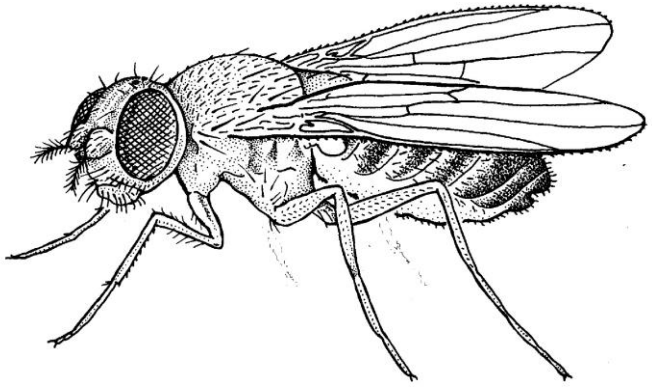
Drosophila Information Service



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

Preface

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

The production of DIS volume 97 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. Except for the special issues that contained mutant and stock information now provided in detail by FlyBase and similar material in the annual volumes, all issues are now freely-accessible from our web site: www.ou.edu/journals/dis.

One special effort deserves grateful acknowledgment. Danny Miller, working with Scott Hawley and Casey Bergman at the University of Kansas Medical Center Stowers Institute for Medical Research, is leading the electronic accession of remaining material from very early copies of DIS (*e.g.*, early stock lists and original mutation descriptions). Many of the first DIS issues are rare and not readily available in libraries. Danny and his associates are working on a project to trace the historical development of some of the original balancer chromosomes that are critical to many *Drosophila* experiments. Recently, he has gotten the loan of personal copies of several early volumes from Dan Lindsley (University of California, San Diego), and we have obtained others from Ron Woodruff (Bowling Green State University, Ohio) and from our own collection. We expect to have this expansion of content from the very early issues on line soon.

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

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

Jenna J. Hellack, Associate Editor
Department of Biology, Emeritus
University of Central Oklahoma, Edmond
Department of Biology, Adjunct Professor
University of Oklahoma, Norman

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

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

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Research Notes

Absence of strong signal of background selection affecting nucleotide diversity in *Drosophila pseudoobscura*.

Hish, Alexander J., and Mohamed A.F. Noor. Biology Department, Duke University, Durham, NC 27708 USA; noor@duke.edu.

Summary

Nucleotide variation correlates positively with regional rates of recombination in the genomes of many species, and intensive effort has been devoted to deciphering the evolutionary forces driving this association. The effects of sweeps associated with the spread of advantageous mutations and background selection associated with the removal of detrimental mutations are exceedingly hard to decouple. Here, we examine patterns of nucleotide variation in a subset of *Drosophila pseudoobscura* genes that are unlikely to have been affected by one extreme class of selective sweeps, and test for residual signal that may be attributable to background selection. While our statistical power is low due to the number of genes fitting our criteria and small numbers of sequences, the proportion of variation explained by our tests is extremely low and sometimes opposite in direction of predictions of background selection. We tentatively conclude that most of the association of nucleotide variation with recombination rate in this species is probably not attributable to background selection.

Introduction

One of the most consistent patterns observed in molecular evolutionary studies of diverse species is that regions of the genome experiencing low recombination also exhibit little sequence variation among individuals (see review in Smukowski and Noor, 2011). Researchers have been seeking the cause of this pattern since it was first observed in *Drosophila melanogaster* over 20 years ago (Aguade *et al.*, 1989; Stephan and Langley, 1989; Begun and Aquadro, 1992). A positive association between recombination rate and nucleotide diversity could result from recombination being mutagenic, for which there is some evidence in yeast with respect to mitotic recombination (Strathern *et al.*, 1995). However, results of detailed studies in *Drosophila* species in particular suggest that much of this pattern is explained by natural selection eliminating variation in regions of low recombination (*e.g.*, Begun and Aquadro, 1992; Begun *et al.*, 2007; McGaugh *et al.*, 2012).

At least two forms of natural selection can explain the positive association between recombination rate and nucleotide diversity. First, selective sweeps reduce variation at closely-linked neutral loci. Alleles linked to the advantageous mutation become fixed along with the spreading mutation, and a single haplotype replaces multiple ancestral haplotypes (Maynard Smith and Haigh, 1974; Kaplan *et al.*, 1989). This effect varies with the degree to which the mutation and neutral loci are linked, since the size of the spreading haplotype will be larger in regions of reduced recombination. An alternative explanation is background selection (BgS), wherein deleterious mutations reduce variation at closely-linked neutral loci because alleles linked to the mutation are eliminated from the population when the mutation is eliminated (Charlesworth *et al.*, 1993). Similar to the effect of a selective sweep, the BgS effect will be amplified in regions of reduced recombination, as the haplotypes being eliminated are larger where recombination is low, therefore reducing the effective population size at more loci.

The similar predictions of the two models have led to disagreement over the relative impact of BgS on reducing genetic diversity (see reviews in Stephan, 2010; Charlesworth, 2013). Unambiguous effects of selective sweeps are documented repeatedly-- researchers observe new haplotypes that spread in local

populations and the associated reductions in variation at nearby sites (*e.g.*, see review in Sabeti *et al.*, 2006). In contrast, although results ***consistent with*** particular formulations of the BgS model have been observed, unambiguously ascribing a local reduction in nucleotide variation to BgS as distinct from selective sweeps has remained an elusive goal (Innan and Stephan, 2003; Loewe and Charlesworth, 2007; Kaiser and Charlesworth, 2009). For example, a recent analysis of diversity in the human genome found a large reduction in average diversity near conserved sites, but was unable to definitively ascribe these effects to background selection as distinct from possible confounding effects of positive selection (McVicker *et al.*, 2009). Because the selective sweep model predicts a shift of the site frequency spectrum towards low-frequency or rare nucleotide variants, whereas the spectrum produced by BgS models is essentially neutral, much attention has been paid to these types of analyses in distinguishing the models. Nonetheless, efforts using these methods have failed to definitively determine that a particular region of the genome has lost variation as a direct result of BgS.

This study seeks to identify effects of BgS as distinct from effects of selective sweeps using data from *Drosophila pseudoobscura*. Previous work showed that this species exhibits a positive association between recombination rate and nucleotide diversity (Kulathinal *et al.*, 2008; McGaugh *et al.*, 2012). Here, we focused our analyses on genes without fixed differences between *D. pseudoobscura* and its close relative *D. miranda*, and stratified these genes *a priori* based on population genetic parameters that correlate with degree of purifying selection (and thus, presumably, background selection) on their coding regions. We then tested for associations between these correlates of purifying selection on coding sequence and nucleotide variation in introns. Our test is conservative in some respects, because background selection can occur in genes that bear fixed sequence differences between related species, but our focus on such genes excludes at least one class of selective sweeps ("hard, complete sweeps", strong selection driving new mutations to rapid fixation; see Pritchard *et al.*, 2010).

Methods

Candidate Gene Identification

To seek possible effects of BgS, we identified genes in the *D. pseudoobscura* genome that were unlikely to have been affected by "hard, complete" selective sweeps. We focused our analyses on genes bearing one intron less than 1000 bp in length, because linkage disequilibrium (and thus likely effects of BgS) decays rapidly in *Drosophila*, even in regions of low recombination (*e.g.*, Langley *et al.*, 2000).

We used aligned coding sequence data from 11 *D. pseudoobscura* and 3 *D. miranda* individuals (sequence data downloaded from Pseudobase: http://pseudobase.biology.duke.edu/; McGaugh *et al.*, 2012; McGaugh and Noor, 2012) to identify all genes with no fixed differences between the two species in coding regions or introns. This limitation on fixed differences precludes the possibility that a novel advantageous mutation arose and spread to fixation in the lineage leading to *D. pseudoobscura* after divergence from *D. miranda*. To increase sample size, 15 genes were included in the study that contained small numbers of fixed differences between *D. pseudoobscura* and *D. miranda*. In this subset, all fixed differences were inferred to have arisen in *D. miranda* via parsimony: the *D. pseudoobscura* variant was shared with the outgroup species *D. lowei*. Therefore, similar to the case discussed above, a recent, hard, complete selective sweep in *D. pseudoobscura* was unlikely to have occurred in these genes. The final set of 43 genes fitting these criteria, along with their FlyBase identifiers and associated statistics, is presented in a Supplementary Table in Dryad (http://datadryad.org/).

Statistical Analyses

For the final suite of candidate genes, we used DnaSP (Librado and Rozas, 2009) to obtain various population genetic parameters including the average pairwise difference in sequence within introns among individuals within species (intron π), intron Tajima's D (Tajima, 1989), d_{xy} (Nei, 1987) between *D. pseudoobscura* and distant outgroup species *D. lowei* in the coding region, and Ka/Ks (Nei and Gojobori, 1986) between *D. pseudoobscura* and *D. lowei* in the coding region. We then performed a series of regressions to test if the data supported patterns predicted the BgS model.

Results

We predicted that genes that experienced BgS would have reduced intronic nucleotide diversity (low intron π) and disproportionately negative Tajima's D (indicating population size expansion and/or purifying selection). We found that the average values of intron π from our candidate genes were close to those estimated for the genome as a whole (autosomes: 0.016 for candidate genes *vs.* 0.015 for whole genome). Most genes in *D. pseudoobscura* have a negative Tajima's D (Moriyama and Powell, 1996; Hamblin and Aquadro, 1999; Machado *et al.*, 2002), so we predicted a ***disproportionately*** negative Tajima's D, relative to other *D. pseudoobscura* genes, in those genes experiencing BgS (Charlesworth *et al.*, 1993; Charlesworth *et al.*, 1995). We tested the association of each of these metrics to three parameters correlated with the expected degree of BgS: Ka/Ks, d_{xy}, and recombination.

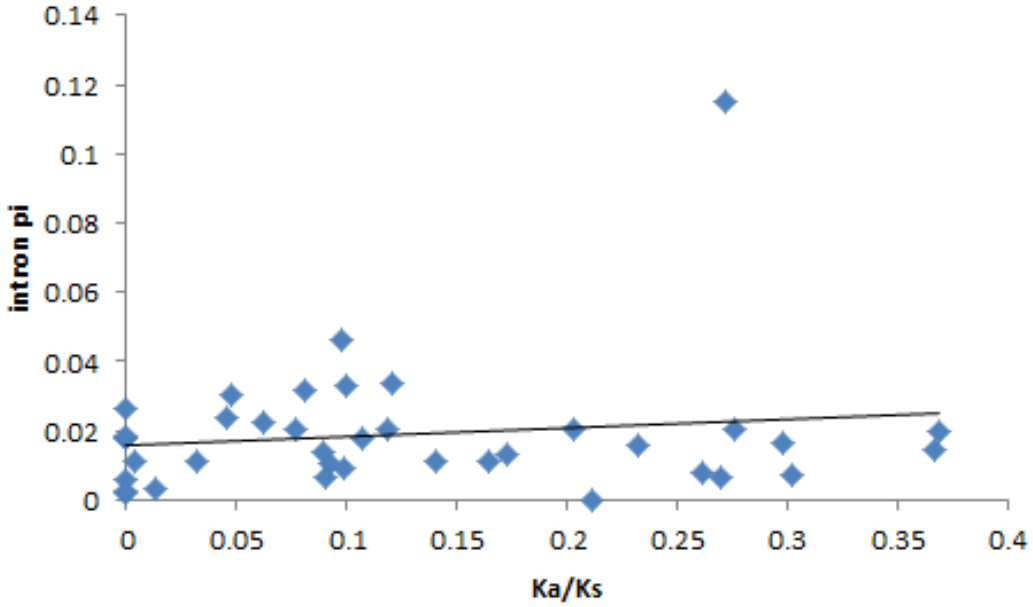

Figure 1. Correlation of intron pi with coding region Ka/Ks (N = 38).

First, we tested for associations between intronic nucleotide diversity and Ka/Ks (Nei and Gojobori, 1986) between *D. pseudoobscura* and outgroup *D. lowei*. Ka/Ks provides a measure of evolutionary constraint on the coding region, with lower values indicating greater constraint and potentially greater background selection. Therefore, BgS predicts low values of intronic diversity to be associated with low values of Ka/Ks. We excluded genes with high (>1, 1 gene excluded) or moderate (>0.5, 4 genes excluded) values of Ka/Ks so as to limit possible effects of positive selection. Our data follows the trend predicted by BgS, but is not statistically significant ($r^2 = 0.02$, $p = 0.38$, Figure 1). Removing the one extreme outlier with $\pi = 0.11$ made the trend negative, opposite in prediction to BgS. Results were qualitatively similar when log(Ka/Ks) was used and when X-chromosomal and autosomal genes were split for separate analysis. We also tested for an association of Tajima's D of intron sequences to Ka/Ks, and again, all associations were weak and not statistically significant (see table in Dryad: http://datadryad.org/).

Second, we tested for associations between intronic diversity and the average number of nucleotide substitutions per coding region site between *D. pseudoobscura* and *D. lowei* (d_{xy}; see Nei, 1987). Similar to Ka/Ks, d_{xy} potentially provides a measure of evolutionary constraint (low values indicating sequence similarity over a long evolutionary timespan), but d_{xy} estimates conservation more directly without *a priori* assumptions about synonymous or nonsynonymous differences. Again, BgS predicts low values of intronic diversity to be associated with low values of d_{xy} (indicating high constraint). As above, results followed the trend predicted

by BgS, but very weakly and with low statistical support (r^2 = 0.02, p = 0.42, Figure 2). Removing the one extreme outlier with π = 0.11 again made the trend negative, opposite in prediction to BgS. Results were qualitatively similar again for the association between Tajima's D and d_{xy}, and when the X-chromosomal and autosomal genes were split for analyses (see data in Dryad: http://datadryad.org/).

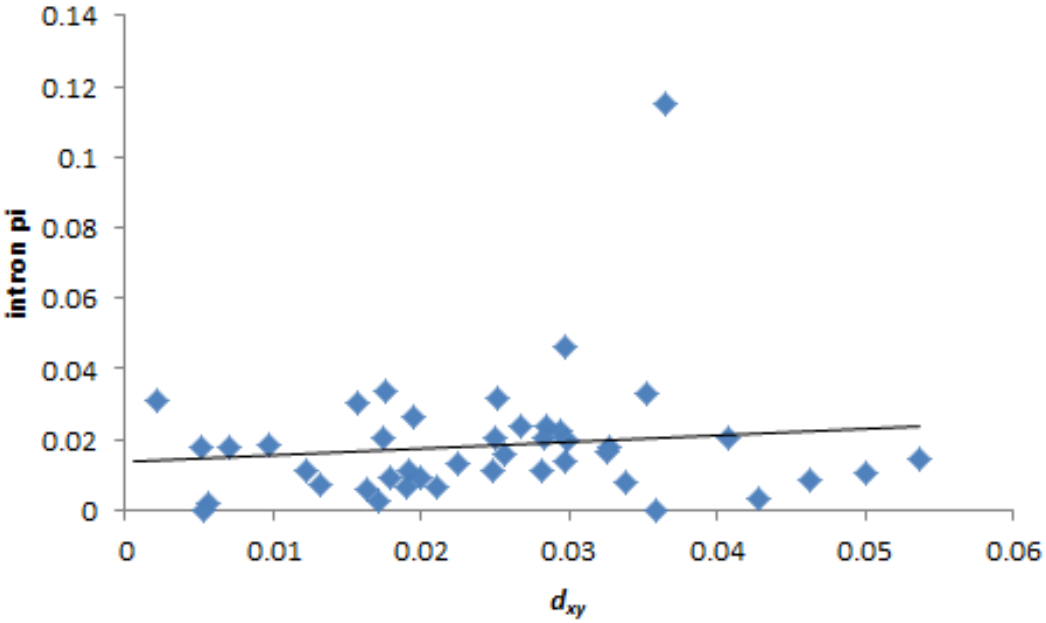

Figure 2. Correlation of intron pi with coding region d_{xy} (N = 43).

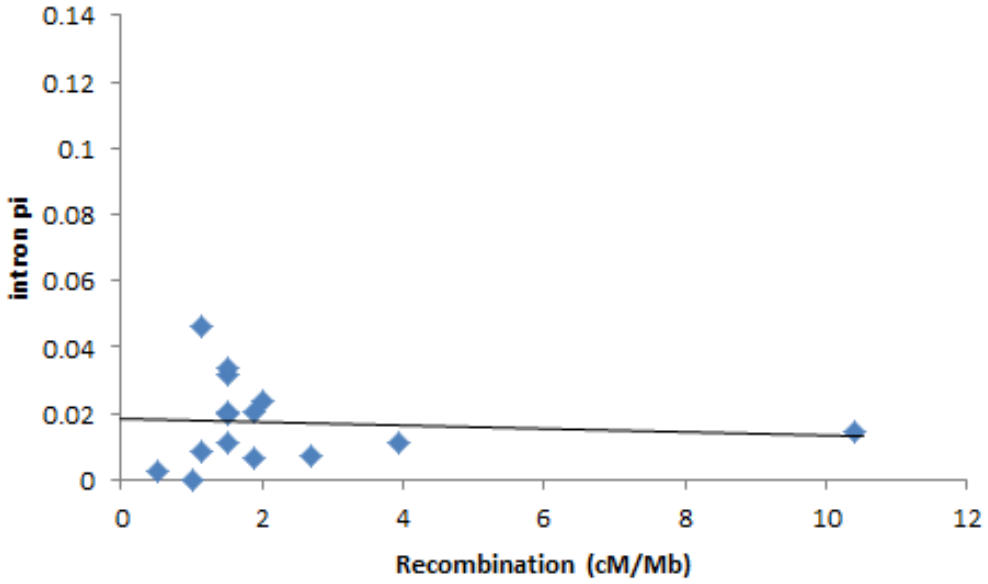

Figure 3. Correlation of intron pi with recombination rate for X-chromosome genes (N = 15).

Finally, we tested for associations between intronic diversity and recombination rate (measured in Kosambi cM/Mb). The recombination datasets for *D. pseudoobscura* consist of two very similar

recombination maps (McGaugh *et al.*, 2012), and we used the average of the values from the two maps. Assuming similar density of targets for purifying selection, background selection should be most intense in regions of low recombination (Charlesworth *et al.*, 1993), and so we predict a decrease in neutral variation (*i.e.*, intronic diversity) with a decrease in recombination rate. Instead, we observed a non-significant, negative association between recombination rate and intronic diversity, both for X-chromosomal ($r^2 = 0.01$, $p = 0.75$, Figure 3) and autosomal genes ($r^2 = 0.19$, $p = 0.38$). The association between recombination rate and Tajima's D was also non-significant ($r^2 = 0.12$, $p = 0.14$).

Discussion

Our efforts failed to identify statistically significant, or even suggestive, evidence that background selection (BgS) reduced a detectable amount of variation in the 43 genes studied in *Drosophila pseudoobscura*. There is a strong correlation of recombination rate to diversity in *D. pseudoobscura* and many other species (*e.g.*, Begun and Aquadro, 1992; Hellmann *et al.*, 2003; Roselius *et al.*, 2005; Smukowski and Noor, 2011; McGaugh *et al.*, 2012); however, there is persuasive evidence that much of this correlation is caused by selective sweeps or other forms of directional selection (Sella *et al.*, 2009; Slotte *et al.*, 2010; McGaugh *et al.*, 2012). Therefore, it is possible that positive selection explains most of this correlation, and the contribution of BgS may be quite small.

While we focus our analyses on *D. pseudoobscura*, its genome may be more weakly affected by BgS than other genomes because of its high recombination rate. The average recombination rate in *D. pseudoobscura* is roughly four-fold higher than that in *D. melanogaster* (Ortiz-Barrientos *et al.*, 2006), and higher still than in mammals. Since large effects of BgS are not expected in species with high recombination rates (Nordborg *et al.*, 1996), our result may not be unexpected. Further, *D. pseudoobscura* has a very large effective population size (Schaeffer, 1995), and this may minimize effects of BgS on Tajima's D or other metrics (Charlesworth *et al.*, 1993; Charlesworth *et al.*, 1995).

Conversely, our tests were conservative in many respects, and we may therefore lack power to identify BgS. First, our sample size of genes was greatly limited by restricting the analysis to those genes with a single intron and no derived fixed differences between species. Although our sample size was larger than several classic studies which detected associations of recombination rate with nucleotide diversity (Begun and Aquadro, 1992 surveyed only 20 genes), effects of BgS may be often more elusive than those of selective sweeps, except in regions of severely reduced recombination (Charlesworth *et al.*, 1993; Charlesworth *et al.*, 1995). In addition to a small number of genes, our estimates of intron π and Tajima's D were based on a limited number of available sequences (McGaugh *et al.*, 2012), some of which were also short sequences, and thus the estimates may be imprecise or insufficient for detection of BgS effects (Charlesworth *et al.*, 1995).

Second, beyond reducing our sample size and statistical power, our limitation to genes with no fixed interspecies differences may slightly bias us against detecting effects of BgS. BgS can reduce the effective population size of a region, and thus potentially facilitate weakly disadvantageous variants going to fixation. As such, we may have excluded some genes because they bore fixed differences, when those differences spread in part as a result of BgS.

Had our approach detected a signature consistent with BgS, we would have needed to address the possibility that partial sweeps contributed to the results observed. Recent examinations have found evidence that "soft" or "partial" sweeps may be quite common (Messer and Petrov, 2013). Similarly, we cannot exclude possible selective sweeps outside the genes but near them, though LD decays rapidly in *Drosophila*, even in regions of low recombination (Langley *et al.*, 2000). While we cannot rule out abundant soft or partial sweeps in *D. pseudoobscura*, partial sweeps are predicted to produce positive Tajima's D measures, whereas genome wide patterns of variation in *D. pseudoobscura* exhibit a negative Tajima's D (Moriyama and Powell, 1996; Hamblin and Aquadro, 1999; Machado *et al.*, 2002).

Although multiple instances of selective sweeps have been identified, unambiguously demonstrating effects of background selection as distinct from effects of selective sweeps remains an ongoing, yet elusive, goal. We hope that new approaches, even if conservative, may provide further insight into the potential impact of BgS on genome wide or gene-specific patterns of nucleotide variation.

Acknowledgments: This research was performed as part of an undergraduate independent study fellowship to AJH. We thank S. McGaugh for sharing her data and for a first-pass at identifying candidate genes bearing no fixed differences. We also thank C. Smukowski Heil for providing an estimate of pi for the autosomes, and comments on the manuscript.

References: Aguade, M., N. Miyashita, and C.H. Langley 1989, Genetics 122: 607-615; Begun, D.J., and C.F. Aquadro 1992, Nature 356: 519-520; Begun, D.J., *et al.*, 2007, PLoS Biology 5: e310; Charlesworth, B., 2013, Journal of Heredity 104: 161-171; Charlesworth, B., M.T. Morgan, and D. Charlesworth 1993, Genetics 134: 1289-1303; Charlesworth, D., B. Charlesworth, and M.T. Morgan 1995, Genetics 141: 1619-1632; Hamblin, M.T., and C.F. Aquadro 1999, Genetics 153: 859-869; Hellmann, I., *et al.*, 2003, American Journal of Human Genetics 72: 1527-1535; Innan, H., and W. Stephan 2003, Genetics 165: 2307-2312; Kaiser, V.B., and B. Charlesworth 2009, Trends in Genetics 25: 9-12; Kaplan, N.L., R.R. Hudson, and C.H. Langley 1989, Genetics 123: 887-899; Kulathinal, R.J., S.M. Bennett, C.L. Fitzpatrick, and M.A.F. Noor 2008, Proceedings of the National Academy of Sciences USA 105: 10051-10056; Langley, C.H., *et al.*, 2000, Genetics 156: 1837-1852; Librado, P., and J. Rozas 2009, Bioinformatics 25: 1451-1452; Loewe, L., and B. Charlesworth 2007, Genetics 175: 1381-1393; Machado, C.A., R.M. Kliman, J.A. Markert, and J. Hey 2002, Mol. Biol. Evol. 19: 472-488; Maynard Smith, J., and J. Haigh 1974, Genet. Res. 23: 23-35; McGaugh, S.E., *et al.*, 2012, PLoS Biology 10: e1001422; McGaugh, S.E., and M.A.F. Noor 2012, Philosophical Transactions of the Royal Society of London Series B 367: 422-429; McVicker, G., D. Gordon, C. Davis, and P. Green 2009, PLoS Genetics 5: e1000471; Messer, P.W., and D.A. Petrov 2013, Trends Ecol. Evol. 28: 659-669; Moriyama, E.N., and J.R. Powell 1996, Mol. Biol. Evol. 13: 261-277; Nei, M., 1987, *Molecular Evolutionary Genetics*. Columbia University Press; Nei, M., and T. Gojobori 1986, Mol. Biol. Evol. 3: 418-426; Nordborg, M., B. Charlesworth, and D. Charlesworth 1996, Genet. Res., Camb. 67: 159-174; Ortiz-Barrientos, D., A.S. Chang, and M.A.F. Noor 2006, Genet. Res., Camb. 87: 23-31; Pritchard, J.K., J.K. Pickrell, and G. Coop 2010, Curr. Biol. 20: R208-215; Roselius, K., W. Stephan, and T. Stadler 2005, Genetics 171: 753-763; Sabeti, P.C., *et al.*, 2006, Science 312: 1614-1620; Schaeffer, S., 1995, Pp. 329-352 *in* L. Levine, ed. *Genetics of Natural Populations: The Continuing Importance of Theodosius Dobzhansky*. Columbia University Press, New York; Sella, G., D.A. Petrov, M. Przeworski, and P. Andolfatto 2009, PLoS Genetics 5: e1000495; Slotte, T., J.P. Foxe, K.M. Hazzouri, and S.I. Wright 2010, Mol. Biol. Evol. 27: 1813-1821; Smukowski, C.S., and M.A. Noor 2011, Heredity 107: 496-508; Stephan, W., 2010, Phil. Trans. Roy. Soc. Lond. B 365: 1245-1253; Stephan, W., and C.H. Langley 1989, Genetics 121: 89-99; Strathern, J.N., B.K. Shafer, and C.B. McGill 1995, Genetics 140: 965-972; Tajima, F., 1989, Genetics 123: 585-595.

Antistress ability of *Myristrica fragrans* (Japatrae) a nutmeg to detoxify reactive oxygen species in stress-induced *Drosophila melanogaster.*

Riya, Peter, H.B. Vasanth Patil, and B.Y. Sathish Kumar*. Post Graduate Department of Bio-technology, JSS College (An Autonomous College of University of Mysore), Ooty Road, Mysore 570 025, Karnataka, India; E-mail: bysathish@gmail.com.

Abstract

Stress describes a positive or negative condition, which has an impact on an organism's genome, transcriptome, proteome, and phenome well-being. Origin of stress may vary but its effect is deleterious. The anti-stress property of *Myristrica fragrans* (Leaf powder) in combating stress in stress-induced *D. melanogaster* a fruit fly were experimented where four groups of flies were reared simultaneously. The Control flies reared on normal media followed by media containing MTX (Second group), the third group on the media containing MTX and 0.5 gm of japatrae, finally flies on only 0.5 gm of japatrae in media. Then the flies were assayed for stress related marker enzymes like SOD, CAT, and GPx. Reduction in level of ROS by *Myristrica fragrans* in stress-induced fruit flies has increased the ability to scavenge them and lowering the

free radical concentration there by balancing the expression of stress related marker enzymes in the stress-induced flies. Key words: Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), Methotrexate (MTX), Reactive oxygen species (ROS).

Introduction

Hans Selye termed stress as an inadequate physiological response to any demand. Stress is simply a fact of nature, forces from the inside or outside world affecting the individual. The stress of exhilarating, creatively successful work is beneficial, while that of failure, humiliation, or infection is detrimental (Koolhaas, 2011). The word stress is derived from Latin word "stringi", which means "to be drawn tight". In medical terms stress is described as '*a physical or psychological stimulus that can produce mental tension or physiological reactions that may lead to illness.'* It is related to both external and internal factors. Acute, episodic, and chronic stress are the different types of stress (Lyle and Alma Dell Smith, 2013).

Acute stress is the most common form of stress. Stomach, gut and bowel problems, such as heartburn, acid stomach, flatulence, diarrhea, muscular problems including tension headache, back pain, anxiety are the causes of acute stress. Episodic acute stress is common for people with acute stress reactions to be over aroused, short-tempered, irritable, anxious, and tense. Often they describe themselves as having "a lot of nervous energy." Always in a hurry, they tend to be abrupt, and sometimes their irritability comes across as hostility. Some chronic stresses stem from traumatic, early childhood experiences that become internalized and remain forever painful and present. Some experiences profoundly affect personality. Chronic stress kills through suicide, violence, heart attack, stroke, and, perhaps, even cancer (Melinda Grossman, 2008; Lyle and Alma Dell Smith, 2013).

Myristrica fragrans is a bushy, evergreen and aromatic tree with oblong leaves and pale yellow flowers, followed by round fleshy fruits, containing a brown seed. Nutmeg is a spicy bitter, astringent, and warming herb that is a digestive tonic. It helps to control vomiting and relaxes spasms. Its topical application has anti-inflammatory effects (Herbal encyclopedia). So due to this beneficial property we selected this plant to investigate anti stress property on *D. melanogaster*, which were grown under the condition of stress, where stress induction was done by feeding the fruit flies in the medium containing MTX.

MTX is thought to affect cancer and rheumatoid arthritis by two different pathways. For cancer, MTX allosterically inhibits dihydrofolate reductase (DHFR), an enzyme that participates in the tetrahydrofolate synthesis. The affinity of MTX for DHFR is about one thousand-fold that of folate. DHFR catalyses the conversion of dihydrofolate to the active tetrahydrofolate. Folic acid is needed for the *de novo* synthesis of the nucleoside thymidine, required for DNA synthesis. Also, folate is needed for purine base synthesis, so all purine synthesis will be inhibited. Methotrexate, therefore, inhibits the synthesis of DNA, RNA, thymidylates, and proteins thus causing drug induced oxidative stress (Cronstein, 2005). In view of their abundance as normal by-products of metabolism, ROS (Reactive oxygen species), such as singlet oxygen, superoxide, peroxyl radicals, and peroxynitrite, are considered as probably the main source of spontaneous DNA damage (Evans *et al*., 2004). To prevent ROS from rising to excessive levels, cells are equipped with a variety of antioxidant defense systems. Such systems include the enzymes SOD, CAT, and GPx (Jan Vijg, 2007). SOD an enzyme that catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. CAT a common enzyme found in living organisms exposed to oxygen which has high turnover number of all enzymes. One CAT molecule can convert millions of molecules of hydrogen peroxide to water and oxygen each second. Thus, these enzymes are an important antioxidant defense in nearly all cells exposed to oxygen. Oxidative stress dependent upon superoxide radical can account for a number of acute and chronic disease states. Biological role of GPx is to protect the organism from oxidative damage similar to that of CAT (Weydert and Cullen, 2010).

In the present study we have cultured *D. melanogaster* in different groups where in the first group fruit flies are reared on normal rava cream agar media as well as in the medium containing MTX in different concentrations (second group) to induce the stress in the flies, and in the third group flies were reared on medium containing both plant sample as well as different concentrations of MTX. And the flies were cultured in the media containing only plant sample (*Myristrica fragrans*) in the fourth group. The enzymatic assay of

SOD, CAT, and GPx were done for all the groups of flies, and the results of all the groups are compared with each other in order to investigate the ability of the plant sample in reducing stress in stress-induced flies.

The purpose/objective of the present study is to evaluate the anti-stress property of *Myristrica fragrans* on stress-induced fruit flies. In this study we concluded that the plant sample used here has the ability to balance between ROS and antioxidant defense system, which is confirmed by ROS scavenging enzymatic studies. This may open up new avenues of research in a search of plants to combat against environmental stress.

Materials and Methods

Culturing of fruit flies

The *Drosophila* Stock Centre, Department of Zoology, University of Mysore, provided the stocks of wild type of *D. melanogaster*. Further the stocks were cultured in our laboratory at 26 ± 1°C. The flies are grown on a flour-based medium gelled with agar and seeded with baker's yeast and are sub cultured to fresh medium for every 15-20 days (Ashburner and Thompson, 1978).

Stress induction and reduction study

Any alteration in the food creates an environmental stress in an organism. MTX an anti cancerous drug that induces stress in fruit flies was used and mixed along with the media in different concentration in range of 5 ppm, 10 ppm, 15 ppm, and 20 ppm, and 25 ppm in second group of flies over the first group (Control) where fruit flies are cultured in cream rava agar medium without MTX and plant sample. To investigate stress reduction parameter by plant sample 0.5 g of japatrae is added to the medium containing MTX at different concentrations in third group. Finally fruit flies are cultured only in the medium with 0.5 g of plant sample in fourth group. Later the stress induction and reduction parameters were found by the estimating antioxidant defense enzymes in every group of flies.

Enzyme collection

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

SOD assay

SOD enzyme (EC 1.15.1.1) was assayed using a slightly modified procedure originally described by Beauchamp and Fridovich (1971). Mix 3 ml of cocktail solution containing 250 mM Phosphate buffer pH 7.8 (0.8 ml), 100 mM Methionine (1 ml), 100 mM Riboflavin (0.5 ml), 5 mM EDTA (0.1 ml), 750 µM NBT (0.5 ml), Enzyme extract (0.1 ml) total volume is 3 ml. A blank was set without the enzyme and NBT to calibrate the spectrophotometer. Another control was prepared having NBT but no enzyme and is taken as a reference control. Then all the tubes were exposed to 400W bulb for 15 minutes and these colored solutions absorbances were read at 560 nm immediately to know the activity, and later on to know the specific activity. Protein estimation is done by the method described by Lowry (1951) and is expressed in units/mg of protein.

CAT assay

CAT enzyme (EC.1.11.1.6) is assayed by following the method of Beers and Sizer (1952). 0.1 ml of crude Enzyme extract was mixed with 2.9 ml of 30% of hydrogen peroxide (freshly prepared using 50 mM phosphate buffer). The absorbance was measured by spectrophotometer at 240nm. Decrease in the absorbance indicates the action CAT on hydrogen peroxide. Protein estimation is done by method described by Lowry (1951) in order to determine specific activity and the same is expressed in units/mg of protein.

GPx assay

GPx (EC.1.11.1.9) is assayed according to the procedure of Rotruck *et al*. (1973) with some modifications. The reaction mixture consisting of 0.4 ml of 0.4 M sodium phosphate buffer (pH 7.0), 0.1 ml of 10 mM sodium azide, 0.2 ml of 4 mM reduced glutathione, 0.1 ml of 2.5 mM H_2O_2, 0.2 ml of distilled water and 0.1 ml of enzyme was incubated at 37ºC for 15 min. The reaction was terminated with 0.5 ml of 10% TCA and after centrifugation, 2 ml of the supernatant was added to 3 ml of phosphate buffer and 1 ml of DTNB (5,5-dithiobis 2-nitrobenzoic acid) reagent (0.04% DTNB in 1% sodium citrate). The color developed was read at 412 nm and the activity is calculated by determining the amount of glutathione utilized. Protein estimation is done by the method described by Lowry (1951) in order to determine specific activity and the same is expressed in units/mg of protein.

Results

Activity of SOD, CAT, and GPx activity in flies exposed to MTX

The normal flies were taken as control files and their enzymatic assay was done so as to compare with that of stress-induced flies by different concentrations of MTX where the specific activity of the SOD, CAT, and GPx increases gradually with the increase in the concentration of MTX over the control flies (Figures 1, 2, 3).

Enzyme activity in the stress-induced flies treated with plant sample

Addition of plant sample to the medium containing different concentrations of MTX has shown a considerable decrease in the level of antioxidant enzymes by suppressing the elevated level of ROS even though it is not equivalent to that of control flies (Figures 4, 5, 6; Table 1).

Variation of enzyme activity in normal flies treated with plant sample alone

Comparative study of the fruit flies grown in the media containing only 0.5 g of plant sample with that of control is carried out. The enzyme activity is different in the fourth group of flies reared on the media containing only 0.5 g of the plant sample (Table 2). There is increased CAT activity in the fourth group flies grown on medium containing only 0.5 g of japatrae over the control flies (Figure 8), and the activity of SOD and GPx is found to be decreased in the same flies grown on medium containing only 0.5 g of japatrae to that of control flies (Figure 7, 9; Table 2). The above result shows that CAT, SOD, and GPx activity has increased when MTX is added and the plant sample is found to reduce the stress.

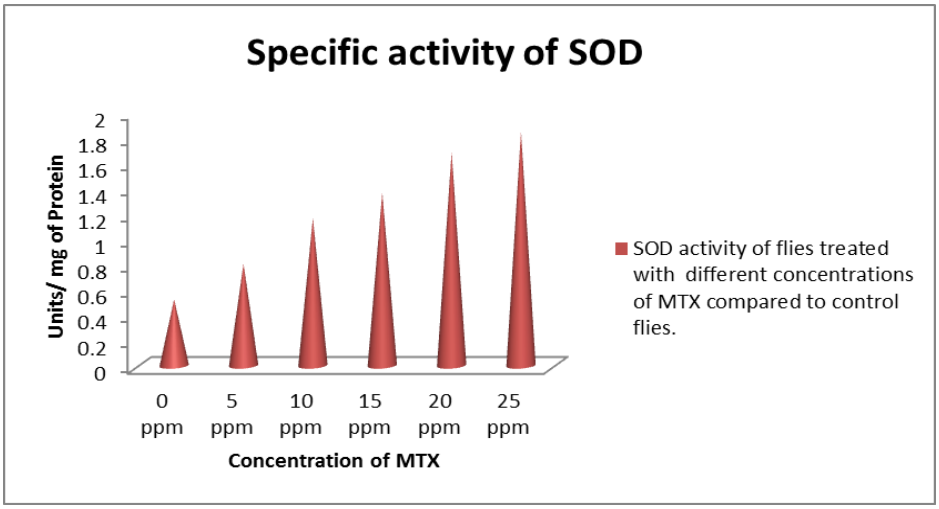

Figure 1. Activity of SOD (Second group) increases gradually in the flies reared on media containing increased concentrations of Methotrexate when compared with control flies.

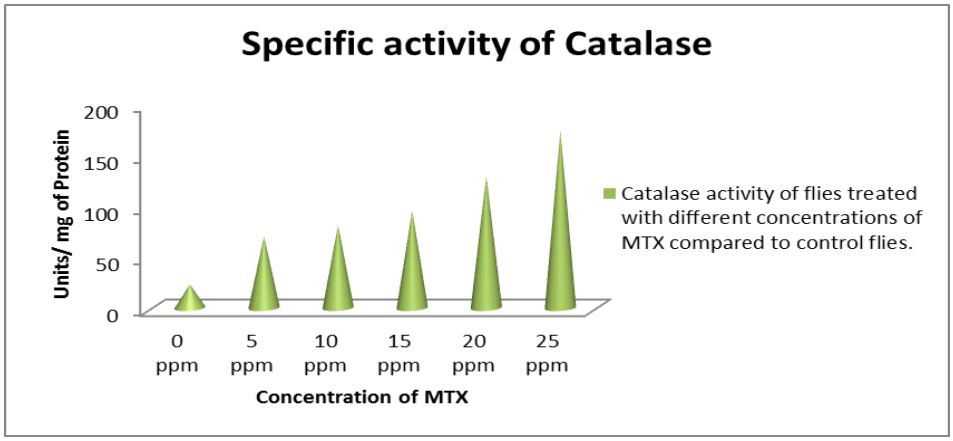

Figure 2. Activity of CAT (Second group) increases gradually in flies reared on media containing increasing concentrations of Methotrexate when compared with control flies.

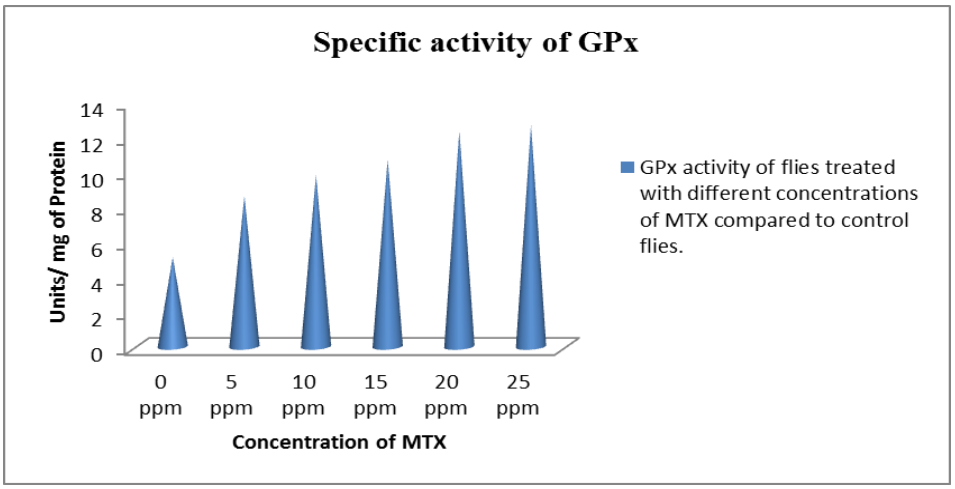

Figure 3. Activity of GPx (Second group) increases gradually in flies reared on media containing increasing concentrations of Methotrexate when compared with control flies.

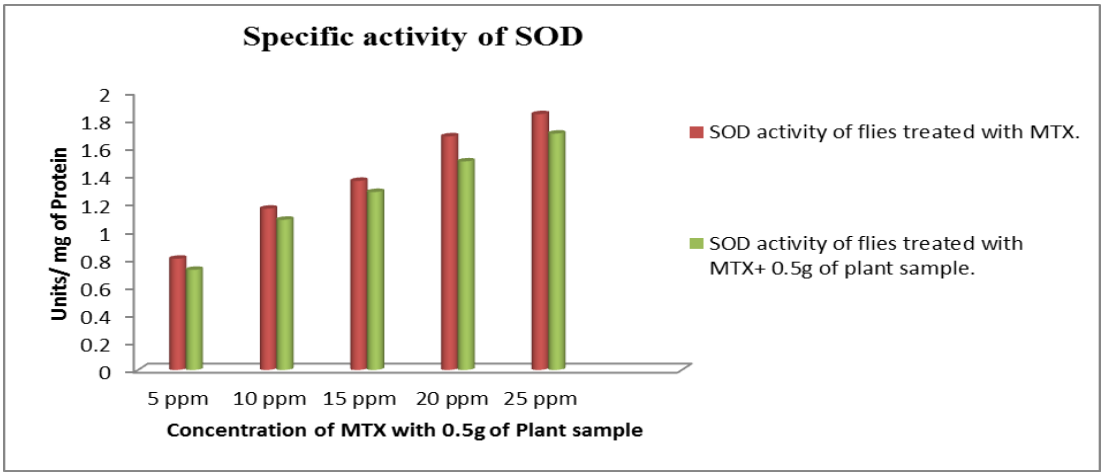

Figure 4. Activity of SOD (Third group) increases gradually in flies reared on media containing increasing concentrations of Methotrexate and reduction of activity in the presence of 0.5 g of plant sample.

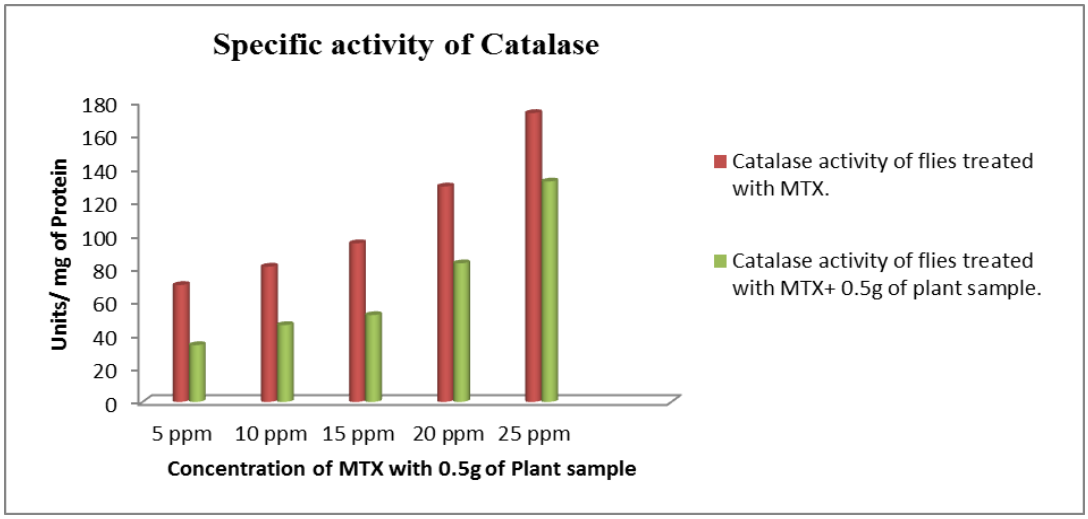

Figure 5. Activity of CAT (Third group) increases gradually in flies reared on media containing increasing concentrations of Methotrexate and reduction of activity in the presence of 0.5 g of plant sample.

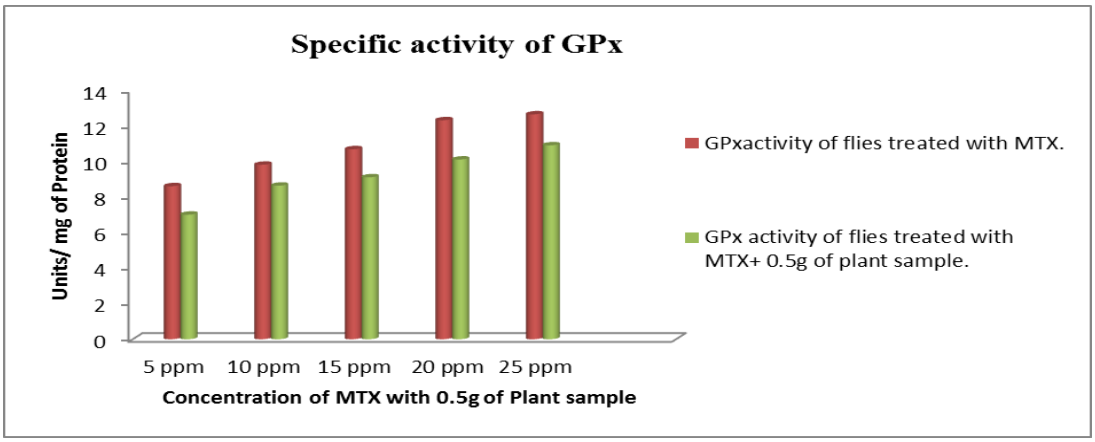

Figure 6. Activity of GPx (Third group) increases gradually in flies reared on media containing increasing concentrations of Methotrexate and reduction of activity in the presence of 0.5 g of plant sample.

Table 1. Comparison table of SOD, CAT and GPx activity of stress induced (MTX) flies (Second group) and 0.5 gm of plant sample + MTX at different concentrations (Third group).

	Concentration of MTX	5 ppm	10 ppm	15 ppm	20 ppm	25 ppm
SOD activity (units/mg protein)	MTX alone	0.80	1.16	1.36	1.68	1.84
	MTX+0.5 gm plant sample	0.72	1.08	1.28	1.5	1.7
Catalase activity (units/mg protein)	MTX alone	70	81	95	129	173
	MTX+0.5 gm plant sample	34.5	46	51.7	82.5	132
Gpx activity(units/mg protein)	MTX alone	8.6	9.81	10.68	12.31	12.64
	MTX+0.5 gm plant sample	7.00	8.63	9.10	10.1	10.9

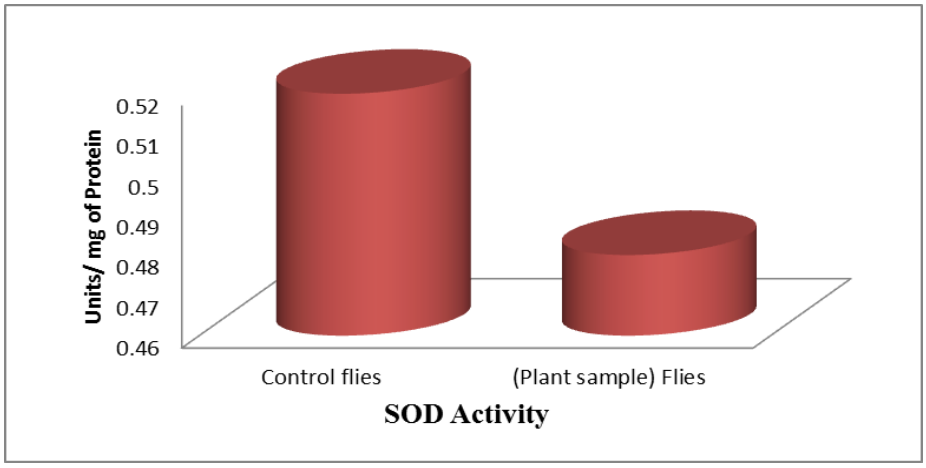

Figure 7. SOD activity of the fruit flies in Control (First group) and plant sample (Fourth group) comparative study.

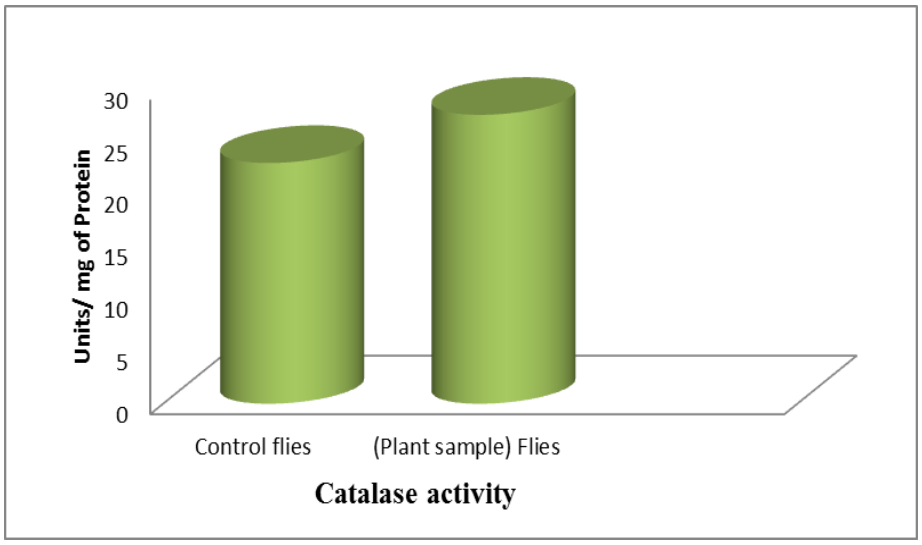

Figure 8. CAT activity of the fruit flies in Control (First group) and plant sample (Fourth group) comparative study.

Table 2. Variation of enzyme activity in normal *D. melanogaster* (First group) and the flies treated only with plant sample (Fourth group).

	SOD activity in units/mg of protein	Catalase activity in units/mg of protein	Gpx activity in units/mg of protein
Control flies	0.52	23	5.1
Flies treated with plant sample	0.48	27.6	4.9

Discussion

Stress is defined as a condition that disturbs the normal function of the biological system or a condition that decreases fitness. It was a physical or psychological stimulus that can produce mental tension or physiological reactions that may lead to illness. It is a well-known fact that stress of any nature produces a

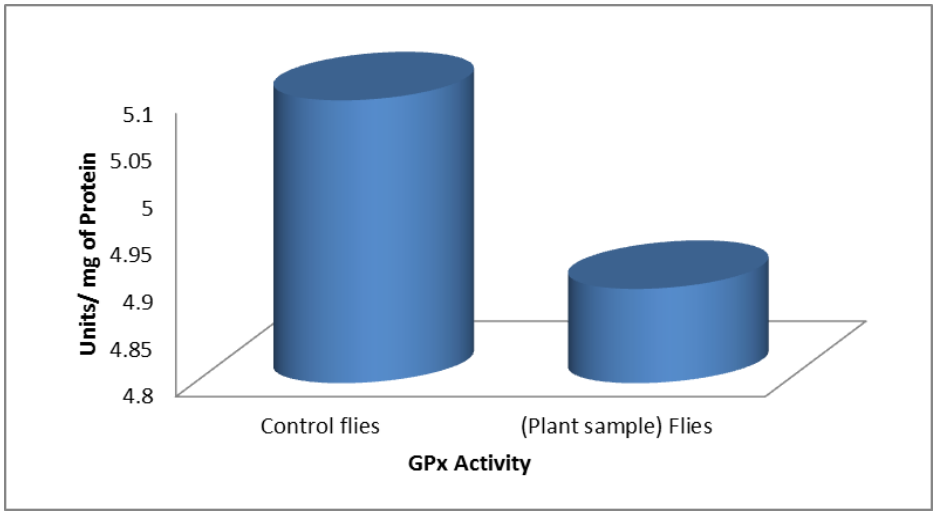

Figure 9. GPx activity of the fruit flies in Control (First group) and plant sample (Fourth group) comparative study.

non-specific state in the organism, *i.e*., the state of stress or "stress syndrome", which was characterized by adrenal hypertrophy, depletion of adrenal ascorbic acid and cortisol, and a decrease in the size of lymphoid tissue (Singh *et al*., 1978). Any damaging or potentially damaging stimulus (stressor) besides having its own specific effects induces the secretion of adrenal corticosteroids and catecholamines, cardiovascular alterations, and gastrointestinal lesions. The change observed in the stress syndrome has been explained on the basis of activation of hypothalamo-hypophysealadrenal axis. The corticoids, thus released, help animals in combating stressful situation (Selye, 1938).

Oxidative stress is a condition characterized by elevated levels of intracellular ROS. Either are, or break down to form, free radicals. ROS include superoxide anion (O2-), singlet oxygen (O2), hydroxyl radical (OH-), and hydrogen peroxide (H_2O_2), that are capable of reacting with, and damaging not only DNA, even proteins, and lipids as well (Jan Vijg, 2007).

The results of the current study demonstrated an increase in the activity of the stress related marker enzymes in stress-induced fruit flies viz., SOD, which dismutases the highly reactive superoxide anion to the less reactive species H_2O_2 (Chelikani *et al*., 2004), CAT, a haeme containing enzyme, and GPx, which scavenges hydrogen peroxide or tert-butyl hydroperoxide into water and molecular oxygen (Christine *et al*., 2010). Under normal conditions, ROS are cleared from the cell by the actions of SOD, CAT and GPx. low level of intracellular ROS have been identified as second messengers in signaling pathways and implicated in transcriptional regulation to promote cell growth, but higher doses of ROS result in growth arrest and cell death. Oxidative damage to proteins plays a crucial role in ageing, because oxidized proteins lose catalytic function and are preferentially hydrolyzed (Jan Vijg, 2007).

The activity of SOD, CAT, and GPx increases significantly in a concentration dependent manner after inducing stress by MTX. One possible reason is that the stress inducing agent MTX an anticancerous drug, which acts by inhibiting the metabolism of folic acid where it is needed for the *de novo* synthesis of nucleocides, may cause drug induced oxidative stress and much ROS is produced. In order to antagonize ROS, the level of the defensive enzymes such as CAT, SOD, and GPx increases under stressful condition (Figures 1, 2, 3) (Maehly and Chance, 1954). Similar result of increase in the antioxidant enzyme levels in the medium fed with same concentration of MTX is obtained by Deepthi and Sathish (2011). There are considerable studies on free radical mediated changes to biological systems due to stress created by surrounding environment. Environmental stress causes generation of free radicals in higher concentration that would cause irreversible mutations in DNA. But, however, the expression of these defective genes can be buffered by the action of Hsp90 a molecular chaperone (Queitsch *et al*., 2002; Rutherford, 2003). The action of chaperones camouflages the adverse effect of polymorphic variants or accumulated somatic mutations that

would normally result in protein folding defects. Although Hsp90 is highly abundant and can be further induced by heat stress, it can be overwhelmed when more and more proteins are destabilized when oxidative stress is more as a part of aerobic life and metabolism under stress (Jan Vijg, 2007).

As per our results, the plant powder used in this study is found to reduce the stress induced by MTX. This was confirmed by comparing the activity of antioxidant enzymes in second group of flies with that of third group flies containing both MTX as well as 0.5 g of plant sample (Figures 4, 5, and 6; Table-1). Similarly, *Convolvulus pluricaulis*, *Glycyrrhiza glabra*, and *Rauwolfia serpentine* have reduced the level of antioxidant enzymes in stress-induced fruit flies treated with respective plant sample in different concentrations (Arun and Sathish, 2010; Sowmya and Sathish, 2010; Deepthi and Sathish, 2011). The antioxidant enzyme activity in the third group of flies was found to be decreased slightly, indicating that the plant sample is effective in suppressing the stress. Herbs are not second best to "chemical medicine" in helping to fight stress. On the contrary, herbs are much more intelligent, have many more components that all work together, and of course, they have evolved together with our likewise immensely complicated human bodies, side by side.

In fourth group the flies were reared in the media containing only 0.5 gm of plant sample where the specific activity of the SOD and GPx is less than that of control (Figures 7 and 9). The hypothetical reason behind this is may be the plant molecules are reducing the level of ROS much less to that of normal level which in turn reduces the antioxidant enzyme levels. Whereas the fruit flies reared in the medium containing *Convolvulus pluricaulis* and *Glycyrrhiza glabra* the activity of SOD in control and fourth group of flies were almost similar to that of control (Arun and Sathish, 2010; Sowmya and Sathish, 2010).

In the current study specific activities of CAT in both control and fourth group flies were almost the same. But the specific activity of CAT has decreased in flies reared on medium containing *Glycyrrhiza glabra* and there is a slight increase in CAT activity in the flies with *Convolvulus pluricaulis* to that of control flies (Arun, and Sathish, 2010; Sowmya and Sathish, 2010). Especially when it comes to reducing the effects of stress on the body - which is so more than "just a chemical reaction"! - herbs are a perfect solution to reduce stress related build ups of toxins, to calm the overactive mind, to help break down adrenaline, to strengthen the heart and breathing systems, all of which are under attack by ongoing stress (Wang *et al*., 2005).

The stress induction in *Drosophila* was confirmed by the increased activity of cellular defensive enzymes like SOD, CAT, and GPx. As per the results plant powder is found to reduce the stress-induced by MTX in fruit flies. This may open up a new avenue of research in identifying the plants which possess anti-stress property and exploiting its action by using *D. melanogaster* as model organism.

Conclusion

The plant sample used as an anti-stress agent can be used to combat stress related disorders. The anti-stress property was confirmed by employing the SOD, CAT, and GPx activity assay, compared to the stressor induced group. The stressor group treated with plant sample showed decreases in the level of ROS thereby reducing antioxidant enzyme activity. Thus, *Myristrica fragrans* (japatrae) tends to balance between ROS and a variety of enzyme systems that can deactivate ROS, thereby it aids in improving and maintaining the health of *D. melanogaster* even under stressful conditions. This experiment has a profound implication for the broad scope of applications of anti-stress molecules to humans before which fruit flies can be used as models to study its power of action.

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Occurrence of the genus *Zygothrica* (Diptera, Drosophilidae) in a high-altitude forest in northeastern Brazil.

Monteiro, L.S.[1], W.B.M. Cabral[1], M.A. Montes[2], A.C.L. Garcia[1], and C. Rohde[1]. [1]Laboratório de Genética, Centro Acadêmico de Vitória, Universidade Federal de Pernambuco, Rua do Alto do Reservatório s/n, 55608-680, Vitória de Santo Antão, PE, Brazil; [2]Departamento de Biologia, Universidade Federal Rural de Pernambuco, Rua Dom Manuel de Medeiros s/n, 52171-900, Recife, PE, Brazil. Corresponding author: claudia.rohde@yahoo.com.br

Introduction

The genus *Zygothrica* includes drosophilids native to the Neotropical region, apart from some species found in Africa and in the Indo-Pacific region (Prigent and Toda, 2006). From the ecological standpoint, representatives of this genus have traditionally been associated with fungi (Malogolowkin, 1952; Grimaldi, 1987, 1990), though some species use flowers as food resources (Grimaldi, 1987).

In the Neotropical region *Zygothrica* is the second genus in the Drosophilidae family in terms of diversity, after *Drosophila* (Bächli, 2014). The taxonomic history of the genus dates back to 1830, when C.R.W. Wiedemann described the type species *Zygothrica*, *Z. dispar* (Wiedemann 1830), as a subgenus of *Achias* (Platystomatidae). Almost one hundred years later, in a pioneering description of several *Zygothrica* species, Sturtevant (1920) enlarged the taxonomic knowledge on this genus. Following this line of study, Duda (1952) and Burla (1956) collected specimens in Costa Rica and in Brazil, respectively, and described a large number of species in the genus. Subsequently, new species were later described and reviewed by Grimaldi (1987, 1990). Today, *Zygothrica* comprises 124 species (Bächli, 2014), 54 of which occur in Brazil (Gottschalk *et al.*, 2008). In spite of that, Grimaldi (1987) believes that only half of the total number of estimated *Zygothrica* species has been described.

In Brazil, representatives of the *Zygothrica* genus have been increasingly captured in recent years (De Toni *et al.*, 2007; Döge *et al.*, 2007; Gottschalk *et al.*, 2007; Schmitz *et al.*, 2007; Döge *et al.*, 2008; Mata *et al.*, 2008; Gottschalk *et al.*, 2009; Bizzo *et al.*, 2010; Hochmüller *et al.*, 2010; Garcia *et al.*, 2012; Poppe *et al.*, 2012; Roque *et al.*, 2013; Poppe *et al.*, 2014). However, in some of the country's regions, such as the northeast, there is a paucity of information on the genus (Gottschalk *et al.*, 2008). In northeastern Brazil, north of the São Francisco River, two subregions of the Atlantic Forest were outlined, *Pernambuco* and *Brejos de Altitude*. The latter is characterized by wet forest islands surrounded by *Caatinga*, a semiarid biome. These areas are located in plateaus between 500 and 1,000 m above sea level, where orographic rainfall ensures

precipitation levels above 1,100 mm yearly (Andrade-Lima, 1960, 1961).

The present study assesses the richness and abundance of the *Zygothrica* genus in an area in the subregion *Brejos de Altitude*, in the state of Pernambuco, northeastern Brazil.

Materials and Methods

Drosophilids were collected in a high-altitude forest near *Mata do Mucuri* Conservation Unit (08°30'46.7"S; 35°43'19.6"W), municipality of Bonito, state of Pernambuco, northeastern Brazil (Figure 1). Mean altitude in the area of study is 800 m above sea level. The prevailing climate is type As' (rainy tropical with dry season in summer) according to the Köppen classification system. Mean rainfall is 1,157 mm, mean temperature is 21.5ºC and relative humidity is between 40% and 70%. Rain distribution defines two clear seasons: the rainy, between April and August, and the dry season, during the rest of the year (Santiago *et al.*, 2004).

Collections were carried out in August 2010 (end of the rainy season), December 2010 (dry season), and April 2011 (beginning of the rainy season). Drosophilids were captured using 20 traps built as described by Tidon and Sene (1988), containing banana baits. Traps were left hanging in the study site 1.5 m above the ground, 10 m away from one another, for two consecutive days.

The specimens of the *Zygothrica* genus were identified at species level by means of analysis on the external morphology and the male genitalia (Malogolowkin, 1952; Burla, 1954, 1956; Grimaldi, 1987).

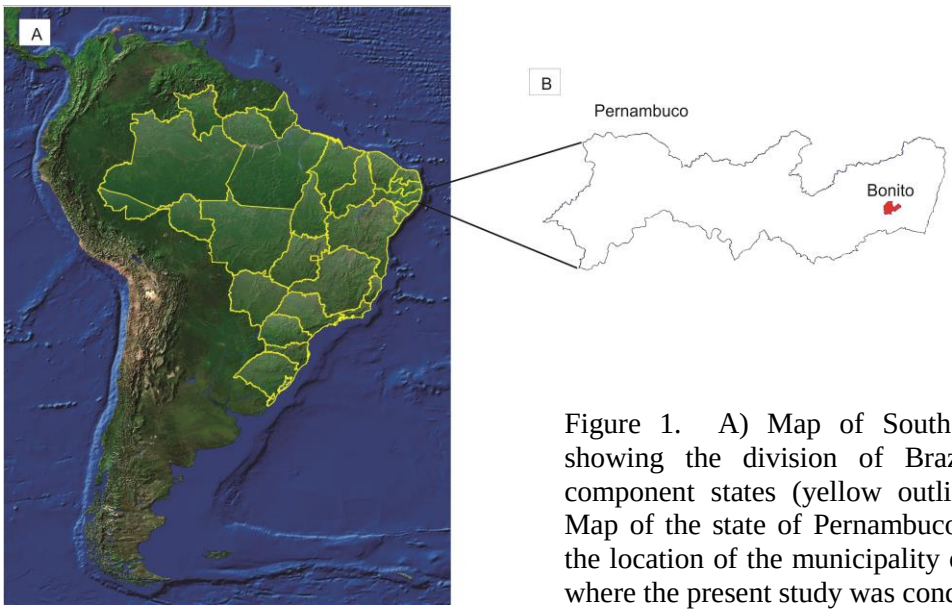

Figure 1. A) Map of South America showing the division of Brazil in its component states (yellow outlines); B) Map of the state of Pernambuco showing the location of the municipality of Bonito, where the present study was conducted.

Results and Discussion

In total, 8,802 drosophilids were collected, and of these 1,782 were *Zygothrica*, represented by: 1,752 individuals of *Z. orbitalis* (Sturtevant 1916); 24 of *Z. dispar* (Burla 1956); and six of *Z. prodispar* Duda 1925 (Table 1).

Table 1. Absolute abundance of *Zygothrica* species in the municipality of Bonito, state of Pernambuco, Brazil.

	August 2010	December 2010	April 2011
Z. orbitalis	1,749	0	3
Z. dispar	23	0	1
Z. prodispar	6	0	0
Other species	3,853	252	2,115

Zygothrica represented 20.24% of the drosophilids collected in the area of *Brejo de Altitude* surveyed. This value is high, compared with samplings carried out in other subregions of the Brazilian Atlantic Forest, where relative frequencies of *Zygothrica* representatives reached up to 11% of all drosophilids collected and, as a rule, represented less than 1% of samples (De Toni *et al.*, 2007; Döge *et al.*, 2007; Gottschalk *et al.*, 2007; Schmitz *et al.*, 2007; Döge *et al.*, 2008; Gottschalk *et al.*, 2009; Bizzo *et al.*, 2010; Garcia *et al.*, 2012). The relative abundance of the genus was also under 1% of drosophilids captured in samplings previously conducted in the *Cerrado* (Mata *et al.*, 2008; Roque *et al.*, 2013) and *Pampa* (Poppe *et al*., 2012, 2014) biomes.

In the present study, the most abundant species of the *Zygothrica* genus was *Z. orbitalis*, followed by *Z. dispar* and *Z. prodispar* (Table 1). This abundance pattern of the first two species has also been reported in other studies carried out in the Brazilian Atlantic Forest (Gottschalk *et al.*, 2007; Döge *et al.*, 2007).

Here, *Z. orbitalis*, *Z. dispar*, and *Z. prodispar* were more abundant in the higher rainfall period (Table 1). The highest abundance of *Zygothrica*, in this period, may be associated with greater availability of food resources and increased humidity. Nevertheless, in other drosophilids samplings in Brazil, these three species did not exhibit any preference for wetter periods (De Toni *et al.*, 2007; Bizzo *et al.*, 2010; Poppe *et al.*, 2014).

Prior to the present investigation, *Z. orbitalis*, *Z. dispar*, and *Z. prodispar* had been sampled in the Brazilian Atlantic Forest, but only in the subregions located in southern and southeastern Brazil (Frota-Pessoa, 1952; Burla, 1956; Grimaldi, 1987; Val and Kaneshiro, 1988; De Toni *et al.*, 2007; Döge *et al.*, 2007; Gottschalk *et al.*, 2007; Schmitz *et al.*, 2007; Döge *et al.*, 2008; Gottschalk *et al.*, 2009; Bizzo *et al.*, 2010; Garcia *et al.*, 2012). This study presents the first record of these species in an Atlantic Forest area north of the São Francisco River, in the subregion *Brejos de Altitude*, and underlines the high abundance of *Z. orbitalis* in the site surveyed.

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Seasonal distribution of Drosophilids at Jnanabharathi Campus, Bangalore University, Bangalore, Karnataka, India.

Ramesh B.Y.,[1] B.K. Neethu,[2] and B.P. Harini.[2*]..[1]Centre for Applied Genetics, Bangalore University, Bangalore-560 056, Karnataka, India; [2]Department of Zoology, Bangalore University, Bangalore-560 056, Karnataka, India; E-mail- dr.bpharini@gmail.com.

Abstract

Biodiversity is defined as the variety and abundances of species in different habitats. Drosophilids are regarded as potential indicators which are extremely necessary to achieve the conservation, and which are an effective group to test as potential indicators at different ecological gradients. In this the diversity and distribution of the Drosophilids have been affected enormously where human habitat is frequently sensed in Ghandhibhavan when compared to Madhuvana. Irrespective of vegetation, seasonal variation also has on impact on population density of *Drosophila*. This shows that climatic conditions existing in different seasons of the year may be a critical factor in population fluctions.

Introduction

Drosophila is the most abundant genus and comprises of 2240 species (around 64% of the total species). India with its vast array of vegetation and climates harbors a variety of *Drosophila* (Bächli, 1998). More than 3750 different species are now recognized belonging to the family Drosophilidae (Wheeler, 1981); about one-third (1048) of these species have been newly described since the publication of Wheeler's catalogue (Wheeler, 1986). The Indian subcontinent, with it subtropical climate and varied physiographic conditions, including variable altitudes and luxuriant flora, offers an adobe for the rich and wide distribution of *Drosophila* fauna. During recent years, considerable data have been accumulated regarding faunal composition of Drosophilid species as a result of extensive field collections in different ecological habitat by Ayala (1970).

Drosophila is being used for study of population fluctuations as they are highly sensitive to slight environmental modifications that are reflected in the size of natural populations, structure and ecology. It is known that temperature and rainfall affect viability, fertility, developmental time, and other factors that influence the rate of population growth and survival. *Drosophila* studies include intra-inter relationships, such as population density, population age, distribution, competition, and relationship between Drosophilids and their hosts and predators (Guruprasad *et al*., 2009). Drosophilid flies are good tools to improve the understanding of patterns and processes related to biodiversity, and the understanding of how human activities affect biodiversity at various temporal and spatial scales. Such tools permit the elaboration of more accurate and efficient conservation strategies, as well as the improvement of the projection about what might happen in the future. *Drosophila* has been used as a model in studies on bioindication (Parsons, 1991, 1995; Mata *et al*., 2008) and also biological invasions (Tidon *et al*., 2003).

Similarly, biotic factors like the kind of vegetation that form natural gradients and changes associated to latitude, for example, are also important (Powell, 1997). Therefore, the composition and structure of a Drosophilid assemblage depends on the habitat in which it was established. The better understanding of how different species are affected by current climates and why they sometimes respond differently to climate change is necessary for predicting future effects of climate change (Weatherhead, 2005).

In view of this an attempt has been made to understand the existing species diversity of *Drosophila* populations in nature at different localities, flies were particularly collected in two different localities, *i.e*., Madhuvana and Ghandhibhavana, at different seasons (summer, winter, rainy) in Jnanabharathi campus of Bangalore University, Bangalore, Karnataka, India during the year 2012-2013.

Materials and Methods

Drosophila flies were collected at Jnanabharathi campus in the month of October (2012), *i.e*. rainy season, January (2013) which falls in winter season, and in the month of April (2013) summer season at two different localities of Madhuvana and Gandhibhavana situated in Jnanabharathi campus, Bangalore University, respectively. The collections were made uniformly in the fourth week of each month for the assessment of fly's distribution pattern, during which the temperature recorded was 26°C-28°C (rainy), 28°C-31°C (winter), to 32°C-34°C (winter) and relative humidity varied from 19% to 65%. The method used to collect the flies was by net sweeping (Markow and O'Grady, 2006). The rotten mixed fruits were spread in the evening of the previous day. The fermented fruit was spread in each distant trap collection spot. Such bait as fermenting fruits retains its attractive odor for a long time. The collections were made early in the morning by sweeping in each trap at least three times and transferring to six quarter pint milk bottles filled with standard agar medium sprayed with yeast. The collected flies were brought to the laboratory, etherized, categorized, counted and species were identified under Olympus Stereozoom Microscope. The males were studied as such but the individual females, which could not be identified, were isolated and allowed to breed in separate vials containing standard laboratory food medium. The progeny obtained from such single gravid female were used for species identification.

Table 1. Number of *Drosophila* species collected from two different localities of Jnanabharathi campus.

Locality→	Madhuvana			Gandhibhavana		
Seasons→ Species↓	Summer	Rainy	Winter	Summer	Rainy	Winter
D. melanogaster	65	117	39	78	111	54
D. malerkotliana	68	78	32	44	56	21
D. simulans	47	45	21	33	21	10
D. rajashekari	56	45	37	41	45	37
D. bipectinata	38	65	34	31	71	34
D. nasuta	125	113	83	76	81	47
D. neonasuta	89	97	75	89	103	75
D. phorticella setiata	134	91	103	122	88	91
D. brundavensis	2	0	0	0	0	0

Results

Distribution of different species of *Drosophila* and their numbers were found, during collections along with temperature in Jnanabharathi campus, Bangalore, during 2012-2013. A total of 9 species (Madhuvana and Ghandhibhavana) were encountered in the collected site that belonged to 2 sub genera, namely, Sophophora and *Drosophila.* The collection of Drosophilid flies from two locations of Jnanabharathi campus at different seasons have yielded 3158 flies, out of which 1699 flies were collected from Madhuvana and 1469 from Ghandhibhavana during year 2012-2013. The data (Table 1) from the present survey have revealed 9 different species of *Drosophila* with no record of new species in different localities of Jnanabharathi campus. Of these *Drosophila phorticella setiata* was more abundant than the other eight species. *Drosophila brundavensis* was the least recorded.

However *Drosophila phorticella setiata* were found to be abundant in both the localities of the collected site, *i.e.*, Ghandhibhavana and as well as Madhuvana (Table 2). The commonly found 7 species in the assessments were *Drosophila melanogaster, Drosophila bipectinata, Drosophila simulans, Drosophila malerkotliana, Drosophila neonasuta, Drosophila nasuta, Drosophila phorticella setiata.* Of the 9 species

captured, *Drosophila brundavensis* was recorded only in Madhuvana. The most commonly found species with increasingly more in number next to *D* phorticella *setiata* were *D. melanogaster*, *D. malerkotliana,* and *D. simulans,* which means that these four species are more adapted to the prevailing environment (Figure 1). The sampling size varied with the season and temperature. The flies collected in rainy was abundant and winter was least, respectively, while in summer the collection was intermediate in number. Thus, the temperature has an impact on the development and distribution of the flies (Figures 1 and 2).

Table 2. Represents the Simpson-Weiner values for the species diversity.

	Madhuvana	Gandhibhavana
Taxa	9	8
Individuals	1699	1459
Simpson 1-D	0.858	0.852
Shannon-Weiner	2.017	1.983

As per Table 2, Simpson index value is 0.858 in Madhuvana and 0.852 in Ghandhibhavana, and Shannon Wiener values are 2.017 in Madhuvana and 1.983 in Ghandhibhavana. The Simpson values represent higher diversity, as "0" represents infinite diversity and "1" represents no diversity. However, it is vice versa in the case of Shannon Weiner-indices as the Weiner-indices more than "1" represents higher diversity (Ludwing and Reynold, 1998).

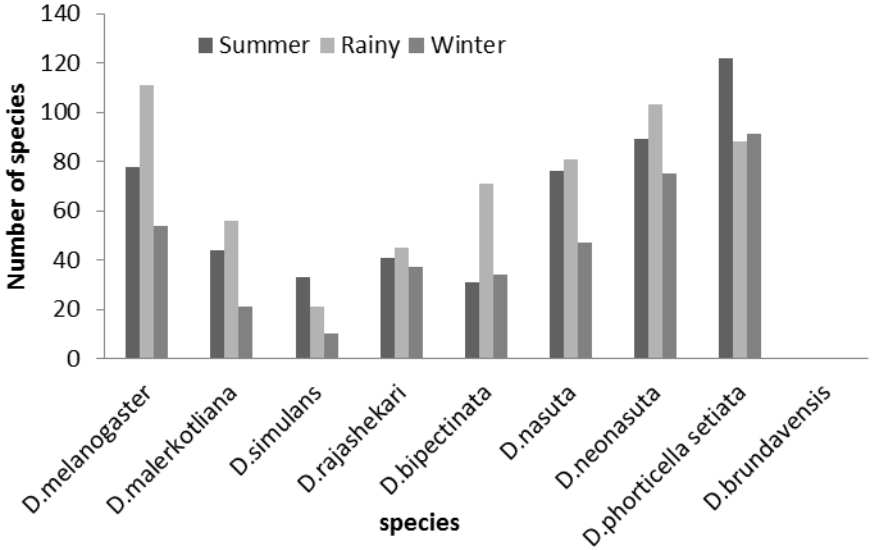

Figure 1 Top Left. Total number of flies collected at Madhuvana in different seasons.

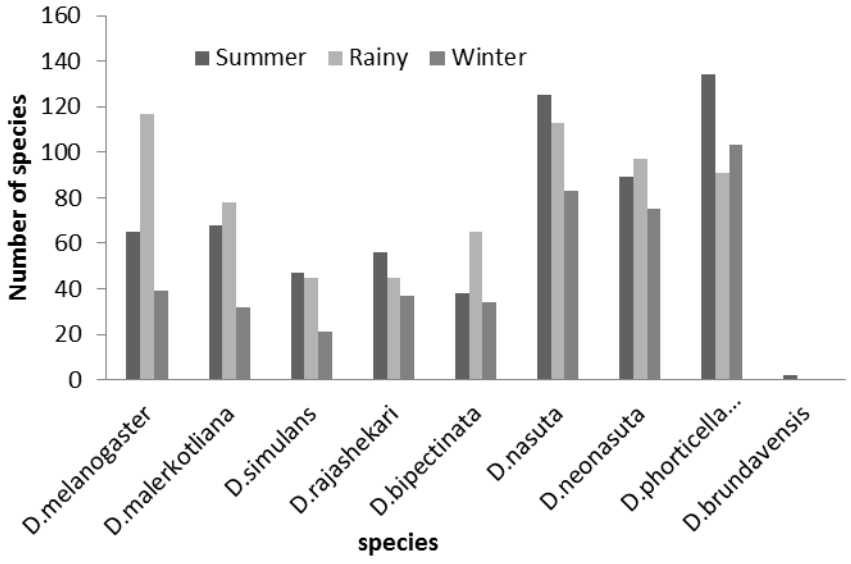

Figure 2 Bottom Left. Total number of flies collected at Gandhibhavana in different seasons.

Discussion

The changes in the natural environment caused by the alteration of seasons would result in the change in the relative frequency of different species from season to season. In tropical areas, especially in Brazil, changes in the environment are caused by the alteration between the dry and rainy seasons (Dobzhansky and Pavan, 1950). Species that are habitat specialists make up much of biodiversity, but the evolutionary factors that limit their distributions

have rarely been considered. Such species are likely to be constrained in their evolutionary responses to future climate changes (Kellermann *et al.*, 2009).

At first sight the flies collected from Madhuvana are increasingly higher in number, as it contains fruiting vegetation. But in Ghandhibhavana the number of flies collected was less as there is very little fruiting vegetation. But as far as the species diversity are connected the Madhuvana and Ghandhibhavana consists in a total of 9 species, which are common in both the collected localities of the present study. A better understanding of how different species are affected by current climates and why they sometimes respond differently to climate change is necessary for predicting future effects of climate change (Weatherhead, 2005).

Interestingly, it was also observed that the flies were recorded more in number during rainy season when compared to summer and winter. However, in winter season flies were least recorded. This ensures that the distribution of the flies is mainly effected in nature due to the variation in the temperature. The present study also implies that the climatic variables such as humidity, rainfall, and temperature are determining factors in the occurrence of Drosophilid species as suggested (Pavan, 1959). The diversity and distribution of the Drosophilids have been affected enormously where human habitat is frequently sensed in Gandhibhavana when compared to Madhuvana. Irrespective of vegetation, seasonal variations also have an impact on population density of Drosophilids. Thus assemblages of Drosophilids are less frequent in numbers at Gandhibhavana, which means that it is prone to be a disturbed gradient with human habitat than Madhuvana.

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Reduced male fertility of the Canton-S strain due to spermiogenic failure.

Kanamori, T.[§], Y. Yasuno[§], T. Tomaru, and T. Takano-Shimizu-Kouno*. Drosophila Genetic Resource Center, Kyoto Institute of Technology, Saga Ippongi-cho, Ukyo-ku, Kyoto 616-8354, Japan; [§]These two authors contributed equally; *author for correspondence (E-mail: fruitfly@kit.ac.jp).

Introduction

Male fertility is a quantitative trait composed of several components and appears to vary considerably among individuals; therefore, it is not a simple matter to quantitatively define the wild type. On the other hand, for a detailed analysis of the reproductive process, standard and marker strains with a normal phenotype are essential. Here, to test the adequacy of strains often used in the study of spermatogenesis, we studied the male fertility of eight strains of *Drosophila melanogaster*, finding a significantly reduced fertility of the Canton-S strain.

Materials and Methods

Flies

We studied eight strains for male fertility: three strains from Bloomington Drosophila Stock Center, w^{1118} (BDSC stock number 5905) and two strains from the *Drosophila melanogaster* Genetic Reference Panel (DGRP 208, BDSC # 25174, and DGRP 301, BDSC # 25175; Mackay *et al.*, 2012); four strains from Drosophila Genetic Resource Center, Kyoto Institute of Technology, Canton-S (DGRC# 105666), Canton-S-brn (DGRC# 109019), *w[*]; P{w[+mC]=dj-GFP.S}AS1/CyO, P{ry[+t7.2]=sevRas1.V12}FK1* (abbreviated here as *dj-GFP*, DGRC # 108217), and *w*; P{ProtamineB-eGFP}1/CyO* (*ProtamineB-eGFP*, DGRC # 109173); and a highly inbred *y w* strain (TT16). *dj-GFP* and *ProtamineB-eGFP* are used as a sperm tail and a sperm nucleus marker, respectively.

Male fertility test

Three- to five-day-old males were individually placed with a single female of the same age in a vial (day 0) and males were removed the next day (day 1). The females were transferred to new vials on days 2, 4, 7, 10, 14, 18, and 22 and then removed on day 29. They were thus allowed to lay eggs for 29 days in a total of eight vials, and all offspring were counted. We used DGRP 208 and *y w* strains as female parents and made twelve replicate crosses for each combination of female and male strains. All crosses (2 female strains × 8 male strains × 12 replicates = 192) were done simultaneously.

Microscopic examination

Squashed testes were prepared essentially as described in Pisano *et al*. (1993). Adult testes were dissected in 0.7% NaCl and squashed with a coverslip. Sample slides were quickly frozen in liquid nitrogen and the coverslips were removed with a razor blade. Samples were fixed by methanol at –20°C for 5 min and then by acetone at –20°C for 1 min. They were immersed in PBST, washed twice in PBS, and mounted with Vectashield containing DAPI (Vector Laboratories). Microscopic examination of testes was performed under a Nikon Eclipse 80i microscope, and micrographs were processed with Adobe Photoshop CS6.

Results and Discussion

The numbers of male and female offspring were not significantly different in any combination of female and male strains, and we pooled them. In general, the number of offspring from *y w* females was smaller than that of DGRP 208 females (Figure 1); the average number of offspring per cross was 128.1 ± 7.0 in *y w* and 174.1 ± 9.3 in DGRP 208. Indeed, the two-way analysis of variance revealed a highly significant effect of female parents (F = 23.1, $d.f.$ = 1/176, P < 0.001). Offspring were not observed at all in the last vials of 160 out of 192 crosses, and only 32 crosses, including 4 crosses in which parental females died

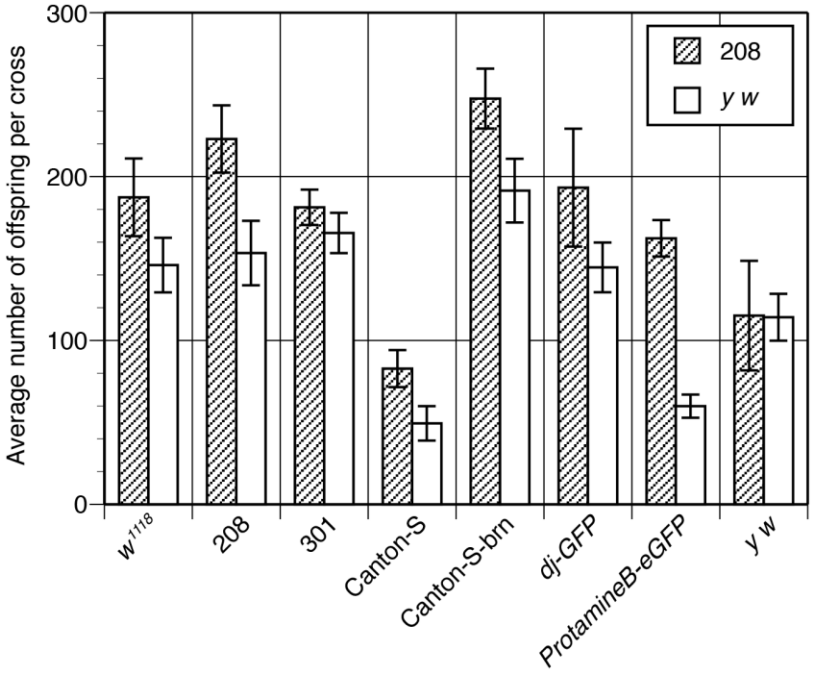

Figure 1. Variation in male fertility among eight strains. The male fertility is defined as the total number of offspring of a single-pair cross, where DGRP 208 (hatched bars) and *y w* (open bars) were used as the female parents. Error bars shown are standard error of mean.

before making the eighth vial, produced offspring in the last vials with an average of 8.3. Therefore, we counted most, if not all, of the offspring, presumably from a single mating. The total number of offspring per cross was used as an index of the male fertility.

The male fertility varied among the eight strains (two-way ANOVA: F = 13.41, *d.f.* = 7/176, P < 0.001); specifically, Canton-S had a reduced fertility. Indeed, the male fertility of the Canton-S strain was significantly lower than *ProtamineB-eGFP* (approximate test of equality of means using the Games-Howell method, the actual difference of 79.5 > the minimum significant difference, MSD, of 74.9 at a 5% experiment-wise level of significance), DGRP 301 (98.4 > MSD of 73.8), DGRP 208 (140.1 > MSD of 113.7) and Canton-S-brn (164.8 > MSD of 103.4) in crosses with DGRP 208 females, and *dj-GFP* (95.2 > MSD of 88.2), w^{1118} (96.6 > MSD of 94.7), DGRP 301 (116.2 > MSD of 76.5), and Canton-S-brn (142.0 > MSD of 107.4) in crosses with *y w* females.

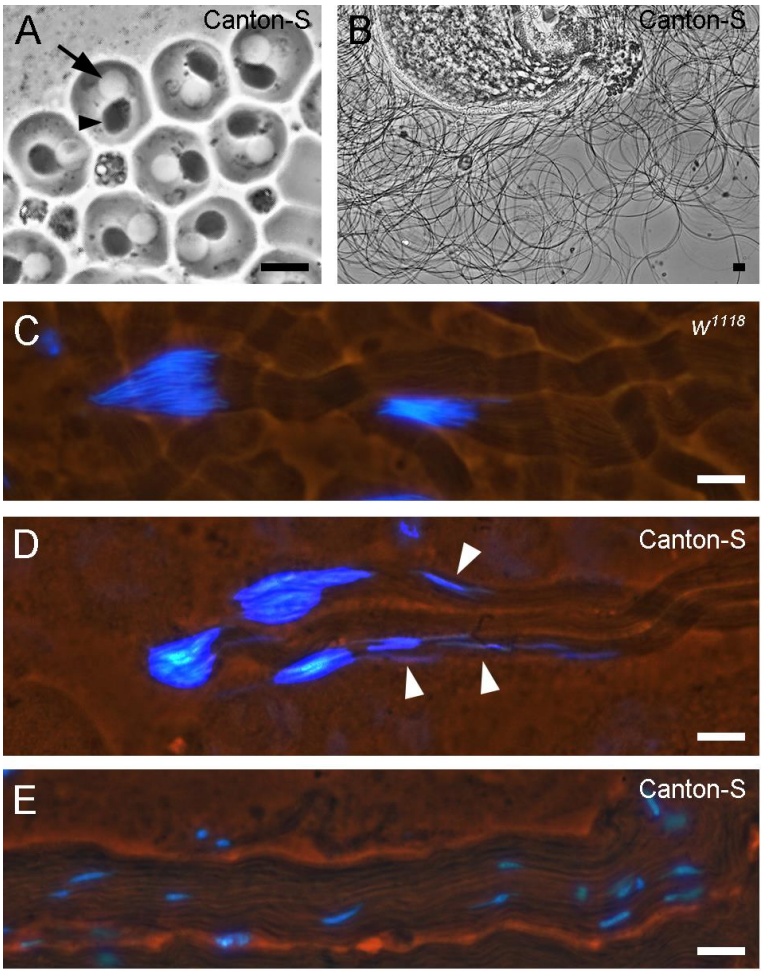

Figure 2. Post-meiotic spermatid differentiation is impaired in Canton-S males. (A–B) are phase-contrast images and (C–E) are DAPI-stained images with illumination of weak transmitted light (phase-contrast). (A) Canton-S onion-stage spermatids showing normal size nuclei (arrow) and nebenkern (arrowhead). (B) Canton-S motile mature sperm dissected from a seminal vesicle. (C) Normal elongated spermatid of w^{1118}, showing aligned nuclei at the head end of the cysts. (D) Abnormal elongated spermatid of Canton-S, showing disorganized alignment of nuclei in the head region. Arrowheads indicate spermatid nuclei that were scattered caudally. (E) Abnormal elongated spermatid of Canton-S, showing scattered and irregular-shaped nuclei in the tail end. Scale bars = 10 μm.

The male fertility still varied significantly even if the Canton-S strain was removed from the analysis (F = 7.45, *d.f.* = 6/154, P < 0.001), although individual comparisons were not statistically significant except for three cases of *y w* females (DGRP 301 *vs. ProtamineB-eGFP*, 105.7 > MSD of 68.6; Canton-S-brn *vs. ProtamineB-eGFP*, 131.5 > MSD of 103.4; *dj-GFP vs. ProtamineB-eGFP*, 84.7 > MSD of 82.3).

To investigate the cause of fertility reduction in Canton-S males, live and fixed testes were examined under light microscopy. Germ cell development of Canton-S males appeared to be normal under phase-contrast optics; spermatocyte growth, meiosis, and spermatid elongation occurred properly, and motile mature sperm were observed in the seminal vesicles (Figure 2 A–B). However, DAPI staining showed conspicuous abnormalities during spermiogenesis. While spermatid nuclei from normal males such as w^{1118} elongated synchronously and were aligned at the head end of the elongated cysts (Figure 2C), spermatid nuclei from Canton-S males were often misaligned and scattered in the tail region of the cyst (Figure 2D). The caudally displaced nuclei were irregular in shape (Figure 2E). This suggests that the reduced fertility of Canton-S males can be attributed, at least partly, to the spermiogenic failure.

Conclusion

The male fertility significantly varied among the eight strains studied here. In particular, the fertility of Canton-S, which has been used as a wild-type control in many studies, was reduced to one-half to one-third of those of most strains. Because Canton-S-brn has a high male fertility, a mutation responsible for the reduced male fertility likely occurred after the Canton-S strain and the Canton-S-brn diverged from each other in about 1980 (Boussy *et al.*, 1998). In contrast, *dj-GFP* and *ProtamineB-eGFP* had the normal range of male fertility, although the fertility of *ProtamineB-eGFP* was lower than normal when crossed with *y w* females. The reduced fertility of the Canton-S strain can be explained, at least partly, by the spermiogenic failure.

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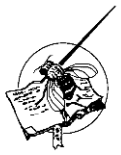

Flavors supplemented in diet regulate the hatchability and viability in *Drosophila.*

Neethu, B.K.[1], Yarajarla Ramesh Babu[2], and B.P. Harini[1*]. [1]Department of Zoology, Drosophila Culture Laboratory, Bangalore University, Bangalore-560 056, Karnataka, India; [2]Centre for Applied Genetics, Bangalore University, Bangalore-560 056, Karnataka, India; E.mail- dr.bpharini@gmail.com.

Abstract

Food additives are substances added to food to preserve flavor or enhance its taste and appearance. The ways of food additives classification, source of nature, food coloring, flavors, taste, which were collected from literature based on structural and biochemical characteristics with description of source and possible effects on *Drosophila* organisms, have been presented. The study reveals significant differences with reference to hatchability and viability on exposure to variant food additives with varying concentrations in *Drosophila melanogaster*. Of the three additives used namely, ajinomoto, turmeric, and vanilla, vanilla has a significant effect on hatchability and viability. Keywords: Food additives, *Drosophila melanogaster*, hatchability, and viability.

Introduction

Food additive is any substance, which is added to, or used as food at any stage to affect its keeping quality, texture, consistency, taste, color, alkalinity or acidity, or to serve any other technological function in relation to food, and includes processing aids in so far as they are added to food, which are common in the food production, have been described in the present review. Food additives and preservatives have been used for thousands of years. In industrialized nations, the last 50 years have seen a significant increase in the number of preservatives and additives introduced to foods before they go to market. The growth in the use of food additives has increased enormously in the past 30 years, totaling now over 200,000 tons per year. Therefore, it has been estimated that today about 75% of the Western diet is made up of various processed foods. Each person is now consuming on average 3.6-4.5 kg of food additives per year. With the great increase in the use of food additives, there also has emerged considerable scientific data linking food additive intolerance with various physical and mental disorders, particularly with childhood hyperactivity and hypersensitivity (Smith, 1991).

To regulate these additives and inform consumers, each additive is assigned a unique number, termed as "E numbers", which is used in Europe for all approved additives. This numbering scheme has now been adopted and extended by the Codex Alimentarius Commission to internationally identify all additives (Bucci, 1995) regardless of whether they are approved for use. This is usually done for the purpose of enriching the

food with supposed beneficial ingredients it did not have originally, which is why the question has been posed: Are these functional foods beneficial or detrimental to our health? There has been growing concern on whether or not these functional foods are any better for the human body as opposed to untreated foods, and for good reason.

Different types of additive are used for different purposes, though many individual additives perform more than one function. For the purposes of both classification and regulation, they are grouped according to their primary function. Ajinomoto is the monosodium glutamate (MSG/E621) added as a flavor enhancer to soups, sauces, and meat-preparation products. Its popularity originates from the tastiness of the Far East cuisine (Populin *et al.,* 2007). Its use has been controversial in the past 30 years because of reports of adverse reactions in people who ate foods containing MSG and were involved in severe food reactions. In 1968 there appeared the first report about the so called "Chinese Restaurant Syndrome" described as a triad of palpitations, generalized weakness, and sensory numbness originating at the nape of the neck with radiation to the arms and back (Kwok, 1968). Turmeric has been used commonly as a spice, food additive, and an herbal medicine worldwide, known as a bioactive polyphenolic extract of Turmeric, curcumin has a broad range of health benefit properties for humans. Curcumin (E100) is the major component of turmeric rhizomes of the *Curcuma longa*, a popular perennial plant cultivated in India, China, and Indonesia, becoming also increasingly popular in the Western society in products such as nutritional food supplements. The active component of turmeric is curcumin, a polyphenolic photochemical with anti-inflammatory, antiamyloid, antiseptic, antitumor, and antioxidative properties. Vanilla is a flavoring derived from orchids of the genus *Vanilla*, primarily from the Mexican species, flat-leaved vanilla (*V. planifolia*). Food additives are used either to facilitate or complement a wide variety of production methods in the modern food supply. Their two most basic functions are that they either make food safer by preserving it from bacteria and preventing oxidation and other chemical changes, or they make food look or taste better or feel more pleasing in the mouth.

The fruit fly, *Drosophila* belongs to the family Drosophilidae, which is recorded as rapid breeder with a lot of eggs and short life cycle. *Drosophila melanogaster* has been a model for examining fundamentally important problems in biology, especially developmental biology (Rubin and Lewis, 2000; Nichols, 2006). *Drosophila* as a model for genetics research prompted scientists to develop media that produced consistently high numbers of offspring. Though food additives may be linked with diseases and health risks, they also preserve nutrient value by providing vitamins, minerals, and other nutrients to foods such as flour, cereal, margarine, and milk which normally would not retain such high levels. Flies have previously been used as an effective model for the role of diet in the regulation of several important physiological parameters, including sleep (Linford *et al*., 2012), baseline locomotion (Parashar and Rogina, 2009), fertility (Lushchak *et al*., 2012), and feeding behaviors (Skorupa *et al*., 2008).

The effect of food additives is well known, but their effects on reproduction and development have received little attention. The primary goal of this investigation was to test the effects of food additives on hatchability and viability of *Drosophila melanogaster*.

Materials and Methods

The fly stocks were routinely cultured in standard wheat cream agar medium in uncrowded condition at 22±1°C (rearing temperature), 12:12 h light and dark periods, and relative humidity of 70%. The *D. melanogaster* (Oregon k) stocks were obtained from National *Drosophila* Stock Centre, Mysore, India. The test flies were cultured in wheat cream agar medium along with different concentrations of the food additives at ambient temperature. For experiments, five pairs of 10 replicates of *D. melanogaster* flies were fed with 3 different concentrations of 0.02, 0.06, and 1.0 g/L of food additives, namely ajinomoto, turmeric, and vanilla, to culture media. Along with treated, the control flies were also setup without additives. Further the data were recorded for the rate of development from the date of egg laying till the adult emergence and subsequently the same set of vials were screened for adult viability through a series of development from larval hatchability, pupariation, and adult eclosion (Ashburner and Roote, 2000; Flagg, 2005).

Results

In the present data flies fed with food additives, namely, ajinomoto, turmeric, and vanilla along with the control flies at different concentrations 0.02, 0.06, and 1.0 have been significantly affected by the food additives in terms of hatchability and viability than control flies.

Ajinomoto most commonly is found in the form of monosodium glutamate, which has been used for several centuries in the Far East as a condiment in savory products. It is a normal constituent of all proteins, an essential amino acid and present in the body. It showed reduced viability in 0.02% concentration when compared to 0.06 g/L and 1.0 g/L, whereas hatchability of flies was more in 0.06% and 1.0% concentration when compared to control ($P < 0.05$).

Turmeric, which is a coloring agent, showed reduced viability with increase in concentration with that of control, i.e., the viability of flies was more in 0.02% concentration when compared to 0.06% and 1.0% with that of control. Hatchability was more in 0.02% and 0.06% but it was less in 1.0% with that of control ($P < 0.05$).

Flies treated with vanilla have been recorded with increased hatchability in all the concentrations and viability was higher compared to the other food additives. The mean hatchability from egg to larvae was much less in ajinomoto and turmeric when compared to vanilla and also the viability of flies was significantly more in vanilla with that of control as shown in Table 1. Therefore, the data reveal that vanilla, a flavoring agent which imparts odor and with defined chemical substances, has sensed with increased potential with reference to egg to adult eclosion significantly in the model organisms.

Table 1. Mean (± SE) Mean life history traits of *Drosophila melanogaster* on exposure to different food additives.

Dose↓	Ajinomoto				Turmeric				Vanilla			
Traits→	Egg	Larva	Pupa	Adult	Egg	Larva	Pupa	Adult	Egg	Larva	Pupa	Adult
Control	10.33 ± 0.86	6.33 ± 0.55	4.66 ± 0.49	2.66 ± 0.40	10.33 ± 0.86	6.33 ± 0.55	4.66 ± 0.49	2.66 ± 0.40	10.33 ± 0.86	6.33 ± 0.55	4.66 ± 0.49	2.66 ± 0.40
0.02	16.20 ± 1.39	6.60 ± 0.63	3.30 ± 0.39	1.60 ± 0.33	37.77 ± 2.10	25.76 ± 3.32	8.60 ± 1.35	4.30 ± 1.35	25.50 ± 3.24	20.50 ± 2.65	5.00 ± 0.82	2.60 ± 0.85
0.06	17.10 ± 1.62	12.30 ± 1.63	6.30 ± 0.55	4.30 ± 0.51	26.33 ± 3.50	20.50 ± 2.65	5.74 ± 0.50	2.53 ± 0.64	34.30 ± 2.76	21.00 ± 2.67	5.60 ± 0.56	2.66 ± 0.40
0.1	25.20 ± 1.61	20.60 ± 1.33	8.60 ± 1.20	7.80 ± 1.09	25.17 ± 2.50	9.75 ± 1.41	4.86 ± 0.64	1.58 ± 0.62	39.30 ± 1.52	27.20 ± 2.76	8.60 ± 1.35	4.30 ± 0.84
ANOVA	F = 11.164	F = 23.182	F = 8.821	F = 16.033	F = 4.632	F = 5.763	F = 3.735	F = 3.014	F = 7.699	F = 9.677	F = 4.173	F = 3.199
	P < 0.05	P < 0.05	P < 0.05	P < 0.05	P < 0.05	P < 0.05	P < 0.05	P < 0.05	P < 0.05	P < 0.05	P < 0.05	P < 0.05

Discussion

Food additives are substances added to products to perform specific technological functions. These functions include preserving, increasing shelf-life, or inhibiting the growth of pathogens, or adding coloring and flavoring to food for interest and variety. It is also a substance or a mixture of substances other than basic foodstuffs, which are present in food as a result of production, processing, or packing. Food additives are added to foods in precise amounts during processing. Existing studies have provided conclusive evidence that a large number of food additives and preservatives produce harmful effects upon ingestion, especially among children. In fact, it is extremely difficult to remove many of these compounds from the body, and the cumulative effects of food additive consumption through a lifetime can lead to a troublesome buildup of toxins over the years, similar to how a septic tank collects grime (Hackworth, 2007).

However, one of the largest and most useful contributions to science remains the genetic sequence of *Drosophila*, the entirety of which has been completed and extensively researched.

Additionally, it gives reasons to why *Drosophila* is practical and efficient for lab experimentation (Roberts, 2006).

The most obvious way by which environmental variation may influence body condition and fecundity is via nutritional effects resulting from variability in food type availability. In general terms, diet effect can be classified as either quantitative (*i.e*., food availability) or qualitative (*i.e*., food composition). With all of the synthetic preservatives and artificial compounds present in the foods on the market today, one cannot help but wonder what side effects may arise when these substances are ingested, particularly if this is done so for extended periods of time.

In contrast to the profound effects of dietary content on endurance, the addition of food additives in the diet has certainly induced the changes in fecundity, hatchability, and viability statistically significant as shown in (Figure 1). The most significant effects were seen in ajinomoto, turmeric, and vanilla, *i.e*., from egg to larvae and pupae to adult, whereas flies in vanilla supplemented diet showed more viability. Boric acid was widely used as a food preservative from the 1870s to the 1920s, but was banned after World War I due to its toxicity, as demonstrated in animal and human studies. During World War II, the urgent need for cheap, available food preservatives led to it being used again, but it was finally banned in the 1950s (McCann *et al*., 2007). These do not include edible substances and products intended to be consumed as such, or substances that have exclusively a sweet, sour or salty taste, *i.e*., ordinary food ingredients such as sugar, lemon juice, vinegar, or salt. The present study has an impact on the overall hatchability and viability in which the concentration of all food additive, vanilla supplemented was considered to be essence for the development of flies.

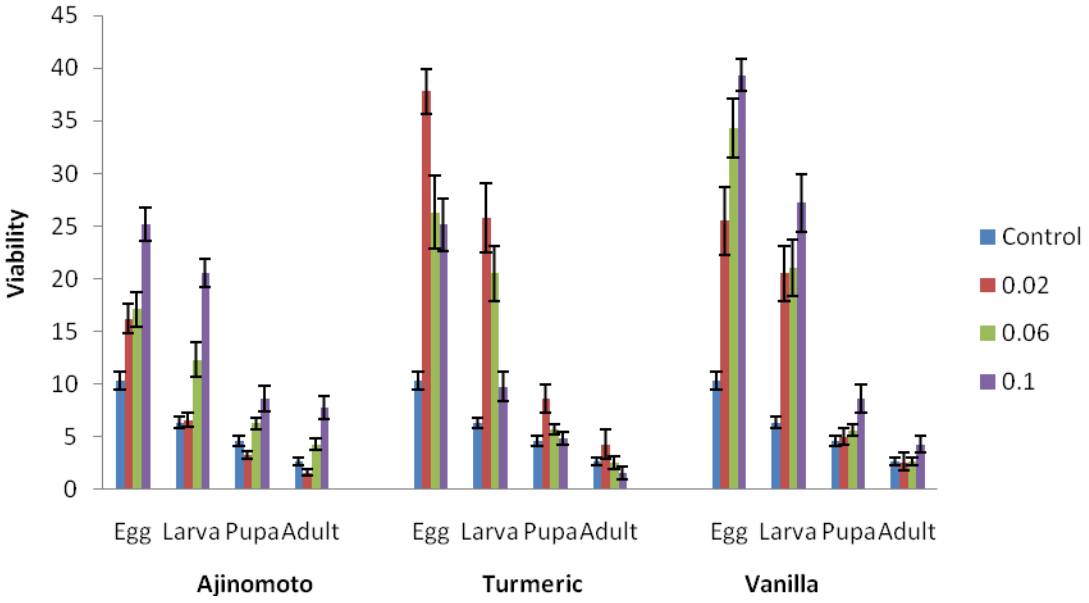

Figure 1. Mean ± SE life history traits of *Drosophila melanogaster* on exposure to three different concentrations of food additives.

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Drosophila suzukii (Matsumura) found on the Greek island Crete.

Máca, Jan. Na Potoce 276, CZ-39181 Veselí n. Luz., Czech Republic.

On demand of dipterist Libor Dvořák (Municipal Museum, Mariánské lázně, Czech Republic), Czech coleopterist Stanislav Benedikt used during his short stay on Crete a very simple beer trap to collect Diptera. The trap consisted of a beer bottle, half-full of beer; it was hung on a shrub branch in the garrigue growth, close to Myrtos at the southeastern shore of the island, 100 m above sea level. The trap had been exposed for one week; drowned dipterans were removed and conserved on 25 March 2014. The author of the present note determined collected drosophilids and the specimens are kept in his collection.

Pertinent data are thus: Greece, Crete, Myrtos env., garrigue, beer trap, 25.3.2014, S. Benedikt leg., J. Máca det. and coll. Nine species were ascertained (Table 1).

Table 1. Pertinent data are thus: Greece, Crete, Myrtos env, garrigue, beer trap, 25.3.2014, S. Benedikt leg. J. Máca det. and coll. Nine species were ascertained, as follows:

Species	Males number	Females number
Drosophila (s. str.) *immigrans* Sturtevant	17	14
Drosophila (s. str.) *hydei* Wollaston	3	3
Drosophila (Dorsilopha) *busckii* Coquillett	1	1
Drosophila (Sophophora) *melanogaster* Meigen	2	12
Drosophila (Sophophora) *simulans* Sturtevant	11	13
Drosophila (Sophophora) *suzukii* (Matsumura)	3	2
Drosophila (Sophophora) *obscura* Fallén	2	1
Drosophila (Sophophora) *subobscura* Collin in Gordon	19	10
Zaprionus tuberculatus Malloch	0	2

The East-Asian *Drosophila* (*S.*) *suzukii* is the most important record. This species invaded both North America and Europe in 2008 and it has been reported also from Brazil (Deprá *et al*., 2014). Unlike other *Drosophila* species, females of *D. suzukii* are able to use their ovipositors to damage intact fruit by perforating its skin and thus initiating its fermentation. Considerable damage of various kinds of fruit has been reported mainly from USA, but also from Italy and France (see, *e.g.*, Cini *et al*., 2012, for more references). In Europe, *D. suzukii* has been reported mostly from its western part: Spain, France, Italy (Calabria *et al*., 2012), Switzerland, Slovenia, Croatia, Austria, Belgium (Cini *et al*., 2012), Netherlands, Great Britain, Portugal, and Hungary (Anonymus, 2012), Germany (Rhineland colonized in 2012, great part of the whole country in 2013; Vogt, 2014), Bosnia-Hercegovina (Ioriatti *et al*., 2014), and Czech Republic (western part; Březiková *et al*., in press). (All countries but only selected quotations of literature are given). The collection from Crete represents the first finding of this colonizing species in Greece and the easternmost European record. Rota-Stabelli *et al*. (2013) have shown some further European countries endangered by possible invasion of *D. suzukii*: Poland, Romania, Ukraine, and Russia, yet at the time of writing the present note (November 2014) this species still did not overcome the boundaries of Germany and Bohemia, with exception of a single record

close to the motorway at the Balaton lake in Hungary (not established). Thus, the countries of Eastern Mediterranean (Levant) seem to be currently at least as endangered as is central Europe.

Precariousness of the predictions concerning the spreading of colonizing species can be illustrated also by the following fact: drosophilid *Chymomyza amoena* (Loew), a previously detected invader, was found in Europe for the first time in 1975, and soon afterwards (1980) it was collected in Serbia. Until now *C. amoena* has invaded most European countries and the western part of Asian Russia up to the Altay Mts. (Máca and Bächli, 1994; Sidorenko and Ivannikov, 2001; detailed information on the distribution see Bächli, 2014), nevertheless it has not been as yet ascertained in Greece, although at least some parts of this country are reasonably well explored (Máca, 2011).

Acknowledgments: Technical help of S. Benedikt (Starý Plzenec, Czech Republic) and L. Dvořák (Mariánské Lázně, Czech Republic) has been much appreciated.

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First report of Drosophilid diversity in an ecotone adjoining Bannerghatta National Park (Karnataka, India).

Dilip A.S, D.S. Pranesh Sujaymeendra[1], R. Alexander, K. Avinash, S. Phalke, and M. Jayashankar*[2]. A Rocha India; Taralu Field Study Centre, #257, Taralu Estate, Taralu Post, Uttarahalli, Bengaluru South Bengaluru- 560082; [1]Jubilant Biosys, Yeshwanthpur, Bengaluru; [2]Department of Entomology and Nematology, Indian Institute of Horticultural Research, Bengaluru -560 089 and Research Advisor, A Rocha India; *Correspondence: jay81zoology@gmail.com.

Drosophila species are thought to have a role as biological indicators of habitat change (Parsons, 1991) and are used as models to study the biological effects of global warming (Francisco Rodríguez-Trelles *et al.*, 1998). *Drosophila* actually means "lover of dew", hence its cosmopolitan distribution is limited by availability of water and temperature. The fruit flies live primarily on rotting plants and fruits and oviposit on unripened/slightly ripened fruit ensuring primary food supply for the newly hatched maggots. Coincidently this habit of the flies has made them major pests (Demerec, 1950; Lutz, 1948; Wilson, 1999; In Miller, 2000; CABI, 2014).

Forests serve as excellent ecosystems for Drosophild diversity world over and there are numerous records in India (Upadhyay and Singh, 2006; Guru Prasad and Pankaj Pathak, 2011; Harini and Pranesh, 2011). During the present study a preliminary effort to record the species diversity of drosophilds was undertaken in the Taralu village (10° 17'-10° 19' N; 76° 39'-76° 44' E) adjacent to the Bannerghatta National Park (BNP) south of Bengaluru, Karnataka, India. Despite its proximity to BNP and dense vegetation no records of drosophilids are available nor has any species inventory been undertaken. Hence, a preliminary study to record the drosophilid diversity was undertaken during the summer months of April and May, 2014. The traditionally followed bottle trapping was used with a smash of ripened fruits of *Musa paradisca* (Banana), *Pyrus malus* (apple), and *Punica granatum* (pomegranate) were used as baited traps. On a first time experimental basis four such traps were tied to twigs of trees 5 feet above the ground and left open in an

orchard at random positions with equidistance. The trapped flies were carefully extracted after securing the mouth of the traps with a cotton cloth and preserved in 70% alcohol. Four successive collections were done in the following week. The species identification was made according to taxonomic groups by employing several keys of Sturtevant (1927), Patterson and Stone (1952), Throckmorton (1962), and Bock and Wheeler (1972).

Table 1. Sub genus of the species recorded at Taralu.

Sl. No.	subgenus	Species
1		*D. takahashii*
2		*D. melanogaster*
3		*D. ananassae*
4	Sophophora	*D. bipectinata*
5		*D. nagarholensis*
6		*D. malerkotliana*
7		*D. rajashekari*
8	Melanogaster	*D. immigrans*
9	Zaprionus	*Phorticella striata*

Nine species of *Drosophila* were recorded in the present bait trapping studies (Table 1). The trap count of species in the order of percent traps *P. striata* (29 %) > *D. rajashekari* (21%) > *D. malerkotliana* (13%) > *D. bipectinata* (9%) > *D. melanogaster* (8%) > *D. nagarholensis* (7%) > *D. ananassae* (5%) > *D. immigrans* (4%) > *D. takahashii* (3%). *P. striata* and *D. bipectinata* were recorded all through the observation period. The latter is a non-drosophilid genus of family Drosophilidae which is endemic to South India (Sajjan and Krishnamurthy, 1975). A rich diversity index (Simpson_1-D = 0.82) was obtaine; however, moderate richness and the evenness of the community was obtained (Shannon_H =1.94) from the analysis using PAST software (version 3). Ecodistributional analysis of *Drosophila* implies the biodiversity of *Drosophila* of a given locality and also the principles underlying adaptive radiation and central mechanisms involved in speciation (Hegde *et al*., 2001; Guru Prasad and Hegde, 2010). In this regard the present report is a first time record of drosophilid diversity from the selected study area. The results certainly need to be related to the proximity of the study area to the Bannerghatta National Park (BNP) one of the last largest remaining scrub forests of the country, placed on the confluence of the Eastern and the Western Ghats (Varma *et al*., 2009). Hence, future studies on eco-dynamics including drosophild diversity of the entire region is relevant and needs to be undertaken.

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Comparison of *ebony* gene from three *ebony* mutants.

Rossi, Fabiana, Luis A. Quesada-Allué, and Martín M. Pérez. Department of Biological Chemistry, FCEyN, University of Buenos Aires, IIBBA CONICET, and Fundación Instituto Leloir, Patricias Argentinas 435, Buenos Aires 1405, Argentina; mperez@leloir.org.ar.

Drosophila melanogaster ebony mutant is easily recognizable by its black pigmentation instead of the normal brown color of the wild type strains. This mutant is defective for the synthesis of β-alanyl derivatives

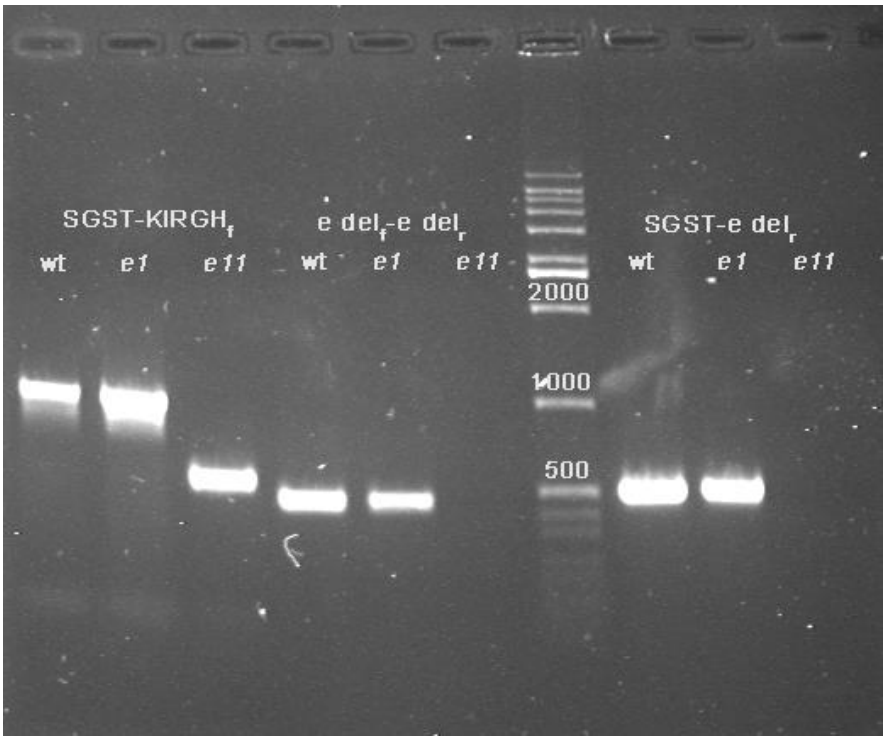

Figure 1. PCR amplification of wt (CS) e^1 and e^{11} cDNA with different primers (see Figure 2). M: DNA markers (numbers indicate the base pairs).

such as N-β-alanyldopamine (NBAD) or N-β-alanyl-histamine (carcinine), which are the products of the conjugation of β-alanine with dopamine or histamine, respectively. Besides body color defect, other well-known features of this mutant are the neurological and behavioral disorders, such as abnormal electroretinograms, lacking "on" and "off" transients (Hotta and Benzer, 1969), reduced phototaxis (Benzer, 1967), and abnormal circadian rhythm (Newby and Jackson, 1991). For many years, Ebony was known as an epidermal enzyme responsible only for tanning and sclerotization of brown cuticles (Wright, 1987). We previously demonstrated that NBAD-synthase is an enzyme induced in epidermis with a narrow window of expression at the beginning of pupariation (Pérez *et al.*, 2002; Wappner *et al.*, 1996a). The enzyme is also induced during the transition from pharate adult to imago and during the first hours after the ecdysis (Pérez *et al*., 2004; Pérez *et al.*, 2010). Recently, we documented the expression of NBAD-synthase in epidermal tissues of *D. melanogaster* embryos (Pérez *et al.*, 2010). In addition to the expression of NBAD-synthase in epidermis, we found that this activity is also expressed constitutively in nervous system (Pérez *et al.*, 2004), suggesting a role in the metabolism of neurotransmitters. Furthermore, it has been postulated that this enzyme functions in a metabolic pathway that may terminate the action of histamine in photoreceptor cells (Borycz *et al.*, 2002; Richardt *et al.*, 2003). Our biochemical and immunohistochemical results demonstrated that NBAD-synthase is widely expressed in the brain. Thus the expression of Ebony in brain regions other than retinas suggests that this enzyme not only plays a role in the metabolism of histamine in visual system but also in the metabolism of other neurotransmitters like dopamine and possibly octopamine and serotonin (Pérez *et al.*, 2002; Richardt *et al.*, 2003; Pérez *et al*., 2004). We previously analyzed NBAD-synthase *in vitro* activity in the *ebony* mutants e^1 and e^4 and we found that they are unable to synthesize β-alanyderivatives (Pérez *et al*., 1997, 2001). We have also cloned and sequenced the e^4 mutant gene, showing that it has a 447 base pair deletion in its first exon, synthesizing a protein without activity due to the lacking of 149 amino acids (Pérez *et al.*, 2001). Some slight physiological differences exist among the different *ebony* mutants, with e^4 being the less drastic phenotype (Newby and Jackson, 1991; Rossi *et al*., data not shown; FlyBase). To address the reason for this discrepancy we characterized molecularly e^1 and e^{11} to better understand their phenotypes.

Flies were from Bloomington stock center, e^1 (stock number 1658), e^4 (stock number 507), and e^{11} (stock number 497); reared on standard corn meal yeast agar medium.

Cell-free synthase activities in slightly purified homogenates from *D. melanogaster* were measured as previously described (Pérez *et al.*, 2002). Briefly, the standard assay for NBAD synthesis contained 2 mM ATP, 5 mM $MgCl_2$, 0.1 mM dopamine, 10 µM β-alanine and 3×10^5 counts/min (cpm) of [14C]β-alanine and 3 µl of enzymatic extract in a final volume of 50 µl, in 50 mM Na-tetraborate-Boric acid buffer, pH 8.3. Catecholamine derivatives were isolated using alumina columns and analyzed by reversed phase HPLC

(Econosphere- C-18, Altech) as previously described (Pérez *et al.*, 2002).

RNA was extracted with TRI-Reagent (SIGMA), purified and used to synthesize cDNA with reverse transcriptase (SuperScript II, Invitrogen) and specific primers. Amplification was with a combination of specific primers named, SGST, KIRGH, EHRQ, (according to the putative amino acid sequence of specific *ebony* regions), eDEL (forward and reverse, which flank the e^4 sequence deletion) and C-term (Figure 2). After electrophoresis, the DNA bands were eluted with Gene Clean (Bio 101), cloned in easy T vector (Promega) and sequenced. Sequence analysis was made with Blast program (NCBI).

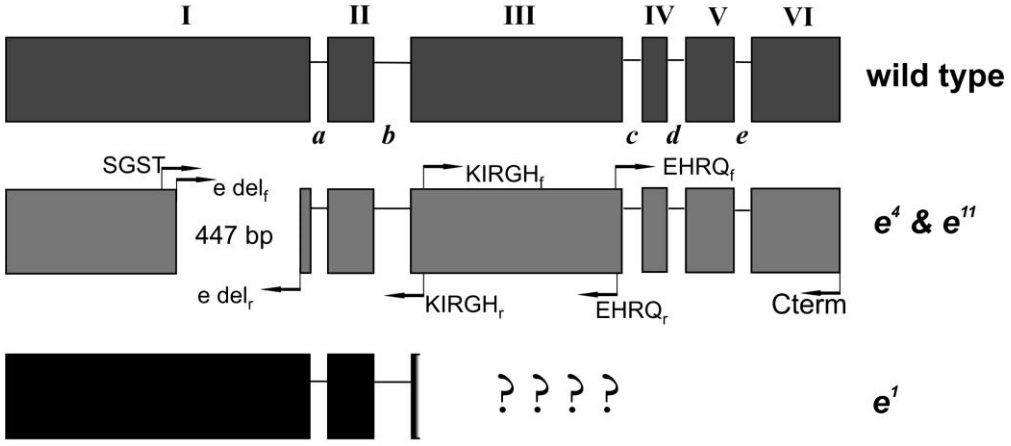

Figure 2. Scheme of e^1, e^4 and e^{11} coding sequence. Boxes represent exons, lines represent introns. Roman numerals indicate exon number, italic lowercase letters indicate introns. Letters and arrows (in e^4 and e^{11} exons) indicate primers and direction of amplification; e del$_f$ and e del$_r$: primers that amplified the sequence deleted in e^4 and e^{11}.

Our results confirm, as expected, that none of these 3 *ebony* mutants were able to synthesize NBAD (not shown). Analyzing the cDNA sequence, we observed that the nature of e^1 defect is different from the previously sequenced e^4 (Pérez *et al*., 2001). The nucleotide sequence of e^1 in the first exon is similar to that of wild type flies. However, we were unable to clone and sequence the e^1 cDNA from the beginning of the 3rd exon to the C-terminal region of the gene. We used different sets of primers (including several poliT primers) and different approaches, but we never were able to amplify and clone this region, thus suggesting that something more complex such as an insertion or inversion occurs. It does not seem a deletion, because otherwise it should amplify the cDNA using a poliT primer. The e^{11} sequence, surprisingly, was similar to that of e^4, with the same deletion of 447 bp in the first exon. As is reported in FlyBase, e^4 flies are the most viable and generally best of the dark alleles, such as e^1 and e^{11}. This suggests that probably e^{11} carries other mutations, in the non-coding sequence of the *ebony* gene or in another site of the genome.

References: Benzer, S., 1967, Proc. Natl. Acad. Sci. USA 58: 1112-1119; Borycz, J., J.A. Borycz, M. Loubani, and I.A. Meinertzhagen 2002, The Journal of Neuroscience 22: 10549-10557; Hotta, Y., and S. Benzer 1969, Nature 222 (5191): 354-356; Newby, L.M., and Jackson 1991, FR. J Neurogenet. 85-101; Pérez, M., N. Castillo Marin, and L.A. Quesada Allué 1997, Dros. Inf. Serv. 80: 39-41; Pérez, M., and L.A. Quesada-Allué 2001, Dros. Inf. Serv. 84: 9-12; Pérez, M., P. Wappner, and L.A. Quesada-Allué 2002, Insect Biochemistry and Molecular Biology 32: 617-625; Pérez, M., J. Schachter, and L.A. Quesada-Allué 2004, Neuroscience Letters 368: 186-191; Pérez, Martín M., Julieta Schachter, Jimena Berni, and Luis A. Quesada-Allué 2010, Journal of Insect Physiology 56: 8-13; Richardt, A., T. Kemme, S. Wagner, D. Schwarzer, M.A. Marahiel, anf B.T. Hovemann 2003, Journal of Biological Chemistry 278: 41160-6; Wappner, P., K.J. Kramer, F. Manso, T.L. Hopkins, and L.A. Quesada-Allué 1996, Insect Biochemistry and Molecular Biology 26: 585-592; Wright, T.R.F., 1987, Advances in Genetics 24: 127-222.

First record of Drosophilids at Nandi Hills, South Karnataka, India.

Pranesh Sujaymeendra, D.S.,* and B.P. Harini. Department of Zoology, Jananbharathi campus, Bangalore University, Bangalore 560056; *Corresponding Author.

Introduction

Biodiversity is a buzz word, which is attracting the researchers to understand the diversity of biological systems. Insects are supposed to be the major contributors and comprise 80% of faunal constellation. More than 3750 different species are now recognised belonging to the family Drosophilidae (Wheeler, 1981); about one-third (1048) of these species have been newly described since the publication of Wheelers catalogue (Wheeler, 1986). The Indian subcontinent, with its subtropical climate and varied physiographic conditions, including variable altitudes and luxuriant flora, offers an abode for the rich and wide distribution of *Drosophila* fauna. During recent years, considerable data have been accumulated regarding faunal composition of Drosophilid species as a result of extensive field collections in different ecological habitat by Ayala (1970a). The Drosophilid flies thus obtained have been utilized for various studies, namely: taxonomic, ecological, genetical, behavioral, and its distribution. A better understanding of how different species are affected by current climates and why they sometimes respond differently to climate change is necessary for predicting future effects of climate change (Weatherhead, 2005). In view of this, this first record surveys Drosophilid fauna distributed in Nandi Hills, South Karnataka, India during 2012 at variable altitudes and to record the impact of seasonal stress on Drosophilid composition.

Materials and Methods

Chikkaballapur District - The collection was made from Nandi hills located just 10 km from Chikkaballapur town and approximately 60 km from the city of Bangalore, Karnataka, India. The hill is visible as a compact block of three rocky outcrops, one of them, Nandidurg, raising some 1478 m. It lies between 12º 53' N – 78º 12' E and is 4,851 ft above sea level. It consists of semi evergreen forest with Nilgiri plantations. An investigation has been presented for different seasons at three altitudes (901 m, 1093 m and 1395 m).

At 901 m: The foot of the hill was occupied with *Magnifera Indica* along with trees such as *Acacia concinna, Acacia catechu, Anacardium occidentale, Vitis ripari, Ficus religosa, Ficus bengalensis, Pongamia glabra, Tectona grandis, Phyllanthus* species and many shrubs including cactus. At 1093 m: The most commonly found are *Andrographis serpelliflolia, Lantana camera, Zizipus jujube, Vitex negundo*. At 1395 m: At the top of the hill includes *Acacia catechu, Vitex negundo, Ficus religosa, Autocarpus integrifolia, Anacardium occidentale, Zizipus jujube.*

Collection procedure

Both bottle trapping and net sweeping methods were used. For bottle trapping, milk bottles of 250 ml capacity containing smashed ripe bananas sprayed with yeast were tied to the twigs underneath to small bushes and trees. Five bottles were kept at each altitude. The following day the mouth of the bottle was plugged with cotton and brought to the laboratory. These flies that were collected in the bottles were transferred to the fresh bottles containing cream of wheat agar medium as food. Net sweeping was also done for collecting the flies using banana rotting fruits with equal quantity of approximately 250 g, which were placed beneath the shaded areas of trees and bushes one day before collection. Such bait as fermenting fruits retains its attractive odor for a long time. The collections were made early in the morning by sweeping in each trap at least three times and transferring samples to six quarter milk bottles filled with standard agar medium sprayed with yeast. Likewise from each collection spot, the flies were collected in ten bottles. Then the traps were brought to the laboratory, flies were isolated, counted, and categorized. The species identification was made according to taxonomic

groups by employing several keys of Sturtevant (1927), Patterson and Stone (1952), Throckmorton (1927), and Bock and Wheeler (1972). The study was conducted during the months of May, August, and December.

Table 1. *Drosophila* collected at the different seasons of Nandi Hills (2012).

Species	Summer A	Rainy A	Winter A
D. melanogaster	133	105	81
D. malerkotliana	65	74	72
D. simulans	136	65	102
D. elegans	1	4	1
D. eugracilis	3	3	0
D. nagarholensis	43	56	6
D. rajashekari	135	72	41
D. bipectinata	76	131	44
D. n. nasuta	67	222	40
D. s. neonasuta	46	148	33
Phorticella striata	46	91	80
D. varietas	6	7	2
Total	757	978	502

Results

The total number of flies recorded at Nandi Hills during 2012 was 2237 with diversified species (Table 1), of which the number of flies observed was higher during rainy season with 978 flies, followed by summer with 757 flies, and winter with 502 flies, respectively.

Of the species recorded, frequency of *D. simulans* was more abundant with 136 flies and the least observed was *D. elegans* with only 1 fly during summer. The density of the species during summer is as follows (Figure 1 a): D. sim > D. raj > D. mel > D. bip > D. nas > D. mal > D. neo > P. str > D. nag > D. var > D. eug > D.ele. In case of rainy season, more flies were observed in *D. n. nasuta* with 222 flies and least in *D. eugracilis* with 3 flies. The density of the species during rainy is as follows: (Figure 1 b): D. nas > D. neo > D. bip > D. mel > P. str > D. mal > D. raj > D. sim > D. nag > D. var > D. ele > D. eug. During winter, more were seen in *D. simulans* with 102 flies and least in *D. elegans* with only 1 fly. *D. eugacilis* was not found during this season. The density of the species during winter is as follows (Figure 1 c): D. sim > D. mel > P. str > D. mal > D. bip > D. raj > D. nas > D. neo >D. nag > D. var > D. ele.

More flies were collected in the mid altitudes during all the seasons of the year with slight variations in the case of *D. rajashekari, D. s. neonasuta,* and *D. simulans* in the high altitudes during summer, rainy, and winter season, respectively.

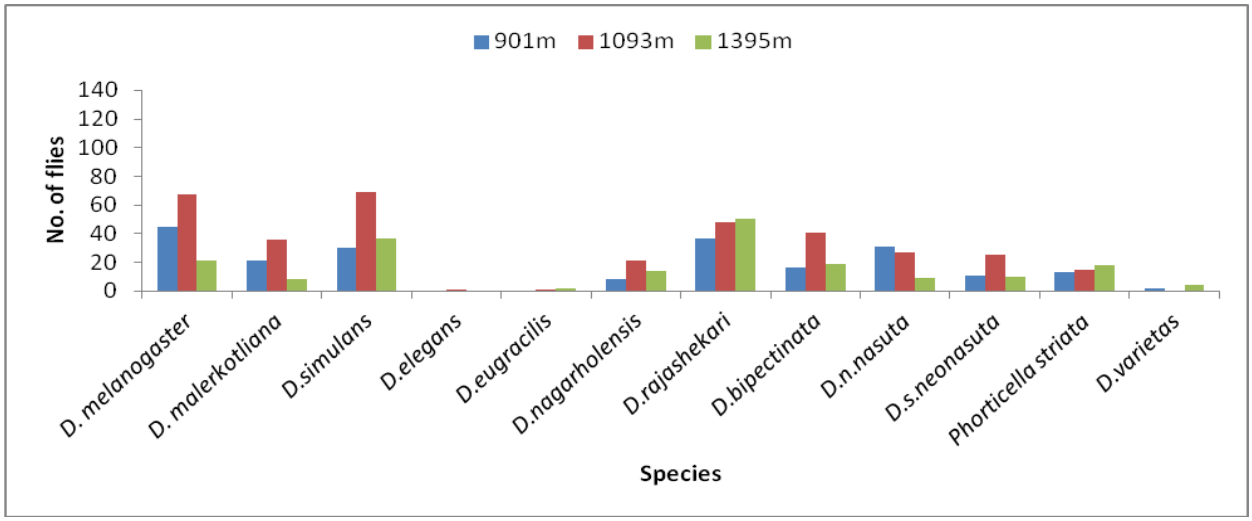

Figure 1 a. Population density of *Drosophila* at different altitudes of Nandi hills (2012) during summer season.

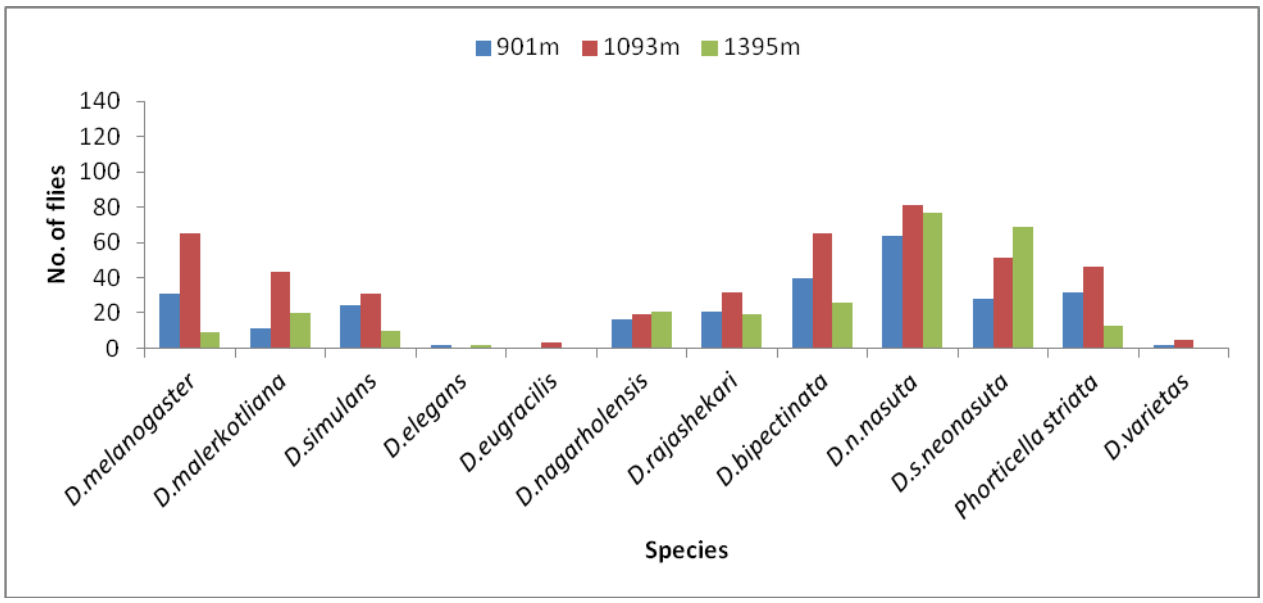

Figure 1 b. Population density of *Drosophila* at different altitudes of Nandi hills (2012) during rainy season.

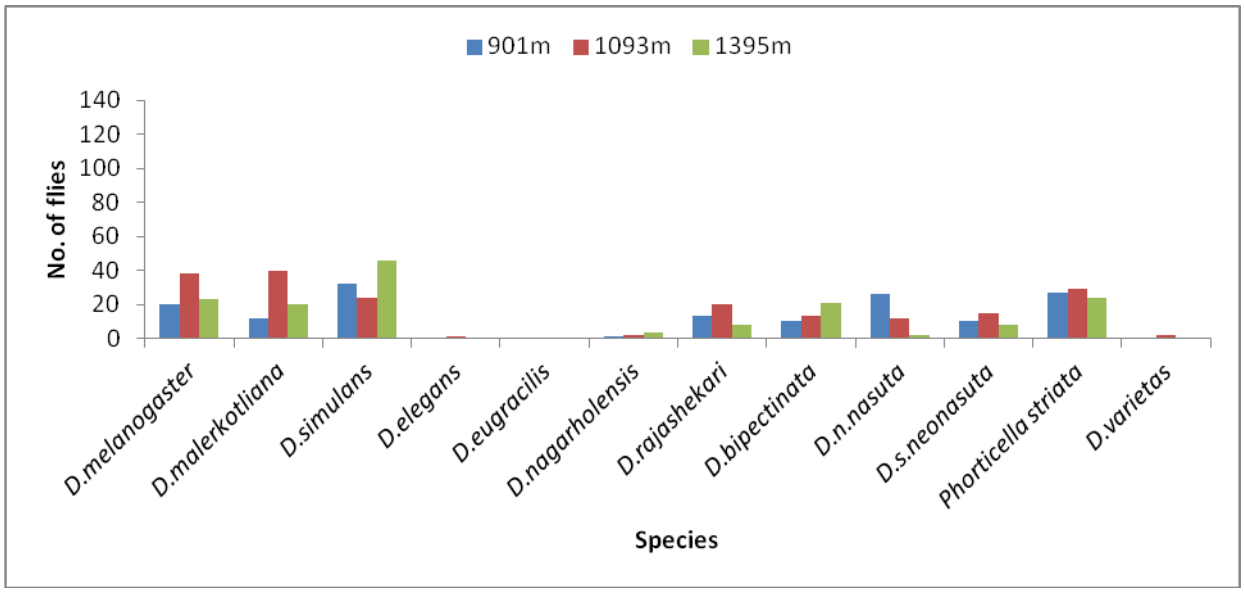

Figure 1 c. Population density of *Drosophila* at different altitudes of Nandi hills (2012) during winter season.

A total of 12 species were found in Nandi Hills (Table 1). Interestingly, the number of *Drosophila* flies decreased with increasing altitude, which indicates that the *Drosophila* community is affected by elevation (Guruprasad, 2006). In addition to this, the abundance of *Drosophila* collected at different altitudes was also recorded to observe species richness and their abundance at various altitudes. *D. melanogaster, D. simulans, Phorticella striata, D. s. neonasuta, and D. n. nasuta* could be assigned as dominant species as they were available at all the altitudes, although the collection of the flies were more in rainy, lesser in winter, and intermediate in summer season. With respect to the vegetation, at the middle region more number of flies were collected, which is proportional to the vegetation, when compared to the other altitudes region.

References: Ayala, F.J., 1970, New York. pp. 121-158; Guruprasad, B.R., and S.N. Hegde 2006, Dros. Inf. Serv. 89: 10-11; Throckmorton, L.H., 1975, In: *Handbook of Genetics* (King, R.C., ed.). Plenum Press, New York, pp. 421-467; TaxoDros, 2010, The database on taxonomy of Drosophilidae. Bächli, G., (org). [2010 April 18]. Available from http://taxodros.unizh.ch/; Weatherhead, P.J., 2005, Oecologia 144: 168-175; Wheeler, M.R., 1981, The Drosophilidae: A taxonomic overview. In: *The Genetics and Biology of* Drosophila (Ashburner, M., H.L. Carson, and J.N. Thompson, Jr., Eds.), Academic Press, New York. 3a: 1-97; Wheeler, M.R., 1986, Additions to the catalog of the world's Drosophilidae. In: *The Genetics and Biology of* Drosophila. (Ashburner, M., H.L. Carson, and J.N. Thompson, Jr., Eds.) Academic Press, New York. 3e: 395–409.

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 31 December of the year of the issue. The annual issue will, therefore, include material submitted during that calendar year. To help us meet this target date, we request that submissions be sent by 15 December if possible, but articles are accepted at any time. Receipt by 31 December is a firm deadline, due to printer submission schedules.

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)-325-2001; email jthompson@ou.edu; FAX (405)-325-7560.

Submission: Manuscripts should be submitted in Word, with pictures preferably in *jpg. To help minimize editorial costs, proofs will not be sent to authors unless there is some question that needs to be clarified or they are specifically requested by the authors at the time of submission. The editor reserves the right to make minor grammatical, spelling, and stylistic changes if necessary to conform to DIS format and good English usage. 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.

New sample of drosophilids from the Font Groga site, Barcelona (Spain).

Pineda, L., C. Esteve, M. Pascual, and F. Mestres*. Dept. Genètica, Universitat de Barcelona, Barcelona (Spain). *Corresponding author: fmestres@ub.edu

A new sample of drosophilids was obtained from Font Groga (Barcelona) on 9th October 2013. This site has been described in detail in Araúz *et al.* (2009) and Canals *et al.* (2013). Flies were netted over 12 baits containing fermenting bananas placed along a trail from 4 to 7 pm. The number of flies classified according to species and sex is presented in Table 1.

Table 1. Number and percentage of adult flies collected in Font Groga (Barcelona, Spain) on 9th October 2013.

Species	Total	Percentage
D. subobscura (♂)	58	12.18
D. subobscura (♀)	240	50.42
D. simulans (♂)	33	6.93
D. menalo/simulans (♀)	87	18.28
D. suzukii (♂)	13	2.73
D. suzukii (♀)	25	5.25
D. immigrans (♂)	1	0.21
D. immigrans (♀)	11	2.31
D. phalerata (♂)	1	0.21
D. phalerata (♀)	5	1.05
Scaptomiza sp.	2	0.42
Total	476	100

The most abundant species is *D. subobscura* (62.60%). This is expected because the sample was obtained during its autumn peak of expansion (Krimbas, 1993). Also interesting is to find again *D. suzukii*, and in a percentage similar (9.20%) to that obtained in 2012 sample (Canals *et al.*, 2013). This species invaded recently many European regions (Calabria *et al.*, 2010) and seems it is well established.

We have finally estimated the species diversity using *H'* (Shannon diversity index) and *J* (Shannon uniformity index). The values obtained were 0.990 and 0.615, respectively. They are similar to those estimates obtained in the same site by Calabria (2012) in autumn 2007 and higher than those of Canals *et al.* (2013) in late autumn 2012.

References: Araúz, P.A., F. Mestres, C. Pegueroles, C. Arenas, G. Tzannidakis, C.B. Krimbas, and L. Serra 2009, J. Zool. Syst. Evol. Res. 47: 25-34; Calabria, G., 2012, Ph. D. Dissertation, Universitat de Barcelona (Spain); Calabria, G., J. Máca, G. Bächli, L. Serra, and M. Pascual 2010, J. Appl. Entomol. 136: 139-147; Canals, J., J. Balanyà, and F. Mestres 2013, Dros. Inf. Serv. 96: 185-186; Krimbas, C.B., 1993, *D. subobscura*. *Biology, Genetics and Inversion Polymorphism*, Verlag Dr. Kovac, Hamburg, Germany.

Chromosomal polymorphism of *D. subobscura*: no differences between wild males and sons of wild females.

Zivanovic, G.[1], C. Arenas[2], and F. Mestres[3,*]. [1]Dept. of Genetics, Institute for Biological Research "Sinisa Stankovic". University of Belgrade, Serbia; [2]Dept. d'Estadística, Universitat de Barcelona, Barcelona, Spain; [3]Dept. Genètica, Universitat de Barcelona, Barcelona, Spain. *Corresponding author: fmestres@ub.edu

When analyzing the chromosomal polymorphism of *D. subobscura* natural populations it is assumed that the information provided by wild males and sons of wild females is equivalent. Thus, using both in the analysis it is possible to increase the sample size. However, it is important to verify whether there are significant differences between both groups or not. The aim of this research has been to statistically compare the results of chromosomal polymorphism of both groups. We have used data from Avala Mountain (Serbia) where *D. subobscura* flies were collected from the 30th May to the 5th June 2011. Avala is located 18 km south of Belgrade and the trapping place is a forest with polydominant communities of *Fagetum submontanum*

Table 1. Frequencies of chromosomal arrangements for wild males and sons of wild females (Avala Mountain population). The number of individuals is indicated by *n.*

Chromosome arrangements	Wild males		Sons of wild females	
	n	%	*n*	%
A_{st}	7	31.8	13	46.4
A_1	10	45.5	8	28.6
A_2	5	22.7	7	25.0
Total	22		28	
J_{st}	7	15.9	15	26.8
J_1	37	84.1	41	73.2
Total	44		56	
U_{st}	2	4.5	6	10.7
U_{1+2}	24	54.6	33	58.9
U_{1+2+6}	13	29.5	12	21.4
U_{1+8+2}	5	11.4	5	8.9
Total	44		56	
E_{st}	7	15.9	15	26.8
E_{1+2}	1	2.3	1	1.8
E_{1+2+9}	20	45.5	25	44.6
$E_{1+2+9+12}$	3	6.8	1	1.8
E_8	13	29.5	14	25.0
Total	44		56	
O_{st}	6	13.6	12	21.4
O_{3+4}	18	40.9	26	46.4
O_{3+4+1}	3	6.8	8	14.3
O_{3+4+2}	2	4.5	/	/
O_{3+4+5}	1	2.3	1	1.8
O_{3+4+6}	1	2.3	1	1.8
O_{3+4+7}	1	2.3	/	/
O_{3+4+8}	5	11.4	2	3.6
O_{3+4+17}	1	2.3	/	/
O_{3+4+22}	6	13.6	6	10.7
Total	44		56	

mixtum at 450 m a.s.l. (Zivanovic and Mestres, 2010). Males and sons of wild females were crossed with virgin females of the Küsnacht strain. Third instar larvae from F_1 were dissected to obtain the salivary glands and the polytene chromosomes were stained and squashed in aceto-orcein solution. In Table 1, we present the chromosomal polymorphism obtained for both groups (males and sons of wild females).

Fisher's exact test was used to compare the chromosomal composition of wild males and sons of wild females (statistically significant *p-value* < 0.05). This test is more precise than chi-squared when the expected frequencies are small (Zivanovic *et al.*, 2014). The corresponding *p-values* were obtained by bootstrap procedure (100000 runs). No significant differences were observed for any chromosome of the karyotype: A (*p-value* = 0.485), J (*p-value* = 0.230), U (*p-value* =0.572), E (*p-value* = 0.536), and O (*p-value* = 0.338). Thus, it seems that the two groups can be grouped together to obtain the chromosomal polymorphism of the population.

References: Zivanovic, G., and F. Mestres 2010, Hereditas 147: 70-81; Zivanovic, G., C. Arenas, and F. Mestres 2014, Russ. J. Genet. 50: 638-644.

Monthly fluctuations in abundance of *Drosophila willistoni* and the relationship with rainfall in the northern region of the Brazilian Atlantic Forest.

Garcia, Ana Cristina Lauer[1], Jordany Gomes da Silva[1], Danúbia Guimarães Silva[1], Martín Alejandro Montes[2], and Claudia Rohde[1]. [1]Laboratório de Genética, Centro Acadêmico de Vitória, Universidade Federal de Pernambuco, Rua do Alto do Reservatório s/n, 55608-680, Vitória de Santo Antão, PE, Brazil; [2]Departamento de Biologia, Universidade Federal Rural de Pernambuco,

Rua Dom Manuel de Medeiros s/n, 52171-900, Recife, PE, Brazil. Corresponding author: alauergarcia@yahoo.com.br

Introduction

The Atlantic Forest comprises communities of trees that reach 35 m in height, which are surrounded by treelets, bushes, and a variety of epiphytical species (Klein, 1978). The biome stretches over a large region, between latitude 4ºS and 32ºS, along a wide array of climatic zones and vegetation physiognomies, from tropical to subtropical (Tabarelli *et al*., 2005).

The Atlantic Forest is subject to a diversity of abiotic conditions that promote endemisms and high species diversity (Campanili and Schaffer, 2010). However, such diversity is exposed to severe conservation issues. The Atlantic Forest stretches along the 4,000-km Brazilian coastline, which is the country's area with the highest population density and where most economic activities take place (Campanili and Prochnow, 2006). These factors are responsible for the historical destruction of the biome. Today, there remains less than 9% of the original cover of the Atlantic Forest (Fundação SOS Mata Atlântica, 2013).

The Neotropical regions, mainly in their forested areas, are home to approximately one third of the world's insect species. These organisms account for over 50% of the genes, of biomass volumes, and of energy transfer phenomena in terrestrial ecosystems. Therefore, conservation of insect populations plays an essential role in the preservation of these habitats (Brown, 1989). More specifically in the Atlantic Forest, insects stand out compared to other organisms due to their high species richness and considerable abundance of individuals. In this sense, drosophilids have become the object of an increasing number of ecological studies (De Toni *et al*., 2007; Cavasini *et al*., 2008; Döge *et al*., 2008; Garcia *et al*., 2008; Mata *et al*., 2008; Gottschalk *et al*., 2009; Bizzo *et al*., 2010; Rohde *et al*., 2010; Garcia *et al*., 2012). Such preference is explained mainly in light of the fact that these flies are extremely sensitive to environmental changes. However, drosophilids make an interesting model also because of practical advantages, which include high population numbers, small size of individuals, short lifecycle, and low capture effort (Powell, 1997; Mata *et al*., 2008; Garcia *et al*., 2014).

Drosophila willistoni is one of the most abundant drosophilids in the Brazilian Atlantic Forest. In spite of that, the species has been but poorly investigated in the northernmost parts of the biome (Gottschalk *et al*., 2008). It belongs to the *willistoni* group, which comprises 23 species and is widely distributed, from the south of the United States down to Uruguay and northern Argentina (Spassky *et al*., 1971; Ehrman and Powell, 1982). Despite this abundance, most ecological studies have not properly addressed the identification of these individuals to species level, since they belong to a group of cryptic species (the *willistoni* subgroup).

The present study investigates the monthly fluctuations in abundance of *D. willistoni* along one year and assesses the relationship of this species' abundance with rainfall in the northern region of the Atlantic Forest.

Materials and Methods

Drosophilids were collected once a month for a year, starting in May 2012, in Dois Irmãos Park (8°0'25.9"S, 34°56'49.1"W), municipality of Recife, state of Pernambuco (PE), Brazil. The area is a conservation unit under the responsibility of the state of PE. It covers approximately 370 ha, where altitude varies between 30 and 80 m a.s.l, and topography is undulated (Machado *et al*., 1998). Vegetation cover is classified as dense ombrophilous forest. Normally, the rainy season starts in March and ends in August, and mean yearly rainfall is 2,400 mm (Andrade-Lima, 1961). The area has been completely enclosed by the city of Recife in its growth process, becoming an "island" of Atlantic Forest inside an urban center (Figure 1).

Drosophilids were captured using the trap described by Tidon and Sene (1988). In each collection, 10 traps containing banana baits were hung from trees 1.5 m above the ground level. Traps were spaced 30 m away from one another along the transect, and were left in place for three consecutive days.

The individuals that belonged to the *willistoni* subgroup were initially sorted from the remaining flies based on external morphology characteristics. The representatives of *D. willistoni* were identified to species

level with the analysis of male genitalia (Wheeler and Kambysellis, 1966), according to Malogolowkin (1952) and Rohde *et al*. (2010). Females of the *willistoni* subgroup were differentially classified to species level based on the number of males identified.

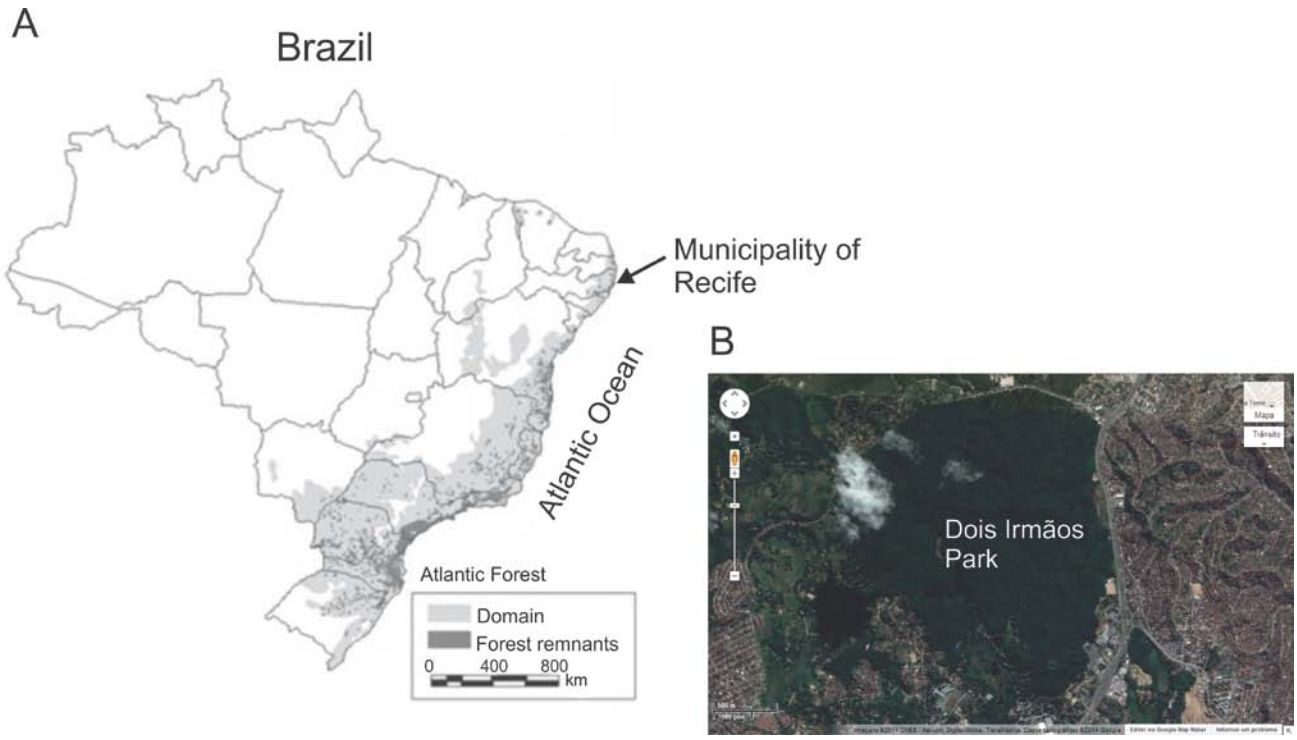

Figure 1. (A) Map of Brazil showing the municipality of Recife and the distribution of the Atlantic Forest biome domain and remnants, according to Fundação SOS Mata Atlântica and Instituto Nacional de Pesquisas Espaciais (2010). (B) Satellite image showing Dois Irmãos Park, where drosophilids were collected, and part of the city of Recife. Source: Google Earth (2014).

Along the collection period, rainfall data in the study site were obtained from the local climate and hydrological resources agency (APAC, 2014). The relationship between abundance of *D. willistoni* and rainfall was evaluated using the Pearson Correlation criterion (r). Qualitative correlation was assessed using the criteria described by Callegari-Jacques (2003), where r values are 0 (no correlation), 0.01 - 0.3 (weak), 0.31 - 0.6 (moderate), 0.61 - 0.9 (strong), 0.91 - 0.99 (very strong), and r = 1 (full correlation). The test was carried out using the Biostat software (Ayres *et al*., 2007).

Results and Discussion

In total, 15,301 drosophilids were collected, of which 13.70% were identified as *D. willistoni*. The lowest relative abundance of the species was recorded in January 2013 (4.66%). In July, September and October, abundance values accounted for over 30% of the individuals collected. The highest number of individuals of the species was captured in July (44.05%). This peak in abundance virtually coincided with the maximum rainfall value (Figure 2).

Significant and strong correlation was observed between absolute abundance of *D. willistoni* and rainfall ($r = 0.66$; $p = 0.02$).

Studies on the ecology of drosophilids that differentiate the representatives of the *willistoni* subgroup to species levels are scarce (Garcia *et al*., 2014). Still rarer are those that aim to shed more light on the

seasonal fluctuations in the numbers of these flies along periods of up to one year. Research has more consistently addressed the abundance of *D. willistoni* in the southern parts of the Atlantic Forest (De Toni and Hofmann, 1995; Garcia *et al*., 2008; Garcia *et al*., 2012). These studies, as observed in the present investigation, for the northern region of the Atlantic Forest, reported that the abundance of *D. willistoni* was greater in periods with higher rainfall.

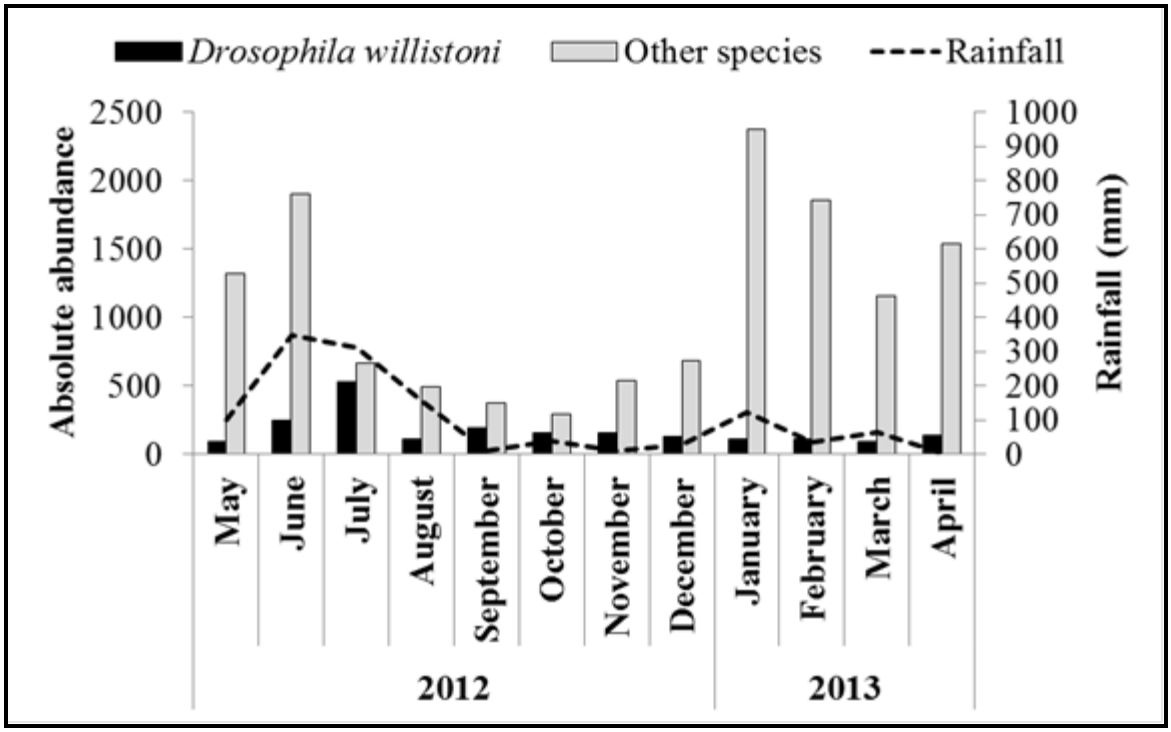

Figure 2. Oscillation in absolute abundance of *Drosophila willistoni* and rainfall along a 12-month period in Dois Irmãos Park, Recife, PE, Brazil.

Garcia *et al*. (2014) investigated the representativeness of *D. willistoni* in periods of varying rainfall in the northern region of the Atlantic Forest in Brazil. The authors detected that the greatest abundance of the species was during the rainy season. The same pattern was observed in the Caatinga biome. Other studies reported similar findings for the Cerrado biome (Valadão *et al*., 2010; Roque *et al*., 2013), but not for the Pampa biome (Poppe *et al*., 2013).

The present study contributes with new information about the representativeness and monthly fluctuations of *D. willistoni* in the northern part of the Atlantic Forest. Since species respond differently to abiotic factors, knowing how they affect populations is essential in order to understand interspecies interactions.

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Lack of evidence for directional selection on *Sex combs reduced* gene in *Drosophila* species differing in sex comb morphology.

Mittleman, Briana E., Amanda B. Abrams, and Mohamed A.F. Noor. Biology Department, Duke University, Durham, NC, USA; bem29@duke.edu.

Introduction

Sex combs are secondary sex characteristics located on the first and second tarsals segments in males of some *Drosophila* species. They are characterized by a set of one or two modulated transverse bristle rows which point distally, and are oriented along the proximo-distal axis of the prothoracic legs. Sex combs can influence male mating success (*e.g.*, Ng and Kopp, 2008). While sex combs may have originally evolved for pushing female wings apart prior to copulation, the combs are now used for varying purposes across *Drosophila* subspecies (Spieth, 1952). For example, in the *obscura* group, sex combs aid in the abdominal attachment of the male to the female. When sex combs have been reduced, copulation rates have consequently dropped (Spieth, 1952).

Dramatic phenotypic variations exist in sex comb morphology between species of *Drosophila*, but the genetic underpinnings of this variation are still poorly understood (Graze *et al.*, 2007). In this study, we focus on three species in the *obscura* group of *Drosophila* differing in the number of sex comb rows per foreleg and number of teeth per comb. Figure 1 illustrates the variation in sex combs of the species examined here. *D. subobscura* males have large sex combs on both the first and second tarsal segments, with the combs lying perpendicular to the tarsus (Beckenbach and Prevosti, 1986). *D. pseudoobscura* have smaller sex combs on both segments than *D. subobscura* (Beckenbach and Prevosti, 1986). Finally, *D. azteca* has a still smaller sex

comb, bearing only 4-5 teeth (Sturtevant and Dobzhansky, 1936), with only a single comb on the tarsal section. Sizes of sex combs vary within species as well.

The *Sex combs reduced* (*Scr*) gene influences the development of sex combs (Kopp, 2011). Here, we examine DNA sequences of *Scr* to infer types of selection operating on the coding region using the ratio of nonsynonymous to synonymous differences within *vs*. between species. Our ultimate goal is to assess the genetic and evolutionary contribution *Scr* may have had on sex comb morphology in *D. subobscura*, *D. pseudoobscura*, and *D. azteca.*

Figure 1. The male sex combs of *D. azteca* (top), *D. pseudoobscura* (lower left), and *D. subobscura* (lower right).

Methods

Stocks:

D. azteca strains used were MSH4 and MSH7, collected from Mt. St. Helena (MSH), California in 2013 by A. Hish. *D. subobscura* strains used were MSH12, also collected from Mt. St. Helena, California (MSH) in 2013, and Seattle, Washington 6, collected in 2011. *Scr* sequences of 11 *D. pseudoobscura* strains were extracted from the genome sequences in McGaugh *et al.* (2012) and are available online in Pseudobase (http://pseudobase.biology.duke.edu/): refer to website for original stock/ collection details.

DNA isolation:

We isolated genomic DNA from *D. subobscura* and *D. azteca* using a standard fly squish protocol (Gloor and Engel, 1992). The *Scr* gene was amplified via PCR using primers (5' - CCTGCTATCCGCAGCAGATGAATC - 3') and (5' - CCAGGACTGTGCATCGGGAC – 3') in 25µl reaction, amplifying a region of approximately 850 bases from the largest exon. The sizes of the PCR products were confirmed on a 1% TBE agarose gel. Samples were purified using ExoSAP-IT (Affymetrix) reactions. The samples were sent to Eton Bioscience for sequencing and submitted to GenBank as accessions KM596822-KM596825.

Data Analysis:

Sample DNA sequences were aligned with the *Scr* sequences from *D. pseudosoobscura* from McGaugh *et al*. (2012) using the ClustalW program in BioEdit (Thompson *et al*., 1994). Analysis was performed using DNAsp (Rozas and Rozas, 1999). A McDonald Kreitman (1991) test and Ka/Ks calculation (Nei and Gojobori, 1986) were done to compare sequences of each pair of the three species and test for evidence of natural selection.

Results

Results of the McDonald Kreitman test are presented in Figure 2. No within-species nonsynonymous variation was detected in the region of *Scr* we examined, and only 2-3 nonsynonmous variants differentiated the species studied. No significant difference was observed in the nonsynonymous to synonymous ratio within

vs. between species. Similarly, Ka and the Ka/Ks ratio were very low (Table 1), indicating strong purifying selection but not showing any clear signal of positive selection on the coding regions of *Scr*.

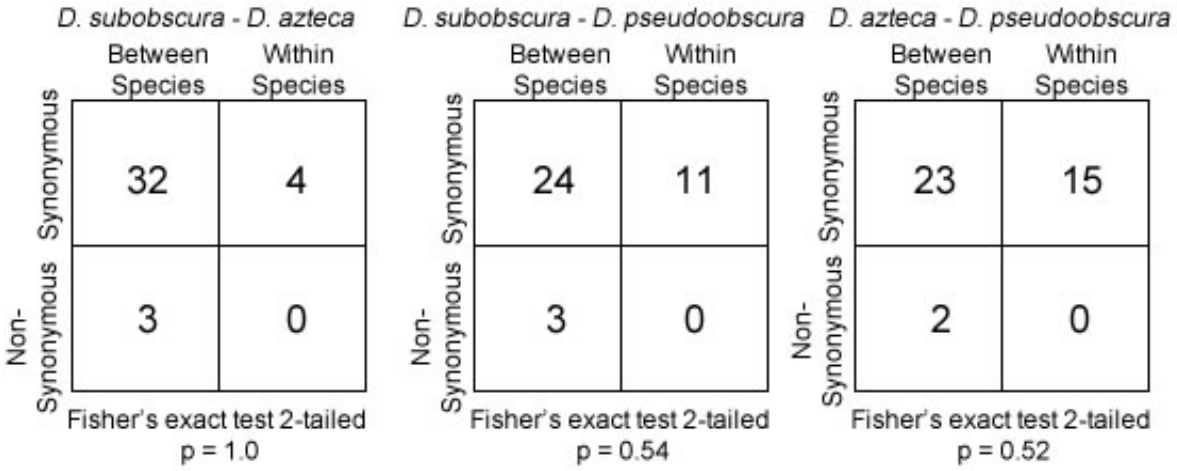

Figure 2. McDonald Kreitman test results.

Table 1. Estimates of Ka, Ks, and Ka/Ks in interspecies comparisons.

	D. subobscura – D. azteca	*D. subobscura – D. pseudoobscura*	*D. azteca – D. pseudoobscura*
Ka	0.0079	0.0087	0.0087
Ks	0.237	0.221	0.225
Ka/Ks	0.028	0.034	0.018

Discussion

While the three *Drosophila* species studied vary dramatically in sex comb morphology, our analysis indicates strong selective constraint but no statistically significant signal for positive selection acting on coding sequence variation in the first exon of the *Sex combs reduced* gene. Despite this result, our analyses cannot completely rule out three means by which *Scr* variation may still affect sex comb variation in these species: 1) via the small amount of nonsynonymous variation observed in our sequences, 2) via differences in the other (much smaller) exon of this gene, or 3) via noncoding regulatory differences among these species. Other genes have also been implicated in sex comb diversity either via correlations in expression (Kopp, 2011) or genetic mapping (Graze *et al.*, 2007). Future research can focus on these amongst other possibilities.

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Disabling *Cdc42* disrupts bristle patterning.

Held, Lewis I., Jr., Samuel A. Billingsley, and Jaime O. Muñoz. Department of Biological Sciences, Texas Tech University, Lubbock, Texas 79409.

Cell rearrangements are known to be involved in sex-comb rotation (Atallah *et al.*, 2009), and they have been implicated in (1) aligning bristles in transverse rows (t-rows) on the 1st-leg tibia (Held, 2002) and (2) ensuring uniform spaces between bristles in longitudinal rows (l-rows) on the 2nd-leg basitarsus (Held *et al.*, 1986). We investigated the role of cell movements in leg development by studying the effects of a dominant-negative allele of *Cdc42*.

Cdc42 encodes a Ras-related GTPase in the Rho subfamily. Other members of this group include *Rac* and *Rho* (Machacek *et al.*, 2009). Genes in the Rho subfamily have been shown to regulate cell polarity, cell shape, and cell movements by modifying the actin cytoskeleton in various ways that are characteristic of the specific genes (Etienne-Manneville and Hall, 2002).

Consistent with the presumption that *Cdc42* mediates cell movements and the hypothesis that cell movements drive sex-comb rotation, t-row fine-tuning, and l-row bristle spacing, we did indeed find disruptions in sex-comb orientation, t-row alignment, and l-row bristle intervals, depending upon the time when we inactivated the *Cdc42* gene.

Materials and Methods

Flies were raised on Ward's *Drosophila* Instant Medium augmented with Fleischmann's live baker's yeast. Culture vials were monitored to prevent larval overcrowding, which can cause developmental delays. Adults were preserved in 70% ethanol. Legs were mounted in Faure's fluid (Lee and Gerhart, 1973) between cover glasses and examined at 400× magnification in a Nikon compound microscope.

One limitation of this mounting technique is that the rigor mortis of the legs constrains their orientation when sandwiched between cover slips. Roughly half of the mounted forelegs have their sex comb suitably positioned (facing up or down versus sideways) so as to permit an assessment of whether the comb has an abnormal "S" shape (see Results). Our minimum sample size was 6 legs per time point (1st-leg pairs of 3 flies), but we examined more flies when needed. Mutant individuals in the earliest cohorts died before eclosion, so those that developed to the pharate adult stage had to be removed from the pupal case before mounting their legs.

Cdc42 was artificially switched OFF at different times by a standard method that involves the transgenic (yeast-derived) components *Gal80ts*, *Gal4*, and *UAS* (Leung and Waddell, 2004), plus incubators at different temperatures (McGuire *et al.*, 2003). The temperature-sensitive (*ts*) mutation *Gal80ts* prevents Gal80 from inhibiting the transcription factor Gal4 at the restrictive temperature of 29°C (Elsaesser *et al.*, 2010) but not at the permissive temperature of 18°C (McGuire *et al.*, 2004). When Gal4 is active, it binds (in *trans*) to an "upstream activating sequence" (*UAS*) and stimulates transcription of any gene attached (in *cis*) to the *UAS* element.

Experimental individuals were obtained as *Dll-Gal4/UAS-Cdc42^{N17}; tub-Gal80ts/+* F_1 progeny of a cross between *w$^+$*; *UAS-Cdc42^{N17}* males and *Dll-Gal4/CyO; tub-Gal80ts* virgin females. Eggs were harvested in one-day laying periods at 22°C (to circumvent paternal sterility at 29°C). In these offspring the expression of the *Gal4* gene is restricted (by its enhancer-trap locus) to the distal tibia and tarsus where its host gene *Distal-less* (*Dll*) is expressed (Wu and Cohen, 1999). *N17* is a dominant-negative allele of *Cdc42* (Luo *et al.*, 1994), which can suppress all *Cdc42* function even though it is only present in heterozygous condition.

At 18°C, *Cdc42* should function normally because *Gal80ts* blocks *Gal4* from activating *UAS* so that there is no transcription of the dominant-negative *Cdc42^{N17}* allele. At 29°C, *Cdc42* should be disabled because *Gal80ts* cannot prevent *Gal4* from activating *UAS* and allowing transcription of *Cdc42^{N17}*. When *Dll-Gal4/UAS-Cdc42^{N17}; tub-Gal80ts/+* larvae or pupae are shifted from 18°C to 29°C, therefore, their *Cdc42* gene

should stop functioning. However, there could be an appreciable time lag until the residual Cdc42 GTPase activity dwindles to a null state—the phenomenon of "perdurance" (Garcia-Bellido and Merriam, 1971; Szabad, 1998).

In order to establish an independent control for assessing delays or other epiphenomena, we conducted a parallel series of temperature shifts using the *Hox* gene *Scr*, whose temporal profile of sensitive periods is known from previous pulse experiments (Held, 2010). For that purpose, we shifted *Dll-Gal4/+*; *UAS-Scr-dsRNAi/tub-Gal80ts* offspring (from a cross of y^1 sc^* v^1; *UAS-Scr-dsRNAi* males with *Dll-Gal4/CyO; tub-Gal80ts* virgin females) from 18°C to 29°C at different times. *Scr* functions at 18°C (*Gal80ts* blocks *Gal4* from activating *UAS*, thus aborting the *Scr RNAi*), whereas *Scr* is blocked at 29°C (*Gal80ts* allows *Gal4* to activate *UAS*, thus expressing *Scr RNAi*)—hence causing 1st legs to look like 2nd legs, which lack sex combs and t-rows.

We also tested whether the loss-of-function effects of *Rac1* and *Rho1* match those of *Cdc42*. For that purpose we performed crosses like those described above, using *UAS-*$Rac1^{N17}$ (on Chromosome 3) or *UAS-*$Rho1^{N19}$ (on Chromosome 1), instead of *UAS-*Cdc^{N17}. We found that these dominant-negative mutations had stronger effects than Cdc^{N17}: $Rac1^{N17}$ individuals that are shifted at or before puparium formation had necrotic, unevenly narrowed legs with clumped, disoriented bristles, while $Rho1^{N19}$ individuals died as pre-pharate pupae, with a few (N = 2) developing to a late enough stage for us to assess the size of their (miniscule) wings. Bristle clumping has been reported for $Cdc42^{N17}$, and tiny wings have been seen with both $Cdc42^{N17}$ and $Cdc42^{L89}$ (Baron *et al.*, 2000). No further data on *Rac1* or *Rho1* are presented here.

Elapsed times at 18°C were converted to equivalent times at 25°C, which is the standard temperature for staging *Drosophila* (Ashburner, 1989), by dividing these numbers by 2.0, which is the ratio of 18°C/25°C rates (Held, 1990). Normalized times computed in this way are reported as ages "@ 25°C." Other abbreviations: h (hours), PF (puparium formation), APF (after PF, with minus signs denoting times before PF), BPF (before PF), ta1-ta5 (tarsal segments 1-5), WPP (white prepupae), and pers. comm. (personal communication).

The WPP stage (0 h APF) marks the start of the pupal period and lasts ~1 h @ 25°C, after which the pupal case quickly turns brown, so it is useful for the precise staging of cohorts. For shifts APF, WPP were collected from 18°C culture bottles and put in moistened test tubes. These tubes were then either placed in a covered 29°C water bath immediately (for a shift time of 0 h APF) or returned to the 18°C incubator for varying durations before transfer to the 29°C bath at a later time. Hence, the ages in our APF cohorts (0, 12, or 24 h APF @ 25°C) varied by $^+$/- 0.5 h @ 25°C.

For shifts before PF, culture bottles were transferred from 18°C to a covered 29°C water bath (for faster equilibration than afforded by air) for ~3 h and then placed on a dry shelf at 29°C. (Leaving bottles at 100% humidity causes delays because larvae seek a dry surface on which to pupariate and will wander for hours if no suitable location is available.) Batches of pupae (WPP and older stages) were then collected from these bottles at either 2-h or 12-h intervals. Hence, the ages in our BPF cohorts (0 to -2, -2 to -4, -4 to -6, -6 to -8, -8 to -10, -10 to -12, -12 to -24, and -24 to -36 h APF @ 25°C) varied by either $^+$/- 1 h or $^+$/- 6 h at 29°C, which is roughly equivalent to developmental times at the standard temperature of 25°C (Held, 2010).

Results and Discussion

When *Dll-Gal4/UAS-*$Cdc42^{N17}$*; tub-Gal80ts/+* flies were raised at 18°C (the "*Cdc42*-ON" state), they eclosed and looked wild-type. However, no such individuals survived to the pharate adult stage when they were raised at 29°C (the "*Cdc42*-OFF" state), following one-day egg-laying periods that were conducted at 22°C (instead of 29°C) in order to circumvent paternal sterility; only their curly-winged (*Dll-Gal4/CyO; tub-Gal80ts/+*) siblings eclosed. This mortality might be due to a subset of *Dll* enhancers that are driving *Gal4* (and hence $Cdc42^{N17}$) in vital organs (of unknown identity) in addition to the legs (Galindo *et al.*, 2011) because null *Dll* mutants die as embryos (Cohen and Jürgens, 1989).

When *Dll-Gal4/UAS-*$Cdc42^{N17}$*; tub-Gal80ts/+* males were shifted from 18°C to 29°C (switching *Cdc42* from ON to OFF) at 12 h APF @ 25°C or earlier, we were surprised to find that they typically displayed S-shaped sex combs (Figure 1, upper panel). This shape bears a striking resemblance to a normal

phase of sex-comb rotation. In wild-type *D. melanogaster* the sex comb begins as an ordinary (horizontal) t-row. It starts rotating toward a longitudinal (vertical) orientation at 16 h APF (Held *et al.*, 2004). By 23 h APF, the comb's midsection has completed about half of its 90° turn, but the termini lag behind, thus giving the comb a sinusoidal shape overall (Atallah *et al.*, 2009). This "S" phase lasts until ~28 h APF, when the midsection has attained an angle of ~65°. Evidently, *Cdc42* function is needed in order for sex combs to proceed beyond this "S" phase.

Figure 1. Effects of disabling *Cdc42* (upper panel) or *Scr* (lower panel) on the male foreleg basitarsus. Numbers along the top denote ages at the time of upshift (in hours after pupariation) from 18°C to 29°C, as normalized to developmental time @ 25°C. Disabling *Cdc42* at any time from -36 to +12 h APF @ 25°C causes sinusoidally shaped ("S") sex combs. Disabling *Scr* at any time from -4 to +12 h APF @ 25°C causes the top end of the comb to curve ventrally like an inverted "J" (≈ upper half of an "S"), while shifts between -12 and -4 h APF reduce comb size and inhibit comb rotation, and shifts between -36 and -12 h APF eliminate the sex comb and all t-rows (basitarsal and tibial) as part of a homeotic transformation of the 1st leg into a 2nd-leg identity (Held, 2010). Basitarsal length stays constant in the *Scr* series, but it decreases by ~50% in the *Cdc42* series for shifts before -8 h APF. Another distinctive (diagnostic) feature of the *Cdc42* series was necrotic (melanotic) tissue at various leg joints (*e.g.*, the tibia-ta1 joint in the "-2 to -4" leg). The intersegmental membranes at such sites were often puffed out like balloons as well (omitted here because they obscure bristle clarity). These effects on joints might be due to a combination of (1) *Cdc42*'s regulation of

(Figure 1, continued). *Rho1* (Machacek *et al*., 2009) and (2) the roles of *RhoGEFs* and *RhoGAPs* in joint development (Greenberg and Hatini, 2011). See Materials and Methods for procedures and genotypes. In all photos the anterior face of the segment is shown, with proximal at the top, distal at the bottom, dorsal to the left, and ventral to the right. All photos are at the same magnification. Scale bar (lower left) = 100 microns. A minimum of six legs was examined for each time point, and the depicted legs are typical examples. Legs from cohorts shifted at 0 to -2, -4 to -6, and -8 to -10 h APF obey the trends shown here and are omitted for the sake of conciseness. Legs from the +24 h APF cohort (not shown) look wild-type, like flies raised continuously at 18°C ("18° Control"). For ease of comparison, the images of all left legs were flipped horizontally to appear as right legs. Combs that are shaped like an inverted-J or "cane" have been described for a variety of other mutants and artificially selected lines, and readers should consult Malagón *et al*. (2014) for incisive mechanistic explanations.

Figure 2. Effects of disabling *Cdc42* on the foreleg tibia. Numbers along the white banner denote ages at the time of upshift (in hours after pupariation) from 18°C to 29°C, as normalized to developmental time @ 25°C.

(Figure 2, continued) The distal half of the foreleg tibia in wild-type flies has ~6 transverse ("t-") rows, numbered 1 to 6 from distal to proximal, in a triangular area. These rows are used as brushes to clean the eyes (Szebenyi, 1969). When *Cdc42* is disabled at 12 h APF, t-rows 3-6 are slightly disorganized. Shifts at 0 or -2 to -4 h APF disrupt t-row 2 as well, and earlier shifts disrupt the entire t-row area. In the youngest cohorts (-12 to -24 and -24 to -36 h APF) bristle density declines severely on the tibia—a phenotype previously reported for the wing, where the sparseness stems from an inhibitory effect of *Cdc42* on the *Notch* pathway that regulates bristle spacing (Baron *et al*., 2000). This link could explain why we occasionally saw duplicated bristle shafts and missing sockets, since the *Notch* pathway also mediates bristle differentiation (Held, 2002). Disorderly t-rows have also been reported for loss-of-function mutations in *Scr* (Held, 2010) and *Egfr* (Held, 2002), and in both cases the sensitive period is after PF. See Materials and Methods for procedures and genotypes. In all photos the anterior face of the tibia is shown, with dorsal to the left, and ventral to the right. All photos are at the same magnification. Scale bar (upper left) = 100 microns. A minimum of six legs from male flies was examined for each time point, and the depicted legs are typical examples. Legs from cohorts shifted at 0 to -2, -4 to -6, and -8 to -10 h APF obey the trends shown here and are omitted to reduce clutter. Legs from the +24 h APF cohort (not shown) look wild-type, like flies raised continuously at 18°C ("18° Control"). For ease of comparison, the images of all left legs were flipped horizontally to appear as right legs.

Figure 3. Effects of disabling *Cdc42* on the midleg basitarsus. Numbers along the top denote ages at the time of upshift (in hours after pupariation) from 18°C to 29°C, as normalized to developmental time @ 25°C. See Materials and Methods for procedures and genotypes. In all photos the anterior face of the basitarsus is shown, with proximal at the top, distal at the bottom, dorsal to the left, and ventral to the right. The row along the right edge of each basitarsus is row 8 (Hannah-Alava, 1958). The bristles of this row have the most orderly spacing of any longitudinal row on wild-type legs (Held, 1979). With successively earlier shifts before PF, the spacing regularity declines, reaching its most chaotic state in the -24 to -36 h APF cohort. Note, however, that such basitarsi are about half the length of control segments, and growth reduction alone—as witnessed for *Cdc42-null* alleles in wings and eyes (Baron *et al*., 2000; Genova *et al*., 2000)—can disrupt bristle spacing (Held, 1990). Likewise, the bristles on these legs are misaligned and are often missing sockets and bracts, so it is hard to determine which effects are direct consequences of *Cdc42* dysfunction (filopodia-associated) and which are coincidental side-effects (not filopodia-associated). All photos are at the same magnification. Scale bar (lower left) = 100 microns. See text for further discussion. A minimum of six legs

(Figure 3, continued). from male flies was examined for each time point, and the depicted legs are typical examples. Legs from cohorts shifted at 0 to -2, -4 to -6, and -8 to -10 h APF obey the trends shown here and are omitted to reduce clutter. Legs from the +24 h APF cohort (not shown) look wild-type, like flies raised continuously at 18°C ("18° Control"). For ease of comparison, the images of all left legs were flipped horizontally to appear as right legs.

Based on Cdc42's role in actin dynamics (Kozma *et al.*, 1995; Nobes and Hall, 1995; Tapon and Hall, 1997; Genova *et al.*, 2000), we propose that Cdc42 is enabling bristle cells to form filopodia, and that these filopodia are needed to drive the sex comb past its "S" phase of rotation, possibly via intercellular signaling (Fairchild and Barna, 2014). Filopodia have been seen on *Drosophila* bristle cells, where they mediate lateral inhibition (de Joussineau *et al.*, 2003). Filopodia are also thought to help align scale cells in moths (Nardi and Magee-Adams, 1986) and pigment cells in zebrafish (Mahalwar *et al.*, 2014), so they might be playing a fine-tuning role here, too. However, the sex comb's bristle cells do not appear to require Cdc42 for alignment *per se* (*i.e.*, chain formation), since most S-shaped combs had single files of bristles. We did see gaps, clumps, and displaced bristles, but they were rare except in younger cohorts.

The *Scr* series also yielded contiguous sex combs, but the comb shapes were different. When *Dll-Gal4/+*; *UAS-Scr-dsRNAi/tub-Gal80ts* males were shifted from 18°C to 29°C (switching *Scr* from ON to OFF) between -4 and 12 h APF @ 25°C, the proximal ends of their sex combs typically curved ventrally. In Figure 1 (lower panel) we denote this shape by an inverted "J" because it resembles the top half of the "S" shape in the *Cdc42* series (Figure 1, upper panel). A similar J-shaped bend occurs when *Scr* is turned OFF by pulses (instead of shifts) between 0 and 18 h APF @ 25°C (Held, 2010). (*N.B.:* The bar labeled "bent" in Figure 2b of that paper was plotted incorrectly; it should range from 6 to 12 h APF.) This "J" distortion disappears with shifts before shifts at -4 h APF, presumably because sex comb size and rotation diminish with successively earlier shifts, as the 1st leg adopts a 2nd-leg identity due to the loss of *Scr* function.

Evidently, *Cdc42* and *Scr* are both permissive agents for rotation, but their roles differ. Both genes appear to enable the proximal end of the comb to overcome a "bottleneck" for cell movements that occurs during the "S" phase (Atallah *et al.*, 2009). The shorter combs from earlier *Scr-RNAi* shifts may avoid this bottleneck by pivoting past the distalmost t-row, instead of colliding with it (Atallah *et al.*, 2009)—a trend that is seen with combs which have been artificially selected for fewer bristles (Malagón *et al.*, 2014).

If so, then why do the shortest combs (7-8 bristles) of our *Cdc42* series (-24 to -36 h APF cohort; N = 8 legs) remain S-shaped? One idea (E. Larsen, pers. comm.) is that these combs lose bristle cells during or after rotation; another possibility (J. Atallah, pers. comm.) is that they get snagged on the tip of the t-row somehow. We don't know why *Cdc42* (but not *Scr*) is needed for the distal end of the "S" to straighten (= a side-effect of $Cdc42^{N17}$'s impact on joints?; *cf.*, necrosis at "-2 to -4" joints in Figures 1, 2, and 3), nor why *Scr* (but not *Cdc42*) is needed for earlier stages of rotation.

The t-rows tell a different story from the sex comb. *Cdc42* is evidently required there in order for bristle cells to form contiguous chains, rather than just for straightening the chains after they arise. Shifts at 24 h APF @ 25°C have no effect, but those at 12 h APF disrupt tibial t-rows 3-6 (9 of 14 legs), where the rows are numbered from distal to proximal (Figure 2). Disruptions include gaps, kinks, zigzags, clumps, and triradii. Shifts between -2 and 0 h APF affect t-rows 2-6 (21 of 22 legs), and earlier shifts (between -4 and -36 h APF) affect all tibial t-rows. A similar wave of disruptions occurs when Epidermal Growth Factor Receptor is disabled (Held, 2002).

Basitarsal t-rows are less regular than tibial t-rows in wild-type flies, so they are less reliable as indicators of misalignment. Nevertheless, they did prove to be informative. They were relatively normal in cohorts shifted at ≥ 12 h APF but were disrupted (like those on the tibia) by shifts ≤ 0 h APF.

Why should $Cdc42^{N17}$ block the concatenation ("self-assembly") of bristles in t-rows (tibial and basitarsal) but not in the sex comb? Conceivably, t-row bristles need more Cdc42 activity than do sex comb bristles, or maybe the *Dll-Gal4* driver is expressed more strongly there, in which case the difference would merely be an artifact (J. Atallah, pers. comm.).

Based on peculiar correlations between bristle spacing and bristle polarity, one of us long ago proposed that bristle cells use filopodia to space themselves at regular intervals in the rows on fly legs (Held *et*

al., 1986). Thus, we wondered whether disabling a putative regulator of filopodia (*Cdc42*) might disrupt bristle spacing. The most orderly longitudinal row on the legs is row 8 on the 2nd-leg basitarsus. Its bristles exhibit a military precision in their intervals.

As shown in Figure 3, we did indeed find spacing irregularities in Row 8 in the earlier cohorts. However, the affected legs also display other anomalies (*e.g*., stunted growth and/or evagination, zigzag bristles, and missing sockets) that confound the analysis. A cleaner test of this hypothesis would be to use a bristle-specific driver (*e.g*., *scabrous*- or *neuralized-Gal4*) with *UAS-Cdc42*^{N17} instead of *Dll-Gal4* (N. Malagon, pers. comm.)—an approach which is now under way.

Acknowledgments: Starter stocks were provided by Konrad Basler (*Dll-Gal4/CyO*), Teresa Orenic (*Gal80ts*), Jeffrey Thomas (*UAS-Cdc42*^{N17} inserted on Chromosome 2), and the Bloomington Stock Center (#50662: *UAS-Scr-dsRNAi* inserted on Chromosome 3). The manuscript draft was constructively critiqued by Joel Atallah, Artyom Kopp, Ellen Larsen, Nicolas Malagon, and Jeffrey Thomas.

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Adult sex ratio in *Drosophila melanogaster* developed in different nutritive conditions.

Filipović, Ljupka[1], Sofija Pavković-Lučić[1], and Tatjana Savić[2]. [1]University of Belgrade, Faculty of Biology, Studentski trg 16, 11000 Belgrade, Serbia; [2]University of Belgrade, Institute for Biological Research "Siniša Stanković", Despota Stefana Blvd. 142, 11000 Belgrade, Serbia.

In most of the animal species, there is approximately equal proportion of females and males (Hamilton, 1967). Sometimes, when one sex is in excess, sex ratio is disturbed. Biased sex ratio is well known for many *Drosophila* species (James and Jaenike, 1990; Montchamp-Moreau and Joly, 1997; Jaenike,

2001; Long and Pischedda, 2005; Price *et al*., 2010; Dyer, 2012). It was investigated in relation to inbreeding (Robinson *et al*., 2014), "selfish" genetic elements (Székely *et al.*, 2014), irradiation and mutagenesis (Ivanov, 2002), addition of antidepressant drug into the food (Fakoorziba *et al.*, 2012), larval density (Santos *et al.*, 1994) and population size (Grechanÿ and Pogodaeva, 1996), female age (Hu *et al.*, 2012) and age of their mates (Long and Pischedda, 2005), sex-differential maturation time, and sex-biased mortality (Székely *et al.*, 2014).

In this note, we examined sex ratio in *Drosophila melanogaster* exposed to different nutritive conditions during development. Flies were collected in their natural habitat and maintained over 13 years on five substrates: standard cornmeal-sugar-agar-yeast substrate (ST), apple (A), banana (B), carrot (C), and tomato (T) (Kekić and Pavković-Lučić, 2003). Flies were kept in optimal laboratory conditions (12 h:12 h light: dark cycle, temperature of ~25°C, relative humidity of 60%, 300 lux of illumination). Thirty to fifty pairs, 4-5 days old, were crossed and laid eggs on their own substrate.

Three experimental groups were formed. In the first experimental group, eggs were transferred and developed on their own substrate. In the second experimental group, eggs from each particular strain were transferred to ST substrate, usually used in laboratory conditions. In the third experimental group, eggs of flies maintained on carrot substrate were transferred to apple substrate, and *vice versa*, since flies reared on "carrot" and "apple" evinced significant difference in developmental time (Filipović *et al*., 2014). There were 5-7 replicates with 60 eggs *per* substrate and *per* experimental group. The emerged males and females were counted. Sex ratio, as the proportion of males and females, was analyzed using *Z-test*.

Proportions of eclosed males and females in three experimental groups are presented in Figure 1. In most combinations, approximately equal proportions of males and females were observed. Significant difference in sex ratio was recorded only for C-ST flies (Z = -5.282, $P < 0.01$), where males were more numerous (58.51%), and for C-A flies (Z = 2.190, $P< 0.05$), where females were more numerous (53.53%) (Figure 1).

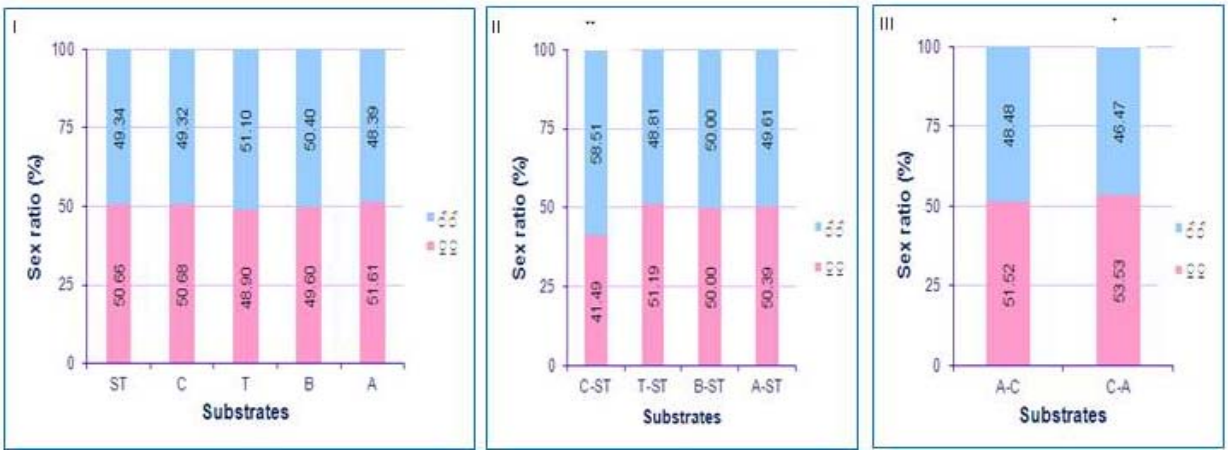

Figure 1. Sex ratio of eclosed flies in the first experimental group (I), second experimental group (II), and third experimental group (III). Abbreviations: ST, cornmeal-sugar-agar-yeast substrate; C, carrot substrate; T, tomato substrate; B, banana substrate; A, apple substrate; C-ST, flies transferred from "carrot" to standard substrate; T-ST, flies transferred from "tomato" to standard substrate; B-ST, flies transferred from "banana" to standard substrate; A-ST, flies transferred from "apple" to standard substrate; A-C, flies "originated" from apple substrate and transferred to carrot substrate; C-A, flies "originated" from carrot substrate and transferred to apple substrate.

It was previously reported that dietary restriction may disturb sex ratio (och Felix Zajitschek, 2012), as well as diet of females (Hu *et al*., 2012). Deviation from 1:1 sex ratio observed in our experiment was recorded only for flies maintained on carrot substrate after transferring to the new nutritional environments. Such sex ratio distortion may arise at least partially as a consequence of different sex-specific mortality in earlier developmental stages in flies maintained on carrot, *i.e*., one sex may be more sensitive to different nutritive conditions during development. This assumption should be further tested in the context of sex-specific nutritional requirements during development and adaptations to new nutritive environments.

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Reanalysis of polytene chromosomes in *Drosophila mojavensis* populations from Santa Catalina Island, California, USA.

Delprat, A.[1], W.J. Etges[2], and A. Ruiz[1]. [1]Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain 08193; [2]Program in Ecology and Evolutionary Biology, Department of Biological Sciences, University of Arkansas, Fayetteville, AR 72701; Alejandra.Delprat@uab.cat; wetges@uark.edu; Alfredo.Ruiz@uab.cat.

One of the four major geographical and host plant associated population groups comprising *Drosophila mojavensis* resides on Santa Catalina Island, California (Heed, 1982; Ruiz *et al*., 1990; Wasserman, 1992; Etges *et al*., 1999). Host cacti used include *Opuntia littoralis*, *O. oricola*, and *O. demissa* (*O. oricola* × *O. ficus-indica hybrids*) (Barbour *et al*., 2007; Beckenbach *et al*., 2008) as other mainland hosts are absent on Santa Catalina Island. Based on initial observations of polytene chromosomes from larvae of a moderate (n = 30) number of wild-caught females in 1981, these flies were reported to be homokaryotypic for second chromosome 2abcfghqrs (ST) and third chromosome 3abd (ST) similar to mainland California populations in the Mojave Desert (Ruiz *et al*., 1990).

In recent analyses of chromosomal evolution using the sequenced genome of Santa Catalina Island *D. mojavensis* (Drosophila 12 Genomes Consortium, 2007) and the recently sequenced *D. buzzatii* genome (Guillén, 2014; Guillén *et al*., submitted), inversion breakpoint analyses of the third chromosome suggested that these Santa Catalina Island flies were actually homozygous for an alternate gene arrangement $3f^2$ (MU = Mulege). Here we analyzed the karyotypes of the sequenced strain from Santa Catalina Island provided by the UC San Diego Drosophila Species Stock Center, stock number 15081-1352.00 and another stock collected from Santa Catalina Island in 2004 by Brian Counterman (SC05) derived from 113 wild-caught adults, including 63 adults reared from *Opuntia* cactus rots. We also made a series of crosses with other populations and conclude that the third chromosome in Santa Catalina Island populations of *D. mojavensis* is uniformly homozygous for gene arrangement $3f^2$ (MU).

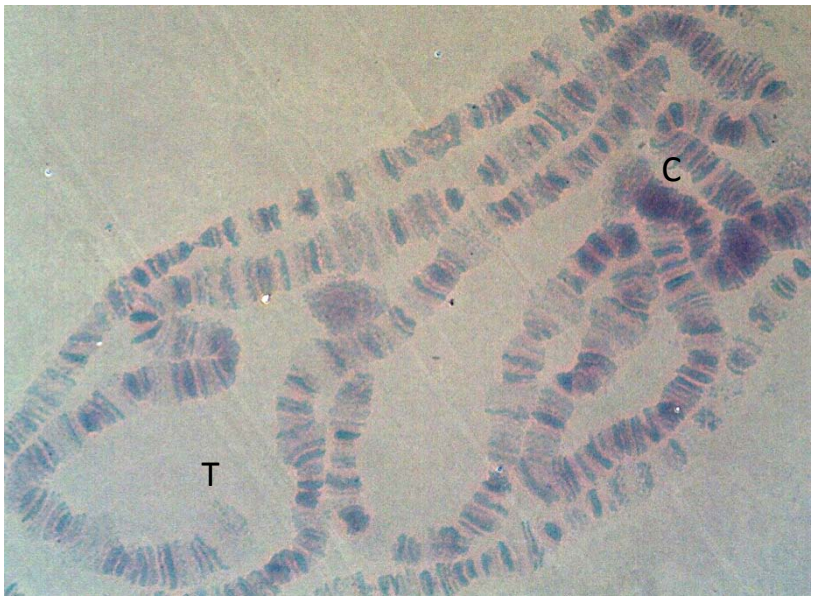
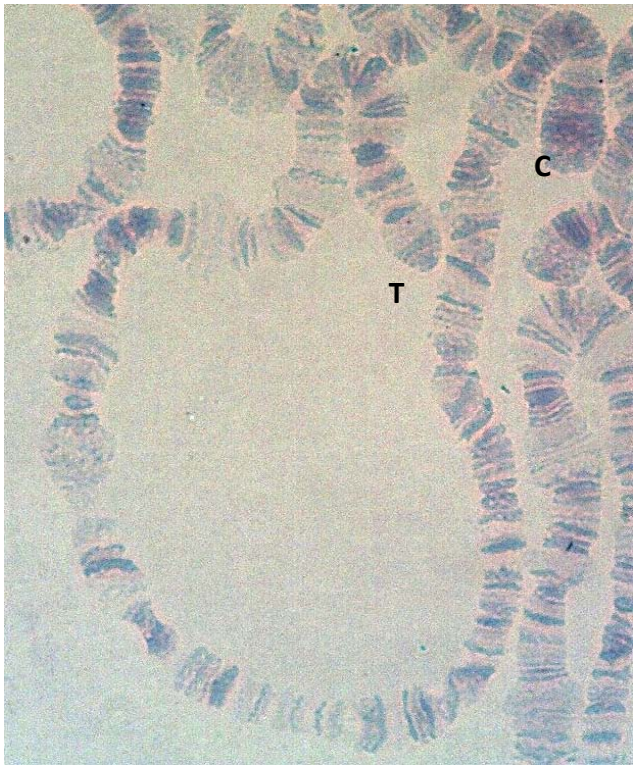


Figure 1. Chromosome 2 ST/ST from Santa Catalina Island. T: Telomere; C: Centromere.

We analyzed 10 larvae from the genome strain. They all had the same karyotype, *i.e.*, homozygous for arrangements 2ST and $3f^2$ (Figures 1, 2; Table 1). Karyotypes of another 30 larvae were analyzed from the more recently collected SC05 stock and revealed the same chromosome configuration, *i.e.*, all were 2ST/ST 3 f^2/f^2 homozygotes (Table 1). Confirmation that Santa Catalina Island $3f^2$ chromosomes were the same as those observed in other parts of the species range was shown in Santa Catalina Island × Punta Prieta reciprocal crosses where third chromosome homozygotes were completely syntenic (Figure 3).



The identity of $3f^2$ was also confirmed by comparing the Santa Catalina Island chromosome 3 with a drawing (Figure 4) of a chromosome $3f^2$ (MU) from stock A564 Santa Rita, Baja California made in 1983 by A. Ruiz.

Figure 2. Chromosome $3f^2/f^2$ from Santa Catalina Island. T: Telomere; C: Centromere.

The other stocks used were: A900 from Santa Rosa Mountains, Arizona; A997 from the Providence Mountains, eastern Mojave Reserve, California; A975 from S. Bahía de Concepción, Baja California; A976 from Santiago BCS, Baja California; PP08 from Punta Prieta, Baja California; LB09 from Las Bocas, Sonora; and PO88 from Punta Onah, Sonora.

We analyzed 4 larvae from strain A975. They were all homozygous 2ST/ST and 3ST/ST. Then we made crosses with 10 virgin females from Santa Catalina Island and 10 males from each of the other stocks of *D. mojavensis*. For the crosses SC05 × Punta Prieta and SC05 × Providence Mtns., we made reciprocal crosses with 10-20 adults of each sex. We cytologically analyzed the larvae of the F_1 and the results are shown in Table 1. A $2q^5$/ST heterozygote ($2q^5$ = LP, the La Paz gene arrangement) is shown in Figure 5. $3f^2$/ST heterokaryotypes from two different crosses with Santa Catalina Island are shown in Figure 6.

Discussion

Based on previous cytological evidence (Ruiz *et al.*, 1990), the sequenced genome of Santa Catalina Island *D. mojavensis* was considered to correspond to the ST arrangements on both chromosomes 2 and 3

(Schaeffer *et al.*, 2008). However, our reanalysis of *D. mojavensis* populations on Santa Catalina Island, California, shows that they are homozygous for second chromosome gene arrangement ST and third chromosome gene arrangement $3f^2$. Thus, the *D. mojavensis* third chromosome drawing shown in Figure 12 of Schaeffer *et al*. (2008) does not actually correspond to the photomap depicted underneath.

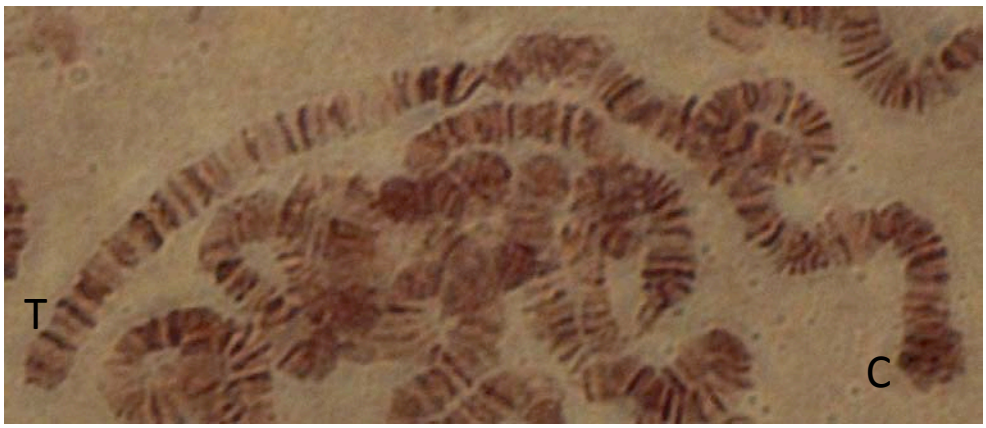


Figure 3. Third chromosome $3f^2/f^2$ homozygotes from the cross Santa Catalina Island × Punta Prieta (PP08), Baja California showing complete synteny. T: Telomere; C: Centromere.

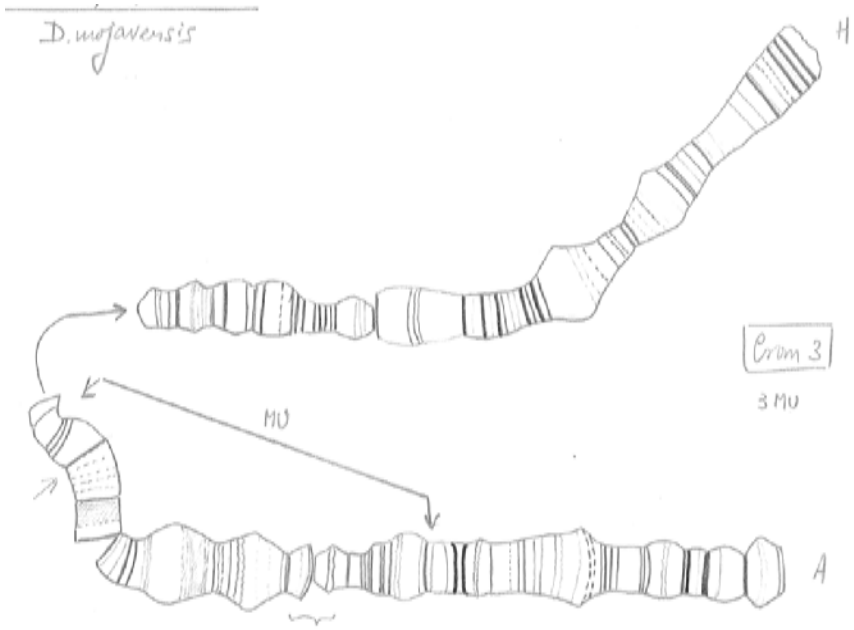


Figure 4. Chromosome $3f^2$ (MU) from A564 Santa Rita, Baja California.

As previous surveys have suggested that all mainland California, Mojave Desert populations, as well as those in southern Arizona are homozygous 3ST/ST or nearly so (Etges and Heed, 1987; Ruiz *et al.*, 1990; Etges *et al.*, 1999), our observations suggest a Baja California origin of the Santa Catalina insular populations rather than mainland California. Northern Baja California *D. mojavensis* populations are polymorphic for $3f^2$ and 3ST, but are currently limited further northwards due to the distribution of its host plant, agria cactus, *Stenocereus gummosus*. No coastal, *Opuntia*-using populations of *D. mojavensis* are currently known in southern California. It is also possible that mainland California and Arizona populations were more polymorphic for the third chromosome in the past and, after colonization of Santa Catalina Island, underwent a reduction in inversion polymorphism with the island founders subsequently going to fixation for $3f^2$. Further, statistical parsimony analysis of mtDNA COI sequence variation suggested Baja California as the probable source of Santa Catalina Island *D. mojavensis* populations (Richmond *et al*., 2013).

Our remaining observations about karyotypic diversity among populations of *D. mojavensis* are largely in agreement with past surveys. The following observations are tentative, however, due to moderate sample sizes. F_1 larvae from crosses involving mainland Sonora populations, *i.e*., SCI × LB09 and SCI × PO88, were all heterozygous for chromosome 2, $2q^5$/ST and 3 chromosome 3, $3f^2$/ST, indicating that these stocks are likely homozygous for $2q^5$ (LP) and 3ST. The cross involving an Arizona population SCI × A900

produced F_1 larvae with both $2q^5$/ST and 2ST/ST, but all were $3f^2$/ST suggesting this stock is fixed for 3ST but still segregating for second chromosome arrangements 2ST and $2q^5$. This stock was collected and karyotyped in 1975 and 1985 and was fixed for 2ST in both collections (Etges and Heed, 1987) suggesting possible laboratory contamination.

Table 1. Chromosomal constitution of *Drosophila mojavensis* stocks and the progeny in crosses in this study.

Populations	Chr 2	Chr 3	n[1]
Santa Catalina Island - 15081-1352.00	2ST/ST	$3f^2/f^2$	10
Santa Catalina Island - SC05	2ST/ST	$3f^2/f^2$	30
South of Bahía de Concepción, Baja California - A975	2ST/ST	3ST/ST	4
Providence Mountains, CA - A997	2ST/ST	3ST/ST	28
Crosses			
Santa Catalina Island × Las Bocas, Sonora - LB09	$2q^5$/ST	$3f^2$/ST	19
Santa Catalina Island × Santa Rosa Mountains, AZ - A900	$2q^5$/ST	$3f^2$/ST	7
	2ST/ST	$3f^2$/ST	3
Santa Catalina Island × Punta Onah, Sonora - PO88	$2q^5$/ST	$3f^2$/ST	10
Santa Catalina Island × S. Bahía de Concepción, BC - A975	2ST/ST	$3f^2$/ST	5
Santa Catalina Island × Santiago, Baja California Sur - A976	$2q^5$/ST	$3f^2$/ST	8
Santa Catalina Island × Punta Prieta, Baja California - PP08	??	$3f^2$/ST	4
		$3f^2/f^2$	10
Santa Catalina Island × Providence Mountains, CA - A997	2ST/ST	$3f^2$/ST	15

[1] number of larvae karyotyped.

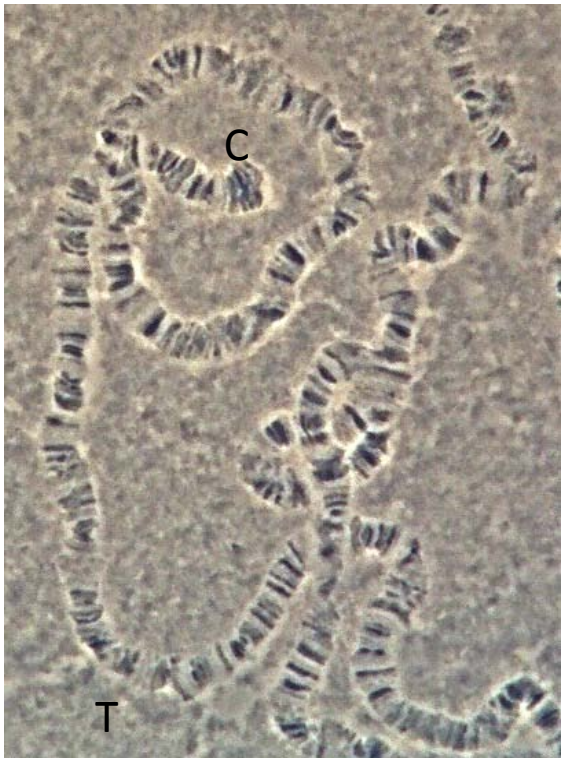


Figure 5. An F_1 heterozygous $2q^5$/ST karyotype from the cross SCI × PO88.

In Baja California stocks, all four larvae from South of Bahía de Concepción, Baja California (A975) were homozygous for 2ST and 3ST, and all SCI × A975 F1s were homozygous for 2ST and heterozygous for ($3f^2$/ST) suggesting this stock is homozygous for 2ST and 3ST. Offspring of wild-caught females from this region in Baja California collected in 1971 and 1974 (Johnson, 1980) were polymorphic for 2ST, $2q^5$ (LP), and SL (San Lucas gene arrangement) suggesting the A975 stock has lost polymorphism in laboratory culture. Similarly, F_1 larvae from crossing SCI to the stock derived from Santiago, Baja California Sur (A976) were all chromosome 2 heterozygotes, $2q^5$/ST, and chromosome 3 heterozygotes, $3f^2$/ST, suggesting this stock is fixed for $2q^5$ and 3ST. However, a collection of wild flies in 1982 revealed that the

frequency of $2q^5 = 0.98$ and $3f^2 = 0.94$, n = 82 (Etges *et al*., 1999) suggesting that our sample sizes were low, and inversion frequencies have changed in culture over many years.

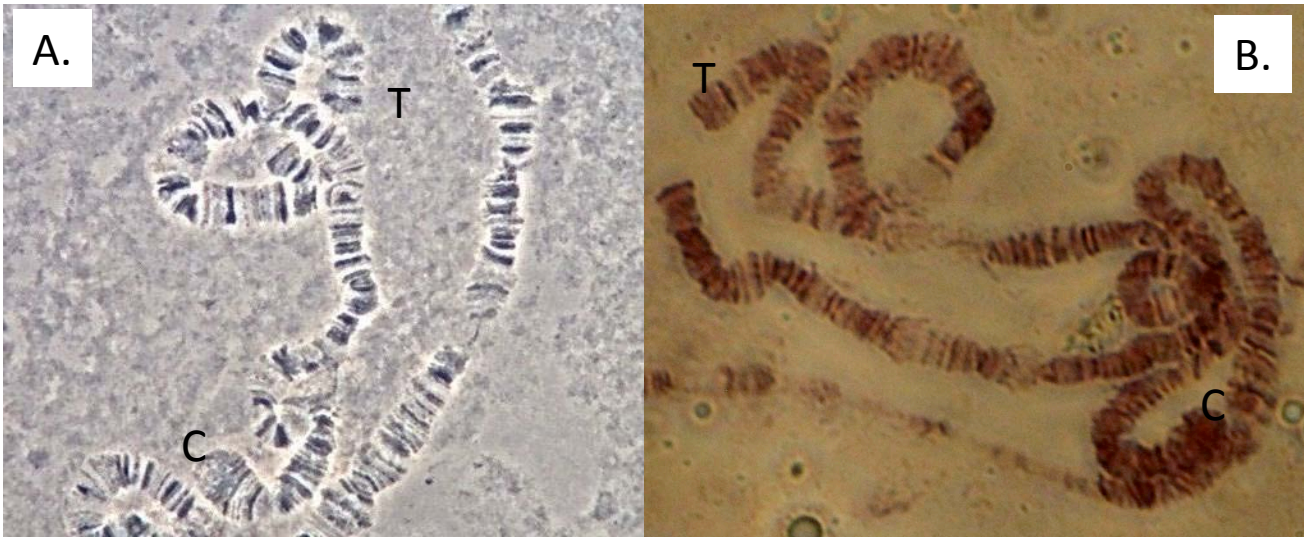


Figure 6. F_1 heterozygous $3f^2$/ST karyotypes from A. SCI × LB09, and B. SCI × Providence Mtns. crosses. T: Telomere; C: Centromere.

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Strain dependent effect of developmental lead exposure on mating latency in *Drosophila melanogaster*.

Wilson, D.[1], B. Possidente[2*], and H.V.B. Hirsch[1]. [1]Biology Department, SUNY Albany, Albany, NY 12222 USA; [2]Biology Department, Skidmore College, Saratoga Springs, NY, 12866 USA; [*]email: bposside@skidmore.edu.

Lead pollution remains a serious public health problem because of continued use in commercial products, release into the environment from industrial sources, and residual background levels from past use (*cf*., Sanders *et al*., 2009). Although it is certain that lead exposure is a public health hazard, we do not understand the mechanisms mediating its harmful biological effects. *Drosophila* can provide a useful model system for investigating these mechanisms (*cf*., Hirsch *et al*., 2012). Hirsch *et al*. (2003), for example, demonstrated that developmental exposure to lead acts in a dose-dependent manner on a number of *Drosophila* traits associated with reproduction, where low levels increase fecundity and female receptivity to mating but higher levels decrease these behaviors. Here we examined effects of developmental exposure to low levels of lead on latency to copulation in two isogenic strains to determine whether there is evidence of genetic variation altering the response to lead treatment for this trait.

Flies from two isogenic strains numbered 60 and 63 that were derived from the "Dover" wild-type strain (*cf*., Possidente, 1999) were raised from eggs oviposited onto Carolina Instant *Drosophila* Medium made with either distilled water (control), 2 ppm lead acetate, or 20 ppm lead acetate. Flies were raised at 25°C in a 12:12 LD photoperiod, collected within five hours of eclosion, and anesthetized with cold for sexing. They were subsequently placed into new vials with fresh medium: in all cases, the lead concentration matched that present before eclosion. Virgin females were housed in groups of three to five while virgin males were housed individually. At four days of age the flies were transferred to fresh control medium for 24 hours and then tested on day five. The vials were coded so that the tester was blind to each mated pair's treatment history. Copulation latency was tested at 25°C by adding a single virgin male and female from the same treatment group to a fresh vial of control medium and timing the interval to copulation. Flies were tested in successive blocks of six pairs representing one pair from each treatment group (two strains by three lead levels). Some blocks contained less than six pairs depending on available sample sizes for each treatment. Pairs that failed to mate within 30 minutes (approximately 25%) were not included in the analysis. Mating success was not affected significantly by lead treatment ($p > 0.05$, non-parametric crosstabs procedure). Table 1 shows the mean copulation latency for each genotype in each treatment group.

Table 1. Effects of PbAc on mean copulation latency (seconds) ± SEM (n pairs).

Treatment	Strain 60	Strain 63
Control 0 ppm	905 ± 128 (7)	998 ± 119 (6)
Lead at 2 ppm	769 ± 82 (13)	1187 ± 104 (6)
Lead at 20 ppm	757 ± 121 (12)	1228 ± 160 (6)

Analysis of variance was used to test main effects of strain, PbAc treatment, and their interaction. There was no significant main effect of lead. There was a significant main effect of strain ($p < 0.03$) with flies from strain 60 mating faster than flies from strain 63. There was a significant interaction between lead and genotype treatments ($p < 0.03$) as lead treatment increased mating speed in strain 60 and decreased it in strain 63. These results are consistent with lead acting in a genotype-dependent manner on behavior in *Drosophila.* This illustrates why averaging results of lead exposure across a diverse population may obscure variations resulting, for example, from genotype-dependent differences in susceptibility to lead or other toxicants. Our results for mating latency are consistent with those of Hirsch *et al*. (2009) who examined effects of developmental lead exposure (15 ppm) on locomotor behavior in 75 recombinant inbred lines of D. *melanogaster*. While they found no overall effect of lead they did observe a significant strain by treatment interaction.

Our results are consistent with Hoffmann and Parsons' (1991) proposal that environmental stress may increase additive genetic variance. Ruden *et al*. (2003) proposed a molecular mechanism that may mediate

such responses to stress and showed that developmental effects of lead exposure (15 ppm) in the same recombinant inbred strains assayed by Hirsch *et al*. (2009) significantly alters transcription at approximately 2,500 loci out of about 19,000 assayed (Ruden *et al*., 2009), including the *cacophony* locus that alters courtship song frequency in males (Schilcher, 1976). It is possible, therefore, that genotype-dependent variation in response to lead exposure results from differential effects on gene expression and possibly differences in sets of loci that respond to lead. Finally, our data are also consistent with the possibility that lead is an endocrine disruptor (Hirsch *et al*., 2010).

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Drosophila fauna of Dharwad District with a report of *Drosophila latifshahi* from South India.

Srinath, B.S.,[1] and N. Shivanna[2*]. [1]Department of Zoology, [2]Department of Applied Genetics, Karnatak University, Dharwad; [*]Corresponding author: drnshivanna@rediffmail.com

Abstract

Drosophila species were collected from different localities of Dharwad district. It revealed a total of 21 species belonging to different groups which includes a rare species *D. latifshahi*, Gupta and Raychaudri, belonging to *Polychaeta* subgroup, for the first time from South India.

Introduction

The fruit fly, *Drosophila* is considered as a model organism for the studies of ecology, evolution, and population genetics. At present the total number of Drosophilids described all over the world is about 4217, which includes 1178 species belonging to genus *Drosophila*, among which 751 species belong to subgenus *Drosophila* and 335 species belong to subgenus *Sophophora*, whereas the remaining 92 species belong to other subgenera (Bachli, 2014). Some species are cosmopolitan and some are endemic to certain regions.

According to Fartyal and Singh (2001) a total of 283 species of Drosophilids have been reported. Among genus *Drosophila* 140 species were reported in India of which south India has only 50 species (Hegde *et al*., 2001). Most of the faunal analysis of *Drosophila* was mainly concentrated on the surrounding areas of Mysore in South Karnataka (Reddy and Krishnamurthy, 1974) and Dandeli and Ambikanagar of North Kanara district in northern part of Karnataka (Nagaraj and Krishnamurthy, 1980; Vasudev *et al*., 2001) and the Western Ghats, which includes Bababudangiri and Kemmannugundi hill ranges (Prakash and Reddy, 1978 1979), Biligirirangana hills (Ranganath and Krishnamurthy, 1972), Charmadi ghat (Gowda and Krishnamurthy, 1972), three different localities of Maharashtra state (Hegde and Krishnamurthy, 1980), Kodagu, Mysore, and Dakshina Kannada (Mangalore) districts (Prakash and Ramachandra, 2008), and so forth. Most of the other localities in South India have remained unexplored. In view of this present study, a survey of *Drosophila* in Dharwad District was carried out.

Materials and Methods

Study Area: Dharwad district lies in the north western sector of Karnataka state with varied climate consisting of rugged foothills (part of Western Ghats) and plain lands (maidan). It lies between the latitudinal parallels of 15° 15' and 15° 35' North and longitudes of 75° 00' and 75° 20' East. Dharwad district is divided into 5 different taluks (Dharwad, Kalghatgi, Hubli, Navalgund, and Kundagol).

Collection of *Drosophila* was carried out from different localities of all the 5 taluks of Dharwad district. Bottle trapping and net sweeping methods were employed for collection as described by Hegde *et al.* (2001). Bottles of the flies collected were brought to the laboratory and were segregated according to their sexes. Male flies were identified by referring to keys given in Markow and Grady (2006). The female flies were cultured for next progeny using wheat cream agar medium to identify the flies (Shivanna *et al.*, 1996).

Results

A total of 21 species were identified from different localities of Dharwad District. Among 21 species 11 species belonged to subgenus *Sophophora* while 6 species belonged to subgenus *Drosophila*; 2 species belonged to genus *Scaptodrosophila*; the remaining species belong to genus *Zaprionus* and *Phorticella*. Among 11 species belonging to subgenus *Sophophora*, 3 belong to *ananassae* subgroup, 4 belong to *montium* subgroup whereas the other individual species belonged to *melanogaster*, *suzukii*, *eugracilis*, *takahashii* subgroups. In the case of subgenus *Drosophila*, 2 species belong to *nasuta* and *polychaeta* subgroups, whereas remaining individual species belong to *immigrans* and *repleta* subgroups, respectively (Table 1). The two rare species belonging to *Polychaeta* subgroup, *D. daruma* Okada and *D. latifshahi* Gupta and Raychaudri, were reported for the first time in Dharwad district, Karnataka of South India.

A few morphological characters of *D. latifshahi* are as below.

Length of imago: male 2.4 mm; female 3.2 mm

Head: Arista has 5 ventral and 4 dorsal branches excluding terminal fork.

Antenna: Dark yellowish; carina broad and sulcate; anterior orbital bristle larger than middle orbital but smaller than posterior orbital bristle; anterior orbital bristle proclinate; middle and posterior orbital bristle reclinate; eye dark reddish.

Thorax: Dark yellowish in color; Acrostichal hairs in 7 – 8 irregular rows; extra pair of dorsocentral bristles present prior to anterior dorsocentral bristle; anterior dorsocentral smaller than posterior dorsocentral (Figure 1a). Sterno-index 0.8; legs yellowish with 3 prominent bristles on the femoral region among which 2 are larger; sex combs absent; preapicals on all tibia (Figure 1b).

Wing: Costa with 2 breaks; humeral crossvein not clear; anterior and posterior crossveins clear (Figure 1d).

Wing Indices:

Male; C – index 1.8; 4V index – 2.1; 4C index – 1.2; 5X index – 1.6

Female; C – index 1.9; 4V index – 2.4; 4C index – 1.4; 5X index – 1.5

Wing indices calculated according to Okada (1956).

Abdomen: Tergites are darkish; Sternites pale.

Periphallic Organ: Genital arch narrower; broader at anterior side with about 32 bristles. Primary surstylus present with 6 – 7 long primary teeth, Secondary surstylus absent; Anal plate oval, elongated and independent of genital arch and detached from surstylus with about 59 – 60 bristles. Toe pointed and heel prominent (Figure 1c).

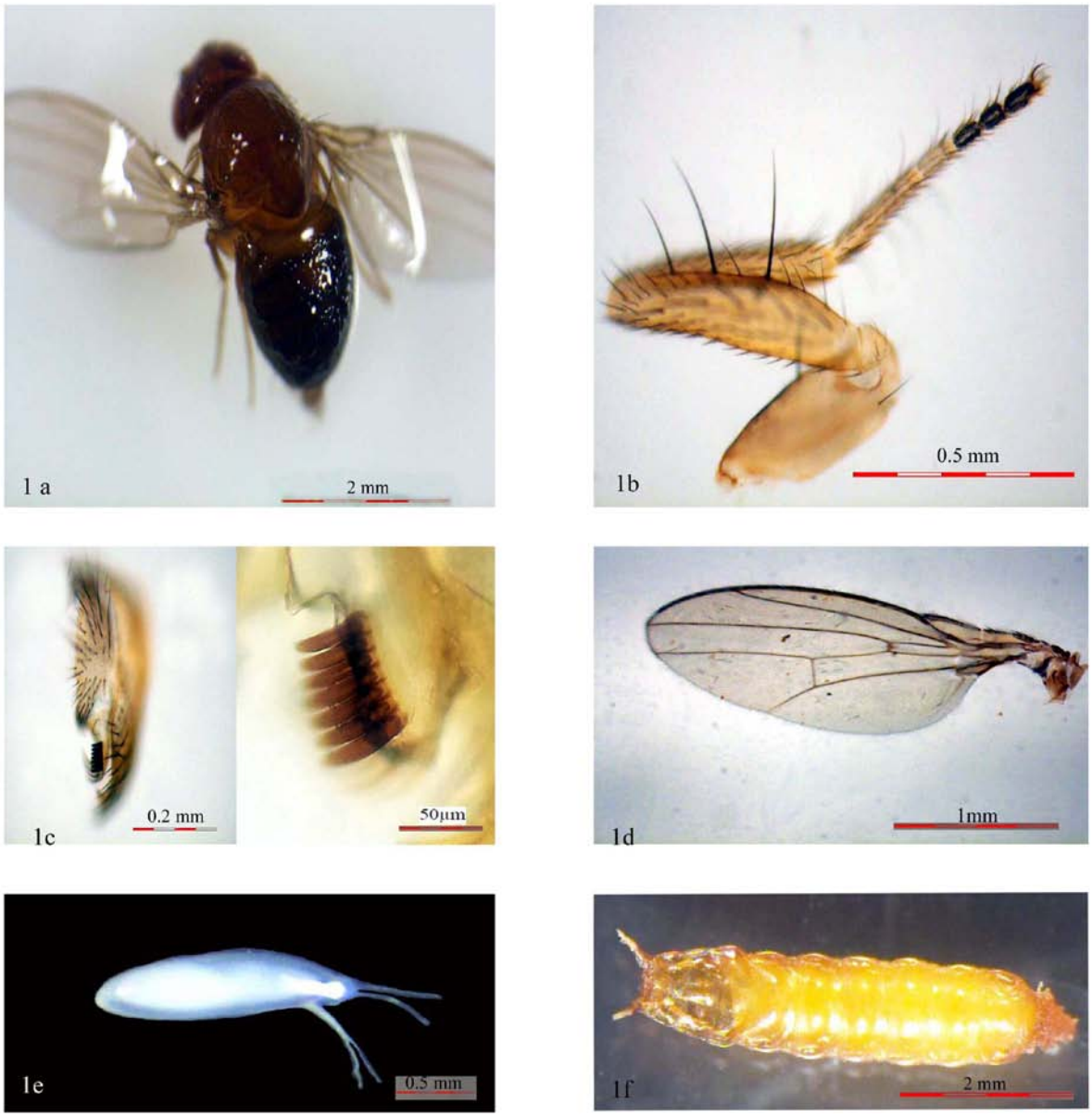

Figure 1. 1a, imago fly; 1b, foreleg; 1c, periphallic organ; periphallic organ primary teeth enlarged; 1d, wing venation; 1e, egg; 1f, pupa.

Phallic organ: Aedal apodeme as long as aedeagus; posterior parameres absent.

Egg: egg with 4 filaments (Figure 1e); Egg guide: Slender at anterior and broader at posterior end with about 14 small marginal teeth; Pupa: yellowish consists of 16 – 20 spiracles (Figure 1f).

Specimens examined: 2 ♂♂, 1♀, India: Karnataka, Dharwad, Jogyellapur, 15°23’31.25’’ N and 75°00’45.96’’ E, 2240 ft. Coll. Srinath B.S 2012

Distribution: India, Bangladesh, China

Table 1. Different species of *Drosophila* collected from Dharwad District.

Genus *Drosophila* Fallen 1823
Subgenus *Sophophora* Sturtevant 1939
Species group *melanogaster*
Subgroup *melanogaster*
1. *Drosophila melanogaster*# Meigen 1830
Subgroup *ananassae*
2. *D. ananassae*# Doleschall 1858
3. *D. malerkotliana* Parshad and Paika 1964
4. *D. bipectinata* Duda 1923
Subgroup *suzukii*
5. *D. rajasekari* Reddy and Krishnamurthy 1968
Subgroup *eugracilis*
6. *D. eugracilis* Bock and Wheeler 1972
Subgroup *montium*
7. *D. kikkawai* Burla 1954
8. *D. jambulina* Parshad and Paika 1964
9. *D. punjabiensis* Parshad and Paika 1964
10. *D. bhagamandalensis* Muniyappa, Reddy and Krishnamurthy 1981
Subgroup *takahashii*
11. *D. takahashii* Sturtevant 1927
Subgenus *Drosophila* Sturtevant 1939
Species group *immigrans*
Subgroup *immigrans*
12. *D. immigrans* Sturtevant 1921
Subgroup *nasuta*
13. *D nasuta nasuta* Lamb 1914
14. *D sulfurigaster neonasuta* Sajjan and Krishnamurthy 1973
Species group *repleta*
15. *D. repleta* # Wollaston 1858
Species group *polychaeta*
16. *D. daruma** Okada 1956
17. *D. latifshahi* * Gupta and Raychaudhuri 1970
Genus *Scaptodrosophila* Grimaldi 1990
18. *Scaptodrosophila nigra* Grimshaw 1901
19. *S. krishnamurthyii* Sajjan and Reddy 1975
Genus *Zaprionus* Coquillet 1902
Subgenus *Anaprionus*
20. *Zaprionus bogoriensis* Mainx 1958
Genus *Phorticella* Duda 1923
Subgenus *Xenophorticella* Duda 1929
21. *Phorticella striata* Sajjan and Krishnamurthy 1975

16 * First time reported in South India; 17* present report; # Cosmopolitan species

Discussion

Family Drosophilidae with its varied diversity has still remained unexplored in most parts of India. Dharwad district with its varied climate and vegetation has proved to be a good example in collection of some

rare and endemic species, out of which 3 species are cosmopolitan and a remaining 18 species are endemic to South Asia. Most of the species collected have revealed to belong to subgenus *Sophophora* and *Drosophila*. Among the *Sophophoran* subgenus all the species belong to *melanogaster* species group. This is on par with the conclusion of Bock and Wheeler (1972) that the *melanogaster* species group must have originated from the South Asian or Indian subcontinent. This result may provide further insights into the evolutionary origin and diversification of *melanogaster* species group. Further the diversity of the *Drosophila* subgenus has also proved to be interesting with the Indian species of *D. daruma* reported for the first time from South India (Srinath and Shivanna, 2012). *Z. bogoriensis* belonging to subgenus *Anaprionus* is also a rare species, which was reported by Yassin and David (2010) from Bangalore, India; they also discussed its morphological characters.

Gupta and Raychaudri (1970) described *D. latifshahi* for the first time from Chakia forest in North India. They categorized this species under subgenus *Scaptodrosophila.* The *Polychaeta* species group is characterised with 3 pairs of dorsocentral bristles and surstylus with more or less pubescent flap (Toda and Peng, 1989). Later Toda and Peng (1989) reported this species for the first time from Guangdong province, China. They reclassified the taxonomic status of this species and categorized it under *Polychaeta* species group of the subgenus *Drosophila*. The species collected from Dharwad district in North Karnataka of South India has the similar characteristic feature of *Polychaeta* species group. It is one of the rare species which was not reported by earlier workers from South India.

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Latitudinal clines of allozymes in Indian natural populations of *Drosophila ananassae.*

Kumar, S., and A.K. Singh. Genetics Laboratory, Department of Zoology, Banaras Hindu University, Varanasi-221 005 India; Email: aksbhu23@rediffmail.com.

Animal species are known to live in environments that vary through time and space. In many situations, such environmental heterogeneity can act as a strong selective force causing adaptive differentiation among populations. Evolutionary biologists try to quantify the magnitude of adaptive differentiation among

populations and also to scrutinize gene loci responsible for such adaptive differentiation in different populations. A number of species of animals including *Drosophila* has been examined for their genetic differentiation between populations. Many latitudinal clines have been demonstrated for various quantitative traits in *Drosophila* species. Latitudinal clines are also reported for chromosomal polymorphism and show climatic selection over chromosomal arrangements in many species of *Drosophila*. Geneticists have also tried to demonstrate that chromosomal arrangements are not solely responsible for these clines and have proved that many quantitative traits (*viz*. body size, starvation resistance, and chill coma recovery) exhibit significant linear clines in inversion free species of *Drosophila simulans* (Arthor *et al*., 2008). Numerous clinal studies have been performed in invertebrate species including *Drosophila* (Hoffmann and Weeks, 2007). These studies not only look into action of climatic selection but also allow genetic variation in these traits to be linked to particular genes.

Study with regard to electrophoretic variants of enzymes has also shown interesting aspects for *Drosophila* workers (Mulley *et al*., 1979; Cavener and Clegg, 1981; Santos *et al*., 1989; Prout and Barker, 1993; Moraes and Sene, 2002). Oakeshott *et al*. (1982) selected four gene loci, *Adh, α-Gpdh, Est-6* and *Pgm* in *D. melanogaster* and found them to be polymorphic. They could observe latitudinal clines for three enzyme loci except for *Pgm.* The results obtained helped them to conclude that selection operates on the *Adh* and *α-Gpdh* loci due to the advantage of heterozygotes. Mulley *et al*. (1979) found association between allozyme and environmental variables in the Australian populations of *D. buzzatii.* They detected significant association between genotypes and environment for five of the six loci studied. Bubliy *et al.* (1994, 1999) analysed several natural populations of *D. melanogaster* coming from different parts of the world for *Adh, α-Gpdh, Est-6, Odh, G6pd*, and *Pgd* enzyme polymorphism. Their study clearly indicated the clinal variation with respect to allelic frequency of respective enzyme and geographical locations. Based on the results they concluded that allozyme polymorphisms are maintained by climatic selection. Negative correlation with latitude were found for *Adh-S* and *α-Gpdh-F* allele frequencies by Land *et al.* (2000) in natural populations of *D. melanogaster* collected from central and South America. Umina *et al*. (2005) reported geographical clines in genetic polymorphism, which was evidence for climatic selection and which was expected to shift with climate changes. They showed that the classic latitudinal cline in the *Adh* polymorphism of *D. melanogaster* shifted over 20 years in eastern coastal Australia. Moraes and Sene (2002) studied temporal and spatial intra-population allozyme variation in two natural populations of cactophilic species of *Drosophila*: *D. antonietae* and *D. gouveai*. Their results suggested that environmental variation influences temporal variation in allozyme polymorphism. They could not find cyclical variation in allozyme polymorphisms but detected an association between genetic distance and rain precipitation.

Drosophila ananassae, which was initially described from Indonesia by Doleschall in 1858, is a cosmopolitan and domestic species. It belongs to the *ananassae* species complex of the *ananassae* subgroup in the *melanogaster* species group (Bock and Wheeler, 1972). It occupies a unique status in the genus *Drosophila* due to certain peculiarities in its genetic behavior (Singh, 2010). Chromosomal polymorphism has been extensively studied in natural and laboratory populations of this species (Singh, 2010). This species has also been involved to see allozyme polymorphism in different Indian natural populations (Parkash *et al*., 1994; Kumar and Singh, 2012, 2013, 2014; Krishnamoorti and Singh, 2013; Singh *et al*., 2013). In the present study we wish to report that some of the enzyme loci in *D. ananassae* show graded variation in the frequency of their alleles when compared with populations derived from different geographical locations (varying latitude).

D. ananassae flies were collected from fifteen different eco-geographical regions of India by using net sweeping method from fruits and vegetable markets. Place of collection and their latitudinal position are given in Table 1. After bringing the flies to laboratory, naturally impregnated females were cultured in separate vials to establish isofemale lines. The isofemale lines were maintained on simple yeast-agar culture medium at 24±1°C with 12 hour cycle of dark-light period. Individual flies from isofemale lines were used to analyze allozyme polymorphism. For allozyme analysis, a single fly was homogenized in 50 µl 20 mM Tris buffer (pH 7.4) sample buffer and the homogenate was centrifuged at 12000 rpm at 4°C for 10 minutes (Kumar and Singh, 2013). Supernatant was separated into two aliquots and subjected to 8% native polyacrylamide gel electrophoresis in 25 mM Tris and 250 mM Glycine electrode buffer (pH 8.2) at 200V for 4 hour at 4°C. In-gel staining for enzymes was performed according to Shaw and Prasad (1970) and Ayala *et al.* (1972). The

locus and allele designations were done following the standardized genetic nomenclature for enzyme coding loci (Lakovaara and Saura, 1971).

Table 1. Geographical localities, their abbreviation and latitude of natural populations of *D. ananassae.*

S. No.	Populations	Abbreviation	Latitude
1	Kanniyakumari	KKR	8.08
2	Madurai	MDR	9.93
3	Thrissur	TSR	10.52
4	Dharmapuri	DMP	12.13
5	Bellary	BLY	15.15
6	Hyderabad	HYD	17.38
7	Solapur	SLP	17.68
8	Washi	WSI	18.58
9	Akola	AKL	20.44
10	Ranchi	RNC	23.35
11	Varanasi	VNS	25.2
12	Lucknow	LKO	26.51
13	Jaipur	JPR	26.55
14	Agra	AGR	27.11
15	Delhi	DLH	28.4

Table 2. Correlation coefficient (*r*) for slow allele of 12 enzyme loci and latitude of 15 natural populations of *D. ananassae.*

S. No.	Locus	Correlation coefficient (r)
1	*Acph1*	-0.256
2	*Acph2*	0.366
3	*Xdh*	0.814
4	*Aph2*	0.944
5	*Aph3*	0.007
6	*Ao1*	0.009
7	*Ao2*	0.840
8	*Est2*	-0.37
9	*Est3*	-0.151
10	*Est5*	-0.772
11	*Mdh*	0.861
12	*Me*	-0.177

Figure 1. Graphs (1a to 1e) showing clinal variation between latitude and frequency of slow allele of five enzyme loci.

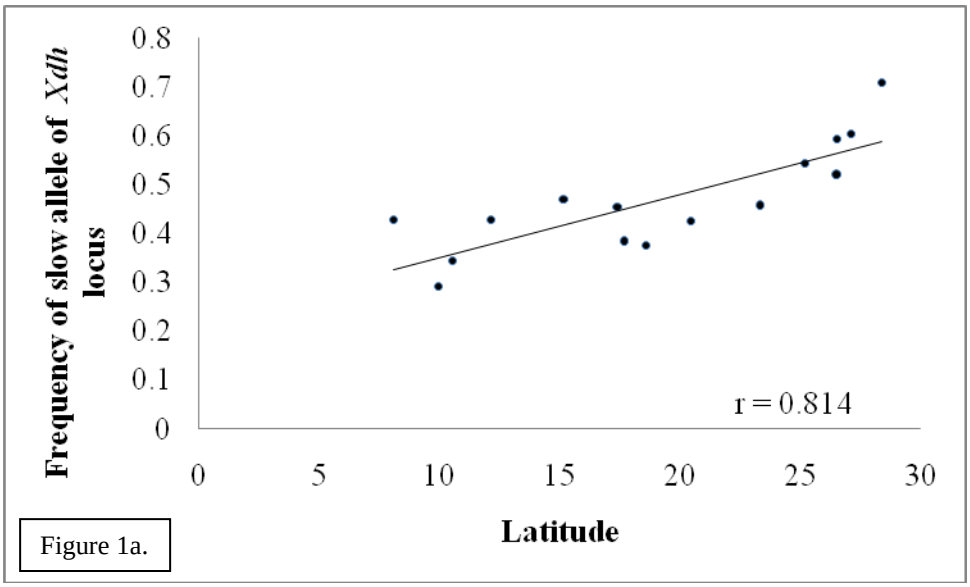

Figure 1a.

Correlation coefficient was computed between latitude and slow migrating alleles of twelve enzyme loci (Table 2). Out of twelve enzymes tested, significant correlation could be observed with five enzyme loci (Figure 1a to 1e). *Est5* locus shows significant negative correlation whereas four enzyme loci, *i.e.*, *Xdh*, *Aph2*, *AO2* and *Mdh* showed significant positive correlation. *Aph3* and *AO1* showed very low *r* values (0.007 and 0.009, respectively) indicating almost no correlation in this regard. Loci encoding for enzymes *Acph1*, *Acph2*,

Est2, *Est3*, and *Me* did not show significant departure from zero indicating insignificant correlation between the frequency of slow allele and latitude. The present results reveal that all polymorphic enzymes may not be subject of natural selection. Therefore, only those enzyme loci that show significant correlation between the allele frequency and latitude could be considered of adaptive significance.

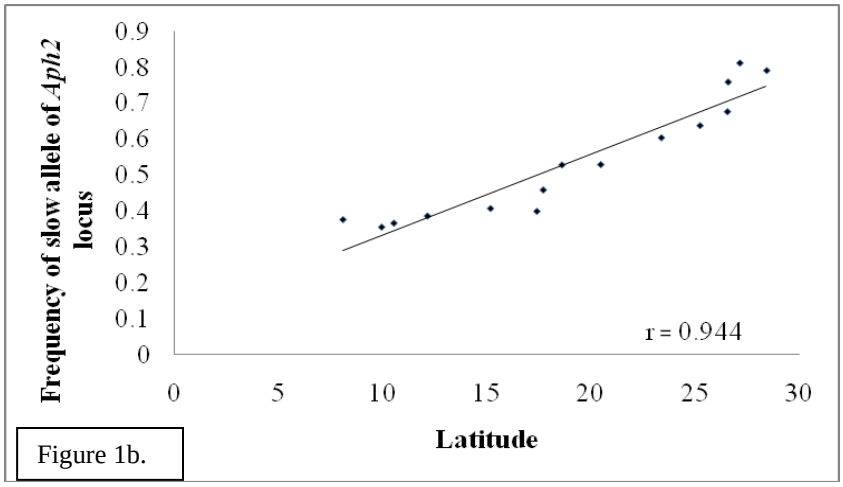

Figure 1b.

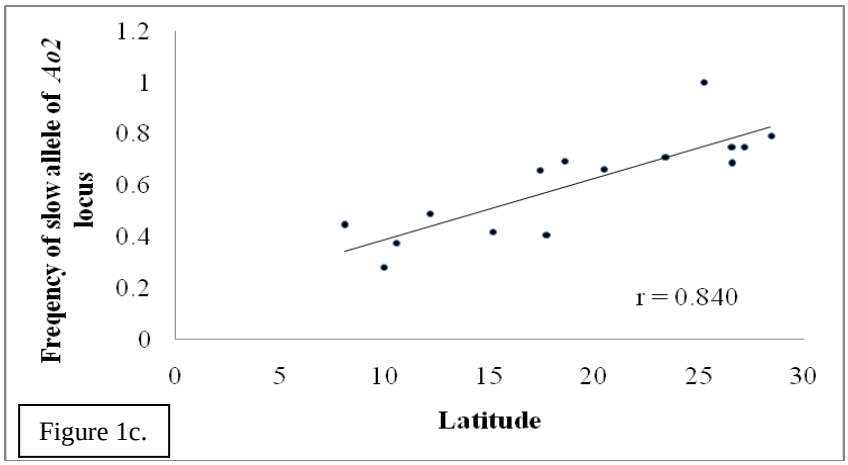

Figure 1c.

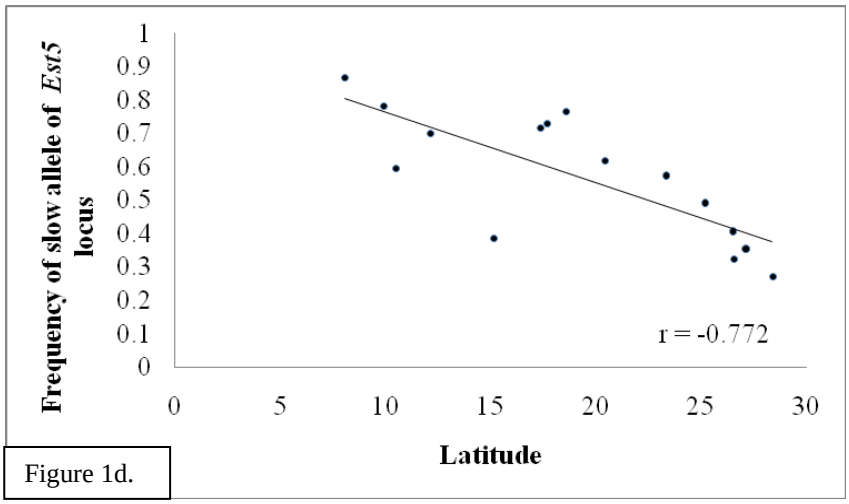

Figure 1d.

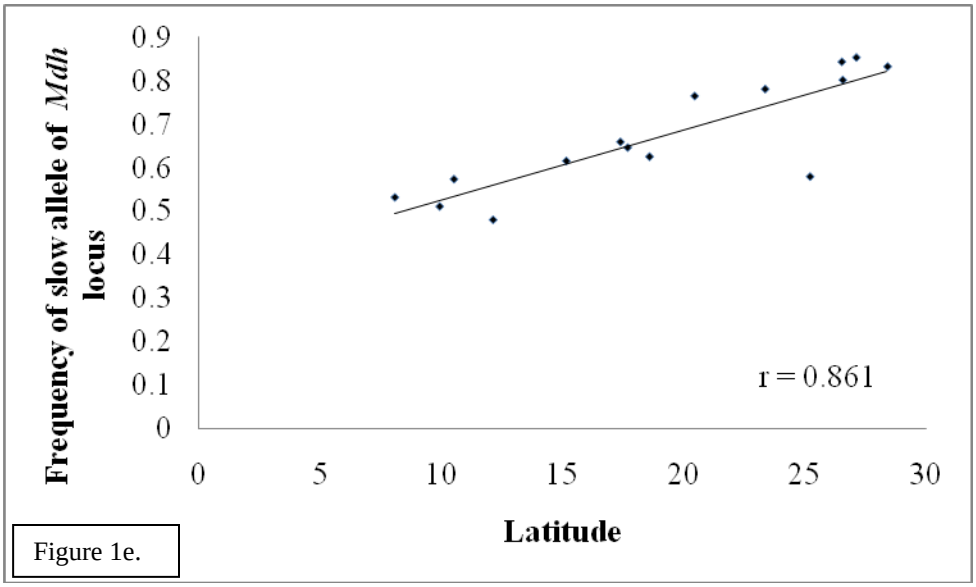

Figure 1e.

Acknowledgment: We are thankful to UGC (University Grants Commission), New Delhi, for providing financial support in the form of major research project to AKS and research fellowship to SK.

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Cannibalism and "partial carnivorism" in *Drosophila* sp. larvae.

Bhattacharyya, Dipita. Cytogenetics Laboratory, P.G. Department of Zoology, Maulana Azad College. 8, Rafi Ahmed Kidwai Road, Kolkata-700013, India.

The seminal observation of cannibalism in *Drosophila* sp. larvae in our laboratory is purely accidental rather than being the resultant of meticulous execution of a well-designed research plan. Initially, as post graduate students, we were in the process of conducting an experiment to separate the two mutants - one, X-linked (white), and the other, an autosomal (ebony) from a double mutant strain, (*i.e.*, white ebony). During

this time, due to some unavoidable circumstances, severe mite infection compelled us to discard some of the severely infected vials and regularly monitor the remaining vials under a dissection microscope (magnification 20× and 40×) in order to establish the cultures completely free of mites.

At this time of rigorous monitoring, it was discovered that a few of the larvae seemed to somehow associate with some of the dead bodies of the adult flies in the vial. It had been observed earlier as well. However, unaware of the fact that comparable observation was reported in Canton-S flies in the previous year, (Vijendraverma *et al.*, 2013), the thought of a possible cannibalistic behavior was of course discarded. However this time, having kept the vials under frequent observation under the dissection microscope, we had direct evidence that some of the larvae were feeding on the dead adults (parental flies). In fact, further studies revealed that larvae, especially first and second instar, fed on other larvae and pupae as well. Such repeated observations, coupled with the aforesaid publication, tempted us to extend our observations in different experimental situations.

Observations

Some of the notable observations that are worth mentioning are as follows:

- Cannibalistic behavior initiated from the first instar larvae itself and continued till the early third instar stage.
- There is a gradual decrease in the cannibalistic behavior as the mid-third instar larvae start crawling out from the culture medium, up along the wall of the glass vial.
- Finally, in the late third instar stage, there is complete absence of cannibalism as the larvae gradually stops feeding, turns motionless and enters into the pupal stage. These pupae in turn might serve as an easy target for the cannibalistic larvae, if they form near the surface of the culture medium.

The Exhibition of Cannibalism Was Characterized as Follows

The larvae were found to approach the victimized counterpart, followed by continuous scrapping of the cuticle of the victim with their jaws (Figure 1, a-b), until they were successful to make a lesion. They then entered into the body and started eating the soft internal body parts, leaving only the empty exoskeleton of the victim either on the food surface or deep in the culture medium (Figure 1, c-d). They were also found to have consumed the wings of the dead parental flies.

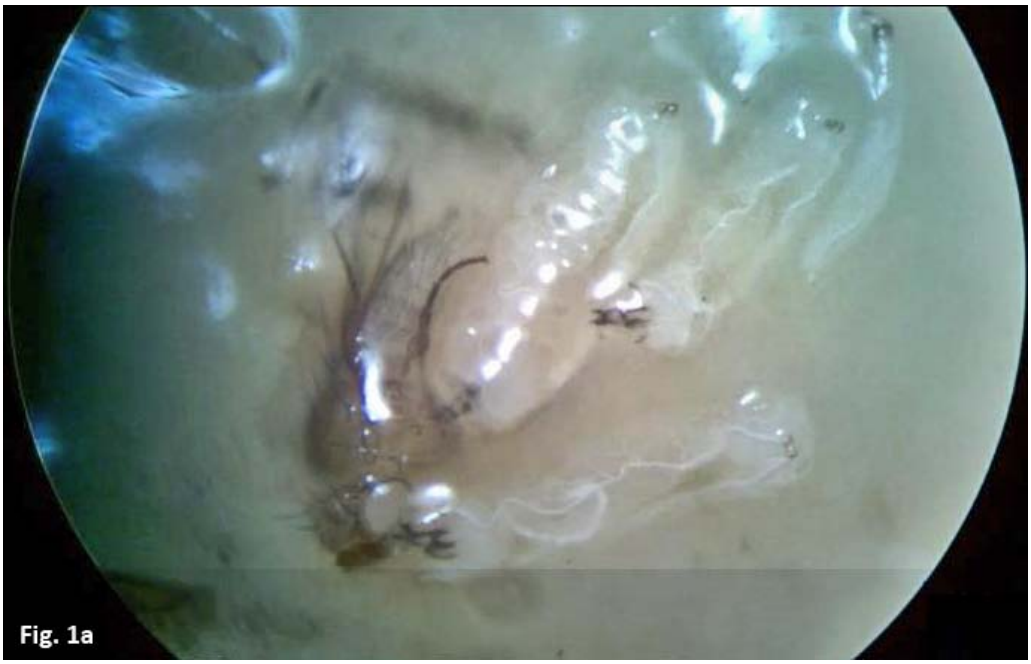


Figure 1. (a) White ebony larvae approaching the victimized counterpart (adult parental fly) before feeding on the same, (b) White ebony second instar larva scraping against the cuticle of the victimized counterpart (a first instar larva), (c) White ebony larva feeding on the soft internal body part of a dead adult, (d) Remnant of the victim on the culture medium.

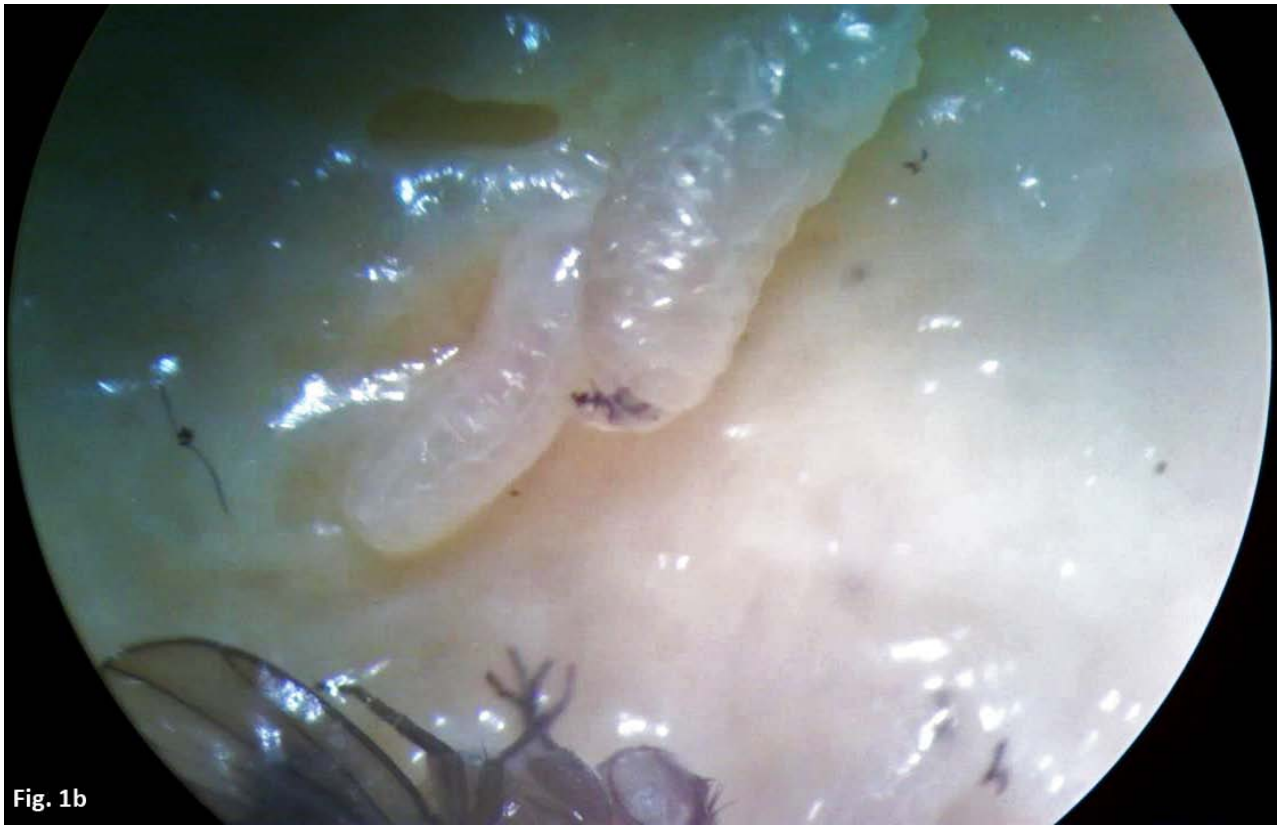
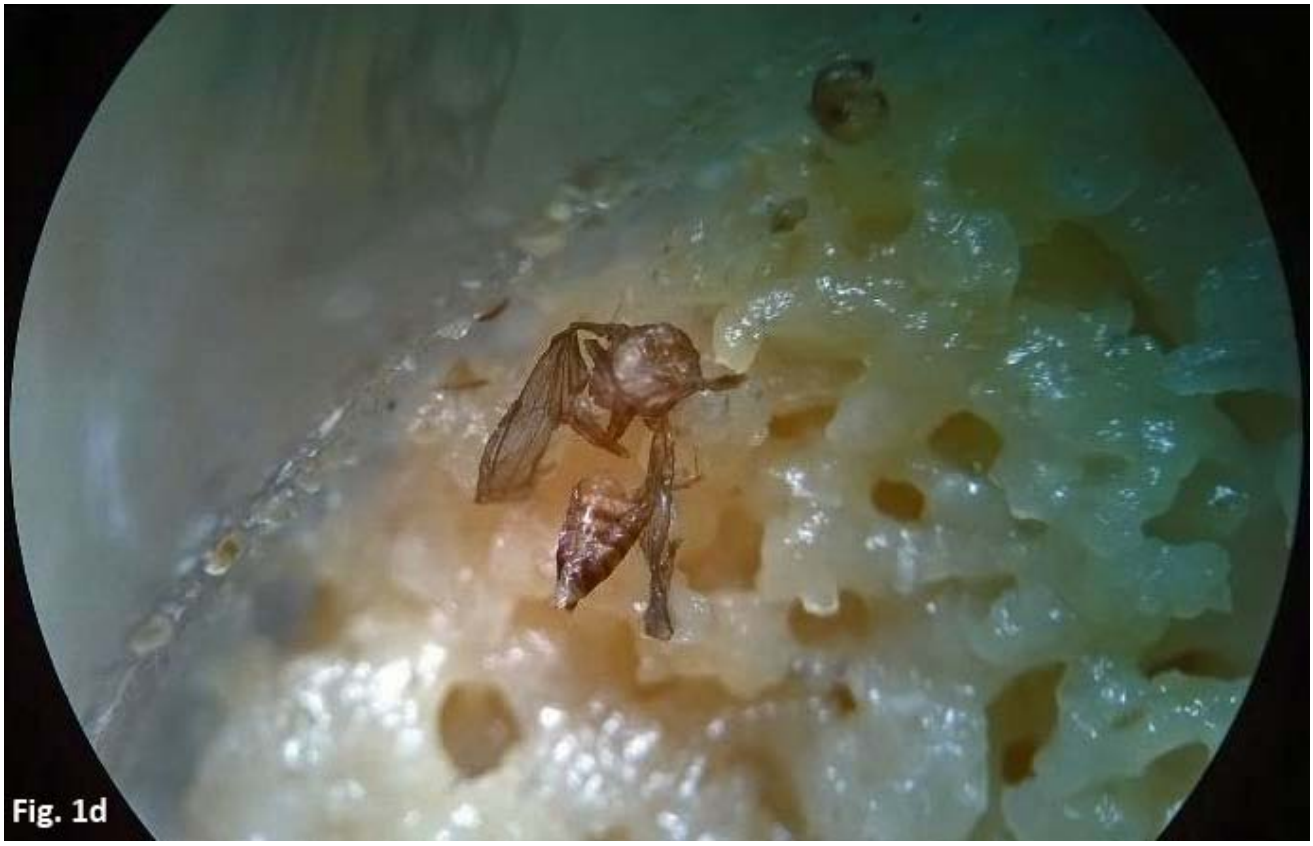


Fig. 1b

Fig. 1c



Experiments

As the larval cannibalism was first observed in a double mutant strain, extension of observations to wild type (Oregon R) culture became necessary to determine whether these two mutants somehow influenced the cannibalistic behavior of the larvae, especially when the mutant, white, has been known to have pleiotropic effects, influencing both adult and larval characteristics (Lindsley and Grell, 1968). Interestingly, identical results with respect to cannibalism were also observed in the wild-type stock (Figure 2, a-b). These observations exclude the possibility of influence of these two markers on the cannibalistic behavior.

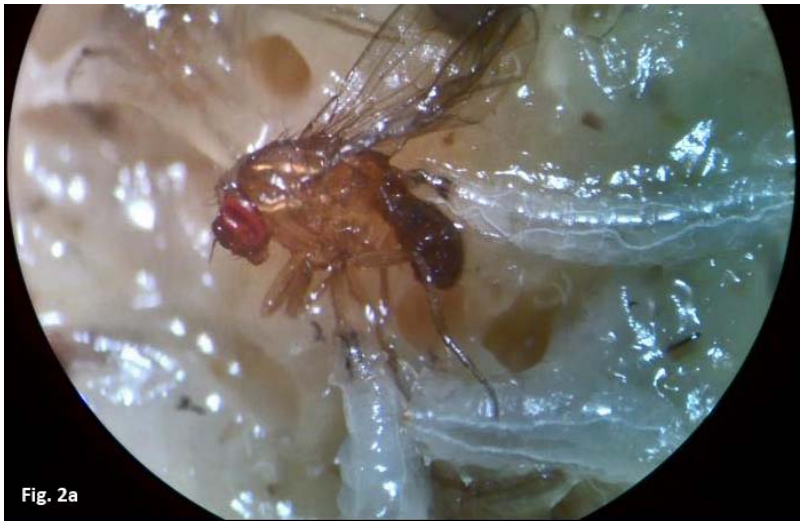


Figure 2. (a) Cannibalism as observed in the wild-type (Oregon R) stock, (b) Having made a successful lesion, the larva enters into the body of the victimized counterpart and feeds on the soft internal body parts.

Next, a series of simple experiments were designed to answer the following questions:

- *In cannibalism, among the larvae, is the cannibalism the resultant of specific competition for a limited resource?*
- *Whether the cannibalistic behavior of the larvae is limited to their own parents?*
- *Whether such behavior is species specific? If the answer is a negative one, is there any preference?*
- *Is (Are) the victim(s) of cannibal larvae only restricted to the members belonging to the same genus?*
- *Whether such a phenomenon is limited only to one species under the genus* Drosophila*?*

Experiments conducted to answer the above questions

- *In cannibalism, among the larvae, is the cannibalism the resultant of specific competition for a limited resource?*

When *D. melanogaster* larvae were cultured in normal maize-jaggery-yeast-agar medium, in the following vials, at 22±1°C,

1. Uncrowded (5 males and 10 females, kept for 2 days),
2. Crowded (10 males and 20 females, kept together for 3 days), and
3. Overcrowded (10 males and 20 females, kept together for 5 days),

cannibalistic behavior was found to remain unaltered significantly in all three situations.

The experimental observations cited above, point to the general conclusion that cannibalistic behavior in the larvae might not be the resultant of competition for limited food resources. But at the same time, further experimental verifications are required by altering the parameters to reach any definite satisfactory conclusion,

as food limitation was considered to be one of the major cause of killing conspecifics in spiders (Wise, 2006) and *Drosophila* sp. (Vijendraverma *et al.,* 2013).

- *Whether the cannibalistic behavior of the larvae is limited to their own parents?*

All the adult flies from the vials were discarded after allowing them to mate for three days. After the emergence of first instar larvae, etherized adult flies from separate vials were introduced into the vials. The first instar larvae were found to attack the etherized flies within 10-15 minutes and on the next day, only exoskeletons were found, either on the food surface or in some cases inside the culture medium (observed from the sides of the vials).

- *Whether such behavior is species specific? If the answer is a negative one, is there any preference?*

The same experiment was repeated with the locally collected species. When etherized locally collected adult flies were introduced into the vials containing only *D. melanogaster* larvae, the larvae were found to feed on them as well. Next, when equal number of both *D. melanogaster* and locally collected *Drosophila* sp adults were added together in vials culturing only *D. melanogaster* larvae, they fed equally upon all the flies, irrespective of the species.

- *Is (Are) the victim(s) of cannibal larvae only restricted to the members belonging to the same genus?*

In an extension of this series of experiments, adult houseflies (*Musca domestica*; family Muscidae), adult flesh flies (*Parasarcophaga ruficornis*; family Sarcophagidae), and sliced raw chicken meat were supplemented separately to vials containing only *D. melanogaster* first instar larvae. The larvae fed on these equally, as was seen in the earlier cases (Figure 3, a-c).

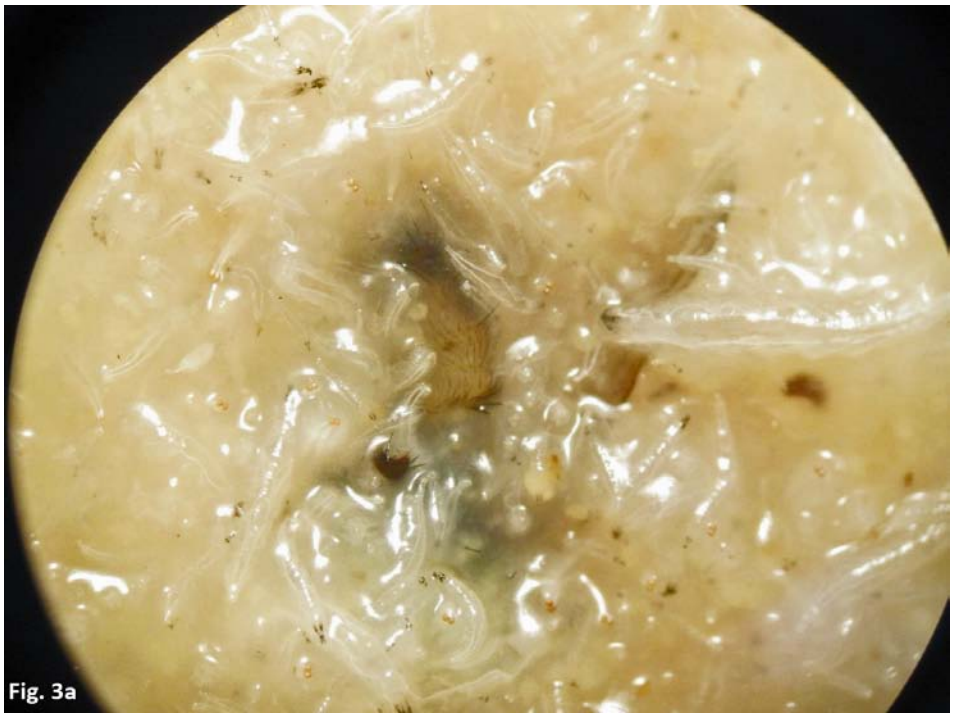


Figure 3. (a) *D. melanogaster* larvae feeding on *Musca domestica* added to vial, (b) *D. melanogaster* larvae feeding on the body of adult flesh fly added to vial (eye pigment having been consumed already), (c) Wild type *D. melanogaster* larvae feeding on sliced raw chicken meat.

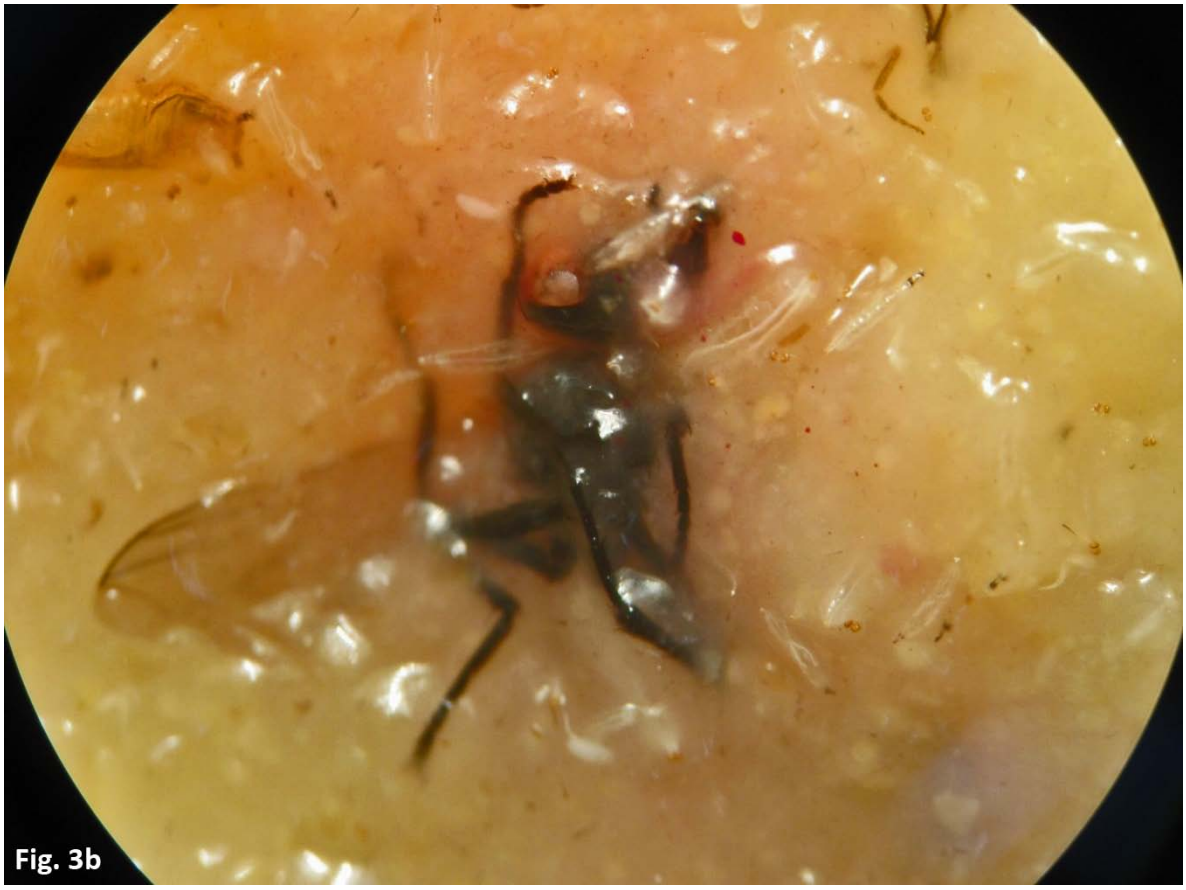


Fig. 3b

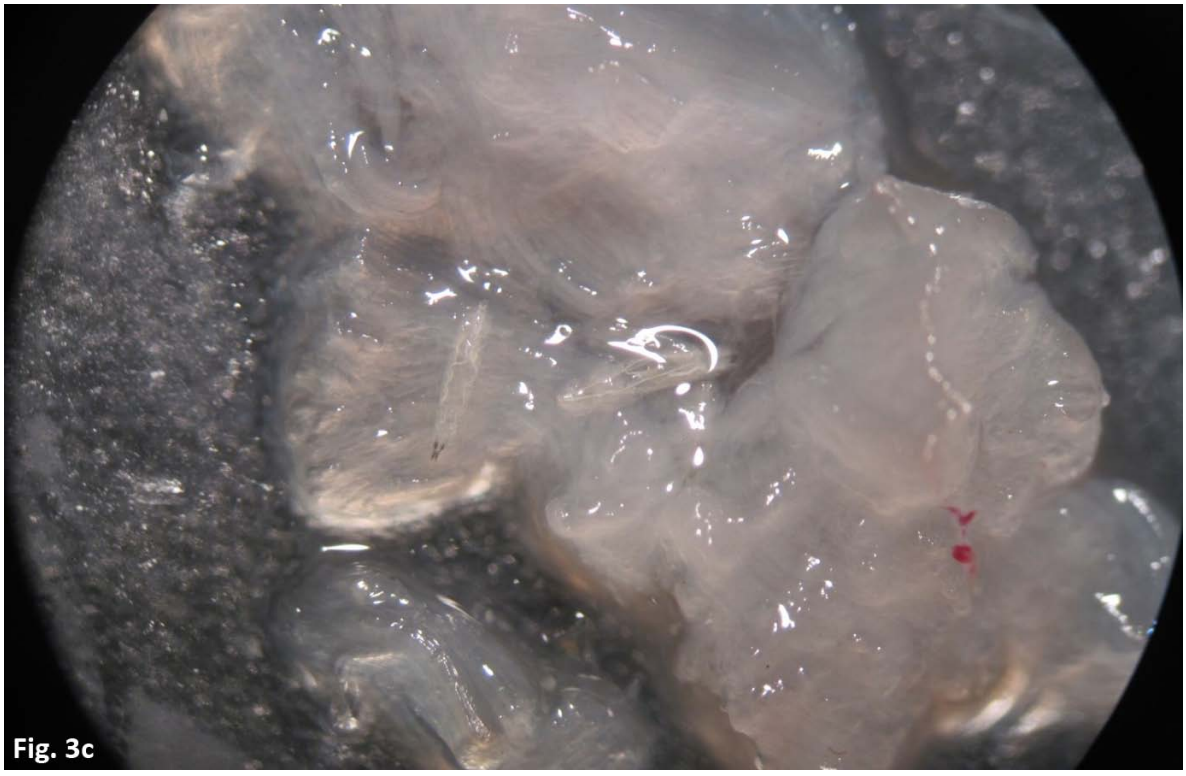


Fig. 3c

- *Whether such a phenomenon is limited only to one species under the genus* Drosophila*?*

Comparable cannibalistic behavior was also observed in locally collected *Drosophila* species, in all the experimental situations performed earlier using *D. melanogaster* larvae (Figure 4, a-b).

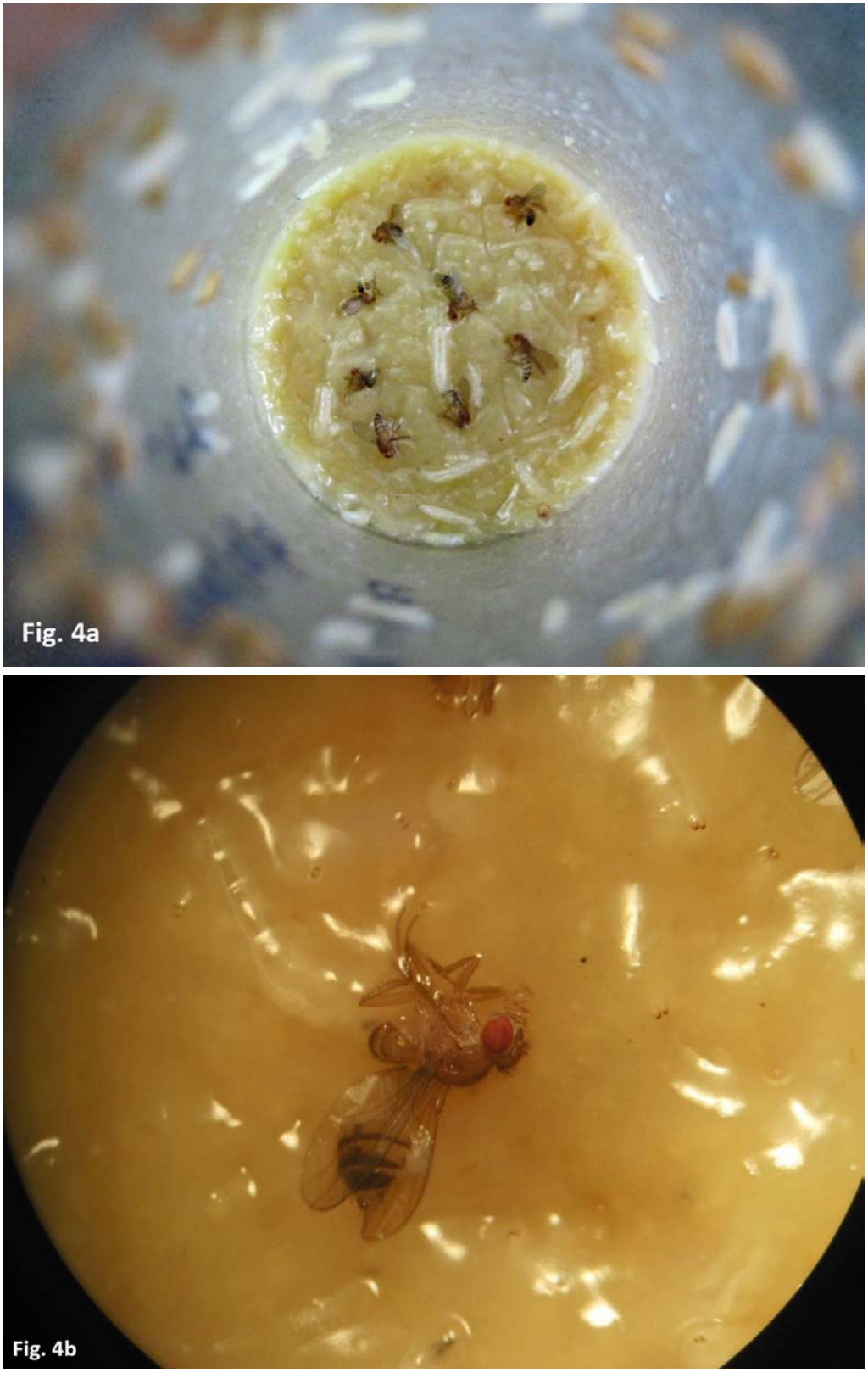


Figure 4. (a) Cannibalistic behavior observed in locally collected *Drosophila sp*. larvae, as seen after supplementing dead bodies of adult flies to the vial, (b) A closer view depicting larvae of the locally collected *Drosophila* sp., feeding on the adult of the same species.

All the experimental observations so far tempted us to speculate that cannibalistic behavior of the larvae is not restricted to *D. melanogaster* only, it is not the resultant of intraspecific competition among larvae for limited amount of food resources, and there is no species and genus specificity with respect to larval cannibalism. The last point we would like to add is that the feeding on *Musca* sp., *Parasarcophaga* sp., and raw chicken meat although allowed us to use the term, "partial carnivorism" in *Drosophila* larvae; we thus far failed to culture the flies in vials containing only raw chicken meat.

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The natural breeding sites of *Drosophila funebris* in Chile.

Jara, Claudia[1], Sergio Flores[2], Luis Pino[1], María Cristina Medina[3], Eduardo Alvarez[1], Francisco Del Pino[1], and Raúl Godoy-Herrera[1]. [1]Programa de Genética Humana, ICBM, Facultad de Medicina, Universidad de Chile, Santiago; [2]Departamento de Antropología, Facultad de Ciencias Sociales, Universidad de Chile; [3]Departamento de Biología y Ciencias Naturales, Facultad de Ciencias, Universidad de Playa Ancha de Ciencias de la Educación, Valparaíso.

Drosophila funebris, an Holoartic, cosmopolitan species, can be collected in Chile to South of the latitude 43°- 44°. There, natural populations of the species live in extreme climatic conditions near the Strait of Magellan (53° 10´S) and in Caleta Josefina in Tierra del Fuego (53° 40´S). *D. funebris* is one of the Southernmost *Drosophilidae* (Brncic and Dobzhansky, 1957; Brncic and Sanchez, 1958). On the other hand, *D. funebris* adult flies can emerge from fungi, walnut husks, decaying fruits, potatoes, and onions (Carson, 1965).

In Central Valley of Chile, latitude 29°- 37° S, *D. funebris* larvae and adults exploit a variety of decaying materials in a diversity of habitats very different to those of Tierra del Fuego, attesting to the versatility and flexibility of *D. funebris* genome. For example, populations of *D. funebris* utilize decaying pumpkin (*Cucurbita maxima*), prickly pear decaying tissue (*Opuntia ficus-indica*), and decaying tissue of a Chilean cactus (*Echinopsis chilensis*). Depending on the locality, the three types of breeding sites may be separated for a few meters as in Pelequén (34° 28´S) and Melipilla (33° 31' S) or scattered over a surface of about 50 km^2 as in Til-Til (33° 06' S).

The Pelequén, Melipilla, and Til-Til localities also differ in climate. For example, in annual rain: (i) Pelequén, 563.4 mm; (ii) Melipilla, 397.7 mm; (iii) Til-Til, 318.7 mm. Annual mean temperature in these three localities is: (i) Pelequén, 13.5°C, (ii) Melipilla, 14°C, and (iii) Til-Til, 17°C.

On the other hand, depending on the type of decaying fruit, larvae of D. *funebris* coexist with larvae of other *Drosophila* species. In pumpkin, *D. funebris* shares the fruit with *D. immigrans*. In prickly pear tissues, *D. funebris* lives together with the Chilean endemic *D. pavani* and the cosmopolitan *D. buzzatii*. From decaying cactus tissue *E. chilensis*, adults of *D. funebris* emerge together with *D. busckii* and *D. buzzatii*.

The ecology of Chilean natural populations of *D. funebris* offers an opportunity to investigate the role of ecological factors in the origin of new species. Likewise, these populations are a good biological material to study behavioral barriers that restrict gene flow. Recent results (unpublished data) suggest that there are gene flow restrictions between natural populations of *D. funebris* that live in sympatry on different substrates (pumpkin, cactus, and prickly pear). These restrictions also exist between allopatric populations of *D. funebris* reared on the same type of fruit. That is, sexes prefer to copulate with individuals emerged from the same type

of food and locality. Chilean populations of *D. funebris* are good to investigate the role of *Drosophila* breeding sites in isolation between populations and our understanding of the speciation process in animal species.

Acknowledgments: Thanks are due to Enlace Proyect ELN012/14, Universidad de Chile.

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The development of hooks in larvae of the two isolates of *Drosophila gaucha*.

Álvarez, Eduardo[1], Luis Pino[1], Claudia Jara[1], María Cristina Medina[2], Francisco Del Pino[1], and Raúl Godoy-Herrera[1]. [1]Programa de Genética Humana, ICBM, Facultad de Medicina, Universidad de Chile, Chile; [2]Departamento de Biología y Ciencias Naturales, Facultad de Ciencias, Universidad de Playa Ancha, Valparaíso, Chile.

Complex behaviors as food ingestion depend on movements of mouth anatomical components coordinated by the nervous system. In *Drosophila* larvae, the hook movement has a crucial role in ingestion of feeding items allowing food to get into the larval gut (Sewell, Burnet, and Connolly, 1974). Thus, investigations focused on the development of larval hooks are important to understand properly the larval age-related changes in feeding rate (Godoy-Herrera, Burnet, and Connolly, 2005). On the other hand, a comprehension of population variation in development patterns of *Drosophila* larva hooks may clarify the role of genetic and ecological factors in the origin and maintenance of morphological differences linked with food ingestion. We investigated the genetics of morphological changes of hooks through the whole of larval period in two isolates of *Drosophila gaucha* separated by over 1200 km. Specifically, we measured hook width through the whole of larval period of *D. gaucha*, a Neotropical, Latin American endemic species, belonging to the *mesophragmatica* group of species of *Drosophila* (Brncic and Koref-Santibañez, 1957). Populations of the species distribute from South Brazil (Campos de Jordan, CJ), through Uruguay and Argentina (Buenos Aires, BA). We conjectured that geographic variation in development patterns of hook morphology of *D. gaucha* larvae could be indicative of inter population genetic differences in larval feeding rates (Okada, 1963).

Climatic differences

The populations examined live in contrasting environments. The climate in Buenos Aires is temperate-humid (1147 mm of mean rain per year; annual mean temperature is 17.6ºC; 25 m over the sea level). In Campos de Jordan (Brazil) the climate is tropical-height (1700 m over the sea level; 1566 mm of mean rain per year; annual mean temperature 13.6ºC; Campos de Jordan is the only place in Brazil where snow falls in winter).

Crosses and collection of larvae

We established cultures of the BA and CJ isolates at 18ºC. After 12 months, once the cultures were well established, we collected virgin individuals of the two sexes. Fifteen-day-old virgin males and females of the two populations were reciprocally crossed. Homogametic crosses within the strains were also made as controls for the inter-population crosses. We examined N = 50 larvae per each: (i) parental population, (ii) the two reciprocal F_1, (iii) the two out four F_2, and (iv) each one of the 4 out 8 backcrosses. That is, N = 50 per each of the 10 groups of genotypes. Groups of 30-40 inseminated females were allowed to oviposit for 3-4 h on plastic spoons filled with culture medium. Larvae eclosed around 48 h after the eggs are laid. Larvae were collected at successive 24 h intervals after emergence until 192 h of larval age.

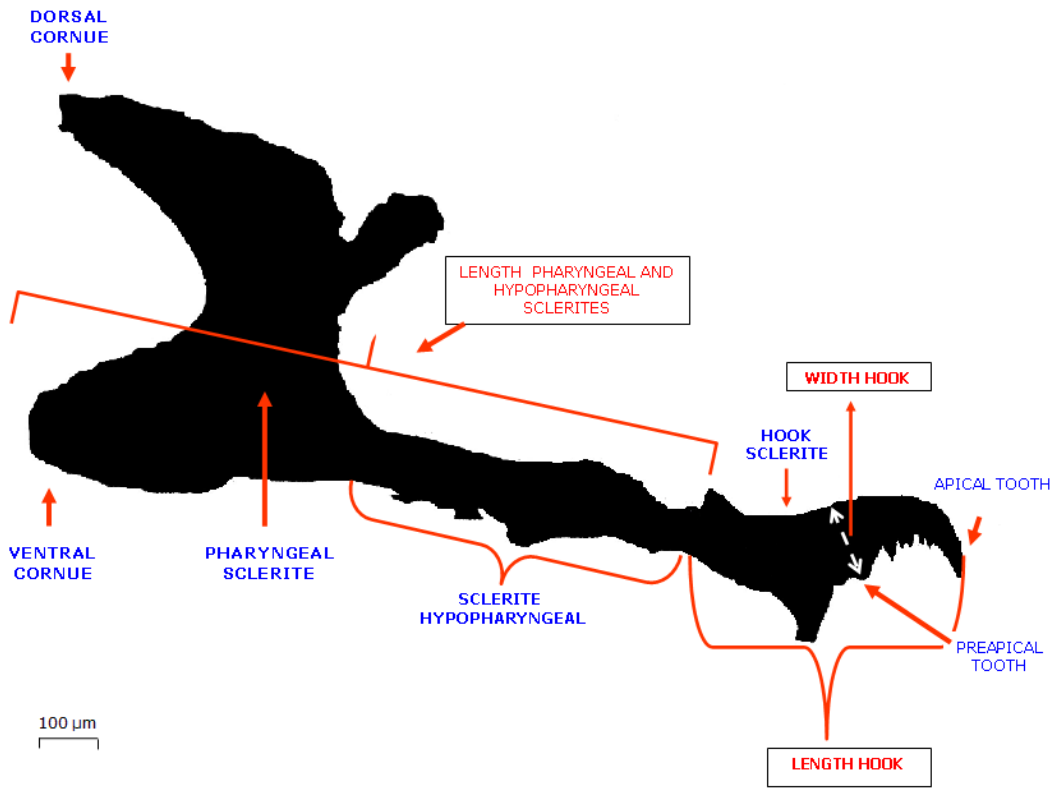

Figure 1. Hook of *Drosophila* larva. The traits measured are indicated by arrows. Hook width is indicated by white arrows.

Larval hooks

Larval hooks, N = 50 pairs of hooks per larval age and group of genotypes, were dissected at successive 24 h until 192 h of larval development. Figure 1 shows the hook traits measured. Here, we communicate the data on the development of larval hook width.

Figure 2 shows the changes in hook width between 24 to 192 h of larval development in the Buenos Aires and Campos de Jordan parental strains of *D. gaucha*, reciprocal F_1, F_2, and four backcrosses. In larvae of the BA and CJ parents and the two F_1 increase in hook width occurs at 72 and 144 h of larval age. By contrast, in the F_2 and backcross larvae the hook width increase occurs continuously from 24 until 192 h of larval age (Figure 2). These findings suggest that recombination of the BA and CJ genetic heritage substantially modified the development patterns of larval hooks in the F_2 and backcrosses generations.

We made an ANOVA to know whether the growing patterns of hooks in larvae of the ten groups of genotypes analyzed were statistically different. The F-values were: (i) between generations, $F_{3, 312} = 3.08$, $P < 0.05$, (ii) between the BA and CJ parents, $F_{1, 78} = 0.34$, $P > 0.05$, (iii) the F_1 versus the parents, $F_{2, 234} = 0.07$, $P > 0.05$, (iv) the F_2 versus mean backcrosses, $F_{1, 78} = 4.22$, $P < 0.05$, (v) between the four backcrosses, $F_{3, 234} = 0.03$, $P > 0.05$. The results suggest genetic segregation for development of hook width in larvae of *D. gaucha*.

We carried on the analysis by making further scaling tests to estimate whether the data fitted to an additive-dominant model (Mather and Jinks, 1971). Means and variances of all generations were used. The joint scaling test was applied. It compares the observed generation means with expected values derived from the estimates of the [*m*], [*d*], and [*h*] (details in Mather and Jinks, 1971). The comparison between observed and expected means assumes a χ^2 distribution with degrees of freedom three less than the number of family

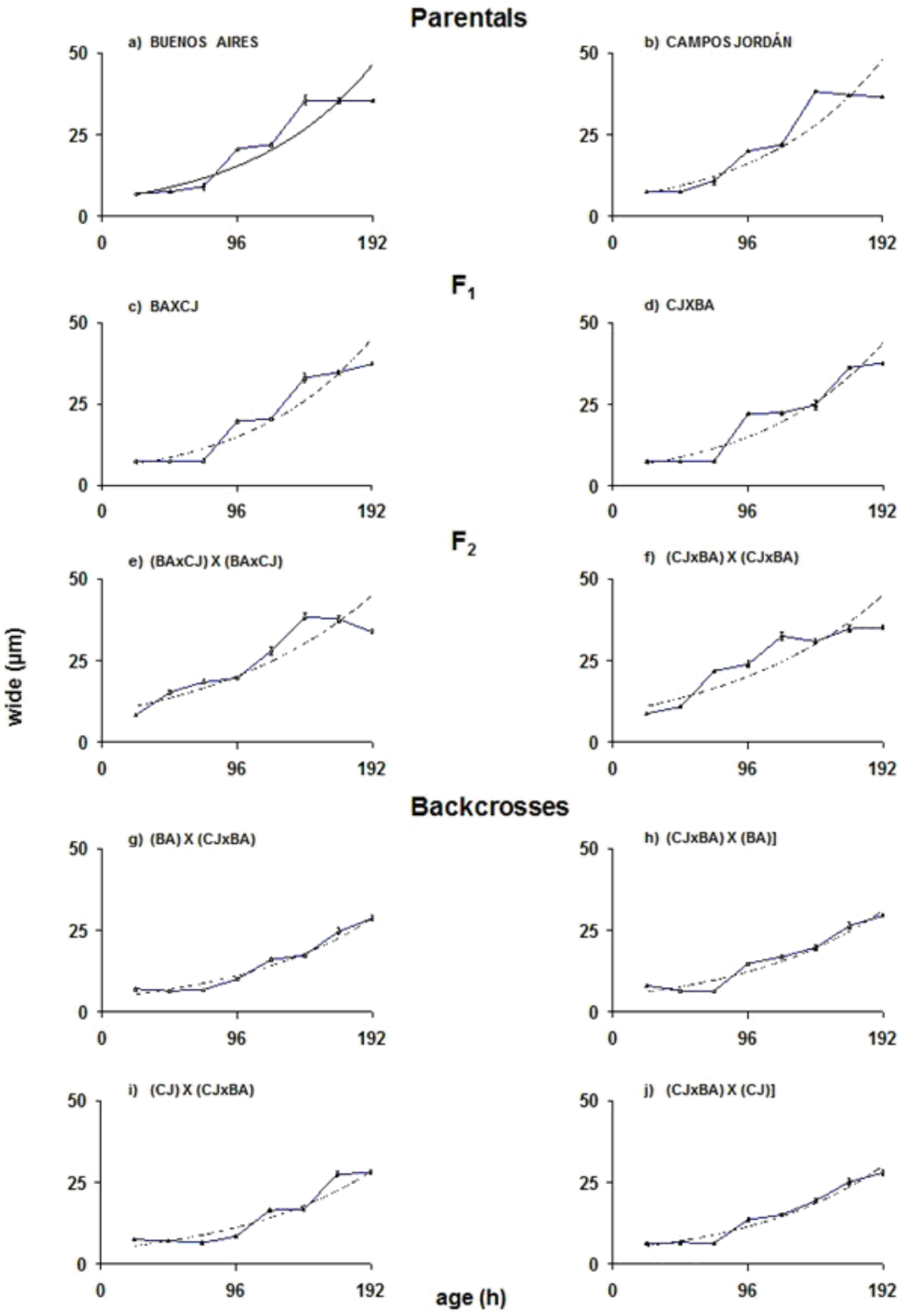

Figure 2. The development of hook width in larvae of *D. gaucha*. (i) parents: Buenos Aires (BA) and Campos de Jordan (CJ), (ii) the two reciprocal F_1, the two reciprocal F_2 and (iv) the four backcrosses. Other details in the text.

means available. We obtained a $\chi^2 = 2.37$, *df* = 7, *NS*, suggesting a genetic architecture for larval growing of hook width based principally on additive and dominant components.

The results support the conclusion that the Buenos Aires and Campos de Jordan populations of *D. gaucha* differ genetically in growing patterns of larval hooks. Thus, the F_2 and backcross larvae show hook width growing patterns different from those of the parental and F_1 larvae (Figure 2). These inter population differences emerge by introgression of genetic inheritance of the BA and CJ populations of *D. gaucha*. In fact, the two isolates show similar hook width growing patterns, but the F_2 and backcross larvae differ statistically with respect to the parents (Figure 2). We conclude that larvae of the two populations examined exhibit genetic differences for growing patterns of hook width. This is a morphological structure essential for ingestion of food.

Acknowledgments: Authors thank Enlace Proyect ELN012/14 from University of Chile.

References: Brncic, D., and S. Koref-Santibañez 1957, Evolution 11: 300-311; Godoy-Herrera, R., B. Burnet, and K. Connolly 2004, Heredity 92: 14-19; Mather, K., and J. Jinks 1971, *Biometrical Genetics*. Chapman and Hall, London; Okada, T., 1963, Evolution 17: 84-98; Sewell, D., B. Burnet, and K. Connolly 1976, Genet. Res. 24: 163-173.

Length of feeding breaks in larvae of six species of the *mesophragmatica* group of *Drosophila*.

Del Pino, Francisco[1], Claudia Jara[1], Luis Pino[1], María Cristina Medina[2], Eduardo Alvarez[1], and Raúl Godoy-Herrera[1]. [1]Programa de Genética Humana, ICBM, Facultad de Medicina, Universidad de Chile; [2]Departamento de Biología, Facultad de Ciencias, Universidad de Playa Ancha de Ciencias de la Educación, Valparaíso, Chile.

The structure of feeding behavior of *Drosophila* larva – that is the arrangement and relations between the elements that participate in food ingestion – merits particular attention in view of the fact that the rate of feeding is linked with the larval growth and adult reproduction (Arizmendi *et al*., 2008). Feeding of *Drosophila* larvae is conformed by periods of intense activity of the hooks in which food gets into the larval gut (feeding bout), interrupted by breaks that affect the rate at which food is ingested (feeding breaks; Green *et al*., 1983). In the nature, it is also observed that a *Drosophila* larva feeds on decaying fruits by a continuous rhythm of raking movements of the hooks interrupted by frequent breaks (personal observations). We are interested in comparing the structure of larval feeding behavior of *Drosophila* in a phyletic group of species of *Drosophila*. To address this goal we examined duration of break length at 24 h of larval development, that is when feeding rate is low, and at 120 h of larval age when ingestion of food is maximized (Sewell, Burnet, and Connolly, 1975). We studied six species of the *mesophragmatica* group of *Drosophila*. The phylogenetic relationships between the species and the larval foraging behavior are known (Brncic and Koref-Santibañez, 1957; Del Pino and Godoy-Herrera, 1999; Godoy-Herrera, Burnet, and Connolly, 2005). These investigations may be of importance to understand the evolution of organization and functioning of the brain in a *Drosophila* larva.

The species studied were *Drosophila pavani*, *Drosophila gaucha*, *Drosophila brncici*, *Drosophila gasici*, *Drosophila mesophragmatica*, and *Drosophila viracochi*. With the exception of *D. gaucha* that lives in South Brazil, Uruguay, and Argentina, the other species can be collected in Andean habitats and *D. pavani* in Central Valley of Chile (Brncic and Koref-Santibañez, 1957). The flies were all reared in a constant environment under permanent light at 18ºC in 300 cc glass bottles containing about 50 cc of Burdick's medium (1954). The six species have similar development times, to molt, wander and pupate (Koref-Santibañez, 1964; Del Pino and Godoy-Herrera, 1999).

Foraging behavior at 24 and 120 h of larval age was recorded individually under stereomicroscope, N = 50 larvae per species and age. Larvae tested were transferred to a Petri dish with agar covered by a film of fresh yeast paste. For each larva a new Petri dish was used. Observations were made at 22ºC, 90% humidity

between 14.30 and 18 h. We record the behavior of each larva continuously as a sequence of key presses using a keyboard connected to a Toshiba notebook (details in Ruiz-Dubreuil *et al.,* 1996). Cessation of movement by the mouth hooks for a period exceeding 2 seconds was defined as a break in feeding.

Analyses for normality and homogeneity of variances of feeding breaks were performed. The analysis showed that the variances of the six species were heterogeneous. A log transformation of the data yielded homogeneous variances (Bartlett's test; χ^2values at 24 and 120 h fluctuated between 5.9 and 7.6, *df* = 49 per species and age, NS). The analysis carried on by making a *t-test* to examine differences in feeding break length between larvae of 24 and 120 h of age within a species.

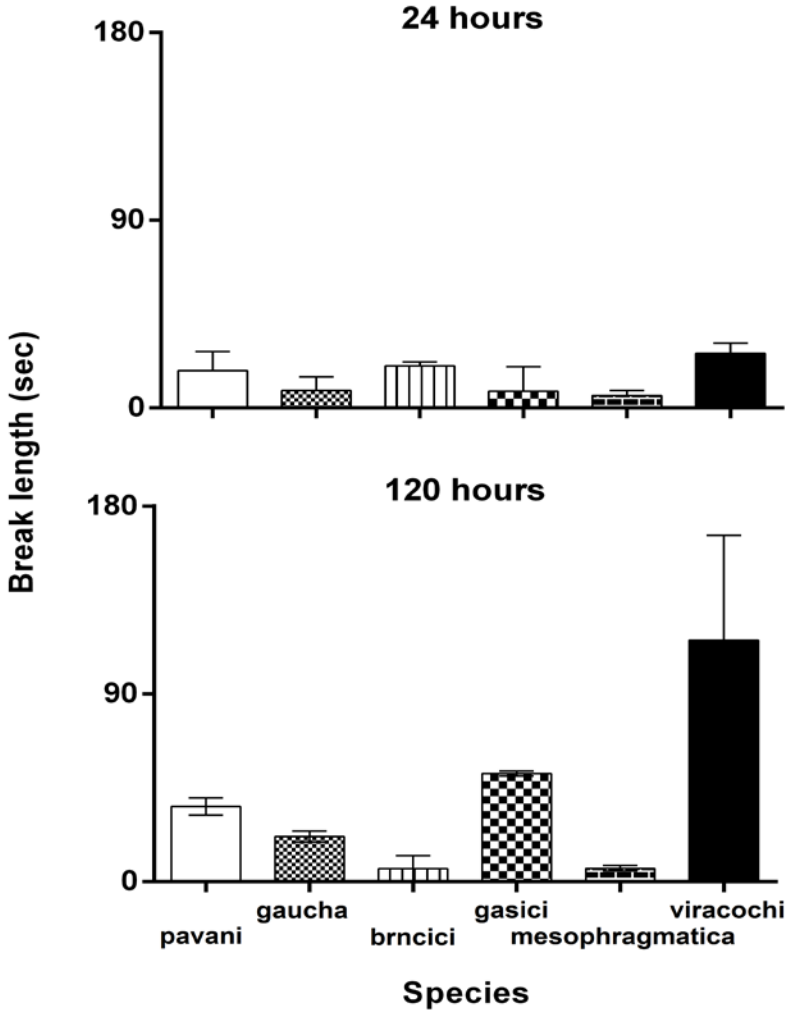

Figure 1. Duration of break length (sec; mean ± ES) in larvae of 24 and 120 hours of development. The larvae were of *D. pavani*, *D. gaucha*, *D. brncici*, *D. gasici*, *D. mesophragmatica*, and *D. viracochi*, N = 50 larvae per age and species.

Figure 1 shows that with the exception of *D. brncici* larvae, which showed feeding break duration greater at 24 than 120 h of age (*t-test* = 2.14, *df* = 98, $P < 0.05$), those of *D. pavani*, *D. gaucha*, *D. gasici*, and *D. viracochi* increased duration of feeding break length at 120 h (*t-test* values fluctuated between 3.14 (*D. gaucha*) and 5.37 (*D. viracochi*), *df* = 98, $P < 0.05$). Larvae of *D. mesophragmatica* at 24 and 120 h of age showed similar duration of feeding break length (*t-test* = 1.66, *df* = 98, $P > 0.05$).

We also compared duration of the feeding bout length in larvae of the two ages of the six species. We found the feeding bout lengths were consistently greater at 120 than at 24 h of larval age in the six species (*t-test* yielded values that fluctuated between 12.45 (*D. gasici*) and 35.79 (*D. mesophragmatica*), *df* = 98, $P < 0.05$).

Feeding breaks interrupt the rate at which food is ingested by *Drosophila* larvae. In the *mesophragmatica* species group, the larvae at 24 h of age show feeding rates lower than at 120 h of development (Del Pino and Godoy-Herrera, 1999), suggesting that feeding break length could decrease as larval development goes by. However, our results indicate that duration of feeding break length is greater at 120 h than at 24 h of development in five out of six of the species examined (Figure 1). Feeding breaks can also be recorded as number of events in time. Here we only recorded the duration. Perhaps, the frequency of feeding breaks decreases in 120-hours-old larvae. This is a question that we ought to answer. On the other hand, feeding bout length in 24-hours-old larvae in the six species of the *mesophragmatica* group are smaller than in larvae of 120 h. Thus, this parameter, but not feeding break length, is in agreement with the increase of

feeding rates between 24 and 148 h of larval development in the *mesophragmatica* group (Del Pino and Godoy-Herrera, 1999). In short, investigations on structure of behaviors deal with ecological resources as food may provide indications on evolution of behavior and brain of *Drosophila* larvae.

Acknowledgments: Thanks are due to Enlace Proyect ELN012/14, Universidad de Chile.

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Male age effect on fitness is independent of inversion system in *Drosophila ananassae*.

Prathibha, M., S.C. Jayaramu*, and M.S. Krishna. Drosophila Stock Centre, Department of Studies in Zoology and Yuvaraja's College, University of Mysore, Mysore -570006, Karnataka, India; E-mail: prathibhajayaramu@gmail.com; *Corresponding author**:** jayaramu21@gmail.com; drosokrish@-gmail.com.

Abstract

Male age influence on fecundity and fertility has been studied in monomorphic (inversion free) and polymorphic (with inversion) strains of *Drosophila ananassae*. It was noticed that in both monomorphic and polymorphic strains, females mated to old males showed greater fecundity and fertility than females mated to young or middle aged males. Thus, in *D. ananassae* male age effect on fitness is independent of inversion system. Key Words: *Drosophila ananassae*, monomorphic, polymorphic, male age, fitness trait.

Introduction

In studies of sexual selection evolutionary response to selection also depends on the amount of genetic variation present in the population of a given species. The genetic variation could be a consequence of either point mutations or due to gross changes in the karyotype. Karyotypic changes are brought about due to either numerical (ploidy) or structural (chromosomal) aberrations. Numerical changes are found mostly in plants and structural changes are common in animals. Structural changes include deletions, duplications, inversions, and translocations. Although all these four kinds of aberrations are of common occurrence in the animal kingdom, all groups of organisms do not have all four kinds of them. According to White (1977) chromosomal rearrangements have played a major role in evolution and the phenomenon has occurred many times in the evolutionary history so as to produce new variants.

Male age is a trait that has received a lot of attention as a potential cue that females might use to derive both direct and indirect benefits (Trivers, 1972; Hansen and Price, 1995; Kokko and Lindstrom, 1996). Theory suggests that males should be favored due to their proven survival ability with only the fittest males able to survive to old age, ensuring a higher average genetic quality (Trivers, 1972; Brooks and Kemp, 2001). Simply reaching old age is, therefore, a reliable way of displaying both genetic superiority in current environmental conditions and lack of mutations accumulated at the prezygotic stage that could reduce survival (Manning, 1985). This hypothesis is supported by empirical evidence in beetles (Conner, 1989; Pervez and Richmond, 2004), field crickets (Zuk, 1988), and warblers (Hasselquist *et al.*, 1996). But the reverse has also been found in bush crickets (Ritchie *et al.*, 1995) and in sand flies, (Jones *et al.*, 2000). Hansen and Price (1995) suggest three main reasons that an individual's fitness decreases with age. First, the older the male, the

greater the possibility of accumulating mutations in the germ line that could offset the advantages of genes for longevity (Crow, 1993). Second, in continually evolving populations with long generation periods, younger males would be preferred due to more recent selection acting on their parents, making them better adapted to current environmental conditions. Third and importantly, with regard to resource allocation, males that have invested heavily in survival and fertility at a younger age may have a lower viability and residual reproductive value when old as a result of negative genetic correlations between early and late fitness components (Cordts and Partridge, 1996). However, models incorporating resource allocation and genetic variation suggest that there could still be positive correlations between early and late fecundity and survival in many circumstances (Kokko, 1998). Further, very few studies have been carried out to study male age influence on offspring qualities (Pervez *et al.*, 2004; Paukku and Kotiaho, 2005; Prokop *et al.*, 2007). Even in these studies, they have found both negative and positive influence of male age on offspring qualities. The difference in conclusions of these models is a probable result of their contrary approaches towards the change in physiological state of males with age and the existence of life history tradeoffs. Unless more species and genera are involved, it is difficult to understand the concept. Hence more studies are needed in this regard. To test these, model species of *Drosophila* as they do not show parental care is more suitable.

D. ananassae has been selected as the experimental model in the present investigation, because of its following characteristics. It is a cosmopolitan domestic species belonging to the *melanogaster* group of *ananassae* subgroup and *ananassae* species complex (Bock and Wheeler, 1972). This species occupies a unique status in the whole of genus *Drosophila* due to certain peculiarities in its genetical behaviour (Singh, 1985a, b). Presence of male crossing over, high levels of inversion polymorphism, and high mutability are the features which make it useful for certain genetic studies. Therefore, the present study has been undertaken in *D. ananassae* to study the role of inversion system in male age effect of mating activity and female fitness.

Materials and Methods

Monomorphic (inversion free) and polymorphic (with inversion) strains of *D. ananassae* were established from out-bred populations collected at semi domestic localities of Mysore, Karnataka, India have been used to study male age effect on mating success and female fitness. To study polytene chromosomes and to screen for the presence of inversions, the larvae were dissected in 0.7% sodium chloride solution. These salivary glands were fixed in 1N HCl for five minutes. The stained glands were individually placed on slides with two drops of 45% acetic acid. Then salivary glands were squashed by placing a clean cover glass and by applying uniform pressure. This achieves uniform spreading of the chromosomes. After squashing, edges of the cover slip were sealed with paraffin lanolin mixture. The observation of slides was done under Leitz Ortholux II scientific and clinical microscope both at low (10×) and high (45×) magnification. If inversion loop is present in the larvae then the mother of those larvae was treated as polymorphic. If there is no inversion loop in the larvae then the mother of those larvae was treated as monomorphic. Presence or absence of inversions was checked for 5 generations. Like this polymorphic and monomorphic strains were established. These experimental stocks were maintained at 21±1°C at relative humidity of 70% using 12:12 L:D cycle. At the 5^{th} generation after testing the inversion, virgin females and bachelor males were isolated within 3 hr of their eclosion separately from monomorphic and polymorphic strains, were aged as required, and were cultured using the same laboratory condition until they were used in the experiments.

Selection of male age classes

Before assigning male age classes we studied the longevity of males in monomorphic and polymorphic strains of *D. ananassae* by transferring individually unmated male into a vial containing wheat cream agar medium once in a week and maintained them in the same condition. This process was continued until their death, and longevity was recorded. A total of 50 replicates were made for each of monomorphic and polymorphic strains, and mean longevity data showed 60± days for monomorphic and 63±2 days for polymorphic strain. Since mean longevity of *D. ananassae* ranges from 60-65 days we assigned days for young, middle, and old aged males as follows [(Young age male (2-3 days), middle age male (24-25 days), and old age male (46-47 days)]. In addition to this, we also collected 5-6 days old virgin females from the respective stocks, and these were used in the present experiment.

Male age influence on fecundity and fertility in monomorphic and polymorphic strains

We used unmated young, middle, and old aged males and 5-6 days old virgin females of monomorphic and polymorphic strains to study male age influence on fitness. A female along with a male (young/middle/old age) were individually transferred into an Elens-Wattiaux mating chamber and observed for 1 hr. Any pair unmated within 1 hr was discarded. All the mated females by each male were individually transferred to a new vial once in 24 hr until their death to study fecundity and fertility. Total number of eggs and progeny produced were recorded. Experiments were done separately for both monomorphic and polymorphic strains.

A total of 50 trials were used separately for each of the three male age classes. Two Way ANOVA followed by Tukey's Honest post hoc test (Tukey's test) was carried out on data of fecundity using SPSS 10.0 Programme.

Results and Discussion

Male age influence on fitness

Figures 1a and 2a, and Tables 1b and 2b, show mean values of fecundity and fertility data of both monomorphic and polymorphic strains. Females mated to old males had greater fecundity and fertility than females mated with either middle or young males. Two-way ANOVA followed by Tukey's honest post-hoc test carried out on above data showed significant variation between male age classes between strains.

The concept of fitness has played a key role in the development of evolutionary biology (Haymer and Hartl, 1982). Fitness in general is a property that can be attributed to an individual, a genotype, or a species and refers to the ability of an organism to leave surviving offspring (Ayala, 1965). Fecundity and productivity are the two intrinsic factors contributing to fitness (Soliman, 1973). Evolutionary biologists relate certain biological phenomenon with the measurement of fitness. Population geneticists call these biological phenomena "components of fitness" (Dobzhansky *et al.*, 1963; Marinkovic, 1967).

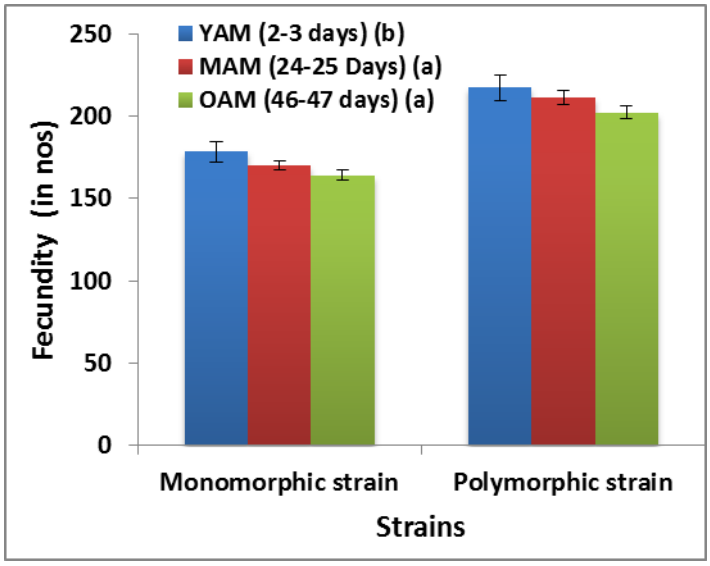

Figure 1a. Male age influence on fecundity in monomorphic and polymorphic strains of *D. ananassae* (Values are mean ± SE).

These fitness characters are influenced by the factors such as species, genotype, body size, age, and by her mate or male's effects as well as by environmental factors such as crowding and temperature (Robertson, 1957; Markow and Akney, 1984; Hoffmann and Harshman, 1985; Partridge *et al.*, 1986; David *et al.*, 1983; David, 1988). In the present investigation it was noticed that female mated with old age male had significantly greater fecundity and fertility than female mated with either young or middle aged males (Figure 1a and 2a, and Table 1b and 2b).

It is not known whether reaching old age should have trading with other fitness traits or it should have positively related to other fitness traits; this might have a bearing on sexual selection. In the present study we have not noticed trade-off between early and late effect of male age on fitness traits in fecundity and fertility of females mated with early and late age males in *D. ananassae*. In other words in *D. ananassae* females preference for old age male could be due to obtaining direct fitness advantage rather than tradeoff between age

Table 1a. Two Way ANOVA of fecundity of female mated to males of different age classes in monomorphic and polymorphic strains of *D. ananassae.*

Fecundity	Source	Type III Sum of Squares	df	Mean Square	F-Values
Fecundity (in no)	Strains	116308.830	1	116308.830	43.07**
	Male age	10569.247	2	5284.623	0.143^{NS}
	Strains*Male age	145.500	2	72.750	.027*
	Error	793862.220	294	2700.212	
	Total	11819775	300		

* Significant at 0.05 level, ** Significant at 0.0001 level, NS- Non significant

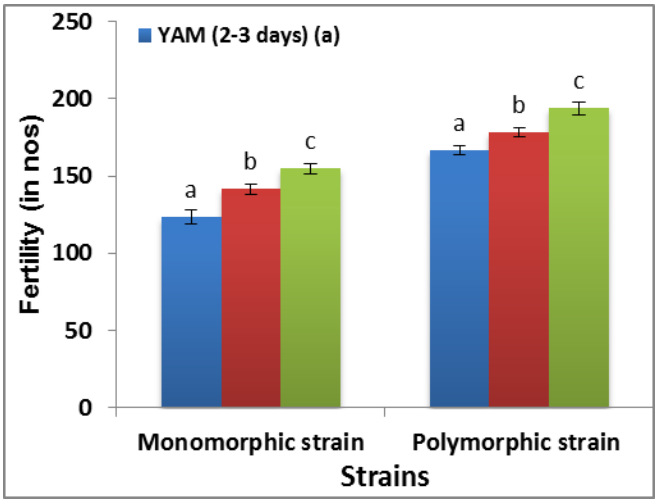

Figure 2a. Male age influence on fertility in monomorphic and polymorphic strains of *D. ananassae* (Values are mean ± SE).

Table 2b. Two Way ANOVA of fertility of female mated to males of different age classes in monomorphic and polymorphic strains of *D. ananassae.*

Fecundity	Source	Type III Sum of Squares	df	Mean Square	F-Values
Fecundity (in no)	Strains	117572.403	1	117572.403	72.770**
	Male age	43162.747	2	21581.373	13.358**
	Strains*Male age	482.027	2	241.013	$.861^{NS}$
	Error	475005.420	294	1615.665	
	Total	8296715.000	300		

* Significant at 0.05 level, ** Significant at 0.0001 level, NS- Non significant

and fitness traits. This is in contrast to the model of Hansen and Price (1995) mutation accumulation theory and antagonistic pleiotropy theory (Charlesworth, 2001; Partridge and Gems, 2002). These models and theories are based on the idea that mutations that have deleterious effects only later in life after the normal breeding age will have a greater chance of persisting and accumulating in the population in the face of natural selection. The antagonistic pleiotropy theory suggests in addition that the late acting deleterious genes may have beneficial effects early in life and are thus favored by selection and will be actively accumulated in populations despite their deleterious effects late in life. However, evidence is accumulating on age-dependent expression of genes affecting reproduction. For example, about half of the genes down regulated with age in *Drosophila melanogaster* are linked to reproduction (Girardot *et al.*, 2006), and female fecundity seems to be

determined by different loci at different ages, with no genetic correlation between early and late age fecundities (Leips *et al.*, 2006). In our study we also found that polymorphic strains had greater reproductive success over monomorphic strains (Singh, 1989; Singh and Chatterjee, 1988; Singh and Som, 2001). This confirms the earlier studies of greater fitness of polymorphic strains over monomorphic strains (Dobzhansky and Levene, 1951; Jayaramu, 2009). Thus, these studies in *D. ananassae* suggest that females of *D. ananassae* discriminate males on the basis of age and older males had greater reproductive success over young or middle age males which is found to be independent from influence of inversion system.

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Reproductive advantage of middle aged females in monomorphic and polymorphic strains of *Drosophila ananassae.*

Jayaramu, S.C. Drosophila Stock Center, Manasagangotri and Yuvaraja's College, University of Mysore, Mysore 570006, Karnataka State, India; Corresponding author E-mail: jayaramu21@gmail.com.

Abstract

Monomorphic (inversion free) and polymorphic (with inversion) strains of *Drosophila ananassae* established from out-bred populations collected at Mysore has been used to study female age influence on mating success, mating latency, courtship activities, copulation duration, and female wing length. It was noticed that polymorphic strains had greater mating success, mated faster, performed greater courtship

activities, and copulated longer than monomorphic strains. It was also noticed that in both monomorphic and polymorphic strains middle aged females had significantly greater mating success, mated faster than young or old aged females. Males showed significantly greater intensities of courtship activities, *i.e*., tapping, scissoring, vibration, circling, licking to middle aged females compared to young or old aged females, copulated longer over young or old aged females. Middle aged females showed significantly less rejection responses, *i.e*., ignoring, extruding, decamping to male compared to young or old aged females. However, insignificant variation was found in female wing length among females of different age classes. Thus, in *D. ananassae* middle aged females had reproductive advantage than young or old aged females, which is independent of inversion system. Key Words: *Drosophila ananassae*, female age, male mate preference, wing length, monomorphic and polymorphic.

Introduction

Most compelling studies on sexual selection have largely concentrated on females, as they are often the selective sex that chooses from amongst males (reviewed by Milinski, 2001). Now it is becoming increasingly apparent that in many species males, nevertheless, have a high cost of reproduction (mating) due to costs arising from factors such as energetically expensive courtship displays (Jones and Hunter, 1993) and the productions of ejaculates (Dewsbury, 1982; Galvani and Johnstone, 1998). For this behavior to be adaptive, these individuals are expected to benefit one way or another.

In general, mate choice is less common in males, being reported in only 58 insect species, distributed among 11 orders and 37 families (Bonduriansky, 2001). There is now a growing number of observations of male choice which is seen in a wide range of taxa including insects (Bonduriansky, 2001; Byrne and Rice, 2006), birds (Jones and Hunter, 1993), and fishes (Amundsen and Forsgren, 2001). These studies suggest that male mate choice is predicted to be adaptive when variance in female fitness is large and males experience a cost to mating such that they cannot inseminate all females encountered (Burley, 1977; Parker, 1983; Owens and Thompson, 1994; Johnstone *et al*., 1996; Kokko and Monaghan, 2001). However, the empirical evidence is limited.

The most obvious character influencing the reproductive value of the female is her fecundity (Bonduriansky, 2001). When mating opportunities are constrained, males that show a preference for more fecund females will benefit directly by increasing the number of offspring they produce (Katvala and Kaitala, 2001). However, models of the evolution of male choice suggest that male choice will tend to breakdown when males target arbitrary female traits rather than those that reliably signal fecundity (Kokko and Johnstone, 2002; Chenoweth *et al*., 2006; Servedio and Lande, 2006). Therefore, male preference for female traits may be an indirect way of assessing female fecundity. The characters used by the males to select the females are generally virginity, her size, age, and gravid status (Bonduriansky, 2001). Thus, there is a need to understand how male choice is related to such traits.

D. ananassae is one of the species that belongs to the genus *Drosophila* and has been employed in the present investigation. It is a cosmopolitan domestic species belonging to *melanogaster* group of *ananassae* subgroup and *ananassae* species complex (Bock and Wheeler, 1972). This species occupies a unique status in the whole of genus *Drosophila* due to certain peculiarities in its genetical behavior (Singh 1985a, b). Absence of male crossing over, high level of inversion polymorphism, and high mutability are the features which make it useful for certain genetic studies. Female age influence on reproduction and male mating success has not been studied. Therefore, monomorphic and polymorphic strains of *D. ananassae* have been employed in the present investigation to study relationship of female age, reproductive success, and inversion system.

Materials and Methods

Experimental stocks of monomorphic and polymorphic strains of *D. ananassae* were established from isofemale lines of *D. ananassae* obtained from out-bred populations collected at semi domestic localities in Mysore, Karnataka, India (Prathibha and Krishna, 2010). These isofemale lines were maintained at 21±1ºC and relative humidity of 70%. When larvae appeared, eight third instar larvae from each isofemale line were used to analyze presence or absence of inversions. This procedure was continued for 3 generations to establish

monomorphic (inversion free) and polymorphic (with inversion) strains and to allow flies to acclimatize in the laboratory. In the 4th generation 5-6 day old flies were used to collect synchronized eggs (± 30 min) using Delcour's procedure (1969). Eggs (100) were seeded in a vial containing wheat cream agar medium. When adults emerged, virgin females and unmated males were isolated within 3 hr of their eclosion and were aspirated into a new vial containing wheat cream agar medium. These flies were aged as required for the experiment. Young aged females (2-3 day old), middle aged females (17-18 days old), and old aged females (32-33 days). These females were kept individually in culture vials containing wheat cream agar medium and were transferred to a new vial containing wheat cream agar medium once in a week until they were used in the experiment. Male flies were aged in the same environment for 5-6 days.

Female age influence on mating success in monomorphic and polymorphic strains

Young, middle, old aged females and 5-6 day old bachelor males of monomorphic and polymorphic strains of *D. ananassae* were used to study whether or not inversion system influences male mating preference for female age. Two females (young, middle/young, old/middle, old) and 5-6 day old male were aspirated into an Elens-Wattiaux (1964) mating chamber. Indian ink was painted to one of the females on the thorax. (The effect of paint was tested before commencing the experiment by painting young female in one trial and middle/old aged female in an alternate trial and allowing them to mate. In 27 out of 50 trials, middle aged females were mated, and in the remaining 23 out of 50 trials old aged females were mated ($\chi^2 = 0.32$; df = 1; $p > 0.05$). Results showed insignificant difference suggesting that painting did not have influence on the performance of the flies). Each pair was observed for 1 hr. When mating occurred, pairs in copulation was aspirated out from the mating chamber and aspirated into a new vial containing wheat cream agar medium. A total of 50 trials were made for each combination and Chi-square analysis was carried out to the male mate choice data. Separate experiments were made for monomorphic and polymorphic strains.

Female age influence on wing length in monomorphic and polymorphic strains

Virgin young/middle/old aged females were individually sacrificed to count female wing length following the procedure of Krishna and Hegde (1997). From the same female, wing length was also measured using 100× following the procedure of Hegde and Krishna (1997). A total of 50 trials were made separately for each of the three female age classes.

Female age influence on mating activities in monomorphic and polymorphic strains

To study relation between female age related changes in mating activities, fecundity, fertility, and inversion system, a male (5-6 days old) along with a female (young/ middle/ old) were aspirated into an Elens-Wattiaux (1964) mating chamber and observation was made for 1 hr. Mating latency (time between introduction of male and female together into a mating chamber until initiation of copulation of each pair) and copulation duration (time between initiation of copulation to termination of copulation of each pair) were recorded. We also quantified courtship acts such as tapping, scissoring, vibration, licking, circling, ignoring, extruding, and decamping following the procedure of Hegde and Krishna (1997).

Separate experiments were carried out for monomorphic and polymorphic strains. Two-way ANOVA followed by Tukey's Honest Post hoc test were carried out on mating latency, courtship activities, copulation duration data using SPSS 10.1 software.

Results

In both monomorphic and polymorphic strains of *D. ananassae* males generally chose to mate with middle aged females more frequently than young or old aged females. Middle aged female success in crosses involving young and middle aged females was 31 ($\chi^2 = 1.40$, df = 1; $P > 0.05$; N = 50) in monomorphic strains and 35 ($\chi^2 = 0.4$, df = 1; $P > 0.05$; N = 50) in polymorphic strains. Middle aged female success in crosses involving old and middle aged females was 35 ($\chi^2 = 0.4$, df = 1; $P > 0.05$; N = 50) in monomorphic strains and 36 ($\chi^2 = 0.45$, df = 1; $P > 0.05$; N = 50) in polymorphic strains. Young aged female success in crosses involving young and old aged females was 32 ($\chi^2 = 0.28$, df = 1; $P > 0.05$; N = 50) in monomorphic strains and 34 ($\chi^2 = 0.36$, df = 1; $P > 0.05$; N = 50) in polymorphic strains.

Table 1. Mean wing length of mated female of monomorphic and polymorphic strains in male choice experiment of *D. ananassae*.

Male	Strains	Females in male Choice experiment	Number of females mated	Wing length (in mm)	t-value
5-6 days male	Mono	YAF	32	1.97 ± .02	0.28^{NS}
		OAF	18	1.99 ± .02	
	Poly	YAF	34	2.00 ± .02	2.12*
		OAF	16	2.10 ± .03	
	Mono	YAF	19	1.98 ± .03	0.19 ^{NS}
		MAF	31	1.99 ± .02	
	Poly	YAF	15	2.05 ± .04	0.32 ^{NS}
		MAF	35	2.07 ± .02	
	Mono	MAF	35	1.99 ± .02	0.27 ^{NS}
		OAF	15	1.98 ± .03	
	Poly	MAF	36	2.08 ± .02	0.12 ^{NS}
		OAF	14	2.08 ± .04	

*P< 0.005; NS – insignificant
Note: 1. YAF- Young age female; OAF- Old age female; MAF- Middle age female.
2. Mono- Monomorphic strain, Poly- Polymorphic strain.

Polymorphic strains had greater mean wing length of Table 1 revealed that in both monomorphic and polymorphic strains insignificant variation was observed in mean wing length of mated female among female age classes (Young, middle, and old), between strains (mono-morphic and polymorphic) and also interaction between female age and strains (young, middle, old and monomorphic, polymor-phic).

Mean courtship, mating activities of females of different age classes mated to males in monomorphic and polymorphic strains are provided in Table 2. It was noticed that, in both monomorphic and polymorphic strains, males mated with middle aged female had taken shortest time for mating initiation, copulated longest, performed greatest courtship activities, *i.e*., tapping, scissoring, vibration, licking, circling. While males mated with old aged female had taken longest time for initiation of mating, copulated shortest, and showed least courtship activities, middle aged females showed least rejection responses to courting male. Old aged females show highest rejection responses to courting males. Male and female courtship activities in polymorphic strain, although not showing significant variation in all the activities studied, had greater activities compared to monomorphic strains. Two way ANOVA followed by Tukey's Honest Post hoc test (Tukey's test) carried out on courtship, mating activities data showed significant variation between strains and between female age classes, however insignificant difference in the interaction between strains and female age classes except licking and ignoring. Tukey's test showed middle aged females took significantly shorter time for mating initiation, males performed greatest courtship activities, copulated longest, and middle aged females had significantly greater compared to young or old aged female. Similarly males performed significantly greater courtship activities to young aged females and copulated longest than old aged female.

Discussion

In *Drosophila* high cost of reproduction is due to cost arising from factors such as energetically expensive courtship displays, the production of ejaculates, and time loss during displaying different courtship (Bonduriansky, 2001). In both monomorphic and polymorphic strains, males of *D. ananassae* prefer to mate with middle aged females more frequently over young or old aged females. This suggests that in *D. ananassae* female age is an important determinant of male mate choice. In other words males of *D ananassae* do not show the same levels of interest in females of different age classes she encountered. This supports the suggestion of Gowaty *et al*. (2003) who, while working on *D. melanogaster*, have also pointed out that males of *Drosophila* do not show the same level of interest in all the females he encountered and provided evidence that males have also evolved to mate selectively. Our study also confirms the earlier studies of existence of male mate choice for females in other insects, too (Bonduriansky, 2001). Thus these studies in *Drosophila* and other insects suggest that male preference for female traits may be an indirect way of assessing female fitness.

Table 2. Female age influence on courtship and mating activities in monomorphic and polymorphic strains of *D. ananassae* (Values are Mean ± SE).

Parameters	Strains	Female age (in days) Young (2-3 days)	Middle (17-18 days)	Old (32-33 days)	F-values F-1 = between strains (df-1, 294)	F-2 = between ages (df-2, 294)	F-3 = between strains & ages (df-6, 294)
Mating latency	Mono	24.36 ± .77^{b}	14.48 ± .48^{a}	32.66 ± .82^{c}	53.14**	300.96**	.27 ^{NS}
	Poly	19.64 ± .58^{b}	10.78 ± .26^{a}	28.14 ± 1.10^{c}			
Tapping	Mono	8.44 ± .19^{b}	10.36 ± .27^{c}	6.96 ± .21^{a}	63.54**	90.83**	.86 ^{NS}
	Poly	9.92 ± .23^{b}	12.54 ± .40^{c}	8.64 ± .27^{a}			
Scissoring	Mono	9.54 ± .31^{b}	11.12 ± .35^{c}	7.86 ± .18^{a}	36.69**	62.73**	.75 ^{NS}
	Poly	10.88 ± .41^{b}	13.16 ± .29^{c}	9.24 ± .32^{a}			
Vibration	Mono	10.22 ± .26^{b}	12.78 ± .37^{c}	7.58 ± .22^{a}	35.36**	114.38**	.53^{NS}
	Poly	11.62 ± .34^{b}	14.14 ± .40	9.54 ± .29^{a}			
Circling	Mono	4.12 ± .10^{b}	5.16 ± .17^{c}	3.18 ± .16^{a}	4.92*	80.12**	1.04^{NS}
	Poly	4.26 ± .13^{b}	5.76 ± .25^{c}	3.38 ± .15^{a}			
Licking	Mono	3.86 ± .19^{b}	4.46 ± .18^{c}	2.88 ± .13^{a}	32.08**	67.39**	5.61*
	Poly	4.68 ± .22^{b}	6.02 ± .26^{c}	3.16 ± .10^{a}			
Ignoring	Mono	4.52 ± .14^{b}	3.24 ± .17^{a}	7.68 ± .17^{c}	42.04**	354.59**	3.18*
	Poly	4.12 ± .20^{b}	2.14 ± .09^{a}	6.52 ± .18^{c}			
Extruding	Mono	4.24 ± .10 ^{b}	2.70 ± .12^{a}	5.94 ± .25^{c}	16.53**	111.42**	.78^{NS}
	Poly	3.42 ± .20^{b}	2.32 ± .17^{a}	5.12 ± .28^{c}			
Decamping	Mono	3.22 ± .13^{b}	2.38 ± .07^{a}	4.94 ± .23^{c}	5.65*	74.92**	.96^{NS}
	Poly	2.98 ± .22^{b}	2.18 ± .14^{a}	4.26 ± .26^{c}			
Copulation duration	Mono	3.96 ± 0.04^{b}	4.13 ± .01^{c}	3.44 ± 0.03^{a}	20.88**	187.71**	.95^{NS}
	Poly	4.08 ± 0.04^{b}	4.34 ± .03^{c}	3.55 ± 0.04^{a}			

*P< 0.005; **P< 0.001; NS – insignificant

Note: 1) Different letter superscript in each row indicates significant by Tukey's test. 2) Mating latency and copulation duration are measured in minutes while other Parameters are measured in numbers. 3) Mono- Monomorphic strain, Poly- Polymorphic strain.

Like female mate choice even in male mate choice too, it was difficult to separate between male choice and female-female competition and differences in female motivation to mate (Byrne and Rice, 2006). Therefore, we can hypothesize that in *D. ananassae* middle aged females are more eager to mate than young or old age females. This agrees with earlier studies of sexual selection in different species of *Drosophila* too (Speith, 1952; Manning, 1961; Hegde and Krishna, 1997; Byrne and Rice, 2006; Prathibha and Krishna, 2010).

Females of young, middle, and old age of *D. ananassae* used in our study were virgins and reared in same condition throughout their experiment suggesting that the observed greater mating success of middle aged females may not be due to difference in the life history and rearing condition. Instead it could be the result of age specific reproductive success in females.

Studies in insects have also found a positive correlation between female size and measure of wing length (Branquart and Hemptinne, 2000). Even in *Drosophila* studies of Robertson (1957) have found positive correlation between female sizes. In the present study we sacrificed the flies which were used for measuring wing length to understand the relationship between female ages wing length. It was found that there was an insignificant difference in mean female wing length among females of different age classes in monomorphic as well as polymorphic strains (Table 1). This suggests that female age has no influence on female body size.

In *Drosophila* successful mating depends of male activity and female receptivity (Manning, 1961; Spieth, 1968). Table 2 shows in both monomorphic and polymorphic strains of *D. ananassae* middle aged females mated faster, copulated longer than young or old aged females. This suggests that middle age females were more receptive than young or old aged females. This supports earlier studies of *Drosophila* suggesting that females which mate faster and longer are more receptive than the females which mate slower and copulate shorter. This is because mating latency (time taken for initiation of mating) and copulation duration (time between initiation of copulation to termination of each pair) are good estimates of female receptivity (Spieth and Ringo, 1981; Hegde and Krishna, 1997).

In turn males of *D. ananassae* showed greater courtship activities to middle aged females compared to young or old aged females suggesting influence of female age on male courtship activities in too (Table 2). Through these courtship activities males of *D. ananassae* not only convey chemical, auditory, visual signals to middle aged females better and try to convince the middle aged female faster for mating than young or old aged females. This agrees with earlier studies of *Drosophila* that males which perform greater courtship activities are better mates and obtained greater mating success than those males which do not show high level of courtship activities (Hegde and Krishna, 1997).

Thus these studies suggest that males of *D. ananassae* exercised mate choice to obtain direct fecundity benefits, and we also found occurrence of age specific reproductive success in females of *D. ananassae* which is independent from influence of inversion system.

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Genetic evidence for differential activities of $G\alpha_O$ isoforms in *Drosophila melanogaster*.

Di Gioacchino, V., T. Barwell, and L. Seroude*. Department of Biology, Queen's University, Kingston, Ontario, Canada K7L 3N6; *Corresponding author, e-mail: seroudel@queensu.ca.

$G\alpha_o47A$ encodes two isoforms of the $G\alpha_O$ subunit ($G\alpha_O1$ and $G\alpha_O2$) in *Drosophila melanogaster* (de Sousa *et al.*, 1989; Schmidt *et al.*, 1989; Thambi *et al.*, 1989; Yoon *et al.*, 1989). These two isoforms are

98% identical and differ from each other by only 7 single residue changes within the 21 N-terminal amino acids (Figure 1). Although this region is evolutionarily conserved, it is amorphous (Slep *et al.*, 2008); hence, molecular modeling cannot predict what structural consequences these differences might have. Studies on this gene have only used genetic constructs encoding one of the isoforms ($G\alpha_O2$, referred to as cDNA class II in Yoon *et al.* (1989)). We have cloned the open reading frames encoding $G\alpha_O2$ and $G\alpha_O1$ (class I cDNA in Yoon *et al.* (1989)) into the pINDY5 *P* element injection vector (Kazemi-Esfarjani and Benzer, 2000) under the control of a UAS promoter (Di Gioacchino, 2014). Each construct was injected into w^{1118} embryos to generate 8 and 12 strains with independent insertions for $G\alpha_O1$ and $G\alpha_O2$, respectively.

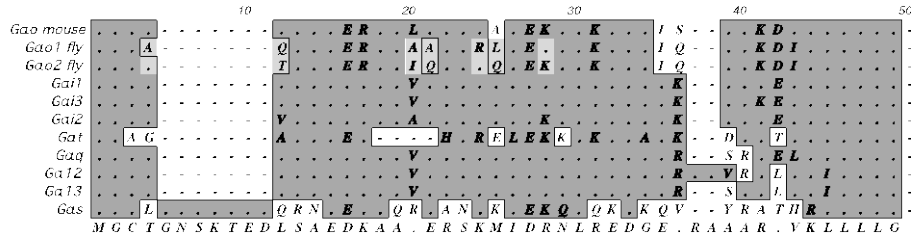

Figure 1. Sequence alignment of heterotrimeric Gα subunit N-termini using methods of Slep *et al.* (2008) and *D. melanogaster* sequences from Yoon *et al.* (1989). Dark grey indicates similar residues, dots indicate identical residues, and light grey highlights the 7 differences between the *Drosophila* isoforms. The last 6 residues correspond to the first ß-sheet motif.

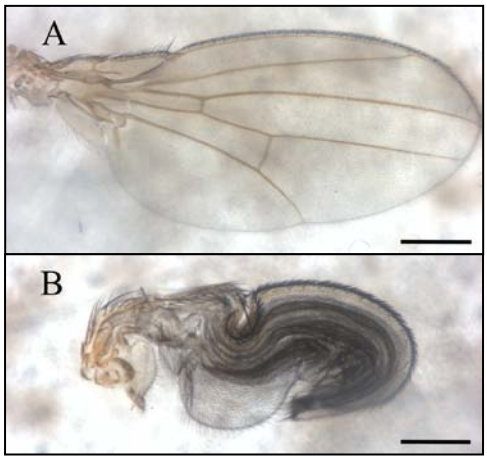

Figure 2. (A) A wild-type wing (32× magnification, scale bar = 0.4 mm), and (B) a crumpled wing (64× magnification, scale bar = 0.2 mm) from two DJ858/+; VD131/+ individuals.

Driven by the DJ858 GAL4 enhancer trap (Seroude, 2002), expression of *UAS-Gα$_O$2* caused a crumpled wing phenotype (Figure 2). This phenotype has been previously observed when $G\alpha_O2$ overexpression was driven by *MS1096-GAL4* (Katanayeva, *et al.*, 2010). Crumpled wings result from the failure of wing maturation, caused by a $G\alpha_O$ subunit competing with and antagonizing the function of a $G\alpha_S$ subunit in a heterotrimer with Gβ13F and Gγ1. DJ858-driven expression of *UAS-Gα$_O$2* caused the crumpled wing phenotype with 7 of the 12 insertions, at varying levels of penetrance (Figure 3). In contrast, only 1 of 8 independent *UAS-Gα$_O$1* insertions caused this phenotype. Unless the insertional biases of the two *P* elements are fundamentally different from each other, the range of expression levels for each construct should be comparable. This suggests that the two isoforms have different biological activities.

One of the DJ858/+;$G\alpha_O2$/+ genotypes (VD134) also caused a noticeable level of pupal lethality and only a few escapers could be recovered. Obvious lethality was not observed with any of the DJ858/+;$G\alpha_O1$/+ genotypes. This observation suggests that $G\alpha_O2$ can cause lethality in addition to the crumpled wing phenotype. Attempts to increase the copy number of both transgenes were unsuccessful since DJ858;$G\alpha_O1$ or DJ858;$G\alpha_O2$ flies exhibit a high rate of lethality. Therefore, increased expression of *UAS-Gα$_O$1* transgenes can also be lethal to the flies.

It was also found that the presence of the TM3, Sb balancer exacerbated the crumpled wing phenotype caused by the expression of *UAS-Gα$_O$2*. $G\alpha_O2$ flies carrying the balancer showed a noticeably higher frequency of the crumpled wing phenotype compared to flies that had a wild-type third chromosome (Figure 4). The addition of the TM3, Sb balancer in a subset of *UAS-Gα$_O$1* genotypes did not induce the crumpled wing phenotype. In addition, there were significantly fewer $G\alpha_O2$ progeny carrying the balancer than expected. These observations indicate that the TM3, Sb chromosome carries one or more mutations that are epistatic to $G\alpha_O2$ expression with respect to both the crumpled wing phenotype and lethality.

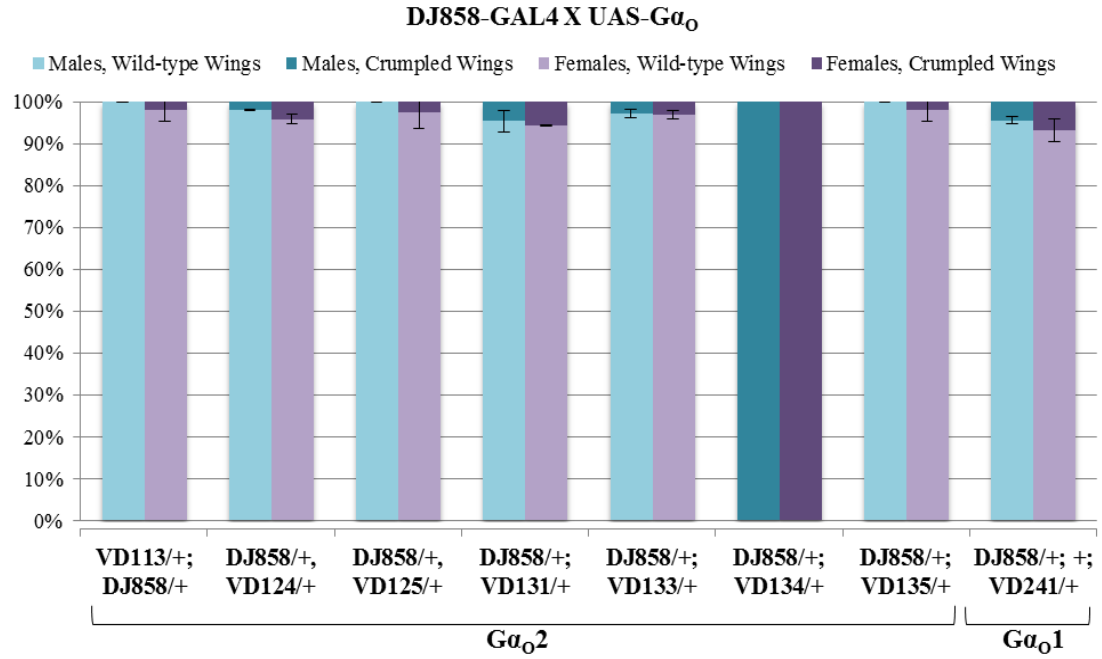

Figure 3. Frequency of the crumpled wing phenotype in progeny carrying *DJ858-GAL4* and either *UAS-Gα$_O$2* or *UAS-Gα$_O$1*. Strains that did not display the phenotype were omitted. Average of two independent experiments; an average of 90 individuals was obtained per replicate, with the exception of VD134 (replicate 1: 4, replicate 2: 3).

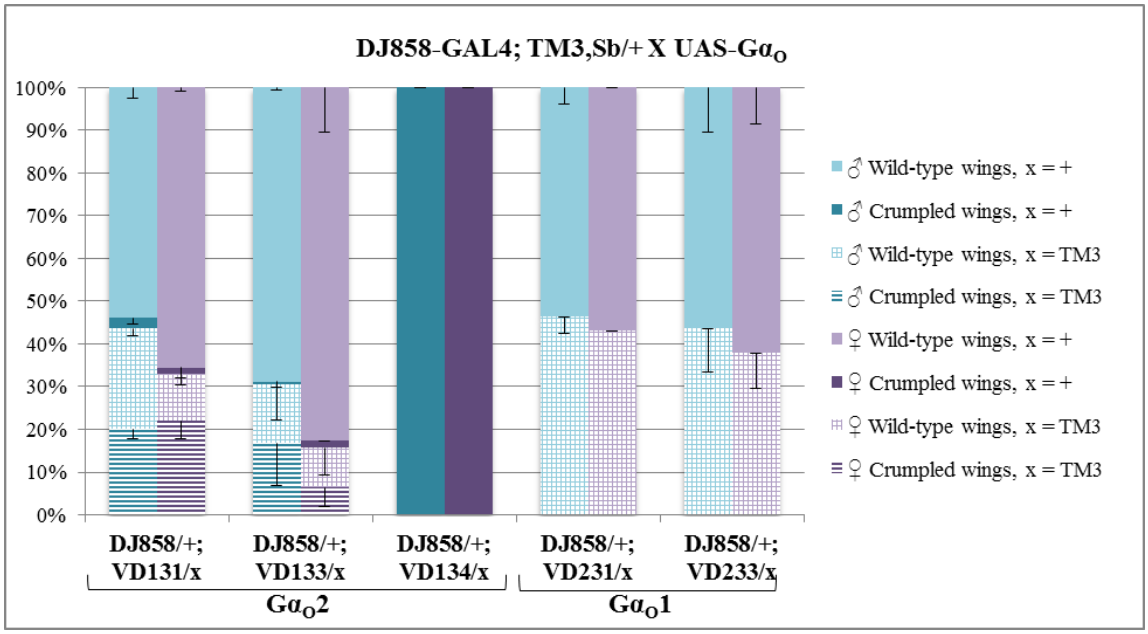

Figure 4. Gα$_O$-TM3,Sb epistasis: frequencies of the wing and bristle phenotypes in progeny carrying *DJ858-GAL4*, *UAS-Gα$_O$2* or *UAS-Gα$_O$1*, and the TM3,Sb balancer or a wild-type third chromosome ("x"). Average of two independent experiments; an average of 110 individuals was obtained per replicate, with the exception of VD134 (replicate 1: 1, replicate 2: 3). χ^2 tests for progeny with and without TM3, Sb: VD131 p♂ > 0.05, p♀ < 0.001; VD133 p♂ < 0.005, p♀ < 0.001; VD231 p♂ > 0.05, p♀ > 0.05; VD233 rep. 1 p♂ < 0.05, p♀ < 0.05, rep. 2 p♂ > 0.05, p♀ > 0.05.

In order to function properly, Gα subunits need to interact with G-protein coupled receptors, β and γ subunits, GTP nucleotides and regulators of G-protein signalling (RGS). Many of the domains that play a key role in these interactions have been well characterized and are evolutionarily conserved (Slep *et al.*, 2008). It has been demonstrated that the 21 N-terminal residues of an α subunit are involved in its interaction with the βγ heterodimer (Navon and Fung, 1987). However, the specific residues that are critical for that interaction have not yet been identified. The presence of two $G\alpha_O$ isoforms in *Drosophila* provides a unique opportunity to investigate structure-function relationships in the N-terminus. Preliminary findings in this report indicate that these two isoforms are not biologically equivalent. Overexpression of both isoforms can cause crumpled wings and lethality, although to a different degree. Furthermore, only $G\alpha_O2$ exhibits epistatic interaction with TM3, Sb. Therefore, these differential biological effects are likely due to one or more of the 7 single residue changes that differentiate $G\alpha_O2$ from $G\alpha_O1$. However, it remains to be shown that these changes are directly involved in interactions with βγ subunits.

It is worth noting that one of the TM3 break points (76C - 71C) is located at the same cytological position as *Gβ76C* (St. Pierre *et al.*, 2014), bringing forth the possibility that TM3 does not carry a wild-type *Gβ76C* allele. It is tempting to speculate that $G\alpha_O2$ and Gβ76C compose the same G-protein. A mutation in *Gβ76C* could reduce the formation of that heterotrimer, increasing the amount of "free" $G\alpha_O$ in cells overexpressing $G\alpha_O2$. This would enhance its ability to antagonize $G\alpha_S$, ultimately leading to an increase in the penetrance of the crumpled wing phenotype.

Materials and Methods

Strains

w^{1118} (w[1118]; +; +) (Bloomington Fly Stock Center, Indiana University, Stock #3605); DJ858 (w[1118]; P{w[+mW.hs]=GawB}DJ858) (Seroude *et al.*, 2002); TM3,Sb balancer from 2475 (w*; $T(2;3)ap^{Xa}$, ap^{Xa}/CyO; TM3, Sb^1) (Bloomington Fly Stock Center, Indiana University, Stock #2475).

$G\alpha_O$ transgenic strains

P element-mediated transformation was performed according to standard protocol without removal of the chorion (Robertson *et al.*, 1988). 200 embryos were injected with the UAS-$G\alpha_O1$ construct, resulting in 11% fertile adults and a 3.5% transformation efficiency. 250 embryos were injected with the UAS-$G\alpha_O2$ construct, resulting in 17.2% fertile adults and a 3.6% transformation efficiency. $G\alpha_O1$ strains were named "VD2xy" and $G\alpha_O2$ strains were named "VD1xy"; "x" refers to the chromosomal location and "y" distinguishes between strains with the insertion on the same chromosome.

Crosses

All crosses were performed for 3-5 days at 25°C on food composed of 0.01% molasses, 8.2% cornmeal, 3.4% yeast extract, 0.94% agar, 0.18% benzoic acid, and 0.66% propionic acid. Progeny were scored 3-5 days after the first progeny emerged. *$G\alpha_O$ expression:* Virgin homozygous DJ858 females were crossed with males from each independent $G\alpha_O$ strain. *$G\alpha_O$-TM3 epistasis:* Virgin DJ858; TM3, Sb/+ females were crossed with homozygous males from $G\alpha_O2$ (strains VD131, VD133, and VD134) or $G\alpha_O1$ (strains VD231 and VD233).

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Male age effect on mating success is independent of inversion system in *Drosophila ananassae.*

Prathibha, M. Drosophila Stock Centre, Department of Studies in Zoology and Yuvaraja's College, University of Mysore, Mysore -570006, Karnataka, India.

Abstract

Male age influence on mating success, courtship, and mating activities has been studied in monomorphic (inversion free) and polymorphic (with inversion) strains of *Drosophila ananassae*. It was noticed that in both monomorphic and polymorphic strains, females of *D. ananassae* were able to discriminate males on the basis of male age and preferred to mate with old aged males more frequently than young or middle aged males using female choice experiments. Old aged males significantly mated faster, performed greater courtship activities, copulated longer than females mated to young or middle aged males. Thus, in *D. ananassae* male age influence on mating success is independent of inversion system. Key Words: *Drosophila ananassae*, monomorphic, polymorphic, male age, female preference, courtship activities.

Introduction

In the field of behavioral biology from the time of Darwin females of a species exercise their choice in mating to obtain potential male was shown to exist in many taxa (Andersson, 1994**)**. Females use number of male traits to select male such as size, mating care, courtship song, pigmentation, and so forth (Somashekar *et al.*, 2011). In recent years it was shown that females of a species could also use male age as a cue to select male in the mating to derive either direct or indirect benefits (Trivers, 1972; Hansen and Price, 1995; Kokko and Lindstrom, 1996**)**. Different models have been proposed for female preference to male age in different organisms. Good gene model is one such model which predicts that females of a species should select the old age male to obtain indirect genetics benefits. This model is based on the assumption that old males have proven their survival ability since only the fittest males should be able to survive to old age suggesting they have higher genetic quality. Therefore, females mating with such males could obtain better offspring qualities than the females mated with young age males (Brooks and Kemp, 2001). Some empirical evidence has also been found for female preference for old male in a few taxa (Wedell *et al.*, 2002; Simmons and Zuk, 1997; Simmons, 1995; Manning, 1989). In contrast to this the others are of the opinion that the female should select young males as they have accumulated fewer germ line mutations (Crow, 1993), that the presence of negative genetic correlations between traits results in trade-offs between early and late fitness components (Cordts and Partridge, 1996) or in long lived organisms, and that offspring born to younger parents are better adapted to current environmental conditions, because their parents were exposed to recent selection (Hansen and Price, 1995). When factors such as the allocation of resources to sexual traits, variations in male condition and age specific survival probability were included, the models showed that preferences for older males could evolve (Kokko, 1997; 1998; Hansen and Price, 1999; Beck and Powell, 2000; Beck and Promislow, 2007; Beck *et al.*, 2002). However these models also predicted that preferences for younger males are equally possible, depending upon the conditions specified. The difference in conclusions of these models is a probable result of their contrary approaches towards the change in physiological state of males with age and the existence of life history tradeoffs. Unless more species and genera are involved, it is difficult to understand the concept. Hence more studies are needed in this regard. To test these, model species of *Drosophila* as they do not show parental care is more suitable.

D. ananassae has been selected as the experimental model in the present investigation because of its following characteristics. It is a cosmopolitan domestic species belonging to *melanogaster* group of *ananassae* sub group and *ananassae* species complex (Bock and Wheeler, 1972). This species occupies a unique status in the whole of the genus *Drosophila* due to certain peculiarities in its genetical behavior (Singh, 1985a, b). Presence of male crossing over, high level of inversion polymorphism and high mutability are the

features which make it useful for certain genetic studies. Therefore, the present study has been undertaken in *D. ananassae* to study the role of inversion system in male age effect of mating activity.

Materials and Methods

Monomorphic (Inversion free) and polymorphic (with inversion) strains of *D. ananassae* that were established from out-bred populations collected at semi domestic localities of Mysore, Karnataka, India have been used to study male age effects on mating success. To study polytene chromosomes and to screen for the presence of inversions, the larvae were dissected in 0.7% sodium chloride solution. These salivary glands were fixed in 1N HCl for five minutes. The stained glands were individually placed on slides with two drops of 45% acetic acid. Then salivary glands were squashed by placing a clean cover glass and by applying uniform pressure. This achieves uniform spreading of the chromosomes. After squashing, edges of the cover slip were sealed with paraffin lanolin mixture. The observation of slides were done under Leitz Ortholux II scientific and clinical microscope both at low (10×) and high (45×) magnification. If inversion loop is present in the larvae then the mother of those larvae was treated as polymorphic. If there is no inversion loop in the larvae then the mother of those larvae was treated as monomorphic. Presence or absence of inversions was checked for 5 generations. Like this polymorphic and monomorphic strains were established. These experimental stocks were maintained at 21±1°C at relative humidity of 70% using 12:12 L:D cycle. At the 5^{th} generation after testing the inversion, virgin females and bachelor males were isolated within 3 hr of their eclosion separately from monomorphic and polymorphic strains and aged as required and cultured using the same laboratory condition until they were used in the experiments.

Selection of male age classes

Before assigning male age classes, we studied the longevity of males in monomorphic and polymorphic strains of *D. ananassae* by transferring an individually unmated male into a vial containing wheat cream agar medium once in a week and maintaining them in the same condition. This process was continued until their death and longevity was recorded. A total of 50 replicates were made for each of monomorphic and polymorphic strains and mean longevity data showed 60 ± days for monomorphic and 63 ± 2 days for polymorphic strain. Since mean longevity of *D. ananassae* ranges from 60-65 days, we assigned days for young, middle, and old aged male as follows [Young age male (2-3days), middle age male (24-25 days), and old age male (46-47days)]. In addition to this, we also collected 5-6 days old virgin females from the respective stocks and these were used in the present experiment.

Male age influence on male mating success in monomorphic and polymorphic strains

Five-six day old virgin females and unmated young, middle, old aged males of monomorphic and polymorphic strains were used to study male age influence on male mating success using female mate choice experiment. A female along with two males of different male age classes were individually transferred into an Elens-Wattiaux mating chamber (Elens and Wattiaux, 1964). (The effect of paint was tested before commencing the experiment by painting one of the two young /middle/old age males and allowing them to mate. This pair was observed for 1 hr. Between young age males in 24 out of 50 trials, males painted were mated and in the remaining 26 out of 50 trials males that were not painted were mated ($\chi^2 = 0.08$, df = 1; $P > 0.05$), for middle age males in 23 out of 50 trials, males painted were mated and in the remaining 27 out of 50 trials unpainted males were mated ($\chi^2 = 0.32$, df = 1; $P > 0.05$), and for old age males in 24 out of 50 trials, male painted were mated while in the remaining 26 out of 50 trials, males not painted were mated ($\chi^2 = 0.08$, df = 1; $P > 0.05$). Thus, these results indicated that painting of one of the competing males in female mate choice experiments did not have an effect on the performance of the flies). Observation was made for 1 hr. When mating occurred, pairs in copulation were aspirated out from the mating chamber. Rejected males in the female mate choice experiment were also transferred to new vials. Separate experiments were conducted for both monomorphic and polymorphic strains. A total of 50 trials were made separately for each combination of female mate choice experiment, and Chi-square analysis was applied to the data of female mate choice experiments. We also measured wing length of 50 selected and rejected males in each combination of the

female mate choice experiment following the procedure of Hegde and Krishna (1997), and Paired 't' test was applied to the data.

Male age influence on mating activities in monomorphic and polymorphic strains

We used unmated young, middle, and old aged males and 5-6 days old virgin females of monomorphic and polymorphic strains to study male age influence on male mating activities. A female along with a male (young/middle/old age) were individually transferred into an Elens-Wattiaux mating chamber and observed for 1 hr. Pair unmated within 1 hr were discarded. We recorded mating latency (time between introduction of male and female together into mating chamber until initiation of copulation of each pair) and copulation duration (time between initiation of copulation to termination of copulation of each pair). We also quantified courtship acts such as tapping, scissoring, vibration, licking, circling, ignoring, extruding, and decamping following the procedure of Hegde and Krishna (1997). The behavior of males and females was recorded simultaneously but separately by two observers for 1 hr; the number of pairs mated was also recorded.

A total of 50 trials were used separately for each of the three male age classes. Two way ANOVA followed by Tukey's Honest post hoc test (Tukey's test) was carried out on data of mating activities, courtship activities using SPSS 10.0 Programme. Experiments were done separately for both monomorphic and polymorphic strains.

Results

Male age influence on male mating success

In both monomorphic and polymorphic strains, females of *D. ananassae* generally chose to mate older of the 2 competing males of different age classes (Figures 1a, 1b, and 1c; Tables 1a, 1b, and 1c). In monomorphic strain: in 70% of cases, older males were successful in crosses involving young and old aged males ($\chi 2 = 8.00$, df = 1; $P < 0.05$; N = 50); in 68% of cases middle aged males were successful in crosses involving young and middle aged males ($\chi 2 = 6.48$, df = 1; $P < 0.05$; N = 50). In 64% of cases, old aged males were successful in crosses involving middle and old aged males ($\chi 2 = 3.92$, df = 1; $P < 0.05$; N = 50). While in polymorphic strain: In 82% of cases, older males were successful in crosses involving young and old aged males ($\chi 2 = 20.48$, df = 1; $P < 0.05$; N = 50); in 72% of cases middle aged males were successful in crosses involving young and middle aged males ($\chi 2 = 9.68$, df = 1; $P < 0.05$; N = 50). In 66% of cases, old aged males were successful in crosses involving middle and old aged males ($\chi 2 = 5.12$, df = 1; $P < 0.05$; N = 50).

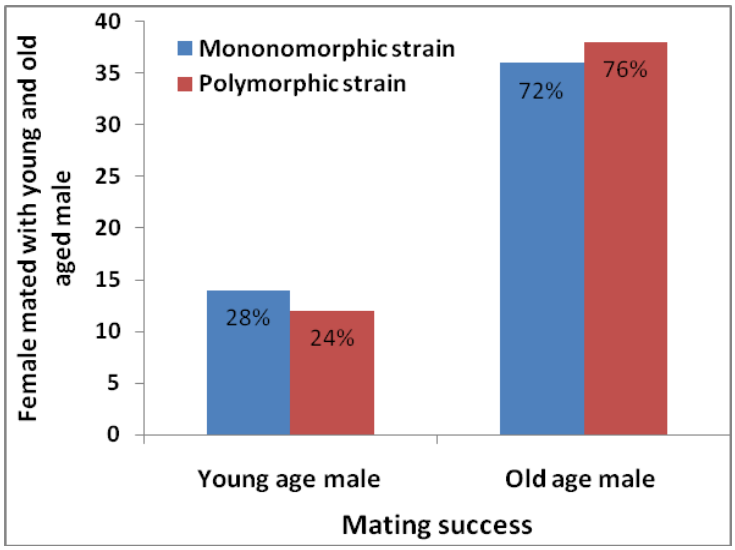

Figure 1a. Female preference for young and old aged males in monomorphic and polymorphic strains of *D. ananassae.*

Table 1a. Female preference for young and old aged males in monomorphic and polymorphic strains of *D. ananassae.*

Strains	Female	Males	Female mated with YAM	Female mated with OAM	X^2 - Value
Monomorphic	5-6 days	YAM + OAM	14 (28%)	36 (72%)	9.68*
Polymorphic	5-6 days	YAM + OAM	12 (24%)	38 (76%)	13.52*

*Significant at 0.01 level; YAM-Young age male; OAM- Old age male

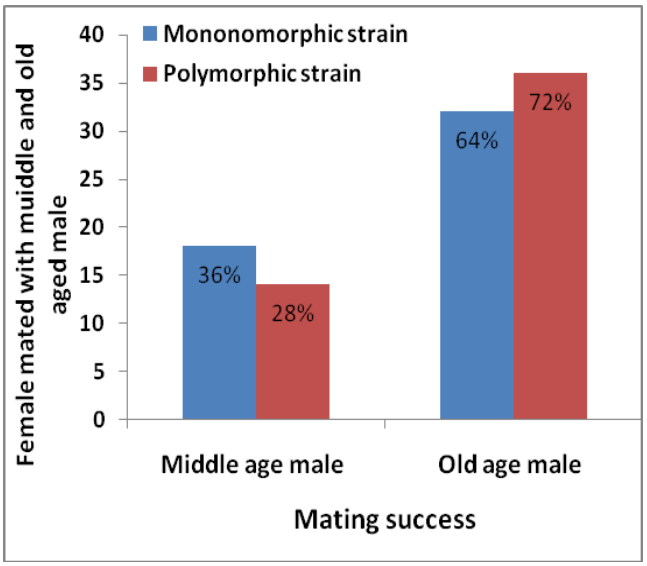

Figure 1b. Female preference for middle and old aged males in monomorphic and polymorphic strains of *D. ananassae.*

Table 1b. Female preference for middle and old aged males in monomorphic and polymorphic strains of *D. ananassae.*

Strains	Female	Males	Female mated with MAM	Female mated with OAM	X^2 - Value
Monomorphic	5-6 days	MAM + OAM	18 (36%)	32 (64%)	3.92*
Polymorphic	5-6 days	MAM + OAM	14 (28%)	36 (72%)	9.68**

* Significant at 0.05 level, **Significant at 0.01 level; MAM- Middle age male; OAM- Old age male.

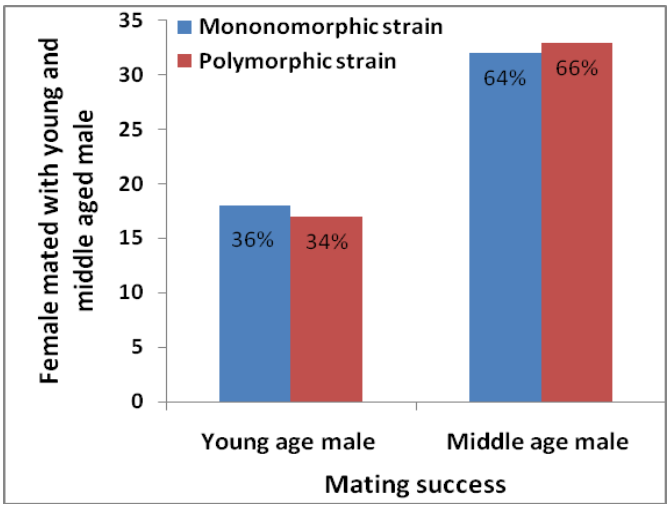

Figure 1c. Female preference for young and middle aged males in monomorphic and polymorphic strains of *D. ananassae.*

Table 1c. Female preference for young and middle aged males in monomorphic and polymorphic strains of *D. ananassae.*

Strains	Female	Males	Female mated with YAM	Female mated with MAM	χ^2 - Value
Monomorphic	5-6 days	YAM + MAM	18 (36%)	32 (64%)	3.92*
Polymorphic	5-6 days	YAM + MAM	17 (34%)	33 (66%)	5.12*

* Significant at 0.05 level; YAM-Young age male; MAM- Middle age male

Male age influence on selected and rejected male

Mean wing length of rejected males was slightly greater than that of mean wing length of selected males in all the combinations of monomorphic and polymorphic strains studied (Table 2). Paired 't' test carried out on mean wing length data of selected and rejected males showed insignificant variation in both monomorphic and polymorphic strains.

Table 2. Mean wing length (in mm) of selected and rejected males of monomorphic and polymorphic strains of *D. ananassae* in female choice experiment (Values are mean ± SE).

Strains	Crosses		Wing length (in mm)		
	Female	Males	Selected male	Rejected male	t- value
Monomorphic	5-6 days	YAM + OAM	1.701 ± .006	1.710 ± .005	1.79 ^{NS}
Polymorphic		YAM + OAM	1.738 ± .006	1.744 ± .007	0.85 ^{NS}
Monomorphic	5-6 days	MAM + OAM	1.716 ± .004	1.718 ± .005	0.74 ^{NS}
Polymorphic		MAM + OAM	1.751 ± .016	1.763 ± .020	0.63 ^{NS}
Monomorphic	5-6 days	YAM + MAM	1.719 ± .006	1.721 ± .006	1.40 ^{NS}
Polymorphic		YAM + MAM	1.767 ± .015	1.772 ± .014	0.36 ^{NS}

NS- Non significant; df -49.

Male age influence on courtship and mating activities

Table 3 shows mean values of courtship and mating data of both monomorphic and polymorphic strains. It was noticed that old aged male had the lowest mating latency, copulated longer, and performed greater male courtship activities (tapping, scissoring, vibration, licking, circling), while the reverse trend was found in the above characters in young aged males. Females showed least rejection responses (ignoring, extruding, and decamping) to old aged males, while she showed greater rejection responses to young aged males. Two Way ANOVA followed by Tukey's test carried out on mean mating latency, copulation duration, courtship activities data using SPSS 10.0 programme showed significant variation in all the above characters among males of different age classes, between monomorphic and polymorphic strains, and also interaction between age and strains (Table 3). Tukey's test showed that in both monomorphic and polymorphic strains old aged males had significantly lesser mating latency, greater copulation duration, mating activities than young or middle aged males except vibration. Vibration by young and middle aged males was found to be insignificant by Tukey's test. Similarly, middle aged males had significantly greater mating latency, copulation duration, mating activities compared to young aged males. Females showed significantly greater rejection responses to young aged males compared to old and middle aged males. Polymorphic strains showed significantly greater courtship, mating characters compared to monomorphic strains.

Table 3. Male age influence on male courtship and mating activities in monomorphic and polymorphic strains of *D. ananassae* (Values are Mean ± SE).

Parameters	Strains	Males			F-values		
		Young (2-3days)	Middle (24-25days)	Old (46-47days)	F1 = between strains (df-1, 294)	F2 = between ages (df-2, 294)	F3 = between strains & ages (df-6, 294)
Mating latency	Mono	24.68 ± .79a	19.90 ± .64b	14.56 ± .51c	121.87**	120.86**	1.25NS
	Poly	18.86 ± .68a	13.74 ± .52b	10.20 ± .37c			
Tapping	Mono	8.92 ± .21a	10.88 ± .28b	11.86± .29c	11.49**	45.70**	.15NS
	Poly	9.84 ± .34a	11.56 ± .34	12.88 ± .37c			
Scissoring	Mono	9.96 ± .35a	11.08 ± .31b	12.08 ±. 36c	8.55**	19.43**	.23NS
	Poly	11.56 ± .4a	12.14 ± .38b	13.12 ± .42c			
Vibration	Mono	8.50 ± .35a	9.04 ± .39a	11.00 ± .47b	10.16**	25.30**	.81NS
	Poly	9.02 ± .41a	10.34 ± .56a	12.60 ± .39b			
Circling	Mono	3.40 ± .15a	3.98 ± .14b	4.78 ± .15c	67.65**	63.53**	10.99**
	Poly	3.76 ± .18a	5.52 ± .20b	7.08 ± .33c			
Licking	Mono	3.08 ± .19a	4.00 ± .21b	4.66 ± .16c	9.90**	27.53**	.28NS
	Poly	3.74 ± .26a	4.36 ± .21b	5.24 ± .17c			
Ignoring	Mono	5.54 ± .24a	4.32 ± .16 b	3.54 ± .20c	7.32*	47.75**	.12NS
	Poly	5.04 ± .25a	3.96 ± .17 b	2.98 ± .16c			
Extruding	Mono	4.94 ± .19a	3.50 ± .14b	2.74 ± .13c	9.35**	91.52**	.33NS
	Poly	4.42 ± .20a	3.24 ± .13b	2.32 ± .12c			
Decamping	Mono	3.80 ± .16a	3.12 ± .17b	2.48 ± .11c	5.81*	38.19**	.16NS
	Poly	3.46 ± .15a	2.92 ± .16b	2.12 ± .12c			
Copulation duration	Mono	3. 41 ± .03a	3.64 ± .04b	4.04 ± .04c	96.33**	84.54**	2.87NS
	Poly	3.74 ± .05a	4.15 ± .04b	4.35 ± .05c			

*P< 0.05; **P< 0.001; NS-insignificant.
Notes: 1) Different letter in superscript in each row indicates significant by Tukey's test. 2) Mating latency and copulation duration are measured in minutes while other parameters are measured in numbers.

Discussion

In *Drosophila ananassae* it was noticed from mating success that in both monomorphic and polymorphic strains, the female was able to discriminate males on the basis of male age and she preferred to mate more frequently with old aged males than with young or middle aged males (Figures 1a, 1b, and 1c; Tables 1a, 1b, and 1c). This suggests that female preference for old age is independent of inversion system. Our study also supports female preference for old age male studies in *D. melanogaster* and *D. simulans* (Moulin *et al*., 2001) and in *D. pseudoobscura* (Avent *et al*., 2008). That suggests that females of these species were able to discriminate males on male age classes and she mates with old aged males more frequently over young aged males. This result is also consistent with some previous observations in other insects and birds (Singh and Som, 2001; Conner, 1989; Hasselquist, 1996) and also with the theoretical models of good gene hypothesis (Kokko, 1997, 1998) suggesting that female preference for old aged male can be an evolutionarily stable strategy (Avent *et al*., 2008). In contrast to this, studies of Hansen and Price (1995) and Price and Hansen (1998) in *D. melanogaster* have argued that females prefer to mate with young males more frequently than old aged males. However, empirical evidence in this regard is absent in *Drosophila.*

In studies of female mate choice it is common that in addition to female preference, it also involves male-male competition (Avent *et al*., 2008). Therefore, it was difficult to say whether observed mating success resulted due to female preference or the result of male-male competition. However, in studies of female choice it was suggested that male-male competition can reinforce female mate preferences rather than operating in an antagonistic fashion (Moore and Moore, 1999).

In studies of *Drosophila* it was found that male size is important of his success in mating (Partridge *et al.,* 1987; Santos *et al.,* 1992; Hegde and Krishna, 1997). Insignificant variation was also found in mean wing length of selected and rejected males and even in some combination mean wing length of rejected males were slightly longer than that of selected males (Table 2). In the present study wing lengths of selected and rejected males were also measured. This suggests that observed greater mating success of old aged males was not due to difference in the mean wing length between young, middle, and old aged males. Instead it could be attributed to male age itself.

Rearing condition provided for young, middle, and old aged males in the experiment were the same and all these males were unmated. Therefore the observed greater mating success of old aged males cannot be attributed to differences in the rearing condition, male experience, and male mating history as found in some previous experiments in insects (Jones and Elgar, 2004; Svetec and Ferveur, 2005).

Young, middle, and old aged males of *D. ananassae* used here were fully mature and all showed courtship activities. Males below 2nd day were immature; they do not show any courtship activities and males above 46-47 days started showing decline in courtship activities. Therefore, the observed lesser mating success of young or middle aged males may not be attributed to young and middle aged males being immature.

In *Drosophila* the success of males in mating also depends on mating activities performed to convince the female (Bastock, 1956; Hegde and Krishna, 1997). In our study in both monomorphic and polymorphic strains, old aged males mated faster (as the time is reverse of the speed), copulated longer, and performed greater courtship activities than young or middle aged males (Table 3). Through these activities old aged males convinced the female faster and had greater mating success because through these courtship acts the male communicates to the female through visual, chemical, tactile, and auditory stimuli. As a result he increases the female receptivity (Speith, 1966; Tompkins *et al.,* 1982; Hegde and Krishna, 1997). This confirms the earlier studies of courtship in different species of *Drosophila* suggesting that flies which show greater activities during courtship have greater mating success than males which show less activities (Hegde and Krishna, 1997). Females of *D. ananassae* showed less rejection activities, *i.e*., extruding, decamping, ignoring to old aged males compared to young or middle aged males (Table 3). This suggests influence of male age on female receptivity. This confirms earlier studies of *Drosophila* where females which show less rejection responses, have greater receptivity, and have greater mating success than the females which show greater rejection to courting males (Hegde and Krishna, 1997).

Studies of sexual behavior in *Drosophila* suggest that courtship activity of male and female culminates in copulation (Spiess, 1970). In our study we noticed that old aged males copulated longer compared to young or middle aged males. This suggests a male age influence on copulation duration. This supports the work of Avent *et al.* (2008) who while working in *D. pseudoobscura* has also found longer copulation duration of old age males. It is not known whether the delayed mating could be the reason for older male to copulate longer or not. There is a strong theoretical reason to expect for greater duration of copulation of old age males was that old males have not encountered females for many days to be investing more resources in the first female he encounters (Wedell *et al.,* 2002), or old age males have lower residual reproduction value and may increase their ejaculate investment per mating with increasing age (Roff, 1992). Also, old males may have accumulated a larger quantity of ejaculate (sperm and seminal fluids) that takes a longer time to transfer to the females (Jones *et al.,* 2007). In *D. pseudoobscura* it was found that males that were kept as virgins for 14 days had high ejaculate quantities (Avent *et al*., 2008). This suggests that sperm quantity may increase with increasing male age.

This confirms the earlier studies of greater mating activities of polymorphic strain over monomorphic strain (Dobzhansky and Levene, 1951; Jayaramu, 2009). Thus, in *D. ananassae* male age influence on mating success is independent of inversion system.

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Greater fitness of middle aged females in monomorphic and polymorphic strains of *Drosophila ananassae.*

Jayaramu, S.C., and M. Prathibha*. Drosophila Stock Center, Department of Studies in Zoology, University of Mysore, Manasagangotri, Mysore 570006, Karnataka State, India; E-mail corresponding author: *prathibhajayaramu@gmail.com; jayaramu21@gmail.com.

Abstract

Monomorphic (inversion free) and polymorphic (with inversion) strains of *Drosophila ananassae* established from out-bred population collected at Mysore have been used to study female age influence on fecundity, fertility, and ovariole number. It was noticed that polymorphic strains had greater fecundity, fertility, and ovariole number than monomorphic strains. It was also noticed that in both monomorphic and polymorphic strains middle aged females had significantly greater fecundity, fertility, ovariole number than young or old aged females. Thus, in *D. ananassae* middle aged females had higher reproductive fitness than young or old aged females, which is independent of inversion system. Key Words: *Drosophila ananassae*, female age, male mate preference, ovariole number, monomorphic and polymorphic.

Introduction

Male mate choice is expected in systems where males allocate valuable resources to parental investment, in response to variation in female quality or where the costs of mate search and/or assessment are

low (Bounduriansky, 2001). Further, assuming the costs of male mate choice are not too great (Altmann, 1997), it would benefit males to exercise mate choice: 1) if male reproductive success is limited by more factors than simply the number of female mates and 2) if females differ in quality or, more specifically, in their reproductive potential (Byrne and Rice, 2006). Both of the preceding criteria need to be in place so that males can mate selectively to be evolutionarily advantageous. If all females in a male's pool of potential mates have equal reproductive potential, males should not preferentially mate with one over another, as maximizing only the number of female mates would give males the highest reproductive pay off (Bateman, 1948). If females differ in reproductive potential, males might exercise some degree of mate choice. Therefore, males must operate under time constraints, as well as possibly dwindling energy or sperm reserves, or both. Males that exercise mate selectivity might, therefore, have a reproductive advantage by allocating their time, sperm, and energy wisely. This is possible with the females (Andersson, 1994) that will provide them with the greatest gain in reproductive success.

Male mating preferences have now been documented in insects (Kvarnemo and Simmons, 1999; Bonduriansky, 2001), fish (Berglund and Rosenqvist 2003; Kvarnemo *et al.,* 2007), lizards (LeBas and Marshall, 2000; Orrell and Jenssen, 2002), birds (Torres and Velando, 2005; Kraaijeveld *et al.*, 2007), and mammals (Domb and Pagel, 2001; Parga, 2006).

Male mating preferences may often serve to increase their reproductive success, as the characteristics of females that they prefer are often correlated with individual differences in fecundity or with the proximity of females to conception (Jones *et al.,* 2001; Ruscio and Adkins-Regan, 2003). Few studies have yet investigated the additional possibility that male mate choice may also affect the quality of offspring, but a recent study of house mice, *Mus domesticus,* suggests that this may be the case: when males were experimentally mated with females they did not prefer, they produced offspring with lower viability and poorer performance than those allowed to mate with preferred females (Drickamer *et al.*, 2003; Gowaty *et al.*, 2003).

The characters used by the males to select the females are generally virginity, her size, age, and gravid status (Bonduriansky, 2001). The most obvious character influencing the reproductive value of the female is her fecundity (Bonduriansky, 2001). When mating opportunities are constrained, males that show a preference for more fecund females will benefit directly by increasing the number of offspring they produce (Katvala and Kaitala, 2001). Most compelling studies of male choice suggest that female mating success is often associated with traits that are correlated with female fecundity (Bonduriansky, 2001; Byrne and Rice, 2006), while in others mate assessment may occur through display traits such as colorations, pheromones, or ornament and morphological features (Amundsen, 2000b; LeBas *et al.,* 2003). However, models of the evolution of male choice suggest that male choice will tend to break down when males target arbitrary female traits rather than those that reliably signal fecundity (Kokko and Johnstone, 2002; Chenoweth *et al.,* 2006). Therefore, male preference for female traits may be an indirect way of assessing female fecundity. Thus, there is a need to understand how male choice is related to such traits.

Therefore, the present study of maternal age influence on offspring fitness has been undertaken in *D. ananassae,* a cosmopolitan domestic species of *Drosophila* belonging to *melanogaster* group of *ananassae* subgroup.

For this purpose *D. ananassae* has been selected as the experimental model because of its following characteristics. It is a cosmopolitan domestic species belonging to *melanogaster* group of *ananassae* sub group and *ananassae* species complex (Bock and Wheeler, 1972). This species occupies a unique status in the whole of genus *Drosophila* due to certain peculiarities in its genetic behavior (Singh, 1985a, b). Presence of male crossing over, high level of inversion polymorphism and high mutability are the features which make it useful for certain genetic studies. Further in this species female age influence on mating success and offspring fitness has not been studied. Therefore, the present investigation was undertaken in *D. ananassae* to address the following questions: 1. Are males of *D. ananassae* able to discriminate females on the basis of female age? If so, what is its effect on female fitness and offspring quality? 2. Whether or not successful mothers produce successful offspring? 3. Whether or not inversion system has influence on inheritance of characters from female to their offspring in relation to female age?

Materials and Methods

Experimental stocks of monomorphic and polymorphic strains of *D. ananassae* were established from isofemale lines of *D. ananassae* obtained from out-bred populations collected at semi domestic localities in Mysore, Karnataka, India (Prathibha and Krishna, 2010). These isofemale lines were maintained at 21±1ºC and relative humidity of 70%. When larvae appeared, eight third instar larvae from each isofemale line were used to analyze presence or absence of inversions. This procedure was continued for 3 generations to establish monomorphic (inversion free) and polymorphic (with inversion) strains and to allow flies to acclimatize in the laboratory. In the 4th generation 5-6 days old flies were used to collect synchronized eggs (± 30 min) using Delcour's procedure (1969). Eggs (100) were seeded in a vial containing wheat cream agar medium. When adults emerged, virgin females and unmated males were isolated within 3 hr of their eclosion and were aspirated into a new vial containing wheat cream agar medium. These flies were aged as required for the experiment: young aged females (2-3 day old), middle aged females (17-18 days old), and old aged females (32-33 days). These females were kept individually in a culture vials containing wheat cream agar medium and were transferred to a new vial containing wheat cream agar medium once a week until they were used in the experiment. Male flies were aged in the same environment for 5-6 days.

Female age influence on fecundity and fertility in monomorphic and polymorphic strains

To study relation between female age related changes in fecundity and fertility and inversion system, a male (5-6 days old) along with a female (young/ middle/ old) were aspirated into an Elens-Wattiaux mating chamber (1964) and observation was made for 1 hr. Soon after mating, the mated female was transferred individually into a fresh vial containing wheat cream agar media once in 24 hr to study fecundity and fertility, and this was continued for 16 days. Total number of eggs laid by each pair and total number of flies emerged from each pair was recorded. A total of 50 trials were made separately for each of the three female age classes. Separate experiments were carried out for monomorphic and polymorphic strains. Two Way ANOVA followed by Tukey's Honest Post hoc test was carried out on fecundity and fertility data using SPSS 10.1 software.

Female age influence on ovariole number in monomorphic and polymorphic strains

Virgin young/middle/old aged females were individually sacrificed to count number of ovarioles following the procedure of Krishna and Hegde (1997). To count ovariole number, each female was dissected in a drop of physiological saline using binocular stereomicroscope; ovarioles were separated from one another from the left ovary with the help of fine needles. Number of ovarioles in each female was counted. Mean ovariole number data were also subjected to two way ANOVA followed by Tukey's Honest Post hoc test. Separate experiments were carried out for monomorphic and polymorphic strains.

Results

Mean fecundity and fertility of young, middle aged, and old females mated to males in monomorphic and polymorphic strains are provided in Figure 1a and Table 1b. Two way ANOVA followed by Tukey's Honest Post hoc test (Tukey's test) carried out on fecundity and fertility data showed significant variation between strains and between female age classes. However, it shows insignificant difference in the interaction between strains and female age classes. Tukey's test showed middle aged females had significantly greater fecundity and fertility compared to young or old aged female. Young aged females had greater fecundity and fertility than old aged females.

Polymorphic strains had greater mean number of ovarioles compared to monomorphic strains. In both monomorphic and polymorphic strains mean number of ovarioles was found to be highest in middle aged female while lowest in old aged females (Figure 2a and Table 2b). Mean ovarioles data of monomorphic and polymorphic strains subjected to two way ANOVA followed by tukey's test showed significant variation between female age classes (young, middle, and old) between strains (monomorphic and polymorphic) and also interaction between female age and strains. Tukey's test showed that middle aged females had significantly greater ovariole number compared to young or old aged females. Similarly young aged females

had significantly greater ovariole number than old aged females. Polymorphic strains had significantly greater ovariole number compared to monomorphic strains by Tukey's test.

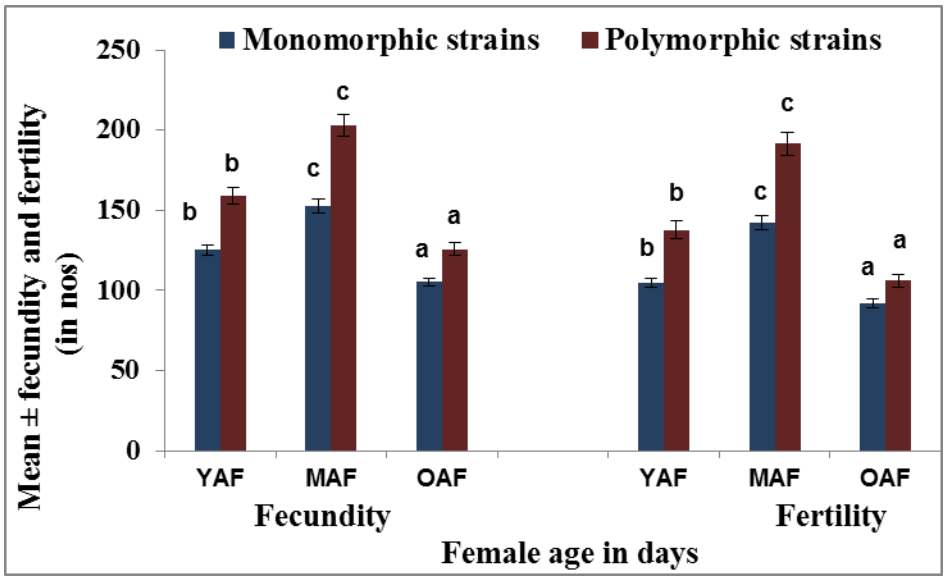

Figure 1a. Female age influence on female fecundity and fertility in monomorphic and polymorphic strains of *D. ananassae* (Values are mean ± SE). **YAF-** Young age female**; OAF-** Old age female; **MAF-** Middle age female. Different letter on bar graph indicates significant by Tukey's test.

Table 1b. Female age influence on female fecundity and fertility in monomorphic and polymorphic strains of *D. ananassae*.

Parameters	Source	Type III Sum of Squares	df	Mean Square	F-Values
Fecundity (in no)	Strains	90688.853	1	90688.853	85.001**
	Male age	196104.807	2	98052.403	91.903**
	Strains × Male age	10778.447	2	5389.223	5.051*
	Error	313671.280	294	1066.909	
	Total	6926866.000	300		
Fertility (in no)	Strains	77666.430	1	77666.430	73.746**
	Male age	240433.047	2	120216.523	114.148**
	Strains*Male age	15115.220	2	7558.110	7.177*
	Error	309629.220	294	1053.161	
	Total	5636435.000	300		

* Significant at 0.05 level, **Significant at 0.0001 level.

Discussion

Figure 1a and Table 1b show that in both monomorphic and polymorphic strains middle aged females had significantly greater fecundity and fertility over young or old aged females suggesting that females of the same species show variation in reproductive potential across the age. This confirms the earlier works suggesting that female age is also one of the traits known to influence female fecundity (Bonduriansky, 2001; Jones, *et al*., 2001; Schamel *et al*., 2004; Marinkovic, 1967; Singh and Chaterjee, 1987, 1988; Katvala and Kaitala, 2001; Long *et al*., 1982); working on the evolution of male mate choice in insects has also found influence of female age on female fecundity.

Since ovariole number is positively correlated with fecundity, we sacrificed females of different age classes to study ovariole number variation, if any, across the female ages. Figure 2a and Table 2b show that middle aged females had significantly greater ovariole number over young or old aged females in monomorphic and polymorphic strains and followed the similar pattern of fecundity variation across the

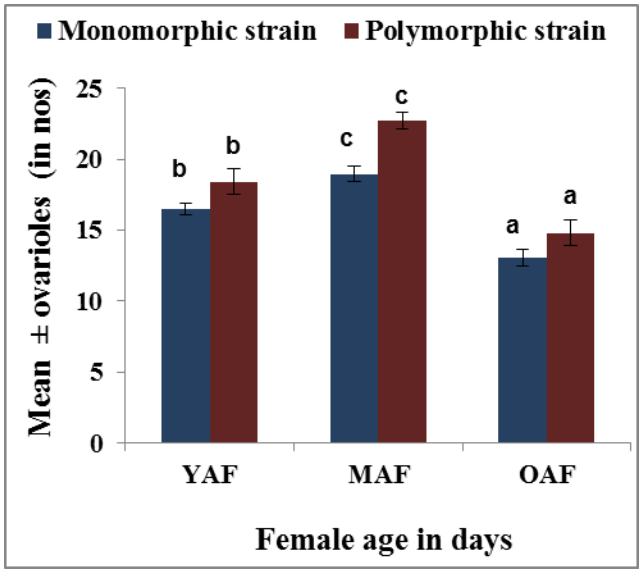

Figure 2a. Female age influence on female ovariole number in monomorphic and polymorphic strains of *D. ananassae*. **YAF-** Young age female**; OAF-** Old age female; **MAF-** Middle age female.

Table 2b. Female age influence on female ovarioles number in monomorphic and polymorphic strains of *D. ananassae.*

Ovarioles	Source	Type III Sum of Squares	df	Mean Square	F-Values
Ovarioles (in no)	Strains	461.280	1	461.280	68.045**
	Male age	2394.647	2	1197.323	176.621**
	Strains×Male age	63.780	2	31.890	4.704*
	Error	1993.040	294	6.779	
	Total	95880.000	300		

* Significant at 0.05 level, **Significant at 0.0001 level.

female ages. This again confirms the earlier studies of female age influence on reproductive success in other insects, too (Bonduriansky, 2001; Branquart and Hemptinne, 2000; Partridge *et al*., 1987). From the results it was also noticed that in *D. ananassae* middle aged females had significantly greater ovariole number, fecundity, and fertility compared to young or old aged females in both the strains studied. Therefore, it is advantageous for the male to mate with middle aged females compared to young or old aged females. However, it is not known why older females had less ovarioles compared to young or middle aged females? Whether they reabsorbed as female age, we do not have answer for this.

Thus, these studies suggest that males of *D. ananassae* exercised mate choice to obtain direct fitness benefits, and we also found occurrence of age specific reproductive success in females of *D. ananassae* which is independent from influence of inversion system.

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Newly recorded inversion and re-annotation of inversion breakpoints in *Drosophila cardini* species.

Wildemann, B., A.F. Maris, and C. De Toni. Departamento de Biologia Celular, Embriologia e Genética – Universidade Federal de Santa Catarina, Florianópolis, Brasil; E-mail: detoni@ccb.ufsc.br.

Abstract

This report presents a new paracentric inversion in the middle of the chromosome X in *Drosophila neocardini*. Also, a careful analysis was carried out of chromosomal inversion breakpoints that were previously described for *Drosophila polymorpha*. Photo comparison, and the release of a newly designed photomap, allowed us to introduce changes to the breakpoints of In(2R)A and In(2R)D. All individuals analyzed were collected in two conserved areas in Santa Catarina/south of Brazil. Key words: chromosomal polymorphism, cytogenetic, polytene chromosomes.

Introduction

Chromosomal inversions were first discovered by Sturtevant in 1917 as the recombination modifier in *Drosophila melanogaster*, that occurs when a chromosome breaks at two points and the segment bounded by the breakpoints is reinserted in the reversed orientation (Navarro *et al*., 2000). This inversion mutation that reordered genes in the genome became a powerful genetic marker to assess naturally occurring genetic variation (Wallace *et al*., 2013). Also, the ability to identify inversions with simple cytological analysis proved to be very important in these early studies (Painter, 1933).

The cytological analysis involves a technique with polytene chromosomes present in the nuclei of salivary glands of *Drosophila* species. These chromosomes allow easy identification of the arrangements in heterozygotes, since they in this case result in an inversion loop. Different types of inversion are classified into two basic categories: pericentric inversions that include a centromere region, and paracentric inversions, that do not. Paracentric inversions are also the more common type of chromosomal inversion (Krimbas and Powell, 1992).

Natural populations of *Drosophila* species show a wealth of gene arrangement polymorphisms and may play a significant role in adaptation, speciation, and sex chromosome evolution (Kirkpatrick, 2010). The variation of chromosomal inversions in a population allows us to estimate the genetic diversity of the species, while the comparison between populations provides an overview of the gene flow and habitat fragmentation with regard to where these populations reside (Feder *et al*., 2011).

The *cardini* group belongs to the subgenus *Drosophila* and was established by Sturtevant (1942). It currently includes 16 species of a large geographical distribution in Neotropical America (Heed, 1962). The group comprises two subgroups: *dunni* (*D. antillea, D. arawakana, D. belladunni, D. caribiana, D. dunni, D. nigrodunni, D. similis*) and *cardini* (*D. bedichecki, D. acutilabella, D. cardini, D. cardinoides, D. neocardini, D. neomorpha, D. parthenogenetica, D. polymorpha, D. procardinoides*).

Drosophila neocardini, described by Streisinger (1946), and *Drosophila polymorpha*, described by Dobzhansky and Pavan (1943), from *cardini* subgroup, are extremely similar with respect to their morphology and ecological requirements (Rohde and Valente, 1996; Medeiros and Klaczko, 2004). Both species are common in Neotropical forests and the specific differentiation between them is made through the analysis of the internal male genitalia and the pattern of abdominal pigmentation. Their nuclei consist of four chromosome pairs: submetacentric chromosomes 2 (chr2R, chr2L) and 3 (chr3R, chr3L), the sexual pair composed of the acrocentric XX, (chrX) and the Y chromosome (chrY), which is heterochromatic and not distinguishable from the chromocenter, and a dot pair, being chromosome 4 (chr4) (Rohde and Valente, 1996; Cordeiro *et al*., 2014).

Studies involving chromosomal inversions in the *cardini* group indicate that *D. neocardini* does not have many arrangements described so far, whereas *D. polymorpha* shows the highest number of polymorphisms of the group (Da Cunha *et al*., 1953; De Toni *et al*., 2001a; Cordeiro *et al*., 2014). Thus far, 19 rearrangements have been described for *D. polymorpha* and three for *D. neocardini* (see Table 1).

The current study reports a new paracentric inversion in *D. neocardini* and also a careful re-annotation of the breakpoints of two inversions previously described for *D. polymorpha.* These remarks were noticed in the course of a major work involving inversion polymorphisms in the *cardini* group species. Data presented here are considered relevant in order to avoid further misinterpretation and, thus, guarantee effective results regarding the *cardini* group's genetic research.

Table 1. Arrangements described for *Drosophila polymorpha* and *Drosophila neocardini.*

Species	Heterozygous rearrangements	First description
Drosophila polymorpha	In(2R)A	Rohde and Valente, 1996
	In(X)A, In(X)B, In(2L)A, In(2L)B, In(2R)B, In(2R)C, In(2R)D, In(3R)A	De Toni *et al.,* 2001b
	In(X)C, In(3L)A, In(3L)B, In(3L)C, In(3R)B, In(3R)C, In(3R)D, In(3R)E, In(2R)A+C, In(2R)A+D	Cordeiro *et al.*, 2014
Drosophila neocardini	In(3L)A	De Toni *et al.,* 2001a
	In(3L)B, In(3L)C	Cordeiro *et al.*, 2014

Material and Methods

Adult *Drosophila* population samples were obtained through fermented banana bait traps (Roque *et al*., 2011) from conserved areas in Santa Catarina, south of Brazil during the year 2013. Isofemale lines of flies from *cardini* group were established and samples were analyzed by observing the banding patterns of polytene chromosomes. Slides with salivary glands were processed according to Ashburner (1967). Images were captured using a digital camera attached to a binocular microscope connected to a computer, and processed with the Adobe® Photoshop® program (Adobe® Photoshop® CS5 extended, v 12.0 x32).

The newly discovered inversion and the correction of inversion breakpoints were established using the reference photomaps for *D. polymorpha* and *D. neocardini* proposed by Cordeiro *et al*. (2014). At least 10 nuclei where analyzed in order to achieve a conclusive notation for the breakpoints.

Results and Dicussion

Cytological analyses of inversion breakpoints in polytene chromosomes formed the first step for further investigation on the regions of gene rearrangements. Also, registering inversions and defining their breakpoints has led to studies such as inversion polymorphisms and genetic variations in populations.

We report here the first record of an inversion in the chrX in *D. neocardini*. According to the sections established in the photomap of this species (Cordeiro *et al*., 2014), the new paracentric inversion, now named In(X)A, comprises sections 13a proximal and 9b distal (Figure 1).

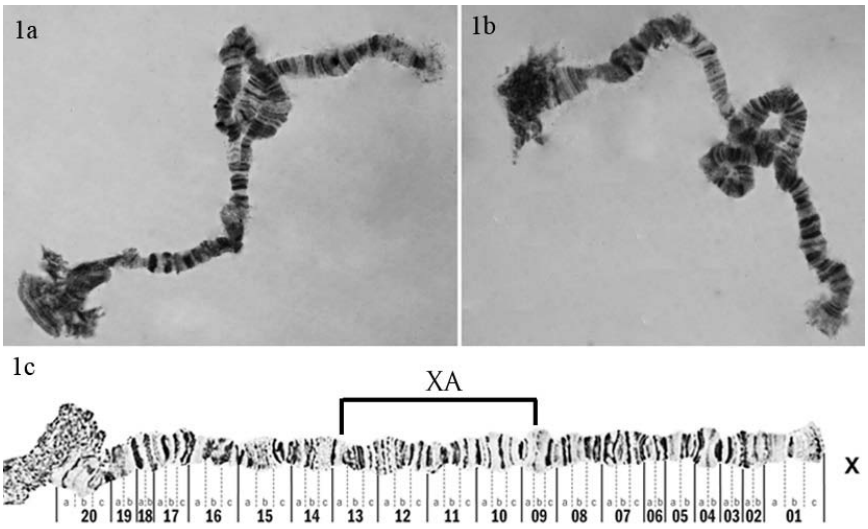

Figure 1. 1a, 1b) Polytene chrX of *Drosophila neocardini* and its new paracentric inversion, In(X)A, in heterozygous state showing the inversion loop. 1c) Photomap of chrX of *D. neocardini* designed by Cordeiro *et al*. (2014). Brackets point to the sections that comprise the new inversion.

Studies involving inversion polymorphisms in *D. polymorpha* reveal that the chromosome arm 2R is the most polymorphic and In(2R)A is the most commonly recorded in the populations investigated (De toni, *et al*., 2001b; Rohde and Valente, 1996; Cordeiro *et al*., 2014). Rohde and Valente (1996) were the first to describe the In(2R)A in *D. polymorpha*, comprising of sections 53 proximal and 48 distal. Wildemann and De Toni (2011) described a new inversion in the chr2R in the same species, and named it In(2R)E (sections 50a proximal and 48c distal). Moreover, the frequent appearance of In(2R)A (Rohde and Valente, 1996) and In(2R)E (Wildemann and De Toni, 2011) during this study, has led us to compare and re-evaluate the breakpoints to those described previously, since they are located in very close regions of the chromosome. Photographic comparison between In(2R)A, In(2R)E, and the 2R inversions found in this study has allowed us to confirm that, in fact, In(2R)A and In(2R)E are the same. Therefore, we are suggesting a new proximal breakpoint for In(2R)A (In(2R)E), which comprises of the sections 52b proximal (instead of 53 or 50a, respectively), and distal sections 48b (Figure 2).

A similar misinterpretation occurred for In(2R)D in *D. polymorpha*. This inversion was first described by De Toni *et al*. (2001b), involving section 45 distal and 47 proximal. Photographic comparison allowed us to confirm that in the case of In(2R)D, in fact, its distal breakpoint comprises of the section 45b, instead of 44, and its proximal breakpoint is 47a (Figure 3).

Detecting inversions and determining their breakpoints allows us to focus studies on the importance of arrangements such as described above, based on the fact that it has been proposed that inversions persist in natural populations as recombination-protected co-adapted gene complexes (Dobzhansky, 1970). Moreover, if two subsequent inversions with similar or identical breakpoints are overlooked, a phylogenetic link could be missed as a result. Thus, when two different inversions are mistakenly identified as the same one, it creates a false phylogenetic link between two unrelated species (Rohde and Valente, 2012).

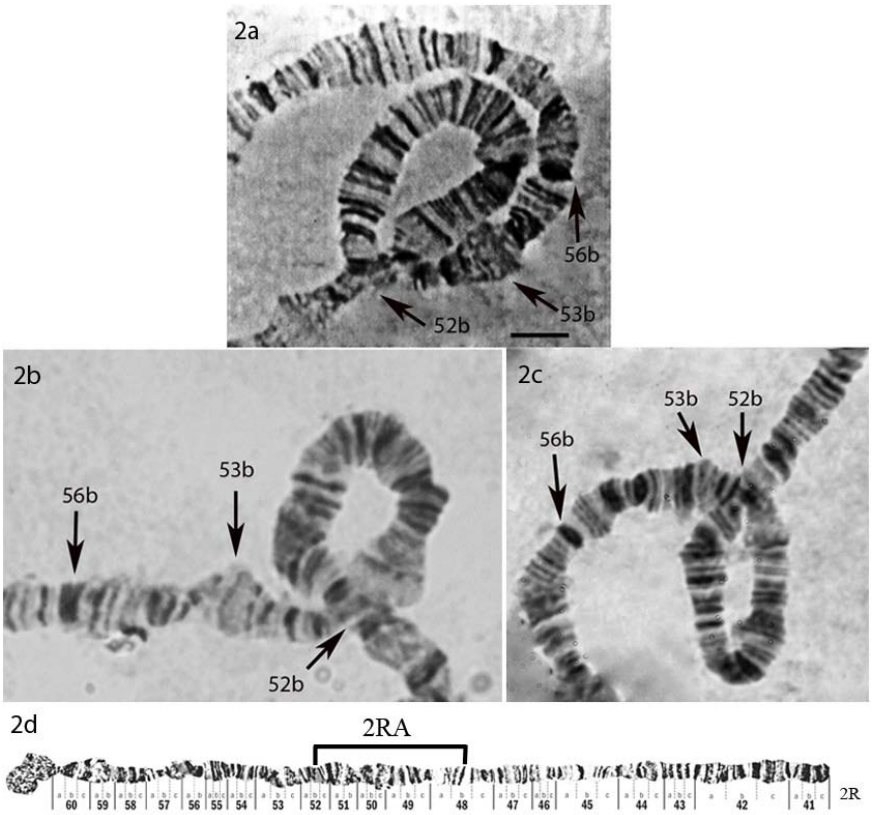

Figure 2. Comparison of In(2R)A in *Drosophila polymorpha*. 2a) In(2R)A (Rohde and Valente, 1996). 2b) In(2R)E (Wildemann and De Toni, 2011). 2c) Inversion 2R from current work. Arrows indicate sections that are key features for the identification of its chromosome regions. 2d) Photo-map of chr2R of *D. polymorpha* designed by Cordeiro *et al*. (2014). Brackets point to the re-evaluated sections that comprise In(2R)A. Bar represents 10µm.

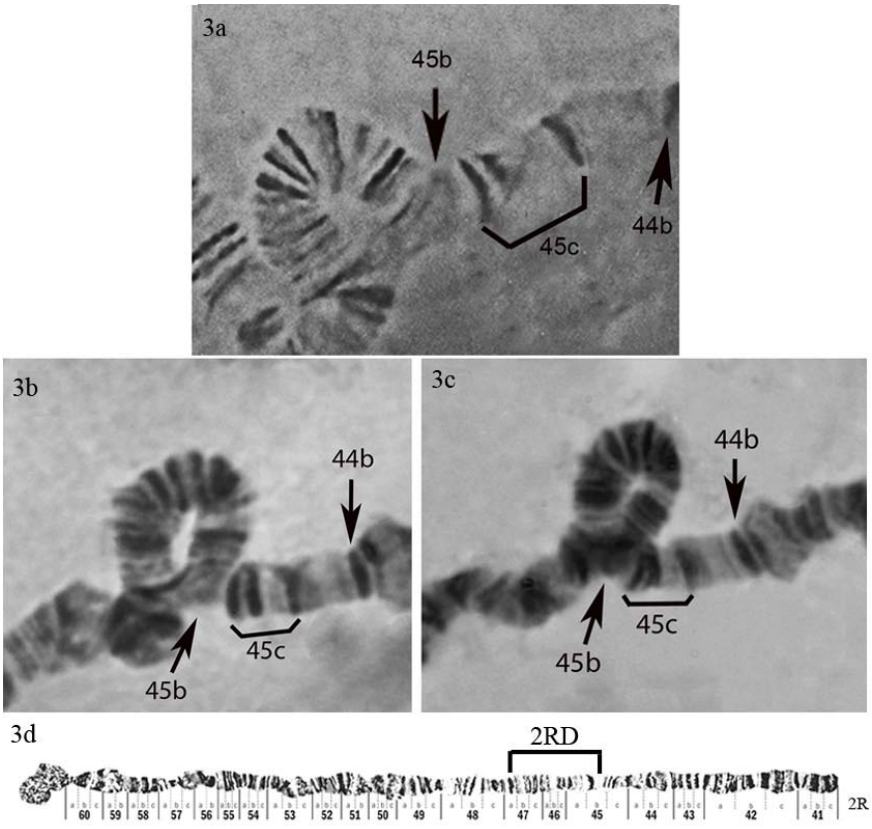

Figure 3. Comparison of In(2R)D in *Drosophila polymorpha*. 3a) In(2R)D (Cordeiro *et al*., 2014). 3b, 3c) In(2R)D from current work. Arrows and brackets indicate sections that are key features in the identification of its chromosome region. 3d) Photo-map of chr2R of *D. polymorpha* designed by Cordeiro *et al*. (2014). Brackets point to the re-evaluated sections that comprise In(2R)D.

The misinterpretation confirmed here with regard to the first records of breakpoints in case of In(2R)A suggests that it was a newly discovered inversion. However, we confirmed by means of photographic comparison and a careful analysis of the new version of *D. polymorpha*'s photomap (Cordeiro *et al*., 2014) that it was only necessary to re-evaluate In(2R)A breakpoints. The corrections were only possible due to the improvement in the photomap that shows the chromosome regions in larger detail.

Although in total *D. polymorpha* present 19 documented rearrangements (Table 1), this work only reevaluated two inversion breakpoints present in the chr2R, since they were common inversions observed in collections of populations from the south of Brazil.

All these results and observations show that chromosomal polymorphisms deserve further investigation. Detailed descriptions of inversions, molecular isolation and the analysis of chromosomal breakpoint sequences can provide an overview of the gene content in the region of the inversion. Also, molecular analysis can detect if any gene expressions have been altered due to the new arrangement that is formed (Wesley and Eanes, 1994).

Acknowledgments: This work was supported by Coordenação de Aperfeiçoamento Pessoal de Nível Superior - CAPES e Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina – FAPESC. The authors are very grateful for the assistance provided by Dr. Gordon Craggs in revising the English version of this work.

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First register of *Drosophila carcinophila* at South America, Brazil.

Nicoladeli, A.T., H.R. Nunes, M.F. Ramirez, C.J. Cavalcante, C.J. Carvalho-Pinto, and D.C. De Toni. Laboratório de drosofilídeos, sala 304C, Departamento de Biologia Celular, Embriologia e Genética, Universidade Federal de Santa Catarina, Campus Universitário Trindade, 88040-900, Florianópolis, Santa Catarina, Brasil; e-mail: detoni@ccb.ufsc.br.

This academic work describes the occurrence of *Drosophila carcinophila* in Southern Brazil, in Southern Brazilian coastal strand vegetation, in the neighborhood of Rio Vermelho, Florianópolis, Santa Catarina State (Figure 1).

For the monthly gathering was utilized 6 traps made out of PET bottles, with the volume of 2 litters**,** as adaption of the Ferreira's **(**1978) traps. As a bait, it was utilized chicken liver and bovine meat placed in each trap, in a portion of approximately 25 grams of each (total of 50 grams of bait). Before being put in the traps, the baits are kept in an average environment temperature in a period of 48 hours, inside a closed plastic container, to avoid contact with insects. The traps were exposed to the environment for a 4-day period in each month, during 12 months. The collections were made between November 2012 and October 2013. The

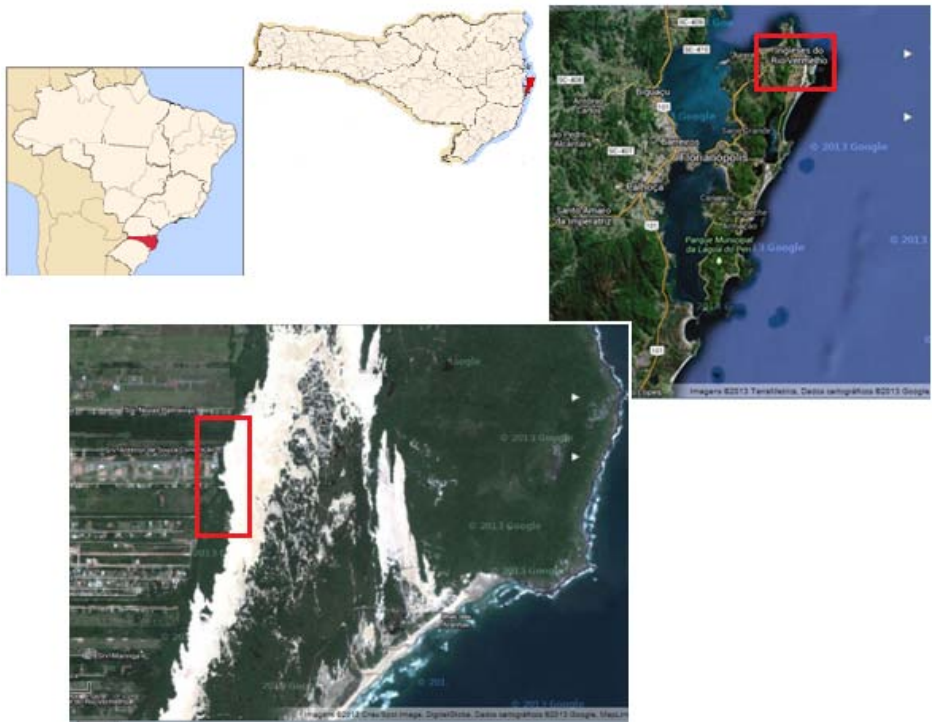

Figure 1. Map of the study site in Brazil, showing Santa Catarina State in red. Map of Santa Catarina State, highlighting the collection area at Florianópolis in red, with satellite images of the region limited by the red rectangles (Reference: http://maps.google.com.br/).

Table 1. Abundance. dominancy indexes and frequency of Diptera families collected in the Rio Vermelho dunes. Florianópolis. Santa Catarina state.

Family	Abundancy	Dominancy index (%)	Frequency (%)
Calliphoridae	179	2.33	100
Sarcophagidae	572	7.46	100
Muscidae	117	1.53	100
Phoridae	4081	53.20	100
Ulidiidae	1036	13.51	100
Sepsidae	8	0.10	33.33
Piophilidae	33	0.43	66.67
Fanniidae	648	8.45	100
Sphaeroceridae	40	0.52	58.33
Micropezidae	32	0.42	100
Chloropidae	694	9.05	100
Drosophilidae	139	1.81	91.67
Psychodidae	3	0.04	25
Neriidae	3	0.04	16.67
Anisopodidae	1	0.01	8.33
Cecidomyiidae	59	0.77	83.33
Ceratopogonidae	4	0.05	25
Sciaridae	3	0.04	16.67
Other unidentified Diptera	19	0.25	58.33
Total	7671		

Abundance and identification of Drosophilidae species and species groups.

Identification	Abundance
Group repleta	30
Drosophila flexa	6
Group mesophragmatica	1
Group tripunctata	8
Group cardini	2
Group annalinea	1
Drosophila paraguayenis	1
Drosophila suzukii	1
Zaprionus indianus	1
Zygotricha sp	1
Drosophila cuaso	1
Drosophila carcinophia	4
Total	57

insects were caught and removed from the traps every 24 hours over each monthly collection period. We collected only the subjects imprisoned in the upper part of the trap; they were collected after the exposure period of approximately 24 hours. Thereby, due to the character of the trap, their were put only adult individuals (winged), considering that possible larvae being in the bottom of the trap will not be included in the analysis. The 6 traps were hung to the vegetation in the selected spots all over the dunes using strings, being kept a distance between the traps and the soil of 60 cm and 1.30 m. Every 24 hours, during the monthly periods, the traps were removed and transported to a handling place near the study area. In this place, the traps were cautiously opened, separating the part where the captured insects were kept from the base containing the bait. The dead insects were transferred to petri dishes to be properly identified under a stereoscopic microscope. After this, the traps were reassembled utilizing the same initial baits and replaced in their respective collection spots.

Table 1 shows the achieved results with the collection, and Table 2 details the *Drosophila* specimens identified.

As observed in Table 2, with this type of trap, besides *D. carcinophila*, (Figures 2 and 3) were collected other individuals of the *repleta* group and a new-found invader species, *D. suzukii*, also found in Santa Catarina (Depra *et al.*, 2014).

Figure 2. Adult male of *Drosophila carcinophila.*

This register leads to strong evidence that *D. carcinophila* is distributed all over South American and most parts of Brazilian territory coming from Caribbean islands, its center of original habitat since the 1970's, when it was collected by Carson *et al.* (1971).

That points out the necessity of doing taxonomic surveys, including different types of baits, to collect restricted ecology *Drosophila* species.

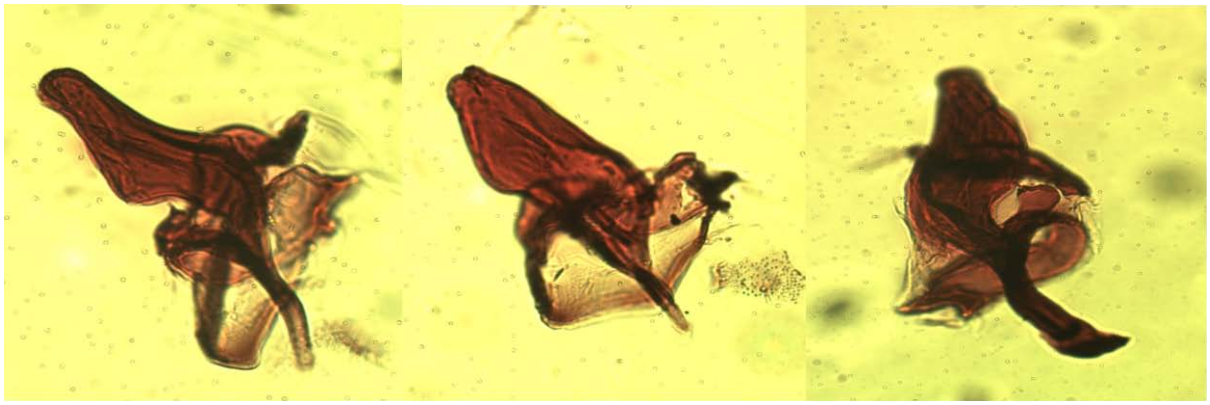

Figure 3. Lateral, dorsum-lateral, and lateral-ventral views from aedeagus of *Drosophila carcinophila.*

References: Carson, H.L., and C.E. Hartt 1971, The Ecology of *Drosophila* Breeding Sites. Harold L. Lyon Arboretum Lecture Number 2, University of Hawaii; Depra, M., J.L. Poppe, H.J. Schmitz, D.C. De Toni, and V.L.S. Valente 2014, J. Pest Sci. 2: 1-4; Ferreira, M.J.M., 1978, Revta. Bras. Biol. 38(2): 445-454.

First record of *Drosophila suzukii* in the Brazilian Savanna.

Paula, M.A., P.H.S. Lopes, and R. Tidon. Instituto de Ciências Biológicas, Universidade de Brasília, CP 04457, Brasília, Brazil 70910-900. *Corresponding author: rotidon@pq.cnpq.br.

Introduction

Drosophilid species are not usually considered pests, because they consume microorganisms associated with tissue decay. In the Neotropics, these flies normally infest overripe, fallen, decaying fruits. Some species, however, lay their eggs inside sound fruits before harvest. This is the case of *Zaprionus indianus*, which established itself in South America in the late 20th century and emerged as a pest due to the significant economic damage it caused in fig plantations (Vilela, 1999; Stein *et al*., 2003). In February 2013, another exotic species able to lay eggs inside healthy fruit was documented for the first time in South America: *Drosophila suzukii* Matsumura (Deprá *et al*., 2014). This latter species is considered a pest of soft-skinned fruit crops in a number of countries, because it infests a wide host range using its characteristic serrated ovipositor (Mitsui *et al*., 2006). Estimations of losses due to *D. suzukii*, only for three northwestern states in the USA, reached U$511 million, certainly adding substantial costs to fruit production (Werts and Green, 2014).

Drosophila suzukii, an Oriental species from the *D. melanogaster* group, is also known as Spotted Wing *Drosophila* due to the dark spots on male wings (Acheapong, 2010). In 2008 it invaded Europe, and two years later it arrived in North America (Rota-Stabelli *et al*., 2013). The first occurrences in Brazil, in late 2013, were recorded in southern states, where the climate is subtropical according to Köppen's classification (Deprá *et al*., 2014). In this study, we document *D. suzukii* in the Brazilian Savanna, a tropical biome located in the center of South America.

Materials and Methods

The Brazilian savanna, locally known as the Cerrado biome, covers most of the interior of Brazil and consists of savanna of quite variable structure on well-drained terrain, with forests or other moist vegetation

following the watercourses (Oliveira-Filho and Ratter, 2002). Fieldwork was conducted in the IBGE Ecological Reserve (15º56'S; 47º53'W), a part of an environmental protection area extending over 10,000 ha and located 35 km south of Brasilia, the capital of Brazil. We have been collecting drosophilid communities in this reserve since 1998, and from 2012 we have been monitoring these assemblages bimonthly, using an experimental design of 90 banana-baited traps (Roque *et al*., 2011) that remain in the field for four days. These traps are distributed, as replicates, in forest and savanna vegetation.

The drosophilids captured in this study were transported live to the laboratory and identified by identification keys, descriptions, and in some cases, by the male terminalia (Dobzhansky and Pavan, 1943; Freire-Maia and Pavan, 1949; Pavan and Cunha, 1947; Val, 1982; Vilela and Bachli, 1990; Vilela, 1992; Walsh *et al.*, 2011).

Results

In December 2013, we captured 12,120 specimens in our traps, among them a male of *Drosophila suzukii* recognized by its spotted wings (Figure 1). This was the first record of this species in the Brazilian Savanna. In February 2014, we did not find any specimen of *D. suzukii* among the 16,700 individuals and 41 species identified in this collection, but two months later (April) we captured a female characterized by its distinctive saw-like ovipositor. Both the male and the female were collected in savanna vegetation. In the collections of June, August, and October (2014) we did not catch any specimen of *D. suzukii*. Table 1 shows a summary of these collections.

Exotic species accounted for 47% of the sample collected from October 2013 to August 2014 and were mostly represented by *Zaprionus indianus* (29%), *Drosophila simulans* (10%), and *D. malerkotliana* (7%). The most abundant Neotropical species were those of the *D. willistoni* subgroup (38%) and *D. sturtevanti* (10%). The remaining Neotropical species (41 nominal species and 27 morphospecies) altogether contributed the remaining 5% of the specimens.

Table 1. Abundance of drosophilids from gallery forests (45 traps) and savannas (45 traps) in the IBGE Ecological Reserve, from October 2013 to August 2014.

	Oct	Dec	Feb	Apr	Jun	Aug	Total
Sgr. Drosophila willistoni	4	527	9739	3765	115	4	14154
*Zaprionus indianus**	7	10235	375	112	52	93	10874
*D. simulans**	32	772	719	89	95	2008	3715
D. sturtevanti	1	204	2453	551	318	40	3567
*D. malerkotliana**	0	86	2605	40	2	1	2734
*Scaptodrosophila latifasciaeformis**	0	4	44	12	2	0	62
*D. immigrans**	0	25	8	1	0	1	35
*D. ananassae**	0	1	1	2	0	0	4
*D. kikkawai**	0	2	0	1	1	0	4
*D. busckii**	0	0	0	1	0	1	2
*Drosophila suzukii**	0	1	0	1	0	0	2
Other Neotropical species	79	263	756	364	130	160	1764
TOTAL	123	12120	16700	4939	715	2308	36917

**Exotic species*

Discussion

The Brazilian Savanna is a hotspot of biodiversity, due to its high plant endemism and anthropogenic threats. We have been collecting drosophilids in this region since 1998, especially in the IBGE Ecological Reserve (Tidon *et al.*, 2003; Tidon, 2006; Mata *et al.*, 2008; Roque and Tidon, 2008; Roque *et al.*, 2013), and currently there are 128 species of Drosophilidae recorded in this biome (Roque and Tidon, 2013). It is worrying that during the period of this study (2013-2014) almost half of the drosophilid specimens belonged to exotic species. *Zaprionus indianus*, *Drosophila simulans*, and *D. malerkotliana* have spread not only to the

Brazilian Savanna, but also to several other Neotropical biomes (Gottschalk *et al.*, 2008). Therefore, they have reached the status of invasive species. On the other hand, the other exotic species are locally rare and/or geographically restricted; they have not spread and nor do they dominate Neotropical drosophilid assemblages. The fate of *D. suzukii* is still uncertain. In Brazil and in other areas invaded by this species, the first records were near seaports, suggesting that propagules arrived as immature stages in fruits imported from Asia (Rota-Stabelli, 2013). Nevertheless, our collection sites are at least 1,000 km from the nearest seaport, on the Atlantic coast. Therefore, these propagules could have dispersed from southern Brazil or from North America.

Figure 1. Male of *Drosophila suzukii* collected in the Brazilian Savanna.

It is possible that the presence of *D. suzukii* in our traps was occasional, since we captured only two specimens and *D. suzukii* seems to prefer a moderate climate (Walsh *et al*., 2011; Deprá *et al*., 2014). However, this species can be considered highly thermal tolerant because viable populations resist hot summers in Spain as well as cold mountain regions in Japan (Cini *et al*., 2012). It is possible that, like *Zaprionus indianus* (Mata *et al*., 2010), *D. suzukii* can change its ecological niche and establish viable populations in this savanna area.

The drosophilid populations in the Brazilian savanna, for most species, occur from December to April, when there are more breeding sites available due to the rainy season. As we are monitoring the IBGE Ecological Reserve every two months, we will be able to detect if the presence of *D. suzukii* in these assemblages was occasional or if it is establishing itself in the center of the Neotropical Region.

Acknowledgments: We are grateful to the Universidade de Brasília and Reserva Ecológica do IBGE for logistical support. This work was funded with grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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Parasitoid wasps in the Brazilian Savanna: adding complexity to the *Drosophila*-fruit system.

Schneider, D.I., H. Valadão, and R. Tidon. Instituto de Ciências Biológicas, Universidade de Brasília, CP 04457, Brasília, Brazil 70910-900; *Corresponding author: rotidon@pq.cnpq.br.

Introduction

The *Drosophila*-wasp system is suited to disentangling physiological, genetic, and ecological interactions occurring among the partners of a host-parasitoid association. Moreover, understanding parasitoids may also contribute to comprehending the ecology and evolution of *Drosophila*, which cannot miss out the possible impact of parasites. Several species of wasps parasitize immature stages of drosophilids. They are considered parasitoids, because they can kill the host: they oviposit on fly larvae or pupae, and at the end of development the imago that emerges from the drosophilid pupa is a wasp.

In 1986, Carton *et al.* provided a substantial paper on the hymenopteran parasitoids associated with drosophilids. This overview raised a number of questions that were studied throughout subsequent decades, particularly host immune resistance and factors of parasitoid virulence and their possible pleiotropic effects (Prevost, 2009). The field ecology of these wasps, on the other hand, has been less studied. Geographical distribution, taxonomy, and host range are insufficiently documented for the four major groups of *Drosophila* parasitoids, especially in the tropical regions of America and Africa, where many new species remain to be described (Fleury *et al.*, 2009).

In this study, we analyze wasps and drosophilids that emerged from fruits collected in four ecological reserves in the Federal District of Brazil, located in the center of the Brazilian savanna.

Material and Methods

The landscape of the Brazilian savanna, locally known as the Cerrado biome, is a mosaic of vegetation types ranging from grasslands to forests (Ratter *et al.*, 1997). The average annual rainfall is 1500 mm, but the rains are heavily concentrated in the wet season, between November and March (Eiten, 1972), when most plants are fructifying. This study was conducted in four protected areas of the Federal District, located in the center of the biome: Estação Ecológica de Águas Emendadas - ESECAE (15° 34'26" S, 47° 34'58" W), Parque Nacional de Brasília - PNB (15° 43'56" S, 47° 55'53" W), Jardim Botânico de Brasília - JBB (15° 52'42" S, 47° 50'17" W), and Reserva Ecológica do IBGE (15° 56'31" S, 47° 52'41" W).

During each collection event, two collectors searched for 120 minutes for fallen fruit on the ground in each protected area. On these occasions, fruits of various species were collected, intact or partially degraded in different stages of decay. No more than 50 fruits from each plant species were collected in each area/day, but collectors rarely found more than this amount of fruit. Fruits were identified using field guides (Silva-Júnior, 2005; Silva-Júnior and Pereira, 2009; Kuhlmann, 2012), and in some cases a specialist was consulted. In the laboratory, the fruits were stored individually in plastic containers with vermiculite moistened with a solution of Nipagin ®, an inhibitor of filamentous fungi. These containers were covered with a thin, translucent piece of cloth to retain the insects that emerged from the fruit. All fruits were stored at a constant temperature (25°C). The flies and wasps that emerged from the fruits were removed every other day and stored in microtubules with 70% alcohol. The identification of the flies was based on taxonomic keys (Freire-Maia and Pavan, 1949), descriptions (Chassagnard and Tsacas, 1993), and on the male terminalia in the case of cryptic species (Vilela and Bächli, 1990). Wasps were determined based on taxonomic keys to the families (Triplehorn and Johnson, 2011) and to the genera (Burks, 1971; Wharton *et al.*, 1997; Buffington and Ronquist, 2006). Voucher specimens were deposited in the drosophilid collection of the Evolutionary Biology Laboratory of the *Instituto de Ciências Biológicas* at the *Universidade de Brasília*.

Results

We observed wasps emerging from eight species of plants: *Alibertia edulis* (Rubiaceae), *Cariocar brasiliense* (Cariocaraceae), *Diospyros hispida* (Ebenaceae), *Garcinia gardneriana* (Clusiaceae), *Erythroxylum suberosum* (Erythroxylaceae), *Syzygium jambos* (Myrtaceae), besides a species of *Syagrus sp*. (Arecaceae) and a non-identified yellow fruit. The abundance of emerged wasps and drosophilids (from the genera *Drosophila*, *Scaptodrosophila* and *Zaprionus*) is shown in Table 1.

Wasp samples were dominated by the genera *Aphaereta*, *Eurytona*, and *Leptopilina*, whereas drosophilids were represented mostly by *Drosophila paulistorum*, *D. cardini*, *D. simulans*, and *Scaptodrosophila latifasciaeformis* (Figure 1).

Table 1. Abundance of wasps and drosophilids emerging from rotting fruits collected in four protected areas of the *Federal District*, located in the center of the Brazilian savanna, from October 2010 to March 2011.

	Alibertia edulis	*Cariocar brasiliensis*	*Diospyros hispida*	*Erythroxylum suberosum*	*Garcinia gardneriana*	*Syagrus sp.*	*Syzygium jambos*	yellow fruit	total
Aphaereta	1	4	0	0	9	1	6	19	40
Eurytoma	0	0	20	0	0	0	0	0	20
Leptopilina	0	0	0	0	0	0	16	1	17
Nordlandiella	0	2	0	0	0	0	0	2	4
Bracon	0	0	0	0	0	0	0	2	2
Hormius	0	0	0	0	0	0	0	2	2
Apanteles	0	0	0	1	0	0	0	0	1
Total wasps	1	6	20	1	9	1	22	26	86
D. cardini	0	2	0	0	0	0	0	0	2
D. mediopunctata	1	0	0	0	0	0	0	1	2
D. mediostriata	0	1	0	0	0	0	0	0	1
D. mercatorum	0	3	0	0	0	0	0	0	3
D. nebulosa	0	56	0	0	0	0	2	22	80
D. paulistorum	0	1	0	0	0	0	0	0	1
D. willistoni	0	17	0	0	35	0	0	23	75
S. latifasciaeformis	0	0	0	0	6	0	0	0	6
D. malerkotliana	0	5	0	0	0	0	0	46	51
D. simulans	0	4	0	0	0	0	5	0	9
Z. indianus	2	32	0	0	0	0	9	3	46
Total drosophilids	3	121	0	0	41	0	16	95	276

E. suberosum (n=1, October 2010); *Syagrus sp*. (n=1, November 2010); *S. jambos* (n=6, November 2010); *A. edulis* (n=1, January 2011); *C. brasiliensis* (n=3, January 2011); *D. hispida* (n=2, February and March 2011), *G. gardneriana* (n=4, March 2011), yellow fruit (n=15, December 2010 to March 2011).

Discussion

Four of the seven genera of wasps belong to the family Braconidae, one of the most species-rich families of insects. The vast majority of braconids are primary parasitoids of other insects, especially upon the larval stages of Coleoptera, Diptera, and Lepidoptera but also including some hemimetabolus insects (aphids, Heteroptera, Embiidina) (Tree of Life, 2014). Almost one half of the emerged wasps belong to the cosmopolitan genus *Aphaereta* (Alysiinae), which includes 16 described New World species reared from at least 15 families of Diptera, mainly calyptrate muscoid flies (Wharton *et al*., 1997). The accurate identification of *Aphaereta* species is extremely difficult, because these species are exceedingly variable, and some of them attack a wide range of hosts in a variety of situations, promoting interpopulation variation (Wharton, 1977). The cosmopolitan genus *Bracon* (Braconinae) is extremely large, and in most parts of the world represents the vast majority of braconines collected. They are parasitoids of a wide range of coleopterous, lepidopterous, dipterous, and stem boring sawflies (Symphita) (Quicke and Sharkey, 1989). In

our samples, however, *Bracon* corresponded to only 2% of the wasps. Wasps of the genus *Hormius* (Hormiinae) also corresponded to 2% of the sample, and the parasitoids include gregarious ectoparasites of Lepidoptera living in silken retreats, mainly Gelechiidae and Tortricidae (Quicke, 2015). Little is known about this genus, despite specimens being fairly common. Finally, the wasps of the genus *Apanteles* (Microgastrinae), a common and extremely diverse genus (probably nearly 1000 species in New World), are mostly associated with Lepidoptera (Wharton *et al*., 1997); their relative abundance in this study was about 1%.

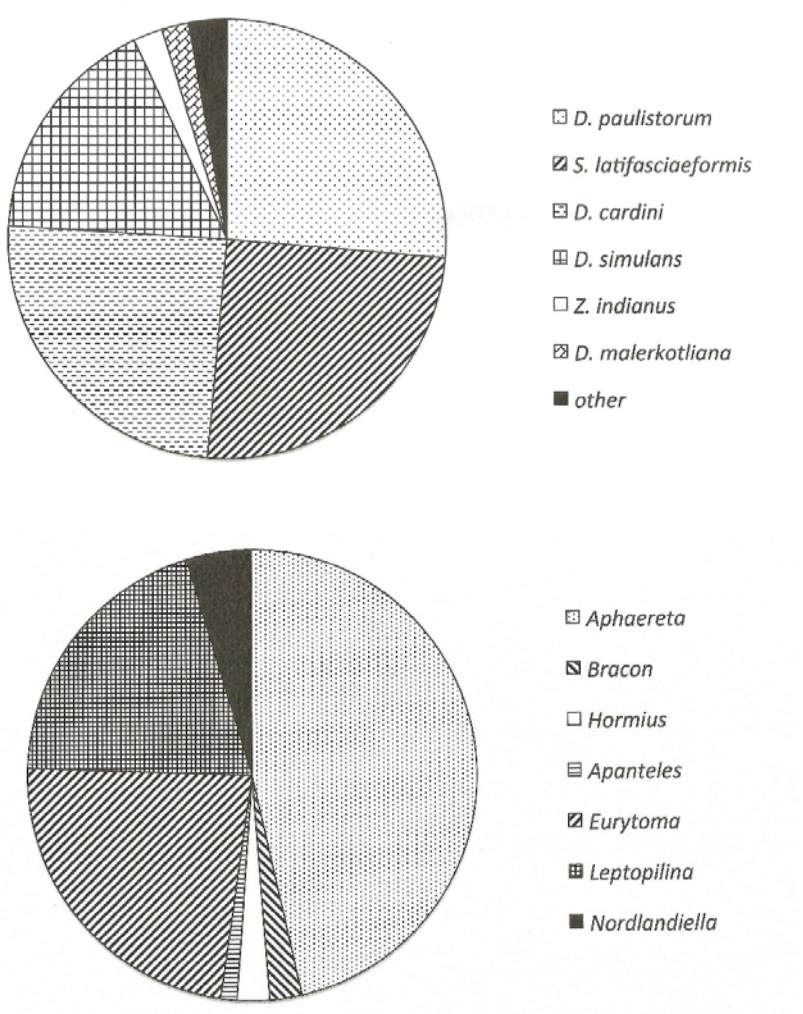

Figure 1. Relative abundance of drosophilids (above) and parasitoids (below) emerging from rotting fruits collected in four protected areas of the *Federal District*, located in the center of the Brazilian savanna, from October 2010 to March 2011.

The genus *Eurytoma* (Eurytomidae, Eurytominae) was the second in abundance (23% of specimens). This is the largest and most difficult genus in the family Eurytomidae, and includes hundreds of species throughout the world. Many of them are placed in *Eurytoma* not because they are greatly like the type-species, but because it has not as yet been possible to place them elsewhere. Almost all the generic characteristics of *Eurytoma* are, as a result, subject to exceptions. Some species of *Eurytoma* are phytophagous, others pass part of their larval development as parasites and then complete their growth by feeding on plant tissues (Burks, 1971). Most species of *Eurytoma* attack gall formers, especially Tephritidae, Cecidomyiidae, and Cynipidae. A few species parasitize Coleoptera or Lepidoptera. There are also species that parasitize sawflies, bees, wasps, and even primary parasites such as Braconidae and Echneumonidae (Gibson *et al*., 1997).

Finally, we found two genera of the family Figitidae. Both belong to the subfamily Eucoilinae, which are solitary endoparasitoids that oviposit in the larval stage of cyclorrhaphous Diptera. Eucoilines have been reared from hosts representing a wide range of microhabitats, and several species have been reared from flies breeding in fruit (Naturewatch, 2014). *Leptopilina* is one of the best-know genera of *Drosophila* parasitoids (Prevost, 2009), and it has already been collected in the Brazilian Savanna infesting *Zaprionus indianus* (Marchiori *et al.*, 2003). In this study it corresponded to 20% of the wasps emerged in fruits. *Nordlandiella*, on the other hand, is a less well-known genus and corresponded to about 5% of our sample.

Here we recorded seven wasp genera and 11 drosophilid species that emerged from fruits collected in the Brazilian Savanna. Therefore, we have no direct information on the parasitoid-host relationship, only an indication of the number of drosophilid species that can potentially be parasitized (Offenberger and Klarenberg, 1994). Besides, the identification of the wasps at the species level was virtually impossible. Our

goal, in future work, is to invest taxonomic efforts in parasitoid identification and/or descriptions, and to reveal the complex relationships among plant species (fruits), Drosophilids, and parasitoids.

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Records of *Zaprionus indianus* and *Drosophila suzukii indicus* as invasive fruit pests from mid valley region of Garhwal Uttarakhand, India.

Fartyal, Rajendra Singh*, Manisha Sarswat, Nema Lhamo, Pradeep Chandra Sati, and Asha. Systematics, Cytogenetics & Molecular Systematics Laboratory, Department of Zoology & Biotechnology, Chauras Campus, HNB Garhwal University (A Central Univ.), P.O. Kilkileshwar, Kirtinagar, Garhwal-249 161, Uttarakhand, India; *Corresponding author: rs.fartyal@hnbgu.ac.in; fartyalrs@gmail.com.

Abstract

The present work is the first record of *Zaprionus indianus* Gupta and *Drosophila suzukii indicus* Parshad and Paika (Diptera: Drosophilidae) as invasive fruit pests from mid valley region of Garhwal, Uttarakhand. Different life stages of these flies were observed in Sweet orange (*Citrus sinensis* L.) and Guava (*Psedium guajava*). The female *Drosophila suzukii indicus*, widely known as spotted wing *Drosophila*, with

its serrated ovipositor lays eggs inside unwounded ripening fruits, while *Zaprionus indianus* females are unable to lay eggs through the skin of fruits and need injuries or wounds to lay their eggs. Here, we provide the description of the pests, their biology, life cycle along with extent of infestation which could be useful in devising future management plans and monitoring techniques.

Introduction

Zaprionus indianus Gupta 1970 and *Drosophila suzukii indicus* Parshad and Paika 1964 (Diptera: Drosophilidae) are among the most widely documented invasive fruit pest species of the family Drosophilidae. *Z. indianus* mostly attacks ripe and damaged fruit unlike *D. suzukii indicus*. The females of *Z. indianus* are incapable to insert eggs through the fruit surfaces; thus they mostly oviposit on the surface of previously damaged fruits (Tidon *et al*., 2003; Steck, 2005). *Z. indianus* adult females are also known to deposit eggs on the ostiole of fresh fruits like *Ficus carica* from where maggots penetrate the supple and fleshy internal core of the fruits (Vilela *et al*., 2001). They primarily feed on the yeast, like *Candida tropicalis* (Gomes *et al*., 2003) and bacteria found on decomposing fruits.

Drosophila suzukii and *Drosophila suzukii indicus* commonly known as spotted wing *Drosophila* is among those species that have been identified to oviposit in healthy, unwounded fruits in contrast to *Z. indianus*, which is mostly found on damaged or overripe fruits. The distinctive characteristics of *D. suzukii* make it an arduous pest are its proclivity towards fresh ripening (as opposed to overripe) fruit (Mitsui *et al*., 2006) and more importantly the presence of prominent serrated ovipositor of the female, which upon insertion causes physical damage to the fruit. Subsequently, these oviposition wounds caused by *D. suzukii* flies lead to secondary infections by insects and several other pathogens like bacteria, fungus, and yeasts, causing increased losses (De Camargo and Phaff, 1957; Molina *et al*., 1974; Louise *et al*., 1996). Also, the eggs laid often develop into larvae within the fruit, leading to fruit rot and overall reduced yields. Recently, Depra *et al*. (2014) gave the first record of *D. suzukii* from Brazil and raised the concerns towards its rapid dispersal throughout the region and potential menace to fruit culture.

Identification of Pests

Zaprionus indianus can be easily identified from other known species of this genus due to some distinguishing features (Figure 1a). It is somewhat yellowish, with even number of discrete silver-white stripes on head and thorax and 4 to 6 distinct composite spines on the anterior femora. These spines are not present on small tubercles and have a second short branch at its base which functions as a rest for the tibia of the folded leg. As compared to other species, the narrow black bands around the silvery thoracic stripes do not get wide on the scutellum, and the scutellum lacks a white tip (Van der Linde, 2006).

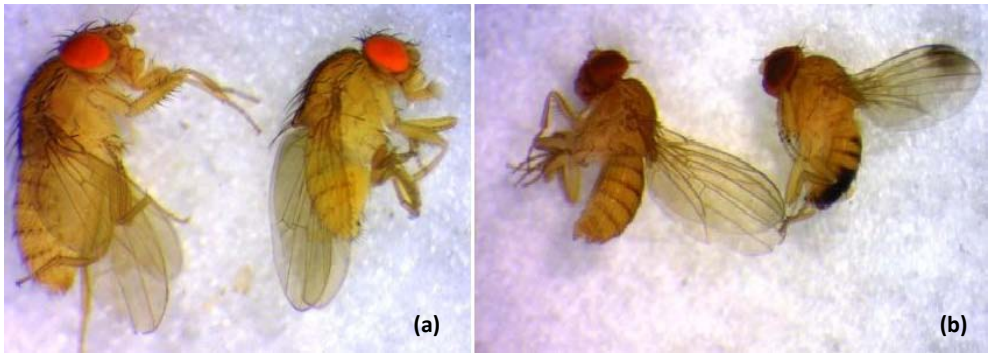

Figure 1. (a) *Zaprionus indianus* female and male fly, (b) *Drosophila suzukii indicus* female and male fly, respectively.

Adult *Drosophila suzukii indicus* are small (2-3 mm) flies with yellowish-brown thorax and abdomen and red eyes (Figure 1b). They also have black stripes on the abdomen and males have a distinguishing dark spot on the leading front near the tip of each wing. The females have a well serrated ovipositor, which aids in penetrating the most thin-skinned fruits leaving a small wound or depression on the surface.

Life Cycle

Zaprionus indianus females produce around 60-70 offspring on an average in their entire lifespan. The eggs laid are milky white in color and are typically in small masses laid by several females, mostly in damaged or fallen fruits with the exposed fruit pulp. The eggs are also laid in ostioles of some fruits while they are still on the tree, especially in figs (*Ficus* spp.). Transitions through the entire lifecycle (from egg to adult) may also be influenced by temperature. The average temperature of the study site during the sampling was around 18°-20°C, where the egg stage was for about 1-2 days, larval stage for 12-15 days, and pupal for 4-5 days. *D. suzukii indicus* on other hand had higher fecundity than *Zaprionus indianus*. Females on an average can lay up to a total of 400-500 eggs during their lifetime. These eggs hatch inside the fruits anywhere between 2-3 days, the larvae also matures within the fruit in about 5 to 15 days. Pupa mostly resides within the fruit for almost 4 to 12 days (Figure 2).

Figure 2. Life cycle of pests within infested fruits, (a) breeding, (b) emergence of larvae, and (c) pupae and larvae isolated from fruits.

This region experiences sub-tropical to temperate climatic conditions favoring sudden outbursts in abundance of this species. Similarly, population expansion after invasion of a few individuals favored by cooler climate more similar to the native range and areas previously invaded by this fly have also been reported from Brazil (Depra *et al*., 2014). Further, due to the short generation time and optimum temperature conditions in the region, these species achieve exponential growths within one season causing extensive damage to fruit crops.

Fruit Damage

Z. indianus was mostly found associated with damaged fruit on trees or felled off rotten fruits of sweet orange (*Citrus sinensis* L.). The larvae were even able to invade the soft tissue of over-ripened guava (*Psedium guajava*). On the other hand, *D. suzukii indicus* was able to lay eggs on healthy, unwounded fruit due to the serrated female ovipositor, preferring the ripening orange fruits over the over-ripened ones. Mostly the damage caused by *D. suzukii indicus* was due to its larvae feeding on fruit flesh. Moreover, the scar left behind due to insertion of the ovipositor into the skin of the fruit caused physical damage and called for secondary infections of pathogens fungi, yeasts, and bacteria leading to rapid deterioration and further losses (Figure 3). Another important observation was that the flies transferred and infested different fruits as seasons progressed and were not limited to one. The fact is currently looked upon in detail and will be communicated in further publications.

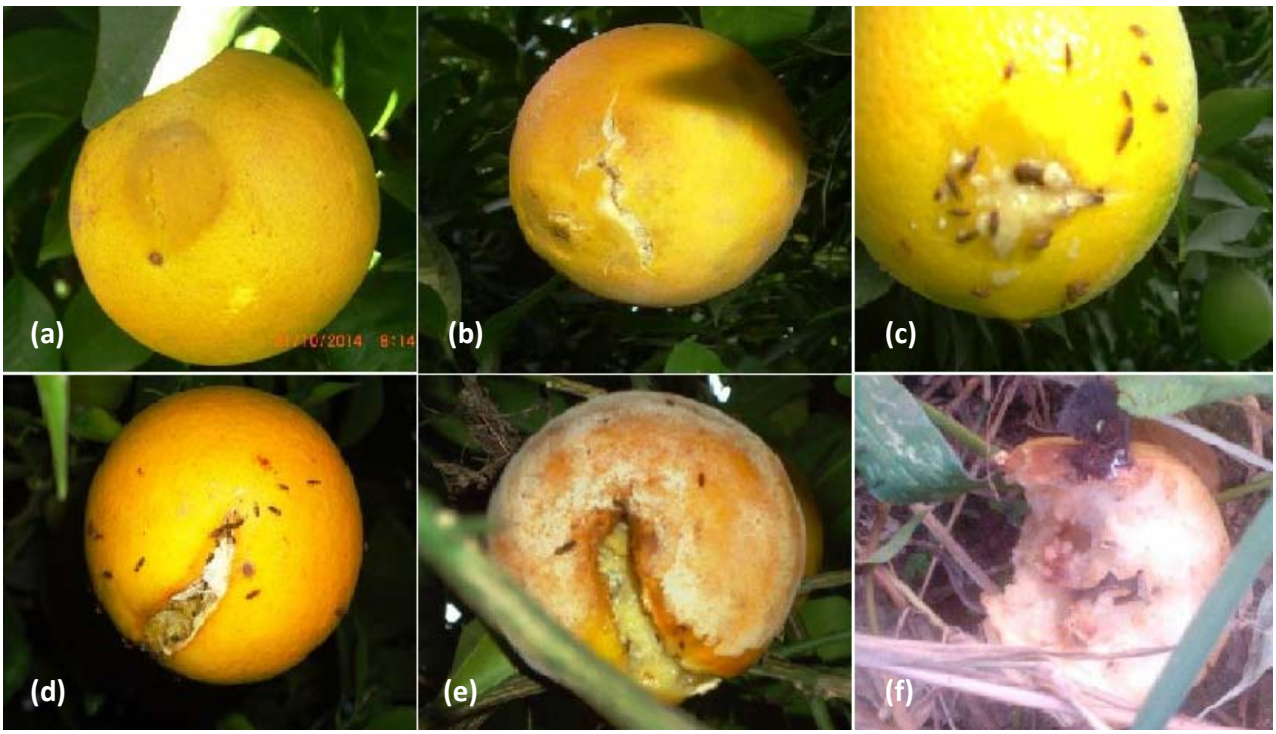

Figure 3. Different stages of fruit damage, (a) - (b) initial wound on sweet orange (*Citrus sinensis* L.), (c) - (d) infestation by pests, (e) secondary damage by fungal infection, and (f) *Z. indianus* larvae on fallen guava (*Psedium guajava*).

Eggs and larvae of the fly cannot be easily detected inside the fruits and some eggs and larvae can even survive periods of refrigeration (Kanzawa, 1939), which is often a menace to international fruit trade. This has been a plausible reason behind spread of *D. suzukii* to Europe and the USA (Rota-Stabelli *et al*., 2013) and recent invasion to Brazil (Depra *et al*., 2014). Thus, we also collected some fresh fruits from the vicinity of damaged ones. These fruits, along with some damaged by birds or other predators as well as the ones lying on the ground, were collected, brought to the laboratory, and were placed individually into sealed culture bottles (Figure 4) and kept at optimum temperature corresponding to the habitat. The bottles were examined every day for emergence of different life forms. We observed emergence of larvae even from some of those fruits that had no signs of physical damage. Subsequently, the adults that emerged were removed from bottles and identified. There were no records of emergence of the parasitoids throughout the culture period.

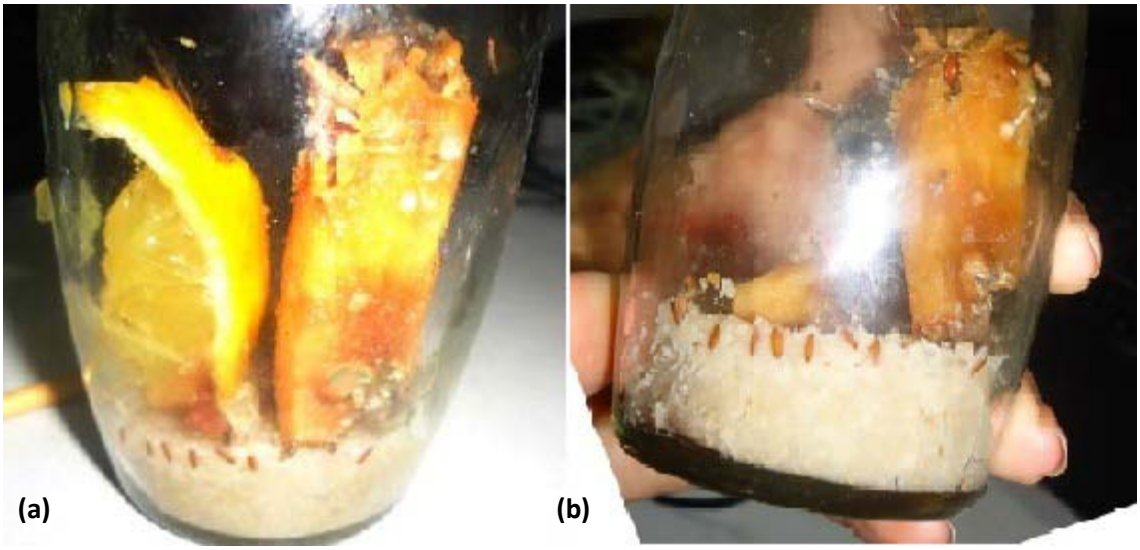

Figure 4. Laboratory culture of infested fruits depicting pupal and adult emergence, (a) sweet orange (*Citrus sinensis* L.), and (b) guava (*Psedium guajava*).

Conclusion

The present study provides the first records of *Zaprionus indianus* and *Drosophila suzukii indicus* as invasive fruit pests from mid valley region of Garhwal, Uttarakhand. The understanding of their basic biology, ecology, and distribution in this region would be helpful for development of efficient management strategies in future.

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The frequency of *In(3R)P* from the Guam population of *Drosophila melanogaster* is the highest ever reported in the world.

Yutaka, Inoue[1], and Masayoshi Watada[2]*. [1]Osaka University, Toyonaka, Osaka 560-0043, Japan, and [2]Ehime University, Matsuyama, Ehime 790-8577, Japan; *Corresponding author: watada@mserv.sci.ehime-u.ac.jp.

Natural populations of *Drosophila melanogaster* were found to show the inversion polymorphisms on the four major autosome arms in world-wide scale. These naturally occurring inversions are all the paracentric type. Most of them are found only once in a particular local population, being called "*Unique"* inversions, and some are observed repeatedly in many populations. In view of the frequency and geographical distribution, Inoue and Igarashi (1994) categorized these repeatedly observed inversions into four classes (*Common Cosmopolitans*, *Rare Cosmopolitans*, *Quasi Cosmopolitans*, and *Endemics*). *Common Cosmopolitan* inversions are usually maintained in most populations all over the world, on occasion being more frequent than the standard rearrangement, and show frequency clines by latitudes over large geographical regions. Four inversions, *In(2L)t*, *In(2R)NS*, *In(3L)P*, and *In(3R)P* are in this category, each of which exists in the left and right arms of two major autosomes. *Rare Cosmopolitan* inversions, *In(3R)C* and *In(3R)Mo*, are also distributed all over the world, but their frequencies are usually low, not enough to show the geographical cline. *Quasi Cosmopolitan* inversions are also widespread in the world, but just enough to say "cosmopolitan" in their distribution. They always appeared with very low frequencies and were absent in many populations. *Endemic* inversions are found more than once in a given population but appear in a geographically region-limited manner. Occasionally they show higher frequencies than *Rare Cosmopolitans.*

In the present study we report the recent result of two successive year surveys from the population of Guam island belonging to the Mariana Islands in the Pacific Ocean, and four Japanese populations (see Table 1). Wild caught females were individually transferred to culture vials and allowed to lay eggs. Established strains were kept at 25°C for examination. Inversions were determined through direct observation of the chromosomes of one larva from each female by identifying the inversion homozygotes. This method gives two genomes sampled per each female. Cytological analysis was made on salivary gland chromosomes stained with the lactic-acetic orcein method. Breakpoints and nomenclature of inversions were established by comparing with representations of Bridges' map (Lefevre, 1976). Average frequencies of total cosmopolitan inversions per major autosome arm (Arm Average in Table 1) were also calculated to compare the degree of inversion polymorphisms, because significant positive correlations were observed among these frequencies for all four major autosome arms (Inoue *et al.*, 1994).

Table 1. Frequencies of the cosmopolitan inversions in Guam and Japanese populations.

Locality (Year)	N*	*In(2L)t*	*In(2R)NS*	*In(3L)P*	*In(3R)P*	*In(3R)C*	*In(3R)Mo*	Arm Average**
1. Guam (2012)	42	0.404	0.190	0.190	1.000	0	0	0.446
2. Guam (2013)	262	0.523	0.230	0.172	0.940	0	0	0.484
3. Kyoto (2012)	122	0.165	0.157	0.008	0.107	0.074	0.025	0.110
4. Kobe (2013)	200	0.175	0.130	0.030	0.235	0.030	0.020	0.168
5. Ogasawara (2012)	68	0.148	0.044	0.018	0.471	0	0	0.170
6. Iriomote (2012)	72	0.444	0.236	0.347	0.444	0	0	0.368

* Number of genomes examined

** Average frequency of inversions per major autosome arm

Table 1 shows the frequencies of the cosmopolitan inversions in the present study. The Guam sample in 2012 (No. 1) was collected by the banana bait traps in the agricultural field of Guam University, and the

sample in 2013 (No. 2) was collected in and around the agricultural field of the university. These two samples showed almost the same results. The *In(2L)t* frequencies were 0.404 in 2012 and 0.523 in 2013, which showed that the ratio of the standard chromosome and *In(2L)t* was about half and half in the left arm of the second chromosome. Both the *In(2R)NS* and *In(3L)P* frequencies were about half of *In(2L)t* in the two samples. The point of the present report is the frequency of *In(3R)P*. In the sample of 2012, all right arms of the third chromosome were fixed by *In(3R)P*. In order to confirm the fixation of *In(3R)P* in the Guam population, we examined more chromosomes the following year and found that all right arms of the third chromosome were not fixed, but most of all 3R arms had *In(3R)P*, being 0.940 of the frequency. *Rare Cosmopolitan* inversions, *In(3R)C* and *In(3R)Mo*, were not found in the two samples. The arm averages were 0.446 and 0.484, being almost the same. Additionally *In(3R)K* was found by the collection of 2013 in the heterozygous condition with the standard chromosome, which is classified to *Quasi Cosmopolitan* inversions.

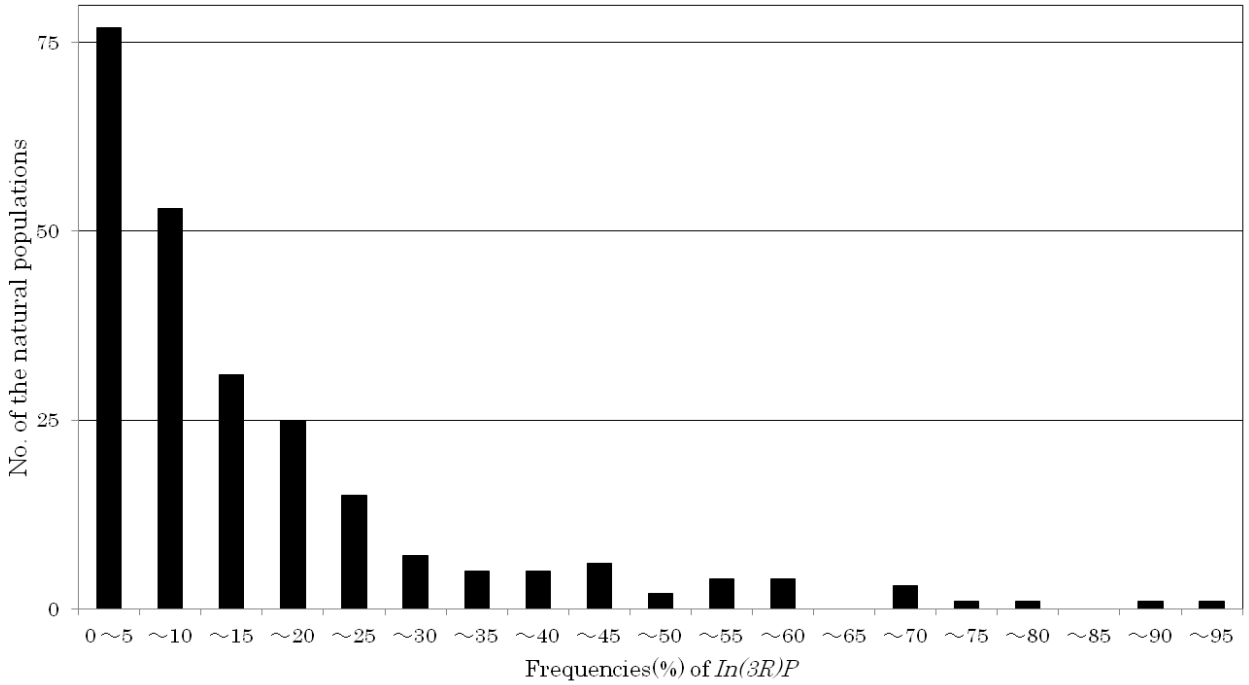

Figure 1. The distribution of the maximum In(3R)P frequencies in a total of the 241 separated local populations all over the world analyzed in 1944 ~ 2013.

Kyoto (No. 3) and Kobe (No. 4) exist in the center of Japanese Mainland. In comparison with Guam (No. 1 and 2), these Japanese populations had a much smaller amount of inversions, being 0.110 and 0.168 of the arm average. Especially *In(3L)P* showed very low frequencies in both cases (0.008 and 0.030). Inoue *et al.* (2002) also reported very low frequencies of *In(3L)P* in the Japanese mainland populations. But *Rare Cosmopolitan* inversions, *In(3R)C* and *In(3R)Mo*, were observed constantly, which were not found in the two Guam populations. Additionally two *Unique* inversions, *In(2R)46B;55F* and *In(2L)23F;26B*, were found twice in Kyoto populations and once in the Kobe population, respectively. They are *Unique* inversions. Moreover, the *In(2R)O* which is found recently in Japan and increasing its distribution gradually (Inoue *et al.* 2011), appeared with a frequency of 0.05 in Kyoto and 0.025 in Kobe. Ogasawara (No. 5) is the islands, which are located 1,000 km south of Tokyo. The arm average was 0.170, which was similar to Kyoto (No. 3) and Kobe (No. 4). But the Ogasawara population showed very low frequency of *In(2R)NS* (0.044) as well as *In(3L)P*, and had no *Rare Cosmopolitan* inversions, *In(3R)C* and *In(3R)Mo*. So in Ogasawara major cosmopolitan inversions were only *In(2L)t* and *In(3R)P*, which was the same result as the past Ogasawara data of 2000 and 2004 (Inoue and Watada 2006). Iriomote (No. 6) belongs to the South-west Islands of Japan. According to the latitudinal cline

(Mettler *et al.*, 1977; Knibb *et al.*, 1981) that the populations near the equator incline to have more inversions, the Iriomote population was found to have much more inversions than the mainland and Ogasawara populations (No. 3, 4, and 5). Its arm average (0.368) was less than that of the Guam populations, but the frequency of *In(3L)P* (0.347) was the highest in the present study. It confirmed the result of Inoue *et al.* (2002) that *In(3R)P* is relatively adaptive in the South-West Islands in comparison with the other regions. *Rare Cosmopolitan* inversions, *In(3R)C* and *In(3R)Mo*, were not found there as well as Guam (No. 1 and 2) and Ogasawara (No. 5).

Figure 1 shows the distributions of *In(3R)P* frequencies by almost all the data from 1944 to 2013 all over the world (see the references). The samples of less than 40 genomes examined were not used in Figure 1. A total of 241 separate local populations were used, each of which was the highest frequency in each given locality. The only one highest value among the data at differing sampling time was used in each locality. The numbers of populations of *In(3R)P* frequencies were counted by every 5% level. A total of 77 local natural populations was put between 0 ~ 5% level, 53 populations between 5 ~ 10% level, 31 populations between 10 ~ 15% level, 25 populations between 15 ~ 20% level, and 15 populations between 20 ~ 25% level. After that level, the number of populations decreased gradually to the zero point. Only a few populations showing more than 65% were found in the South-west Islands of Japan, Florida in the U.S.A., and New Guinea, among which the highest frequency (0.890) was from the Iriomote 1979 population (Inoue *et al.*, 1994). Thus, the present data of 0.940 from the Guam 2013 population with a total of 262 genomes examined is so far the highest frequency for *In(3R)P* in the world.

Acknowledgment: We thank Dr. Masanobu Itoh, T. K. Katoh, and Rika Ogoshi for collections of *D. melanogaster* and their technical assistances.

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Organically grown banana fruit effects on reproductive fitness of *Phorticella straiata.*

Krishna, M.S. *Drosophila* Stock Center, Department of Studies in Zoology, University of Mysore, Manasagangotri, Mysore - 560006. Karnataka, India; drosokrish@gmail.com.

Introduction

Diet is one of the external factors known to affect on an organism's growth, development, reproduction, and survival (Sisodia and Singh, 2012). The diet consumed by an individual can be grouped

broadly into quantitative, which is dependent on food availability, and qualitative, which is dependent on nutritional constituents of the food. It is believed that consumption of diet exposed to pesticides has detrimental effects on the health of an individual. It has been shown that individuals exposed to high levels of pesticides face increased risks of developing cancers such as prostate cancer (Alavanja *et al.,* 2003).

Nowadays, popularity and consumption of organic fruits and vegetables is growing at an exponential rate due to a belief that such foods are healthier and free of pesticides. Further studies on comparison between organically and conventionally grown foods have shown that organic samples have fewer pesticide residues when compared to conventionally grown food (Baker *et al.,* 2002). A recent study has shown positive effects of organic foods on the health of *D. melanogaster*, where flies reared in organically grown food showed overall better health (Chhabra *et al.,* 2013). More studies are required in other species or genera to understand health benefits of organic fruits. Therefore, the present study has been undertaken in *P. straiata* to study effects of organic banana on reproductive performance

P. straiata (Nirmala and Krishnamurthy, 1974) is a Drosophilid insect discovered from Karnataka, India belonging to group Drosophilidae. This species also has all characteristics of a good laboratory tool to analyze genetic and evolutionary problems as that of the genus *Drosophila* (Sarat and Hegde, 2003).

Materials and Methods

Establishment of experimental stock

The experimental stock of *P. straiata* was obtained from the progenies of 50 isofemale lines collected from Chamundi hills, Mysore, India. In each generation flies obtained from these culture bottles were mixed together and redistributed to 20 different culture bottles containing wheat cream agar media (100 g of jaggery, 100 g of wheat powder, 8g of *Agar* was boiled in 1000 ml of double distilled water and 7.5 ml of Propionic acid was added) each with 20 flies (10 males and 10 females). These flies were maintained at 22°±1°C with a relative humidity of 70% in a 12 hr dark: 12 hr light cycle. This procedure was carried out for three generations to acclimatize flies to lab conditions. At the fourth generation, eggs were collected using Delcour's procedure (1969). Eggs (100) were seeded to each culture bottle containing wheat-cream agar media / organically banana based media / conventionally banana fruit based media (1 kg of fine paste of organically / conventionally banana was boiled in 1 l of double distilled water containing 10% *Agar*. To this 7.5 ml of Propionic acid was added). When pupae were formed, females and males were isolated and were allowed to eclose and aged for five days to test for virginity. These flies were used for present experiments. Organic fruits were purchased from organic food product distributors, *Hasiru Organic Shop*, Mysore, which is a unit of Karnataka State Natural Farming Movement.

Organically grown banana fruit effect on larval feeding in P. straiata

Third instar larvae obtained from eggs collected (±2 hours) from wheat-cream agar media grown flies using Delcour's procedure (1969) were used to study feeding behavior. Each larva was placed in a vial containing wheat-cream agar / organically grown fruit based media / conventionally grown fruit based media and observed under a stereomicroscope. The back and forth movement of the proboscis was recorded for a minute. A total of 50 replicates were run separately for each of organic / conventional / wheat-cream agar media grown larvae. Separate experiments were carried out for larvae of organic / conventional banana/ wheat-cream agar media.

Organically grown banana fruit effect on reproductive performance in P. straiata

Four-day-old virgin females and unmated males from the wheat-cream agar / organic banana based media / conventional banana based media were aspirated into an Elens-Wattiaux chamber (Elens and Wattiaux, 1964). Each pair was observed for an hour. The pairs which did not mate within this time limit were discarded. Mating latency (time between introduction of a pair of male and female flies into the Elens-Wattiaux chamber until the initiation of copulation of each pair) and copulation duration (time between initiation to termination of copulation of each pair) were recorded. Mated pairs were transferred once in 24 hr to a new vial containing 5 ml of normal media until death. Total number of eggs laid and progeny obtained

were recorded. A total of fifty replicates were performed separately for wheat-cream agar media / organically / conventionally fruit based media grown flies.

Organically grown banana fruit effect on ovariole number in P. straiata

Four-day-old virgin females were etherized and killed. The thorax of these flies was dissected out using a pair of fine dissection needles in physiological saline under a binocular stereomicroscope. The ovaries were separated and the total number of ovarioles in either the right or the left ovary was noted (Hegde and Krishna, 1997).

Results

Figure 1 provides the larval feeding rate in different diets. It was found that highest larval feeding rate occurred in flies grown in organic fruit based media compared to conventional fruit based media and wheat cream agar media. Flies fed on conventional fruit based media performed least among the three diets studied. One-way ANOVA followed by Tukey's *post hoc* test carried out using SPSS version 10.0 on the above data showed significant variation in feeding rate between diets (Table 1). Tukey's *post hoc* test also showed that flies grown on organic banana media had significantly greater feeding rate than those flies grown in conventional fruit based media grown flies and wheat cream agar media grown flies.

Table 1. One way ANOVA on feeding rate, mating latency, copulation duration, fecundity and ovarioles number in *P. straita.*

Parameter	Source	Type III Sum of Squares	df	Mean Square	F value
Feeding rate (in no.)	Diet	34421.08	2	17210.540	524.214**
	Error	4826.180	147	32.831	
	Total	2073687.00	150		
Mating latency (in min)	Diet	85.13024	2	42.56512067	338.3274**
	Error	18.49414	147	0.125810463	
	Total	2843.6	150		
Copulation duration (in min)	Diet	45.62028	2	22.81014067	88.43846**
	Error	37.9144	147	0.257921061	
	Total	1486.729	150		
Fecundity (in no)	Diet	27033.24	2	13516.62	250.1735**
	Error	7942.26	147	54.02897959	
	Total	876477	150		
Ovarioles number (in no.)	Diet	1654.24	2	827.12	57.17097**
	Error	2126.72	147	14.46748299	
	Total	134850	150		

**Significant at 0.01 level (P < 0.01)

Mating latency of flies grown in different diets is provided in Figure 2. It was observed that flies grown in organic banana based media had taken lowest time to initiate copulation whereas flies grown in wheat cream agar based media had taken highest time to initiate copulation. One-way ANOVA followed by Tukey's *post hoc* test carried out on the above data showed significant variation in mating latency between

flies grown in different diets (Table 1). Flies grown on organic banana fruit took significantly greater time to initiate copulation than those flies grown on convention banana fruit or wheat cream agar media by Tukey's *post hoc* test.

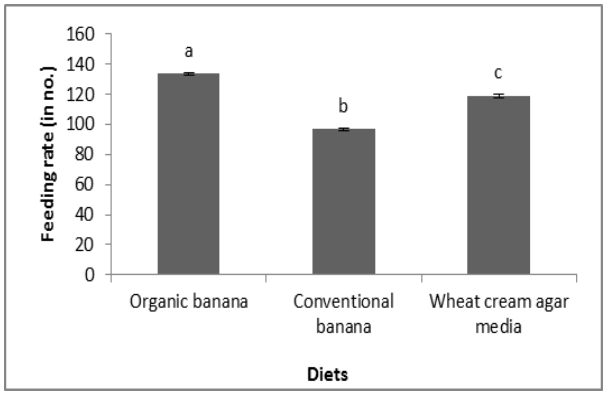

Figure 1. Diet effect on feeding rate in *P. straiata.* (Different letters on the bar graph indicate significant at 0.05 level by Tukey's *post hoc* test).

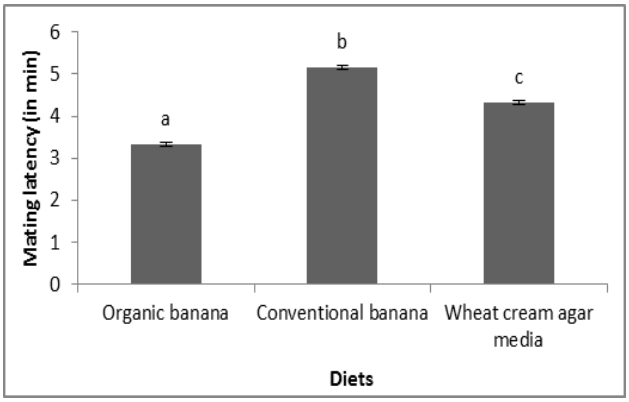

Figure 2. Diet effect on mating latency in *P. straiata.* (Different letters on the bar graph indicate significant at 0.05 level by Tukey's *post hoc* test).

Copulation duration of flies reared on different diets (Figure 3) shows that flies reared on organic banana fruit copulated longer while flies grown on wheat cream agar based media had copulated for shorter period. One-way ANOVA followed by Tukey's *post hoc* test carried out using SPSS version 10.0 (Table 1) showed significant variations in copulation duration of flies reared between different diets. Tukey's *post hoc* test also showed that flies grown in organic banana copulated significantly longer than those flies reared in conventional banana based media and wheat cream agar media.

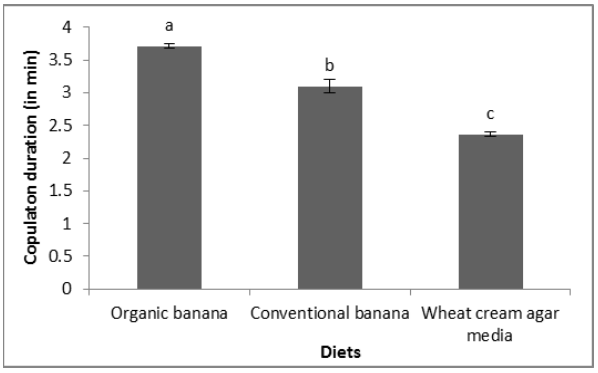

Figure 3. Diet effect on copulation duration in *P. straiata.* (Different letters on the bar graph indicate significant at 0.05 level by Tukey's *post hoc* test).

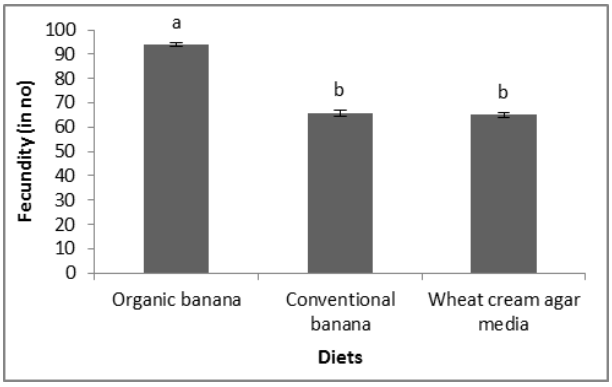

Figure 4. Diet effect of female on fecundity in *P. straiata.* (Different letters on the bar graph indicate significant at 0.05 level by Tukey's *post hoc* test).

Fecundity data of flies reared in different diets is provided in Figure 4. It was noticed that egg production was greater in flies reared on organic banana compared to flies reared on conventional banana and wheat cream agar media. Fecundity data subjected to one-way ANOVA followed by Tukey's *post hoc* test showed significant variation in fecundity between flies reared in different diets (Table 1). Tukey's *post hoc*

test also showed that flies reared on organic banana had significantly greater egg production than those flies reared in conventional banana and wheat cream agar media.

Female flies reared in organic media had greater number of ovarioles when compared to both conventional banana fruit based media as well as wheat-cream agar media (Figure 5). One-way ANOVA followed by Tukey's *post hoc* test carried out on the above data showed significant variation in ovarioles between different diets (Table 1). Flies reared in organic banana media had significantly greater number of ovarioles than flies reared in conventional banana fruit based and wheat cream agar media.

Mean wing length of female grown in different diets is provided in figure (not shown). It was noticed that mean wing length of females reared in organic banana had slightly larger wing length than those females grown in conventional banana and wheat cream agar media. Mean wing length data of females reared in different diets subjected to one-way ANOVA showed insignificant variation in female wing length between different media.

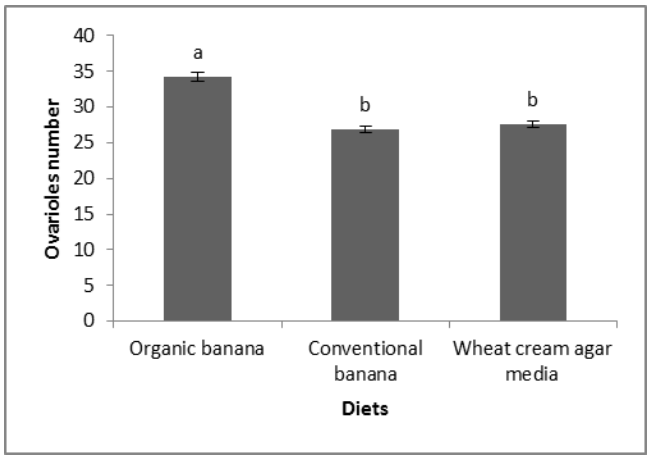

Figure 5. Diet effect on ovarioles in *P. straiata.* (Different letters on the bar graph indicate significant at 0.05 level by Tukey's *post hoc* test).

Discussion

Diet is one of the important environmental factors known to affect organism's growth and development (Sisodia and Singh, 2012). The quality and quantity of food consumed by the organism has an impact on health and reproductive fitness of an organism. Therefore, in the present study *P. straiata* flies were fed with a diet made of organic banana / conventional banana/ wheat cream agar based media to study its effect on reproductive performance.

In *P. straiata* larval stage is the active feeding stage; they feed continuously accompanied by a massive increase in mass. This is true with other *Dipteran* species too (Melcher *et al.*, 2007). However, inhibition threshold exists for feeding on new or full tasting food (Melcher et al., 2007). Such inhibition threshold is not observed in larvae of *P. straiata* fed on organic banana fruit when compared to larvae feeding on conventional banana fruit or wheat cream agar media (Figure 1 and Table 1). The greater the larval feeding, the greater is the quantity of food taken inside the organism. Therefore, larvae of *P. straiata* had taken significantly greater quantity of organic banana than larvae grown on conventional grown banana fruit and wheat cream agar media.

All biological processes are directly related to reproduction that plays an important role in determining fitness. It was shown that reproductive capacity of an organism is a good index of fitness that go through repeated cycles of rapid population growth, and it is evolved as a way of species to maximize their potential of survival. Quality and quantity of food taken by an organism has an influence on reproductive performance (Sisodia and Singh, 2012). In the present study, effect of organic banana fruit on fitness traits, such as mating latency, copulation duration, fecundity, and ovariole number, has been tested in *P. straiata.*

Time taken to initiate copulation forms mating latency; it is an important component of mating behavior in *P. straiata.* Flies which took a shorter time to initiate copulation were fast maters than those flies which took longer time to initiate copulation. In the present study in *P. straiata* organic banana fruit grown males took the shortest time to initiate copulation while males grown on wheat cream agar media had taken the longest time to initiate copulation (Figure 2 and Table 1). This suggests that males grown on organic banana fruit were fast maters whereas males grown on wheat cream agar media were slow mated. This difference in mating latency between different diets could be attributed to the difference in the nutrients level. Organic banana fruit had greater nutrient qualities than conventional banana fruit (Chabra *et al*., 2013). In species of

Drosophila it was also shown that mating latency is also affected by body size, age, and diet (Hegde and Krishna, 1997; Somashekar and Krishna 2011; Singh and Sisodia, 2012). In *P. straiata* courtship activities of male and female culminate in copulation (Latha and Krishna, 2014). Longer copulation is an adaptation of males which could reduce the risk of sperm competition with future ejaculations with the help of mating plug which prevents the female from remating before oviposition (Gilchrist and Partridge, 2000). In the present study it was found that flies grown on organic banana fruit had copulated significantly longer compared to flies grown on non-organic banana and wheat cream agar media (Figure 3 and Table 1). Our results in *P. straiata* confirm work of organic banana fruit on reproduction (Chabra *et al*., 2013). Longer the duration of copulation, greater is the transfer of accessory gland proteins and sperm to the mated female (Hegde and Krishna, 1997; Somashekar and Krishna, 2011).

Fecundity is the most obvious trait that influences the reproductive ability of female usually considered as female fitness component. It is known that fecundity is influenced by age, body size, and diet of an organism (Krishna and Hegde 1997). In *P. straiata* flies grown on organic banana fruit based media had greater number of ovarioles compared to flies grown in other two media (Figure 4 and Table 1). In the present study, flies used were of same age and were grown in same conditions but foods were different. Therefore, in the present study quality of food had influenced fecundity in *P. straiata*. Ovariole number was positively correlated with fecundity (Krishna and Hegde, 1997). They also pointed out that greater the ovariole number the greater is the fecundity. Therefore, in the present study ovariole numbers of flies grown on different diets were analyzed in *P. straiata*. Flies grown on organic banana fruit had significantly greater number of ovarioles than those flies grown in conventional banana fruit and wheat cream agar media (Figure 5 and Table 1). Thus these studies in *P. straiata* suggest that organic fruit has positive effect on reproduction**.** Organic banana fruit flies had greater reproductive performance and fitness.

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Female reproductive traits of the model Hawaiian fly *Drosophila grimshawi.*

Craddock, Elysse M. School of Natural and Social Sciences, Purchase College, State Univ. of New York, Purchase, N.Y. 10577-1400; E-mail: elysse.craddock@purchase.edu.

Introduction

The evolutionary success of the endemic Hawaiian Drosophilidae, a monophyletic group of ~1,000 species, owes much to the diversity of reproductive strategies of these flies (Kambysellis and Heed, 1971), in conjunction with adaptation of female reproductive function and oviposition behavior to a broad array of breeding substrates and plant hosts (Heed, 1968; Montgomery, 1975; Kambysellis and Craddock, 1997). Members of the well-studied picture wing group of over a hundred species are typically large, long-lived flies

with high fecundity, greater than that observed in other groups of Hawaiian flies. At the outset of cytological analyses of the picture wing group, Hamp Carson arbitrarily chose the species *Drosophila grimshawi* Oldenberg of the *grimshawi* species group as the 'standard' picture wing species (Carson, Clayton, and Stalker, 1967). Polytene chromosome banding sequences of all subsequently studied Hawaiian species were compared to the *D. grimshawi* Standard sequence, and the number and locations of inversions required to derive each sequence from the Standard determined. These inversion relationships were used to derive a chromosomal phylogeny of evolutionary relationships among the studied species (Carson, 1970), long before the advent of molecular phylogenies. In planning the expansion of genome sequencing efforts beyond the model fly *Drosophila melanogaster*, it was natural to select Carson's standard species, *D. grimshawi*, as the representative of the large group of endemic Hawaiian *Drosophila* (*Drosophila* 12 Genomes Consortium, 2007).

D. grimshawi, the genomic model for the Hawaiian *Drosophila*, is somewhat anomalous, however, in that it is not a single-island endemic, which is the characteristic situation for these flies. Originally, this species was thought to occupy all six high islands of the archipelago. The taxon on the youngest island, Hawaii, was then described as a distinct species, *Drosophila pullipes*, by Hardy and Kaneshiro (1972). Subsequently, phylogenetic analyses of the clade indicated that the Kauai and Oahu populations were much more closely related to *D. pullipes* than to those on Maui, Molokai, and Lanai (Piano *et al.*, 1997). These data led to a taxonomic revision which distinguished the Kauai and Oahu populations as a separate but morphologically cryptic species, *Drosophila craddockae* (Kaneshiro and Kambysellis, 1999). The clade thus comprises three closely related species. Importantly, two species, *D. craddockae* and *D. pullipes*, are strict ecological specialists that breed in decaying bark of *Wikstroemia* (family Thymeleacae); *D. grimshawi*, on the other hand, is a generalist that has been reared from ten other families of Hawaiian plants, but not Thymeleacae (Heed, 1968; Montgomery, 1975). The distribution of the species *D. grimshawi* on the three islands of the Maui Nui complex includes four geographically disjunct metapopulations, namely those on the volcanoes of East Maui, West Maui, Molokai, and Lanai. Although currently separate, all islands were connected by land bridges into one composite island, Maui Nui, as recently as 0.3 – 0.4 Myr ago (Carson and Clague, 1995). Despite this, it cannot be assumed that montane forest suitable for *Drosophila* populations extended to the lowland land bridges at times of lower sea level such that the current population isolates were contiguous; nonetheless, the chances for migration and gene exchange among the *D. grimshawi* populations on the separate volcanoes were potentially greater then than at the present. The sequenced genome of *D. grimshawi* is from the East Maui population, specifically from the G1 laboratory stock which was the first successfully established stock of a Hawaiian species. Remarkably, this strain has been in continuous laboratory culture for half a century since the original isofemale was collected from Auwahi, East Maui in 1965.

Now that natural populations are sparse and it is uncommon to encounter this species in the field, the relatively recent collection of several *D. grimshawi* females and establishment of additional laboratory stocks at the University of Hawaii from East Maui, West Maui, and Molokai populations, provided an opportunity to examine ovarian traits and compare them with those of the long established G1 strain. All stocks are isofemale lines, with each derived from a single field-inseminated female. Consequently, all have experienced a population bottleneck. Nonetheless, it is possible that these samples from three disjunct populations display genetic differences in reproductive, behavioral, and other traits, given their current geographic isolation.

Materials and Methods

Besides the G1 stock of *D. grimshawi*, I examined females from four lab strains (ZA6Z2, ZA6Z3, ZA6Z4, and ZA010-5) from a different East Maui site, Makawao Forest Reserve, one strain from north of Hanaula, Waikapu, on West Maui (WM1005), and two strains from a side gulch SE of Kawela Gulch Road on Molokai (MoA, Mo010). Because of the high fecundity of *D. grimshawi*, and its generalist habit, all have readily adapted to the standard laboratory culture protocol for Hawaiian *Drosophila*. This entails supplementation of the Wheeler-Clayton medium for adults and the modified cornmeal medium for larvae with a brew of fermented leaves and stems of *Clermontia* (family Campanulaceae), a common host plant for many Hawaiian species including *D. grimshawi*. Samples of young adult females 4-5 weeks old from each of

the eight strains were withdrawn from the culture jars and sedated by chilling in the freezer for a few minutes, before dissection to remove their ovaries and obtain ovariole counts. Developmental rates in Hawaiian flies are extremely variable (despite uniform nutrition, light-dark cycle, and temperature), such that a proportion of approximately one-month old females still had quite immature previtellogenic ovaries, making accurate determination of ovariole numbers unreliable. Thus, no data were obtained from many dissected flies, which precluded obtaining equal sample sizes for all stocks. The data presented here for Makawao Forest Reserve are based on five females from the ZA6Z2 isoline, six females from ZA6Z3, three females from ZA6Z4, and two females from ZA010-5. The Molokai sample included 11 females from the MoA line, and seven females from the Mo010 stock.

Flies were dissected in chilled Waddington's Ringer solution and the two ovaries placed on separate microscope slides, before removal of the membrane that ensheathes each ovary and teasing apart of individual ovarioles with very fine needles to a point at which I could be sure of a completely accurate count (see Figure 1B). Ovariole counts were made separately for both ovaries of an individual female. In cases where some of the ovarioles already contained mature eggs at their posterior end, the number of mature eggs per ovariole was noted.

Thorax length in mm was used as a proxy for body size. A digital image of a right lateral view of the thorax of each female was acquired, and for calibration a digital image of a slide micrometer, taken at the same magnification. Image J was used to obtain the length from the anterior end of the thorax to the tip of the scutellum. Similarly, a sample of mature eggs dissected from ovaries was imaged and egg length and length of the respiratory filaments determined from the digital images using Image J. Sample means and standard errors of the mean (S.E.M.) of ovariole number per ovary and per fly, and female thorax length, were calculated in Excel for each of the four populations. Comparisons of these traits among populations were made using ANOVA, and between the two East Maui samples via *t*-tests.

Results

Figure 1 shows the morphology of eggs and ovaries of *D. grimshawi*. The most striking feature of the mature chorionated egg of this species is the four long respiratory appendages that are more than twice the length of the egg (Figure 1D). As shown in Figures 1A and C, these elongated respiratory filaments extend anteriorly within the ovariole beyond the germarium region. This is typical of all the Hawaiian bark-breeding picture wing species (Kambysellis and Heed, 1971; Kambysellis, 1993; Kambysellis and Craddock, 1997). Eggs of *D. grimshawi* range in length from 0.88 - 0.91 mm (mean 0.90 $\pm$ 0.01 mm), with respiratory filaments measured at lengths from 1.7 to 2.1 mm. (The filaments are fragile and the tips easily break off during handling; their curved nature also makes their precise measurement somewhat problematic.)

Table 1. Data on mean female thorax lengths (± S.E.M.), and two ovarian traits, numbers of ovarioles per ovary (mean ± S.E.M., and range) and maximum number of mature eggs observed per ovariole, in samples of lab-reared *D. grimshawi* females derived from four field populations in Maui Nui.

Island & Locality	*Isofemale Line(s)*	*Mean Thorax Length (mm)*	*N*[a]	*Mean # Ovarioles per Ovary*	*Range*	*Max # Mature Eggs Observed per Ovariole*
East Maui, Auwahi	G1	2.11 ± 0.07	50	21.2 ± 0.34	16 - 26	(2)
East Maui, Makawao Forest Reserve	ZA6Z2, 3, 4; ZA010-5	2.20 ± 0.07	32	21.8 ± 0.50	15 - 28	(1)
West Maui, Hanaula	WM1005	2.20 ± 0.06	40	22.1 ± 0.51	16 -29	4
Molokai, SE of Kawela Gulch Road	MoA; Mo010	2.19 ± 0.02	36	20.6 ± 0.47	15 - 26	3

[a]Number of ovaries scored to determine ovariole numbers. The number of females measured for thorax length is exactly half this number.

Table 1 presents data on mean thorax length, mean number of ovarioles/ovary, variation in number of ovarioles/ovary, and maximum number of mature eggs per ovariole observed in samples of females from the East Maui G1 strain (the Standard) and seven additional lab strains of *D. grimshawi*, comprising a second recently collected population on East Maui and populations on West Maui and Molokai. Within the species

and within each sample there is considerable variation among individuals in number of ovarioles per ovary, which varies over an almost two-fold range from 15 to 29 (Table 1). Kambysellis and Heed (1971) noted a comparable inter-individual variation in ovariole numbers for samples of field collected flies of other Hawaiian species and attributed this to variable nutrition.

Comparing the Auwahi and Makawao Forest Reserve strains from East Maui, there is no significant difference in female thorax length (t_{39df} = 0.88; P > 0.05) or in number of ovarioles per ovary (t_{80df} = - 0.93, P > 0.05), despite the temporal and geographic difference in the source samples. Moreover, comparing all four *D. grimshawi* populations via a one-way Analysis of Variance, no significant differences were observed in either thorax length (ANOVA, $F_{3,75}$ = 0.56, P = 0.64) or in number of ovarioles per ovary (ANOVA, $F_{3,154}$ = 2.02, P = 0.11). Thus, based on these pilot data, there is no significant genetic differentiation in these two traits among the East Maui, West Maui, and Molokai populations of *D. grimshawi*.

With respect to the maximum number of mature eggs per ovariole, three, or three or four mature eggs per active ovariole, were commonly observed in lab flies from the Molokai and West Maui strains, respectively. The majority of females dissected from the East Maui strains were still young flies (≤ one month old), and therefore either had not yet completed maturation of any eggs, or had at most one mature egg per ovariole. Some of the older females examined in these samples had already oviposited their first batch of eggs and were in the early stages of developing their next batch of eggs, so again the potential maximum number of eggs per ovariole could not be assessed.

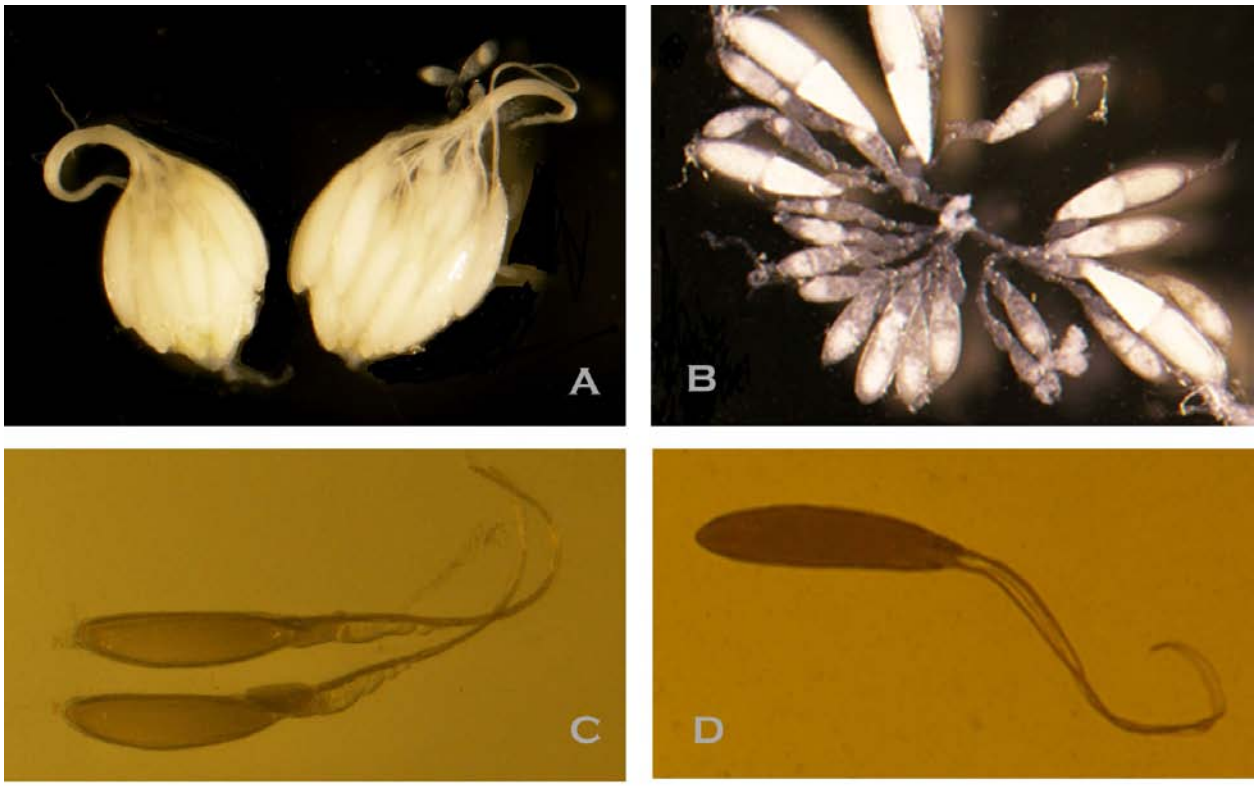

Figure 1. A. A pair of *D. grimshawi* ovaries dissected from a mature female, containing many mature eggs in the most posterior egg chambers. Note that the extremely long respiratory filaments extend to the anterior tip of the germarium and beyond (at top). B. An ovary of a young female teased apart to display the 22 ovarioles which have been left connected at the anterior germarium (center). The most advanced egg chambers are in mid vitellogenesis. C. Two dissected ovarioles each containing one mature egg. Note the younger egg chambers (to the right) lying alongside the long respiratory filaments. D. Mature egg dissected from an ovary showing the four long respiratory filaments at the anterior end of the egg.

Discussion

Based on examination of ovaries of lab-reared flies derived from four different populations of *D. grimshawi* from three islands in the Maui Nui complex, no consistent differentiation was found for a key female reproductive trait, the number of ovarioles per ovary. Combining data from the four geographic populations, the average for the species is 21.4 $\pm$ 0.22 ovarioles per ovary, or 42.8 $\pm$ 0.59 ovarioles per fly. As noted for *D. melanogaster*, ovariole number is a phenotypically plastic trait, subject to both genetic and environmental effects (Wayne *et al.*, 1997). The highest number scored in an individual *D. grimshawi* (from the West Maui isoline) was 55, with 29 ovarioles in one ovary and 26 in the other. The lowest number of ovarioles per fly was 31, with 16 ovarioles in one ovary and 15 in the other. While there is some variation in the number of ovarioles between the left and right ovaries of an individual, it is minimal, generally no more than two or three. It should be noted that the data presented here are for laboratory flies raised on protein-rich media. Kambysellis and Heed (1971) noted that ovariole numbers of lab-reared flies were greater than those of their field-collected parents, presumably because competition in nature for nutritional resources is more intense than in the lab. No ovariole numbers for field-collected *D. grimshawi* were presented in Kambysellis and Heed (1971), as this species is not as common as others in field collections. This is surprising, given that *D. grimshawi* is a generalist with a broad host range with respect to ovipositional resources. It might be assumed that larvae and adults of this species could feed on microbes from an equally broad array of plant resources, and would therefore be able to sustain larger populations than those of ecologically specialized species that are limited by the rarity of their host plants. On the other hand, as a generalist, *D. grimshawi* competes with many other sympatric *Drosophila* species using the same breeding resources, and this factor may limit population sizes in the field.

It should be noted that the value of 28 ovarioles/fly for *D. grimshawi* published in Markow and O'Grady (2007) is erroneous. No standard errors or sample sizes are provided, so the source of this value is unclear. In this species it is misleading to simply count the number of mature eggs observed in a female as an indicator of the number of ovarioles, since ovariole function in *D. grimshawi* is asynchronous.

Wayne *et al.* (1997) estimated the mean number of ovarioles per ovary for *D. melanogaster* at 15.1 from a sample of 1152 ovaries. Thus, ovariole numbers in *D. grimshawi* (21.4 per ovary) are higher than those in *D. melanogaster* (David, 1970; Wayne *et al*., 1997), but the difference is not as great as the dramatic difference in body size between these two species. It is important to note, however, that ovariole number and thorax length are not genetically correlated (Telonis-Scott *et al.,* 2005), although the two traits may be environmentally correlated. Given that *D. grimshawi* is the largest of the 12 *Drosophila* species for which full genome sequences are available, it is not surprising that its eggs are significantly larger than those of the other 11 species (Markow *et al.*, 2009). *D. grimshawi* eggs are twice as long as those of *D. melanogaster*, and twice the weight, and have an outer endochorion that is eight times as thick (Margaritis *et al.*, 1983). In addition, there are four rather than two respiratory appendages at the anterior end of eggs of *D. grimshawi* that are extraordinarily long. For further details of the ultrastructure of the *D. grimshawi* chorion, the reader is referred to the scanning electron micrographs presented in Margaritis *et al.* (1983), Kambysellis (1993), and Figure 1 of Piano *et al*. (1997), along with the accompanying descriptions.

Ovariole number is a fitness-related trait that shows genetic variation within species but is under relatively strong stabilizing selection (Wayne and Mackay, 1998). But this trait is just one component of the extremely high potential fecundity of *D. grimshawi* compared to *D. melanogaster.* In common with many other members of the *grimshawi* and *planitibia* species groups, the eggs and ovaries of *D. grimshawi* fit into reproductive type IIIb, one of the seven discrete reproductive types recognized among the Hawaiian species (Kambysellis and Craddock, 1997), and the one with the highest fecundity of all among the diverse array of reproductive strategies in the endemic Hawaiian *Drosophila*. Besides high ovariole numbers, this reproductive type is characterized by development of up to 4 mature eggs per ovariole. In fact, the maximum number observed in the sample of females from the eight lab stocks of *D. grimshawi* examined in this study was four mature eggs per ovariole. One other factor besides the ovarian structure and function of *D. grimshawi* is their exceptionally long reproductive lifetimes. Although no systematic attempts have been made to collect data on adult longevities, it is known that under optimal conditions many picture wing species can survive in the lab for over a year. Moreover, lab females can continue to mature and lay eggs. Carson *et al.* (1970) reported that

a field-captured inseminated female of *D. grimshawi* continued to produce fertile eggs for almost a year without additional insemination. This example emphasizes the abundance of sperm that can be stored and remain fertile for many months in the spermathecae and ventral receptacle of this species, as well as the enormous numbers of eggs produced per female over her long reproductive life. At any point in time, a mature female of *D. grimshawi* can potentially carry an egg load of 100 or so mature eggs in her ovaries (Craddock and Kambysellis, 1997). For comparative purposes, the egg load parameter (the number of ovarioles per fly times the number of mature eggs per ovariole) provides only a rough measure of potential female fecundity, given the asynchronous nature of ovariole function in these Hawaiian picture wing species and the lack of solid data on reproductive longevities. Of course, realized fecundity is typically less than potential fecundity. By all measures, however, the potential lifetime fecundity of *D. grimshawi* far exceeds that of nonHawaiian species and in particular, that of *D. melanogaster* and the other ten *Drosophila* species with complete genome sequences. The availability of these sequence data now provides the chance to address many important questions about the molecular basis of evolutionary differences in longevity, reproductive, developmental, and other traits within the genus *Drosophila.*

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Drosophila suzukii has been found in tropical Atlantic Rainforest in southeastern Brazil.

Bitner-Mathé, B.C.*, J. Victorino, and F.S. Faria. Departamento de Genética, Instituto de Biologia, Universidade Federal do Rio de Janeiro, 21941 902, Cidade Universitária, Rio de Janeiro, Brazil; *E-mail: bcbitner@biologia.ufrj.br.

Drosophila suzukii (Matsumura, 1931) belongs to the *Drosophila melanogaster* species group, probably native to the southeastern Palaearctic region (Bächli, 2013). Its ability to feed and breed in healthy fruits led it to become an agricultural pest. *D. suzukii* is an invasive species, being recorded in North America

(Hauser *et al.*, 2009; Hauser, 2011) and Europe (Calabria *et al.*, 2012). In the Neotropical Region, there is a report that P. O'Grady had collected *D. suzukii* in Costa Rica and Ecuador in the late 1990s (Calabria *et al.*, 2012). More recently, Deprá *et al.* (2014) recorded this new invader in areas of subtropical Atlantic Rainforest in states of Santa Catarina and Rio Grande do Sul, southern Brazil. Vilela and Mori (2014) found it in blueberries produced in São Joaquim, state of Santa Catarina, that were bought at a São Paulo city grocery store. These authors point out that despite being a cold-adapted species, after having arrived to the southeastern state of São Paulo, this invasive fly will probably expand its territory to other Brazilian states and South American countries.

The present report concerns the first finding of *Drosophila suzukii* in the tropical Atlantic Rainforest. Collections were performed between 12th and 20th of November 2014 in the *Parque Nacional da Serra dos Órgãos* (PARNASO), Petrópolis, state of Rio de Janeiro, southeastern Brazil. The collection localities have about 800 m altitude with tropical highland climates. During November 2014, the average temperature in this region was 18.9^{o}C, with minimum of 14.8^{o}C and maximum of 23.0^{o}C (Climate-data 2014). Our sampling effort consisted in ten transects (A-J) of about 300 m: A- 22° 30' 16.8''S, 43°07'09.7''W; B- 22° 30' 20.0''S, 43°06'47.5''W; C- 22° 30' 31.6''S, 43°06'23.8''W; D- 22° 29' 42.2''S, 43°07'27.4''W; E- 22° 29' 38.8''S, 43°07'04.6''W; F- 22° 29' 20.5''S, 43°07'27.8''W; G- 22° 29' 07.5''S, 43°07'15.0''W; H- 22° 27' 36.2''S, 43°05'37.0''W; I- 22° 27' 49.6''S, 43°05'18.2''W; J- 22° 27' 57.1''S, 43°04'55.6''W. Except for transects named H and I, the localities surveyed in this study are areas of Atlantic Rainforest in good state of conservation. For each transect, five banana-baited traps were placed spaced 50-60 m apart. In transect "J", in a total of 299 flies, two males and one female of *D. suzukii* were collected. Although we have made similar effort to collect flies in all localities, no other specimen of *D. suzukii* was obtained. Species identification was based on external morphology and on the terminalia of both sexes (Bock and Wheeler, 1972; Vilela and Mori, 2014). An isofemale line was obtained from the wild collected female. Then, wild flies were preserved in a solution of 6 ethanol: 4 water: 1 acid acetic: 1 glycerin for further DNA analysis.

Most previous reports indicate the presence of *D. suzukii* in temperate and subtropical regions. Here we register the presence of this species in a tropical region, showing its high potential of spread and reinforcing the importance of monitoring this species for the knowledge of its colonization process in the Neotropics.

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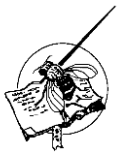

Oxidative stress and longevity: Evidence from a long-lived strain of *Drosophila melanogaster.*

Deepashree, S., S. Niveditha, T. Shivanandappa, and S.R. Ramesh*. Department of Studies in Zoology, University of Mysore, Manasagangotri, Mysuru – 570006, India; e-mail: rameshuom@gmail.com; *Corresponding author. Tel.: +91 821 2419779.

Oxidative stress is one of the inescapable outcomes of the cellular processes. Reactive oxygen species (ROS) is one such contributor to the oxidative stress. Oxidative stress is implicated in aging and

neurodegenerative disorders. Free radical theory of aging also supports the concept that ROS inflicts damage to the system (Harman, 1956). Although aging is a multifactorial process, oxidative stress remains an important factor. *Drosophila melanogaster* is often used as a model for understanding the role of oxidative stress in aging. Extensive selection experiments, transgenic variants, and antioxidant enzyme studies in *Drosophila* have been made to explore the relationship between oxidative stress and longevity (Arking, 2005).

Our study was aimed at understanding the relationship between longevity, oxidative stress, and antioxidant enzymes in *D. melanogaster* using ethanol as the inducer of oxidative stress. Similarities in behavior between flies and humans when exposed to alcohol are noteworthy. Although inebriation in humans is a complex process, understanding the simple behavioral and genetic aspects of intoxication in flies would help us to deduce the mechanisms and consequences. Innumerable studies have been carried out on consumption of alcohol, preference for alcohol, sensitivity to alcohol, sedation effects of alcohol, and effects of alcohol on locomotion (Moore *et al.*, 1998; Rothenfluh and Heberlein, 2002; Jahromi *et al.*, 2014).

Two strains of *D. melanogaster*, normal lifespan strain (NLS) that lives approximately for 60 days and long lifespan strain (LLS) that lives approximately for more than 100 days, were employed for the present study. These lines were isolated from *D. melanogaster* (Oregon K strain) flies that were obtained from the Drosophila Stock Center, University of Mysore, Manasagangotri, Mysore. These fly stocks were maintained in a vivarium at 22±1°C on standard wheat cream agar medium with 12:12 light and dark cycles. Synchronized eggs were collected (Delcour, 1969) and were raised under uniform conditions of temperature, humidity, food medium, and density. The flies obtained from these cultures were used for the experiments. In the current study, effects of ethanol-induced oxidative stress in NLS and LLS in relation to longevity were investigated.

Both NLS and LLS flies were exposed to different concentrations of ethanol for 24 h using 5% sucrose as a medium (Montooth *et al.*, 2006). Assays were performed separately for males and females of both NLS and LLS. Using dose-response curve, the LC_{50} was calculated. Oxidative stress resistance assay was performed to decipher the sensitivity and resistance between the two strains. The LC_{50} dose of ethanol was used for the assays to induce stress in NLS and LLS flies.

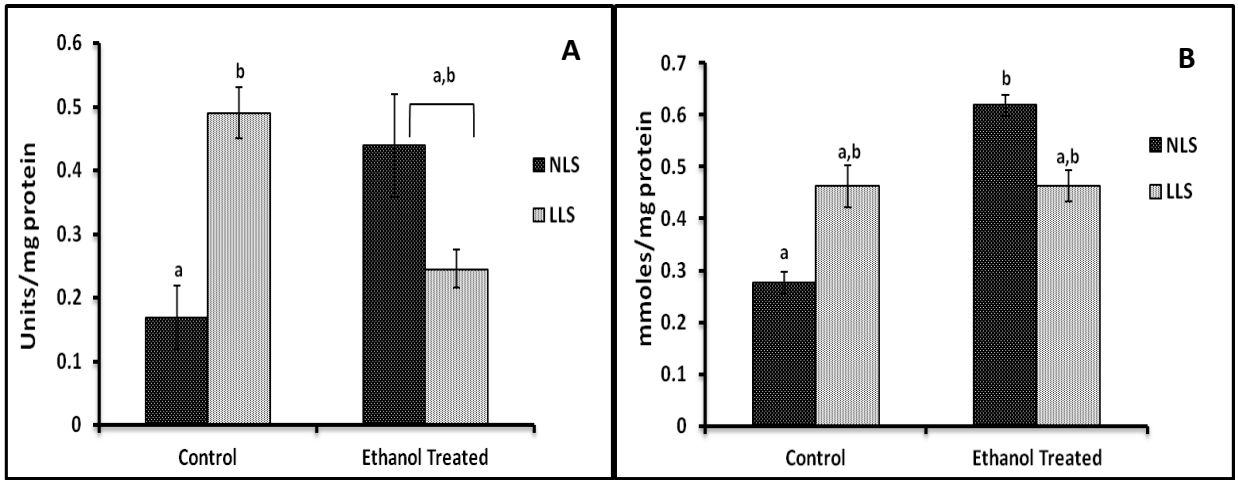

Figure 1. Enzyme activities in control and ethanol treated normal lifespan strain (NLS) and long lifespan strain (LLS) *D. melanogaster* males. Each value represents the Mean±SE. Bars with different alphabets above them differ significantly at $P < 0.05$. A, Superoxide dismutase activity; B, Catalase activity.

Negative geotaxis assay was carried out to evaluate the effect on the locomotory behavior (Feany and Bender, 2000). Altered locomotion of flies exposed to ethanol was similar to humans (Moore *et al.*, 1998). For superoxide dismutase assay, pyrogallol auto-oxidation method (Marklund and Marklund, 1974) and for catalase assay the procedure of Aebi (1984) was followed. Glutathione in the deproteinized supernatant was

estimated by Ellman's reagent with a standard curve (Ellman, 1959). Acetylcholinesterase activity was determined by following the Ellman method (Ellman *et al.*, 1961). Alcohol dehydrogenase activity was measured following the method of Vallee and Hoch (1955). Induction of ROS was measured using 2',7'-dichlorofluorescein diacetate (Driver *et al.*, 2000).

Our results are consistent with those of Arking *et al.* (2000) and Arking (2005), who have reported positive correlation between oxidative stress resistance and antioxidant enzyme activities in long-living strains of *Drosophila*. However, we have noted higher alcohol-induced tolerance in LLS which positively correlates with antioxidant enzyme activities when compared with NLS of *D. melanogaster.* Figures 1A and B show superoxide dismutase and catalase activities in control and ethanol-treated NLS and LLS male flies, respectively. LLS flies showed higher antioxidant enzyme activities when compared to NLS flies.

Our study demonstrates that LLS flies have higher resistance to ethanol-induced oxidative stress when compared with NLS. Similarly, LLS flies show higher resistance to ethanol-induced locomotory behavior than NLS flies. In addition, the present study also revealed that aging affects the resistance to ethanol-induced oxidative stress in both NLS and LLS flies. This is the first report showing the relationship between ethanol-induced oxidative stress and longevity.

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Decalepis hamiltonii root extract protects against Gamma radiation toxicity in *Drosophila melanogaster.*

Pasha, Muzeer, Ganesh Sanjeev[a], T. Shivanandappa, and S.R. Ramesh[*]. Department of Studies in Zoology, University of Mysore, Manasagangotri, Mysuru 570006, Karnataka, India; [a]Department of Studies in Physics, Microtron Centre, Mangalore University, Mangalagangotri, Konaje 574199, Karnataka, India; E-mail: rameshuom@gmail.com; *Corresponding author. Tel.: +91 821 2419779.

Radiation therapy is widely used as theraputic option for cancer treatement (Mackillop *et al.*, 1997). Despite its therapeutic benefit, radiation is toxic and induces oxidative stress through generation of free radicals (Katz *et al*., 1996; Kaur *et al.*, 2000).

The fruit fly, *Drosophila melanogaster* is widely used as an experimental model in biological research as it shares many genes that are orthologous to humans (Mahtab *et al.*, 2007). Further, the age-related functional decline in flies is widely similar in other animals including humans (Grotewiel *et al.*, 2005). *Drosophila* is often used as a model organism in aging research.

For various therapies, the herbal preparations are often preferred as an alternative to the synthetic drugs in view of their safety. Phytochemicals, with free radical scavenging, antioxidant properties, and immune stimulatory effects have been evaluated for their radioprotective effects. Preclinical studies in the past

two decades have shown that many medicinal plants and their phytochemicals possess radioprotective potential (Ahlersova *et al.*, 1998).

The tuberous roots of *Decalepis hamiltonii* (Wight and Arn.) (Family: Asclepiadaceae) are consumed in southern India as pickles and juice in view of their health promoting properties. The roots are also used in folk medicine and ayurvedic preparations as a general vitalizer and blood purifier (Nayar *et al.*,1978). The root extract of *D. hamiltonii* (*Dh*) is a potent cocktail of novel antioxidants and have hepatoprotective and neuroprotective potential (Srivastava *et al.*, 2006; Srivastava and Shivanandappa, 2006, 2010a, b).

Present study was undertaken to determine the possible radioprotective role of *Dh* root extract against gamma radiation toxicity in *D. melanogaster* (Oregon K).

The fly stock was obtained from the Drosophila Stock Center, Department of Studies in Zoology, University of Mysore, Mysore. Experimental stocks comprising 5 day old flies were built up by the serial transfer method, and these flies were maintained on standard wheat cream agar medium at 22 ± 1°C and 70–80% relative humidity in a vivarium.

The adults were fed with a diet containing 0.5%, 1% *Dh* and the control flies were fed with diet without *Dh* root extract. By confining the flies in polypropylene tubes (65 × 25 mm), they were irradiated with gamma rays at 100 Gy, 200 Gy, 400 Gy, 600 Gy, 800 Gy, 1000 Gy, 1200 Gy, and 1400 Gy (Cobalt-60 Gamma radiation, Gamma chamber 5000) 3 times with a gap of 3 hr at a source strength of 14,000 Ci (Curie) that delivers about 9 kGy/hr (kilo Grey per hour).

After exposure to the radiation, the flies were transferred to fresh media bottles and the number of dead flies in each dose was recorded at 24 hr. Based on dose-response data, the median lethal dose (LD_{50}), that causes 50% mortality in 24 hr, was determined. The LD_{50} was calculated by using probit analysis.

From Figure 1 it is evident that exposure of *D. melanogaster* to different doses of gamma radiation produced dose-dependant mortality. At 100 Gy there was no mortality beyond which mortality increased in a dose dependant manner. The LD_{50} for control, 0.5% *Dh*, and 1% *Dh* treated group was found to be 800 Gy, 848 Gy, and 1010 Gy, respectively.

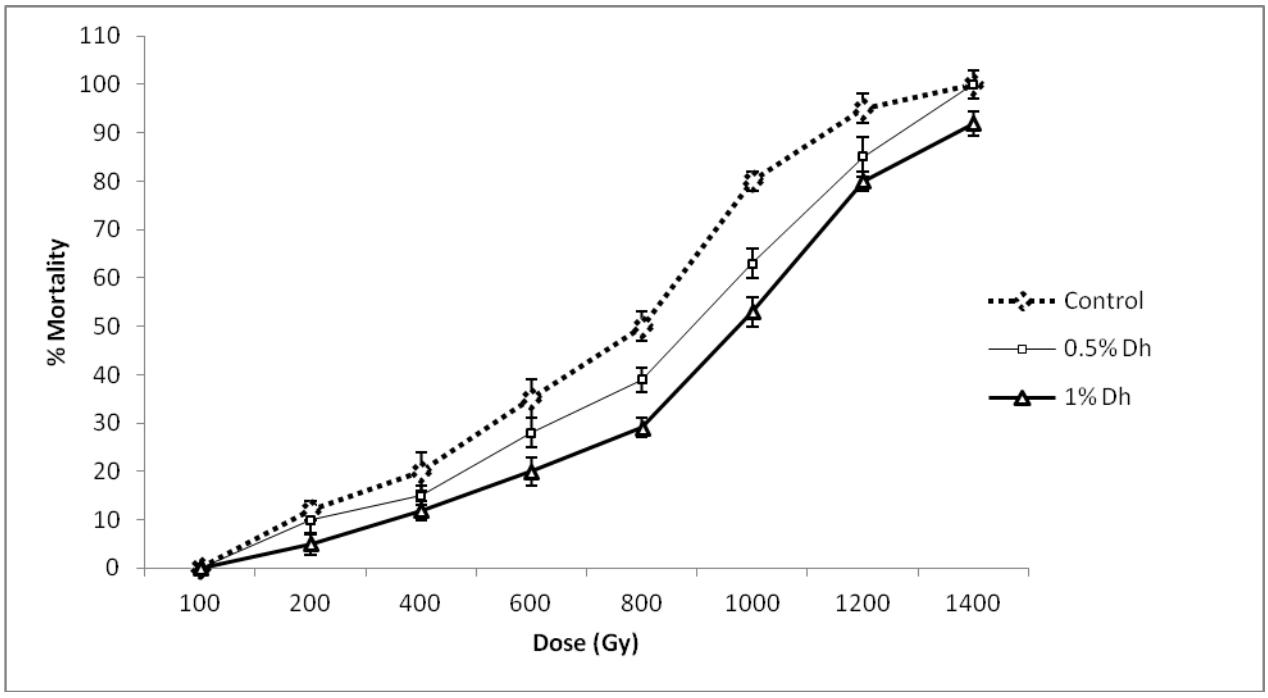

Figure 1. Gamma Radiation induced mortality in *D. melanogaster* in control, 0.5% *Dh*, and 1% *Dh* fed flies at different doses. Values given are Mean ± S.E (Each set contained 25 flies × 6 replicates).

Our results clearly show that *Dh* pretreatment protected *Drosophila* from gamma radiation induced lethality (Figure 1) and increased survivability of *Dh* fed *Drosophila* compared to control group. Therefore, *Dh* pretreated flies exhibit more radiation tolerance/resistance than the control flies. Our study demonstrates the radio protective potential of the edible roots of *Dh*, which has implications in cancer radiation therapy.

Acknowledgments: We thank the Board of Research in Nuclear Science (BRNS), Department of Atomic Energy (DAE), Govt. of India, for financial support (Project No.2011/34/18/BRNS/0587).

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A novel *GMR-Gal4* insertion produces a rough eye phenotype.

Sheaves, Danielle W., and Brian E. Staveley. Department of Biology, Memorial University of Newfoundland, St. John's, Newfoundland & Labrador, Canada, A1B 3X9; telephone (709) 864-4317; telefax (709) 864-3018; Corresponding author: Dr. Brian E. Staveley; e-mail address: bestave@mun.ca.

Introduction

Much of modern research in *Drosophila* depends upon the use of the *UAS/Gal4* system (Brand and Perrimon, 1993) to express various transgenes under defined conditions. For the most part, it has been widely believed that the *Gal4* transgenes produce few negative effects in *Drosophila melanogaster*. However, *GMR-Gal4*^{12} was shown to produce an apoptosis-dependent "rough eye" phenotype (Kramer and Staveley, 2003) and the neural accumulation of the protein product of *Gal4* has been linked to neurodegeneration in *Drosophila* (Rezaval *et al.*, 2007). Apart from transcriptional interactions with the *UAS*-bearing transgenes, highly elevated levels of *Gal4* expression have been shown to lead to stress and immune responses (Liu and Lehmann, 2008). Due to the prominence of *Gal4* in *Drosophila* research, we believe that this phenomenon should be further examined.

GMR-Gal4^{12}, very commonly referred to as simply *GMR-Gal4*, was originally selected from a group of fifteen *GMR-Gal4* transgenic insertion lines (Freeman, 1996). Only two of these lines, including *GMR-Gal4*^{12}, did not display a hemizygous roughened eye phenotype at 25°C. Our group has shown, in *GMR-Gal4*^{12} homozygotes cultured at 25°C and *GMR-Gal4*^{12} hemizygotes raised at 29°C, that an apoptosis-dependent altered developmental process can produce a "rough eye" phenotype (Kramer and Staveley, 2003). To further investigate this phenomenon, we have produced a version of *GMR-Gal4* that we believe may be similar to the other original "rough eye" insertions to help evaluate the consequences of *Gal4* expression.

Materials and Methods

Drosophila media

Our standard cornmeal-yeast-molasses-agar medium is prepared with 65 g/L cornmeal, 10 g/L nutritional yeast, and 5.5 g/L agar in water, cooked by autoclave for 30 minutes (plus depressurization) then

augmented with 50 ml/L fancy grade molasses. To inhibit mold growth, 5 mL of 0.1 g/mL methyl 4-hydroxybenzoate in 95% ethanol and 2.5 mL of propionic acid are added per litre when cooled to 55 to 60°C before being poured into standard plastic shell vials and stored at 4 to 6°C.

Drosophila stocks and culture

The *GMR-Gal4*^{12} (Freeman, 1996) and *UAS-lacZ*$^{Bg4-1-2}$ (Brand and Perrimon, 1993) lines were obtained from the Bloomington Drosophila Stock Center at Indiana University. The *w; Sb PΔ2-3 e/TM6 Ubx e* line (Robertson *et al.*, 1988) was obtained from Dr. William Engels. The *w*^{1118} was obtained from Dr. H. Lipshitz.

P element transposition

The novel insertion of *GMR-Gal4* was generated by crossing *w; Sb PΔ2-3 e/TM6 Ubx e* males to *GMR-Gal4*^{12} females to produce dysgenic males which were, in turn, crossed to *w*^{1118} females at 25°C. Non-dysgenic male progeny were selected against the presence of *Sb PΔ2-3* and crossed to *w*^{1118} females. The F1 were mated and F2 were screened for the "rough eye" phenotype at 25°C. One line was isolated: *GMR-Gal4*^{H1}.

Drosophila crosses and biometric analysis

To generate critical class males, *GMR-Gal4*^{12} or *GMR-Gal4*^{H1} females were mated to either *w*^{1118} males, to produce "responder-less" progeny or *UAS-lacZ* to produce "benign responder" progeny at 25°C. These were collected, aged for three to five days at 25°C, frozen at -80°C, mounted on aluminum studs and desiccated for 24 hours or more before micrography. Scanning electron micrographs were produced by a FEI Quanta 400 Environmental SEM and analyzed using NIH ImageJ software (Abramoff *et al.*, 2004). Images were analysed from 10 individuals (n = 10) and the number of ommatidia and bristles were determined and standard error of the mean calculated.

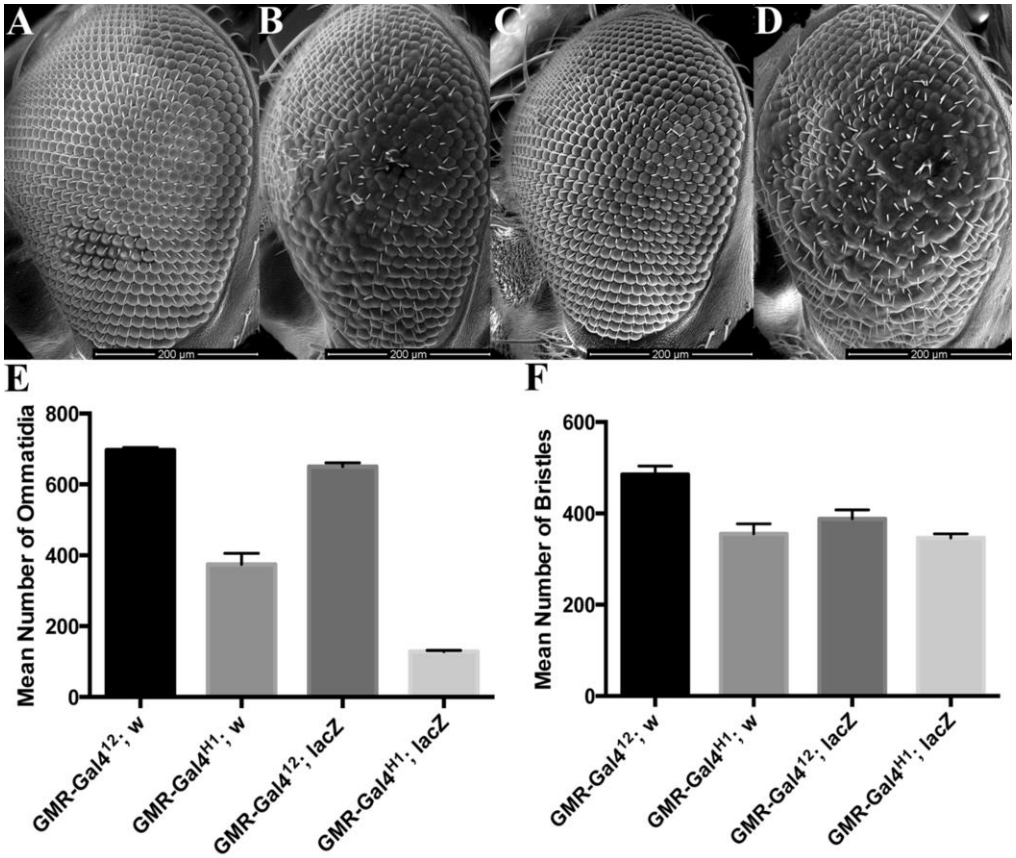

Figure 1: *GMR-Gal4*^{H1} shows a rough eye phenotype with and without *UAS-lacZ*. Scanning electron micrographs of the eyes of (A) *GMR-Gal4*^{12}/+, (B) *GMR-Gal4*^{H1}/+, (C) *GMR-Gal4*^{12}*/UAS-lacZ*, (D) *GMR-Gal4*^{H1}*/UAS-lacZ* males at 25°C. *GMR-Gal4*^{H1} shows a reduced number of (E) ommatidia and (F) interommatidial bristles when compared to the *GMR-Gal4*^{12}. Error bars indicated standard error ($p < 0.05$) and n = 10 for all four classes.

Results and Discussion

By P element transposition, a novel insertion line, *GMR-Gal4^{H1}*, has been generated. This line displays a rough eye phenotype at 25°C as a hemizygote with and without the presence of a *UAS*-controlled responder transgene (Figure 1).

Without a responding transgene, the eyes of *GMR-Gal4^{12}*/+ appear to be relatively normal, with a regular hexagonal array of ommatidia and interommatidial bristles (Figure 1A). With many fused ommatidia and reduced bristles, *GMR-Gal4^{HI}*/+ produces a "roughened" eye (Figure 1B). In the presence of the *UAS-lacZ* transgene, the eyes of *GMR-Gal4^{12}/UAS-lacZ* appear to be fairly normal (Figure 1C), while the eyes of *GMR-Gal4^{HI}/UAS-lacZ* are more severely compromised than in the absence of a responder (Figure 1D). With such an obvious developmental defect, the *GMR-Gal4^{H1}* line provides the opportunity to both suppress and enhance a phenotype that can be readily analysed through biometric means. Often utilised as control for the expression of genes of interest under any of a number of circumstances, the *UAS-lacZ* gene is usually considered to be benign in the developing eye under the control the *Gal4* transgenic drivers. Clearly, this does not seem to hold true under these conditions. Overall, the new insertion line *GMR-Gal4^{H1}* can produce striking phenotypes that seem ideal for further investigation of the toxic effects of *Gal4* expression.

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Relationship between gender difference in longevity and oxidative stress response in *Drosophila melanogaster.*

Niveditha, S., S. Deepashree, S.R. Ramesh, and T. Shivanandappa*. Department of Studies in Zoology, University of Mysore, Manasagangotri, Mysore – 570006, Karnataka, India; *Corresponding author. Tel. +91 8212511885; E-mail: tshivanandappa@yahoo.com.

Females have longer life expectancies than males across many species including humans, laboratory rats, and *Drosophila*. It is not clearly understood as to what genetic or environmental factors are responsible for the gender difference in longevity. According to free radical theory of aging, lifespan appears to be limited by the cumulative effects of oxidative damage from reactive oxygen species (Harman, 1959). However, there is evidence implicating the role of free radical-induced oxidative stress in aging. Previous studies in mammalian systems suggest that shorter lived males express lower levels of antioxidant defenses such as superoxide dismutase, catalase, and consequently suffers higher levels of oxidative stress than females (Ede *et al*., 2002; Tomas-Zapico *et al*., 2006).

Drosophila melanogaster offers a good model system to study the sex difference in longevity since females live significantly longer than males. Ethanol is known to induce free radical-mediated oxidative stress, and sex difference in acute ethanol responses have been reported in *Drosophila* (Das and Vasudevan, 2007; Devineni and Heberlein, 2012). However, differential susceptibility of the sexes to ethanol-induced oxidative stress in relation to their antioxidant status has not been studied in *Drosophila*. Therefore, we have investigated the differential susceptibility to ethanol induced oxidative stress in male and female *D. melanogaster* in relation to the antioxidant enzymes, superoxide dismutase, catalase.

D. melanogaster (Oregon K) flies were obtained from the *Drosophila* Stock Centre, University of Mysore, Karnataka, India. For lifespan studies, newly-eclosed male and female flies were housed separately

in vials supplemented with standard wheat cream-agar medium. Flies were transferred to vials with fresh diet once every 5 days and mortality was recorded at different time intervals till the end of the experiment. The vials containing male and female flies were subjected to oxidative stress using exposure to ethanol. For mortality studies, a group of 10 flies each were transferred to the parafilm sealed vials containing filter paper soaked with 5% sucrose solution containing various concentrations of ethanol (8%, 10%, and 12%), whereas the control group received only 5% sucrose solution (Montooth *et al.*, 2006). Flies were maintained at 22°C throughout the experiments. The number of dead flies was recorded for 24 hours and expressed as percentage mortality. The whole body homogenate of the flies was prepared in respective assay buffer. Reactive oxygen species, glutathione, activity of the antioxidant enzymes, acetylcholinesterase, and alcohol dehydrogenase were determined using standard protocols (Vallee and Hoch, 1955; Ellman, 1959; Ellman *et* al., 1961; Marklund and Marklund, 1974; Aebi, 1983; Cathcart *et al*., 1983).

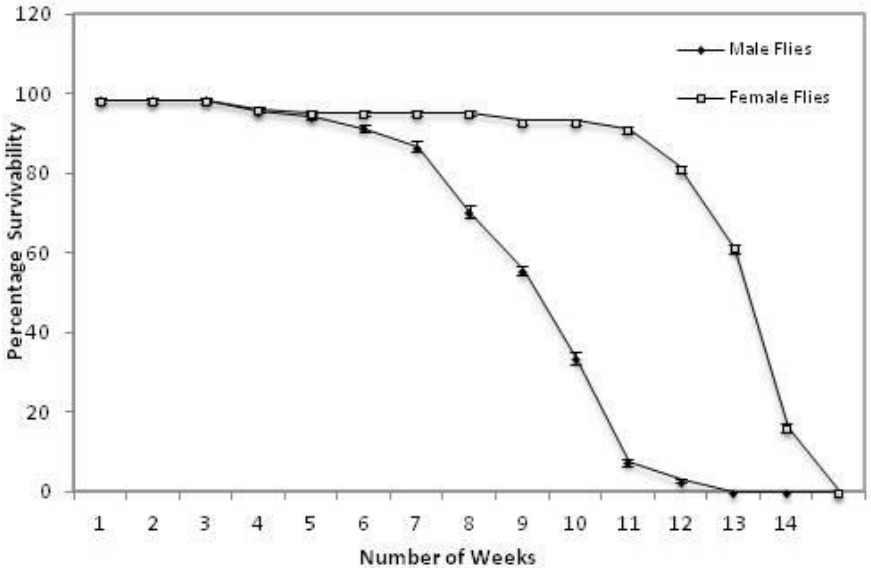

Figure 1. Mean lifespan of male and female *D. melanogaster*.

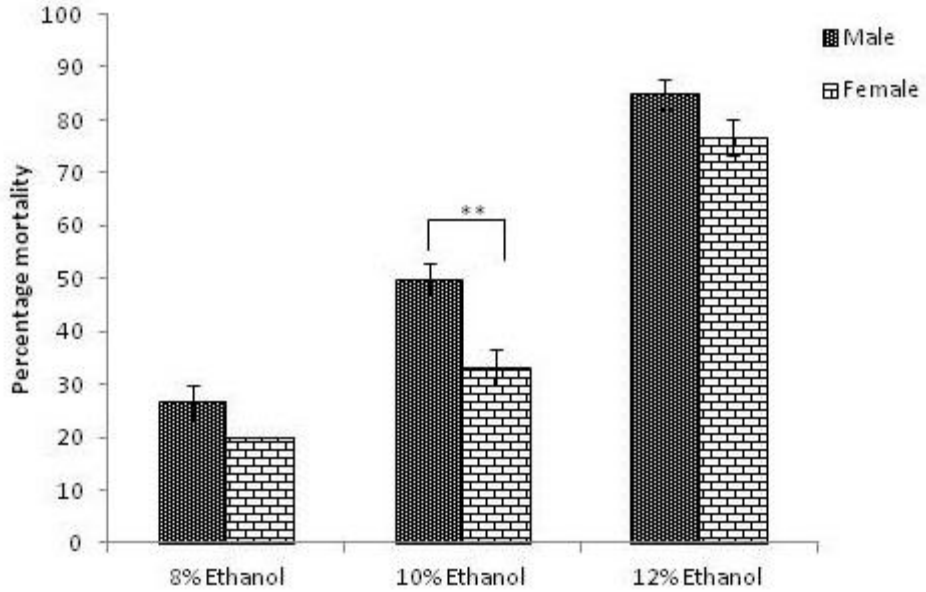

Figure 2. Gender difference in mortality of *D. melanogaster* exposed to ethanol. Values are expressed as Mean ± S.E. Data were analyzed by t-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Figure 1 shows the marked difference in the survivability of male and female *D. melanogaster*. There was a significant difference in the susceptibility of flies to ethanol-induced toxicity and oxidative stress

between the sexes. Females, the longer lived sex, showed greater resistance to ethanol-induced mortality when compared with that of males (Figure 2). Ethanol-induced differential oxidative stress correlates with that of antioxidant status in the sexes (detailed results will be published elsewhere). Our results strongly point out the marked sex difference in ethanol-induced mortality which positively correlates with the antioxidant defense mechanisms in the sexes. Our study presents evidence for the possible role of oxidative stress in the gender difference in longevity of *D. melanogaster*.

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Age based male mate preference in *Phorticella straita.*

Krishna, M.S. Drosophila Stock Center, Department of Studies in Zoology, University of Mysore, Manasagangotri, Mysore 570006, Karnataka State, India; Email: drosokrish@gmail.com.

Introduction

Studies of sexual selection have largely concentrated on females, as they are often the selective sex that chooses from among males (Milinski, 2001). Although female-biased empirical evidence were more in the literature, males are also likely to demonstrate mate choice under some conditions (Dewsbury, 1982). Therefore, male mate choice is also expected in systems where males allocate valuable resources to their partner, in response to variations in female quality, or where the costs of mate searching and/or assessment are low (Bounduriansky, 2001).

It was found that there are now growing numbers of observations of male choice seen in a wide range of taxa, and it has been reported in 58 insect species distributed among 11 orders and 37 families (Bonduriansky, 2001; Byrne and Rice, 2006), birds (Jones and Hunter, 1993), and fish (Amundsen and Forsgren, 2001). Studies of male mate choice in these organisms is predicted to be adaptive when variance in female fitness is large and males experience costs of mating such that they cannot inseminate all females encountered (Burley, 1977; Parker, 1983; Owens and Thompson, 1994; Johnstone *et al.*, 1996; Kokko and Monaghan, 2001). However, the empirical evidence is limited.

It was also suggested that unlike females, males also use characters in females such as virginity, body size, age, and gravid status (Bonduriansky, 2001; Prathibha and Krishna, 2010; Somashekar and Krishna, 2011). However, the most obvious character influencing the reproductive value of a female is her fecundity (Bonduriansky, 2001). If mating opportunities are constrained, of males then he shows a preference for more-fecund females to obtain direct benefit by increasing the number of offspring they produce (Katvala and Kaitala, 2001).

The most-compelling studies of male choice suggest that female mating success is often associated with traits that are correlated with female fecundity (Bonduriansky, 2001; Byrne and Rice, 2006), while in

others mate assessment may occur through display traits, such as coloration, pheromones, or ornamental and morphological features (Amundsen, 2000; Chenoweth and Blows, 2003; Lebas *et al*., 2003). However, models of the evolution of male choice suggest that male choice tends to break down when males target arbitrary female traits rather than those that reliably signal fecundity (Kokko and Johnstone, 2002; Chenowethet *et al.,* 2006; Servedio and Lande, 2006). Therefore, male preference for female traits may be an indirect way of assessing female fecundity. Thus, there is a need to understand how male choice is related to such traits. Therefore the present study has been undertaken in *P. straiata* to understand male choice for female fecundity.

P. straiata (Nirmala and Krishnamurthy, 1975) is a Drosophillid insect discovered from Karnataka, India, belonging to group Drosophillidae. This species also has all characteristics of a good laboratory tool to analyze genetic and evolutionary problems as that of the genus *Drosophila* (Sarath Chandra and Hegde, 2003). In this species the size related mating and reproductive success has been studied by Sarath Chandra and Hegde (2003). They found that larger flies had greater reproductive success than small flies. But for this maiden lone attempt no work has been done on behavioral genetics, cytogenetics, and evolutionary genetics of this species. Also, no work so far has been done to study female age effect on reproductive performance. Therefore, the present investigation has been undertaken in *P. straiata* to test whether males of this species are able to discriminate their mates on the basis of female age classes to obtain fecundity benefit or not.

Materials and Methods

Establishment of experimental stock

The experimental stock of *P. straiata* was obtained from the progenies of 50 isofemale lines collected from Chamundi hills, Mysore, India. In each generation 20 males and 20 females were transferred to the *Drosophila* culture bottle containing wheat cream agar media. These bottles were maintained at 22±1^{o}C and at a relative humidity of 70%. From these bottles, the virgin females and males were isolated within 3 hr of eclosion and were aged as required in the present experiment.

Selection of female age class

The sexually mature *Drosophila* females will perform certain of the behaviors, such as decamping, wings flicking, leg kicking, and ovipositor extrusion on unwanted advances of courting males (Spieth, 1952). The newly emerged females will not show these rejection behaviors (Manning, 1961). Further, the immature females are characterized by unhardened cuticles, folded wings, and slow movements.

Before assigning the age classes, the reproductive activities of females were studied. The observations showed that females were unreceptive on the day of eclosion and show no courting behavior toward males. From 2-32 days, the females were receptive and showed rejection responses, such as decamping, ignoring, wings flicking, and leg kicking. After 32 days, these behaviors began to decline. Therefore 2-32 day old females were considered in the present experiment. Because the females take 15-16 days to lay eggs and remate, three age classes were created, each separated by 15 days: young aged (2-3 days); middle aged (17-18 days); old aged (32-33days). Flies of these three age classes were collected and separately maintained under uniform environmental conditions. Additionally, 5-6 day old unmated males were maintained individually in the same laboratory conditions.

Before beginning the experiment, developmental times of flies collected at different times were also tested, and the results indicated no significant variations. These females of different age classes were kept in groups of 4 flies each in culture vials containing wheat cream agar medium and were transferred to a new vial containing wheat cream agar medium once a week until they were used in the experiment. Male flies were aged for 5-6 days. These flies were also maintained using the above-described laboratory conditions until they were used in the experiment.

Age based male mate preferences

To study male mate preference for female age, 2 virgin females (younger *vs*. middle-aged, younger *vs*. older, or middle-aged *vs*. older) and a 5-6 day-old male were aspirated into an Elens-Wattiaux mating chamber

(1964). Indian ink was painted on the thorax of one of the females. The effect of paint was tested before commencing the experiment by painting young flies in one trial and middle-aged/older females in an alternate trial and allowing them to mate. In 28 of 50 trials, middle aged females mated, and in the remaining 22 of 50 trials older females mated ($\chi 2 = 0.72$; *d.f.* = 1; $p > 0.05$). Results showed insignificant differences suggesting that painting had no influence on the performance of the flies. Each pair was observed for 1 h. When mating occurred, the copulating pair was aspirated out of the mating chamber and placed in a new vial containing wheat cream agar medium. In total, 50 trials were conducted for each combination of female ages. A Chi-square analysis was carried out to examine male mate choice data.

Influence of female age on ovariole number and wing length

Virgin young, middle-aged, and old females were individually sacrificed to count the number of ovarioles and determine the female wing length following the procedures of Krishna and Hegde (1997). To count the number of ovarioles, each female was dissected in a drop of physiological saline using a binocular stereomicroscope; ovarioles of the left ovary were separated from one another with the help of fine needles. The number of ovarioles in each female was counted. From the same female, wing length was also measured using a 100× [microscope] following the procedures of Hegde and Krishna (1997). The mean ovariole number and female wing length data were also subjected to one-way ANOVA followed by Tukey's honest *post-hoc* test. In total, 50 trials were separately conducted for each of the 3 female age classes.

Table 1. Age based male mate preference in *P. straita.*

Male (5-6 days)	Females (N = 50)			Females (N = 50)			Females (N = 50)		
	Young	Old	χ^2 value	Young	Middle aged	χ^2 value	Middle aged	Old	χ^2 value
Number	30	20	2*	15	35	8**	34	16	6.48**
%	60	40		30	70		68	32	

*P < 0.05 level; **P < 0.01 level;

Table 2. One way ANOVA on ovarioles number, fecundity and female wing length in *P. straita.*

Parameter	Source	Type III Sum of Squares	df	Mean Square	F value
Ovarioles (in no)	Age	4449.293333	2	2224.647	176.66**
	Error	1851.08	147	12.59238	
	Total	137606	150		
Fecundity (in no)	Age	49396.41333	2	24698.21	590.33**
	Error	6150.18	147	41.83796	
	Total	903953	150		
Female wing length (in cm)	Age	0.005889333	2	0.002945	2.57^{NS}
	Error	0.168018	147	0.001143	
	Total	903953	150		

**P < 0.05 level; NS- Non significant.

Results

Males of *P. straiata* generally chose to mate with middle aged females more frequently than younger or older females (Table 1). In crosses involving young and middle aged females, in 32 of 50 trials, males mated with middle aged females, whereas in crosses involving older and middle aged females also in 34 of 50 trials males preferred to mate with middle aged females. However, younger female success occurred in 30 of 50 trials in crosses involving young and old females (Table.1).

The mean fecundity of young, middle aged, and old females of *P. straita* is given in Figure 1. It was noted that middle-aged females had greater mean fecundity than young or old females. One-way ANOVA followed by Tukey's honest *post hoc* test showed significant differences in mean fecundity among females of different age classes (Table 2). Middle aged females had significantly greater fecundity compared to younger and older females by Tukey's *post hoc* test .

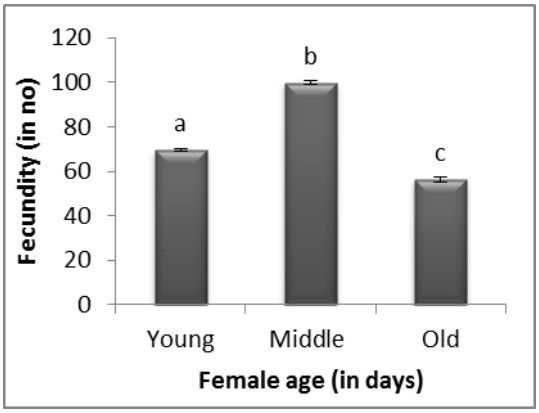

Figure 1. Age effect of female on fecundity in *P. straita.* (Different letters on the bar graph indicates significance at 0.05 level by Tukey's *post hoc* test).

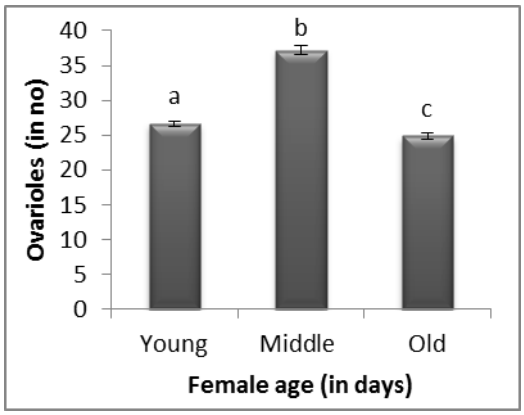

Figure 2. Age effect of female on ovariole number in *P. straita.* (Different letters on the bar graph indicates significance at 0.05 level by Tukey's *post hoc* test).

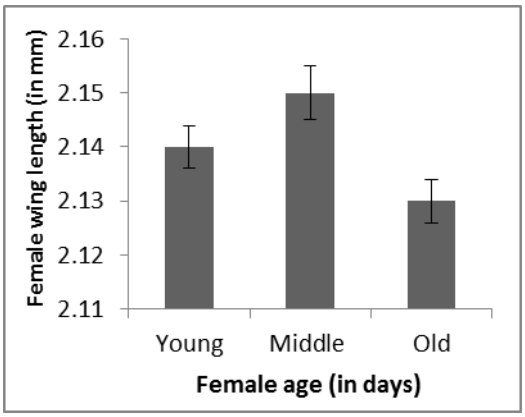

Figure 3. Age effect of female on wing length in *P. straita.* (Different letters on the bar graph indicates significance at 0.05 level by Tukey's *post hoc* test).

Figure 2 shows the mean number of ovarioles of young, middle-aged, and older females of *P. straita*. It was found that highest mean number of ovarioles was found in middle-aged females and was greater than young or old females. The mean number of ovarioles data were subjected to one-way ANOVA followed by Tukey's *post-hoc* test and showed significant variations in the mean numbers of ovarioles among females of different age classes (Table 2). Middle-aged females had a significantly greater mean number of ovarioles compared to young and old females by Tukey's *post hoc* test.

The mean female wing lengths of young, middle-aged, and old of *P. straita* are given in Figure 3. It was noted that differences in mean female wing lengths of different age classes were insignificant (Table 2).

Discussion

Restrictions in mating opportunities in males of *P. straita* may arise for various reasons, because, like males of *Drosophila* species, males of *P. straita* do not show parental care, and males only contribute sperm and components of the ejaculate to the courted female (Sharath Chandra and Hegde, 2003). In *Phorticella straita*, the high cost of reproduction is due to costs arising from such factors as energetically expensive courtship displays, the production of ejaculates, and time lost during different courtship display. It is assumed that in *P. straita* males exercise mate choice because 1) reproductive success of males of *P. straita* is limited by more factors than simply the number of females mates, and 2) females also differ in quality, *i.e*., fecundity. These two criteria need to be in place for male *P. straita* mate selectivity to be evolutionarily advantageous. Table 1 reveals that males of *P. straita* prefer to mate with middle-aged females more frequently than young and old females, suggesting that males of *P. straita* exercise mate choice on the basis of female age. Thus, it appears that female age is an important determinant of male mate choice in *P. straita*. In other words, males of *P. straita* do not show the same levels of interest in females of different age classes they encounter. This confirms earlier studies of the existence of male mate choice for female age in other insects. Our results in *P. straita* also confirms work of Somashekar and Krishna (2011) and Prathibha and Krishna (2010), who, while working in *D. bipectinata* and *D. ananassae*, have also found that females of these species prefer to mate with middle aged females more frequently over young or old females. Gowaty *et al*. (2003), who, while working on *D. melanogaster*, also pointed out that males do not show the same level of interest in all females they encounter and provided evidence that males have also evolved to selectively mate. Therefore, these studies in species of *Drosophila* suggest that male preference for female traits may be an indirect way of assessing female fitness.

Like female mate preference even with male mate choice, it is difficult to separate among male choice, female-female competition, and differences in female motivations to mate (Byrne and Rice, 2006). Therefore, it is hypothesized that in *P. straita*, middle-aged females are more eager to mate than young or old females.

One theory suggests that when males seek direct fecundity benefits, they should discriminate among potential mating partners on the basis of traits which are reliable indicators of fecundity (Fitzpatrick *et al.*, 1995; Servedio and Lande, 2006). On the other hand, models of the evolution of male choice suggest that male choice tends to break down when male target arbitrary female traits rather than those that reliably signal fecundity (Kokko and Johnstone, 2002; Chenoweth *et al.*, 2006). Figure 1 and Table 2 show that in *P. straita* middle-aged females had significantly greater fecundity compared to younger and older females, suggesting that females of the same species show variations in reproductive potential across age. This confirms earlier work suggesting that female age is also one of the traits known to influence female fecundity in species of *Drosophila* (Prathibha and Krishna, 2010; Somashekar and Krishna, 2011).

Since the number of ovarioles is positively correlated with fecundity, we sacrificed females of different age classes of *P. straita* to study variations in the number of ovarioles if any at different female ages. Figure 2 and Table2 show that in *P. straita* middle-aged females had a significantly greater number of ovarioles than young and old females, and this follows a pattern of fecundity across female age. This again confirms earlier studies of the influence of female age on reproductive success in other insects (Bonduriansky, 2001). From the results, it was also noted that in *P. straita*, middle-aged females had a significantly greater number of ovarioles and higher fecundity compared to young and old females. Therefore, it is advantageous for males of *P. straita* to mate with middle-aged females compared to young and old females. However, it is not known why older females of this species had fewer ovarioles than young and middle-aged females. We do not know whether they reabsorb them as the females age or not.

Studies in insects have also found a positive correlation between female size and the number of ovarioles (Branquart and Hemptinne, 2000). Even in *Drosophila*, studies of Robertson (1957) found a positive correlation between female size and the number of ovarioles. Therefore, in the present study in *P. straita* female flies which were sacrificed to study the ovariole number were also used to measure wing length to understand relationships among female age, wing length, and ovariole number. It was found that there was an insignificant difference in the mean female wing length among females of different age classes (Figure 3 and Table 2). This suggests that in *P. straita* female age has no influence on female body size, but has a significant influence on ovariole number. These studies suggest that females of *P. straita* differ in reproductive potential,

i.e., fecundity and ovariole number across different female age classes, but they did not significantly differ in body size across the different female age classes. Therefore, one would expect that males of *P. straita* might exercise some degree of mate choice because of more factors that put an upper limit on male reproductive success. For this behavior to be adaptive, these individuals are expected to benefit by enhanced survival or fecundity. Therefore, it is important that males carefully select females to increase their fitness. The benefits may come in the form of direct enhancement of survival and fecundity (Trivers, 1972). Thus, these studies suggest males of *P. straita* discriminate their mate on the basis of age to obtain direct benefits.

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Biodiversity of Drosophilidae in Biligiriranga Hills wildlife sanctuary.

Savin, P., N. Prashanth, and M.S. Krishna*. *Drosophila* Stock Center, Department of Studies in Zoology, University of Mysore, Manasagangotri, Mysore - 560006. Karnataka, India; drosokrish@gmail.com; *corresponding author.

Species of the genus *Drosophila* feed and breed on rotten fruits. They have been used as a model organism for over 100 years and thousands of scientists around the world work on it. This is because they are a highly tractable genetic model organism for understanding molecular mechanisms of human diseases. Many basic, biological, physiological, and neurological properties are conserved between mammals and *D. melanogaster*, and nearly 75% of human disease-causing genes are believed to have a functional homolog in the fly. However, taxonomic and population studies of these species have progressed little due to the lack of interest of people in this area. Therefore, very few studies have been undertaken to study *Drosophila* fauna from the field in India. Hence the present study has been undertaken in three places of Biligiriranga Hills wildlife sanctuary in Chamarajanagar District of south eastern Karnataka, India, to study *Drosophila* fauna.

In the present study the *Drosophila* fauna was collected from three different places in B,R Hills wildlife sanctuary, which is situated in Chamarajanagar District of south eastern Karnataka, South India, in November, 2014, using regular bottle trapping and banana bait methods. The study revealed a total of ten different species, namely *D. ananassae, D. bipectinata, D. kikkawai, D. malerkotliana, D. takahashii, D. neonasuta, D. varians, D. anomelani, D. sampangiensis*, and *D. nigra* being recorded. The frequency

distribution of the above species in the Western Ghats of south eastern Karnataka, at its border with Tamil Nadu (Erode District) district, is given in Table 1.

Table 1. *Drosophila* fauna of B.R Hills wildlife sanctuary in Chamarajanagar District of south eastern Karnataka, India.

S. No	Name of the species	Frequency distribution at B.R Hills wild life sanctuary							
		B.R Hills Forest		Near Temple (3/4) of the Hill		K. Gudi Forest		Total No. of Flies	
		(F)	(M)	(F)	(M)	(F)	(M)	(F)	(M)
1.	*D. ananassae*	70	78	30	40	80	98	180	216
2.	*D. bipectinata*	30	20	30	20	-	-	60	40
3.	*D. kikkawai*	40	32	18	13	-	-	58	45
4.	*D. malerkotliana*	65	67	38	40	-	-	103	107
5.	*D. takahashii*	30	45	15	25	60	78	105	148
6.	*D. neonasuta*	90	98	-	-	-	-	90	98
7.	*D. varians*	23	28	-	-	-	-	23	28
8.	*D. anomelani*	60	88	35	48	72	60	167	196
9.	*D. sampangiensis*	30	40	-	-	-	-	30	40
10.	*D. nigra*	30	45	-	-	-	-	30	45
	Grand Total	468	541	166	186	212	233	846	963

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Effect of nutritional regime on reproductive performance in *Phorticella straiata.*

Krishna, M.S. *Drosophila* Stock Center, Department of Studies in Zoology, University of Mysore, Manasagangotri, Mysore - 560006. Karnataka, India; drosokrish@gmail.com.

Introduction

It was widely suggested that nutrition is one of the environmental variations that can affect body condition and reproduction. This is because energy required to perform each and every process of life of an organism comes from nutrition; thus, the balance depends on the interplay between matter intake, digestion, and allocation of acquired energy to various functions such as maintenance, growth, and reproduction (Karasov, 1986; Sterner and Schulz, 1998; Taylor *et al.*, 2005). Experimental modifications of animal diets have played a key role in the study of how organisms adjust their energy allocation (Chown and Nicolson, 2004; Cruz-Neto and Bozinovic, 2004). Deficiency or imbalance of fat, carbohydrate, or protein can affect characters such as growth and reproduction. Protein deficiency reduces fecundity and growth in *Drosophila melanogaster* (Wang and Clark, 1995), and in fruit-feeders protein is often a limiting macronutrient (Mattson, 1980; Adams and Gerst, 1991; Hendrichs *et al*., 1991; Markow *et al*., 1999, 2001). When faced with nutritionally imbalanced diets, compensatory feeding for the limiting nutrients results in over ingestion of

other nutrients, as is often seen when insects are confined to food low in protein relative to carbohydrate (Raubenheimer and Simpson, 1999). This may result in increased lipid storage and reduced fitness (Simpson *et al.*, 2004; Warbrick-Smith *et al.*, 2006). Therefore, more studies are required in different species of the same genera to have general concept. Hence the present study has been undertaken in *Phorticella straiata* to study effect of carbohydrate and protein enriched diets on reproductive performance.

Materials and Methods

Establishment of experimental stock

The experimental stock of *Phorticella straiata* was obtained from the progenies of 50 isofemale lines collected from Chamundi Hills, Mysore, India. In each generation flies obtained from these culture bottles were mixed together and redistributed to 20 different culture bottles containing wheat cream agar media (100 g of jaggery, 100 g of wheat powder, 8 g of *Agar* was boiled in 1000 ml of double distilled water, and 7.5 ml of Propionic acid was added) each with 20 flies (10 males and 10 females). These flies were maintained at 22°±1°C with a relative humidity of 70% in a 12 hr dark: 12 hr light cycle. This procedure was carried out for three generations to acclimatize flies to lab condition. At the fourth generation, eggs were collected using Delcour's procedure (1969). Eggs (100) were seeded to each culture bottle containing carbohydrate, protein, and carbohydrate-protein enriched media. Carbohydrate enriched media (20%) was prepared by mixing sucrose and wheat cream media in 1:4 ratio. The protein enriched media (60%) was prepared by mixing casein and wheat cream agar media in 3:2 ratio. The carbohydrate and protein enriched media (30% carbo + 30% protein) was prepared by mixing sucrose:casein:wheat cream agar media in 2:2:1. When pupae were formed, females and males were isolated within three hours of their eclosion and aged for five days to test for virginity. These flies were used for present experiments.

Effect of diet alteration on larval feeding in P. straiata

Third instar larvae obtained from eggs collected (± 2 hours) from wheat-cream agar media grown flies using Delcour's procedure (1969) were used to study feeding behavior. Each larva was placed in a vial containing carbohydrate enriched / protein enriched / carbohydrate and protein enriched media and observed under a stereomicroscope. The back and forth movement of the proboscis was recorded for a minute. A total of 50 replicates were run separately for each of the altered diets.

Effect of diet alteration on reproductive performance in P. straiata

Four-day-old virgin female and unmated male from the carbohydrate enriched / protein enriched / carbohydrate and protein enriched media were aspirated into an Elens-Wattiaux chamber (Elens and Wattiaux, 1964). Each pair was observed for an hour and the pairs which did not mate within this time limit were discarded. Mating latency (time between introduction of a pair of male and female flies into the Elens-Wattiaux chamber until the initiation of copulation between each pair) and copulation duration (time between initiation to termination of copulation of each pair) were recorded. Mated flies were transferred once in 24 hr to new vials containing 5 ml of wheat cream agar media until death of females. Total number of eggs laid was also recorded. A total of 50 replicates were performed separately for flies grown on carbohydrate enriched / protein enriched / carbohydrate and protein enriched media.

Effect of diet alteration on ovariole number in P. straiata

Four-day-old virgin females were etherized and killed. The thorax of these flies was individually dissected out using a pair of fine dissection needles in physiological saline under a binocular stereomicroscope. The ovaries were separated and the total number of ovarioles in either the right or the left ovary was noted following the procedure of Hegde and Krishna (1997).

Results

Figure 1 provides the larval feeding rate in different diets. It was found that highest larval feeding rate occurred in flies grown in protein rich diet compared to carbohydrate rich and carbohydrate and protein rich

diets. One-way ANOVA followed by Tukey's *Post Hoc* test carried out using SPSS version 10.0 on the above data showed significant variation in feeding rate between different diets (Table 1). Tukey's *Post Hoc* test also showed that feeding rate was significantly greater in flies grown in protein enriched media than those flies grown in the other two altered diet media.

Table 1. One way ANOVA on feeding rate, mating latency, copulation duration, fecundity, and ovariole number in *P. straiata.*

Parameter	Source	Type III Sum of Squares	df	Mean Square	F value
Feeding rate (in no.)	Diet	35184.053	2	17592.027	531.816***
	Error	4862.640	147	33.079	
	Total	2090122.000	150		
Mating latency (in min)	Diet	81.413	2	40.707	321.176***
	Error	18.631	147	.127	
	Total	2857.057	150		
Copulation duration (in min)	Diet	46.053	2	23.027	88.686**
	Error	38.167	147	.260	
	Total	1489.434	150		
Fecundity (in no.)	Diet	26429.293	2	13214.647	242.235***
	Error	8019.300	147	54.553	
	Total	873555.000	150		
Ovarioles (in no.)	Diet	1957.213	2	978.607	63.271**
	Error	2273.620	147	15.467	
	Total	137139.000	150		

***Significant at 0.0001 level (P < 0.001); **Significant at 0.001 level (P < 0.001).

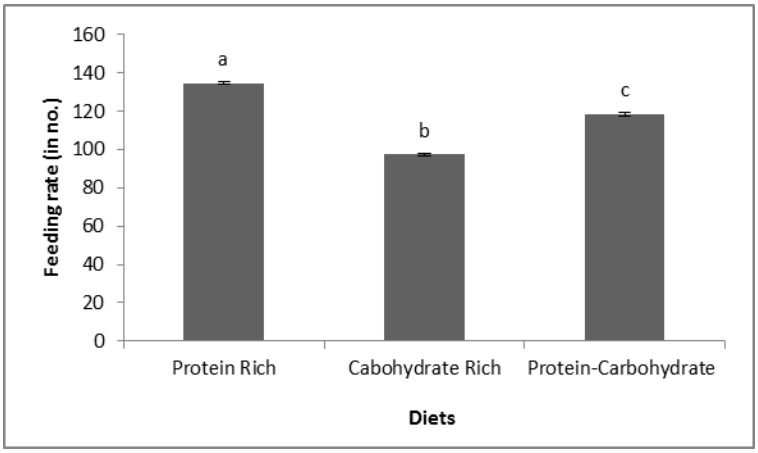

Figure 1. Diet effect on feeding rate in *P. straiata.* (Different letters on the bar graph indicate significance at 0.05 level by Tukey's *post hoc* test).

Mating latency of flies grown in altered diets is provided in Figure 2. It was observed that flies grown in protein rich media had taken least time to initiate copulation when compared to the other two altered media. One way ANOVA followed by Tukey's *Post Hoc* test carried out on the above data using SPSS version 10.0 showed significant variations in mating latency in different diets (Table 1). Flies grown in protein enriched media had taken significantly greater time to initiate copulation compared to flies grown on carbohydrate and carbohydrate and protein enriched media by Tukey's *Post Hoc* test.

Copulation duration data of flies reared in different altered diets is provided in Figure 3. It was noticed that flies grown in protein rich media had copulated longest compared to other two altered media. One way ANOVA followed by Tukey's *Post Hoc* test carried out on the above data using SPSS version 10.0 showed significant variation in copulation duration in different diets (Table 1). Flies grown in protein enrich

media had copulated significantly longer than compared to flies grown on carbohydrate and carbohydrate and protein enriched media by Tukey's *Post Hoc* test.

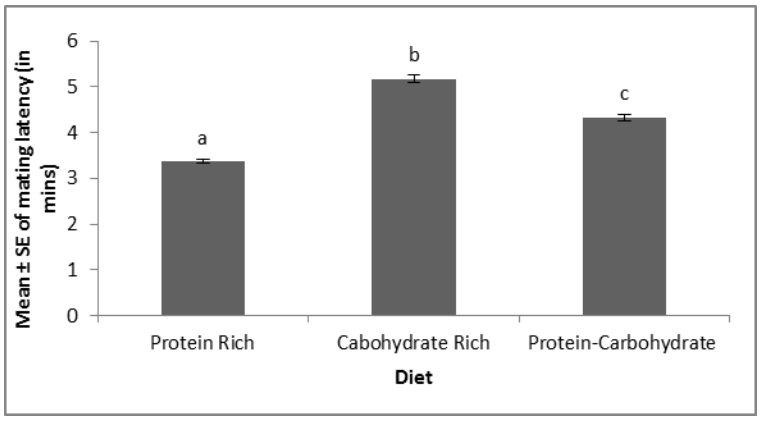

Figure 2. Diet effect on mating latency in *P. straiata.* (Different letters on the bar graph indicate significance at 0.05 level by Tukey's *post hoc* test).

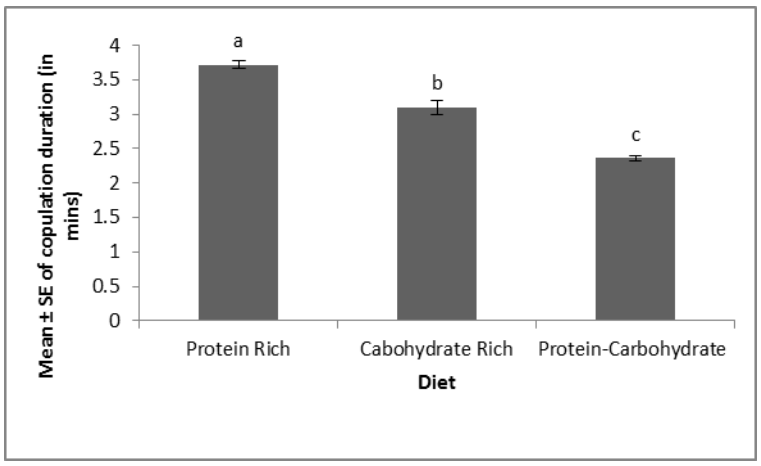

Figure 3. Diet effect on copulation duration in *P. straiata.* (Different letters on the bar graph indicate significance at 0.05 level by Tukey's *post hoc* test).

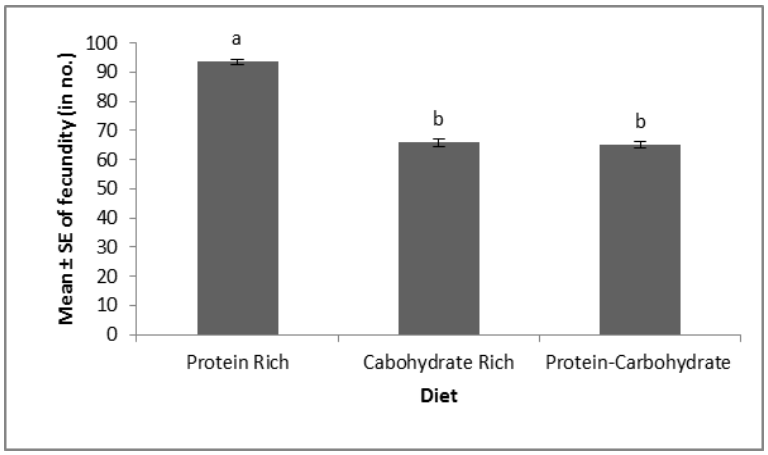

Figure 4. Diet effect on fecundity in *P. straiata.* (Different letters on the bar graph indicate significance at 0.05 level by Tukey's *post hoc* test).

Mean fecundity data of flies reared in different altered diets are given in Figure 4. It was found that flies grown in protein rich media had greater fecundity than those flies grown in carbohydrate and carbohydrate and protein enriched media. One way ANOVA followed by Tukey's *Post Hoc* test carried out on the above data using SPSS version 10.0 showed significant variation in fecundity between different altered

diets (Table 1). Flies grown in protein enrich media had significantly greater fecundity compared to flies grown on carbohydrate and carbohydrate and protein enriched media by Tukey's *Post Hoc* test.

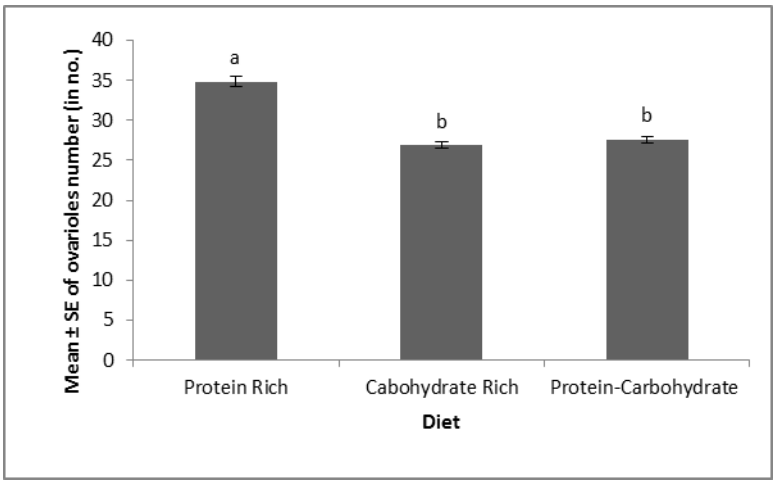

Figure 5. Diet effect on ovarioles in *P. straiata.* (Different letters on the bar graph indicate significance at 0.05 level by Tukey's *post hoc* test).

Figure 5 shows ovariole number data of flies reared in different altered diets. It was found that flies grown in protein rich media had greater ovariole number than those flies grown in carbohydrate and carbohydrate and protein enriched media. One way ANOVA followed by Tukey's *Post Hoc* test carried out on the above data using SPSS version 10.0 also showed significant variation in ovariole number between different altered diets (Table 1). Flies grown in protein enrich media had significantly greater ovarioles number than those flies grown on carbohydrate and carbohydrate and protein enriched media by Tukey's *Post Hoc* test.

Discussion

P. straiata is known to feed and breed on ripe or rotting fruits where protein/carbohydrate ratios vary temporarily and spatially. Therefore, it is possible that quantities of carbohydrate and proteins in the diet of an organism have a direct effect on reproduction of an organism (Sisodia and Singh, 2012). In species of *Drosophila* experiments on feeding rate have suggested that diet type had a significant influence on larval feeding. Such differences may be due to inhibition threshold. *D. melanogaster* larvae feed almost continuously, accompanied by a massive increase in mass. However inhibition threshold exist for feeding on new or foul tasting foods (Melcher *et al.*, 2007). Such inhibition threshold is not observed in larvae fed on protein enriched diet when compared to other two altered diets, since the rate of larval feeding was highest among larvae fed on protein diet.

Reproductive performance of an organism is a good index of fitness in organisms that go through repeated cycles of rapid population growth, and it has evolved as a way for species to maximize their fitness and is known to be influenced by various factors, such as body size, age, diet, and so forth (Partridge *et al*., 1987; Krishna and Hegde, 1997; Somashekar and Krishna, 2010). In the present study it was noticed that in *P. straiata* flies grown on protein rich media had taken less time to initiate copulation compared to flies grown on carbohydrate rich and carbohydrate-protein rich media.

As the time is reverse of speed, flies which took less time to initiate copulation were fast maters, while flies which took greater time to initiate copulation were slow maters (Hegde and Krishna, 1997). Therefore in the present study flies grown on protein rich media were fast maters. This suggests that quality of diet had significant influence on mating latency.

Copulation duration is another important fitness trait. Flies which copulated longer can receive greater quantity of accessory gland proteins and sperm than those flies which copulate shorter (Krishna and Hegde, 1997). Figure 3 shows that flies grown on protein rich media had copulated significantly longer than flies grown on carbohydrate rich and carbohydrate-protein rich media. Further it was also noticed that flies grown on protein rich media had greater egg production compared to egg production in other two nutritional regimes

(Figure 4 and Table 1). A high protein requirement when producing eggs might reflect that synthesis of the egg-yolk protein vitalize in females is dependent on the incorporation of amino acids (Adams and Gerst, 1991; Markow *et al*., 1999). This confirms the work of Sisodia and Singh (2012) in *D. ananassae.* They also found that flies reared in protein rich media had greater fecundity.

In the present study, altered diet effect on ovariole number was also studied in *P. straiata.* This is because both ovarioles number and fecundity are positively related. It was noticed that flies grown on protein rich diet had a significantly greater number of ovarioles than on the other two diets (Figure 5 and Table 1). This suggests that diet has significant effect on fecundity and ovariole numbers. Thus these studies in *P. straiata* suggest that altered diet had significant influence on reproductive performance.

Acknowledgment: The author extends their gratitude to the Chairman, Department of Studies in Zoology, University of Mysore, Manasagangotri, Mysore, and *Drosophila* Stock Center, University of Mysore, for providing facilities to carry out the above work.

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Drosophila suzukii larvae suppress *Aspergillus nidulans* growth particularly at high densities of larvae.

Davis, A.J. Institute for Chemical Ecology, Max Planck Society, Beutenberg Campus, Hans Knoell Str. 07745 Jena, Germany; *and* Animal Ecology Group, Institute of Zoology and Anthropology, Georg August University, Berliner Str. 28, 37073 Göttingen, Germany.

Introduction

Drosophila suzukii Matsumura is an Asian species distributed from northern Japan southwards to the semi tropics and westwards at least as far as India. It has recently been introduced by human agency to the Pacific, North and South America, and Europe (Asquith and Messing, 2012; Calabria *et al.,* 2012; Cini *et al.,* 2012; Hauser, 2011) and is now quite widely spread in these areas. Its arrival has caused great concern in the soft fruit industry, because it lays eggs in fruit that are still on the tree or bush and are not decaying.

Several moulds kill insects including *Drosophila* larvae (Courtney *et al.,* 1990; Hodge *et al.,* 1999; Rohlfs *et al.,* 2005). But, because it lays in fresh fruit, the larvae of *D. suzukii* might not be able to defend themselves against the moulds that occur in fruit decaying on the ground. We, therefore, tested this ability by challenging *D. suzukii* larvae with the mould *Aspergillus nidulans*. Wild type mould of this species produces a number of compounds toxic or fatal to insects including *Drosophila* larvae. But, in addition to the wild type mould, we also challenged larvae with a transgenic strain deficient in toxins. The transgenic strain (Δ laeA) cannot express the gene LaeA that regulates secondary metabolism. Blocking LaeA expression suppresses

several secondary metabolites (Kale *et al.*, 2008; Perrin *et al.*, 2007). We expected that *D. suzukii* larvae might be able to overcome the deficient mould but succumb to the toxin producing wild type.

Methods

The *D. suzukii* larvae came from a strain derived from numerous females collected in July 2004 in suburban Tokyo. They were reared on a malt medium until 2010 and thereafter on a standard medium (Shorrocks, 1971) supplemented with domestic mushrooms (*Agaricus bisporus*). Mushroom provides nutrients that are not otherwise present in standard *Drosophila* media. The mould strains were provided by Nancy P. Keller (University of Wisconsin). They were cultured on malt extract agar at 25°C, L:D 14:10, for 4-5 days. Mature conidia (asexually produced spores) were washed off with 0.9% NaCl in distilled water containing the surfactant Tween 80 (0.1%). The conidia collected were then stored at 4°C for less than a week. Before inoculating the experimental Units, the suspensions were adjusted to a titre of 1000 conidia μ^{-1}.

I put 0, 1, 2, 3, or 4 female *D. suzukii* onto 1000 mm^3 *Drosophila* medium in small beakers of 2000 mm^3. There were 20 replicates of each number of females. After the females had been in the beakers for 24 h, I removed the females and counted the eggs laid. After counting the eggs, I added spores of WT *A. nidulans* at the rate of 3000 per beaker to 10 replicates in each combination. I added spores of LaeA deficient *A. nidulans* at the same concentration to the other 10 replicates in each combination.

The larvae were then allowed to feed and the mould to develop until no further adult flies emerged. The beakers were monitored daily and the growth of the mould recorded. At the end of the experiment, I measured the percentage of the medium surface covered by mould. I also estimated the thickness of the mould on a scale of 1 to 5 where 1 was the highest and 5 the lowest thickness. My index of mould growth is then (percentage cover/thickness). A complete covering of thick mould thus has a growth index of 100, a slight covering of thin mould an index of <<1.

Some larvae might die during development. Therefore, the number of larvae feeding in each replicate is better represented as a combination of the number of eggs laid and the number completing development. The combination I used was (number of eggs + number of puparia/2).

I grouped the replicates for WT or deficient mould into larval number classes in order to estimate the variability of the mould growth index between tubes with similar numbers of larvae. The class "Control" contained those beakers that had not received female flies. Class 0 contained those beakers that received flies but in which no eggs were laid. Class 1 contained those with 1, 2 or 3 larvae, class 2 those in which there were 4-6 larvae, and so on up to class 6. Class 7 contained those beakers with >21 larvae. Most of these contained 22-30 larvae but there was one replicate with 39 and one with 47.

Results

The growth of mould, whether of the toxic or non-toxic genotype, was negatively related to the number of feeding larvae. The correlations were strong and nonlinear (toxic mould; Kendall T = –0.627, 2-tailed $p < 0.001$, n = 45: non-toxic mould; Kendall T = –0.745, 2-tailed $p < 0.001$, n = 48). Strong negative correlations remained even if control beakers were omitted (toxic mould; Kendall T = –0.460, 2-tailed $p < 0.001$, n = 35: non-toxic mould; Kendall T = 0.718, 2-tailed $p < 0.001$, n = 38). The number of eggs per beaker was correlated with the original number of females put into the beaker but the variability around this relationship was very large.

The mould growth index was thus also negatively related to larval number class (Figure 1). Ranked growth index differed very significantly between larval number classes ($F^{8}_{6.581} = 13.896$, $p = 0.002$). There was also an apparent difference between the index for WT and deficient mould ($F^{1}_{7.182} = 5.540$, $p = 0.05$), but this cannot be considered significant with these ranked data. The complete model was also significant ($F^{1}_{8.199} = 25.109$, $p = 0.001$), but the interaction of mould type and larval number class was not. However, the mould growth index was higher for non-toxic moulds at low larval numbers than for toxic moulds.

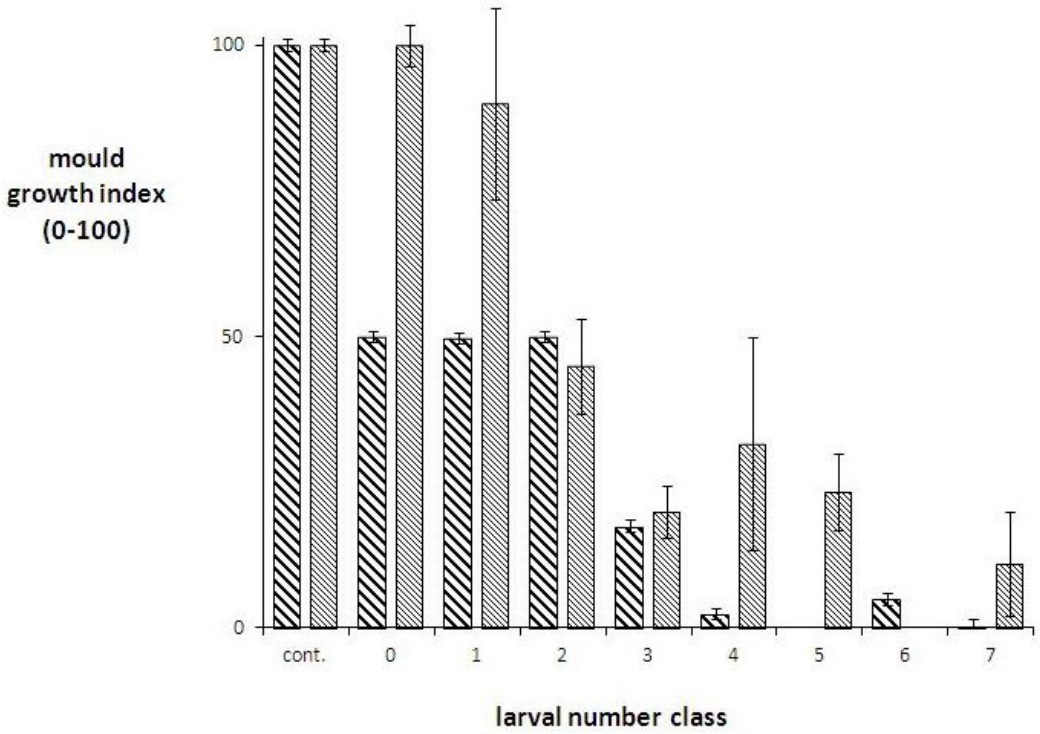

Figure 1. Relationship of median mould growth index to larval number class for toxic (heavy shading) and non-toxic (light shading) mould. Mould growth index is percentage cover divided by thickness. Thickness is a scale of 1-5 where 5 is low. Larval number class C is control (without any flies), class 0 is with flies but where no eggs were laid, class 1 contains larval numbers 1-3, class 2 4-6, and similarly up to class to 6 19-21. Class 7 contains larval numbers >21. Error bars are the standard range (*i.e.*, range/N where N is the number of replicates in that larval number class).

Discussion

Drosophila suzukii larvae are thus able to inhibit the growth of *A. nidulans*, as is *D. melanogaster* (Trienens *et al.*, 2010). This ability of *D. suzukii* is true whether or not the mould is capable of producing toxins. The greater the larval density, the greater the inhibition (Figure 1). However, there is a particularly steep decline in mould growth between larval number class 2 and 3, *i.e*. at about 7 larvae per 1000 cubic millimetres. Larval densities of >15 largely prevent mould growth. The high growth of the non-toxic mould at low larval densities may arise from compensatory growth by this genotype which cannot increase toxin production. Any compensatory growth is overwhelmed, however, at high larval densities.

Drosophila suzukii does not, therefore, suffer great mortality from *A. nidulans* mould as long as larval densities are not very low. It is able to counteract not only toxin deficient *A. nidulans* but also the wild type that contains particularly potent mortality agents. Because *D. suzukii* can deal with these agents, it is also likely to resist other moulds, such as *Penicillium* known to be toxic against insects. The larvae of *D. suzukii* are, therefore, not prevented by moulds from developing in decaying fruit were females to lay in such substrates. Thus, decaying fruit may well be a resource that *D. suzukii* can successfully exploit for breeding. Indeed, D. suzukii breeds in decaying flowers and in sap streams (summarized in Wilson *et al.*, 2013) and I have reared it from decaying fruit baits laid out on the ground in both China and Japan. If this is generally so, *D. suzukii* will be even more difficult to control than is currently envisaged and decaying fruit must also be targeted in any control scheme.

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Retraction

Gürbüzel, Mehmet, 18 July 2014

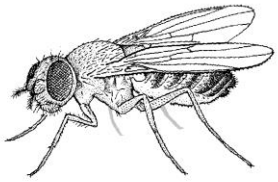
Mehmet Gürbüzel contacted the Editors to explain that "In my earlier studies, I could not write in a good way, due to I did not know the rules of writing. Whereas, '… appropriating another person's ideas or words (spoken or written) without attributing those word or ideas to their true source" according to Brown "plagiarism". When transferring someone else's ideas, I did not change sentences. I used sentences belonging to others, in my own articles. I have cited, but I know now that it is also plagiarism. I very much regret. For that reason, please remove my article in electronic systems."

Gürbüzel, M., 2009, The effects of exogenous estrogen and progesterone on developmental stages of *Drosophila melanogaster*. Dros. Inf. Serv. 92: 60-63.

Gürbüzel, M., and H. Uysal 2009, Toxic effects of Patulin to some developmental stages of *Drosophila melanogaster*. Dros. Inf. Serv. 92: 41-43.

Gürbüzel, M., and H. Uysal 2009, Effects of fumonisin B1 to developmental stages of F2 offspring of *Drosophila melanogaster*. Dros. Inf. Serv. 92: 78-80.

At his request, the Editors are unlinking these three citations from the online index for 2009, volume 92. But, of necessity, the articles themselves must remain in the intact published issue for 2009 and the complete online issue.



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Technique Notes

Improving sampling protocol for assessing drosophilid diversity: spatial independence and sample size.

Mata, R.A.[1], G.A. Santos[1], M. Uehara-Prado[2], and R. Tidon[1]. [1]Instituto de Ciências Biológicas, Universidade de Brasília, CP 04457, Brasília, Brazil 70910-900; [2]Instituto Neotropical: Pesquisa e Conservação. Caixa Postal 19009, 81531-980 Curitiba, PR, Brazil. Corresponding author: rotidon@unb.br.

Introduction

The development of monitoring programs is one of the strategic actions set by the Convention of Biological Diversity (CBD, 2010) to achieve the biological conservation targets. However, such monitoring programs often show problems due to the lack of scientific rigor in the sampling design (Lindemayer and Linkens, 2010). Therefore, robust monitoring programs based on a rigorous scientific protocol have emerged as a high priority area in environmental research (Gardner, 2010; McGeoch *et al*., 2011).

Drosophilid assemblages are suitable candidates for monitoring studies in the Brazilian savanna (Mata *et al*., 2008). However, despite the increasing number of studies on drosophilid diversity in Brazil, to date there has been no robust standardized protocol that allows both a reasonable diversity estimate of a particular area and the comparison among different studies. Here we present a first approach in developing a sampling protocol for assessing drosophilid diversity, by investigating two fundamental aspects of sampling designs: the spatial independence between sampling units and sample size adequacy.

Sampling Design

Pilot samples were conducted in the Ecological Reserve of IBGE, located 35 km south of Brasília, the Brazilian capital, in January and May 2013. On each sampling occasion, four habitat types were sampled: conserved *cerrado* (an open vegetation characteristic of the Brazilian savanna), disturbed *cerrado*, conserved forest, and disturbed forest. In all habitats, five sampling units (SU) were established at least 30 m apart. Each SU contained three traps disposed 10 meters apart, totaling 60 traps per sampling occasion.

The flies were captured using baited traps, which retain the attracted specimens (Roque *et al*., 2011). Traps were baited with mashed bananas, fermented with *Saccharomyces cerevisiae* for 24 hours. The traps were left in the field for four consecutive days, and the flies collected were taken to the laboratory and identified, whenever possible, to the species level. Vouchers of the captured species were deposited at the Collection of the *Laboratório de Biologia Evolutiva da Universidade de Brasilia*.

Spatial autocorrelation analysis was used to determine whether the drosophilid assemblages collected in different distance classes were independent of each other. We used a Mantel test between a similarity matrix (based on the Kulczynski quantitative coefficient) and a geographic distance matrix (based on Euclidian distance). This analysis was run in SAM software (Rangel *et al*., 2010).

The statistical power analysis (run in PASS software; Hintzi, 2013) was used to calculate the adequate sample size to detect richness differences between conserved and disturbed *cerrados* and forests (two-sample t-tests allowing unequal variance; $\alpha = 0.05$, $\beta = 0.80$). The power analysis estimated the sampling effort needed for detecting any difference on drosophilid richness between conserved and disturbed sites, to achieve a statistical power around 80% (1- β).

Spatial Independence and Sample Size Adequacy

The spatial autocorrelation analyzes failed to show any spatial structure between sampling units even in the first distance class both in *cerrados* (Distance centroid = 50m; Pearson's r = 0.143; p = 0.367) and in forests (Distance centroid = 30 m; Pearson's r = 0.319; p = 0.211).

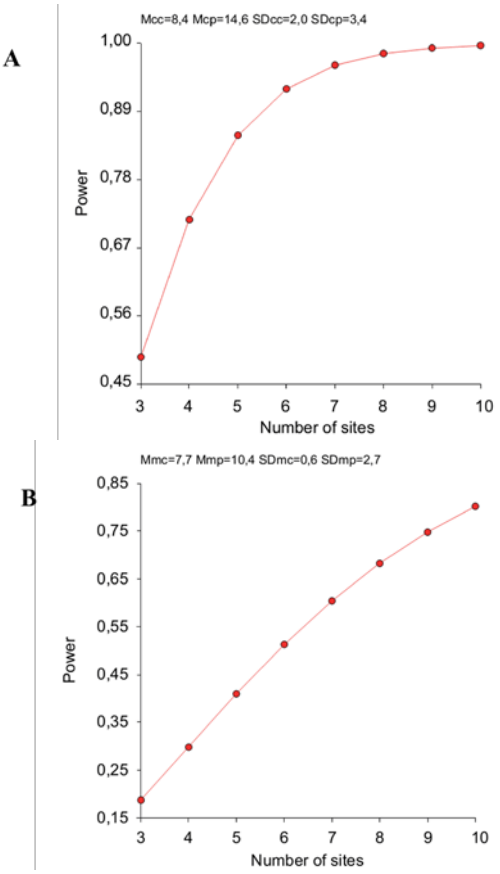

Figure 1. Statistical power increasing as a function of sample size (two-sample T-tests allowing unequal variance; $\alpha = 0.05$, $\beta = 0.80$). A : cerrados; B: forests.

In *cerrados* (Figure 1A), it would require at least five sampling units (composed by three traps each) for each habitat type, while to compare conserved and disturbed forests (Figure 1B), at least ten replicates of each habitat type would be necessary.

From these results we can conclude that – for the Brazilian Savanna – sample independence for drosophilid diversity, ensuring true replicates, can be reached by disposing SUs 50 m apart in *cerrados* and 30 m in forests. Moreover, detecting differences in the drosophilid richness between conserved and disturbed sites requires a sampling effort two times greater in forests than in *cerrados*.

Acknowledgments: We are grateful to the Reserva Ecológica do IBGE for the Field assistance, to Carlos Lodi for collecting coordinates, to Universidade de Brasília for logistical support, and to CAPES and CNPq for financial support.

References: Convention on Biological Diversity (CBD), 2010, Global Biodiversity Outlook 3, Gardner, T. Monitoring Forest Biodiversity, 2010; Hintze, J., 2013, PASS 12. NCSS, www.ncss.com; Lindenmayer, D.B., and G.E. Likens 2010, Biological Conservation 143: 1317-1328; Mata, R.A., M.A. Mcgeoch, and R. Tidon 2008, Biodiversity and Conservation 17: 2899-2916; Mcgeoch, M.A., *et al*., 2011, koedoe 53: 43-51; Rangel, T.F., J.A.F. Diniz-Filho, and L.M. Bini 2010, Ecography 33: 46-50; Roque, F., S.C.F. Oliveira, and R. Tidon 2011, Dros. Inf. Serv. 94: 140-141.

The Dover wild type strain and four derived isogenic lines.

Possidente, B.[1*], C. Norton[2], and D. Possidente[1]. [1*]Biology Department, Skidmore College, Saratoga Springs, NY 12866 USA, email: bposside@skidmore.edu; [2]Biology Department, Saint Catherine University, Department of Biology, St. Paul, MN 55105.

Here we report the availability of a wild type *Drosophila melanogaster* strain.

"Dover" and four derived isogenic lines

The Dover strain was founded in 1978 in Iowa City, IA, USA by J. Dawson Mohler, Department of Biology, University of Iowa. Twenty females were captured in Iowa City from a kitchen on Dover Street. Several eggs from each female were de-chorionated, and the offspring were randomly interbred to found the

"Dover" wild-type strain. Single Dover males were crossed with a balanced lethal marker strain (below), to generate isogenic lines for chromosomes I, II and III. BN and BN' designate pairs of balanced marker chromosomes for chromosome number one, two, or three, and Y represents the Y-chromosome:

Cross 1: B1/B1'; B2/B2'; B3/B3' × +/Y; +/+; +/+ (Dover male)

Cross 2: B1/+; B2/+; B3/+ × B1/Y; B2/B2'; B3/B3'

Cross 3: B1/+; B2/+; B3/+ × +/Y; B2/+; B3/+

Isogenic: +/+; +/+; +/+ × +/Y; +/+; +/+

B1 = FM7 *B g v sn w sc y*
B1'= *y pn cv m f*
B2 = SM1 *sp cn Cy al*
B2'= *Pm*
B3 = TM3 *Sb Ser e bx sep p ri*
B3'= TM2 *e Ubx*

The original Dover wild-type strain and four surviving isogenic lines (53, 60, 63, and 67) were maintained at the University of Iowa from 1978 to 1983 when they were moved to Skidmore College, Saratoga Springs, NY, USA, and maintained by mass culturing in two to five shell vials per strain. Cultures have been maintained on open shelves at an average temperature of approximately 21°C in a room with a 16:8LD photoperiod until 1995 and a 12:12LD photoperiod thereafter. Occasionally, if the population size falls below 10 flies in a vial, flies are transferred from another vial of the same strain to maintain viability of the culture. The Dover isogenic lines have been used to characterize genetic background effects modifying expression of *Antennapedia* (Possidente *et al*., 1990), genetic variance influencing oviposition site preference with respect to the presence of phenylthiocarbamide in the food (Possidente *et al*., 1999), genetic variation and covariation influencing developmental rate (Norton, 1985), and genetic variance affecting latency to copulation in response to developmental lead exposure (Wilson, 2004). The Dover wild-type strain and the four derived isogenic lines are available upon request from B. Possidente.

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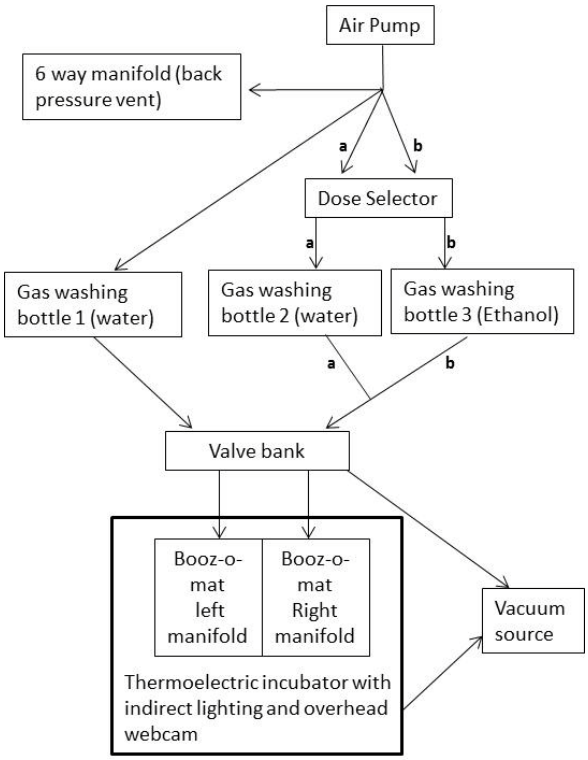
Assaying basal and ethanol-induced locomotion in flies using a custom built apparatus and freely available software.

Kliethermes, Christopher. Drake University, Department of Psychology and Program in Neuroscience, Olin Hall 318, 1344 27th Street, Des Moines IA 50311; Phone: 515 271-3937; Email: c.kliethermes@drake.edu.

Wolf *et al*. (2002) developed an apparatus that can be used to monitor the locomotor stimulant and depressant responses to ethanol in fruit flies. In this assay, approximately 20 flies are loaded into each of 8 tubes and placed into a custom-built, horizontal tube holder though which air or vaporized ethanol is perfused. An overhead video camera connected to a computer is used to record the behavior of the flies before and

during the exposure, and locomotor activity is quantified offline using commercially available DIAS software (Solltech, IA). In implementing this apparatus and protocol in my own lab, I wished to retain its essential feature – the ability to track multiple samples of flies before and during an ethanol exposure – while improving on several aspects, including subject throughput, temperature control, and the ability to present air and ethanol simultaneously to separate groups of flies. The design of this apparatus and the analysis of the data by freely available software are described in this article.

Figure 1. The ethanol delivery system and locomotor activity apparatus. A, air pump; B, gas washing bottles; C, dose selector; D, valve bank; E, incubator; F, computer. Details of each component are provided in the text and other Figures.



Ethanol Vaporization

The modified apparatus consists of two main parts: a vapor delivery system and a behavioral observation area. Figure 1 shows the components of the apparatus, and Figure 2 shows a schematic diagram of the connections. The parts of the vapor delivery system include a commercial grade aquarium pump used as a source of compressed air (Alita Model AL-40), a custom built diverter valve for controlling ethanol dose, three gas washing bottles (Bel-Art Scienceware #110300000), and a bank of valves used to direct the air and ethanol streams as needed.

As indicated in Figure 2, an air stream from the pump is divided into 3 streams: one stream is directed to an adjustable, 6-way gang manifold and is used to vent back pressure; a second stream is directed to gas washing bottle that contains water (bottle 1 in Figure 2) and then to the valve bank (Air stream). The third stream (ethanol stream) is split again, and one half directed towards a water-containing gas washing bottle (bottle 2 in Figure 2) and the other stream to an ethanol-containing gas washing bottle (bottle 3 in Figure 2).

Figure 2. Schematic diagram of the ethanol delivery system and locomotor activity apparatus. Each line represents 3/8" ID tubing, and the direction of the air flow is indicated by the arrows. The dose is selected at the Dose Selector by changing the proportion of air flowing through stream 'a' relative to stream 'b.' Air or ethanol can be directed independently to the left or right manifolds, or concurrently to both manifolds.

Figure 3. The dose selector was built from construction grade lumber and plywood. Two 3/8" ball valves are mounted orthogonally to each other to form a constant output diverter valve. The red handles of these valves are visible in this image. The wooden dowel was secured to both handles with two bolts and a zip tie.

In the apparatus described by Wolf *et al*. (2002), ethanol dose is changed by way of calibrated flow meters, which are used to control the amounts of air directed to an ethanol-containing flask and a water-containing gas washing bottle. The greater the amount of air sent to the ethanol-containing flask, the greater the ethanol concentration downstream. Because of the cost of these meters, a custom built diverter valve (the dose selector) that functions similarly to the flow meters was constructed. The dose selector (Figure 3) consists of two orthogonally mounted 3/8" brass ball valves connected by the handles and attached to a rigid support. A single wooden dowel attached to both of the handles serves as a lever for operating the valves and as the dose indicator. Different ethanol doses are produced by varying the proportions of the air stream sent to two gas washing bottles, one of which contains water and the other contains absolute ethanol (bottles 2 and 3, respectively, in Figure 2). Because the valve handles are connected orthogonally to each other, changing the amount of air flow to bottle 2 inversely and proportionally changes the air flow to bottle 3, allowing for a continuous range of ethanol doses to be produced. The dose is read on an attached scale that ranges from 0 to 90 degrees, which can then be converted to a percentage that reflects the amount of the air stream downstream from the gas washing bottles that carries vaporized ethanol. Downstream from washing bottles 2 and 3, the split streams are unified and sent to the valve bank.

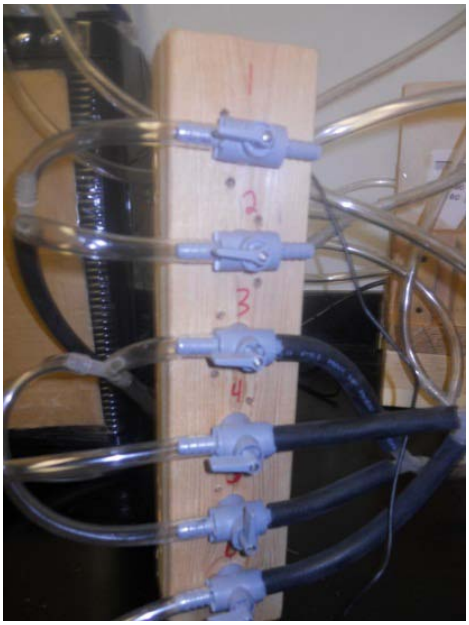


Figure 4. The valve bank consists of a length of lumber into which 6 three-way diverter valves are mounted, and is used to direct the flows of air and ethanol to the left or right manifolds in the testing apparatus. The two barbs visible on each of the six valves were used to direct the output to another valve or to the locomotor apparatus, as appropriate. The third barb on each valve (not visible in the image above) received the input.

The valve bank (Figure 4; see Figure 2 for air flow diagram) consists of six 3-way valves that are used to route the air and ethanol streams to the behavioral observation apparatus. The valve bank functions by splitting the air and vaporized ethanol-containing streams into half, then sending one half of each stream to either the left or right manifold of the testing apparatus. To avoid the difference in flow rate that will result when delivering air or ethanol to a single manifold compared to both manifolds, the valve bank was connected to a vacuum source (a faucet aspirator), which is used to vent a portion of each stream when air and

ethanol are presented concurrently. Three-way valves were used in order to allow the air and ethanol streams to be sent to either the locomotor activity apparatus or to a second apparatus not described here. Two-way valves could be used instead with a slightly different connection configuration; quick connectors could also be used if a fume hood is used to vent the ethanol.

Behavioral Observation Apparatus

The components of the behavioral apparatus consist of two 10-way aquarium manifolds (Sunlight Supply) that can receive an air or ethanol-saturated air stream from the valve bank, a custom built tube holder that accommodates 20 horizontally arrayed tubes, and a temperature controlled reptile egg incubator (Exoterra, PT2499), into which a webcam (Agama V-1325R) and LED panel light (Bescor LED-70) were mounted (see Figure 5). The tube holder measures 6" × 7" overall, and was made from low-glare, translucent polypropylene divided into 10 slots, each 6" × ½", to accommodate the barb spacing on the 10-way manifolds. The barbs were wrapped with PTFE tape, and ¼" ID polypropylene ID acorn nuts, predrilled with three small holes to allow air flow, were threaded onto the barbs. During testing, flies are tapped into the tubes, which are then slid onto the rounded end of the acorn nut.

Two one-half inch holes were drilled through the walls of the incubator to connect the valve bank to the manifolds. A vacuum line connected to a faucet aspirator was inserted into the incubator by way of a third hole drilled into the upper left wall of the incubator. The webcam and light panel were attached to predrilled angle iron connected to one wall of the incubator with bolts, creating an adjustable overhead rigging. The cables for the camera and light were run through a separate hole drilled through the incubator wall.

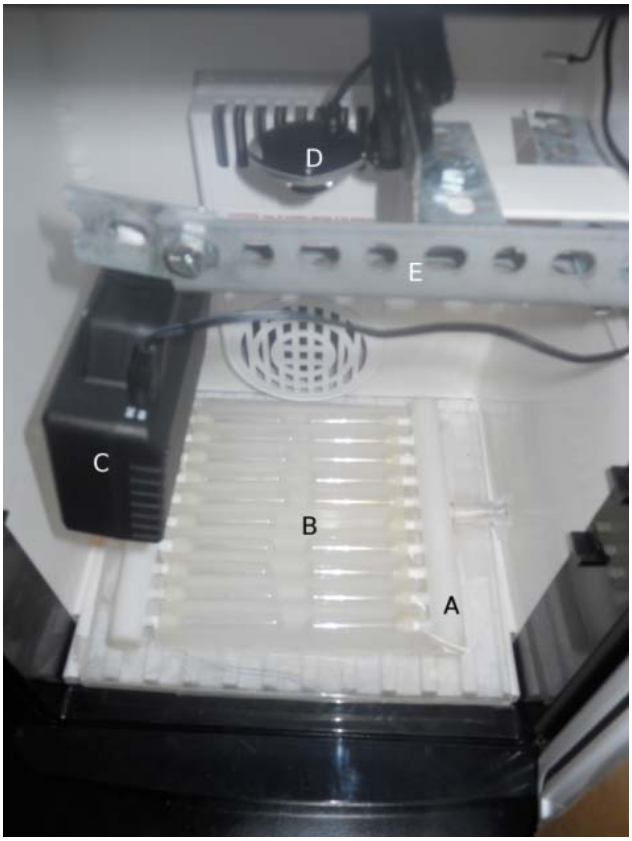


Figure 5. The behavioral apparatus is housed in a thermoelectric incubator containing two manifolds: A, fitted with nylon acorn nuts that fit into the mouths of disposable test tubes; B, used to contain the flies. The tubes are placed horizontally into a custom-built holder in the bottom of the incubator. A LED light panel, C, and webcam, D, are attached to an overhead angle iron rigging, E. The cables for these components exit the incubator through a hole in the upper right corner of the incubator.

Video Recording and Analysis

A webcam (Agama V-1325R) connected to an attached PC running Mint Linux is used to record the experimental sessions. Videos are saved in avi format using GUVCView (http://guvcview.sourceforge.net), which can be configured to record at various frame rates and resolutions. For the experiments conducted so far, a resolution of 640 × 480 at 10 FPS has been adequate to capture the activity of the 5-10 flies per tube that are typically tested. Jpg frames are extracted from the videos using Avidemux (fixounet.free.fr/avidemux) or ImageJ (imagej.nih.gov/ij).

We have used two methods to analyze locomotor activity in this apparatus. The first method uses the procedures and Perl scripts developed by Ramazani *et al*. (2007). Briefly, the "nested_window" script is used to generate a series of images for each user-defined zone, such that movement that occurs between subsequent frames is depicted as white pixels on a black background for each zone. For the apparatus described above, there are 20 zones. A second script, "quantify630," then counts the number of white pixels that are above a user-defined intensity level for each zone, generating a text file oriented with the zones ("cells") as columns

and the locomotor activity that occurs from one frame to the next in rows. In the standard 28 minute videos at 10 FPS collected in my lab, 16,800 jpgs are generated, which results in 16,799 rows × 20 columns outputted by the script. A third perl script is then used to bin the data outputted from the "quantify630" script into more manageable chunks. Baseline activity, area under the curve scores, and other descriptive data are then calculated from these extracted data. The data outputted by the quantify630 script can also be copied and pasted directly into a spreadsheet for analysis, although this operation can be extremely unwieldy if a large number of frames is analyzed.

The second method uses the "MTrack3" plugin for ImageJ. Given a sequence of frames, this plugin detects all objects (subjects) above a user-defined threshold and assembles tracks for each object across frames as a series of time-stamped x-y coordinates. Similar to the commercially available DIAS, empirically-derived values are used to specify the size of the objects to be tracked, the minimum number of frames a single object must be tracked, and the maximum distance that a single object can move from frame to frame and still be counted as the same object. We run MTrack3 in a macro that imports a user-defined number of frames according to a user-defined array of time points (*e.g.*, frames 1-200, 201-400, 401-600). This macro analyzes all of the experiments contained in a directory and outputs the data into separate directories corresponding to the names of the experiments. These data are analyzed using an Awk script, which groups individual object tracks by tube, calculates the total distance moved by all of the flies in a tube at each time point, and divides the total distance by the number of objects and frames that each object was tracked.

While these two methods of analysis produce comparable results under most conditions, the underlying methods of analysis are different and can produce different results. We have found that the nested_window script can produce unexpected results when comparing subjects that differ in body size, such as can occur when comparing strains or male and female flies. This occurs because larger flies will necessarily produce more white pixels from frame to frame than will smaller flies, resulting in larger flies showing more apparent movement than smaller flies between any two frames. To correct this, observed distances traveled can be corrected for body weights by division or analysis of covariance. In contrast, the Mtrack3 plugin calculates movement as the location of the centroid of an object in one frame compared to the location of the same centroid in the next frame. Consequently, this method seems to be less sensitive to differences in body size of the subjects. The scripts and macros used for either of these analyses are available upon request.

Acknowledgments: I would like to acknowledge Tom Kliethermes for modifying the quantify630 script to work with large numbers of files, as well as for writing the script to extract binned activity data generated by this script. And for just being generally swell.

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Co-injected ΦC31 transgenes frequently produce multiple independent germline transformation events in a single *D. melanogaster* embryo.

Albanus, Ricardo D'Oliveira[1]*, David S. Lorberbaum[1,2]*, Andrea I. Ramos[1,2], and Scott Barolo[1,3]. [1]Department of Cell & Developmental Biology and [2]Program in Cellular and Molecular Biology, University of Michigan Medical School, Ann Arbor, MI 48109, USA. [3]Corresponding author: sbarolo@umich.edu. *These authors contributed equally.

Introduction

Injecting *Drosophila melanogaster* embryos with P element or ΦC31 plasmid vectors to generate single-copy transgenic fly lines is a common and well-established procedure (*e.g.*, Rubin and Spradling, 1982; Bischof *et al.*, 2007). Today, many labs employ outside injection services, which charge by the construct, to

generate transgenic fly lines. In standard transformation protocols, transgenic constructs are injected one at a time. However, given the generally high efficiency of ΦC31 transformation, multiple plasmids can be mixed and co-injected, reducing time, effort, and expense compared to multiple single-construct injections. Here, we demonstrate that single embryos injected with a mixture of three ΦC31 vectors can, at a high frequency, produce individual offspring bearing each of the three transgenes. In other words, at least three independent lines, carrying different transgene insertions, can easily be established from the progeny of a single embryo via multiplex injection.

Methods

Three short synthetic DNA constructs of equal length (186 bp) were Gateway-cloned into the 11-kb ΦC31 transformation vector pHPdest-eGFP (Boy *et al.*, 2010) as previously described (Swanson *et al.*, 2008; Ramos and Barolo, 2013). The DNA sequences of the three transgenic constructs, referred to here as A, B, and C, differed from one another by four base pairs. A mixture containing an equal amount (16 µg) of each construct was sent to the commercial service provided by Rainbow Transgenic Flies (Camarillo, CA) for microinjection into the ΦC31 landing site 86Fb on chromosome arm 3R (Bischof *et al.*, 2007). Injection stock: *y w* M{eGFP.vas-int.Dm}ZH-2A; +/+; M{RFP.attP}ZH-86Fb.

Injected females were crossed to w^{1118} males, and the progeny were screened for red eye color, indicating expression of the *mini-white* marker gene from the integrated transgenic vector. We selected three red-eyed male progeny from each of these crosses and individually crossed them to w^{1118} virgin females to establish stocks. After several days, we recovered each red-eyed male, extracted its genomic DNA (Gloor *et al.*, 1993), and amplified the insert sequences with primers anchored in vector DNA (primer sequences are available on request). We then sequenced the PCR products using the same primers (University of Michigan Sequencing Core) and analyzed the sequence with Lasergene software.

Table 1. Transgene sequencing results. Each row shows the identity of the transgene in three male progeny derived from a female embryo co-injected with transgenes A, B, and C.

Injected female	Transformed F_1 #1	Transformed F_1 #2	Transformed F_1 #3
1	**B**	**C**	**A**
2	**A**	**A**	**C**
3	**B**	**B**	**B**
4	**B**	**C**	**C**
5	**A**	**B**	**C**
6	**B**	**C**	**A**

Results

Of 21 viable *y w* females co-injected with transgenes A, B, and C and then crossed to *w* males, 12 produced one or more transformant (red-eyed) progeny. Injected males were not examined in this analysis. Sequencing of PCR-amplified transgenes from genomic DNA (see Methods) revealed the identity of the transgene integrated into the progeny of injected flies. Three red-eyed offspring derived from each of six injected females were genotyped (Table 1). Of the six injected females followed in this analysis, three (#1, 5, 6) produced three males bearing three different transgenes. Two (#2, 4) produced two males with one transgene and one with another, while one female (#3) produced three males carrying the same transgene. The observed frequencies did not significantly deviate from a null assumption of no bias in transgene frequencies derived from a given injected embryo (chi-square test, $p > 0.995$).

Progeny from two additional females, who only produced two red-eyed offspring each, were also genotyped. In each case, both of the offspring carried the same transgene (not shown). This result is consistent with the possibility that these injected embryos, which gave rise to fewer red-eyed offspring, may have undergone transformation in only one germline cell. If so, a relatively high transformation rate is likely necessary in order to achieve the high frequency of "co-transformation" observed here.

Discussion

Our results demonstrate that individual germline cells within an injected embryo can be transformed with different ΦC31 transgenes at a very high frequency, and thus that multiple independent transgenic lines

can be derived from a single injected embryo. Injection of a mixture of transgenic plasmids therefore provides a fast and cheap method of generating multiple transformed fly lines with a relatively small number of microinjections.

The co-injection method requires that transformed progeny be individually genotyped, but this does not slow down the crosses, as red-eyed flies are genotyped after mating. More importantly, the transgenes of all transformed lines should be sequenced, to rule out human error and acquired mutations, regardless of the method of injection. For example, of the 18 flies genotyped here, one showed evidence of mutations in the "A" transgene that was not present in the injected "A" DNA, or in any other fly bearing the same transgene. This may be the result of a PCR amplification error, but alternatively it may reflect a DNA mutation occurring before or after transgene insertion. Mutations aside, without genotyping it is impossible to rule out the possibility that the DNA or the flies could have been mislabeled, either in the lab or by the injection company.

The results presented here almost certainly underestimate the frequency of independent transformation events in different germline cells within a multiplex-injected embryo, for two reasons. First, only three progeny were selected for genotyping; sequencing of additional red-eyed progeny could only have increased the count of embryos giving rise to progeny bearing all three transgenes. Second, only three transgenic vectors were co-injected; it is possible that, for example, the three "B" progeny of injected embryo #3 represent three independent transformation events within that embryo. Taking this into account, it is possible that the number of co-injected plasmids could be increased significantly, further reducing the number of embryos to be injected.

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An efficient and cheap entomological aspirator to collect mycophylic and anthophilic adult Drosophilidae flies.

Machado, Stela[1], João Pedro Junges dos Santos[1], Lizandra Jaqueline Robe[1,2], and Elgion Lucio da Silva Loreto[1]. [1]Universidade Federal de Santa Maria (UFSM), Programa de Pós Graduação em Biodiversidade Animal (PPGBA), Avenida Roraima, 1000, CEP 97105-900 - Santa Maria, RS - Brazil, Phone: +55 55 3220-8912; [2]Universidade Federal do Rio Grande (FURG), Programa de Pós-Graduação em Biologia de Ambientes Aquáticos Continentais (PPGBAC), Instituto de Ciências Biológicas – ICB, Av. Itália, km 08, Campus Carreiros, CEP 96203-900 - Rio Grande, RS – Brazil, Phone: +55 53 3293-5128; Corresponding author: Lizandra Jaqueline Robe, lizbiogen@gmail.com.

Introduction

Traditionally, the methodology used to collect drosophilids in Brazil relies on flies' attraction to traps baited with resources, principally fermented fruits like banana (Tidon and Sene, 1988; Medeiros and Klaczko, 1999). However, this collection method attracts mainly frugivorous species of the genus *Drosophila* (Gottschalk *et al.*, 2008), providing a biased sample of subjacent biodiversity, once species with other feeding preferences are rarely recorded. In fact, *Drosophila* encompasses almost 60% of the 304 reported Brazilian drosophilid species, being followed by far by the mycophylic *Zygothrica* (with 54 species) and *Hirtodrosophila* genera (with only 16 species) (Gottschalk *et al.*, 2008).

Species of *Hirtodrosophila*, *Mycodrosophila*, *Paraliodrosophila*, and *Zygothrica* encompass the putatively monophyletic *Zygothrica* genus group (Grimaldi, 1990), which presents different degrees of association with macroscopic fungi. As only part of these species use fungi as resources for feeding or

oviposition (Courtney *et al*., 1990), we generically named them "mycophylic" species instead of "mycophagous", which is more frequently used. In this case, there seems to be a negative correlation between collection frequency and specialization level in the use of macroscopic fungi as feeding, breeding, or oviposition sites. *Zygothrica* is just the more generalist genus (Courtney *et al.*, 1990; Grimaldi, 1987) and the best recorded mycophylic taxon (Gottschalk *et al.*, 2008), although resource specialization may not be the only factor responsible for this scenario. In fact, reduced or skewed sampling and high levels of unregistered diversity seems to be the case for both, mycophilic (Bolzan, 2011) and anthophilic (Schmitz, 2010) species, which seem to be much more diverse in the Neotropics than previously reported.

The traditional methods used to collect mycophylic drosophilid species are entomological nets, mouth aspiration, or through the collection and storage of resources until adult eclosion (Markow and O'Grady, 2006; Gottschalk *et al.*, 2009; Robe *et al.*, 2014). This last method also seems to be widely used in the collection of antophylic drosophilid species (Vilela, 1984; dos Santos and Vilela 2005; Robe *et al.*, 2013). However, these sampling methodologies have important limitations: entomological nets are frequently hampered by fungi or flower disposition (that sometimes block net passage), and tend to be inefficient when the number of available specimens is low; mouth aspiration adds a health risk, once the collector could aspirate potentially harmful fungus spores or flower pollen, and it is also very inefficient. Once only few flies could be collected before breath breaking. Collection and storage of resources is also a biased sampling strategy, since some species can use fungi/flowers for purposes other than oviposition and these will not be collected at all. So, we developed a cheap entomological aspirator in order to make the collection of mycophagous and/or antophylous drosophilids more safe and efficient.

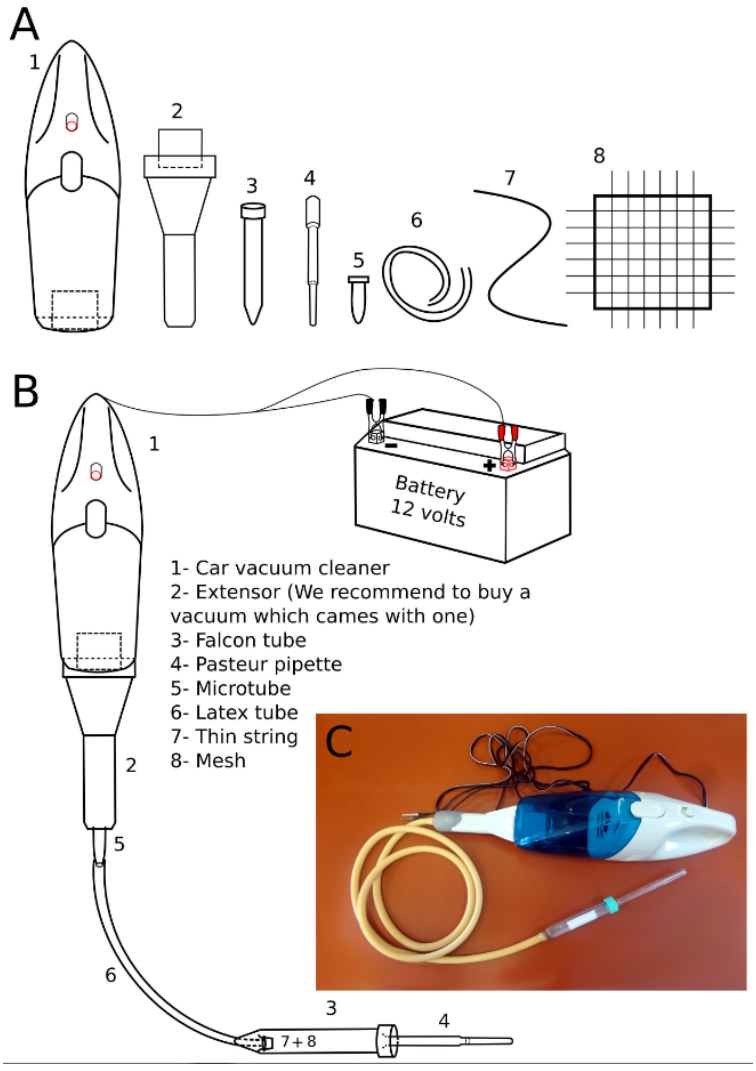

Figure 1. Entomological aspirator scheme showing the required components (A), the assembly design (B), and the final equipment photography (C).

Aspirator Design

The aspirator construction is based on the use of a car vacuum cleaner (preferentially with an extensor), which is coupled to a 1.5 ml microtube (or other small rigid tube), approximately 60-120 cm of a latex or plastic tube with a diameter around 0.5 cm (the first is preferred due to its higher flexibility), a thin string and a fine mesh, a 15 ml Falcon tube, a plastic Pasteur pipette (or other rigid tube as a piece of glass pipette or a Bic pen), epoxy adhesive Araldite and Durepox, two alligator battery clips, and a motorcycle battery (Figure 1).

First of all, the Falcon lid needs to be cut and connected with Araldite to a rigid tube provided, for example, by a Pasteur pipette. Both, the Falcon tube and the Pasteur pipette should have their extremities cut. In parallel, one end of the latex or plastic tube needs to be covered by a mesh with the use of a thin string. This region of the latex or plastic tube should then be passed through the cut end of the

Falcon tube in a way that the mesh is placed within the tube. Araldite glue should be used to connect these pieces firmly, without leaving any air passage. The 1.5 ml microtube needs also to be cut at both of its extremities, and its major diameter end needs to be connected to the vacuum cleaner extensor or, in its absence, directly to the car vacuum cleaner with the use of Durepox. The minor diameter extremity of the cut microtube should be firmly connected to the latex tube. The vacuum cleaner plug to a car's cigarette lighter needs finally to be changed to alligator clips or electrical plugs in order to connect the entire equipment to the motorcycle battery (Figure 1). In order to fasten and easily transport this manufactured entomological aspirator, it is important to leave the extensor unconnected to the main piece, so that the equipment is mounted in the field and readily connected to the battery.

Advantages and Disadvantages

The manufactured entomological aspirator presented here is easily constructed, transported, and handled. Besides, it is safer than a mouth aspirator, allowing the sampling of adult flies in spaces difficult to access with the use of entomological nets. According to flies' availability, it allows effective capture of a great number of species and specimens (tens to hundreds) in a short period of time. This equipment was used by Robe *et al*. (2014) in the sampling of mycophilic drosophilid species across different Brazilian biomes, where it was shown to be highly efficient, leading to the capture of more than 300 individuals encompassing 22 species (besides approximately 180 individuals belonging to undescribed species) in no more than 45 hours (15 collection sites with only 2-4 hours of active search + aspiration activity). The only disadvantages of the equipment refer to battery weights and the need for battery charging before going to the field, although the time of duration of battery generally compensates.

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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 31 December of the year of the issue. The annual issue will, therefore, include material submitted during that calendar year. To help us meet this target date, we request that submissions be sent by 15 December if possible, but articles are accepted at any time. Receipt by 31 December is a firm deadline, due to printer submission schedules.

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)-325-2001; email jthompson@ou.edu; FAX (405)-325-7560.

Submission: Manuscripts should be submitted in Word, with pictures preferably in *jpg. To help minimize editorial costs, proofs will not be sent to authors unless there is some question that needs to be clarified or they are specifically requested by the authors at the time of submission. The editor reserves the right to make minor grammatical, spelling, and stylistic changes if necessary to conform to DIS format and good English usage. 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.

Mutation Notes

Rediscovery and characterization of mus309[D1].

Piergentili, Roberto. Istituto di Biologia e Patologia Molecolari del CNR and Dipartimento di Biologia e Biotecnologie, Sapienza Università di Roma; Rome, Italy.

The *mus309* locus of *Drosophila melanogaster* was first identified in 1981 by Prof. Boyd and coworkers (Boyd *et al.*, 1981) and was originally characterized by three different mutant alleles; they came from a pool of third chromosome, EMS-induced, mutations conferring high sensitivity to mutagens ('mus' meaning mutagen sensitive). Specifically, *mus309* lines are hypersensitive to methyl methanesulfonate (MMS) and nitrogen mustard. It is one of the best characterized *mus(3)* genes, and it is the *Drosophila* homologue of the human gene mutated in Bloom's Syndrome (BS; OMIM entry: #210900). Bloom's Syndrome protein (BLM) is a member of the RecQ family of helicases and its function is to resolve recombinational intermediates, either in meiosis (Walpita *et al.*, 1999) or during somatic DNA repair (Kusano *et al.*, 2001). During a revision of the *Drosophila* stocks of the collection of Emer. Prof. Gatti (Sapienza, Università di Roma), we found three copies of the original *mus309* stocks coming from Prof. Boyd's laboratory. Of them, two were labeled as *mus309[D2]* and *mus309[D3]*, while the third was unreadable as for the allele, but the label clearly stated that it was a *mus309* stock. A complementation test with *mus309[D3]* confirmed the sterility of heterozygous females. Thus, to identify the allele, we sequenced the coding region of both *mus309[D3]* and the undefined line, and compared these sequences with that of the mRNA available in

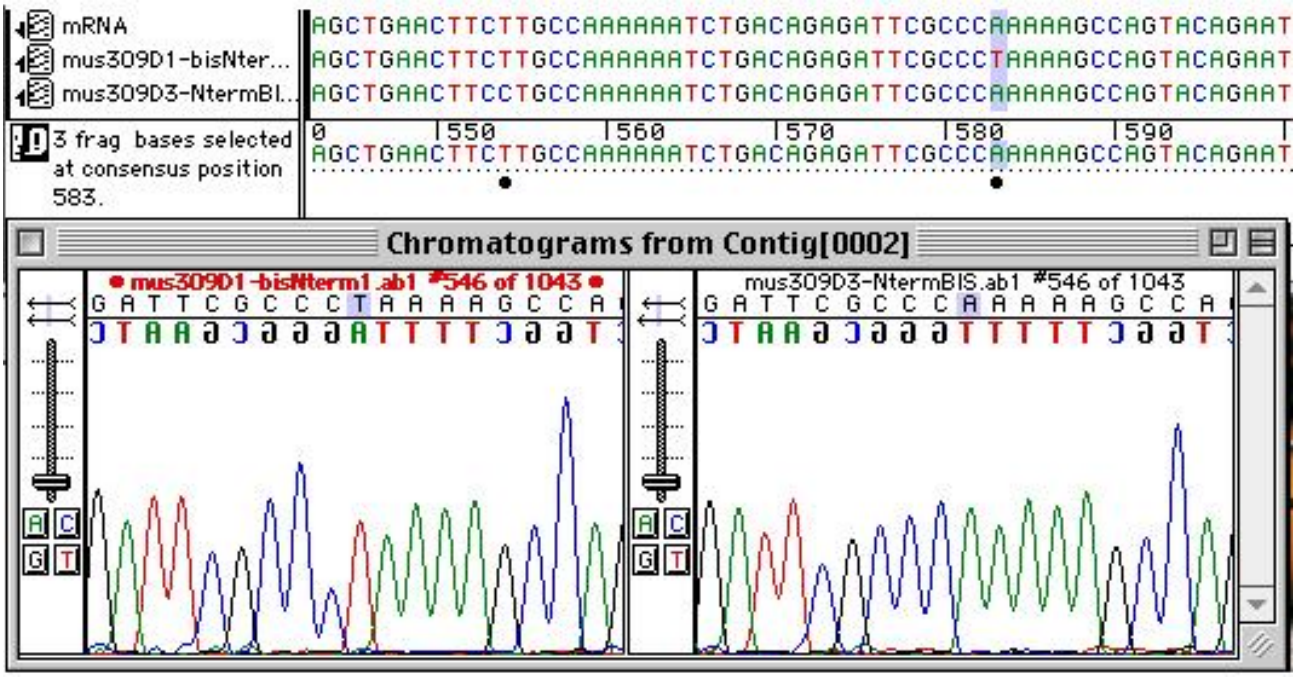

Figure 1. The *mus309[D1]* line presents a precocious stop codon at position Lys-110 inside the dmBLM gene. Top: alignments of mRNA (from FlyBase), *mus309 [D1]*, and *mus309[D3]* in the region of the mutation; the last two sequences were obtained in our laboratory, using external resources. Bottom: chromatograms of the same region from both *mus309* alleles used in the present study.

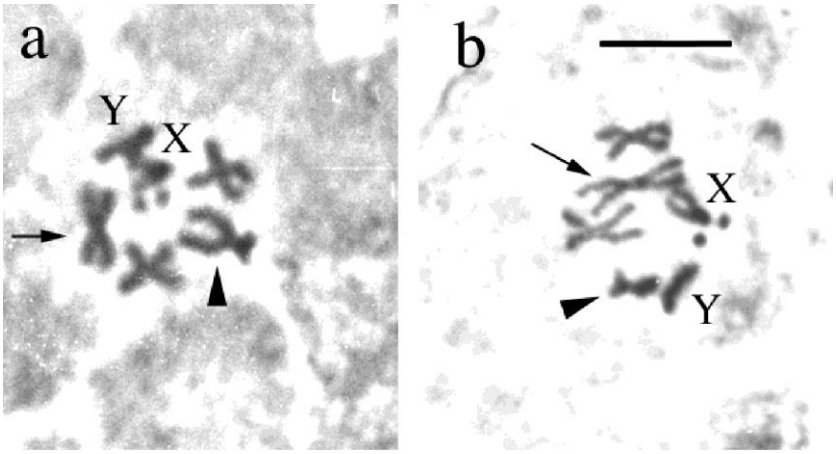

Figure 2. Cytology of *mus309[D1]/ Df(3R)M-kx1* cells. (a) Cells having this genotype show that *mus309* bearing chromosome has a peri-centromeric inversion (arrowhead), if compared to its deficiency bearing homologue (arrow). (b) With a frequency much higher than expected (see also Table 2), a translocation between these two chromosomes occurs, leading to the formation of one big chromosome (derived from the normal one, arrow) plus a small one (derived from the inverted one, arrowhead). The translocations between these two chromosomes always show such a configuration, suggesting that the aberrant recombination always occurs in the same chromosomic region. Both cells in panels come from the same, male brain squash. Bar: 5µm.

FlyBase. As expected, in the *mus309[D3]* sequence both mutations reported in literature are present (Kusano *et al.*, 2001). Instead, the other line showed an A/T transversion changing K110 into a stop codon (Figure 1); this change was confirmed twice, by sequencing both DNA strands.

This mutation is not reported in literature, and due to the age and nature of the stock we found, we are confident that this line indeed represents the *mus309[D1]* line, which was believed lost (McVey *et al.*, 2007). Consequently, from now on, we propose to name this line as *mus309[D1]*. To better characterize the stock, we analyzed the karyotype of the line. Since homozygous flies are now early lethal, we analyzed them in the combination *mus309[D1]/Df(3R)M-kx1*, for which both larvae and adults are available. We found that the *mus309[D1]*-bearing chromosome is characterized by a pericentromeric inversion, clearly recognizable by the shift of the centromeric region towards one chromosome end (Figure 2A). We verified that this inversion is not in the deletion-bearing stock, by crossing it with an Oregon-R line (data not shown).

Table 1. Spontaneous chromosome aberrations in *mus309[D1]* hemizygotes, compared to control.

Line	Total cells scored	Chromatid breaks (%)	Isochromatid breaks (%)	Exchanges (%)	Total aberrations (%)
control (Oregon-R)	1000	0.11	0.38	0.01	0.5
mus309[D1] / Df(3R)M-kx1	605	0.17	7.27	3.3	10.74

Table 2. Distribution of the exchanges inside *mus309/Df(3R)M-kx1* cells.

U-type asymmetric	U-type symmetric	X-type asymmetric	X-type symmetric	More complex exchanges
3	2	4	10	1

Since the *mus309* locus is involved in DNA repair, we also analyzed the line for the presence of spontaneous chromosome damage. For this purpose, we prepared standard brain squashes with colchicine, without adding any chemical compound (MMS, HN2), and stained them with orcein. As illustrated in Table 1, we found that the hemizygous larvae, despite their viability, show a very high number of spontaneous chromosome breaks, compared to controls (overall, more than 20×). As in controls, the class of the

isochromatid breaks (both sister chromatids broken at the same point) is the most represented, but interestingly this genetic combination also shows an excess of chromosome translocations. It is long known that mutated BLM protein in man typically induces an increase in sister chromatid exchanges (Schroeder and German, 1974). Accordingly, we hypothesize that the high number of isochromatid breaks is due to the initiation and subsequent failure of the recombination-mediated repair of single chromatid breaks, which indeed have a frequency comparable to controls. The scored excess of chromosome translocations (> 300× compared to control) further support this idea.

As shown in Table 2, the exchanges scored are not equally distributed among all categories. There is an enrichment in the class of X-type exchanges versus U-type ones. Moreover, X-type symmetric exchanges involve only the inverted chromosome and its homologue (Figure 2B). It is intriguing to conjecture that, because of the imperfect pairing of the two homologues, the inverted chromosome is detected by the cell as something 'unusual'; this in turn would activate the recombination-mediated repair machinery, inducing a high level of somatic recombination between these homologues. The failure to resolve this somatic recombination in a *mus309* mutated background would lead to the over-production of both isochromatid breaks and X-type symmetric exchanges scored.

Acknowledgments: I am grateful to Emer. Prof. M. Gatti (Sapienza – Università di Roma) whose kindness and generosity allowed obtaining the data for the present report.

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A new mutation in *Drosophila parabipectinata.*

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

D. parabipectinata was described by Bock in 1971 and is one of the members of the *D. bipectinata* complex, which is comprised of four closely related and morphologically very similar species: *D. bipectinata*, *D. parabipectinata*, *D. malerkotliana*, and *D. pseudoananassae*. This complex is part of the *ananassae* subgroup of the large *melanogaster* species group (Bock and Wheeler, 1972) of the subgenus *Sophophora*. These species occur throughout Southeast Asia, extending into north eastern Australia, the Indian subcontinent and South Pacific. However, *D. parabipectinata* has restricted geographical distribution as compared to the other three species of the complex. All the four species are sympatric over most of their geographic ranges. All the four species hybridize with each other in the laboratory, and hybrid females are fertile but males are sterile (Mishra and Singh, 2006). *D. parabipectinata* shows incomplete sexual isolation with other members of the *bipectinata* complex (Bock, 1978; Singh *et al*., 1981; Banerjee and Singh, 2012). It shows asymmetrical sexual isolation with *D. bipectinata* and *D. malerkotliana* (Banerjee and Singh, 2012). Results based on interspecific crosses and behavioral studies provide evidence that *D. bipectinata* and *D. parabipectinata* are very closely related species (Bock, 1978; Hegde and Krishnamurthy; 1979, Crossley, 1986; Singh and Singh, 2013, 2014).

A large number of stocks of *D. parabipectinata* established from flies collected from different geographical localities are being maintained in our laboratory. This note describes an x-ray induced mutation in *D. parabipectinata*. For irradiation experiments, the males were taken from a wild type stock collected from Mysore, India, and reared for numerous generations in the laboratory. The newly hatched and two days old wild type males were collected, and these males were kept in a gelatine capsule and were exposed to X-rays under following conditions:-

Target distance – 50 cm
KVP – 120 KVP
Dose rate – 450 r per minute
Total dose received approximately - 1800 r in 4 min.

In each experiment, 50 males were irradiated under similar conditions. The newly-hatched wild irradiated males were allowed to grow for at least 2 to 3 days and were then mated for four days with a first set of 40 virgins (wild type). Similarly 2 day old irradiated males were immediately mated with 50 four day old virgin females. After four days these males were separated and mated with another set of 40 wild virgins. Again after four days, these males were separated and mated again with another set of 40 wild type virgin females. After 12-16 days, F_1 progeny were collected from all the bottles and observed for any variant. Pair mating was made from these F_1 flies. F_2 progeny from vials were carefully examined for any variations from the wild type.

Six males were obtained in one of the vials which showed brownish eye color appearance that resembles *garnet* eye colour sex linked recessive mutation of *D. malerkotliana* (Singh and Singh, 2013). They were crossed with wild type virgin females. Next generation progeny were normal and, when they were pair mated, some of the male progeny showed *garnet* eye color. By making pair matings from these flies, mutant females and males were obtained and a separate stock of garnet eye color could be established. In order to confirm the inheritance pattern, virgin *garnet* eye color females of *D. parabipectinata* were collected from the stock and were mated with wild type virgin males. All the F_1 males showed *garnet* (*g*) eye color phenotype (Figure 1) showing sex linked inheritance. This is the first report of phenotypic marker in this species.

The garnet eye color of *D. parabipectina* shows resemblance with that of garnet eye color mutation of *D. malerkotliana* (Singh and Singh, 2013). Since both the species belong to the *bipectinata* species complex and are closely related and same mutation has been induced by X-rays, the loci may be very susceptible to X-rays in both the species.

Figure 1. Garnet eye color phenotype in *Drosophila parabipectinata*.

Acknowledgments: We are grateful to Apex Hospital and their staff members for providing the X-rays facility. The financial assistance in the form of Meritorious Fellowship to A.S. and UGC-BSR Faculty Fellowship Award to B.N.S. from the University Grants Commission, New Delhi, is gratefully acknowledged.

References: Banerjee, P., and B.N. Singh 2012, Genetica 140: 75–81; Bock, I.R., 1971, Univ. Texas Publ. 7103: 273-280; Bock, I.R., 1978, Aust. J. Biol. Sci. 31: 197-208; Bock, I.R., and M.R. Wheeler 1972, Univ. Texas Publ. 7213: 1–102; Crossley, S.A., 1986, Anim. Behav. 34: 1146-1159; Hegde, S.N., and N.B. Krishnamurthy 1979, Aust. J. Zool. 27: 421-431; Mishra, P.K., and B.N. Singh 2006, J. Zool. Syst. Evol. Res. 44: 175-179; Singh, A., and B.N. Singh 2013, Behav. Proc. 96: 79-87; Singh, A., and B.N. Singh 2014, Indian J. Exp. Biol., in press; Singh, B.N., and A. Singh 2013, Dros. Inf. Serv. 96: 221-222; Singh, B.N., Y.N. Dwivedi, and J.P. Gupta 1981, Indian J. Exp. Biol. 19: 898-900.

A new mutation of PDA synthase, *sepia,* isolated from wild *Drosophila melanogaster*.

Tabios, Myles[1], Louis Boell[2,3], and Floyd A. Reed[1,2]. [1]Department of Biology, University of Hawai'i at Mānoa, Honolulu, HI 96822; [2]Department of Evolutionary Genetics, Max Planck Institute for Evolutionary Biology, 24306 Plön, Germany; [3]Current address: Department of Cell and Developmental Biology, John Innes Centre, Norwich NR4 7UH, UK.

A natural mutation of *sepia* was identified among the descendants of *Drosophila* collected in Kiel, Germany in 2009. The flies were inbred for six generations with a chain of repeatedly isolated single sib-pair matings. The phenotype was recognized as consistent with classical sepia (CG6781; Bridges and Morgan, 1923) and the location was confirmed by a complementation test with *se*[1]. Part of the PDA synthase gene region was amplified and sequenced with a set of primers (5'-CTATCACCACTTGCATCTCTGGACC, 5'-GGAACCGGTTATGGACTGCATTTAT, 56°C annealing, Kim *et al*., 2006) and the nature of the mutation was found to be a 40 base pair frameshift deletion in the second exon from position 3L:8521107..8521146 [+] (bases 461-500 of the CDS), which creates a premature stop codon at codon position 157 (the *sepia* gene product is normally 243 amino acids long). The deleted sequence is 5'-AGAATGCCCGTCTGCTCGCCACCAAAGAATTCCGTACCAC. A set of diagnostic primers was designed with one primer within the deleted region that can test for the presence of the allele in heterozygotes by PCR (5'-GTGGGTAGAGCCAGGAAACC, 5'-TCTGCTCGCCACCAAAGAAT, 60°C annealing). This allele is likely an amorph and is only the second molecularly characterized mutation of *sepia.* The allele, *se*^{Kiel}, has been deposited at the Bloomington Drosophila Stock Center (Bloomington, IN 47405) as stock 55131 and is listed in FlyBase (St. Pierre *et al*., 2014) as http://flybase.org/reports/FBal0294757.html. Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study.

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New mutants of *Drosophila mediopunctata*.

Batista, M.R.D.[a], V.M. Birsenek[1], F. Pradella[2], A.S. Farias[2], and L.B. Klaczko[1].
[1]Laboratório de Biodiversidade Genética e Evolução de *Drosophila*, Departamento de Genética, Evolução e Bioagentes, Instituto de Biologia, Universidade Estadual de Campinas – UNICAMP, Cx. Postal 6109, Campinas, 13083-970 SP, Brazil; [2]Neuroimmunomodulation Group, Departamento de Genética, Evolução e Bioagentes, Instituto de Biologia, Universidade Estadual de Campinas – UNICAMP, Cx. Postal 6109, Campinas, 13083-970 SP, Brazil. E-mail: LBK@unicamp.br

For almost three decades we have been working with *Drosophila mediopunctata*, a species of the *tripunctata* group (Yotoko *et al*., 2003), studying various aspects of its biology. In this note we describe new X-ray induced mutations in this species. We exposed three hundred males from two homokaryotypic strains ([150 males ITA-24P, phenotype: wild; chromosome karyotypes: II: *DA-PA0/DA-PA0*; III: *St/St*; IV: *St/St*; V: *St/St*; X: *St/Y*]; [150 males ITC-229ET, phenotype: wild; chromosome karyotypes: II: *DI-PB0/DI-PB0*; III: *St/St*; IV: *St/St*; V: *St/St*; X: *St/Y*]) to three X-ray doses (2200 rad; 4400 rad; and 6600 rad; equivalent to the absorbed dosage suggested by Marques *et al*., 1991). After one day of recovery, irradiated males were individually crossed with virgin females from the same strain or from a strain (CR-27A or CR-32C) with four visible mutations marking each major autosome (as described by Hatadani *et al*., 2004). In the first generation, we selected dominant visible mutations and those inherited in sexual chromosomes. Then, the F1 progeny were allowed to mate (brother-sister mating) to select recessive mutants in the F2 using the marked chromosomes.

We obtained three new *Delta* (Δ) alleles (Δ_6 and Δ_7 located in *DA-PA0* chromosome from different X-ray mutated males ITA-24P; Δ_8 located in *DI-PB0* from an X-ray mutated male from ITC-229ET). *Delta* is a dominant mutation that produces deltas at junctions of wing veins, or wing veins with margins (Figure 1). We also found flies with yellow body color, named "*louro*" (ll_3) mutation from irradiated strain ITC-229ET flies (Figure 2). This mutation is recessive and linked to the X chromosome. It is probably homologous to *D. melanogaster yellow* gene. Our perspective is to develop new mutations and balanced strains with visible and cytological markers in all chromosomes in this species.

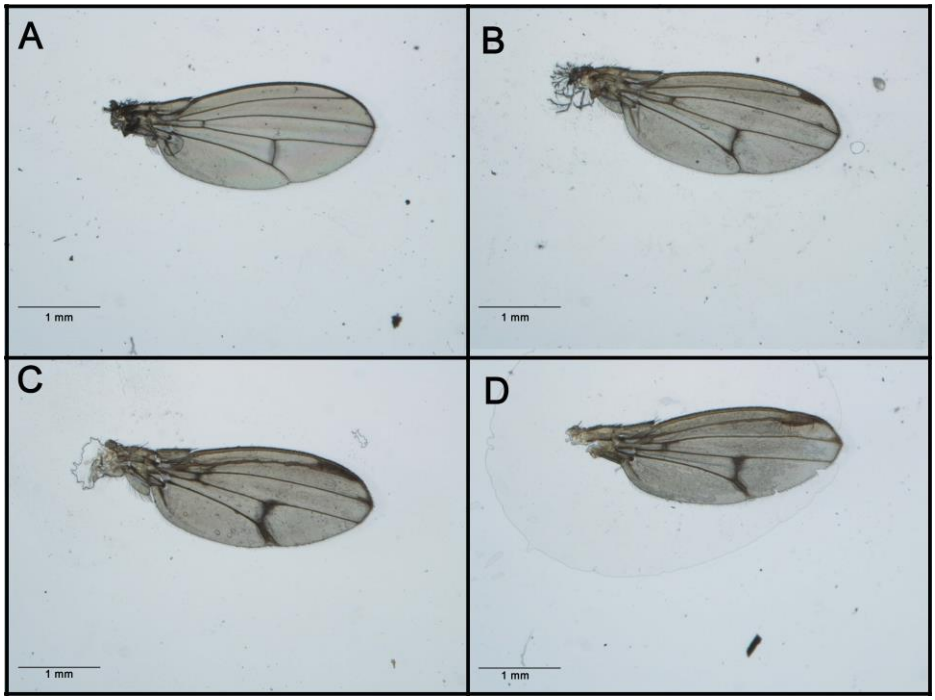


Figure 1. *Drosophila mediopunctata Delta* mutant wings. A) wild type; B) Δ_5; C) Δ_7; D) Δ_8.

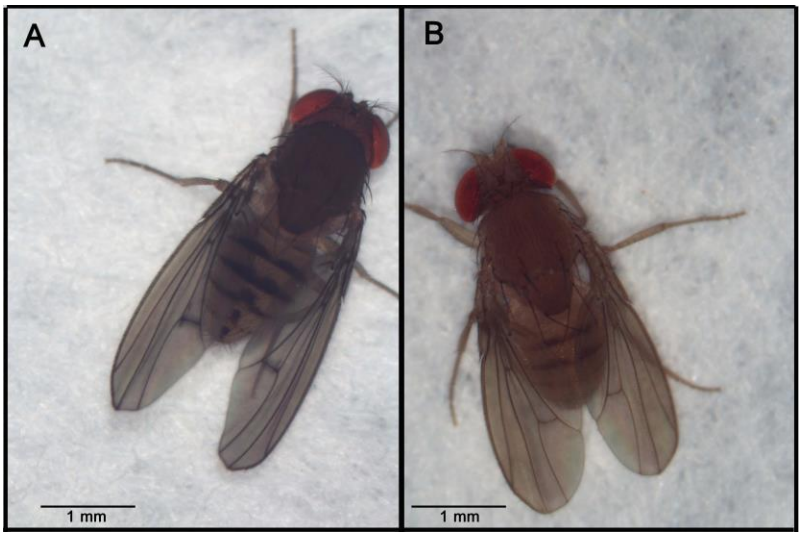
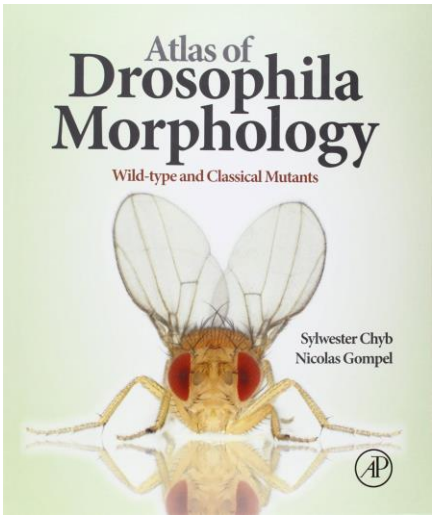


Figure 2. Adult males of *Drosophila mediopunctata*: A) *Drosophila mediopunctata* wild type; B) "*louro*" (*ll)* mutant (yellow body color).

Acknowledgments: We would like to thank Claudete do Couto and Klélia A. de Carvalho for technical help. We also thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq); Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES); Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP); Fundo de Apoio ao Ensino e à Pesquisa (FAEPEX-UNICAMP) for financial support.

References: Hatadani, L.M., J.C.M. Batista, W.N. Sousa, and L.B. Klaczko 2004, Heredity 93: 525-534; Marques, H.V.S., A.B. Carvalho, C.A. Elias, and L.B. Klaczko 1991, Dros. Inf. Serv. 70: 280; Yotoko, K.S.C., H.F. Medeiros, V.N. Solferini, and L.B. Klaczko 2003, Mol. Phylogenet. Evol. 28: 614-619.

New Book Announcement



Atlas of Drosophila Morphology

Chyb, Sylvester, and Nicholas Gompel. 2013. 225 + xvii pp. ISBN: 978-0-12-384688-4. $136.62. Academic Press, Elsevier Publishing.

Review by Lee Ehrman, State University of New York at Purchase.

Useful, extremely so, an adjective summing up this elegantly produced atlas. And this evaluation comes from an aging professor who has instructed Genetics Laboratory, an undergraduate junior level course, for approaching half a century.

Glossily-paged and large (approximately 11¼" × 9¼"), this hardcover is ringed and opens flatly for more-than-one student at-a-time perusal. The wild-type *D. melanogaster* phenotype is presented for all this hemimetabolous insect's life stages, along with that of the D. melanogaster subgroup species: *simulans*, *sechellia*, *mauritiana*, *teissieri*, *erecta*, *orena*, *yakuba*, and *santomea*. Other than possibly the sibling species, *D. simulans*, I suspect that this section will prove less utilized as a source of reference than the six marker mutant sections. These are divided into: bristles, wings, other appendages, eyes (shapes and colors), and bodily alterations. All this is followed by several plates depicting side-by-side comparisons among all these oft-employed teaching and research variants. Twenty-one balancer chromosomes are fully annotated in every known detail, a separate illustrated plate for each amply captioned.

In his gracious Foreword, Peter Lawrence calls this long-overdue atlas, lovely. He is correct. It is easier to use repeatedly, though less comprehensive, than our long relied upon "big red book" (by Lindsley, Grell, and Zimm, 1992, Academic Press: NY, for one edition).

Admittedly biased, I must state that these colorful animals are simply beautiful!

Historical Postscript

Lee Ehrman invited us to add this historical postscript sent to Jim Thompson as a hand-written note, dated 10 July 2014.

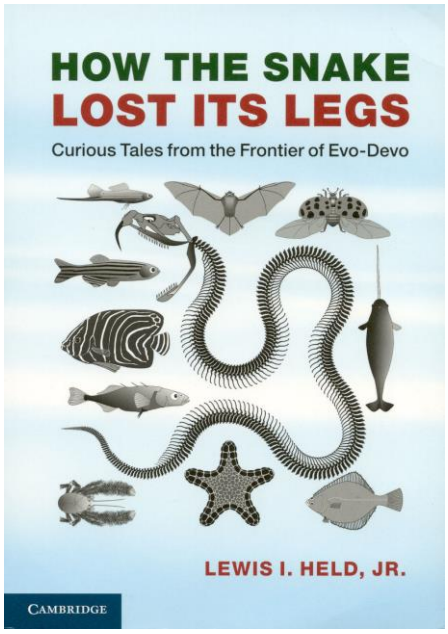
Jim,

You may be interested, along with "DISers", in this story. When I was a graduate student at Columbia, Th. Dobzhansky, my mentor, told me – with a giggle – of a doctoral oral defense at which he was the external examiner. The candidate referred to "… melanogasters and other insects."

Lee Ehrman

School of Natural and Social Sciences
Purchase College
State University of New York
735 Anderson Hill Road
Purchase, NY 10577-1402

New Book Announcement



How the Snake Lost Its Legs: Curious Tales from the Frontier of Evo-Devo.

Held, Lewis I, Jr. 2014, 285 pp. Cambridge University Press, ISBN-13: 978-1-107-62139-8 (paperback); ISBN-10: 1107621399 (hard cover).

Education too often focuses on teaching facts to students. But in science, where our knowledge base is dynamic, learning how to think about unknowns may be as important, if not more so. Nature often does not take the most logical path. Being aware of that simple truth can open our minds to surprising and valuable discoveries. That, in a sense, is a take-home lesson from this wonderful new book by Lewis Held.

Have you ever asked how the fly tattooed its arms or why it twirls its penis? How do animals decorate their skin? How did a bat get its wings or a bee its pollen basket? Why does the centipede always have an odd number of segments? Clear and often surprising answers to these puzzles are rooted in carefully described genetic mechanisms. Detailed diagrams throughout were done by the author. They show the developmental pathways and phenotype changes for characters like the title story about the transition from legged lizards to legless snakes via life in underground tunnels. The fact that many of these insights come from recently recovered fossils or new data from gene sequencing not only makes this an up-to-date, unfolding story, but it shows how quickly our understanding of biological processes and evolutionary history can grow.

Not only is it quite enjoyable to read, it is full of valuable reference sources, as are all of Dr. Held's previous works. The glossary and bibliography make up almost half of the volume and, by themselves, are worth the cost of the book ($35.99 paperback, according to one source).

Teaching Notes

Genetic drift leading to losses or fixations of neutral alleles.

Clendenin, Heather R., Constance R. Santangelo, Rebecca L. Tyo, Joshua C. Carr, Jennifer M. Kiser, and R.C. Woodruff. Department of Biological Sciences, Bowling Green State University, Bowling Green, Ohio 43403.

Peter Buri (1956) studied the genetic drift of two neutral alleles (*bw*^{1} *and bw*^{75}) of the brown (*bw*) locus of *D. melanogaster*. Using 107 bottle populations of eight females and eight males per bottle, in 19 generations Buri was able to observe 28 fixations and 30 losses of the *bw*^{75} allele (see Table 13 in Buri, 1956). We have previously observed that 55 years after the Buri experiment the *bw1* and *bw*^{75} alleles are still almost neutral in the three genotypes: 1) *bw*^{1}/*bw*^{1};*st/st* white-eyed flies, 2) *bw*^{1}/*bw*^{75};*st/st* yellow-eyed flies, and *bw*^{75}/*bw*^{75};*st/st* orange-eyed flies (Woodruff and Boulton, 2011); the *bw*^{1}/*bw*^{1};*st/st* flies had 98% of the fitness of the *bw*^{1}/*bw*^{75};*st/st* and *bw*^{75}/*bw*^{75};*st/st* flies. See Lindsley and Zimm (1992) for a description of the mutant genes used in this study.

It was the objective of this teaching exercise to determine if genetic drift of the *bw*^{1} and *bw*^{75} alleles can be observed in short-term, smaller population experiments (four females and four males per vial), leading to fixations and losses of these alleles.

We began this experiment with a pilot run that had a starting frequency of 0.5 for the *bw*^{1} and *bw*^{75} alleles in five vials and that was run for eight generations. At generation zero, we mated four virgin *bw*^{1}/*bw*^{75};*st/st* virgin females with four *bw*^{1}/*bw*^{75};*st/st* males in each of five vials; in the following, we will not include the *st* (scarlet eyes) gene in our crosses or discussions. The progeny were all counted each generation at day 18 for white-eyed flies (*bw*^{1}/*bw*^{1}), yellow-eyed flies (*bw*^{1}/*bw*^{75}), and orange-eyed flies (*bw*^{75}/*bw*^{75}). Then four females and four males were collected at random each generation, as described in Buri (1956), by lining up the progeny and picking the first four females and the first four males to be used in the next generation for a total of eight generations. Because of genetic drift, we predicted that the frequency of heterozygotes would go down with time (Hedrick, 2011) and that the frequency of the *bw*^{1} and *bw*^{75} alleles would not change significantly over time. The results of this experiment for heterozygotes over time are shown in Figure 1.

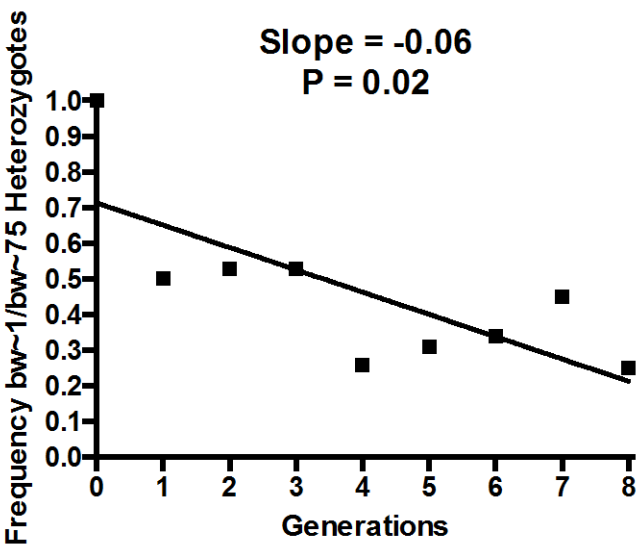


Figure 1. Change in the frequency of heterozygotes over time.

As predicted, the frequency of heterozygotes (*bw*^{1}/*bw*^{75}) did go down with time; the slope was significantly different from zero (P = 0.02). Unexpectedly, the frequency of the *bw*^{1} allele also went down significantly over time (Figure 2). In addition, one line went to fixation for the *bw*^{75} allele (the *bw*^{1} allele was lost).

Next we ran a total of 49 vials for a shorter period of time (three generations). The vials were set up as discussed above and the results for the change in heterozygotes (*bw*^{1}/*bw*^{75}) over time are shown in Figure 3.

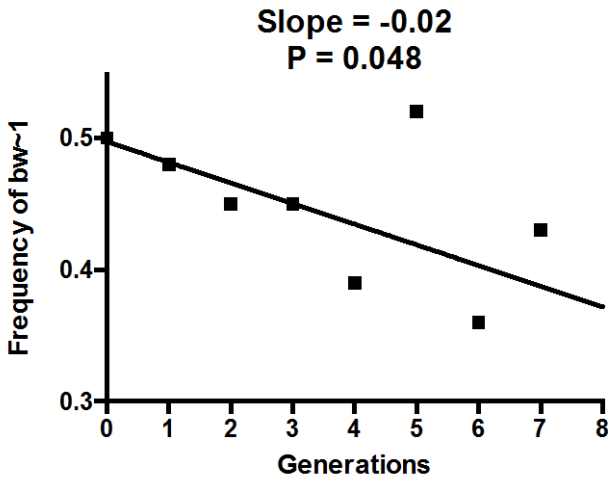


Figure 2. Change in the frequency of the *bw*^{1} allele over time.

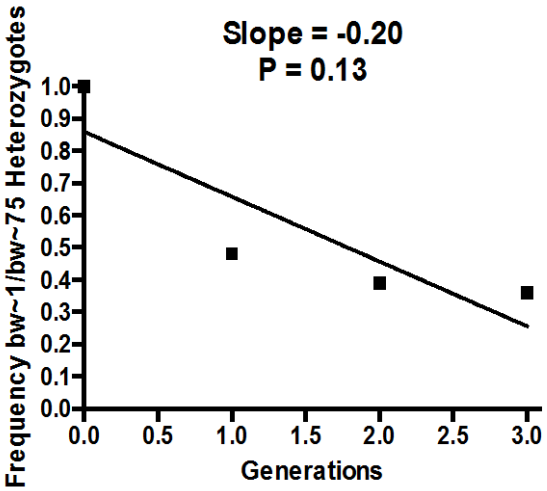


Figure 3. Change in the frequency of heterozygotes over time.

The frequency of heterozygotes did go down with time, but the slope was not significantly different from zero (P = 0.13). In addition, the frequency of the *bw*^{1} allele went down with time (Figure 4), but the decrease over time was also not significant (P = 0.053).

The results of this study show that genetic drift can be observed for the *bw*^{1} and *bw*^{75} alleles, but one would need more than three generations (at least four) to see significant changes in allele frequencies over time. We also observed that it was difficult to differentiate the yellow-eyed (*bw*^{1}/*bw*^{75}) flies from the orange-eyed flies (*bw*^{75}/*bw*^{75}) in older individuals. Hence, in the future we will screen for the losses or fixations of the *bw*^{1} allele by following only the *bw*^{1} allele in white-eyed (*bw*^{1}/*bw*^{1}) flies over time in a much larger number of vials. The down side of this proposed experiment is that we will not be able to follow the change in frequency of heterozygotes over time.

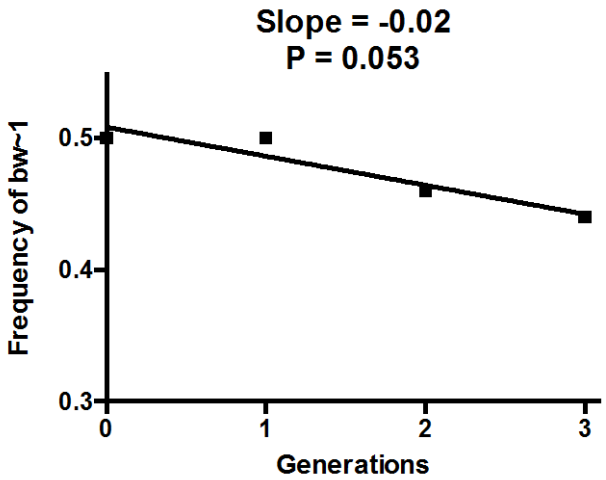


Figure 4. Change in the frequency of the *bw*^{1} allele over time.

A class discussion of the results of this teaching exercise could include: 1) The observed drop in the frequency of heterozygotes for neutral alleles over time is usually faster than expected; for example, Buri (1956) observed that the drop in the frequency of heterozygotes over time in his experiment was more in line with a population size of nine, instead of 16. One might ask students why this would be expected to be true. This is mainly because the number of flies mating within a vial will be less than the total of eight. This reduced number is called the effective population size (see a discussion in Hedrick, 2011). 2) Since the loss of heterozygotes and rare alleles over time is expected to be faster in small populations, this can be a problem for endangered species. Students might be asked why this is true. The loss of genetic variation would not allow endangered species to respond to changes in their environment, such as the introduction of new parasites (Frankham *et al.*, 2010).

References: Buri, P., 1956, Evolution 10: 367-402; Frankham, R., J.D. Ballou, and D.A. Briscoe 2010, *Introduction to Conservation Genetics*. Cambridge University Press; Hedrick, P.W., 2011, *Genetics of Populations*. Jones and Bartlett Publishers, Sundbury, MA; Lindsley, D.L., and G.G. Zimm 1992, *The Genome of* Drosophila melanogaster. Academic Press, New York; Woodruff, R.C., and A.M. Boulton 2011, Dros. Inf. Serv. 94: 167-169.

Heterosis and the recovery of *Drosophila melanogaster* triplo-X females.

Kiser, Jennifer M., Joshua C. Carr, Heather R. Clendenin, Constance R. Santangelo, Rebecca L. Tyo, and R.C. Woodruff. Department of Biological Sciences, Bowling Green State University, Bowling Green, Ohio 43403.

Heterosis (hybrid vigor) improves the fitness of hybrids over their parents, including an increase in reproductive success (Lippmann and Zamir, 2006). We used *D. melanogaster* to test the hypothesis that heterosis also allows for an increased recovery of hybrid progeny that have an extra X chromosome (triplo-X females). Our hypothesis is that triplo-X females, which usually do not survive as adults (Lindsley and Zimm, 1992), will be recovered at a significantly higher frequency in first generation progeny of crosses between males and females from separated, unrelated, stocks, than in subsequent generations. We also predict that hybrid progeny from crosses between separate stocks will have a higher frequency of triplo-X progeny than progeny from crosses between males and females from the same, long-term stock.

To test this hypothesis, we first crossed C(1)DX, *y f* / Y females with w^{1118} / Y males and screened for F1 triplo-X (XXX) females. In this cross, parental females have two X chromosomes attached to a single centromere and contain the markers *y* (yellow body color) and *f* (forked, short bristles). In addition, the males contain the w^{1118} X-linked mutation that causes a white-eyed phenotype. Because of the attached-X chromosome in females, and the Y chromosome, which is present in both males and females, female progeny receive the attached-X chromosome from their mothers and the male progeny receive the w^{1118} X chromosome from their fathers. Females receive their Y chromosome from their fathers, and males receive one from their mothers. See Lindsley and Zimm (1992) for discussions of mutants and the attached-X stock. The F1 triplo-X females from this cross have red eyes, long bristles, and grey body color. The frequency of triplo-X female progeny from this cross was significantly reduced over seven generations (Figure 1, P = 0.04 for the slope of the regression line being zero).

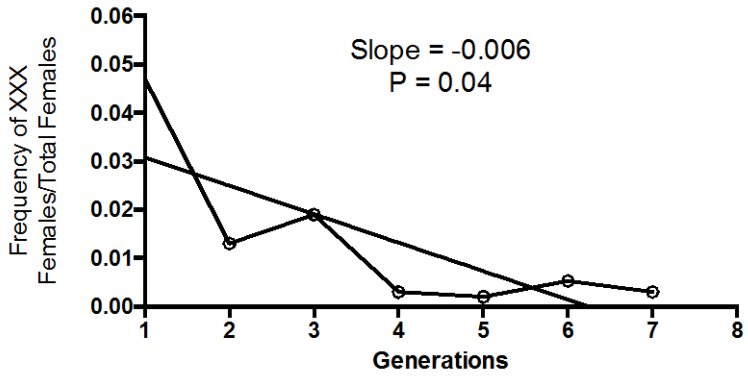


Figure 1. Frequency of triplo-X progeny over time.

We next tested our heterosis hypothesis with five additional crosses. First, we mated C(1)DX, *y f* / Y females with Canton-S (wild type) males that had been maintained in separate stocks and again observed that the triplo-X females were recovered in a significantly higher frequency in the F1 progeny than in the F2 progeny [44/679(6.5%) *vs*. 11/619 (1.8%); P < 0.0001] or the F3 progeny [44/679(6.5%) *vs*. 3/513(0.58%); P < 0.0001) (Figure 2), although the slope of the regression line for the three generations was not significantly different from zero (P = 0.21).

As controls, we also measured the frequency of triplo-X females within two stocks (interline crosses): 1) C(1)DX, *y f* /Y females with Binscy / Y males (the Binscy X contains the Bar-eyed dominant mutation), and

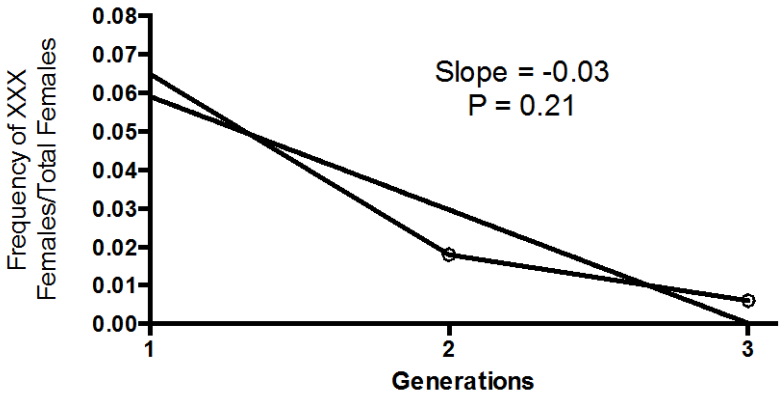


Figure 2. Frequency of triplo-X progeny over time.

2) C(1)DX, *y w f* / Y females with *Per*$^{+}$ (wild type) males. We observed 0 XXX in 1298 progeny in stock 1 and 0 XXX in 673 progeny in stock 2, showing that triplo-X females were recovered within stocks, in the absence of heterosis, at a low frequency.

We then performed inter-stock crosses of stock 1 females (C(1)DX, *y f* / Y) with stock 2 males (*Per*$^{+}$) (Cross 1/2), and stock 2 females (C(1)DX, *y w f* / Y) with stock 1 males (Binscy) (Cross 2/1). It was our hypothesis that the frequencies of triplo-X progeny would be significantly higher in the inter-stock crosses than in the interline controls. In support of this hypothesis we observed 7/309 (2.3%) XXX progeny in Cross 1/2 ($P < 0.0001$, in comparison with 0/1971 from the interline crosses) and 1/317 (0.3%) triplo-X progeny in Cross 2/1 ($P = 0.41$, in comparison with 0/1971 from interline crosses). The latter non-significant results are probably due to the low observed viability of the C(1)DX, *y w f* / Y females, which did not allow for development of the triplo-X progeny. For example, the average number of progeny per vial for C(1)DX, *y f* / Y females was 17.40, whereas for C(1)DX, *y w f* / Y females it was 11.36.

In addition, the frequency of triplo-X progeny from generation one was significantly higher ($P = 0.003$) than from generation two in Cross 1/2, but was not significantly higher in Cross 2/1 ($P = 0.41$) (Figure 3).

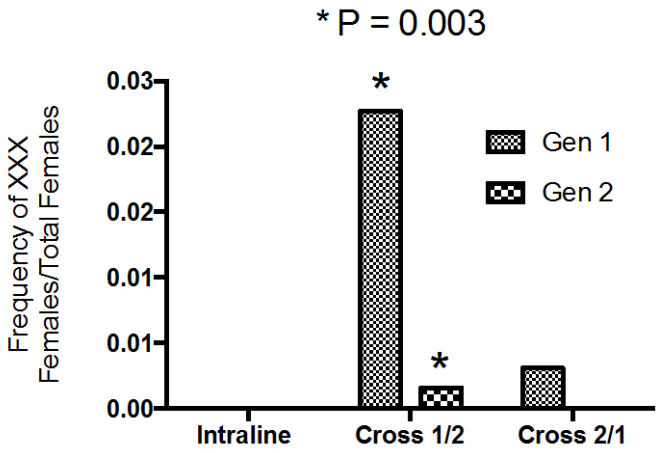


Figure 3. Frequency of triplo-X progeny from crosses within a line and between lines.

In summary, the results from this study support the hypothesis that heterosis increases the recovery of *D. melanogaster* females with an extra sex chromosome (triplo-X females).

A class discussion of the results of this teaching exercise could include: 1) What are the possible genetic mechanisms of heterosis? There are two main mechanisms: the superior fitness of heterozygotes and/or the masking of recessive deleterious mutations in hybrids (Crow, 2008). 2) It is of interest to consider: If heterosis occurs in humans, would this increase the survival of offspring with extra chromosomes, such as trisomy-21 (Down syndrome), trisomy-13 (Patau syndrome), and trisomy-18 (Edwards syndrome) (Schaefer and Thompson, 2014)?

References: Crow, J.F., 2008, Annual Review of Genetics 42: 1-16; Lindsley, D.L., and G.G. Zimm 1992, *The Genome of* Drosophila melanogaster: Academic Press, New York; Lippman, Z.B., and D. Zamir 2006, TRENDS in Genetics 23: 60-66; Schaefer, G.B., and J.N. Thompson, jr. 2014, *Medical Genetics: An Integrated Approach*. McGraw-Hill, New York.

Study of linkage between *miniature* and *singed* genes in *Drosophila melanogaster*.

Mestres, F.[1,*], and C. Arenas[2]. [1]Dept. Genètica, Universitat de Barcelona, Barcelona (Spain); [2]Dept. d'Estadística, Universitat de Barcelona, Barcelona (Spain). [*]Corresponding author: fmestres@ub.edu

We have developed a practical exercise for undergraduate students whose main aim is to identify, using genetic crosses, a pair of *D. melanogaster* mutations (*miniature* and *singed*). Each student receives a vial with the problem strain containing two unknown mutations. The first step is to observe and describe both mutations. Then, the students carry out genetic crosses between mutant and normal strains:

(P) ♀ mutant strain × ♂ normal strain (P) ♀ normal strain × ♂ mutant strain

A different offspring is expected in these crosses: in the first one we will obtain normal females and *m sn* males, whereas in the second all individuals will present normal phenotype. It is possible to deduce that both are sex linked mutations. With this information and to simplify the amount of work, only F_1 individuals from the first cross will be used (m^+sn^+ / *m sn* × *m sn* / Y *chrom.*) to obtain the F_2 generation. The results expected for this F_2 generation would be:

		m sn	Y *chrom.*
Parental type	m^+sn^+	m^+sn^+ / *m sn*	m^+sn^+ / Y *chrom.*
Parental type	*m sn*	*m sn* / *m sn*	*m sn* / Y *chrom.*
Recombinant type	m^+sn	m^+sn / *m sn*	m^+sn / Y *chrom.*
Recombinant type	*m* sn^+	*m* sn^+/ *m sn*	*m* sn^+/ Y *chrom.*

By counting the number of *miniature* (recombinant type), *singed* (recombinant type), *miniature-singed* (parental type) and *normal* (parental type) flies it is possible to estimate the recombination frequency between both genes. Knowing the phenotype, their chromosomal location (X chromosome) and the genetic distance between both mutations, it is possible to identify them by finding all this information in a *Drosophila melanogaster* genetic map (Gardner *et al*., 1991; Russell, 1992; Griffiths *et al*., 1996; Klug and Cummings, 1997; Pierce, 2009).

Additionally, a statistical analysis can be carried out to compare the number of expected F_2 individuals with those observed in the experiment. As the distance between both genes is 15.1 m.u., then the expected percentages for each phenotype would be: *normal* (42.45%), *miniature-signed* (42.45%), *miniature* (7.55%) and *singed* (7.55%). Multiplying the frequency of each class by the total number of individuals obtained in the F_2 it is possible to estimate the expected number of flies for each class. Finally, a χ^2 test can be computed to ascertain whether there are significant differences between expected and observed number of individuals.

References: Gardner, E.J., M.J. Simmons, and D.P. Snustad 1991, *Principles of Genetics*. John Wiley and sons, Inc., N.Y.; Griffiths, A.J.F., J.H. Miller, D.T. Suzuki, R.C. Lewontin, and W.M. Gelbart 1996, *An Introduction to Genetic Analysis*. W.H. Freeman and Co, N.Y. 6th ed.; Klug, W.S., and M.R. Cummings 1997, *Concepts of Genetics*. Prentice Hall International, Inc., Upper Saddle River, N.J.; Pierce, B.A., 2009, *Genetics: a Conceptual Approach*. W.H. Freeman and Co, N.Y. 3rd ed.; Russell, P.J., 1992, *Genetics*. Harper Collins Pub., N.Y. 3rd ed.

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Drosophila melanogaster as a model system to measure the effect of inbreeding depression on the viability of offspring of first cousin matings.

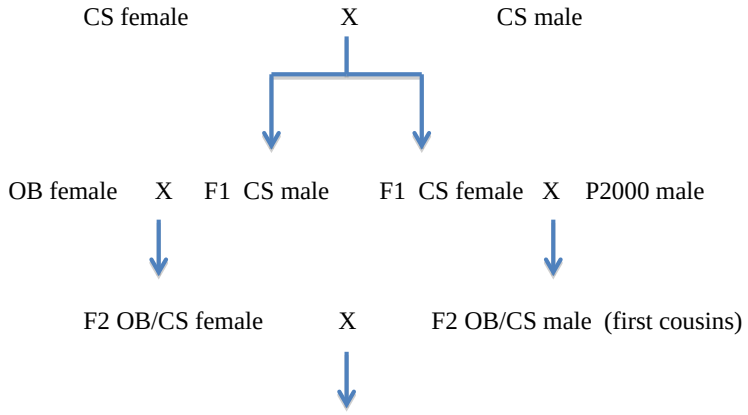
Carr, Joshua C., Jennifer M. Kiser, Heather R. Clendenin, Constance R. Santangelo, Rebecca L. Tyo, and R.C. Woodruff. Department of Biological Sciences, Bowling Green State University, Bowling Green, Ohio 43403

The offspring of first cousin matings in humans have a significant increase in infant mortality and congenital anomalies in comparison to offspring of matings between nonrelatives (Bennett *et al.*, 2002; Bittles and Neel, 1994; Stoltenberg *et al.*, 1999a, b; Helgason *et al.*, 2008). These decreases in fitness of the progeny of first cousin matings are caused mainly by homozygosis of deleterious recessive alleles that are present in close kin (inbreeding depression) (Charlesworth and Willis, 2009; Hedrick, 2011).

Inbreeding depression, and evidence to support it, was described by Charles Darwin in a chapter entitled "On the Good Effects of Crossing and on the Evil Effects of Close Interbreeding" in volume two of his 1868 book, *The Variation of Animals and Plants under Domestication* (Darwin, 1868). Darwin reported that the progeny of cross-fertilized plants were more vigorous that the progeny of self-fertilizing plants (Pannell, 2009; Berra *et al.*, 2010). These observations caused Darwin to be concerned that his offspring with his first cousin, Emma Wedgwood, would have reduced health (Berra *et al.*, 2010). Emma and Charles shared their grandparents Josiah and Sarah Wedgwood. Three of Darwin's ten children died before the age of ten.

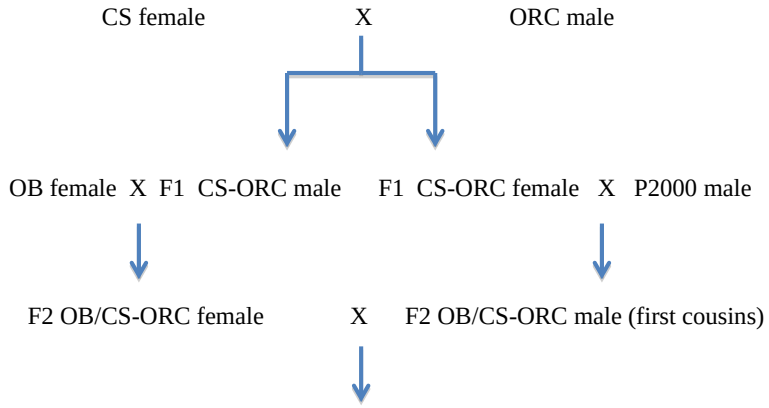
It is the objective of this teaching exercise to determine if *Drosophila melanogaster* can be used as a model system to identify the expected reduction in fitness (measured here by the reduction in viability; the average number of progeny per matings) of the progeny of first cousin marriages. We observed that *D. melanogaster* can be used to observe inbreeding depression in these consanguineous matings.

We performed two first cousin matings: one (Mating Scheme A) with the original, parental cross between flies of the same genetic stock and one (Mating Scheme B) with the original, parental cross between flies of unrelated genetic stocks. We observed inbreeding depression in Mating Scheme A, but not in Mating Scheme B. In the description of these crossing schemes below, the following wild-type stocks were used: Canton-S (CS) is a long-term, wild-type stock collected in Canton, Ohio, in the 1920s (Lindsley and Zimm, 1992); OBL1&2 (OB) was initiated by mixing six mated females from a Perrysburg, Ohio, population that were captured by sweeping bananas in 2010; Perrysburg 2000 (P2000) was initiated by mixing six mated females from a Perrysburg, Ohio, population in 2000; Perrysburg 2013 (P2013) was initiated by mixing six mated females from a Perrysburg, Ohio, population in 2013; and Oregon-R-C (ORC) was obtained from the University of Indiana Stock Center. Each stock was maintained in a half-pint milk bottle on standard cornmeal, molasses, and agar medium. In these mating schemes, single parents were crossed in vials, females were virgins, and the F2 OB/CS flies in Mating Scheme A and the OB/CS-ORC flies in Mating Scheme B were first cousins.



Mating Scheme A:

Count the number of F3 progeny per first cousin mating. As non-cousin controls, F2 OB/CS females were mated with P2013 males (non-cousin control I), F2 OB/CS males were mated with P2013 females (non-cousin control II), and F3 progeny per mating were counted.



Mating Scheme B:

Count the number of F3 progeny per first cousin mating. As non-cousin controls, the F2 OB/CS-ORC females were mated with P2013 males (non-cousin control I), F2 OB/CS-ORC males were mated with P2013 females (non-cousin control II), and F3 progeny per mating were counted.

It was our hypothesis that inbreeding depression would be higher in the cousin crosses of Mating Scheme A than in Mating Scheme B, since the parental cross in Mating Scheme A was between flies from the same CS bottle, whereas in Mating Scheme B the parental cross was females and males from two separate stocks, CS and ORC. This hypothesis was supported by the following results.

Results

Mating Scheme A: A total of 48 F2 vials were scored for the cousin crosses, with a mean ±s standard error of 39.90 ± 1.812 progeny per vial; a total of 45 F2 vials were scored for the non-cousin control I, 46.20 ± 1.721; and a total of 45 vials were scored for the non-cousin control II, 46.22 ± 1.978 (Figure 1). An analysis of variance showed that these results are significantly different (P = 0.02). By t tests, both of the non-cousin crosses were also significantly different from the cousin cross (I, P = 0.01; II, P = 0.02), whereas the two non-cousin crosses were not significantly different (I *vs*. II, P = 0.99). Hence, the viability (progeny count) for first cousin matings is significantly lower than for non-cousin crosses in Mating Scheme A.

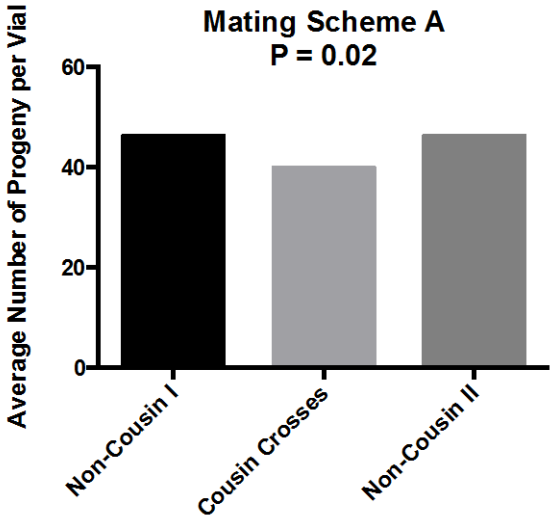


Figure 1. Average number of progeny per vial in first cousin and non-cousin matings. Parental females and males were from the CS stock.

Mating Scheme B: A total of 34 F2 vials were scored for the cousin crosses, with a mean ± standard error of 45.29 ± 3.082 progeny per vial; a total of 34 F2 vials were scored for the non-cousin control I, 46.38 ± 3.172; and a total of 35 vials were scored for the non-cousin control II, 51.83 ± 2.829 (Figure 2). An analysis of variance showed that these results are not significantly different (P = 0.26). By t tests, both of the non-cousin crosses were not significantly different from the cousin cross (I, P = 0.81; II, P = 0.12) and were not significantly different from each other (I *vs*. II, P = 0.20). Hence, the viability (progeny count) for first cousin matings is not significantly lower than for non-cousin crosses in Mating Scheme B.

The significant decrease in progeny numbers in the cousin crosses of Mating Scheme A is probably due to the increased chance of homozygosis of deleterious alleles present in the CS stock used in the parental cross. In Mating Scheme B, the parental cross was from two different stocks (CS and ORC), which will have fewer common alleles that could become homozygous in the F3 progeny. Mating Scheme A would be similar

to parental crosses in humans from Darwin's village of Downe (where individuals are related at some level), whereas the parental crosses of Mating Scheme B would be similar to crosses from individuals from Downe mating with individual from elsewhere in England (where individuals are unrelated).

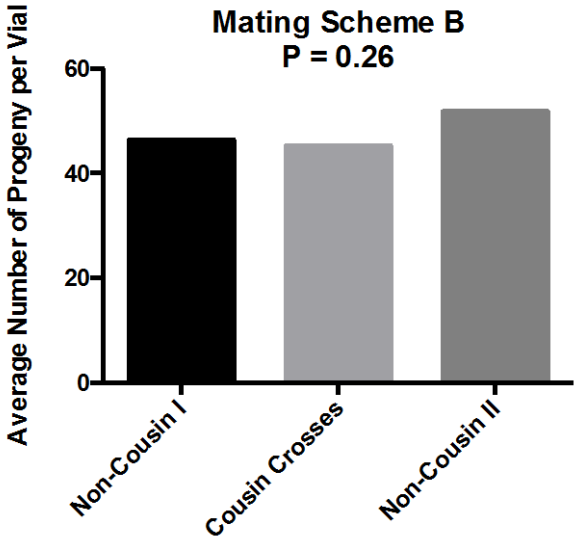


Figure 2. Average number of progeny per vial in first cousin and non-cousin matings. Parental females were from the CS stock, whereas parental males were from the ORC stock.

A class discussion of the results of this teaching exercise could include: 1) Twenty-two states of the USA prohibit marriages between first cousins (Relethford, 2012). Students could read Berra *et al*. (2010) and discuss if these state restrictions are scientifically appropriate. Why in Wisconsin are first cousin marriages allowed if the woman is over 55 years of age? Also ask the students to determine if their state or country allows first cousin marriages. 2) Students could be asked to determine the inbreeding coefficients (F) of first cousin matings (F values go from zero for no inbreeding to one for self-fertilization; see discussions of this topic in Hedrick, 2011; Relethford, 2012; http://www.ihh.kvl.dk/htm/kc/popgen/genetics/4/2.htm). The F value for first cousin matings is 0.0625, whereas the average F value for humans is usually below 0.05, but can be much higher in some parts of the world (Bittles, 2001; Relethford, 2012). 3) Students could be asked to discuss the consequences of inbreeding in a self-fertilizing organism, such as the whiptail lizards. Have them begin with all heterozygous organisms for a gene with two alleles (*A* and *a*) and observe that there is a decrease in heterozygotes (*Aa*) and an increase in homozygotes (*AA* and *aa*) with generations. For example, the frequency of heterozygotes in *n* generations will be $1/2^n$. In four generations, the frequency of heterozygotes will be $1/2^4 = 1/16 = 0.0625$, whereas the frequency of the *AA* or *aa* homozygotes will be $(1-1/2^n) / 2 = 0.9375/2 = 0.46875$ each. The homozygous individuals are the ones that will show inbreeding depression. Students could also be asked if the *A* and *a* alleles change in frequencies over generations of self-fertilization. They do not change frequencies; the *A* and a alleles remain at 0.5 (Hedrick, 2011). For example, after four generations the frequency of the *A* allele is 0.46875 + 0.0.3125 = 0.5. 4) Students could be asked to go to the internet and find other first cousin matings between famous people, such as Queen Victoria and Prince Albert; Albert Einstein and his second wife, Elsa; Saddam Hussein and Sajida Talfah; Jesse James and Zerelda Mimms; Jerry Lee Lewis and Myra Gale Brown; Igor Stravinsky and Katerina Nossenko; Martin Van Buren and Hannah Hoes; H.G. Wells and Isabel Mary Wells; and many more in the royal families of Europe.

References: Bennett, R.L., *et al*., 2002, Journal of Genetic Counseling 11: 97-119; Bittles, A.H., 2001, Clinical Genetics 60: 89-98; Bittles, A.H., and J.V. Neel 1994, Nature Genetics 8: 117-121; Charlesworth, D., and J.H. Willis 2009, Nature Reviews Genetics 10: 783-796; Darwin, C., 1868, *The Variation of Animals and Plants Under Domestication*. Volume II, John Murray, London; Hedrick, P.W., 2011, *Genetics of Populations*. Jones and Bartlett Publishers, Sundbury, MA; Helgason, A. *et al*., 2008, Science 319: 813-816; Lindsley, D.L., and G.G. Zimm 1992, *The Genome of* Drosophila melanogaster. Academic Press, New York; Pannell, J.R., 2009, Biol. Lett. 5: 332-335; Relethford, J.H., 2012, *Human Population Genetics*. John Wiley and Sons, Hoboken, New Jersey; Stoltenberg, C., P. Magnus, A. Skrondal, and R.T. Lie 1999a, American Journal of Medical Genetics 82: 423-428; Stoltenberg, C., P. Magnus, A. Skondal, and R.T. Lie 1999b, American Journal of Public Health 89: 517-523.

Drosophila larval chemotaxis: A practical experiment for the introduction of young children to science.

López-Matas, Mariana[1,2], Moraea Phillips[1,2], and Bàrbara Negre[1,2,3]. [1]EMBL/CRG Research Unit in Systems Biology, Centre for Genomic Regulation (CRG), Dr. Aiguader 88, 08003 Barcelona, Spain; [2]Universitat Pompeu Fabra (UPF), Barcelona, Spain; [3]Corresponding author: bnegre@gmail.com.

Abstract

The importance of introducing children to science and developing their interest in the subject is widely recognised. Here we adapt a well-established chemotaxis assay as an experiment for school children. The students observe the behavior of fruit-fly larvae in response to an attractive odor (*e.g.*, banana). We have performed this experiment successfully with children between the ages of 3 and 9. We also suggest additional steps as optional challenges for older students. A detailed protocol and list of materials are provided.

Presenting the Concept

Drosophila larvae demonstrate robust orientation behavior towards light and odors (Mast, 1911; Sokolowski, 1986; Sawin-McCormack *et al*., 1995; Cobb, 1999). Several assays have been developed to use larvae as a model for the investigation of sensory processing (Louis *et al.*, 2012). Here we present an adaptation of the "petri-dish assay" (Aceves-Piña and Quinn, 1979; Louis *et al.*, 2012) that can be effectively carried out by young children.

Larvae increase their size by approximately 200 times before reaching adulthood, for which they need to feed constantly (Robertson, 1963). Olfaction is thought to play a key role in the localization of food sources. When exposed to an attractive odor source, larvae quickly migrate towards higher odor concentrations. We make use of this behavior to introduce children to the use of *Drosophila* in science and to hypothesis testing.

Activity

We briefly explain the ecology of *Drosophila* and its life cycle (with figures and a food-vial containing larvae at various stages). We ask students about their expectations as to how the larvae find food. *Drosophila* larvae detect light but "do not properly see"; do they use their nose? The children smell the flask with the odor, can they recognise it? As they might find it difficult to recognise the odor, when using banana odor (isoamyl-acetate), having a ripe banana the children can smell and see will help them recognise the odor. The experiment will test whether *Drosophila* larvae like the smell of banana.

We give an overview of the outline of the experiment, followed by step by step instructions and the handing out of material, as they need it. If there is not enough material for students to work alone the experiment can be carried out in pairs.

Each child –or pair of children– is given a vial of larvae in food medium and a tube containing 10 ml of 15% sucrose solution. They pour the sucrose solution into the vial of larvae and leave it to rest for a few minutes, until most larvae have floated to the surface. While they are waiting, empty petri dishes can be handed out. Once the larvae have surfaced, they will pour the larvae in the petri dishes. Make sure they open the petri dish before pouring! Using a paintbrush the larvae can be picked from the liquid (sucrose solution transferred with the larvae from the vial) and transferred to a second petri dish containing agarose. They should transfer as little as possible of the sugar solution with the paintbrush. At this point a drop (10 ul) of test odor is placed close to one edge of the inner surface of the lid (placing the droplet inside a transparent reinforcement ring stuck to the plastic will stop it spreading). The lid is replaced –with the odor droplet

suspended above the agarose surface– and a timer can be set. After a few minutes most larvae will be very close to the odor source, sometimes even having crawled up onto the lid and immersing themselves in it.

The experiment can be complemented with the observation of the different developmental stages (vial with adults, larvae, and pupae) and adults (petri dish with frozen flies). Students can observe them by eye or with a magnifying glass or stereomicroscope. It is also an opportunity to learn how flies live in a laboratory. The activity ends with a short discussion of what they have done and seen and a round of questions encouraged by the supervisor.

We have performed this activity with children aged 3 to 9. They greet with enthusiasm hands-on experiences like this one. For younger children (3-4 years old) we try to have 1 supervisor for every 5 children. With this supervision the experiment can be performed in 45 minutes. If they are to work more independently, we recommend scheduling at least one hour.

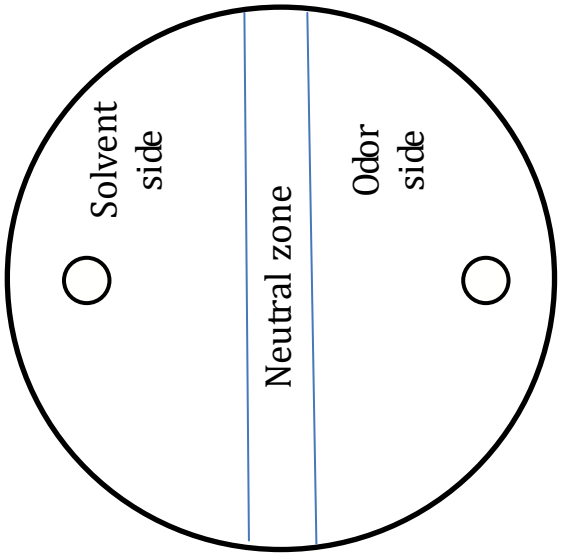
Didactic Possibilities

This activity can also be adapted for older students. We briefly introduce some possibilities to complement the experiment and make it more challenging. This also provides an opportunity to introduce the data collection concepts and the practical application of maths in experiments.

The object of this experiment is to make simple observations of larval behavior, but much more can be learnt. For example, how much do larvae like the odor? Do they prefer one odor to another? Can larvae be repelled by an odor? To answer questions like these we can introduce the use of quantification, comparison of samples, and the use of controls. Some suggestions are indicated below:

Quantification

- *How can we quantify the attraction of the larvae?* We can calculate an attraction index (ATTRAC). This involves marking out beneath the plate two lateral zones and a small neutral zone in the center (Scherer *et al.*, 2003).



The odor is placed on one side of the lid (odor-side) and a drop of paraffin oil on the other (solvent-side) as an odorless control. We spread the larvae over the neutral zone and leave them to roam. After 5 minutes, we count the number of larvae in each zone.

ATTRAC = (number of animals on the odor side
- number of animals on the solvent side)
/ (total number of animals in the plate)

This normalized score ranges from +1 (complete attraction) to -1 (complete repulsion). As the response may change over the duration of the assay, count always at the same time point. 5 minutes is enough time to allow the animals to roam without becoming habituated.

- *How do we know larvae are responding to the odor and not something else in the experimental set up?* We can do a control experiment: analyse the behavior of the larvae when exposed to the same stimulus on either side of the plate (the same odor on both sides or solvent on both sides). In this experiment we should obtain an ATTRAC = 0. If we get a different value, other factors are affecting the behavior of the larvae (maybe light or temperature).

Can we observe different behaviors?

- *Comparison of different concentrations.* How strong does an odor need to be for larvae to detect it? Do they behave differently at different concentrations? We can test a battery of concentrations to see how behavior (and the attraction index) changes.

- *Comparison of different odors.* Are larvae more attracted to odor X than to odor Y? We can compare the individual attraction indices of each odor, or we can do a direct comparison and calculate a preference index. In this case the experiment is carried out with two different odors on either side of the plate. The preference index (PREF) is calculated as the ATTRAC but substituting the solvent by odor 2. In this case a score of +1 indicates complete preference for odor 1, while a score of -1 indicates complete preference for odor 2.

  PREF = (number of animals on odor 1 side
  - number of animals on odor 2 side)
  / (total number of animals in the plate)

- *Comparison of different* Drosophila *species.* Different species have different food preferences and thus behave differently towards attractive odors. We can compare the "attractiveness" of a particular odor in different *Drosophila* species by calculating the attraction index of each species to that odor at any particular concentration. These comparisons can be used to introduce concepts like ecological differences and niche specification.

How do the larvae respond to the odor?

- *How do the larvae detect the odor?* Observe the anatomy of larvae and/or adults with a stereomicroscope or images. What sensory organs do they have? (see for example Gerber and Stocker, 2007, for a description of larval anatomy). Relate these observations to their behavior.

- *How do the larvae move in response to the odor?* Observe the larvae with a magnifying glass or stereomicroscope as they move around the agar plate. Record detailed observations of their behavior and movement. Which part of the body moves the most? Do you observe different head movements? How could this be involved in following an odor trail? (see Gomez-Marin *et al.*, 2011, for a description of larval movements during chemotaxis).

Material

For each experiment (1 or 2 children): 1 vial with 5-6 day old larvae, 1 empty petri dish, 1 petri dish containing 10 ml of 1% agarose (reinforcement rings for the lid are optional), 1 tube with 10 ml of 15% sucrose, 1 small paint-brush, a small container of water to clean the brush.
Additional material to be handled by the supervisors: banana odor (isoamyl acetate, CAS: 123-92-2), paraffin oil, pipette. The odor is best stored in a glass vial with a Teflon cap, but an eppendorf tube is fine for a few days.

Alternative odors: propyl butyrate (CAS: 105-66-8), pineapple odor (ethyl butyrate, CAS: 105-54-4), nail polish remover (ethyl acetate, CAS: 141-78-6).

Preparation of Material

Larvae: Flies are allowed to lay eggs in small food vials for 24 hours. After oviposition, flies are removed and egg-containing vials incubated at 22°C on a 12h-12h light cycle. (These growing conditions are recommended but not essential for the success of the experiment). In 5-6 days larvae reach the 3rd instar stage of development. They are easier to handle than the smaller larvae and also show a robust chemotaxis response. If wandering larvae start to appear on the walls of the food vial remove them with a paintbrush before the experiment, since at this stage they no longer forage and may behave differently towards the odor.

Odors: In this assay we pipetted 10 ul of a 1/40 dilution of isoamyl acetate in paraffin oil. (You can also use a dropper; in this case do the preparation tests with the dropper also). As the response may change depending on species (or even between different strains of the same species) it is advisable to carry out a test beforehand with a battery of dilutions, select the dilution that gives the clearest response. If the odor is too weak, larvae will take longer to respond; if it is too strong, the gradient may not be steep enough for the larvae to detect its direction – which can also result in a low attraction index. Testing of different concentrations can also be carried out by the students as a preparatory experiment.

More details about alternative set ups and tips on this assay can be found in Louis *et al.* (2012).

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Genetic drift leading to fixation of the *bw*^{1} neutral allele of *Drosophila melanogaster*.

Santangelo, Constance R., Kathryn A. Knackstedt, Henry B. Thorpe, Jennifer M. Kiser, Michael A. Balinski, and R.C. Woodruff. Department of Biological Sciences, Bowling Green State University, Bowling Green, Ohio 43403.

We have previously observed a reduction in heterozygosity over time due to genetic drift for two neutral alleles (*bw*^{1} and *bw*^{75}) of the brown locus of *Drosophila melanogaster* in the presence of the scarlet (*st*) mutation (Clendenin *et al.*, 2014; Woodruff and Boulton, 2011). We also screened for losses and fixations of the two neutral alleles over time and observed one fixation for the *bw*^{75} allele (Clendenin, 2014). Yet, it was

difficult to differentiate the *bw*^{1}/*bw*^{75}*; st/st* yellow-eyed phenotype from the *bw*^{75}/*bw*^{75}*; st/st* orange-eyed phenotype among older flies. The *bw*^{1}/*bw*^{1}*; st/st* flies, however, have a distinct white-eye color and should not be confused with other phenotypes. See Lindsley and Zimm (1992) for a description of the mutant genes and alleles used in this study.

Hence, in this study we screened for fixation of the *bw*^{1} allele only, by setting up 99 vials with a starting frequency of 0.5 for the *bw*^{1} and *bw*^{75} alleles, using all *bw*^{1}/*bw*^{75}; *st*/*st* flies and selecting eight flies at random for the next generation by lining up the progeny and picking the first four females and first four males (Buri, 1956). If all subsequent progeny from any one vial are white eyed (*bw*^{1}/*bw*^{1}*; st/st*), there was a fixation for the *bw*^{1} allele. The results of five generations of drift are shown here.

Generation	Number of lines with *bw*^{1} and *bw*^{75} alleles	Number of lines with only *bw*^{1} alleles
1	99	0
2	98	1
3	97	2
4	95	4
5	93	6

Hence, we saw six fixations for the *bw*^{1} allele in five generations, with an average of 46 white-eyed flies per fixation vial. These six fixations compare to one fixation that was observed in the first four generations by Buri (1956), who selected eight females and eight males per vial each generation (in a total of 107 vials).

The results of this study, therefore, support the use of the *bw*^{1} allele for observations of fixations of neutral alleles in small populations of four females and four males as a teaching exercise in a one-semester laboratory course.

A classroom discussion of the results of this study could include:

1) What would be the expected number of fixations of the *bw*^{75} (instead of the *bw*^{1} allele) allele in this study? The number of fixations should be the same as for the *bw*^{1} allele, since they are neutral mutations and the frequencies of each allele was 0.5 at the start of the experiment.

2) What is the predicted probability of fixation of the *bw*^{1} allele over time in this study if all the progeny were added to each vial every generation? Since half of the alleles in the original cross were *bw*^{1}, the probability of fixation would be one half.

3) Woodruff and Boulton (2011) observed that *bw*^{75}/*bw*^{75}*; st/st* and *bw*^{1}/*bw*^{75}*; st/st* flies were about two percent more fit than the *bw*^{1}/*bw*^{1}*; st/st* flies. With that in mind, what would be the expected probability of fixation of the *bw*^{75} allele if all the progeny were added to each vial every generation? Haldane (1927) showed that the probability of fixation of a beneficial mutation (such as *bw*^{75}) is 2*s*, with *s* being the selection coefficient in favor of the *bw*^{75}/*bw*^{75}*; st/st* flies. Hence, the probability of fixation of the *bw*^{75} allele would be about four percent ($2s = 2 * 0.2 = 0.4$) (see Hedrick, 2011, for a discussion of this topic).

4) Students could be asked if the population size that is picked each generation to set up the next generations would influence the probability of fixation of the *bw*^{1} allele. With an increase in population size, there would be a reduction in the frequency of lines that go to fixation for the *bw*^{1} allele (Hedrick, 2011). With this in mind, a class could be divided up into three groups and have one group select at random four females and four males for the next generation, one group select five females and five males, and one group select six females and six males. It is expected that with time the group with the smaller number of females and males picked each generation would have the largest frequency of *bw*^{1} fixations.

5) Finally, students could be asked to go to the literature and see if they can find examples of genetic drift in nature. An example where an organism has lost alleles over time in small populations is the greater prairie chicken in the USA. Compared to older museum specimens, small natural populations of greater prairie chickens have lost a number of microsatellite alleles, which are tandem repeats of two, three, or four nucleotides and are neutral alleles (Bouzat *et al*., 1998).

References: Bouzat, J.L., *et al*. 1998, Am. Nat. 152: 1-6; Buri, P., 1956, Evolution 10: 367-402; Clendenin, H.R., *et al*. 2014, Dros. Inf. Serv. 97: in press; Haldane, J.B.S., 1927, Proc. Camb. Phil. Soc. 23: 838-844; Hedrick, P.W., 2011, *Genetics of Populations*: Jones and Bartlett Publishers, Sundbury, MA; Lindsley, D.L., and G.G. Zimm 1992, *The Genome of* Drosophila melanogaster. Academic Press, New York; Woodruff, R.C., and A.M. Boulton 2011, Dros. Inf. Serv. 94: 167-169.

55th Annual *Drosophila* Research Conference

The 55th Annual *Drosophila* Research Conference was held on 26-30 March 2014 in San Diego, CA. The 2014 Organizing Committee was Daniela Drummond-Barbosa (Johns Hopkins University), Elissa Lei (NIH/National Institute of Diabetes and Digestive and Kidney Diseases), Mihaela Serpe (NIH/Eunice Kennedy Shriver National Institute of Child Health and Human Development), and Mark Van Doren (Johns Hopkins University). 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 two Plenary sessions, 156 platform session talks, 735 posters, and 15 workshops.

Historical Address Speaker

Bruce Alberts (UCSF, San Francisco, CA). Science, Biology, and the World's Future.

Plenary Lectures (in presentation order)

Arthur D. Lander (Dept. of Dev. Cell Biol. and Dept. of Biomedical Engineering, Univ. California, Irvine). Controlling morphogen gradients.

Helen McNeill (Lunenfeld-Tanenbaum Institute, Mt Sinai Hospital, Toronto, ON, Canada, and Dept. of Molecular Genetics, Univ. Toronto, Canada). Fat cadherins in growth, planar polarity and mitochondrial activity.

Xin Chen (Dept. of Biol., Johns Hopkins Univ., Baltimore, MD). Epigenetic regulation of *Drosophila* male germ cell differentiation.

Erica N. Larschan (Molec. Biol., Cellular Biol. & Biochem., Brown Univ., Providence, RI). X marks the spot: Targeting the X chromosome during dosage compensation.

Benjamin H. White (Laboratory of Molecular Biology, National Institute of Mental Health, Betheda, MD). Circuit mechanisms underlying behavioral decisions and motor program sequencing in ecdysis.

David L. Stern (Janelia Farm Research Campus, Howard Hughes Medical Institute, Ashburn, VA). The molecular evolution of morphology and behavior.

Trudy F. Mackay (Dept. of Biological Sciences, North Carolina State Univ., Raleigh). Charting the genotype-phenotype map: Lessons from *Drosophila*.

Aaron DiAntonio (Dept. of Developmental Biology, Washington Univ. School of Medicine, St. Louis, MO). Walking the highwire from synaptic growth to the axon injury response pathway.

Toshie Kai (Temasek Lifesciences Laboratory, Dept. of Biological Science, National University of Singapore). Nuage and piRNAs function for the safeguard of germline genome.

Francois Karch (Dept. Genetic and Evolution, Univ. of Geneva, Switzerland). Multiple layers of complexity in the regulation of the bithorax complex.

Rolf Bodmer (Development and Agi, Sanford-Burnham Medical Research Institute, La Jolla, CA). Cardiomyopathy models.

Patrick H. O'Farrell (Dept. of Biochemistry, Univ. of California, San Francisco). The conflicted existence of the mitochondrial genome.

Workshops (in presentation order)

Ecdysone Workshop
Organizers: Ginger Carney (Texas A&M University) and Laurie von Kalm (University of Central Florida)

Undergraduate Plenary Session and Workshop
Organizers: Alexis Nagengast (Widener University) and Beth Ruedi (Genetics Society of America, Bethesda, MD)

Flies on Drugs – Screening for Therapeutics in *Drosophila*
Organizers: Daniela Zarnescu (University of Arizona, Tucson) and Tin Tin Su (University of Colorado, Boulder)

Developmental Mechanics
Organizers: Guy Tanentzapf (University of British Columbia, Vancouver, Canada) and Rodrigo Fernandez-Gonzalez (University of Toronto, Canada)

Drosophila Male Fertility as a Cell Biological Model
Organizers: Julie Brill (The Hospital for Sick Children, Toronto, Canada) and Mariana Wolfner (Cornell University, Ithaca, NY)

The Practice and Promise of CRISPR/Cas9-mediated Genome Engineering
Organizers: Melissa Harrison (University of Wisconsin, Madison) and Kate O'Connor-Giles (University of Wisconsin, Madison)

Extracellular Epithelial Barriers
Organizers: Edward Blumenthal (Marquette University, Milwaukee, WI) and Bernard Moussian (University of Tübingen, Germany)

Drosophila Metabolomics Workshop
Organizers: Thomas Merritt (Laurentian University, Sudbury, Canada), Daniel Promislow (University of Washington, Seattle), and Jason Tennessen (Indiana University, Bloomington)

Wound Healing and Regeneration
Organizers: Adrian Halme (University of Virginia School of Medicine, Charlottesville) and Rachel Smith-Bolton (University of Illinois, Urbana-Champaign)

Data-driven Mathematical Modeling in *Drosophila* as a Tool for Discovery
Organizer: David M. Umulis (Purdue University, West Lafayette, IN)

Drosophila Research and Pedagogy at Primarily Undergraduate Institutions (PUI)
Organizers: Scott Ferguson (SUNY, Fredonia, NY) and Jennifer Kennell (Vassar College, Poughkeepsie, NY)

Genetics, Genomic and Informatic Resources for Non-*melanogaster* Drosophilidae
Organizers: William Gelbart (Harvard University, Cambridge, MA) and Therese Markow (University of California, San Diego)

Everything You Ever Wanted to Know About Sex
Organizers: Artyom Kopp (University of California, Davis) and Michelle Arbeitman (Florida State University, Tallahassee)

Feeding Behavior, Nutrition and Metabolism
Organizers: Tânia Reis (University of Colorado, Aurora) and William W. Ja (The Scripps Research Institute, Juniper, FL)

Centrosomes and Cilia in Cell Division and Disease
Organizers: Tim Megraw (Florida State University, Tallahassee) and Tomer Avidor-Reiss (University of Toledo, OH)

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 nine 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.

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