAAAGCAAATGCTCAACTGA

8A21    421   ACGTGATAGTTGATGTATACTGTCCGTGGCTTTTTCCAGCGAAGAAGAGTATGGCGCAAC  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AE014298.4    ACGTGATAGTTGATGTATACTGTCCGTGGCTTTTTCCAGCGAAGAAGAGTATGGCGCAAC

8A21    481   AAATGCAAGCCGGTCGCAAATAAATAAAGCCCGCTTTGCAGCCAACACGAATAGTTAGTT  540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AE014298.4    AAATGCAAGCCGGTCGCAAATAAATAAAGCCCGCTTTGCAGCCAACACGAATAGTTAGTT

8A21    541   CCCAACATGACATCATATCGGCCGGAGTTCTCCGCATCGGAGCAGTCGCAAATAGACGCG  600
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AE014298.4    CCCAACATGACATCATATCGGCCGGAGTTCTCCGCATCGGAGCAGTCGCAAATAGACGCG

8A21    601   GAATTCTCTAGATTGGCCAGTAAGTGGAGAACCATGGTGAAATACCCAGCGCAAACAGAT  660
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AE014298.4    GAATTCTCTAGATTGGCCAGTAAGTGGAGAACCATGGTGAAATACCCAGCGCAAACAGAT

8A21    661   AACGAACCTAACCATCCGTCTATCGAACAGGCAACA  696
              |||||||||||| ||||||||||||||||||||||
AE014298.4    AACGAACCTAACTATCCGTCTATCGAACAGGCAACA
```

Figure 1. Alignment the entire fragment amplified from a slide (8A21) and the *Drosophila melanogaster* chromosome X *mal* region DNA (AE014298.4).

8) Add 10 µL of Wizard RNase and 10 µL of Proteinase K (20 mg/mL) and incubate at 37ºC for 20 minutes.
9) Incubate at 95ºC for 5 minutes to denature Proteinase K.
10) Add 200 µL of Wizard Protein Precipitation Solution and vortex for 20 seconds.
11) Chill on ice for 5 minutes.
12) Centrifuge for 4 minutes at 13,000-16,000 ×g.

13) Transfer supernatant to a clean 1.5 mL microfuge tube containing 600 µL of isopropanol.
14) Gently mix by inversion.
15) Centrifuge for 1 minute at 13,000-16,000 ×g.
16) Carefully remove isopropanol with a pipette and discard.
17) Add 600 µL of 70% ethanol and gently mix by inversion.
18) Centrifuge for 1 minute at 13,000-16,000 ×g.
19) Carefully remove ethanol with a pipette and discard.
**20) Add 100 µL 45% acetic acid and mix gently; let stand for 2 minutes.**
**21) Add 300 µL Wizard Rehydration Solution and 1/10 volume 7.5 M ammonium acetate and mix gently.**
**22) Add 2 volumes 95% ethanol and mix gently.**
**23) Incubate at 70ºC for 5 minutes.**
**24) Centrifuge for 1 minute at 13,000-16,000 ×g.**
**25) Carefully remove supernatant with a pipette and discard.**
**26) Add 1000 µL ethanol, mix gently, and centrifuge for 1 minute at 13,000-16,000 ×g.**
**27) Carefully remove supernatant with a pipette and discard.**
28) Invert tube on a paper towel and air-dry the pellet for 10–15 minutes.
29) Add 40 µL of Wizard DNA Rehydration Solution and incubate at 65°C for 1 hour.
30) Store DNA at 2–8°C.

PCR was performed on these extracts using GoTaq® (Promega) and primers that amplify an approximately 744 nt segment at the beginning of the *mal* mRNA (primers: 5'CAGCTGTATGTGTAGGCTATCGTC3' and 5'CCGCATGATCCAGGTAAACACTCT3'). Longer extension times were used (94ºC for 1 minute, 55ºC for 1 minute, 72ºC for 3 minutes for 33 cycles) and hot start PCR was performed using AmpliWax® PCR Gem wax beads (Applied Biosystems). A typical reaction used 10.5 µL of DNA in the lower layer and 12.5 µL of GoTaq and 1 µL of each primer in the upper layer. DNA amplified from two slides using these primers was indistinguishable on agarose gels from similarly amplified DNA from wild-type flies. PCR products were cloned using the TOPO® TA method (Invitrogen) and sequenced (Macrogen, Korea). As shown in Figure 1, this amplified DNA is from the *mal* locus of *Drosophila melanogaster*. All clones from the two slides gave similar results.

It is my hope that this report will make such archived *Drosophila* DNA more accessible.

References: Johnson, D.A., and P.D. Smith 1976, Genetics 83: s36; Johnson, D.A., 1977, Ph.D. Thesis, Emory University, Atlanta, Georgia.

**An organizational strategy for deficiency mapping: A computational approach**.

**Kuntimaddi, Aravinda[*], Mikolaj J. Sulkowski[*], and Daniel N. Cox[†].** Department of Molecular and Microbiology, Krasnow Institute for Advanced Study George Mason University, Manassas, VA 20110 USA. ([*]These authors contributed equally to this work; [†]Author for correspondence: dcox5@gmu.edu).

## Introduction

Deficiency mapping provides *Drosophila* geneticists with the unique ability to physically map mutations by studying a single generation of animals. By crossing a lethal mutation in an unknown

gene with deficiency lines possessing a deletion of a known region of a chromosome, the presence or absence of lethality in the offspring can reveal whether or not the mutation resides in the same location as the chromosomal deficiency. In contrast, researchers studying other model organisms including *Caenorhabditis elegans* and *Mus musculus* must rely on the tedious process of recombination mapping, which involves multiple generations of crosses, scoring large numbers of offspring, and computational analyses. Deficiency mapping, combined with the potential for rapid generation, isolation, and phenotypic analysis of random chemically-induced mutations makes *Drosophila* the organism of choice for rapid forward genetics.

## Purpose

Deficiency mapping is often an arduous task involving a multitude of crosses between the deficiency stocks and the specific mutation to be mapped. If, for example, one is attempting to map a mutation on the second chromosome, they may need to do upwards of 109 crosses. A major problem is that these deficiency stocks contain deletions that often overlap, leading to crosses that may be unnecessary. Thus, it is logical to use the stocks that cover the largest deficiencies, but the problem is that the deficiency stocks are not organized according to the order of deletions along the chromosome. If one attempts to simply carry out this task by creating genetic crosses in order of the deficiency kit stock numbers, this will lead to numerous crosses that are unnecessary, and often unorganized. We have created an efficient method of visually organizing deficiency crosses according to the deletions that they cover on the chromosome.

## Methods

*Sorting the deficiency stocks by chromosomal position*

To illustrate this organizational strategy we have chosen to present information on the second chromosome molecularly defined deficiencies for the *Drosophila* genome, although in principle the same organizational approach could readily be applied to molecularly defined deficiencies covering the X and third chromosomes as well. Importantly, this organizational strategy relies upon known molecularly defined sequence breakpoints for each of the chromosomal deficiencies, such as those generated in the DrosDel deficiency kit (Ryder *et al.*, 2007) available through the Bloomington Stock Center (Bloomington, Indiana).

The first step was to separate the deficiencies between the two chromosomal arms, in this case 2L and 2R. Using Microsoft Excel®, we created two spreadsheets, each sheet used specifically for one chromosomal arm. Next, we simply went through all of the DrosDel deficiency kit stocks and listed the molecularly defined start and end sequence breakpoints and the corresponding stock number in three separate columns as illustrated in Figure 1.

| Starting Breakpoint | Ending Breakpoint | Stock Number |
|---:|---:|---:|
| 159063 | 285763 | 8901 |
| 67365 | 72671 | 9180 |
| 160605 | 285763 | 9177 |
| 67365 | 161120 | 9353 |

**Figure 1.** Organizing deficiency kit stocks into a spreadsheet

The next step is to use an ascending sort function. First, highlight the full data set and then under the "Data" pull-down tab select the "Sort" function followed by the "Sort by starting breakpoint" option which can be summarized as follows: *Data* → *Sort* → *Sort by starting breakpoint.* The two screenshots shown below in Figure 2a and 2b visually demonstrate the steps described above. As shown in Figure 2c, the chromosome deficiency crosses are now sorted according to the starting breakpoint.

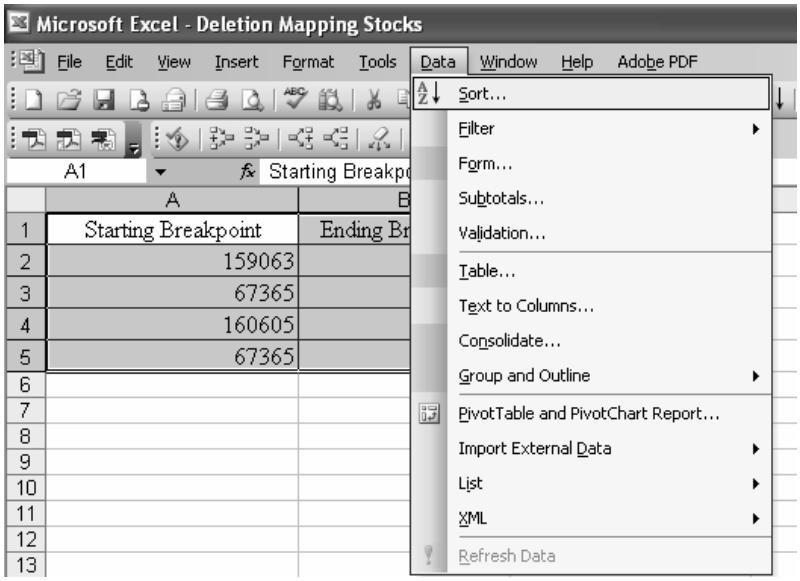


**Figure 2a.** Accessing the sort function through the Data pull-down tab.

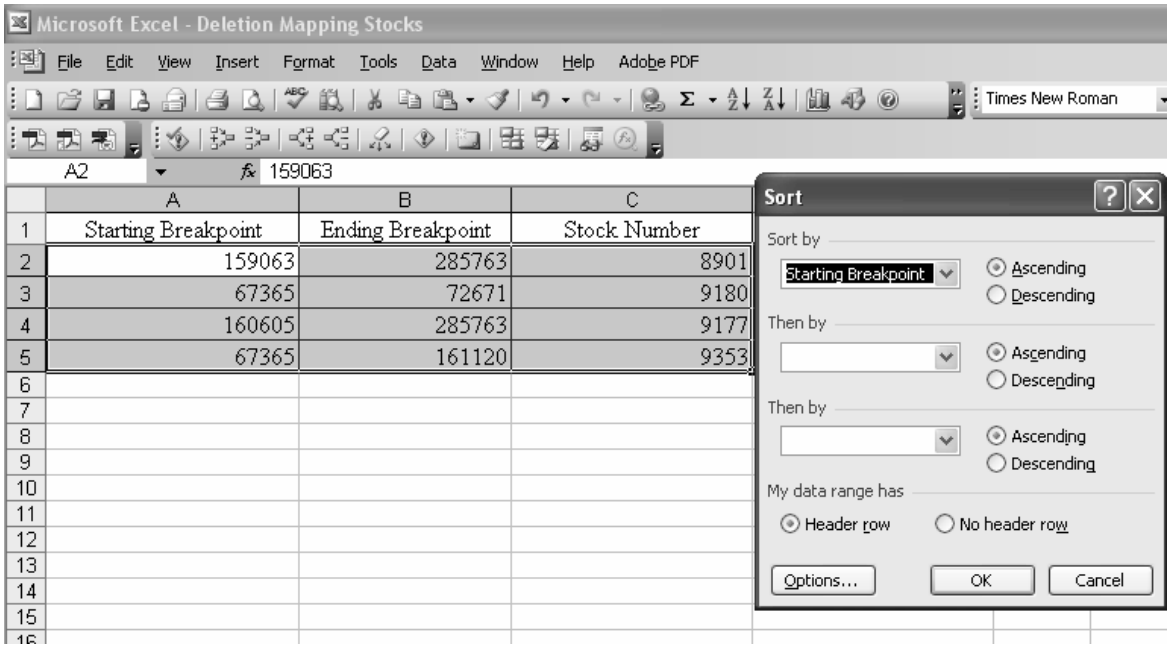


**Figure 2b.** Sorting the starting breakpoint values in ascending order.

| Starting Breakpoint | Ending Breakpoint | Stock Number |
|---|---|---|
| 67365 | 72671 | 9180 |
| 67365 | 161120 | 9353 |
| 159063 | 285763 | 8901 |
| 160605 | 285763 | 9177 |

**Figure 2c.** Deficiency stocks now organized in ascending order according to the starting breakpoint.

*Visually mapping the deficiency stocks*

After sorting the crosses in ascending order, the next step was to visually map the base pair deletions. This visual map does not correlate with physical distance; instead, it shows relative positions of the respective breakpoints. After creating a separate column indicating the visual map of breakpoints, we used the left side of the column to indicate the starting breakpoint and the right side to indicate the ending breakpoint. Next, we simply advanced through the spreadsheet, placing the breakpoints in sequential order as seen in Figure 3. The breakpoints are placed in cells based on ascending numerical value. Each unique number occupies a given column. This creates a visual map with the deficiencies spanning their relative positions. By highlighting the positions occupied by the deficiencies one can indicate the area covered between the breakpoints. The end result is that we can now look at the visual map and immediately see that stocks 9353 and 9177 cover the breakpoints between base pairs 67365 and 285763 (Figure 3). The other two stocks represent sub-deficiencies within this region of the chromosome and are not necessary for the initial deficiency cross mapping. Thus, in this example of four crosses, we have reduced our workload by 50%.

| Stock Number | Visual Map of Breakpoints | | | | | |
|---|---|---|---|---|---|---|
| 9353 | 67365 | | | | 161120 | |
| 9180 | 67365 | 72671 | | | | |
| 8901 | | | 159063 | | | 285763 |
| 9177 | | | | 160605 | | 285763 |

**Figure 3**. Visually mapping the deficiency stocks.

**Discussion**

Thanks to the accumulated efforts of the *Drosophila* community, including Exelixis (Parks *et al.,* 2004) and the recent DrosDel consortium (Ryder *et al.,* 2007), researchers now possess collections of *Drosophila* stocks with deficiencies covering greater than 90% of the fly genome (BDSC; http://flystocks.bio.indiana.edu/Browse/df-dp/dfkit-info.html). The size of each deficiency ranges from the maximum tolerable size of approximately 1 Mb (Ashburner and Bergman, 2005) to relatively small deficiencies of ~100 kb.

We have created a strategy that simplifies the logistics of deficiency mapping. By visually arranging deficiency stocks according to breakpoints, one can create a map representing the relevant information for mapping an unknown mutation. In the example shown in Figure 2, the workload

became reduced by 50 percent after implementing our technique. Thus if one has to scan the entire second chromosome using the DrosDel deficiency kit that has 109 deficiency stocks, our method can reduce the workload by 43 percent, meaning that one only needs to use 62 deficiency stocks for the first round of crosses.

References: Ashburner, M., and C.M. Bergman 2005, Genome Res. 15: 1661-1667; Parks, A.L., K.R. Cook, M. Belvin, N.A. Dompe, R. Fawcett, K. Huppert, L.R. Tan, C.G. Winter, K.P. Bogart, J.E. Deal, M.E. Deal-Herr, D. Grant, M. Marcinko, W.Y. Miyazaki, S. Robertson, K.J., Shaw, M. Tabios, V. Vysotskaia, L. Zhao, R.S. Andrade, K.A. Edgar, E. Howie, K. Killpack, B. Milash, A. Norton, D. Thao, K. Whittaker, M.A. Winner, L. Friedman, J. Margolis, M.A. Singer, C. Kopczynski, D. Curtis, T.C. Kaufman, G.D. Plowman, G. Duyk, and H.L. Francis-Lang 2004, Nat. Genet. 36: 288-292; Ryder, E., M. Ashburner, R. Bautista-Llacer, J. Drummond, J. Webster, G. Johnson, T. Morley, Y.S. Chan, F. Blows, D. Coulson, G. Reuter, H. Baisch, C. Apelt, A. Kauk, T. Rudolph, M. Kube, M. Klimm, C. Nickel, J. Szidonya, P. Maroy, M. Pal, A. Rasmuson-Lestander, K. Ekstrom, H. Stocker, C. Hugentobler, E. Hafen, D. Gubb, G. Pflugfelder, C. Dorner, B. Mechler, H. Schenkel, J. Marhold, F. Serras, M. Corominas, A. Punset, J. Roote, and S. Russell 2007, Genetics 177: 615-629.

## *Drosophila* Proteome Atlas.

**Matsumoto, H.[1], N. Takemori[1,3], J.N. Thompson, jr.[2], M.-T. Yamamoto[3], and N. Komori[1].** [1]Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73190, USA; [2]Department of Zoology, University of Oklahoma, Norman, OK 73019, USA; [3]*Drosophila* Genetic Resource Center, Kyoto Institute of Technology, Saga Ippongi-cho, Ukyo-ku Kyoto, 616-8354, Japan.

Since the first report of the *Drosophila* genome project in 2000 (Adams *et al.*, 2000), DNA sequence information became a valuable asset to research projects using *Drosophila* as a model organism. However, the knowledge of *Drosophila* proteins that are expressed and thereby manifest the function of the genome is far behind the success of its genetics and genomics counterparts. We would like to draw the attention of *Drosophila* researchers to our effort to provide information on proteins based on proteomics inquiries in which proteins are the immediate subject matter. In our recent paper (Takemori *et al.*, 2007) we reported 1) that proteomics profiling of *Drosophila* compound eyes in comparison to brain is a powerful tool to investigate post-translational modification of proteins using tissue-specific calmodulin methylation as an example, and 2) that such proteome information will be a useful asset to the *Drosophila* community. In our protocol, microdissection of the compound eyes and brain from dehydrated tissues provides clean and sufficient materials for analysis on a two-dimensional (2-D) gel electrophoresis (Matsumoto *et al.*, 1982; Matsumoto and Pak, 1984). Furthermore, a multi-stage mass spectrometric analysis of a 2-D gel spot allows us to determine the structure of the modified amino acids at a microscale (at ~100 fmol levels) (Takemori *et al.*, 2006). With a belief that the information obtained in this work and that to be obtained in our future work will benefit other *Drosophila* researchers, we initiated an open access protein database "*Drosophila Proteome Atlas* (DPA)" posted at The University of Oklahoma (Drosophila Information Service) and at Kyoto Institute of Technology (Drosophila Genetic Resource

Center): http://www.ou.edu/journals/dis/ProteomicsDatabase/Proteomics_Home.htm and http://www.dgrc.kit.ac.jp/~jdd/proteome/Proteomics_Home.htm, respectively.

In constructing DPA we start from protein maps displayed on two-dimensional (2-D) gel electrophoresis. Protein is identified by peptide mass fingerprinting followed by the confirmation by MS/MS (Matsumoto *et al.*, 2005). The 2-D gel electrophoresis renders a map of proteins that exist in the sample of interest, giving visually comprehensible images carrying rich information on proteome, including information on post-translational modification of proteins and the quantity of expressed proteins, neither of which can be elucidated from genome information *per se* (Matsumoto and Komori, 2000; Matsumoto *et al.*, 2005). In the long-standing effort to build a biological project from a physiological phenomenon one of the authors started observing the light-induced post-translational modifications of proteins in the compound eyes of *Drosophila in vivo* (Matsumoto *et al.*, 1982; Matsumoto and Pak, 1984). Because of the lack of technical availability it took several years for us to clone the gene that encodes the protein of interest (Yamada *et al.*, 1990). Meanwhile, two key ingredients in proteomics, *i.e.*, genome information and modern mass spectrometry, emerged in different areas of science and technology and, finally, merged as a new interdisciplinary area of bioscience (Matsumoto *et al.*, 1999). Although we started the Atlas from the compound eye in reference to brain, we plan to add more maps of other tissues and organs of *Drosophila* in our future effort, thereby making the *Drosophila Proteome Atlas* increasingly useful to other researchers. It is our belief that proteomics information will add a new dimension to research projects using *Drosophila* and accelerate their progress.

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References: Adams, M.D., S.E. Celniker, R.A. Holt, C.A. Evans, J.D. Gocayne, P.G. Amanatides, S.E. Scherer, P.W. Li, R.A. Hoskins, R.F. Galle, R.A. George, S.E. Lewis, S. Richards, M. Ashburner, S.N. Henderson, G.G. Sutton, J.R. Wortman, M.D. Yandell, Q. Zhang, L.X. Chen, R.C. Brandon, Y.H. Rogers, R.G. Blazej, M. Champe, B.D. Pfeiffer, K.H. Wan, C. Doyle, E.G. Baxter, G. Helt, C.R. Nelson, G.L. Gabor, J.F. Abril, A. Agbayani, H.J. An, C. Andrews-Pfannkoch, D. Baldwin, R.W. Ballew, A. Basu, J. Baxendale, L. Bayraktaroglu, E.M. Beasley, K.Y. Beeson, P.V. Benos, B.P. Berman, D. Bhandari, S. Bolshakov, D. Borkova, M.R. Botchan, J. Bouck, P. Brokstein, P. Brottier, K.C. Burtis, D.A. Busam, H. Butler, E. Cadieu, A. Center, I. Chandra, J.M. Cherry, S. Cawley, C. Dahlke, L.B. Davenport, P. Davies, B. de Pablos, A. Delcher, Z. Deng, A.D. Mays, I. Dew, S.M. Dietz, K. Dodson, L.E. Doup, M. Downes, S. Dugan-Rocha, B.C. Dunkov, P. Dunn, K.J. Durbin, C.C. Evangelista, C. Ferraz, S. Ferriera, W. Fleischmann, C. Fosler, A.E. Gabrielian, N.S. Garg, W.M. Gelbart, K. Glasser, A. Glodek, F. Gong, J.H. Gorrell, Z. Gu, P. Guan, M. Harris, N.L. Harris, D. Harvey, T.J. Heiman, J.R. Hernandez, J. Houck, D. Hostin, K.A. Houston, T.J. Howland, M.H. Wei, C. Ibegwam, M. Jalali, F. Kalush, G.H. Karpen, Z. Ke, J.A. Kennison, K.A. Ketchum, B.E. Kimmel, C.D. Kodira, C. Kraft, S. Kravitz, D. Kulp, Z. Lai, P. Lasko, Y. Lei, A.A. Levitsky, J. Li, Z. Li, Y. Liang, X. Lin, X. Liu, B. Mattei, T.C. McIntosh, M.P. McLeod, D. McPherson, G. Merkulov, N.V. Milshina, C. Mobarry, J. Morris, A. Moshrefi, S.M. Mount, M. Moy, B. Murphy, L. Murphy, D.M. Muzny, D.L. Nelson, D. R. Nelson, K.A. Nelson, K. Nixon, D.R. Nusskern, J.M. Pacleb, M. Palazzolo, G.S. Pittman, S. Pan, J. Pollard, V. Puri, M.G. Reese, K. Reinert, K. Remington, R.D. Saunders, F. Scheeler, H. Shen, B.C. Shue, I. Siden-Kiamos, M. Simpson, M.P. Skupski, T. Smith, E. Spier, A.C. Spradling, M. Stapleton, R. Strong, E. Sun, R. Svirskas, C. Tector, R. Turner, E. Venter, A.H. Wang, X. Wang, Z.Y. Wang, D.A. Wassarman, G.M. Weinstock, J. Weissenbach, S.M. Williams, T. Woodage, K.C. Worley, D. Wu, S. Yang, Q.A. Yao, J. Ye, R.F. Yeh, J.S. Zaveri, M. Zhan, G. Zhang, Q. Zhao, L. Zheng, X.H. Zheng, F.N. Zhong, W. Zhong, X. Zhou, S. Zhu, X. Zhu, H.O. Smith, R.A. Gibbs, E.W. Myers, G.M. Rubin, and J.C. Venter 2000, Science 287(5461): 2185-2195; Matsumoto, H., J.E. O'Tousa, and W.L. Pak 1982, Science 217,

839-841; Matsumoto, H., and W.L. Pak 1984, Science 223: 184-186; Matsumoto, H., E.S. Kahn, and N. Komori 1999, Novartis Found. Symp. 224: 225-244; Matsumoto, H., and N. Komori 2000, Methods Enzymol. 316: 492-511; Matsumoto, H., S. Kurono, M. Matsumoto, and N. Komori 2005, Mass spectrometry of biomolecules in proteomics. In: *Encyclopedia of Molecular and Cell Biology and Molecular Medicine* (Meyers, R.A., ed.), Wiley-VCH Verlag GmbH, Berlin; Takemori, N., N. Komori, and H. Matsumoto 2006, Electrophoresis 27: 1394-1406; Takemori, N., N. Komori, J.N. Thompson, jr., M.T. Yamamoto and H. Matsumoto 2007, Proteomics 7: 2651-2658; Yamada, T., Y. Takeuchi, N. Komori, H. Kobayashi, Y. Sakai, Y. Hotta, and H. Matsumoto 1990, Science 248: 483-486.

## Targeted replacement of *piggyBac* transposable element.

**Park, H., and C. Zeng.** Department of Biological Sciences, University of Wisconsin – Milwaukee, Milwaukee, WI 53211. Email: zeng@uwm.edu.

Since Rubin and Spradling first developed the use of *P*-elements for transgenesis (Rubin and Spradling, 1982), the *P*-element has become an indispensable tool. Gene tagging, enhancer trapping, gene disruption, chromosome engineering, and inducible gene expression have taken advantage of the mobility of *P*-elements (Ryder and Russel, 2003). However, a *P*-element also has drawbacks, such as its strong bias in insertion sites. For reasons that are not entirely clear, it has a preference for some chromosomal locations (hot spots), but avoids others (cold spots) (Spradling *et al*., 1995).

The *piggyBac* element was discovered in the cabbage looper moth and has proven to be an efficient vector for the transformation of many insect species as well as human and mouse cells (Ding *et al.*, 2005). Because *piggyBac* has a different insertion site preference than that of a *P*-element, it has been used in several large scale gene disruption projects as a complement to *P*-elements (Hacker *et al*., 2003; Bellen *et al*., 2004; Thibault *et al*., 2004).

After a *P*-element excises from a chromosomal location, another *P*-element from a second site frequently transposes into the site of the excised *P*-element. This process is called "*P*-element replacement" (Gonzy-Treboul *et al*., 1995) or "targeted transposition" (Heslip and Hodgetts, 1994; Sepp and Auld, 1999). To date, however, similar replacement between *piggyBac* transposons has not been reported. We tested whether a *piggyBac* transposon, PB[$y^+$] on the 2$^{nd}$ chromosome (Bloomington stock ID16249, originally created by Ring and Garza) (Bellen *et al*., 2004), can efficiently transpose into the site of another *piggyBac* element, RB[$w^+$] e03162 (from Exelixis Collection at the Harvard Medical School), which is located in the second intron of the *slowpoke* gene (Thibault *et al*., 2004). We remobilized PB[$y^+$] in the presence of RB[$w^+$] e03162 using the constitutive {α-1 tubulin:*piggyBac* Transposase} transgene (Parks, 2004). Four out of fifteen crosses produced one or more potential replacements based on the marker phenotype. Two of those lines were molecularly characterized by PCR using one primer specific to PB[$y^+$] and the other from the *slowpoke* genomic region, followed by DNA sequencing. We found that in both cases, the replacement appears to be precise, leaving the local genomic DNA outside the transposon unchanged.

Since thousands of *piggyBac* insertions are widely distributed in the *Drosophila* genome and are available from stock centers (Bellen *et al.*, 2004; Thibault *et al*., 2004), the efficient targeted replacement of *piggyBac* opens up many possibilities for precise genetic manipulations and chromosome engineering. For example, a UAS containing *piggyBac* element could be placed at a desirable genomic position that harbors an existing *piggyBac* element without the UAS sequence.

Similarly, FRT or Lox-P sites can be targeted to certain genomic locations by *piggyBac* replacement. It is likely that the targeted *piggyBac* replacement we reported here could also be achieved in other organisms and mammalian cells, making this powerful genetic tool even more versatile.

References: Bellen, H.J., R.W. Levis, G. Liago *et al*., 2004, Genetics 167(2): 761-81; Ding, S., X. Wu, G. Li, *et al*., 2005, Cell 122: 473-483; Gonzy-Treboul, G., J.A. Lepesant, and U. Deutsch 1995, Genes Dev. 9: 1137-1148; Hacker, U., S. Nysted, M.P. Barmchi *et al*., 2003, Proc. Natl. Acad. Sci. 100: 7720-7725; Heslip, T.R., and R.B. Hodgetts 1994, Genetics 138(4): 1127-35; Parks, A., 2004, Personal communication to FlyBase; Rubin, G.M., and A.C. Spradling 1982, Science 218: 348-353; Ryder, E., and S. Russell 2003, Brief. Funct. Genomic. Proteomic. 2(1): 57-71; Sepp, K.J., and V.J. Auld 1999, Genetics 151(3): 1093-101; Spradling, A.C., D.M. Stern, I. Kiss *et al*., 1995, Proc. Natl. Acad. Sci. 92: 10824-10830; Thibault, S.T., M.A. Singer, and J. Margolis 2004, Nature Genetics 36: 283-287.

## Sets of double balancers to facilitate the genetic combination of major chromosomes in *Drosophila melanogaster.*

**Zeng, Chaoyang.** Department of Biological Sciences, University of Wisconsin Milwaukee, Milwaukee, WI 53211, USA; Email: zeng@uwm.edu.

*Drosophila melanogaster* is a powerful genetic tool for basic and applied scientific research. The unique ability to quickly and precisely manipulate major chromosomes (the X, the 2nd and the 3rd) is facilitated by visible markers and ingeniously invented balancers. The ease of such genetic maneuvers contributed greatly to its early success as a model organism, and the methodology has been an essential tool ever since.

We constructed several sets of stocks (Table 1), each containing two balancers for two different chromosomes, to facilitate the combination of unmarked chromosomes from two separate parental sources. Such combination is often necessary in establishing new fly strains that harbor multiple genetic elements. These strains are used in many experiments such as genetic screens, testing genetic interactions, stable combination of UAS and Gal4 insertions, and generating mitotic mosaics.

Table 1. Double balancers for the major chromosomes.

| Name | Genotype |
|---|---|
| DB1 | y w; numb / CyO {Ras} ; fng / TM3 Sb |
| DB2 | y w; numb / CyO ; fng / TM3 Sb {Ras} |
| DB3 | y w; numb / CyO $y^+$ ; fng / TM3 Sb |
| DB4 | y w; numb / CyO ; fng / TM3 Ser $y^+$ |
| DB5 | FM6 y w B ; Pin / CyO |
| DB6 | FM6 y w B ; Ly / TM3 Sb |

Our approach for combining the second and the third chromosome took advantage of the transgenes that confer dominant phenotypes. These phenotypes are easy to score while exerting little or no additional penalties on the strain's robustness. The balancer CyO P{sevRas1.V12}FK1 originated from Gerry Rubin's lab and contains a *P* element that expresses a hyperactive Ras protein exclusively in the eyes (Fortini *et al*., 1992; Karim *et al*., 1996). The transgene added a strong rough eye phenotype to the frequently used CyO balancer. We abbreviated this balancer as CyO {Ras} in this report. The {Ras} transgene was marked by $ry^+$, without obscuring the $w^+$ marker carried by other transposons. Similarly, {Ras} has been transposed into the third chromosome balancer TM3 Sb to make TM3 Sb {Ras} by others. We also used *numb* and *fng*, recessive lethal alleles of two randomly chosen genes on the second and the third chromosomes, respectively, to

construct the double balancers. Another set of double balancers used CyO $y^+$ and TM3 Ser $y^+$, which most likely contain a *P* element expressing the *yellow* gene (Geyer and Corces, 1987; Mardahl *et al*., 1993). All balancers mentioned above were obtained from the Bloomington Stock Center initially. Figure 1 illustrates a scheme using the double balancers DB1 and DB2 to combine A2, the second chromosome from strain A, and B3, the third chromosome of strain B, into a single stock. By differentiating CyO from CyO {Ras}, and TM3 Sb from TM3 Sb {Ras}, the crosses were efficient and reliable.

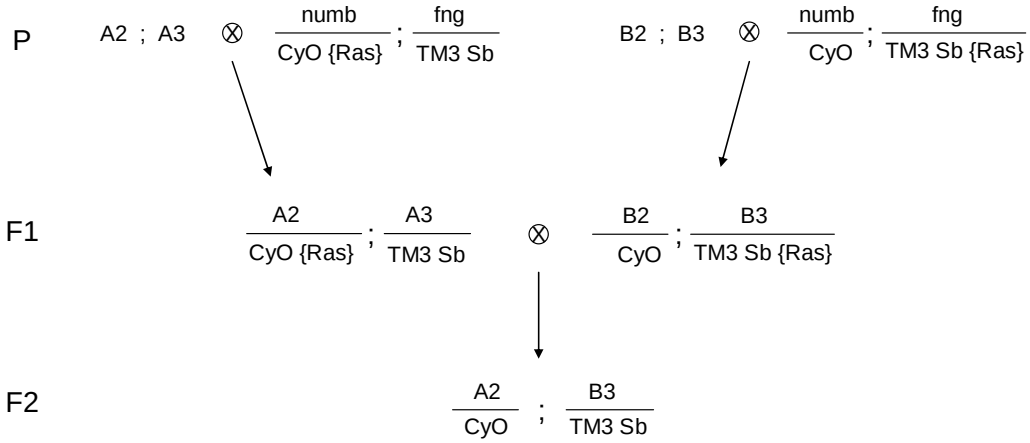


Figure 1. Combining the 2nd chromosome A2 and the 3rd chromosome B3 from strains A and B, respectively. First, cross strain A to DB1 (Table 1) and strain B to DB2. Then, mate the Cy, Sb and rough eye F1s from the two crosses with each other. Finally, select the Cy, Sb and regular eye (not rough) F2s to establish a new stock combining A2 and B3. For combining A3 with B2, the same scheme could be used except A would be crossed to DB2 and B to DB1 first.

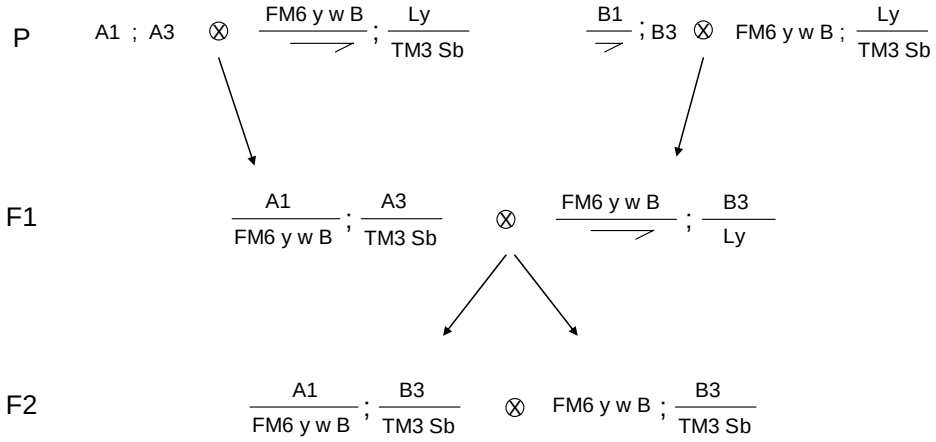


Figure 2. Combining the 1st chromosome A1 and the 3rd chromosome B3 from strains A and B, respectively. First, cross the females from strain A to the DB6 males (Table 1) and the males from strain B to the DB6 females. Then, mate the B, Sb female F1s from the first cross with the B, Ly male F1s from the second cross. Finally, select the heterozygous B and Sb females and the hemizygous B and Sb males in the F2 generation to establish a new stock combining A1 and B3. For combining A3 with B1, the same scheme could be implemented, except that the males from strain A and the females from strain B would be used in the first step.

We also constructed two stocks for double balancing the first and one of the major autosomes (Table 1). Figure 2 presents a scheme for combining A1 and B3 chromosomes into a single strain. The first and the second chromosomes from two parental strains could also be combined into one stock using a scheme similar to the one depicted in Figure 2, but employing stock DB5 instead of DB6.

All the double balancers were in *y w* background as well to further facilitate the manipulation of $w^+$ marked transposons. The double balancers discussed in this report are listed in Table 1 and are available from the author.

References: Fortini, M.E., M.A. Simon, and G.M. Rubin 1992, Nature 355: 559–561; Geyer, P.K., and V.G. Corces 1987, Genes Dev. 1: 996-1004; Karim, F.D., H.C. Chang, M. Therrien, D.A. Wassarman, T. Laverty, and G.M. Rubin 1996, Genetics 143: 315-329; Mardahl, M., R.M. Cripps, R.R. Rinehart, S.I. Bernstein, and G.L. Harris 1993, Dros. Inf. Serv. 72: 141.

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

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

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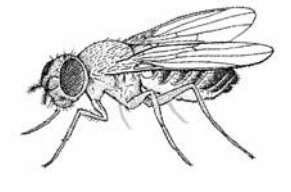

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Teaching Notes


**Ultraviolet (UV) light induced mutations in *Drosophila melanogaster*.**

**Toth, Cynthia L., Jessica L. Heintzelman, and R.C. Woodruff.** Department of Biological Sciences, Bowling Green State University, Bowling Green, OH 43403.

Ultraviolet (UV) light is a potential threat to the integrity of our genetic material. UV is known to cause mutations in prokaryotic and eukaryotic organisms and is a potent inducer of skin cancer in humans (Friedberg *et al*., 1995; Greaves, 2000). Yet, UV does not penetrate tissues very far, and this makes it difficult to determine if UV can induce mutations in germ cells of higher organisms.

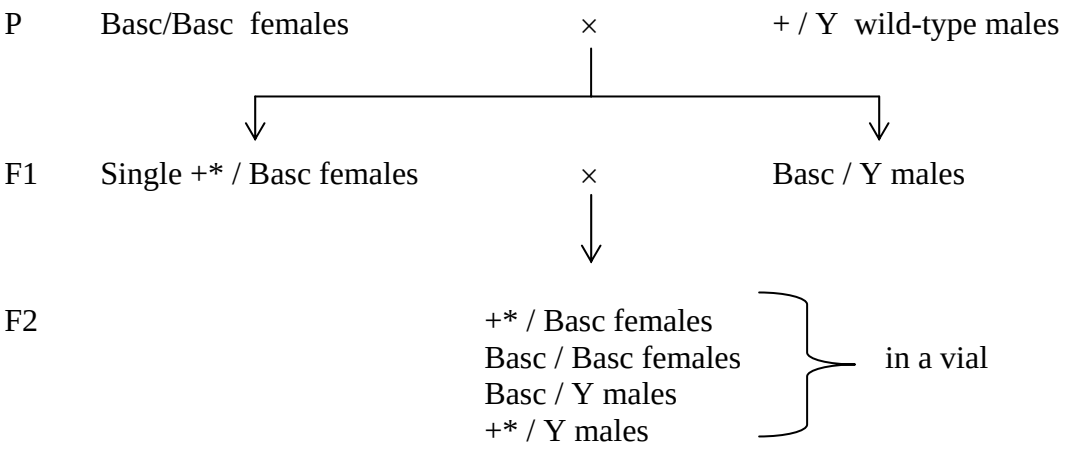
The early observations of the ability of UV to induce germ-line mutations in *Drosophila* were only successful when very young males, before cuticle darkening, were squashed flat to expose their gonads (Mackenzie and Muller, 1940). Even then, only small increases in gene mutations were observed with UV treatments.

The objective of this exercise is to test the ability of UV to induce recessive sex-linked lethal mutations by placing *Drosophila melanogaster* males on a UV transilluminator that is in common use in molecular biology laboratories to view DNA. The rate of mutation in these UV-treated males will be compared to the rate in sibling males that are untreated with UV.

One- to two-day-old *D. melanogaster* wild-type males were etherized and placed directly, abdomens down, on a UV transilluminator. Each male was held down by one or more glass microscope cover slips and was treated with UV for 10 to 60 minutes to determine if UV is toxic to *Drosophila*. In some runs, only the gonad area of the abdomen was exposed to UV by placing a glass cover slip under the flies except for the abdomen. This was done to reduce exposure of the brain and other vital organs to the possible toxic effects of UV. After estimating toxic levels of UV exposure,

males were treated for 11 minutes (whole body) or 45 minutes (gonads only) with UV and were screened for new recessive sex-linked lethal mutations. Males that were not exposed to UV were used as controls.

The UV-treated and control males were mated individually, as shown in the Basc mating scheme below. In this mating scheme, single wild-type males (Canton-S) that do not contain pre-existing X-linked lethals were mated with two or three virgin Basc/Basc females, single F1 heterozygous females were mated with one or two sibling Basc/Y males, and F2 flies were screened for new recessive sex-linked lethal mutations. The absence of +* /Y males (round, red eyes) in the F2 vials means that a new recessive sex-linked lethal mutation has been recovered. Presumptive lethals can be confirmed by mating F2 +* / Basc females with F2 Basc / Y sibling males. A lethal was declared when at least 20 Basc/Y F2 or F3 males and no +* /Y males are recovered. In this mating scheme, B = Bar eye dominant mutation, a = white-apricot eye recessive mutation, sc = multiple X-chromosome inversions, + = wild-type X chromosome from a Canton-S stock, * = UV-treated X chromosome, and Y = Y chromosome in males. The multiple X-chromosome inversions cause the elimination of recombination gametes in the heterozygous F1 females due to duplications and deficiencies of chromosomal material and to acentric and dicentric chromosomes (see Klug *et al*., 2007, for a discussion of this topic). This loss of gametes with recombinant X chromosomes means that a new lethal mutation on the wild-type X chromosome will not be recombined onto the Basc chromosome and be missed as a new mutation. Single F1 +* / Basc females were used in this mating scheme so that a single X-chromosome is tested in each F2 vial. For more details on the Basc mating scheme for recessive sex-linked lethal mutations with *D. melanogaste*r (see Mason *et al*., 1985, 1987).




Toxicity caused by UV was determined by the percentage of males that died after treatments in one day. The toxicity results of UV treatments are shown in Table 1.

From these results, it is clear that long-time whole-body or gonad UV treatment of *D. melanogaster* males is toxic. This, however, does not mean that the UV is reaching the germ cells of these males. UV could be toxic in these *Drosophila* males because it is causing damage to DNA, proteins, and other components of somatic cells, without reaching the gonads. Hence, the males also need to be tested for the ability of UV to induce genetic damage in germ cells.

Hence, we treated *D. melanogaster* male gonads with 11 or 45 minutes of UV and tested for recessive sex-linked lethal mutations using the Basc mating scheme shown above. One lethal mutation was also identified in sibling wild-type males that were not treated with UV (the controls). The results are shown in Table 2.

Table 1. Toxicity Tests.

| Minutes of UV Treatment | Number of Flies Treated | Number of Flies Dead After One Day | % of Flies Dead After One Day |
|---|---|---|---|
| Whole Body Treatments with UV: | | | |
| 0 | 31 | 1 | 3 |
| 10 | 6 | 2 | 33 |
| 11 | 53 | 5 | 9 |
| 12 | 21 | 7 | 33 |
| 13 | 25 | 9 | 36 |
| 14 | 44 | 5 | 11 |
| 15 | 5 | 4 | 80 |
| 20 | 5 | 5 | 100 |
| 25 | 6 | 6 | 100 |
| Gonad Treatments with UV | | | |
| 20 | 31 | 16 | 52 |
| 25 | 37 | 19 | 51 |
| 30 | 33 | 14 | 42 |
| 45 | 28 | 23 | 82 |
| 60 | 25 | 24 | 96 |

Table 2. Recessive Sex-Linked Lethal Mutations in *Drosophila melanogaster*.

| | Lethals | Total Chromosomes Scored | % Lethals |
|---|---|---|---|
| Controls (no UV) | 1 | 627 | 0.2 |
| UV Treatments | | | |
| 11 min whole body | 2 | 340 | 0.6[a] |
| 45 min gonads only | 2 | 79 | 2.5[b] |
| Total | 4 | 419 | 1.0[c] |

[a]Fisher exact P = 0.28.
[b]Fisher exact P = 0.04
[c]Fisher exact P = 0.16

The spontaneous mutation rate is similar to the rate reported in the literature (about one new recessive sex-linked lethal mutation in 1000 X chromosomes) (Mason *et al.*, 1985). The rate of UV-induced mutations for the gonad-only treatment is significantly higher than the spontaneous controls ($P$ = 0.04). The UV rate is not significantly higher than the controls, however, for the whole-body treatments or for the total of the two treatments. These results support UV as a germ-cell mutagen in *D. melanogaster* and support the use of a DNA transilluminator as an appropriate source of mutagenic UV.

A class discussion of the results of these crosses could include the following topics: 1) Would one expect UV to cause mutations in gametes of humans? 2) Go to the literature and determine if UV has been observed to cause chromosome breakage and nondisjunction in *Drosophila*. 3) What is the main cause of DNA damage by UV? 4) Would increased skin pigmentation reduce genetics damage by UV?

References: Friedberg, E.C., G.C. Walker, and W. Siede 1995, *DNA Repair and Mutagenesis*. ASA Press, Washington, D.C.; Greaves, M., 2000, *Cancer The Evolutionary Legacy*. Oxford University Press, Oxford; Klug, Cummings, and Spencer 2007, *Essentials of Genetics*; Mackenzie, K., and H.J. Muller 1940, Proc. Royal Soc. B 129: 491-517; Mason, J.M., R. Valencia, R.C. Woodruff, and S. Zimmering 1985, Environ. Mutagenesis 7: 663-676; Mason, J.M., C.S. Aaron, W.R. Lee, P.D. Smith, A. Thakar, R. Valencia, R.C. Woodruff, F.E. Wurgler, and S. Zimmering 1987, Mutation Research 189: 93-102.

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**Identification of selfish genetic elements in natural populations of *Drosophila melanogaster*. I. P DNA elements.**

**Robinson, Bethany, Natalie Wiseman, and R.C. Woodruff.** Department of Biological Sciences, Bowling Green State University, Bowling Green, OH 43403.

All organisms have large pieces of DNA that have, or have had, the ability to move to new chromosomal locations. These transposable DNA elements make up a large proportion of the genomes of higher organisms, including 46% of the human genome and 5-15% of the *Drosophila melanogaster* genome (Kidwell, 2002; Burt and Trivers, 2006; Drosophila 12 Consortium, 2007). This high a proportion of genomes that could be transposable is surprising, since DNA element movements (insertion and excision events) often cause deleterious mutations and recombination between elements gives rise to chromosome rearrangements, including inversions, deficiencies, duplications, and translocations (Mackay, 1987; Babushok and Kazazian, 2007).

If all of the 4,000,000 transposable DNA elements in humans were active, even if they moved at a very low rate, these transpositions would cause extensive genetic damage that would reduce the health and fitness of humans. Hence, it is not surprising that the vast majority of transposable DNA elements in humans and other higher organisms are nonfunctional, mainly due to nucleotide mutations and deletions within the elements. For example, in humans only about 80 to 100 transposable DNA elements are active (Broushok and Kazazian, 2007). Still, as high as 1 in 10 mutations in humans have a novel insertion, and these DNA elements cause mutations that lead to cancer (Miki *et al*., 1992; Biemont and Vieira, 2006; Burt and Trivers, 2006). The impact of transposable DNA elements on genetic damage in the model organism *D. melanogaster* is even higher (Keightley, 1996).

In *D. melanogaster* there are 93 families of transposable DNA elements, and excisions and insertions of these elements are common inducers of mutation and sterility (Kaminker *et al*., 2002). Some inversions in natural populations of *D. melanogaster* and other *Drosophila* species are also caused by exchanges between DNA elements (Caceres *et al*., 1999). In fact, one of the first isolated visible mutations in *D. melanogaster*, the sex-linked, white-eyed recessive mutation identified by Thomas Hunt Morgan (Morgan, 1910), was caused by a *Doc* DNA element insertion in the *white* gene (Driver *et al*., 1989). One of the classical mutations studied by Mendel, wrinkled pea seeds, was also caused by a DNA insertion event (Bhattacharyya *et al*., 1990).

The first transposable DNA element identified at the genetic and molecular level in *D. melanogaster* was the P DNA element (Bingham *et al*., 1982). The P name for this element came from the observation that parental (P) males that contain the P DNA elements when crossed with maternal (M) females, which do not have active P DNA elements, give rise to a high frequency of P DNA movement in offspring, leading to germ cell death and sterility and to gene and chromosomal mutations in progeny (Henderson, Woodruff and Thompson, 1978; Kidwell and Novy, 1979). This syndrome of genetic damage events caused by P DNA element movement is called hybrid dysgenesis (Kidwell, Kidwell and Sved, 1977). The reciprocal cross of P females with M males, and crosses of M × M or P × P flies does not cause hybrid dysgenesis.

The molecular structure of the P DNA element is given in Figure 1. The functional P DNA element is 2,907 base-pairs long and codes for terminal repeats and a transposase that function in transpositions. There are also truncated P elements that can respond to transposase from other P

DNA elements. The P DNA element is active in germ cells, but not somatic cells, due to differences in splicing out of introns in the two cell types (Laski *et al*., 1986). The P DNA element was also used as the vector for the first gene therapy experiment in eukaryotes (Rubin and Spradling, 1982; Spradling and Rubin, 1982).

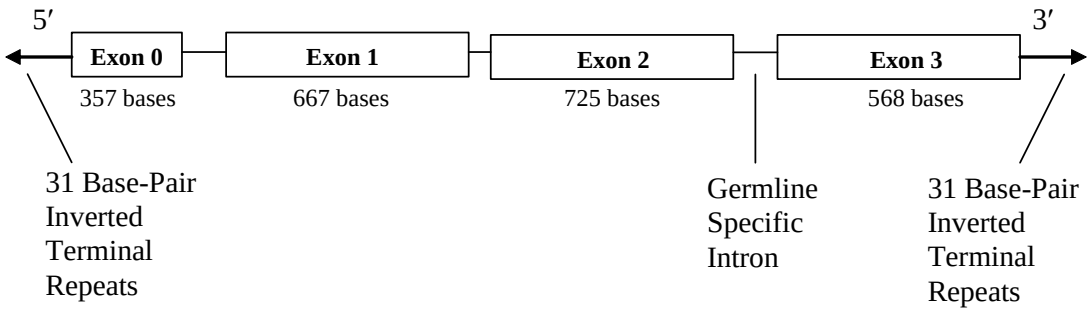



Figure 1. Molecular structure of the P DNA element of *Drosophila melanogaster*.

It is the objective of this teaching exercise to attempt to identify active P DNA elements from natural populations of *D. melanogaster* by screening for P DNA element induced gonadal dysgenesis (atrophied ovaries) in F1 females from crosses of males from nature to laboratory females that do not have active P DNA elements (M line). Woodruff and Thompson (2001) have discussed how to identify active *mariner* DNA elements in natural populations of *D. simulans* by screening for mosaic eye spots caused by the ability of active *mariner* elements from nature to excise a *mariner* insert in the *white* locus.

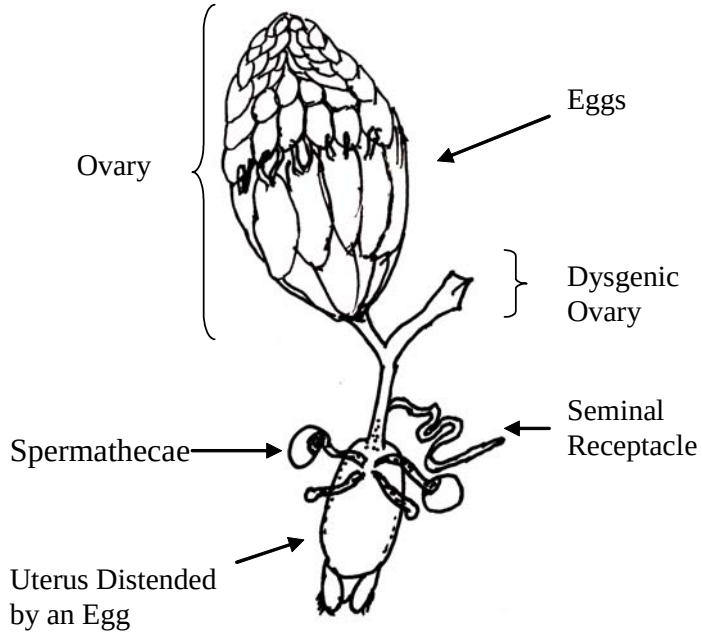



Figure 2. Female reproductive system of *Drosophila melanogaster* with one normal ovary and one dysgenic ovary (atrophied gonad) caused by P DNA element induced germ-cell death. Spermathecae and seminal receptacle are storage organs for sperm. Modified from Miller (1965).

To identify gonadal dysgenesis due to P DNA element induced germ-cell death and atrophy of female gonads, we double mated single *D. melanogaster* males collected in two Northwest Ohio sites in 2007 (Perrysburg, Lucas County, and Jeffers Orchard in Grand Rapid, Wood County) to P DNA element control females (Harwich-w, P line with the white-eyed mutation and active P DNA elements and *y* $w^{67c23}$, M line with yellow-body and white-eyed mutations and without active P DNA

elements). The P DNA element control lines were obtained from Dr. Michael Simmons at the University of Minnesota. These crosses were performed at 29°C, which gives the highest rate of P DNA element movement (Kidwell *et al*., 1977).

2-3 Harwich-w (P) females × Single males from nature × 2-3 *y* $w^{67c23}$ (M) females

↓ ↓

The F1 females were collected, aged for four days at 29°C, and dissected in 0.7% NaCl. The paired gonads of each F1 female were scored for normal or atrophied gonads. Each female gave two data points that were normal/normal, normal/atrophied, or atrophied/atrophied. Figure 2 is a drawing of a paired female gonad, with one being normal in structure with developing eggs and the other atrophied with no eggs. A similar mating scheme has been used to screen for P DNA induced gonadal dysgenesis in crosses with *D. melanogaster* males from Australia (Boussy *et al*., 1998; Itoh *et al*., 1999).

The following crosses were also performed at 29°C to verify that the two control lines were true P and M lines.

2-3 *y* $w^{67c23}$ M females × Harwich-w P males

↓

Expected to show high frequency of gonadal dysgenesis in F1 females

2-3 Harwich-w P females × *y* $w^{67c23}$ M males

↓

Expected to show no, or low, frequency of gonadal dysgenesis in F1 females

**Results**

1. Control Crosses:

As predicted, the crosses of *y* $w^{67c23}$ (M) females with Harwich-w (P) males gave 40 atrophied gonads and no normal gonads among 20 F1 female progeny, and crosses of Harwich-w (P) females with *y* $w^{67c23}$ (M) males gave 40 normal ovaries among 20 F1 female progeny. These results confirm that Harwich-w is a P line with functional P DNA elements and that *y* $w^{67c23}$ is an M line with no functional P DNA elements. Hence, these two control lines can be used to identify gonadal dysgenesis in crosses with natural population males, as shown below.

2. Crosses with Natural Population Males:

The F1 gonadal dysgenesis results from double crosses of Perrysburg and Jeffers Orchard males with *y* $w^{67c23}$ M females and with Harwich-w P females are shown in Table 1 and Figure 3. A total of 20-25 males from each of the populations were each double mated with the two control females, but only eight of the males from Perrysburg and seven from Jeffers Orchard produced

progeny from both crosses. A significant increase in gonadal dysgenesis caused by P DNA element induced germ-cell death and subsequent gonad atrophy was observed in both natural populations.

In the Perrysburg sample, total atrophy in crosses with M females (15.8%) was significantly higher ($P < 0.0001$) than in crosses with P females (0.9%). There were two males (Perrysburg 6 and 7) that had significant increases in gonadal dysgenesis in crosses with M females compared to crosses with P females (33% to 0%, and 39% to 0%, $P < 0.01$). In addition, one male (Perrysburg 8) had a high frequency of gonadal dysgenesis in the cross with M females as compared to P females (39% to 0%), but the increase was not significant because of the low number of tested flies in the latter cross (we were only able to score two F1 progeny due to low fecundity).

Table 1. Gonadal dysgenesis in F1 females.

| Natural Population Males | Gonads from Crosses with *y* $w^{67c23}$ (M) Females | | | Gonads from Crosses with Harwich-w (P) Females | | |
|---|---|---|---|---|---|---|
| Perrysburg, Ohio | Atrophied | Normal | % | Atrophied | Normal | % |
| 1 | 0 | 40 | 0 | 2 | 38 | 5 |
| 2 | 1 | 25 | 4 | 0 | 18 | 0 |
| 3 | 2 | 18 | 10 | 0 | 36 | 0 |
| 4 | 0 | 40 | 0 | 0 | 40 | 0 |
| 5 | 2 | 36 | 5 | 0 | 26 | 0 |
| 6 | 16 | 32 | 33 | 0 | 16 | 0 |
| 7 | 7 | 11 | 39 | 0 | 40 | 0 |
| 8 | 14 | 22 | 39 | 0 | 4 | 0 |
| Total | 42 | 224 | 15.8[a] | 2 | 218 | 0.9[a] |
| Jeffery Orchard | Atrophied | Normal | % | Atrophied | Normal | % |
| 1 | 14 | 10 | 58 | 0 | 36 | 0 |
| 2 | 13 | 26 | 33 | 0 | 40 | 0 |
| 3 | 3 | 37 | 8 | 0 | 40 | 0 |
| 4 | 7 | 17 | 29 | 0 | 40 | 0 |
| 5 | 0 | 14 | 0 | 0 | 40 | 0 |
| 6 | 2 | 38 | 5 | 2 | 38 | 5 |
| 7 | 3 | 37 | 8 | 1 | 39 | 3 |
| Total | 42 | 179 | 19.0[b] | 3 | 273 | 1.1[b] |

[a]$P < 0.0001$, [b]$P < 0.0001$

In the Jeffers Orchard, Grand Rapid sample, total atrophy in crosses with M females (19.0%) was significantly higher ($P < 0.0001$) than in crosses with P females (1.1%). There were three males (Jeffer Orchard 1, 2 and 4) that had significant increases in gonadal dysgenesis in crosses with M females compared to crosses with P females (58% to 0%, 33% to 0%, and 29% to 0%, $P < 0.01$).

These gonadal dysgenesis results indicate that the two Northwest Ohio populations of *D. melanogaster* had active P DNA elements. The frequencies of males with active P DNA elements in the two populations (25% and 43%) and the frequencies of gonadal dysgenesis in the P DNA active lines (15.8% and 19%) are similar to other natural populations (Kidwell, 1994).

A class discussion of the results of these crosses could include the following topics: 1) How could P DNA elements that cause deleterious mutations and chromosomal rearrangements be maintained in natural populations? Would you expect organisms that contain these elements to be selected against? 2) Is there evidence that DNA elements are ever beneficial? You might read about the enzymes that are involved in the movement of segments of DNA to make mature antibody genes in humans (Burt and Trivers, 2006). 3) How could P DNA elements cause germ-cell death? You

might see the photographs of P DNA induced chromosome damage in germ cells of *D. melanogaster* in Henderson, Woodruff and Thompson (1978). 4) Discuss the possible evolutionary implications of DNA elements, such as P DNA elements, that move in germ cells but not in somatic cells. 5) Ask students to go to http://www.med.upenn.edu/genetics/kazazianlab_home.shtml for a list of DNA insertion mutations in humans.

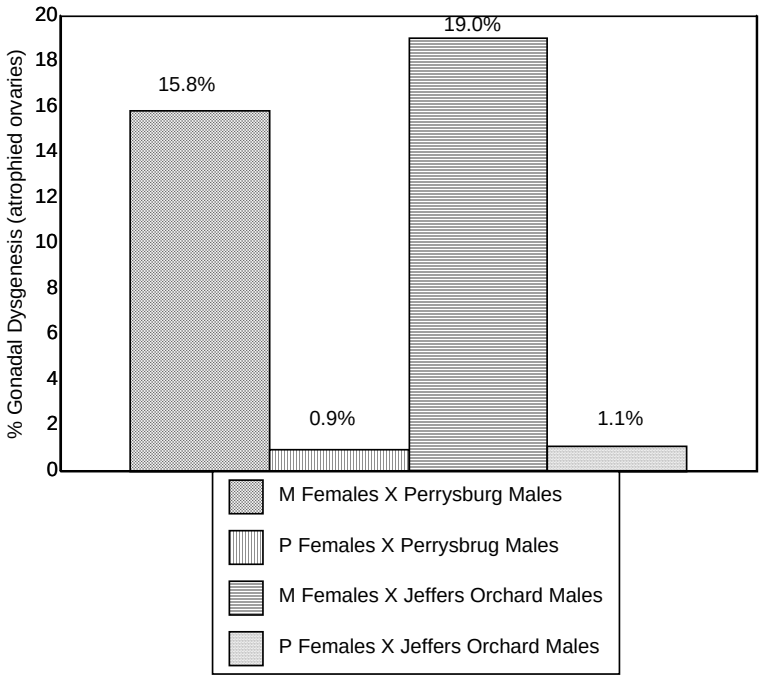



Figure 3. Gonadal dysgenesis levels in crosses of *Drosophila melanogaster* natural population males with females that do or do not contain functional P DNA elements.

References: Babushok, D.V., and H.H. Kazazian, Jr. 2007, Human Mutation 28: 527-539; Bhattacharyya M.K., A.M. Smith, T.H.N. Ellis, and C. Martin 1990, Cell 60: 115-122; Biemont, C., and C. Vieira 2006, Nature 443: 521-524; Bingham, P.M., M.G. Kidwell, and G.M. Rubin 1982, Cell 29: 995-1004; Boussy, I.A., M. Itoh, D. Rand, and R.C. Woodruff 1998, Genetica 104: 45-57; Brouha, B. *et al*., 2003, Proc. Nat. Acad. Sci. USA 100: 5280-5285; Driver, A. *et al*., 1989, Molecular General Genetics 220: 49-52; Burt, A., and R. Trivers 2006, *Genes in Conflict*, Cambridge, Massachusetts: The Belknap Press of Harvard University Press; Caceres *et al*. 1999, Science 285: 415-418; Drosophila 12 Genome Consortium 2007, Nature 450: 203-218; Henderson, S.A., R.C. Woodruff, and J.N. Thompson, Jr. 1978, Genetics 88: 93-107; Itoh, M., R.C. Woodruff, M.A. Leone, and I.A. Boussy 1999, Genetica 106: 231-245; Keightley, P.D., 1996, Genetics 144: 1993-1999; Kidwell, M.G., 1994, J. Heredity 85: 339-346; Kidwell, M.G., 2002, Genetica 115: 49-63; Kidwell, M.G., J.F. Kidwell, and J.A. Sved 1977, Genetics 86: 813-833; Laski, F.A., D.C. Rio, and G.M. Rubin 1986, Cell 44: 7-19; Mackay, T.F.C., 1987, Genetical Research 49: 225-234; Miller, A., 1965, The internal anatomy and histology of the imago of *Drosophila melanogaster*, In: *Biology of* Drosophila (Demerec, M., Ed.) Hafner Publishing Company, New York; Morgan, T.H., 1910, Science 32: 120-122; Rubin, G.M., and A.C. Spradling 1982, Science 218: 348-353; Spradling, A.C., and G.M. Rubin 1982, Science 218: 341-347; Woodruff, R.C., and J.N. Thompson, Jr. 2001, Dros. Inf. Serv. 84: 213-215.

**Changes in linkage disequilibrium over time in *Drosophila melanogaster* for two sex-linked loci.**

**Onasch, Katherine D., and R.C. Woodruff.** Department of Biological Sciences, Bowling Green State University, Bowling Green, OH 43403.

1. Linkage Disequilibrium Definition and Theory:

If a diploid population has two linked genes with two alleles each (*A*, *a* and *B*, *b*), the genes can occur as four possible haplotypes AB, ab, Ab and aB, with haplotypes defined as multilocus genotypes of a chromosome or chromosome region and here underlined. If this population also fits the Hardy-Weinberg assumptions (large population size, no mutations, no selection for or against any of the four haplotypes, no migration into or out of the population, and random matings among individuals with the four haplotypes) the frequencies of the four haplotypes can be estimated from the expected frequencies of alleles at the two loci and from random matings of all combinations of alleles as shown below. Note that *p* is the frequency of the *A* allele, *q* is the frequency of the *a* allele, *s* is the frequency of the *B* allele, and *t* is the frequency of the *b* allele.

| | *A(p)* | *a(q)* |
|---|---|---|
| *B(s)* | AB(ps) | aB(qs) |
| *b(t)* | Ab(pt) | ab(qt) |

With the Hardy-Weinberg assumptions, it is then expected that

$$(AB)(ab) - (aB)(Ab) = 0 \qquad (1)$$

This is called being in linkage equilibrium, because transmission of alleles at the *A* locus are independent of the alleles at the *B* locus and the fitness of all haplotypes are equal (Hedrick, 2005).

As an example, if the frequencies of alleles are $p(A) = 0.3$, $q(a) = 0.7$, $s(B) = 0.4$ and $t(b) = 0.6$ and the frequencies of haplotypes are AB = 0.12, ab = 0.42, aB = 0.28, and Ab = 0.18 in a population of bullfrogs in Lake of the Pines in East Texas, and this population fits the Hardy-Weinberg assumptions, then (AB)(ab) - (aB)(Ab) should be equal to 0. Is this true?

$$\{[(0.3)(0.4)] \times [(0.7)(0.6)]\} - \{[(0.7)(0.4)] \times [(0.3)(0.6)]\} = 0.0504 - 0.0504 = 0$$

Hence this population is at linkage equilibrium, with the frequencies of haplotypes being equal to the frequencies of the two alleles in each haplotype (for example, AB = [(0.3)(0.4)] = 0.12, etc.).

There are genetic and demographic possibilities, however, where the haplotypes are not associated randomly. This can be due to: 1) some combination of alleles at the two loci give haplotypes that are advantageous and increase the fitness of individuals (average number of offspring); 2) some haplotypes have randomly drifted to a higher or lower frequency than the Hardy-Weinberg expectation (genetic drift); 3) frequencies of some haplotypes have increased due to migration from another population or migration out of the population; 4) a haplotype is closely linked to a beneficial gene; 5) new recurrent mutations, or premeiotic clusters of mutations (Woodruff and Thompson, 2002) have increased the frequency of a haplotype(s); or 5) an interaction of one or more of 1-4.

In these population situations, where one or more of the Hardy-Weinberg assumptions are not valid, the excess of some haplotypes above the Hardy-Weinberg expectation, and concomitant decrease of some haplotypes below the Hardy-Weinberg expectation, is called linkage disequilibrium (the nonrandom association of alleles at different loci into gametes) (Lewontin and Kojima, 1960). A

more inclusive term, which includes linked and unlinked loci, is gametic disequilibrium (see discussions of this topic in Halliburton, 2004; Hedrick, 2005; Hartl and Clark, 2006). With linkage disequilibrium:

$$(\underline{AB})(\underline{ab}) - (\underline{aB})(\underline{Ab}) \neq 0 \qquad (2)$$

Historically the amount of linkage disequilibrium is called D, because this is the amount of deviation from a random association of alleles at different loci, and

$$D = (\underline{AB})(\underline{ab}) - (\underline{aB})(\underline{Ab}) \qquad (3)$$

Using this equation for D, in our previous example where $\underline{AB}$ = (0.3)(0.4) = 0.12, $\underline{ab}$ = (0.7)(0.6) = 0.42, $\underline{aB}$ = (0.7)(0.4) = 0.28 and $\underline{Ab}$ = (0.3)(0.6) = 0.18:

$$D = (\underline{AB})(\underline{ab}) - (\underline{aB})(\underline{Ab})$$
$$D = (0.12)(0.42) - (0.28)(0.18)$$
$$D = 0.0504 - 0.0504$$
$$D = 0$$

This bullfrog population in East Texas has no linkage disequilibrium for the four haplotypes.

Now let us look at a population of water snakes on the South Bass Island of Lake Erie in Ohio, where $\underline{AB}$ = 0.47, $\underline{ab}$ = 0.47, $\underline{aB}$ = 0.03 and $\underline{Ab}$ = 0.03. Does this population show linkage disequilibrium for the $\underline{AB}$, $\underline{ab}$, $\underline{aB}$ and $\underline{Ab}$ haplotypes?

$$D = (\underline{AB})(\underline{ab}) - (\underline{aB})(\underline{Ab})$$
$$D = (0.47)(0.47) - (0.03)(0.03)$$
$$D = (0.2209) - (0.0009)$$
$$D = 0.22$$

In addition, since AB = (p)(s) + D; ab = (q)(t) + D; Ab = (p)(t) – D; and aB = (q)(s) – D, the D value can also be determined from:

$$D = AB - (p)(s) \qquad (4)$$

In the above example, D = 0.47 – (0.5)(0.5) = 0.22.

Hence, this water-snake population is in linkage disequilibrium for these four haplotypes. Since water snakes have been observed to swim between Ohio and South Bass Island, one possibility is that linkage disequilibrium was caused by migration.

It should be noted that D can range from + 0.25 to - 0.25. This can be seen from the two possible extremes of haplotype frequencies:

AB = 0.5, ab = 0.5, aB = 0 and Ab = 0 or AB = 0, ab = 0, aB = 0.5 and Ab = 0.5

$$D = (\underline{AB})(\underline{ab}) - (\underline{aB})(\underline{Ab}) \qquad D = (\underline{AB})(\underline{ab}) - (\underline{aB})(\underline{Ab})$$
$$D = (\underline{0.5})(\underline{0.5}) - (\underline{0})(\underline{0}) \qquad D = (\underline{0})(\underline{0}) - (\underline{0.5})(\underline{0.5})$$
$$D = + 0.25 \qquad D = - 0.25$$

See Hedrick (1987, 2005) for other measures of linkage disequilibrium.

2. Expected Changes in Linkage Disequilibrium (D) Over Time if a Population is in Hardy-Weinberg Equilibrium:

If it is assumed that the water-snake population of South Bass Island no longer has migration of snakes from the mainland of Ohio, that the four haplotypes ($\underline{AB}$, $\underline{ab}$, $\underline{Ab}$ and $\underline{aB}$) are neutral in affecting fitness, and that the population fits the other Hardy-Weinberg assumptions, what happens to the previously discussed D value of 0.22 over time? What is the rate of change in D per generation? How many generations would it take for D to be reduced by one-half or to a low level such as 0.01?

Since we previously assumed that the genes of the *A* locus and the *B* locus are linked to the same chromosome in this water-snake population, the reduction in D from 0.22 (call it $D_o$) to zero (linkage equilibrium) will occur over generations (t) based on the recombination rate (r) between the two genes *A* and *B*. In one generation, $D_o$ will be reduced by a value of 1 – r, such that,

$$D_1 = (1 - r)^1 D_0 \quad (5)$$

And for any generation t: $$D_t = (1 - r)^t D_o \quad (6)$$

For example, the value of $D_{50}$ can be determined after 50 generations (t = 50) if the beginning $D_o$ value is known and the recombination frequency (r) between loci *A* and *B* is known. Figure 1 shows that the reduction in D from 0.22 is faster for higher frequencies of recombination (r) between genes. Note that an r value of 0.5 would imply that the two loci are unlinked. The D values in Figure 1, and in Table 1 and Figure 2, are from the Mathematica program.

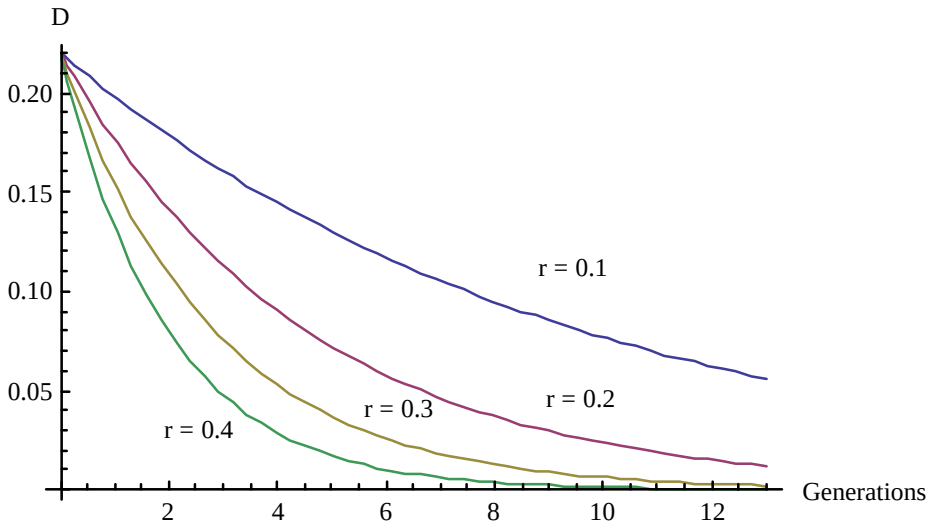



Figure 1. Expected rate of decay of linkage dis-equilibrium (D) over generations with different recombination (r) frequent-cies between two auto-somal linked genes in an organism where recombi-nation occurs in females and males.

One way to show that equation (5) is correct (D drops by 1 – r each generation) is to determine what happens to D in one generation in a population of known haplotype frequencies and recombination frequency, $D_o$ and r, and to compare this D value with the result from equation (4). For example, let us assume that the water-snake population of South Bass Island has haplotype frequencies of AB = 0.47, ab = 0.47, aB = 0.03 and Ab = 0.03 and, hence, $D_o = 0.22$. In addition, it will be assumed that the recombination frequency between the *A* and *B* loci is 20% (r = 0.2). This means that in A B/a b snakes, because of recombination at meiosis, the expected gametes are 47% A B, 47% a b, 3% A b, and 3% a B. If this water-snake population fits the Hardy-Weinberg assumptions, including no migration, the expected frequencies of haplotypes in progeny can be determined from random matings between males and females as shown below. In this table the proportion of possible diploid progeny are shown for each possible union of gametes (eggs and sperm) and are numbered from 1 to 16.

| gametes | AB (0.47) | ab (0.47) | Ab (0.03) | aB (0.03) |
|---|---|---|---|---|
| AB (0.47) | 1 AB/AB (0.2209) | 2 AB/ab (0.2209) | 3 AB/Ab (0.0141) | 4 AB/aB (0.0141) |
| ab (0.47) | 5 ab/AB (0.2209) | 6 ab/ab (0.2209) | 7 ab/Ab (0.0141) | 8 ab/aB (0.0141) |
| Ab (0.03) | 9 Ab/AB (0.0141) | 10 Ab/ab (0.0141) | 11 Ab/Ab (0.0009) | 12 Ab/aB (0.0009) |
| aB (0.03) | 13 aB/AB (0.0141) | 14 aB/ab (0.0141) | 15 aB/Ab (0.0009) | 16 aB/aB (0.0009) |

Based on r = 0.2 for the *A* and *B* loci, one can now determine the expected frequency of AB gametes among each of the 16 possible diploid progeny and the total AB frequency from all possible progeny. Note that recombination is assumed to occur in both sexes. Since AB = ab, one can also determine the frequency of ab, and, since (Ab + aB) = 1 – (AB + ab) and Ab = aB, one can then determine the frequencies of Ab and aB.

From cross results in the above table, the expected frequencies of AB gametes (haplotypes) in the next generation are as follows (details of how frequencies were determined are also given):

Cross results 1 = 0.2209 (all gametes are AB)
Cross results 2 = 0.08836 (0.4 × 0.2209 or ½ nonrecombinants × 0.2209)
Cross results 3 = 0.00705 [(0.4 × 0.0141) + (0.1 × 0.0141)] or [(½ nonrecombinants × 0.0141) + (½ recombinants × 0.0141)]
Cross results 4 = 0.00705 [(0.4 × 0.0141) + (0.1 × 0.0141)]
Cross results 5 = 0.08836 (0.4 × 0.2209)
Cross results 6 = 0 (no AB gametes expected)
Cross results 7 = 0
Cross results 8 = 0
Cross results 9 = 0.00705 [(0.4 × 0.0141) + (0.1 × 0.0141)]
Cross results 10 = 0
Cross results 11 = 0
Cross results 12 = 0.00009 (0.1 × 0.0009)
Cross results 13 = 0.00705 [(0.4 × 0.0141) + (0.1 × 0.0141)]
Cross results 14 = 0
Cross results 15 = 0.00009 (0.1 × 0.0009)
Cross results 16 = 0
Total frequency of AB (1 - 16) = 0.426

Hence, the frequency of AB = 0.426, ab = 0.426, Ab = 0.074, and aB = 0.074. Using these values and equation (3):

$D_1 = (AB)(ab) - (aB)(Ab)$
$D_1 = (0.426)(0.426) - (0.074)(0.074)$
$D_1 = (0.181476) - (0.005476)$
$D_1 = 0.176$

Is this the same $D_1$ value as obtained from equation (5): $D_1 = (1 - r)D_o$? Yes.

$D_1 = (1 - 0.2)0.22$
$D_1 = 0.176$

Hence, the two equations (3) and (5) give the same D results. This means that equation (6) is also correct for determining the value of D for any number of generations.

$$D_t = (1 - r)^t D_o$$

One can also determine how many generations for D to be reduced by one-half or to 0.01 by rearranging equation (6) as follows:

$$t = \frac{\ln \frac{D_t}{D_o}}{\ln(1 - r)} \qquad (7)$$

Hence, for the water-snake population, where $D_o$ is 0.22 and r is 0.2, the number of generations to reduce D by one-half and to 0.01 is:

$$t = \frac{\ln \frac{0.11}{0.22}}{\ln(1 - 0.2)} = 3.11 \quad \text{and} \quad t = \frac{\ln \frac{0.01}{0.22}}{\ln(1 - 0.2)} = 13.85$$

3. Using Two Sex-Linked Loci to Model Changes in Linkage Disequilibrium (D) Over Time in *Drosophila melanogaster*:

The experimental objective of this exercise is to model the change in linkage disequilibrium (D) over time using two visible mutations in *D. melanogaster*. One problem with using recessive autosomal or dominant autosomal alleles of two linked genes is that it is not possible to identify all alleles and, therefore, one cannot determine the frequency of haplotypes each generation. For example, using the wild-type alleles $a^+$ and $b^+$ and their mutant alleles *a* and *b*, one cannot differentiate between the genotypes $\underline{a^+\ b}$ / $\underline{a\ b}$ and $\underline{a^+\ b}$ / $\underline{a^+\ b}$; they both are $a^+$ and *b* in phenotypes, but in the former there is one $a^+$ allele and in the latter there are two $a^+$ alleles. The same problem also occurs for dominant mutations (for example, $\underline{A\ B^+}$ / $\underline{A^+\ B^+}$ has the same phenotype as $\underline{A\ B^+}$ / $\underline{A\ B^+}$). One way to get around this problem is to use protein electrophoresis of allozymes, where one can distinguish among homozygotes and heterozygotes. For example, for the alcohol dehydrogenase locus of *D. melanogaster*, $Adh^F$ / $Adh^F$, $Adh^S$ / $Adh^S$, and $Adh^F$ / $Adh^S$ flies give different banding patterns on starch or cellulose actetate electrophoretic gels (see Thompson *et al*., 2000). This electrophoretic technique has been used to follow changes in linkage disequilibrium (D) over time in *D. melanogaster* for the allozyme loci isocitrate dehydrogenase and phosphoglucomutase (Clegg *et al*., 1980).

In this exercise two sex-linked recessive visible loci (*white* eyes and *singed* bristles) will be used to measure linkage disequilibrium changes over time. This allows for the determination of the frequency of haplotypes in hemizygous males where the X-linked loci are carried against a Y chromosome that does not contain copies of the two genes. Hence, the frequencies of the four haplotypes ($\underline{+\ +}$, $\underline{a\ b}$, $\underline{a\ +}$, and $\underline{+\ b}$) can be determined directly from $\underline{+\ +}$ / Y, $\underline{a\ b}$ / Y, $\underline{a\ +}$ / Y, and $\underline{+\ b}$ / Y males each generation. Hedrick (1976) performed a similar set of experiments using the closely linked (r = 0.015) mutations *y* (yellow body color, 1-0.0) and *w* (white eyes, 1-1.5) of *Drosophila melanogaster*.

The rate of change in linkage disequilibrium (D) over time for two sex-linked loci is different from autosomal loci, however, because there is no recombination for sex-linked genes that are paired against Y chromosomes in males. In fact, the same problem occurs for autosomal loci in *D. melanogaster*, because males do not undergo recombination for any chromosome. The lack of recombination in males for sex-linked genes leads to an expected reduction in the rate of change of D over time as compared with autosomal genes in organisms where recombination occurs in females and males (see discussions in Bennett, 1963; Bennett and Oertel, 1965; Hedrick, 2005). Bennett and Oertel (1965) concluded that for two sex-linked loci the expected reduction in linkage disequilibrium (D) each generation is:

$$\frac{1}{4}\left\{(1-r)+\left(\sqrt{(1-r)(9-r)}\right)\right\}$$

which is about $(1 - r)^{2/3}$

Hence:

$$D_1 = \left[\frac{1}{4}\left\{(1-r)+\left(\sqrt{(1-r)(9-r)}\right)\right\}\right]D_o \qquad (8)$$

and for any generation t:

$$D_t = \left[\frac{1}{4}\left\{(1-r)+\left(\sqrt{(1-r)(9-r)}\right)\right\}\right]^t D_o \qquad (9)$$

For example, for the South Bass Island population of water snakes, where $D_o$ is 0.22 and r is 0.2:

$$D_1 = \left[\frac{1}{4}\left\{\left(1-0.2\right)+\left(\sqrt{(1-0.2)(9-0.2)}\right)\right\}\right]^1 0.22$$

$$D_1 = (0.86332)(0.22)$$

$$D_1 = 0.1899304$$

Notice that this $D_1$ value is higher than the $D_1$ value (0.176) previously obtained for autosomal loci in organisms where recombination occurs in both sexes. The expected reductions in D each generation for two sex-linked genes, as compared to reductions in D for two autosomal genes, where recombination occurs in both sexes, is shown in Table 1 and Figure 2. Note, for example, that at generation 13 the $D_{13}$ value for autosomal loci with recombination in both sexes is 0.0151182, whereas for two sex-linked loci with recombination only in females $D_{13}$ is 0.0377124, a 2.5-fold difference. Linkage disequilibrium is clearly expected to go down slower over time for two sex-linked loci with recombination only in one sex as compared to that for two loci with the same r values, but with recombination in both sexes.

Table 1. Expected changes in linkage disequilibrium (D) over time for two sex-linked loci (*w* and *sn*$^3$), where recombination only occurs in females, and for two autosomal loci, where recombination occurs in both sexes. In both cases, r = 0.2 and $D_o$ is 0.22.

| | D Values | |
|---|---|---|
| Generations | Sex-Linked Loci (*w* and *sn*$^3$); Recombination Only Occurs in Females[a] | Autosomal Loci; Recombination Occurs in Both Sexes[b] |
| G1 | 0.22 | 0.22 |
| G2 | 0.1899304 | 0.176 |
| G3 | 0.1639707 | 0.1408 |
| G4 | 0.1415592 | 0.11264 |
| G5 | 0.1222109 | 0.090112 |
| G6 | 0.1055071 | 0.0720896 |
| G7 | 0.0910866 | 0.0576168 |
| G8 | 0.0786367 | 0.0461373 |
| G9 | 0.0678886 | 0.0369098 |
| G10 | 0.0586096 | 0.0295279 |
| G11 | 0.0505989 | 0.0236223 |
| G12 | 0.0436830 | 0.0188978 |
| G13 | 0.0377124 | 0.0151182 |

[a]D values are derived from equation (9) [b]D value are derived from equation (6)

4. Materials and Methods:

In this exercise, the reduction in linkage disequilibrium (D) over time will be determined for the two sex-linked genes *w* (white eyes; map position 1.5) and *sn*$^3$ (singed-3, very short bristles; map position 21.0), with there being about 20% expected recombination in females each generation between *w* and *sn*$^3$, *i.e.*, r = 0.2 (Lindsley and Zimm, 1992).

This exercise was started by mating 60 $\underline{+\ +}$ / $\underline{w\ sn^3}$ virgin females and 60 males of the proportions 24 $\underline{+\ +}$ / Y, 24 $\underline{w\ sn^3}$ / Y, 6 $\underline{w\ +}$ / Y and 6 $\underline{+\ sn^3}$ / Y in each of four bottles. This gives the beginning frequencies of the four haplotypes in females and males as: $\underline{+\ +}$ = 0.47, $\underline{w\ sn^3}$ = 0.47, $\underline{w\ +}$ =

0.03, and $\underline{+\ sn^3}$ = 0.03, with the frequency of the *w* allele and the $sn^3$ alleles being 0.5 in females and males. In addition, the beginning linkage disequilibrium ($D_o$) value, using equation (3) was:

$D_o = (\underline{AB})(\underline{ab}) - (\underline{aB})(\underline{Ab})$

$D_o = (0.47)(0.47) - (0.03)(0.03)$

$D_o = 0.22$ (same as the water-snake population)

Since $D_o$ is 0.22 and r is 0.2, the expected reductions in D over time for the $w\ sn^3$ crosses are shown in Table 1 and Figure 2 for sex-linked loci. Will the D values that are observed over generations using the *w* and $sn^3$ loci in this exercise be similar to the theoretical expectation?

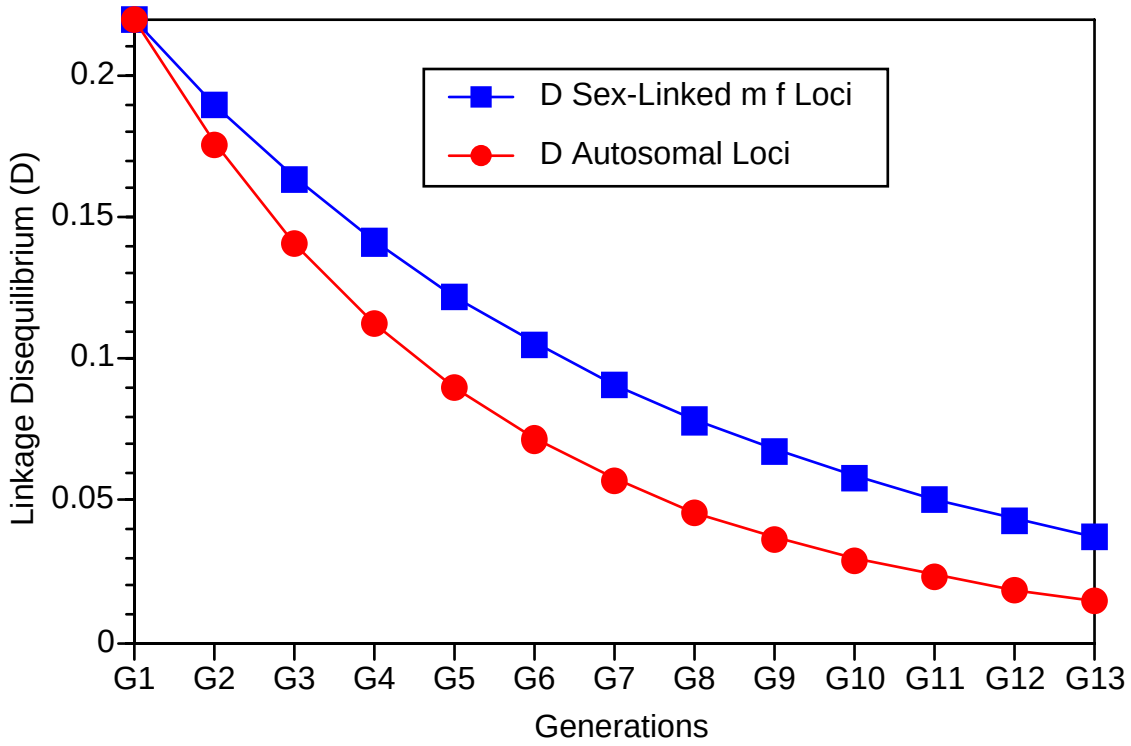



Figure 2. Expected changes in linkage disequilibrium (D) over time for two sex-linked loci (*w* and $sn^3$), where recombination only occurs in females, using equation (9), and for two autosomal loci, where recombination occurs in both sexes, using equation (6). In both cases, r = 0.2 and $D_o$ is 0.22. D values are from Table 1.

After seven days at 25°C, the adults were removed from each bottle and the D haplotype values determined by counting male progeny up to day 14 from time of initiation of the cross. There are four possible phenotypes of males that are equivalent to the four possible haplotypes: $\underline{+\ +}$ / Y = red eyes and long bristles; $\underline{w\ sn^3}$ / Y = white eyes and short bristles; $\underline{w\ +}$ / Y = white eyes and long bristles; and $\underline{+\ sn^3}$ / Y = red eyes and short bristles.

Flies were raised on a standard cornmeal-molasses medium supplemented with yeast. After the initial generation, each subsequent generation bottle was set up with all female and male progeny collected up to day 14 from the time of initiation of each cross, including the males used to measure haplotype frequencies. The progeny were collected and maintained in a bottle until all flies are transferred to a new bottle on day 14. The collection bottle was then discarded.

At the end of the experiment the observed D results over time from the *w sn*$^3$crosses will be compared with the expected theoretical changes of D over time from equation (9). It is our hypothesis that the two curves will not be significantly different. Each bottle will also be tested for significant amounts of linkage disequilibrium by use of the chi-square test as discussed by Hedrick (2005) using the Prism statistical program.

5. Results:

The changes in linkage disequilibrium (D) over time for *w* and *sn*$^3$ are shown in Tables 2 and 3 and in Figure 3. In preliminary crosses we observed 19.22% recombination (551/2,867) between *w* and *sn*$^3$ in our stocks at 25$^o$C and, therefore, used r = 0.1922. Linkage disequilibrium (D) values decreased significantly (P < 0.02) over time in each of the four bottles, but the decrease was significantly (P < 0.05) faster than the theoretical expectation. Part of the reason why the drop in D was faster than theoretical may be because not all haplotypes (*+ +*, *+ sn*$^3$, *w +*, and *w sn*$^3$) had the same viabilities. In crosses of *w + / + sn*$^3$ females with *w +* / Y males, instead of equal proportions of the nonrecombinants haplotype progeny males we observed: 421/1349 (0.31) *w +* / Y males and 688/1349 (0.51) *+ sn*$^3$ / Y males (P < 0.0001). In addition, we did not observe equal frequencies of recombinant male progeny*:* 157/1349 (0.12) *+ +* / Y males and *w sn*$^3$ = 83/1349 (0.06) *w sn*$^3$ / Y males (P < 0.001).

Table 2. Observed changes in genotypes over time.

| Bottle | Generation | Genotypes | | | | D |
|---|---|---|---|---|---|---|
| | | *+ +* | *w sn*$^3$ | *w +* | *+ sn*$^3$ | |
| A | 1 | 84 | 84 | 6 | 6 | 0.22 |
| | 2 | 178 | 96 | 30 | 28 | 0.147 |
| | 3 | 110 | 45 | 37 | 68 | 0.036 |
| | 4 | 184 | 41 | 31 | 65 | 0.054 |
| | 5 | 188 | 42 | 51 | 55 | 0.045 |
| | 6 | 187 | 4 | 39 | 61 | -0.018 |
| | 7 | 224 | 16 | 31 | 28 | 0.012 |
| B | 1 | 84 | 84 | 6 | 6 | 0.22 |
| | 2 | 205 | 122 | 38 | 41 | 0.135 |
| | 3 | 142 | 61 | 33 | 35 | 0.104 |
| | 4 | 141 | 25 | 17 | 25 | 0.072 |
| | 5 | 207 | 35 | 59 | 49 | 0.035 |
| | 6 | 222 | 14 | 39 | 32 | 0.023 |
| | 7 | 266 | 20 | 33 | 51 | 0.027 |
| C | 1 | 84 | 84 | 6 | 6 | 0.22 |
| | 2 | 157 | 122 | 43 | 45 | 0.128 |
| | 3 | 190 | 53 | 25 | 43 | 0.093 |
| | 4 | 192 | 44 | 24 | 42 | 0.082 |
| | 5 | 238 | 30 | 40 | 61 | 0.093 |
| | 6 | 188 | 19 | 33 | 27 | 0.038 |
| | 7 | 219 | 13 | 46 | 30 | 0.013 |
| D | 1 | 84 | 84 | 6 | 6 | 0.22 |
| | 2 | 189 | 127 | 44 | 42 | 0.137 |
| | 3 | 85 | 64 | 15 | 37 | 0.120 |
| | 4 | 142 | 38 | 11 | 29 | 0.104 |
| | 5 | 175 | 55 | 39 | 29 | 0.096 |
| | 6 | 195 | 23 | 37 | 31 | 0.041 |
| | 7 | 190 | 20 | 38 | 29 | 0.034 |

Table 3. Observed changes in linkage disequilibrium (D) over time for the sex-linked genes *w* and *sn*$^3$. The theoretical model is based on a recombination (r) value of 0.1922 between the two loci and $D_o$ is 0.22, using equation (9).

| Generations | Theoretical | Bottle A | Bottle B | Bottle C | Bottle D |
|---|---|---|---|---|---|
| 1 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 |
| 2 | 0.191 | 0.147 | 0.135 | 0.128 | 0.137 |
| 3 | 0.166 | 0.036 | 0.104 | 0.093 | 0.12 |
| 4 | 0.144 | 0.054 | 0.072 | 0.082 | 0.104 |
| 5 | 0.125 | 0.045 | 0.035 | 0.093 | 0.096 |
| 6 | 0.109 | -0.018 | 0.023 | 0.038 | 0.041 |
| 7 | 0.095 | 0.012 | 0.027 | 0.013 | 0.034 |

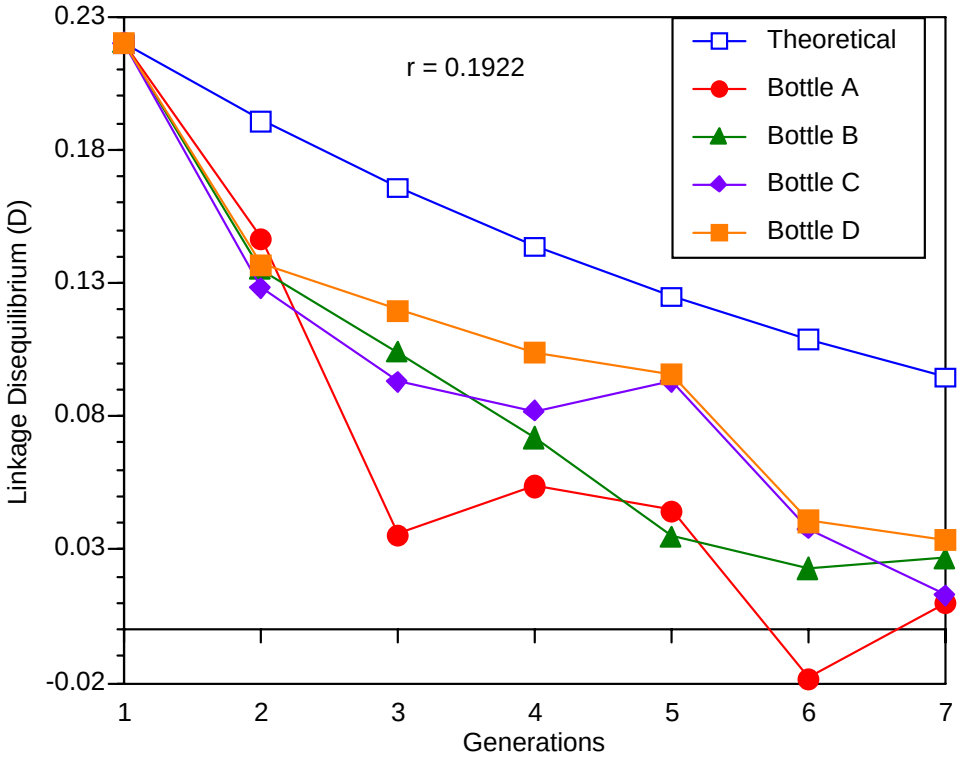



Figure 3. Observed changes in linkage disequilibrium (D) over time for two sex-linked loci (*w* and *sn*$^3$), where r = 0.0.1922 and $D_o$ is 0.22, using equation (9). D values are from Table 3.

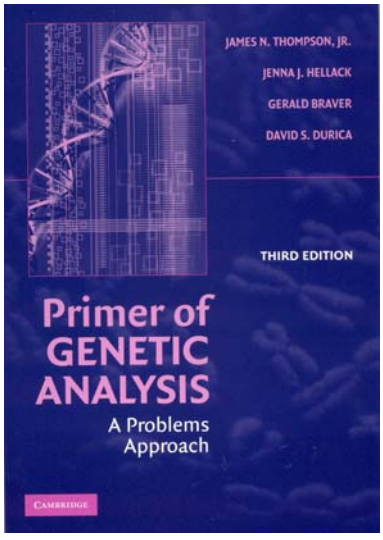
Although linkage disequilibrium (D) dropped from 0.22 to low levels by the seventh generation (0.0102, 0.027, 0.013 and 0.034), there were still significant amounts of linkage disequilibrium in each bottle population ($P < 0.001$).

A class discussion of the results of these crosses could include the following topics: 1) Discuss how linkage disequilibrium is used in evolutionary/population genetics and in human genetics. Linkage disequilibrium is used to locate closely-linked mutant genes in humans that cause genetic disorders (Kruglyak, 1999), to determine effective population sizes (Hedrick, 2005), to estimate the age of mutations in humans and other organisms (Rannala and Bertorelle, 2001), and to

locate genes that have evolved by adaptive selection (Wang *et al*., 2006). These topics related to linkage disequilibrium are discussed in Pritchard and Przeworski (2001), Reich *et al*. (2001), Ardlie *et al*. (2002), Nordborg and Tavar (2002), Weiss and Clark (2002), Schlotterer (2003), and Hinds *et al*. (2006).

References: Ardlie, K.G., *et al*. 2002, Nature Reviews Genetics 3: 299-309; Bennett, J.H., 1963, J. Theroet. Biol. 4: 28-36; Bennett, J.H., and C.R. Oertel 1965, J. Theoret. Biol. 9: 67-76; Clegg, M.T., *et al*.1980, Genetics 94: 217-224; Hartl, D.L., and A.G. Clark 2006, *Principles of Population Genetics*: Sinauer Associates, Inc., Publishers, Sunderland, MA; Halliburton, R., 2004, *Introduction to Population Genetics:* Pearson Prentice Hall: Upper Saddle River, NJ; Hedrick, P.W., 1976, Genetics 83: 551-571; Hedrick, P.W., 1987, Genetics 117: 331-341; Hedrick, P.W., 2005, *Genetics of Populations*: Jones and Bartlett Publishers, Sudbury, MA; Hinds, D.A., *et al*. 2006, Nature Genetics 38: 82-85; Kruglyak, L., 1999, Nature Genetics 22: 139-144; Lewontin, R.C., and K. Kojima 1960, Evolution 14: 450-472; Lindsley, D.L., and G.G. Zimm 1992, *The Genome of* Drosophila melanogaster: Academic Press, New York; Nordborg, M., and S. Tavar 2002, Trends in Genetics 18: 83-90; Pritchard, J.K., and M. Przeworski 2001, Am. J. Hum. Genet. 69: 1-14; Rannala, B., and G. Bertorelle 2001, Human Mutation 18: 87-100; Reich D.E., *et al*. 2001, Nature 411: 199-204; Schlotterer, C., 2003, Trends in Genetics 19: 32-38; Thompson, J.N., jr., R.C. Woodruff, S.B. Gray, G.S. Hendrix, and J.J. Hellack 2000, Dros. Inf. Serv. 83: 203-205; Wang, E.T., *et al*., 2006, Proc. Natl. Acad. Sci. USA 103: 135-140; Weiss, K.M., and A.G. Clark 2002, Trends in Genetics 18: 19-24; Woodruff, R.C., and J.N. Thompson, jr. 2002, Genetica 116: 371-382.

## New Books




**Primer of Genetic Analysis: A Problems Approach, 3rd edition.**

**Thompson, James N., jr., Jenna J. Hellack, Gerald Braver, and David S. Durica.** 2007, *Primer of Genetic Analysis: A Problems Approach*, 3rd edition. Cambridge University Press, U.K.
ISBN: 978-0-521-60365-2 (paperback).

This manual provides students with guided instruction in the analysis and interpretation of genetic principles and practice in problem solving to test their understanding. Each question is accompanied by a detailed explanation. Study Hints and a list of Key Terms introduce topic areas from mitosis and meiosis, to DNA and RNA structure, genetic transmission, probability, pedigree analysis, linkage and mapping in viruses, bacteria, and diploids, mutation, changes in chromosome number and structure, protein synthesis, gene regulation, quantitative genetics, and population genetics. Additional practice problems and a Glossary help students prepare for genetics course examinations or review for tests like the Medical Candidacy Aptitude Test (MCAT), Graduate Record Exam (GRE) subject tests, and other assessments.

# Mutation Notes

## Genes affecting wing planarity of *Drosophila virilis* (I): *curl.*

**Sereda, S.V., and I.A. Kozeretska.** Department of General and Molecular Genetics, Biological Faculty, National Taras Shevchenko University of Kyiv, Ukraine. sergey_sereda@mail.ru.

Organ size and shape are species-specific. Both parameters result from the coordination of cell proliferation, cell death, and arrangement of cells in specific patterns. During the last decades, our knowledge regarding the genetic basis of the cell cycle and cell survival has been greatly advanced, but the systemic relationships between gene expression patterns in cells and their proliferation only now are beginning to be established (Albagli and Pelczar, 2006). The wing of *Drosophila* is an experimental model to study the genetic mechanisms of organ patterning and growth. Due to extensive research work in this area a large number of genes, genetic interactions, several different signalling pathways that regulate strictly specific gene expression pattern in individual and general wing morphogenesis programs in *Drosophila* were described (Baena-Lopez *et al.*, 2006). Nevertheless, our knowledge about genes that affect wing planarity is still incomplete (Molnar *et al.*, 2006). There are several genes of *Drosophila melanogaster,* such as *curled* (*cu*: 3-50.0), *Curly* (*Cy*: 2-6.1), *Upturned* (*U*: 2-70.0), *Curl* (*Cu*: 2-54.6), and so on, known to produce a curled wing phenotype if mutated. But their molecular functions, as well as genetic interactions are still unclear.

A new *D. virilis* mutant was observed in a progeny of dysgenic crosses between strain 9 females (wild type Batumi population) and strain *y Bx w* males [*yellow* (*y*: 1-2.9), *Beadex* (*Bx*: 1-94.5), *white* (*w*: 1-105.0)]. This new wing mutation was genetically mapped by recombination with *Delta* (*Dl*: 2-45.0) and *ebony* (*e*: 2-83.5) to a proximal end region of the 2$^{nd}$ chromosome on the approximate distance of 39 genetic map units left from the *Delta* locus. Hence, more accurate chromosome localization of the new mutation is still unclear. Cytological analysis of salivary gland chromosomes, obtained from heterozygous mutant females, did not reveal obvious chromosome aberrations.

In the crosses with wild type it was shown that the newly-observed mutation is recessive, non sex-linked, controlled by single gene, and non-lethal in the homozygote.

This new mutation affected not only the wing planarity, but as well some more morphological characteristics. In general the mutant wing has a sail-like shape. Wings of the mutant flies are curled upward and diverged with an angle of about 30º relative to the longitudinal axis. High temperature in the last day of pupal life enhances curled character of the wing. The dorsal layer of wing cuticle of the mutant flies, in contrast to the wild type, is crossed with several (from 1 to 4) plications in the proximal area of wing, which are visible in an optical microscope. The normal spatial orientation of the wing hairs in the regions of plicated cuticle is altered (Figure 1).

The postscutellars of mutant flies are erected and crossed. Body color of the mutant flies is dark. Such wing phenotype has not been described earlier for *D. virilis*, but is highly similar to *D. melanogaster curled* mutant phenotype; moreover, a new curled-like mutation of *D. virilis* appeared to be located in the chromosome region that is homologous to *D. melanogaster* chromosome region,

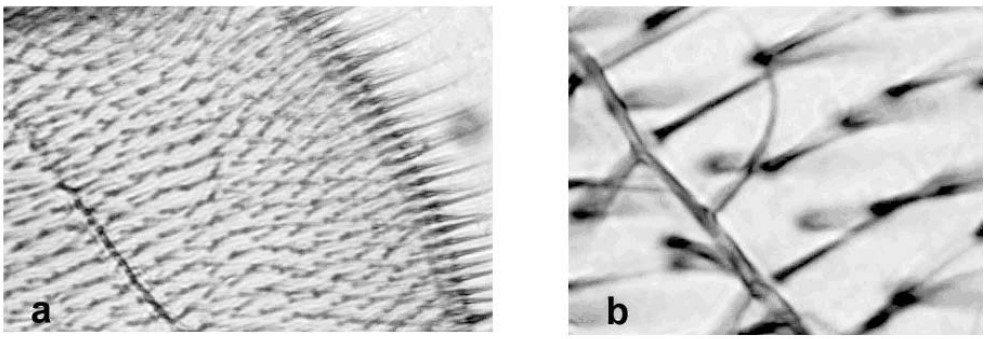



Figure 1. Cuticular plication in *D. virilis curl* wing (a) and wing hairs in the region of cuticular plication (b). Light microscopy.

where *curled* mutation is located. Thus, a new wing mutation of *D. virilis* was named *curled-like* with a symbol *curl*.

To propose a gene that causes the mentioned phenotype, a search for homologous sequences in *D. melanogaster* genome was carried out. Whiting *et al*. (1989) demonstrated sequence homology between proximal end region of the 2nd chromosome of *D. virilis* and 3R(85DE-97EF) region of *D. melanogaster* genome. According to FlyBase (http://www.flybase.org/maps), the *cu (curled)* mutation with the same phenotype is located within the bounds of 3R(85DE-97EF) region. Nucleotide sequence of *cu* genome region (FBgn0000387) contains 6 genes, named CG6629, CG33698, CG4706, Ugt86Dc, Ugt86Dd and Ugt86Di. Information about gene products and their molecular functions is presented in Table 1.

Table 1. Gene products and molecular functions of genes, located in *curled* gene genome region of *D. melanogaster*.

| Gene | Product | Molecular Function | Biological Process | Flybase ID | References |
|---|---|---|---|---|---|
| CG6629 | succinate dehydrogenase, complex, subunit-C, integral membrane protein | succinate dehydrogenase activity | mitochondrial electron transport, succinate to ubiquinone, succinate metabolism, tricarboxylic acid cycle | FBgn0037860 | FlyBase, 1992-FlyBase curation [FBrf0105495] |
| CG33698 | polypeptide | unknown | unknown | FBgn0053698 | FlyBase Genome Annotators, 2005 |
| CG4706 | aconitate hydratase | aconitate hydratase activity | amino acid biosynthesis, tricarboxylic acid cycle | FBgn0037862 | Betran et al., 2002 *Genome Res. 12(12): 1854--1859* |
| Ugt86Dc<br>Ugt86Dd<br>Ugt86Di | UDP-glucuronosyl transferase | glucuronosyltransferase activity | defense response, polysaccharide metabolism, response to toxin, steroid metabolism | FBgn0040257<br>FBgn0040256<br>FBgn0040251 | Theopold et al., 1999 *Biochem. biophys. Res. Commun. 261(3): 923--927* |

Thus, it is impossible to relate molecular functions of mentioned genes to the wing morphogenesis process; moreover, there is no information about expression of these genes during wing formation (Ren *et al*., 2005).

Most likely, the formation of the described phenotype is a result of complex genetic interactions, and the role of mentioned genes in this process is still unclear.

References: Albagli, O, and H. Pelczar 2006, Med. Sci. 22: 695; Baena-Lopez, L.A., and A. García-Bellido 2006, Proc. Natl. Acad. Sci. USA 103: 13734; Molnar, Cristina, Ana Lopez-Varea, Rosario Hernandez, and Jose F. de Celis 2006, Genetics 174: 1635-1659; Ren, Nan, Chunming Zhu, Haeryun Lee, Paul N 2005, Genetics 171: 625-638; Whiting J.H., J., Pliley M.D., J.L. Farmer, and D.E. Jeffery 1989, Genetics 122: 99-109.

## Guide to Authors

Drosophila Information Service prints short research, technique, and teaching articles, descriptions of new mutations, and other material of general interest to *Drosophila* researchers. The current publication schedule for regular issues is annually, with the official publication date being December. The annual issue will include material submitted during the calendar year. To help us meet this target date, we request that submissions be sent by 15 December, but articles are accepted at any time. A 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.

Manuscripts, orders, and inquiries concerning the regular annual DIS issue should be sent to James Thompson, Department of Zoology, University of Oklahoma, Norman, OK 73019. Telephone (405)-325-4821; e-mail jthompson@ou.edu; FAX (405)-325-7560.

**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. 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.

# 48th Annual *Drosophila* Research Conference

The 48th Annual *Drosophila* Research Conference was held on 7-11 March 2007 at the Philadelphia Marriott Downtown, Philadelphia, PA. The 2007 Organizing Committee was Steve DiNardo (University of Pennsylvania School of Medicine), Liz Gavis (Princeton University), Tom Jongens (University of Pennsylvania School of Medicine), and Jessica Treisman (NYU Medical Center). 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 889 presentations, including 156 platform session talks and 733 posters.

## Historical Address Speaker

Spyros Artavanis-Tsakonas (Harvard Medical School/MGH Cancer Center, Boston, MA)

## Plenary Lectures

Ravi Allada (Northwestern University, Evanston, IL). Circadian clocks and sleep.
Thomas Schwarz (Children's Hospital, Boston, MA). Membrane and organelle traffic in neurons.
Kristin Scott (University of California, Berkeley, CA). Taste recognition in *Drosophila*.
Thomas Lecuit (IBDM, Marseille Cedex, France). Regulation of cell surface mechanics underlying tissue morphogenesis.
Lori Wallrath (University of Iowa, Iowa City, IA). Progressive heterochromatin: Players and pathways.
Donald Rio (University of California, Berkeley, CA). Mechanisms of P element transposition and alternative pre-mRNA splicing.
Michael Eisen (University of California, Berkeley, CA). Evolution of gene regulation in Drosophilids.
Mohamed Noor (Duke University, Durham, NC). Signatures of speciation in the *pseudoobscura* species group.
Ulrike Gaul (Rockefeller University, New York, NY). Glial function in nervous system development.
Claude Desplan (New York University, NY). Color vision.
Pernille Rørth (EMBL, Heidelberg, Germany). Regulatory mechanisms controlling directed cell migration.
Eric Rulifson (University of Pennsylvania, Philadelphia, PA). Origin of the brain endocrine axis: Insights for Type 1 Flyabetes.

## Workshops

Ecdysone Workshop
Organizers: Robert Ward (University of Kansas, Lawrence) and Craig Woodward (Mount Holyoke College, South Hadley, MA).

Cell Death

Organizers: Rebecca Hays (University of Kansas, Lawrence), Andreas Bergmann (University of Texas, Houston), and Jamie Rusconi (SUNY, Albany, NY).

A Dozen Fly Genomes: What Have We Learned and What's Next?

Organizers: William M. Gelbart (Harvard University, Cambridge, MA) and Thomas C. Kaufman (Indiana University, Bloomington).

*Drosophila* Research and Pedagogy at Primarily Undergraduate Institutions

Organizer: Don Paetkau (Saint Mary's College, Notre Dame, IN).

Immunity, Hematopoiesis, and Pathogenesis

Organizers: Todd Schlenke (Emory University, Atlanta, GA) and Brian Lazzaro (Cornell University, Ithaca, NY).

Cell Cycle Checkpoints

Organizers: Tin Tin Su (University of Colorado, Boulder) and Claudio Sunkel (University of Porto, Portugal).

RNAi High-Throughput Screening

Organizers: Bernard Mathey-Prevot (Harvard Medical School, Boston, MA) and Steven Suchyta (Ambion, Inc., Austin, TX).

Workshop on RNA Biology

Organizer: A. Javier Lopez (Carnegie Mellon University, Pittsburgh, PA).

Extracellular Matrix Interactions and Signaling

Organizer: Halyna Shcherbata (University of Washington, Seattle).

## 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: Fly Board under the News menu at the FlyBase web site: flybase.bio.indiana.edu.

## *Drosophila* Board Membership as of 48th Annual *Drosophila* Research Conference March 2007

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Northwest: Barbara Taylor (Oregon State University College of Science)
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Bill Gelbart (FlyBase; Harvard University)
Gerry Rubin (BDGP & FlyBase; University of California, Berkeley)
Susan Celniker (BDGP; Lawrence Berkeley National Laboratory, Berkeley)
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Kathy Matthews (Bloomington Stock Center & FlyBase; Indiana University)
Kevin Cook (Bloomington Stock Center & Nomenclature Committee; Indiana University)
Teri Markow (Tucson Species Stock Center; University of Arizona)
Masa Toshi Yamamoto (DGRC, Kyoto)
Jim Thompson (Drosophila Information Service; University of Oklahoma)
Michael Ashburner (Europe & FlyBase; University of Cambridge)
Hugo Bellen (Bloomington Stock Center Advisory Committee & P Element Project; Baylor College of Medicine)
Allan Spradling (P-Element Project; HHMI/Carnegie Institute)
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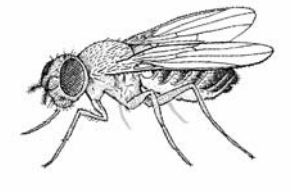

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