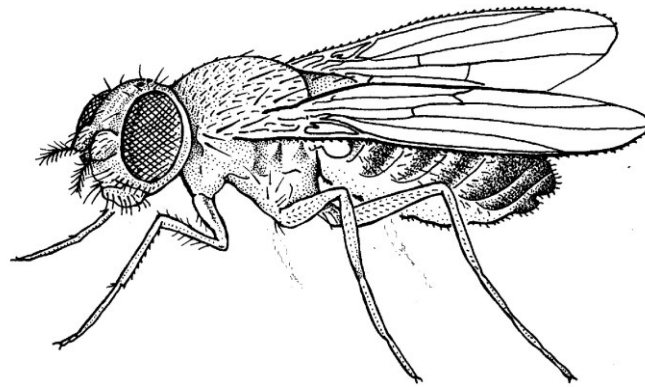


Drosophila Information Service



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Preface

Drosophila Information Service (often called “DIS” by those in the field), was first printed in limited copies in March, 1934. For those first issues, 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 this volume of DIS could not have been completed without the generous efforts of many people. Except for the special issues that contained mutant and stock information now provided in detail by FlyBase and similar material in the early annual volumes, all issues are now freely-accessible from our web site: www.ou.edu/journals/dis. For early issues that only exist as aging typed or mimeographed copies, some notes and announcements have not yet been fully brought on line. But we intend to fill in those gaps for historical purposes in the future. Individual requests for specific entries may be available from Jim Thompson, DIS Editor.

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 for future annual issues as well as any suggestions you have for maintaining this as a useful *Drosophila* research community resource.

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Research Notes

**Spring collection of drosophilids at the Font Gropa site (Barcelona, Spain).**

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A sample of drosophilids was obtained from the Font Gropa site (Barcelona) on 8th May 2018. This site is on a hill (400 m above sea level) located at the edge of Barcelona city. The vegetation of the trapping place is mainly composed of pines (*Pinus pinea*) and ilexes (*Quercus ilex*) with Mediterranean brushwood (*Arbutus*, *Ruscus*, *Erica*, *Hedera*, *Smilax*, and others) (Araúz *et al.*, 2009). Flies were collected from 17:15 to 17:45 pm using 12 baits containing fermenting bananas placed along a trail. The measures for several climatic parameters were obtained from a close meteorological station (Observatori Fabra). For this day, the recorded values were: Maximum temperature: 23°C, Minimum temperature: 14°C, and Rainfall: negligible. It was a cloudy and humid afternoon. Next day in the laboratory, collected individuals were classified according to species and sex (Table 1).

Table 1. Classification of flies according to trapping time, species and sex (Font Gropa site, Barcelona).

SPECIES	TIME				TOTAL
	17:15h	17:30h	17:35h	17:45h	
<i>D. subobscura</i> ♀	20	12	6	25	63
<i>D. subobscura</i> ♂	7	33	11	30	81
<i>D. immigrans</i> ♀	1	0	1	1	3
<i>D. immigrans</i> ♂	0	0	2	0	2
<i>D. sukuzii</i> ♀	0	0	0	1	1
<i>D. hydei</i> ♂	0	0	0	1	1
TOTAL	28	45	20	58	151

The dominant species was *D. subobscura*. This result could be explained by the fact that spring is the season when this species presents its peak of expansion (Krimbas, 1993; Argemí *et al.*, 2003). Furthermore, climatic conditions were excellent for *D. subobscura* on the trapping day. For these reasons, the estimate of N_e for this species based on the number of males and females was rather high (142). It is worth pointing out the absence of individuals from *melanogaster/simulans* group and the low number of specimens from *D. sukuzii*. Likely, both groups of species need hotter and drier conditions than those found on the collecting day. The resulting values of H' (Shannon diversity index) and J (Shannon uniformity index) were 0.219 and 0.158, respectively. They are really different from those recorded at the Front Gropa site in autumn of previous years due to the distinct drosophilid fauna composition (Canal *et al.*, 2013; Pineda *et al.*, 2014; Esteve and Mestres, 2015; Rosselló *et al.*, 2016; Madrenas *et al.*, 2017).

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Revisiting male terminalia of *Drosophila bocainensis* subgroup (Diptera, Drosophilidae) under Scanning Electron Microscopy.

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Introduction

The *Drosophila willistoni* group (Diptera, Drosophilidae) is one of the most studied groups of New World *Sophophora* subgenus. This group comprises the *alagitans*, *bocainensis*, and *willistoni* subgroups (Bächli, 2018).

The *bocainensis* subgroup currently encompasses 12 species: *D. abregolineata* DUDA 1925, *D. bocainensis* PAVAN E DA CUNHA 1947, *D. bocainoides* CARSON 1954, *D. capricorni* DOBZHANSKY E PAVAN 1943, *D. changuinolae* WHEELER E MAGALHÃES 1962, *D. fumipennis* DUDA 1925, *D. mangabeirai* MALOGOLOWKIN 1951, *D. nebulosa* STURTEVANT 1916, *D. parabocainensis* CARSON 1954, *D. pseudobocainensis* WHEELER E MAGALHÃES 1962, *D. subinfumata* DUDA 1925, and *D. sucinea* PETERSON E MAINLAND 1944.

This subgroup is mainly Neotropical, except for a few registers of *D. nebulosa* in the Nearctic region (review in Zanini *et al.*, 2015).

Pavan and Cunha (1947) described *D. bocainensis* and provided drawings of pupae, egg, larvae, and spermathecae. Hsu (1949) presented illustrations and a brief description of the genital arch, surstylus, and prenisetae (former claspers and teeth) of *D. fumipennis*, *D. nebulosa*, *D. sucinea*, and some species of *alagitans* and *willistoni* subgroup.

Wheeler and Magalhães (1952) presented a set of illustrations of the male genitalia of *D. alagitans*, *D. megalagitans*, *D. neoalagitans*, *D. bocainoides*, *D. parabocainoides* (*D. subinfumata* synonym), *D. bocainensis*, *D. pseudobocainensis*, *D. capnoptera*, and *D. changuinolae*. Still, in 1952, Malogolowkin presented a very detailed description of the male and female genitalia of *D. bocainensis*, *D. capricorni*, *D. fumipennis*, *D. nebulosa*, and *D. sucinea*.

Vilela and Bächli (1990) redescribed several species of *Drosophilidae*, including *D. fumipennis* and *D. subinfumata*.

Although there are several studies of the *willistoni* subgroup, *bocainensis* subgroup has been neglected and has a secondary role in the *willistoni* group studies.

In this study, we reviewed and improved the morphological characterization of male terminalia of *D. bocainensis*, *D. capricorni*, *D. fumipennis*, *D. nebulosa*, and *D. sucinea* based on SEM.

Material and Methods

Fly stocks

Fly stocks were reared in a cornmeal medium (Marques *et al.*, 1966) at a constant temperature and humidity (17±1°C; 60% RH). All strains used in this study are listed in Table 1.

Table 1. Species analyzed in this study.

Species	Localities	Collectors
<i>D. bocainensis</i>	Rio Grande do Sul/ Brazil	Maríndia Deprá
<i>D. capricorni</i>	Joinville, SC/ Brazil	Carolina Flores Garcia
<i>D. fumipennis</i>	Florianopolis, SC/ Brazil	Carolina Flores Garcia
<i>D. nebulosa</i>	Porto Alegre, RS/ Brazil	Carolina Flores Garcia
<i>D. sucinea</i>	Mexico City/ Mexico	Stock Center

Scanning Electron Microscopy (SEM) preparation and observation

Male terminalia were treated with 10% KOH (Bächli *et al.*, 2004) and dissected in glycerol, followed by dehydration for 20-30 seconds in 30%, 50%, 75%, and 100 % acetone washes. Whole terminalia and separated pieces were mounted in stubs with carbon tape and metalized with gold in a BALZERS SCD050 sputter coater. Visualization and image capture were performed in a JEOL JSM6060 Scanning Electron Microscope in CME UFRGS (Centro de Microscopia da Universidade Federal do Rio Grande do Sul). We observed approximately 50 seven-day-old specimens of each species.

Terminology and references

The morphological terminology used in this study followed Malogolowkin (1952), Grimaldi (1990), Vilela and Bächli (1990), Bächli *et al.* (2004), and Zanini *et al.* (2015b).

Results

Drosophila bocainensis

The epandrium is covered with short hair and long sparse setae (Figure 1A). Cerci are not fused to epandrium. (Figure 1A). The surstylus presents 17 prenisetae in a sinuous row, with 5-6 posterior prenisetae implanted outside of the other prenisetae plus one preniseta and two setae in the ventral hook (Figure 1E). The deca sternum is highly sclerotized. The hypandrium is longer than the epandrium (Figure 1A). There are discrete paramedian lobes, with an adjacent pair of convergent seta and 2-3 setulae between the lobes (Figures 1G, H, I) and small lateral extensions (Figures 1G, H). From behind of the hypandrium emerge a pair of long, slightly curved, chitinous structures (Figures 1G, H). The aedeagus is humpbacked, linked to hypandrium and apodeme by a membranous tissue; laterodorsal expansions are projected upwards (Figures 1B, C); the distiphallus is rounded at the distal end, with a serrated edge (Figures 1E, F). The aedeagal apodeme is longer than the aedeagus, rod-shaped and distally expanded (Figures B, D). Parameres are fused to apodeme, with a small appendix in the middle section (Figure 1D), very sclerotized and sharply pointed at the distal end (Figure 1D).

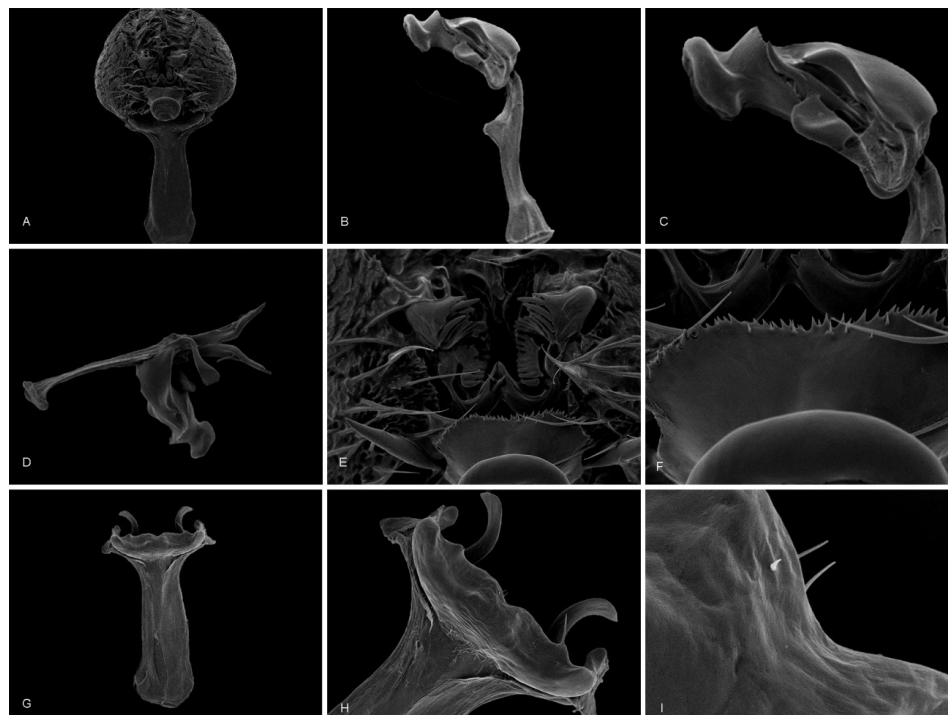


Figure 1. A. External genitalia of *D. bocainensis* (magnification 150×); B. Aedeagus and aedeagal apodeme, lateral view (magnification 200×); C. Aedeagus lateral view (magnification 450×); D. Aedeagal apodeme and paramere (Magnification 200×); E. Surstylus and preniseta (Magnification 600×); F. Distiphallus detail (Mag. 1000×); G. Hypandrium (Mag. 200×); H. Hypandrium detail (Magnification 500×); I. Hypandrium detail (Mag. 4000×).

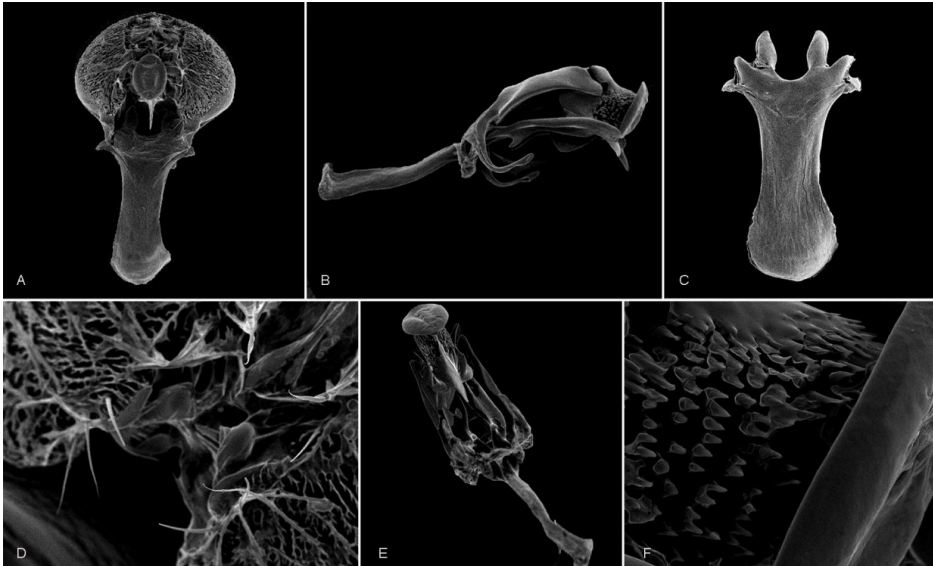


Figure 2. A. External genitalia of *D. capricorni* (magnification 150×); B. Aedeagus and aedeagal apodeme, lateral view (magnification 200×); C. Hypandrium (Mag. 200×); D. Surstylus and preniseta (Magnification 600×); E. Aedeagus and aedeagal apodeme ventral view (Magnification 200×); F. Distiphallus detail (Mag. 1000×);

Drosophila capricorni

The epandrium is covered with short hair and sparse setae (Figure 2A). Cerci are not fused to epandrium (Figure 2A). The surstylus is short, with 9-10 prenisetae in a straight row, plus one larger preniseta and two setae in the ventral hook (Figures 2 A, D). The deca sternum is sclerotized. The hypandrium is larger than epandrium (Figure 2A), with elongated paramedian lobes without setae and bifid lateral extensions (Figure 2C). The aedeagus is humpbacked, linked to hypandrium and apodeme by a membranous tissue; laterodorsal expansions are projected upwards (Figures 2B, E); the distiphallus is covered with tiny spines and rounded at the distal end (Figures 2B, E, F). The aedeagal apodeme is almost the same size as the aedeagus, rod-shaped and distally expanded (Figures 2B, E). Parameres are fused to apodeme and distally sharply pointed (Figure 2B).

Drosophila fumipennis

The epandrium is covered with short hair and long sparse setae (Figure 3A). Cerci are not fused to epandrium (Figure 3A). The surstylus presents 12-13 prenisetae in a sinuous row, plus one preniseta and two setae in the ventral hook (Figure 3B). The hypandrium is longer than epandrium, narrow at anterior $\frac{3}{4}$ and abruptly expanded in the posterior $\frac{1}{4}$ (Figure 3A), with large paramedian lobes (each one with a seta) and large lateral extensions (Figure 3C). The aedeagus is humpbacked, linked to hypandrium and apodeme by a membranous tissue; laterodorsal expansions are projected upwards (Figures 3D, E); the distiphallus is rounded at the distal end (Figures 3D, E). The aedeagal apodeme is longer than the aedeagus, rod-shaped and distally expanded (Figures B, D). Parameres are fused to apodeme, very sclerotized and sharply pointed at the distal end, with the presence of tiny spines (Figure 3F).

Drosophila nebulosa

The epandrium is covered with short hair and sparse setae (Figure 4A). Cerci are not fused to epandrium (Figure 4A). Surstylus presents 13 prenisetae in a sinuous row; 2-3 larger prenisetae implanted almost horizontally, externally to the other 13 prenisetae plus one prenisetae and two setae in a well developed ventral hook (Figures 4E, F). The deca sternum is large and highly sclerotized. The hypandrium is narrow and elongated, longer than epandrium (Figures 4A, D); the paramedian lobes are square shaped and present a seta on each one (Figures 4D). Lateral extensions are small and bifurcated (Figure 4D). From behind the lobes emerge a pair of curved, chitinous structures that bend backward (Figures 4D, G). The aedeagus is humpbacked, linked to hypandrium and apodeme by a membranous tissue; laterodorsal expansions are projected upwards (Figures 4B, C). The distiphallus is rounded at the distal end and covered

dorso-laterally with a thin membrane that wraps it like a cape (Figures 4B, C). The aedeagal apodeme is much longer than the aedeagus and rod-shaped with a large distal expansion (Figures 4B, C).

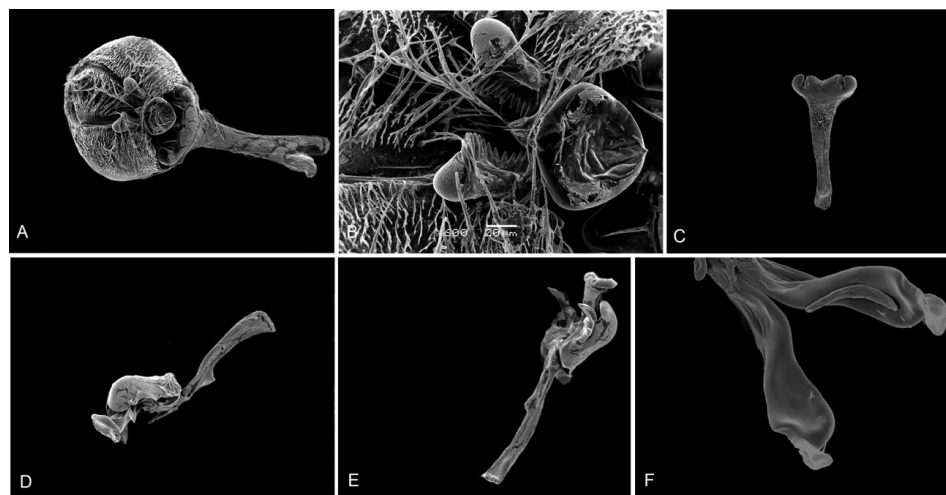


Figure 3. A. External genitalia of *D. fumipennis* (magnification 150×); B. Surstylus and preniseta (Magnification 600×); C. Hypandrium (Magnification 200×); D. Aedeagus and aedeagal apodeme, lateral view (magnification 200×); E. Aedeagus and aedeagal apodeme latero-ventral view (Magnification 200×); F. Paramere detail (Magnification 1000×).

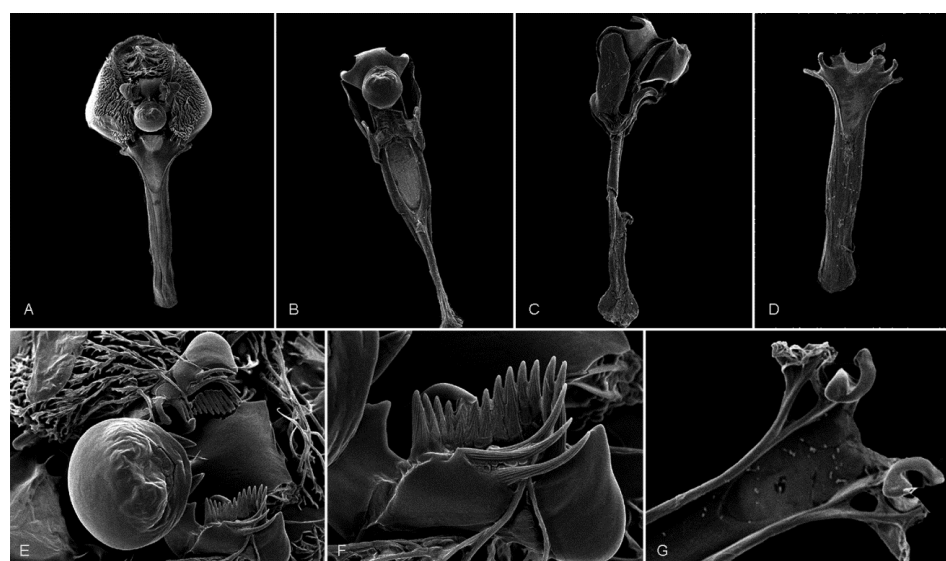


Figure 4. A. External genitalia of *D. nebulosa* (magnification 150×); B. Aedeagus and aedeagal apodeme ventral view (Magnification 200×); C. Aedeagus and aedeagal apodeme, lateral view (magnification 200×); D. Hypandrium (Mag. 200×); E. Surstylus and preniseta (Magnification 600×); F. Surstylus and preniseta detail (Mag. 1000×); G. Hypandrium, dorsal view (Magnification 600×).

Drosophila sucinea

The epandrium is covered with short hair and sparse setae (Figure 5A). Cerci are not fused to epandrium (Figure 5A). The surstylus is short, with 12-13 preniseta in a slightly concave row plus two setae in the ventral hook (Figure 5B). The decasternum is sclerotized and covered with short setae (Figure 5B). The hypandrium is almost the same size as the epandrium (Figure 5A), with very chitinous elongated paramedian lobes twisted at the distal end and two pairs of seta between the lobes and the lateral extensions (Figures 5D, E). The lateral extensions are large, almost half the size of the lobes (Figures 5D, E). The aedeagus is humpbacked, linked to hypandrium and apodeme by a membranous tissue; laterodorsal expansions are projected upwards (Figures 5C, F). The distiphallus is rounded at the distal end (Figures 5C, F). The aedeagal apodeme is smaller than the aedeagus, rod-shaped and slightly expanded at the distal end (Figure 5C). Parameres are fused to apodeme, highly sclerotized and distally sharply pointed (Figure 5C).

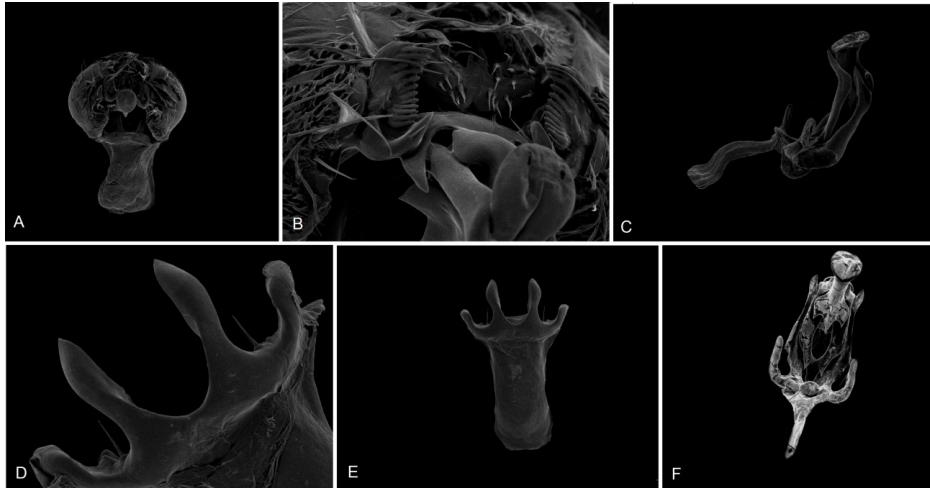


Figure 5. A. External genitalia of *D. sucinea* (magnification 150×); B. Surstylus and preniseta (Magnification 600×); C. Aedeagus and aedeagal apodeme, lateral view (magnification 200×); D. Hypandrium detail (Mag. 200×). E. Hypandrium (Magnification 200×). F. Aedeagus and aedeagal apodeme ventral view (Magnification 200×).

Discussion

Although there are some cryptic species among the bocainensis subgroup (Salzano, 1955; Wheeler and Magalhães, 1952), the species here characterized are easily distinguished by the morphology of the male genitalia. The hypandrium and surstylus present the most remarkable differences, exactly as in the cryptic *willistoni* subgroup (Zanini *et al.*, 2015b).

We can identify two types of hypandrium, long and narrow as in *D. fumipennis* and *D. nebulosa*, and more rectangular, as observed in the remaining species.

An interesting feature observed in *D. nebulosa* and *D. bocainensis* is the chitinous structure present in the hypandrium of both species. Although the structures are not identical, these structures could be homologous. It is also interesting to note that *D. bocainensis* and *D. nebulosa* are the only examined species to present bifurcated lateral extensions.

The aedeagus and aedeagal apodeme are pretty similar in all the analyzed species. The most remarkable features are the serrated edge in *D. bocainensis*, the distiphallus covered with small spines in *D. capricorni*, and paramere with tiny spines, in *D. fumipennis*.

The accurate analysis and description of the male terminalia of the *bocainensis* subgroup could be a starting point for further investigations including this subgroup.

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A high level of heterozygosity at esterase gene loci in natural populations of *Drosophila ananassae*.

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The genetic analyses considering distinct gene loci, particularly those which show moderate variation among the individuals of a population, help to know the extent of genetic variation between the populations of

a species. This kind of analysis first started by considering commonly occurring inversions in different species of *Drosophila*, because of the presence of polytene chromosomes in their larval stage. During 1966, Lewontin and Hubby devised methods to study protein polymorphism in this genus. Through their endeavor, genetic variation at the molecular level could be ascertained among the members of a population as well as among different populations of a *Drosophila* species. During the investigation, it was observed that some of the enzymes are polymorphic and may be even represented by a number of gene loci (Ayala, *et al.*, 1972; Ayala, *et al.*, 1974; Johnson, *et al.*, 1969; Johnson, 1971; Johnson and Schaffer, 1973; Singh, *et al.*, 1982).

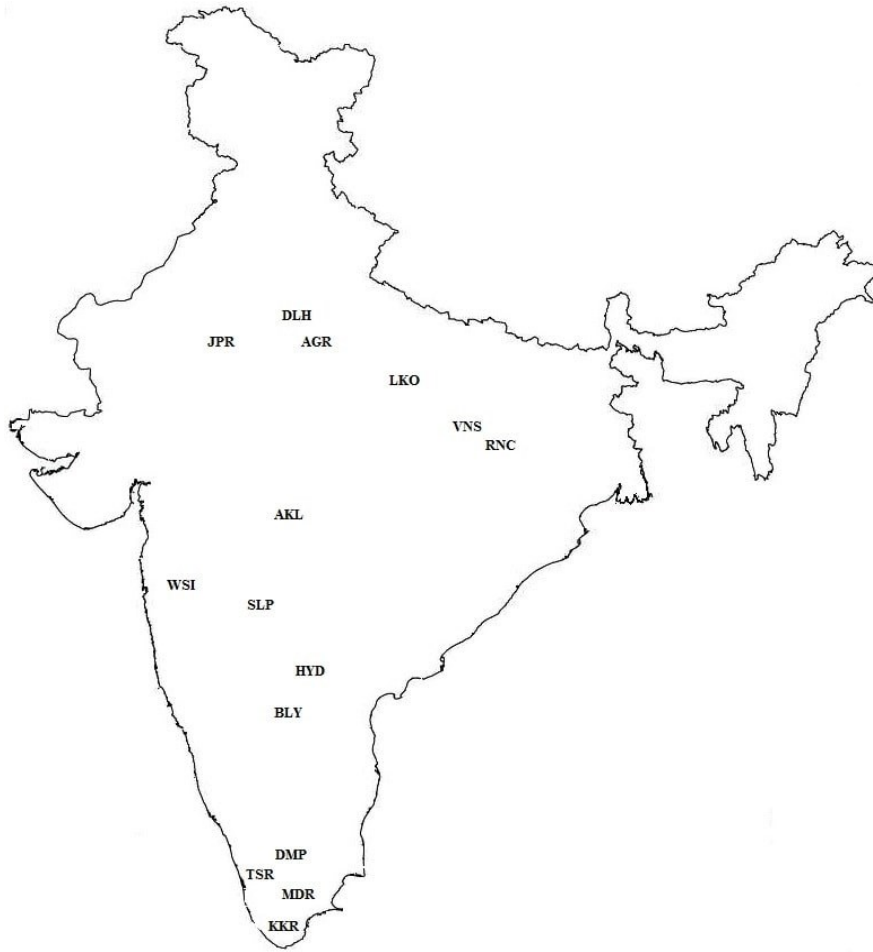


Figure 1. Geographical localities of India from where flies were sampled. The name of the locality and its abbreviation is as follows: Delhi (DLH), Jaipur (JPR), Agra (AGR), Lucknow (LKO), Varanasi (VNS), Ranchi (RNC), Akola (AKL), Wasi (WSI), Solapur (SLP), Hyderabad (HYD), Belari (BLY), Dimapur (DMP), Thirsur (TSR), Madurai (MDR), Kanyakumari (KKR).

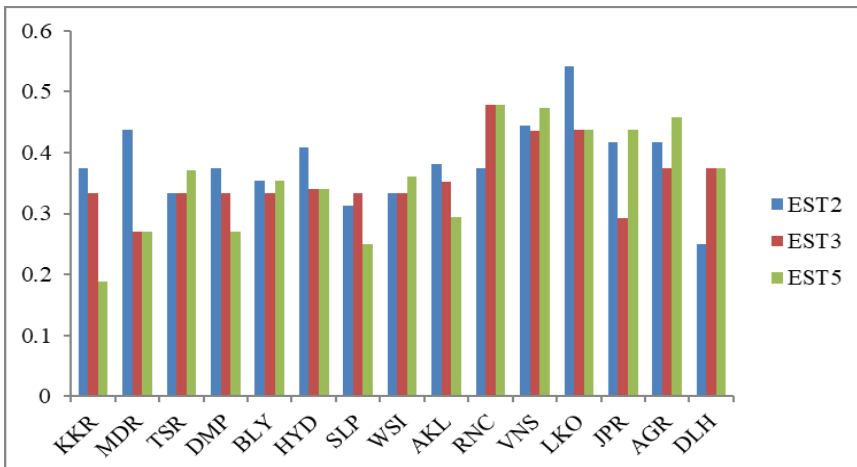


Figure 2. Histogram depicting the extent of heterozygosity for the three esterase gene loci observed in fifteen geographical populations of *D. ananassae*.

Esterases are the enzymes that hydrolyse ester bonds into an acid and an alcohol. A fairly wide range of different esterases are found in the animal kingdom that catalyse varied types of substrates. The occurrence of different forms of an esterase in different ecological conditions might be due to their adaptive significance to the organism. Electrophoresis studies in *Drosophila* have revealed that esterase enzyme is represented by a number of gene loci. Some of its loci may be polymorphic and exhibit allelic variation from population to population (Kumar, 2015; Kumar and Singh, 2016; Krishnamoorti and Singh, 2017; Kumar and Singh, 2017). Allozyme analysis in *D. ananassae* has also revealed that this enzyme has its several discrete gene loci, and a number of them are polymorphic (Kumar and Singh, 2014; Kumar, 2015; Kumar and Singh, 2016; Krishnamoorti and Singh, 2017; Kumar and Singh, 2017; Kumar, Singh and Singh, 2018). The polymorphic level of this enzyme has also been studied in two closely related species of *Drosophila*: *D. bipectinata* and *D. malerkotliana* (Singh, et al., 2015; Singh, et al., 2016).

D. ananassae is a cosmopolitan species and has been one of the largely studied species for its chromosomal polymorphisms (Tobari, 1993; Singh, 2010, 2013). Our analysis for esterase revealed the presence of three polymorphic loci, i.e., *Est2*, *Est3* and *Est5* in *D. ananassae* (Krishnamoorti and Singh, 2017; Kumar and Singh, 2017). *Esterase-1* locus in *D. ananassae* was found to be monomorphic, as it was expressed by a single variant, in all the populations analysed. Its *Est4* locus is either expressed or not and accordingly, it is referred as *Est4 active* and *null* (Krishnamoorti and Singh, 2017). Based on the frequency of different genotypes of the polymorphic *esterase loci* (*Est2*, *Est3* and *Est5*), we measured the level of heterozygosity of all the three esterases in fifteen natural populations of India and the results obtained are given in this note.

Flies were sampled from fifteen geographical localities of India. Figure 1 shows the abbreviated names of the natural habitats from which *D. ananassae* flies were collected. The collected flies were analysed for their allozyme polymorphism. For experimental purposes, native polyacrylamide gel electrophoresis was performed by taking homogenates of single flies. Based on the appearance of electrophoretic variants of the polymorphic loci, all the possible genotypes were ascertained.

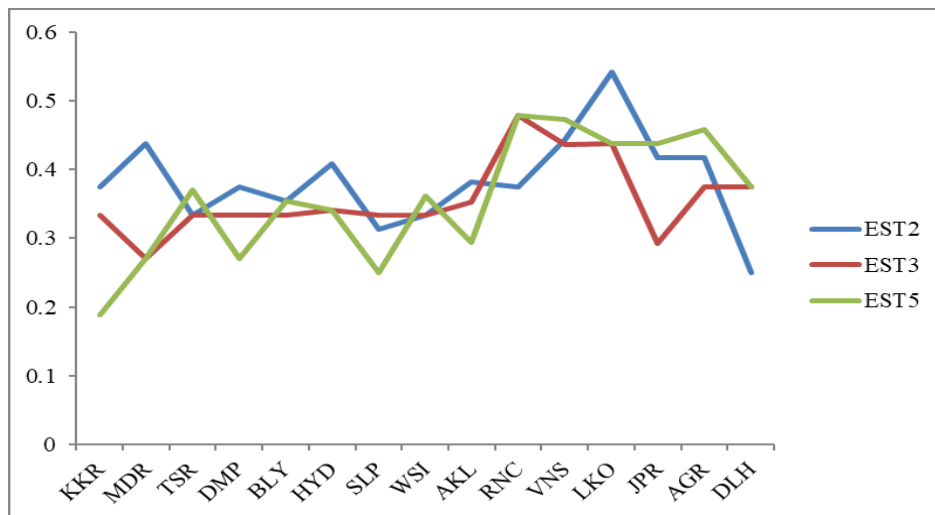


Figure 3. Line diagram of the frequency of heterozygotes for the three esterase loci of *D. ananassae*.

Figure 2 shows the histogram depicting the extent of heterozygosity for the three esterase gene loci observed in fifteen geographically distantly located populations of *D. ananassae*. Figure 3 presents the same information through the line diagram. The results of our study indicate a high frequency of heterozygotes, for all the three polymorphic esterase coding genes. These three gene loci showed the range of variation from 0.250 to 0.542 for *Est-2*, 0.271 to 0.479 for *Est-3* and 0.188 to 0.479 for *Est-5*. The mean heterozygosity was observed to be 0.384 ± 0.017 , 0.357 ± 0.014 and 0.357 ± 0.023 for *Est2*, *Est3* and *Est5*, respectively. Populations that belonged to either extreme north or south of India showed more variation for observed heterozygosity than those that were derived from middle parts of this country. The line diagram of Figure 2

indicates that the level of heterozygosity of all these three enzyme loci did not fluctuate to a large extent between places TSR to AKL.

The high frequency of heterozygosity for esterase coding genes indicates heterozygous superiority in *D. ananassae*. Due to the presence of heterozygotes, there is balancing selection in the population, *i.e.*, the different forms of genotypes have their representation. The extent of heterozygosity is known to vary from population to population. Enzymes that are polymorphic may also be showing variation in the frequency of heterozygotes. Those being more adaptive in heterozygous condition would have a higher frequency of heterozygotes than other polymorphic enzymes. Thus, esterases in *D. ananassae* seem to confer heterozygous superiority for all the loci concerned, although population-wise differences do exist among them.

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Foreign invasive pests *Drosophila suzukii* (Matsamura) and *Zaprionus indianus* Gupta (Diptera: Drosophilidae) threaten fruit production in northwestern Argentina.

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Abstract

The sub-tropical region of northwestern Argentina (Tucuman province) shelters a major soft fruit production and exporting industry. *Drosophila suzukii* (spotted-wing *Drosophila*; SWD) is a major global pest of soft fruits because females can lay eggs under the epidermis of healthy, ripening fruit. Recently, Argentina was invaded by the SWD, which has quickly spread to all cardinal points, showing a great ability of adaptation to different climates and fruit crops. We report for the first time the presence of two invasive drosophilid species, SWD and *Zaprionus indianus* (African fig fly), in the sub-tropical rainforest of the Yungas (Köppen-Geiger climate classification CWa), adjacent to a high-value fruit production region, in the province of Tucumán (northwestern Argentina). Both species were recovered from wild guava fruit (*Psidium guajava*). The SWD was found in healthy, ripe fruit attached to the trees (65%) and in damaged fruit collected from the ground (35%), while *Z. indianus* was only recovered from damaged fruit collected from the ground (100%). *Zaprionus indianus*, SWD, and other drosophilids accounted for 86.6%, 7.1%, and 6.3%, respectively, of the total of drosophilids found. The presence of both invasive insects in the region, especially SWD, is a threat for

the local berry industry. Since SWD can complete its life cycle in guavas, these fruits would allow the sustainability of SWD populations during the seasons in which commercial berry crops are not in production. Berry growers and plant protection agencies should promptly take measures to limit these pests' dispersion to commercial fruit fields. Keywords: Spotted-wing *Drosophila*; African fig fly; *Psidium guajava*

Introduction

Argentina currently exports over 1.9 million tonnes of fruits and vegetables each year, generating revenues of around 1.7 billion dollars. This condition makes Argentina one of the largest produce exporting countries of the southern hemisphere (Fundación Exportar, 2014), being citrus, berries, pome fruits, and stone fruits the most exported. The subtropical region of northwestern Argentina (Tucuman province), where the rainforest is part of the landscape, is a major soft fruit producer and exporter (Funes *et al.*, 2017).

The spotted wing *Drosophila* (SWD), *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), is a highly polyphagous invasive pest from South East Asia (Walsh *et al.*, 2011), detected for the first time in Europe (Cini *et al.*, 2012) and North America in 2008 (Hauser, 2011), and in South America (Brazil) in 2013 (Deprá *et al.*, 2014). From then on, this species has colonized Europe and America affecting a wide range of host plants.

The SWD is considered an important global pest of soft fruits, because females are capable of laying eggs under the epidermis of healthy, ripening fruit, using their powerful, sclerosed and serrated ovipositor. In the last 3 years, Argentina has been literally invaded by the SWD, which has quickly spread to all cardinal points and different fruit crops (Cichón *et al.*, 2015; Santadino *et al.*, 2015; Lue *et al.*, 2017); however, there are no reports on the presence of SWD in sub-tropical regions of northwestern Argentina.

Zaprionus indianus Gupta (Diptera: Drosophilidae) or African fig fly is native to sub-Saharan Africa and, like SWD, has also rapidly spread to tropical and subtropical regions (Silva Commar *et al.*, 2012). This drosophilid infests mainly damaged fruit of about 80 species from over 31 plant families (van der Linde *et al.*, 2006; Yassin and David, 2010). However, valuable crops, such as *Ficus carica* L. (fig) and *Dimocarpus longan* Lour. (longan) have been severely affected by this fly from Brazil to the USA (van der Linde *et al.*, 2006; Vilela and Goñi, 2015). In Argentina, *Z. indianus* was first reported in 2006 from decaying fruits of a wide range of native and cultivated host plants surveyed in northeastern Argentina, including the oriental semiarid lands of Tucumán (Lavagnino *et al.*, 2008), but this finding has received little attention.

Wild and cultivated guava (*Psidium* spp.) species are reported as *Z. indianus* and *D. suzukii* hosts in Brazil (Vilela and Goñi, 2015), México (Lasa *et al.*, 2016), and the USA (van der Linde *et al.*, 2006). Even though in Argentina guavas are not grown with economic purposes, guava trees are very common in the backyards of rural homes for family consumption (Ovruski *et al.*, 2005). Additionally, many local or native people harvest guavas from the subtropical rainforests for the production of juices and jams, which are traded in informal fairs nearby (Telam, 2017). In Tucumán, feral guava (*Psidium guajava* L.) fruit is found in the foothills of the mountain rainforest, known as the Yungas (Grau and Aragón, 2000). The Yungas border the humid piedmont, which hosts most of the soft fruit orchards of the region (Funes *et al.*, 2017). Since there is no information about both invasive drosophilid species in the subtropical region of northwestern Argentina, and considering the potential economic losses that these pests could cause to the fruit industry, the objectives of this study were to determine the species composition of drosophilids infesting “feral” guavas in the Yungas, and their relative abundance and prevalence.

Material and Methods

During a routine tephritid fruit fly monitoring in guava fruit, a large number of unusual drosophilid specimens were observed in the collected samples. The studied area is in Horco Molle (26°45'00”S, 65°20'00”W, 500–600 m elevation; Tucumán province, Argentina), within the “Sierra de San Javier” park, in the southernmost end of the sub-tropical Yungas forest. The site is characterized by disturbed secondary vegetation (exotic and native plant species combined) surrounded mainly by large citrus orchards (Ovruski *et al.*, 2005) and soft fruit crops. Horco Molle's climate is classified as “humid warm-temperate” with a rainy-

warm season from October through April, and a dry-cold season from May through September. Mean annual rainfall ranges from 1300 to 1600 mm, with an average annual temperature of 18°C.

From a group of 30 wild guava trees (*Psidium guajava* L., Myrtaceae) selected in the sampling site, six trees were randomly chosen for the study. Knowing that in this location ripe guava fruit are more abundant in late summer/early autumn (Ovruski *et al.*, 2005), all fruit samples were collected in March, 2016.

Five undamaged early maturing fruit (partially yellow guava, with mottled green spots and soft texture) were harvested from the selected trees, and five damaged, ripe fruit were collected from the ground below each tree canopy. In both cases, fruit were chosen randomly. Each fruit sample was placed individually into a cloth bag (20 cm diameter and 30 cm depth) and transported in a plastic tray to the lab (Laboratorio de Investigaciones Ecoetológicas de Moscas de la Fruta y sus Enemigos Naturales, LIEMEN, Tucumán, Argentina).

Guava fruits were rinsed with a 30% sodium benzoate solution, and weighed. Each fruit sample was placed in a plastic tray (48 × 28 × 15 cm) with a slotted bottom, which was placed over another plastic tray of the same size but without perforations. A 5-cm sand layer was used as pupation substrate in the second tray. Both trays were tightly covered with organdy cloth. The double tray method was used to prevent the contact between fruit and sand, in order to minimize fungal growth and bacterial contamination. Samples were kept in a dark room with no climate control, with temperatures ranging from 22° to 27°C. Sand was sifted once a week to recover drosophilid pupae for a 1-month period, after which all fruit were dissected to search for remaining drosophilid larvae or pupae inside each fruit.

Drosophilid pupae were transferred to glass cups (21 cm diameter, 9 cm depth) filled with sterilized moist vermiculite. Cups were covered with a piece of organdy cloth and held until adult emergence. Adult drosophilid specimens were identified to species using taxonomic keys (Markow and O'Grady, 2006). Species identification was based on external morphology and on the terminalia of both sexes. Voucher specimens were placed in the entomological collection of Fundación Miguel Lillo (FML) in Tucumán, Argentina.

Results and Discussion

Total sampled fruit weight was 3.25 kg, with an individual mean fruit weight of 54.2 ± 9.9 g (SD). This quantity of fruit yielded 387 drosophilid puparia, from which a total of 239 resulted in emerged adults (Table 1), that were identified as *Z. indianus* (207 individuals; 86.6%), SWD (17 individuals; 7.1%), and *Drosophila* spp. (15 individuals; 6.3%; probably *D. melanogaster* and *D. simulans* among others).

Approximately, 65% SWD adults were recovered from guavas collected from the tree canopy, while the remaining 35% were recovered from fruit collected from the ground. Regarding to *Z. indianus* and *Drosophila* spp., 100% of the adults were recovered only from fruit lifted from the ground (Table 1).

SWD is the first drosophilid species found in the subtropical region of northwestern Argentina with capability of laying eggs below the epidermis of healthy, ripe fruit, and of developing in the fruit. In Argentina, this frugivorous fruit fly has recently been recorded in very contrasting environments at different latitudes, fruit species, and climates (Cichón *et al.*, 2015; Santandino *et al.*, 2015; Lue *et al.*, 2017) (Table 2). In fact, the ability of *D. suzukii* to adapt to different environments and hosts has enabled this species to establish in tropical and subtropical regions in both hemispheres (dos Santos *et al.*, 2017).

In the present study, *D. suzukii* was the only drosophilid species recovered from undamaged guava fruit harvested from the plant, which is consistent with the literature. SWD has been reported in several countries infesting a great variety of fresh commercial fruits, such as blueberry, blackberry, raspberry, strawberry, cherry, plum, peach, pear, grape, fig, kiwi, and guava (Van Timmeren and Isaacs, 2014; Wang *et al.*, 2016; Lasa *et al.*, 2017), as well a wide range of non-crop fruits, including guavas (Arnó *et al.*, 2016; Kenis *et al.*, 2016).

On the other hand, *Z. indianus* was the dominant drosophilid species found in damaged, fallen fruit. As reported previously, *Z. indianus* had been recovered from cultivated peaches (*Prunus persica* (L.) Stokes) in Vipos (Lavagnino *et al.*, 2008), located in the semi-arid region of the Tapia-Trancas basin (Tucumán, Argentina). This site is located in the northeastern part of the Tucumán province, where climate is warm semi-arid, with precipitations around 450 mm and permanent water deficit (Zuccardi and Fadda, 1985). Nevertheless, we found *Z. indianus* in a completely different environment. Our sampling site is located ≈60

km south of Vipos, in a very contrasting environment: the Yungas rainforest (humid and perhumid piedmont region), with annual rains ≈ 1000 mm and positive water balance (Zuccardi and Fadda, 1985). Köppen-Geiger climate classification for Vipos is BSh while for Horco Molle it is CWa, which reflects the plasticity of *Z. indianus*, an issue previously discussed by other researchers (da Mata *et al.*, 2010; Calabria *et al.*, 2010).

Table 1. Total and relative abundance and sex ratio of Drosophilidae species, recovered from guava fruits collected from the tree canopy and from the ground in Horco Molle, Tucumán, Argentina.

Sampled Tree	Fruit origin	N° of Fruit	N° of puparia	Drosophilidae species					
				<i>Z. indianus</i>		<i>D. suzukii</i>		<i>Drosophila spp.</i>	
				N° of adults	Sex ratio (%) ^a	N° of adults	Sex ratio (%)	N° of adults	Sex ratio (%)
1	Canopy	5	9	0	-	4	75.0	0	-
	Ground	5	85	50	58.0	0	-	7	-
2	Canopy	5	1	0	-	1	100	0	-
	Ground	5	66	34	44.1	2	50.0	3	-
3	Canopy	5	11	0	-	4	50.0	0	-
	Ground	5	41	25	44.0	1	0	0	-
4	Canopy	5	3	0	-	1	0	0	-
	Ground	5	82	40	40.0	2	50.0	3	-
5	Canopy	5	2	0	-	1	100	0	-
	Ground	5	59	34	32.4	1	100	0	-
6	Canopy	5	15	0	-	0	-	0	-
	Ground	5	13	24	54.2	0	-	2	-

^a Sex ratio = proportion of females on the total number of emerged adults.

Table 2. Name, geographical location, climate type and description, annual precipitation and annual average temperature of sites where the SWD was reported in Argentina, including the present study.

Province	Location name	Location latitude	Associated fruit species	Climate type (Köppen-Geiger) ⁴	Climate description	Annual rain (mm)	Annual average temperature (°C)
Río Negro ¹	Choele Choel	39°17'09"S, 65°39'15"W	Raspberry	Bsk	local steppe	268	15.4
Buenos Aires ²	Lobos	35°11'11"S, 59°05'46"W	Blueberry	Cfa	warm and temperate	1008	16.1
La Rioja ³	Anillaco	28°48'23"S, 66°56'27"W	Pear	BWh	desert	330	20.0
Tucumán	Horco Molle	26°45'00"S, 65°20'00"W	Guava	Cwa	warm and temperate	949	19.4

¹Cichón *et al.*, 2015; ²Santandino *et al.*, 2015; ³Lue *et al.*, 2017; Kottek *et al.*, 2006.

Our findings are very similar to those reported by Fartyal *et al.* (2014), who found both *D. suzukii* and *Z. indianus* affecting sweet orange (*Citrus sinensis* L.) and guavas in subtropical environments of India. They observed that *D. suzukii* was the only drosophilid found in healthy fruit attached to the plant, and that *Z. indianus* only emerged from damaged fruit bearing in the tree or collected from the ground.

The sex ratio, defined as the proportion of adult females on the total number of adults (Table 1), varied from 32.4% to 58.0% for *Z. indianus* in fruit lifted from the ground, which is consistent with previous reports in guava (56%; Lasa *et al.*, 2017). Regarding to SWD, the small number of specimens found was not enough

to make any discussion about sex ratio. In more integral studies, SWD sex ratios were 58% in guavas collected from the tree and 66% in damaged guavas lifted from the ground (Lasa *et al.*, 2017).

An important issue to address in future research is the interaction between both drosophilid species, as shown in other studies. Strawberry fruit injured by SWD adults facilitated the infestation by *Z. indianus*, showing the opportunistic ability of African fruit fly adults to infest damaged fruit (Bernardi *et al.*, 2017).

In hosts like guavas, SWD can complete its life cycle in 15 d under lab conditions, indicating that guavas allow the sustainability of SWD populations during the seasons in which commercial berry crops are not in production (Rebollar-Alviter *et al.*, 2015). As pointed out before, in the sub-tropical region of northwestern Argentina, guavas share the same geographical space with commercial berry orchards. Therefore, our results should be taken as a warning signal for growers and government plant protection agencies.

Our findings reveal the need of increasing the studies about the drosophilid community in this region, including studies on population dynamics, interactions between species, potential natural enemies, geographical distribution, host range within non-crop plants, and potential dispersion of both pest species to neighboring orchards.

Given that SWD is considered a key pest of several fruit crops worldwide (Arnó *et al.*, 2016; Bolda *et al.*, 2010; Wang *et al.*, 2016), its presence in the subtropical region of northwestern Argentina is a threat for the local fruit industry and for native non-crop fruit species. Prompt measures should be taken in order to limit this pest dispersion to commercial orchards and to natural plant sanctuaries.

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Annual record for chromosomal polymorphism in a natural population of *Drosophila pseudoobscura* from Morelos, Mexico.

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Abstract

A seasonal survey of *Drosophila pseudoobscura* was performed in a natural population from Tres Marias, Morelos, Mexico, in order to determine fluctuations in the relative frequencies of inversions in the third chromosome of this species. This study corresponds to an analysis of 1126 third chromosomes, among which we were able to detect 10 different gene arrangements. Two of them, CU and TL, represent the dominant couple and the pair EP/SC as well six minor and sporadic gene arrangements complete the genetic structure of the population. The chromosomal constitution of the population is similar to other nearby populations reported with respect to the number of inversions and dominant pairs. Changes in frequency are related to climatic fluctuations in the locality and ascribed to the adaptability of the chromosomes to climatic conditions as have been observed in many populations. Key words: *Drosophila*, inversions frequencies, temporal changes

Introduction

Based on works of Painter (1934) and Bridges (1935), salivary glands of *Drosophila melanogaster* promoted a continuous series of studies concerning a chromosomal pattern in several species of the genus *Drosophila*. In the western hemisphere such species as *D. pseudoobscura*, *D. persimilis*, *D. robusta*, *D. willistoni*, and *D. nebulosa*, among others, have been extensively studied cytologically. All of them possessed an enormous diversity of chromosomal polymorphism in their genome (Krimbas and Powell, 2000).

We refer now to *D. pseudoobscura*, a species that inhabits temperate climes and with a geographic distribution from southwestern Canada, western region of USA, whole Mexico and Guatemala, and a small colony in Colombia. It inhabits mainly coniferous forests and it has even been found in other habitats with different vegetation. It lives in areas with altitudes above sea level between 1800 and 3000 meters and is easily cultured in the laboratory.

The chromosomal polymorphism in *D. pseudoobscura* is present mainly in its third chromosome with at least 40 different gene arrangements or inversions described among other authors by Dobzhansky and Epling (1944), Olvera *et al.* (1979), and Powell (1992). Concerning its chromosomal constitution a population could be uniform, present only one kind of inversion; even so, the most common is the presence of up to 6-7

different gene sequences in different proportions with a particularity that among them two kinds are the predominant inversions and the remaining are rare or sporadically present. Beside it the number and kind of arrangements varies from population to population.

Relative frequencies of each inversion, in this species, show geographic and/or temporal variation, at this respect, for *D. pseudoobscura* seasonal changes are well documented, for instance Dobzhansky (1956) in a locality of California as well Dobzhansky (1958) in several populations of western USA. In the case of long term changes we have the reports of Dobzhansky (1963), Dobzhansky *et al.* (1964 and 1966), and Anderson *et al.* (1991).

In Mexico there are several studies referring to geographical variation in relative frequencies of inversions present in populations of this species as shown by Guzmán *et al.* (1993 and 2005), Olvera *et al.* (2005), and Salceda *et al.* (2007a, 2007b, and 2015). All these cases related to qualitative changes due to geographic location of such populations. As for changes in relative frequency of different gene arrangements concerning seasonal or temporal variation, we have the study by Espinoza and Salceda (1981) in a population from Saltillo, Coahuila.

In this occasion we are interested in determine changes in relative frequency of inversions of *D. pseudoobscura* occurring in a natural population from Tres Marias, Morelos, Mexico, during an annual cycle in order to find out its behavior.

Materials and Methods

This study was performed in a natural population of *D. pseudoobscura* from a nearby town of Tres Marias in the State of Morelos Mexico at km 47 of Federal Road Mexico-Cuernavaca, a place with an altitude of 3000 meters above sea level. A set of 20-25 plastic buckets containing fermenting bananas was distributed in the area, and, when flies started to visit them, using an entomological net flies were trapped, put into vials with fresh food, and transported to the laboratory. Samples were captured in the morning from sunrise up to 9.00 hours and in the afternoon from 17.00 until dark. Once in the laboratory every single female was cultivated individually in a half pint bottle with fresh food and each male crossed with 2-3 virgin females from the laboratory stock TL/TL. A week later flies were transferred to a new bottle as reserve, and to the original culture some drops of a fresh solution of yeast was added to allow well nourishment of the larvae.

When third state larvae started to emerge, from each culture a single larva was extracted, dissected to obtain salivary glands, stained with an aceto orcein solution, and a smear done and then ready to be analyzed for their karyotype. Once it was done we obtained relative frequencies for each gene arrangement determined and with them a data base performed for further analysis, all performed with help of the figures from Kastritsis and Crumpacker (1966, 1967) as well as our own atlas.

Cultures were kept at $25 \pm 1^{\circ}\text{C}$ and relative humidity of 65%, with regular food made of agar, sugar, corn flour, propionic acid, and tegosept.

Results

The study corresponds to 12 monthly collections and includes an analysis of 1126 third chromosomes for which relative frequencies were determined for each sample. Table 1 shows names and relative frequencies of each inversion and Figure 1 the main changes occurring among the dominant pair of gene arrangements.

From the approximately 40 different inversions already described in *D. pseudoobscura* we detected 10 of them. They are: Cuernavaca (CU), Tree Line (TL), Santa Cruz (SC), Estes Park (EP), Olympic (OL), Oaxaca (OA), Hidalgo (HI), Chiricahua (CH), Tarasco (TA), and Pikes Peak (PP). Not all of them were found at the same time nor with the same frequency as shown later. Inversions CU and TL represent the dominant pair, which together showed a variation from 77 to 97 % of any particular sample. This pair was complemented basically with a second pair represented by inversions SC-EP and occasionally with those remaining gene arrangements. The predominant pair, CU-TL, showed irregular variations along the sampling period; they are fundamentally due to climatic changes that occur along the annual cycle. Our study starts in April in which inversion frequencies found are: 56% for CU, TL 22%, and completing the sample 13% for SC,

Table 1. Relative frequencies of inversions in third chromosome of *Drosophila pseudoobscura* present in a natural population from Tres Marias, Morelos, Mexico.

MONTH	CU %	TL %	SC %	EP %	Others %	N
April	56	22	13	6	4	168
May	70.8	23.8	2.5	1.5	1.5	202
June	62.2	21.4	6.1	9.2	1	98
July	58.2	34.5	1.8	5.5	---	55
August	43.8	34.4	3.1	13	3.1	32
September	45.8	41.7	---	13	---	24
October	69.2	28.3	---	0.8	0.8	120
November	66	30.9	3.1	---	---	94
December	42.3	38.5	11.5	7.7	---	26
January	66.7	25	8.3	---	---	24
February	75.6	19.8	3.8	0.8	---	131
March	67.8	25	2	3.3	---	152
MEAN %	64.7	25.7	4.4	3.6	1.1	
TOTAL N						1126

6% for EP, and 3% the rare ones. This information is the base line for this study. Shortly, we indicate that the successive changes registered doing emphasis on pair CU-TL; in May their relation was 70.8% CU: 23.8% TL, and here starts an increase/decrease relationship until August with proportion 43.8% CU: 34.4% TL, again an increase/decrease to reach in October 69.2% CU: 28.3% TL and by December attaining its minimum with values of 42.3% CU: 38.5% TL when the complementary pair SC-EP reaches their highest values 11.5% for SC and 7.7% for EP. In January begins the increase for the dominant pair with a proportion of 66.7% for CU and 25.0% for TL and in the secondary pair 8.3% of SC and absence of EP. During February the main pair attains its maximum of 75.6% CU and 19.8% TL. Finally in March this relation is 67.8% CU and 25.0% TL completing the cycle. In Figure 1 the trends for relative frequencies for the main inversions CU and TL, which are shown

graphically there, we could see that they suggest that both are related to climatic changes, mainly temperature. Also we observe on it the cumulative frequency of the remaining eight inversions.

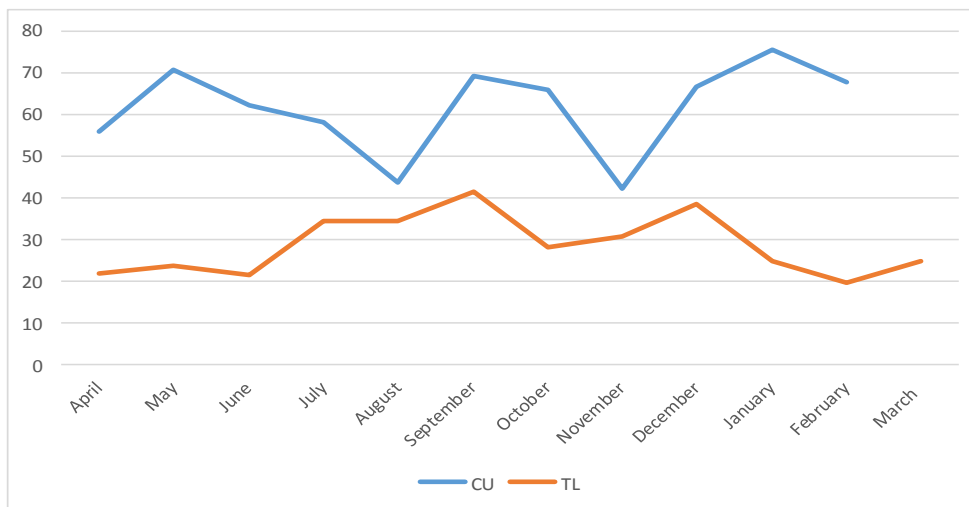


Figure 1. Graphic representation of fluctuation in relative frequency for inversions CU (above) and TL (below) from a natural population of *Drosophila. Pseudoobscura* from Tres Marias, Morelos, Mexico.

Discussion

Among those authors interested in the study of temporal and/or spatial changes in relative frequency of inversions on the third chromosome of *D. pseudoobscura*, in populations from Mexico, we have those reported by Dobzhansky (1944, 1948), Anderson *et al.* (1975), Guzmán *et al.* (1975), and EspinozaA and Salceda (1981). In all these cases, as well as others mentioned in the literature, the population structure is represented by showing a predominant pair of inversions that covers up to 90 percent of the total, being the remaining 10 percent formed for rare inversions. This principal component varies from population to population. This pattern was observed in our population in which arrangements CU and TL constitute the

dominant pair and the remaining eight complete the population structure, even if these eight inversions are not always present, in such cases with variable frequencies as shown in Table 1. Another characteristic of this and other species and/or populations of *Drosophila* is that their principal pair shows fluctuations in relative frequency of its components mostly associated with seasonal changes in the locality. This pattern was observed in our study as is seen in Figure 1.

The main interest in studying the chromosomal polymorphism in natural populations of *Drosophila* species, and in this occasion that of *D. pseudoobscura*, is to know which are the principal or dominant components of its genetic pool. In the population of Morelos they are CU and TL which reached a minimal average of 77% and a maximum of 97%. Among the secondary components is notoriously the pair formed by inversions SC and EP, which were found in all the samples (Table 1). In order to complete the structure, those six remaining inversions were observed sporadically and with low frequencies.

It is necessary to mention the prevalent presence of the CU/TL pair, as has been observed in different Mexican populations, like the ones reported by Dobzhansky *et al.* (1975), Guzmán *et al.* (1993, 2005), Salceda *et al.* (2007b and 2015), although there is no annual record of them since the data refer to unique samples per locality besides a large number of analyzed chromosomes and the absence of a continuous sampling period.

It seems the changes of CU are closely related to the temperature/humidity changes that usually follow the season cycle, since dryer and warmer months are May and February, while the moister and lower temperature ones, occurring either by rain or winter, determine the low frequencies of the inversions. The in-between periods represent the gradual changes that allow the natural adaptation of the population to climate changes. A monthly follow-up of the relative frequencies and specially the CU-TL relationship is indicative of the subsequent adaptations of the population to climatic changes. Common knowledge tells us that one peculiarity of inversions is their great adaptability to those mentioned conditions as has been demonstrated experimentally.

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Comparative study on effects of carbamazepine on mating propensity, life history traits, and mortality among intraspecies of *Drosophila*.

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Abstract

Antiepileptic drugs cause adverse effects on developmental stages and reproductive behavior. The effect on egg to adult viability caused by one of the antiepileptic drugs, carbamazepine (CBZ), were studied in different species of *Drosophila* viz., *D. melanogaster*, *D. nasuta nasuta*, and *D. ananassae*. The present study was carried out to assess the dose dependent response on mating propensity, viability, and mortality in CBZ treated *Drosophila* species. Drug concentrations were supplemented with wheat cream agar media. Treated flies (males and females) were crossed with corresponding untreated flies to assess the dose response effects. Significant difference in mating propensity was observed at high doses. *D. nasuta nasuta* showed significant variation in all traits when compared to other species. Number of offspring that successfully developed from the egg to adulthood was assessed to confirm developmental toxicity. The study showed that flies exposed to CBZ showed dose-dependent reduction in hatchability, pupation, and adult eclosion in all the species with increased doses. CBZ was highly toxic to early larval stages at high dose, while adult mortality was significant in *D. nasuta nasuta* on exposure to all doses. Decrease in the rate of egg development revealed that developmental stages were sensitive to toxic effects of carbamazepine. Key words: Carbamazepine, *Drosophila*, mating propensity, development, mortality, toxicity

Introduction

Carbamazepine (CBZ) is used primarily in the management of epilepsy and is a widely used antiepileptic drug. It is also used in the treatment of neuropathic pains, psychiatric disorders (Ambrosio *et al.*, 2002) and is known to be a potential teratogen with embryotoxic effects. Therapy of pregnant women with CBZ increases the rate of congenital anomalies in the foetus (Oetken *et al.*, 2005; Murabe *et al.*, 2007). Epilepsy patients have to undergo lifelong medication (Avanesian *et al.*, 2009). The adverse effect on development, reproduction and longevity need to be carefully screened using *in vivo* animal test models.

Drosophila provides a powerful platform to perform functional annotations of human genes and disease variants, given the observation that evolutionarily conserved genes tend to have similar molecular functions. In the last two decades, *Drosophila* has been increasingly used to model neurological dysfunction, including neurodegeneration, epilepsy, dementias, stroke, traumatic brain injury, and brain tumors (Ugur *et al.*, 2016).

Direct assessment of teratogenic effects of chemicals in flies has been employed in a limited number of studies (Rand, 2010). The developmental stages of *Drosophila* are well understood and show the fitness of the species. In view of this variation in fitness of life-history parameters including courtship duration, copulation duration, hatchability, pupation, adult eclosion, and mortality were studied on exposure to different concentrations of CBZ among intraspecies of *Drosophila*. Genetic differences are suspected to be a major factor in intraspecies differences within human populations and within test-animal populations (National Research Council, 2000).

The aim of this study was to determine and correlate the adverse effects on exposure to carbamazepine with respect to their propensity to mate and viability at different doses of CBZ within *Drosophila* species. The method of drug ingestion encompasses treating the entire metamorphosis period *i.e.*, egg, larval, and pupa formation by incorporating the test drug into the medium. Adult females lay eggs that hatch as larvae after a day. These larvae grow tremendously over the next 4 days as they voluntarily consume food, and molt twice. During the final larval instar, larvae stop eating, leave the food (wander) and form a puparium, signaling the onset of metamorphosis. The duration of metamorphosis is about 4 days, after which the adult fly emerges. Thus, the life cycle of the fly is such that developmental exposure and the consumption of AEDs-containing food are voluntary, unlike gestational mammals, and occurs mostly during the larval stage of development. Developmental toxicity was evaluated based on the number of days taken for development and number of adults eclosed after treating with the drugs in pre adult stage. Adult flies were systematically examined under a binocular microscope for external morphological anomalies. The external development of flies eliminates the complications of maternal-placenta-fetal interactions seen in mammalian studies (McClure *et al.*, 2011).

The mating behavior and reproductive process in *D. melanogaster* is well studied and provides a useful background. Mating is an important mechanism to propagate the species. Behaviors controlling the propensity to mate can have large effects on fitness (Partridge and Andrews, 1985). Mating behavior of *Drosophila* consists of specific actions, which are accompanied by orientation movements made up of several signals, which are performed sequentially (Spieth and Ringo, 1983). Factors influencing variation in duration of copulation are very complex and species specific traits in *Drosophila* (Kraaijeveld *et al.*, 2008). Adult behavior that involved fine motor coordination for courtship and mating was used to examine aspects of behaviors relevant to human diseases (Becnel *et al.*, 2011). In view of this, the courtship duration and copulation duration among untreated and treated flies (reciprocal crosses) were studied on different species of *Drosophila*. Mating activity is correlated with fitness in species of *Drosophila*. There is considerable variation in courtship and copulation duration among species of *Drosophila*.

The present study was conducted using *Drosophila melanogaster*, *Drosophila ananassae*, and *Drosophila nasuta nasuta* to compare behavioral and developmental effects within the species. These species occupy a unique status in the whole of the genus *Drosophila*, due to certain variations among species. The stocks were obtained from *Drosophila* Stock Centre, University of Mysore, Mysore, India. Data from treated flies can be compared with those from concurrent control flies using statistical tests.

Materials and Methods

Carbamazepine (CBZ) (5H-dibenzazepine-5-carboxamide) 99% CAS No 298- 46 was obtained from Sigma-Aldrich, soluble in proportion of ethylene glycol, alcohol, and water. A modified protocol of Mohammed *et al.* (2009) has been used for drug standardization. The drug concentrations were added into the media and standardization was carried out on adult mortality for seven days, and three doses were obtained namely, low dose (2 mg/ml), mid dose (4 mg/ml), and high dose (8 mg/ml).

The fly stocks, *D. melanogaster*, *D. ananassae*, and *D. nasuta nasuta* were obtained from *Drosophila* Stock Centre, Mysore University, India. They were cultured on standard wheat cream agar medium in uncrowded culture condition at $22 \pm 1^\circ\text{C}$ (rearing temperature) and 12:12 hour photoperiod with a relative humidity of 70% (Ranganath, 1999). The progeny from these stabilized stocks treated with CBZ (2, 4, and 8 mg/ml) were used to assess the mating propensity (courtship duration and copulation duration), viability, and mortality rates and compared to respective controls.

2.1 Mating propensity

For the mating assays, from the uncrowded culture stocks, virgin females and unmated males of *D. melanogaster*, *D. ananassae*, and *D. nasuta nasuta* were isolated, collected, and aged. For the next 3 days the flies were fed on 5 ml media supplemented with the above said doses in 25×100 mm tubes to determine the effect of CBZ on mating behavior.

During morning hours, mating propensity was recorded between 7 to 11 am (Hedge and Krishna, 1997). Courtship duration (time from introduction of male and female together into the mating chamber and until the orientation of male towards the female) and copulation duration (time taken from initiation to

termination of copulation of each pair) were recorded. A minimum of 30 pairs involving each cross were observed. Mating propensity was analysed for four different sets (crosses); Untreated male × Untreated female (C), Treated male × Untreated female (T₁), Untreated male × Treated female (T₂) and Treated male × Treated female (T₃) for each species.

A pair of flies was aspirated into empty glass vials to avoid etherization. The mating activity was observed for 60 minutes. The pairs which do not mate within a stipulated time of 60 minutes were discarded.

2.2 Viability

Virgin females and unmated males were collected and maintained separately in order to age for 5 days and then transferred to media containing CBZ alongside control. The drug was added to a wheat cream agar media in different doses of CBZ (2, 4, and 8mg/ml). The control cultures were raised on the same diet without addition of the drug. 5 ml of media was placed in sample tubes and a pair of flies was transferred to each vial. Likewise, 3 successive transfers were made into fresh food containing the said doses of CBZ once in two days for the three species of *Drosophila*.

Flies were allowed to lay eggs on media containing CBZ alongside control, and the number of eggs laid was recorded. Dilute yeast was added to the vials for larval, pupal development and adult eclosion. The ratio of eggs laid to adults eclosed (viability) was also recorded for all three species of *Drosophila* (Luning, 1966; McClure *et al.*, 2011).

2.3 Mortality

Percent larval, pupal, and adult mortality were assessed from the same set of vials assessed for viability.

2.4 Statistical analysis

The analysis of variance (ANOVA) and Duncan multiple range test (DMRT) were used to record the divergence among different species subjected to variable exposures of AEDS. To compile the data, statistical presentation system software (SPSS) 15.0 was used.

Results

3.1 Mating propensity

Mean courtship duration of *Drosophila* species on exposure to CBZ and control is presented in Table 1. All pairs of *D. melanogaster* for all the given treatments exhibited normal courtship behavior except pairs fed with high dose in T₁ (10.40 ± 6.65) and T₃ (11.50 ± 5.61). *D. ananassae* showed increased duration in T₂ (9.70 ± 0.87) and T₃ (10.40 ± 0.49) at 8 mg/ml.

In the case of *D. nasuta nasuta* differences were significant ($P < 0.0001$) in all the exposed groups compared to control (10.03 ± 0.86 , 16.92 ± 1.76 , and 20.95 ± 2.26). The same treated and untreated flies of reciprocal crosses also demonstrated for copulation time at all dose levels among the three species of *Drosophila*. Mean copulation time showed significant decrease at 8 mg/ml in *D. melanogaster* in T₃ (19.30 ± 1.70), while it was insignificant in *D. ananassae* (2.79 ± 0.23). *D. nasuta nasuta* showed significant ($P = 0.002$) differences at high dose for treated trials T₁ (15.70 ± 0.74), T₂ (14.95 ± 1.15), T₃ (14.44 ± 0.95) compared to control flies.

3.2 Viability

Reduction in the viability was observed in a dose depended manner among the species (Table 2). The mean hatchability of *D. nasuta nasuta* in all the doses (2, 4, and 8 mg/ml) was 148.80 ± 3.79 , 129.20 ± 4.05 , and 108.80 ± 2.08 , respectively. The reduction was significant among the doses at $P < 0.001$. Similarly, mean hatchability *D. ananassae* was 103 ± 2.22 , 107.60 ± 2.46 , and 91.80 ± 4.80 in the respective doses, and the reduction was also significant at $P < 0.001$. There was no significant reduction among the doses in *D. melanogaster*.

Table 1. Mean \pm SE of courtship duration on exposure to carbamazepine in three species of *Drosophila*.

Species	<i>D. melanogaster</i>			<i>D. ananassae</i>			<i>D. nasutanasuta</i>		
Dose \rightarrow Traits \downarrow	2 mg/ml	4 mg/ml	8 mg/ml	2 mg/ml	4 mg/ml	8 mg/ml	2 mg/ml	4 mg/ml	8 mg
C	6.00 \pm 0.93	6.00 \pm 0.93	6.00 \pm 0.93	5.90 \pm 0.73	5.90 \pm 0.73	5.90 \pm 0.73	6.72 \pm 0.58	6.72 \pm 0.58	6.72 \pm 0
T ₁	6.45 \pm 0.84	8.01 \pm 0.55	10.40 \pm 6.65	6.43 \pm 0.93	5.80 \pm 0.56	7.35 \pm 1.36	9.04 \pm 1.10	9.90 \pm 0.86	10.03 \pm
T ₂	7.78 \pm 0.71	7.20 \pm 0.82	9.20 \pm 4.76	5.65 \pm 0.55	5.46 \pm 1.25	9.70 \pm 0.87	11.20 \pm 0.85	11.21 \pm 1.90	16.92 \pm
T ₃	7.98 \pm 0.34	7.32 \pm 0.82	11.50 \pm 5.61	6.75 \pm 0.42	5.22 \pm 1.24	10.40 \pm 0.49	10.60 \pm 0.66	15.83 \pm 1.34	20.95 \pm
ANOVA	F = 1.853	F = 4.438	F = 1.603	F = 3.201	F = 8.381	F = 5.5561	F = 1.603	F = 13.573	F = 21.4
	d.f. = 3,116	d.f. = 3,116	d.f. = 3,116	d.f. = 3,116	d.f. = 3,116	d.f. = 3,116	d.f. = 3,116	d.f. = 3,116	d.f. = 3,
	P = 0.155	P = 0.262	P = 0.620	P = 0.435	P = 0.248	P = 0.023	P < 0.0001	P < 0.0001	P < 0.00
DMRT	C/T ₁ , C/T ₂ ,	C/T ₁ , T ₂ /T ₁ ,	C/T ₁ , C/T ₂ ,	C/T ₁ , C/T ₂ ,	C/T ₁ , C/T ₂ ,	C/T ₁ , C/T ₂ ,	C/T ₁ , C/T ₂ ,	C/T ₁ , C/T ₂ ,	C/T ₁ , C/
	C/T ₃ , T ₂ /T ₁ ,	T ₃ /T ₁	C/T ₃ , T ₂ /T ₃ ,	C/T ₃ , T ₂ /T ₃ ,	C/T ₃ , T ₂ /T ₁ ,	C/T ₃ , T ₂ /T ₁ ,	C/T ₃ , T ₂ /T ₁ ,	C/T ₃ , T ₂ /T ₁ ,	C/T ₃ , T ₂ /T ₃

C- Untreated♂ x Untreated♀; T₁-Treated♂ x Untreated♀; T₂- Untreated♂ x Treated♀; T₃ -Treated♂ x Treated♀

Table 2. Mean \pm SE of copulation duration on exposure to carbamazepine in three species of *Drosophila*.

Species	<i>D. melanogaster</i>			<i>D. ananassae</i>			<i>D. nasutanasuta</i>		
Dose \rightarrow Traits \downarrow	2 mg/ml	4 mg/ml	8 mg/ml	2 mg/ml	4 mg/ml	8 mg/ml	2 mg/ml	4 mg/ml	8 mg/ml
C	24.10 \pm 0.82	24.10 \pm 0.82	24.10 \pm 0.82	3.20 \pm 0.23	3.20 \pm 0.23	3.20 \pm 0.23	18.40 \pm 0.74	18.40 \pm 0.74	18.40 \pm 0.74
T ₁	20.64 \pm 0.56	22.42 \pm 0.81	20.20 \pm 0.78	3.53 \pm 0.26	3.24 \pm 0.37	2.98 \pm 0.23	17.91 \pm 0.36	18.19 \pm 0.79	15.70 \pm 0.74
T ₂	22.70 \pm 1.24	20.00 \pm 0.44	21.00 \pm 0.98	2.95 \pm 0.27	3.15 \pm 0.23	2.91 \pm 0.21	15.43 \pm 0.77	15.57 \pm 1.09	14.95 \pm 1.15
T ₃	23.74 \pm 0.46	23.13 \pm 0.29	19.30 \pm 1.70	3.58 \pm 0.22	3.12 \pm 0.34	2.79 \pm 0.23	16.97 \pm 0.77	16.83 \pm 0.58	14.44 \pm 0.95
ANOVA	F = 0.947	F = 1.069	F = 1.409	F = 1.964	F = 0.226	F = 2.077	F = 0.277	F = 0.905	F = 0.609
	d.f. = 3,116	d.f. = 3,116	d.f. = 3,116	d.f. = 3,116	d.f. = 3,116	d.f. = 3,116	d.f. = 3,116	d.f. = 3,116	d.f. = 3,116
	P=0.428	P=0.374	P=0.025	P=0.937	P=0.877	P=0.120	P=0.841	P=0.614	P=0.002
DMRT	C/T ₁ , C/T ₂ ,	C/T ₂ , C/T ₃ ,	C/T ₁ , T ₂ /T ₁ ,	C/T ₂ , T ₂ /T ₁ ,	NS	C/T ₁ , C/T ₂ ,	C/T ₁ , C/T ₃ ,	C/T ₁ , C/T ₂ ,	C/T ₁ , C/T ₂ ,
	T ₂ /T ₁ , T ₂ /T ₁ ,	T ₂ /T ₁ , T ₃ /T ₁	T ₂ /T ₃ , T ₃ /T ₁	T ₂ /T ₃ , T ₃ /T ₁		C/T ₃	T ₂ /T ₁ , T ₂ /T ₃	T ₂ /T ₃ , T ₃ /T ₁	C/T ₃ , T ₂ /T ₁ ,

C- Untreated♂ x Untreated♀; T₁-Treated♂ x Untreated♀; T₂- Untreated♂ x Treated♀; T₃ -Treated♂ x Treated♀, NS-non significant

Pupation was found to be decreased in *D. melanogaster* and *D. ananassae* at 2 mg/ml (143.80 \pm 4.32), (70.00 \pm 1.93), and 4 mg/ml (141.40 \pm 3.28), (64.40 \pm 3.36), while the reduction was significant in *D. nasuta nasuta* at all the three doses (106.70 \pm 4.28, 103.60 \pm 2.05, and 87.60 \pm 4.95), respectively.

Adult emergence was found to highly reduced among all the species dose dependently. The difference was significant in *D. nasutanasuta* (P < 0.001) and also in *D. ananassae* for all the doses, while the reduction was significant at 4 mg/ml (P < 0.001) and at 8 mg/ml (P < 0.001) in *D. melanogaster* (Table 3). The number of pupae and adults eclosed in *D. ananassae* and *D. nasuta nasuta* were highly reduced when compared to *D. melanogaster*.

3.3 Mortality

The mortality in life stages (larval, pupal, and adult mortality) of different *Drosophila* species in *D. melanogaster* (Figure 1), *D. ananassae* (Figure 2), and *D. nasuta nasuta* (Figure 3) highly increased with increased doses of CBZ. Percent larval mortality was high at 8 mg/ml in *D. melanogaster* (30.48%) (Figure 1) and *D. ananassae* (26.98%) (Figure 2), while the mortality increased for all three doses in *D. nasuta nasuta*

(26.95, 33.41, and 50.64%) (Figure 3), respectively. Percent pupal mortality was similar in *D. melanogaster* and *D. ananassae* for 2 mg/ml, but increased at 8 mg/ml (10.4%) and (12.4%). Mortality also increased in *D. nasuta nasuta* in all the doses, i.e., 2 mg/ml (12.4%), 4 mg/ml (15.37%), and in 8 mg/ml (16.12%). The percentage of adult mortality on exposure to CBZ was significantly increased in *D. nasuta nasuta* at 8 mg/ml (20.14%) and insignificant in *D. melanogaster* and *D. ananassae* at all doses. This shows that larval mortality was high in said three species of *Drosophila* compared to pupal and adult mortality. Pupal mortality was high at high dose in all these species. Increased adult mortality was observed in *D. nasuta nasuta* compared to other two species. Larval stage was more highly sensitive than pupal and adult stages, and *D. nasuta nasuta* was found to be more sensitive to CBZ than *D. melanogaster* and *D. ananassae*.

Table 3. Mean ± S.E of developmental stages on exposure to Carbamazepine in three species of *Drosophila*.

Species	<i>D.melanogaster</i>			<i>D.ananassae</i>			<i>D.nasuta nasuta</i>		
	Hatchability	Pupation	Adults eclosed	Hatchability	pupation	Adults eclosed	Hatchability	pupation	A ec
Control (c)	174.80 ± 0.48	171.00 ± 1.12	163.60 ± 0.87	111.60 ± 1.29	108.20 ± 1.58	106.20 ± 1.29	173.80 ± 1.59	172.20 ± 0.97	166.2
2mg/ml (l)	167.20 ± 3.60	162.60 ± 2.90	118.20 ± 2.48	103.80 ± 2.22	98.20 ± 2.66	91.00 ± 3.71	148.80 ± 3.79	106.20 ± 4.28	98.00
4 mg/ml(m)	179.20 ± 2.98	143.80 ± 4.32	92.20 ± 5.07	107.60± 2.46	70.00 ± 1.93	71.40 ± 4.36	129.20 ± 4.05	103.60 ± 2.05	92.20
8mg/ml (h)	176.80 ± 4.12	141.40 ± 3.28	76.80 ± 4.58	91.80 ± 4.80	64.40 ± 3.36	42.80 ± 4.28	108.80 ± 2.08	87.60 ± 4.95	64.20
ANOVA	F=17.09 d.f = 3, 116 P>0.021	F=34.07 d.f = 3, 116 P<0.003	F=37.09 d.f = 3, 116 P<0.001	F=20.74 d.f = 3, 116 P<0.002	F=31.09 d.f = 3, 116 P<0.001	F=36.09 d.f = 3, 116 P<0.001	F=26.18 d.f = 3, 116 P<0.001	F=41.62 d.f = 3, 116 P<0.001	F=30 d.f = : P<0.001
DMRT	c/l, c/m,c/h, l/m, l/h, m/h	c/l, c/m,c/h, l/m,l/h, m/h	c/l, c/m,c/h, l/m,l/h, m/h	c/l, c/m,c/h, l/m,l/h, m/h	c/l, c/m,c/h, l/m,l/h, m/h	c/l, c/m,c/h, l/m,l/h, m/h	c/l, c/m,c/h, l/m,l/h, m/h	c/l,c/m,c/h, l/m,l/h,m/h	c/l,c/r l/m,l/t

c-control; l-lowdose; m-mid dose; h- highdose.

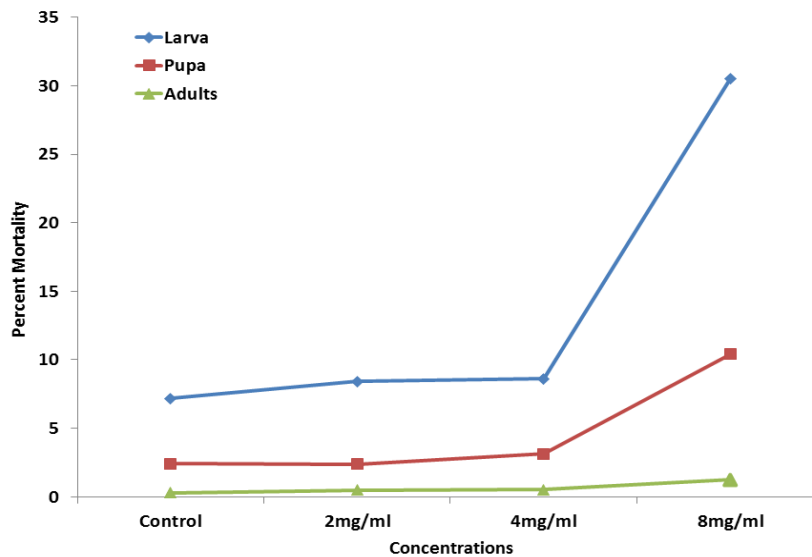


Figure 1. Percent mortality of *Drosophila melanogaster* on exposure to different doses of Carbamazepine.

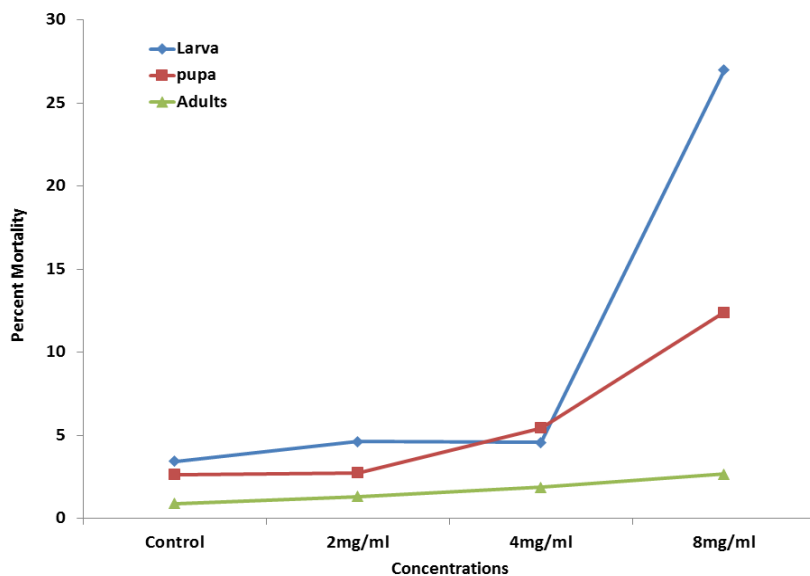


Figure 2. Percent mortality of *Drosophila ananassae* on exposure to different doses of Carbamazepine.

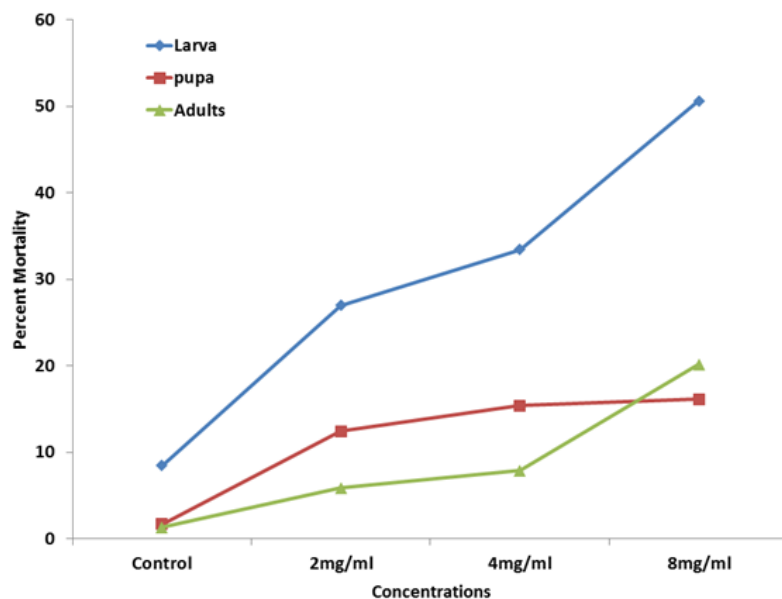


Figure 3. Percent mortality of *Drosophila nasuta nasuta* on exposure to different doses of Carbamazepine.

Discussion

The central nervous system plays a critical role in modulating the physiological and behavioral events associated with normal reproductive function in both male and female mammals (Stoker *et al.*, 2001). Few studies have addressed the issue of sexual activity in animals after AED treatment. Soliman *et al.* (1999) reported that sexual desire was reduced in rats treated with CBZ at very low doses. In the present data, there was decrease in mating propensity in different doses of CBZ and it varied among species of *Drosophila*. Table 1 indicates that CBZ affects the vigour of males and receptivity of females at high doses. Courtship duration was insignificant in *D. melanogaster* and *D. ananassae* while significant in *D. nasuta nasuta* ($P < 0.0001$) at all the doses and experimental crosses T_1 , T_2 , and T_3 on exposure to CBZ.

It is quite natural that copulation is affected when courtship activity is affected. During copulation, sperm from the male are transferred to the female reproductive tract and, therefore, the duration of copulation has a lot of significance in an animals' life (Speith, 1978). Table 2 showed that the copulation duration of three species was affected by different doses of CBZ with significant difference. Copulation duration was

reduced as doses increased; interestingly, it was high in T_3 as compared to T_1 and T_2 . Differences were insignificant on exposure to CBZ for all the species of *Drosophila* for all treated trials but significant for *D. nasuta nasuta* ($P = 0.002$) at high dose.

The mating propensities of flies performing a particular behavior at any given drug level were significantly different. To determine if the observed impairments in mating behavior in CBZ treated flies were due to dysfunction in the male, female, or both, AEDs fed females/males were paired with control males/females. Specifically, the performance of the treated male flies and females (T_3) were reduced compared to the results of pairs C, T_1 , T_2 . This could be due to differences in the dynamics of the interactions between the male and female when only one partner has been exposed to drug. When both partners have been exposed (T_3), the general lack of interest in courtship and copulation are parallel to each other. In contrast, when only the males have been exposed, the males were not interested in the females. Furthermore, there were some instances where the females seemed to seek the male and the male stayed away from the female. When only the female was exposed to drugs, the male vigorously attempted to court whereas females avoided contact with the male. A key gene known to be significantly involved in courtship behaviors is fruitless (*fru*), where almost every stage of the mating process has been shown to be disrupted by certain alleles of the locus (Villegas and Hall, 2008). Antiepileptic drugs that induce the metabolism of gonadal and adrenal steroid hormones induce the synthesis of SHBG, a binding protein for steroid hormones. These alterations in steroid hormones are associated with reproductive endocrine disorders and sexual dysfunction in both males and females (Isojarvi *et al.*, 1995).

The present study showed that *Drosophila* species exposed to doses of CBZ led to reduction in viability with increased doses. The number of offspring that successfully developed from the egg to adulthood was assessed to confirm developmental toxicity. Interestingly, the study also showed that flies reared on media supplemented with different doses of CBZ show a dose-dependent reduction in hatchability, pupation, and adult eclosion in CBZ (Table 3) in all the species. When compared among species, *D. nasuta nasuta* was more severely affected than *D. melanogaster* and *D. ananassae*. Similarly the mean pupation and adult eclosion in *D. nasuta nasuta* was significantly decreased for all the doses assessed, followed by *D. ananassae* and *D. melanogaster*. Reduction in viability showed diverse results among species of *Drosophila* on treatment with different doses of CBZ. In view of the above, the CBZ seem to have species specific effects in *Drosophila*. The viability that arises in response to drugs may help to elucidate the underlying mechanisms in *Drosophila*.

Likewise, significant increased mortality rate was observed in life stages of *Drosophila* on exposure to doses of CBZ. Significant larval mortality was observed in *D. melanogaster* and *D. ananassae* at high dose, while in *D. nasuta nasuta* increased larval and pupal mortality was seen in all the doses while increased adult mortality was observed only at high dose in which larval and pupal mortality is shown here to bear the main responsibility for total mortality, which led to reduction in the number of adult eclosion during *Drosophila* development in all the *Drosophila* species.

There is no absolute safe dose that will provide therapeutic efficacy without the potential risk of toxicity, while evidence suggests that higher drug dosages generally pose a greater risk than do lower doses among species of *Drosophila*. Addition of the fly to the discovery process is predicted to enhance the rate of discovery at reduced costs to potentially identify new targets and therapeutics. The similarities and differences between model animals and humans and will improve the ability to extrapolate risk across species (National Research Council, 2000). In conclusion, the present findings emphasize the *Drosophila* species as a useful model system to unravel the complex etiology of development. Thereby, CBZ exhibit adverse health effects at high doses or after chronic use to humans and are lethal when added to the diet of *Drosophila* and thus reduce the uncertainty associated with human risk assessment.

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Induction of ectopic transverse rows by *Ubx* on fly legs.

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The four-winged “bithorax” fly is familiar to students of genetics. It epitomizes the dramatic phenomenon of homeosis—the conversion of one body part into another. In this case, loss-of-function (LOF) mutations in the gene *Ultrabithorax* (*Ubx*) transform a third thoracic segment (T3) to resemble a second thoracic segment (T2), and in so doing, replace the inconspicuous balancer organs (halteres) with a second pair of wings (Lewis, 1978).

Less well known are the effects of *Ubx*-LOF on the hindlegs. Those legs undergo a similar homeosis to mimic the midlegs—an evolutionary ground state (Casares and Mann, 2001). Thus, *Ubx* dictates T3 (vs. T2) identity both dorsally (haltere vs. wing) and ventrally (hindleg vs. midleg). *Ubx* resides in a cluster of other “*Hox*” genes that collectively control segmental identity in bilaterally symmetric phyla throughout the animal kingdom (Held, 2017).

The *Hox* gene *Sex combs reduced* (*Scr*) governs forelegs just as *Ubx* governs hindlegs. However, certain gain-of-function (GOF) *Scr* phenotypes uncovered recently suggest that *Scr* is playing a subtler role as well (Akam, 1998): when *Scr* is forcibly expressed in midlegs, they acquire transverse rows (t-rows) of bristles not only on their anterior (A) side like forelegs, but also on their posterior (P) side like hindlegs (Held, 2010; Held *et al.*, 2017). These P-side rows suggest that *Scr* is directly inducing t-rows, regardless of segmental identity.

The present study was undertaken to investigate whether *Ubx* has the same ability as *Scr* to elicit t-rows indiscriminately. Indeed, we found that *Ubx* can induce t-rows not only on the P side of forelegs and midlegs—in conformity with T1→T3 and T2→T3 homeosis—but also on the A side of midlegs and hindlegs, where the effect cannot be attributed to homeosis alone.

Materials and Methods

We expressed *Ubx* by the “TARGET” (temporal and regional gene expression targeting) method (McGuire *et al.*, 2004), which relies on the yeast transgenes *Gal4*, *UAS*, and *Gal80^{ts}*. *Gal4* encodes a transcription factor that binds the upstream activating sequence *UAS* (Leung and Waddell, 2004). When *Gal4* is inserted in the *cis*-regulatory region of a “driver” gene, it is expressed at the same time and place as the driver, and any desired “puppet” gene—*e.g.*, the wild-type (WT) *Ubx* gene—can be turned ON congruently by linking it to *UAS*. We used *Distalless(Dll)-Gal4* to elicit *Ubx*^{WT} expression in the tarsus and distal tibia of all

six legs (Kojima, 2004) and *scabrous(sca)-Gal4* to elicit *UbxWT* expression in proneural clusters (Baker and Brown, 2018) all over the fly (Renaud and Simpson, 2001; Troost *et al.*, 2015).

Dll-Gal4:UAS-UbxWT (abbreviated “*Dll>UbxWT*”) flies were obtained (as F₁ offspring) by crossing *Dll-Gal4/CyO; tub-Gal80^{ts}* females X *UAS-UbxWT(isoform-Ia)/TM3, Ser¹* (Bloomington Stock #911) males, and *sca-Gal4:UAS-UbxWT* (“*sca>UbxWT*”) flies were created by crossing *sca-Gal4/CyO; tub-Gal80^{ts}* females X *UAS-UbxWT(isoform-Ia)/TM3, Ser¹* males.

Confusingly, *TM3*-bearing (*Dll-Gal4/+; Gal80^{ts}/TM3, Ser¹*) F₁ males exhibited some of the traits that we expected for *Dll*-driven expression of *Ubx*—*viz.*, scalloped wings (expected for *Ser¹*), abnormal antennae (~100% penetrance), and missing apical bristles (3/10 midlegs)—but other aspects of their phenotype set them apart from *Dll>UbxWT* males—*viz.*, survival to eclosion, normal sex combs, and—to our surprise—extra, inverted leg joints. We traced the joints to the *Ser¹* marker on *TM3* (Bishop *et al.*, 1999; Miller *et al.*, 2016): we found that they also occurred in non-heat-treated *UAS-UbxWT/TM3, Ser¹* (Stock #911) flies. Ectopic joints were mainly confined to tarsal segments 3 and 4, but we also found extra, inverted joints in the tibiae of *Dll-Gal4/+; Gal80^{ts}/TM3, Ser¹* flies from our youngest heat-treated cohorts, presumably due to a quirky interaction between *Dll-Gal4* and *Ser¹* about halfway along the length of the tibia.

We turned *Ubx* ON by shifting F₁ larvae from 18°C (where *Gal80^{ts}* blocks *Gal4*) to 30°C (which disables *Gal80^{ts}* and lets *Gal4* activate *UAS*) at various times before puparium formation (BPF). We then collected pupae from those bottles at 12-h intervals and placed them in humidified petri dishes for the rest of development. Thus, the first batch included individuals aged 0-12 h BPF at the time of shift (average age = 6 h BPF), the second batch 12-24 h BPF (average age = 18 h BPF) and so on, up to 36-48 h BPF. This protocol allowed recovery of all dead pupae and eclosed F₁ adults, regardless of any leg defects that could mire flies in the food. After finding distinct degrees of homeosis (“mild” *vs.* “severe”) in our 12-24 h BPF batch, we repeated the experiment using shorter (6-h) collection intervals and were thereby able to trace the “mild” pupal defects to 12-18 h BPF and the “severe” pupal defects to 18-24 h BPF (see text).

Operationally, we defined “t-row” bristles (normal or ectopic) as bristles whose sockets are aligned transversely and touching one another. By these criteria, as few as two adjacent bristles could be deemed a “t-row”. Distinguishing A *vs.* P t-rows was difficult for basitarsi when t-rows merged. In those cases we used the medial sensillum campaniformia as a marker for the A/P boundary (Held, 2002). In accord with convention, we italicize gene names (*e.g.*, *Ubx*) and use Roman typeface for protein products (*e.g.*, *Ubx*). Abbreviations include “ta1-ta5” for tarsal segments 1 through 5, though ta1 is more commonly called the basitarsus.

Flies were raised on Ward’s *Drosophila* Instant Medium plus live yeast. Experimental individuals were preserved in 70% ethanol, mounted in Faure’s medium (Lee and Gerhart, 1973) between cover slips, and photographed with a Nikon microscope at 200× or 400× magnification.

Results and Discussion

Fly legs arise from imaginal discs that grow inside the larva (Schubiger *et al.*, 2012). *Ubx* is expressed in 3rd-leg discs (Brower, 1987), more strongly in the P compartment, where the t-rows reside, than in the A compartment (Held, 2002; Shroff *et al.*, 2007). *Ubx* is also expressed in the P compartment of wild-type 2nd-leg discs, where it plays a role in determining the distribution of small hairs (trichomes) on the back of the midleg femur (Stern, 1998; Kittelmann *et al.*, 2018). *Ubx* is not normally expressed in 1st-leg discs.

By placing *UAS-UbxWT* under the control of *Dll-Gal4* (in *Dll-Gal4/+; UAS-UbxWT/tub-Gal80^{ts}* flies) we were able to force *Ubx* to be expressed throughout the epidermis of the tarsus and distal tibia in 1st, 2nd, and 3rd leg discs for varying lengths of time before pupariation. The duration of exposure to *Ubx* was varied by shifting larvae from 18°C to 30°C and then collecting the maturing pupae at 12-h intervals. In this way we obtained cohorts whose age at the time of the shift was 0-12, 12-24, 24-36, or 36-48 h BPF (see Materials and Methods). The younger the larvae at the time of the shift, the longer they were exposed to exogenously imposed *Ubx*.

We mainly studied males because the sex comb offers a sensitive barometer of T1→T3 homeosis. This conspicuous row of dark bristles (“teeth”) is found only on male forelegs (Hannah-Alava, 1958) (Figure 1). The greater the foreleg-to-hindleg conversion, the fewer the teeth, and the less rotated the comb was

relative to the transverse axis (Held *et al.*, 2004; Atallah *et al.*, 2009). Regardless of the time of the shift, all of the flies with reduced sex combs were found to have died as pupae at the pharate stage (just before eclosion). In the 0-12 h BPF cohort, for example, all 10 of the *Dll>UbxWT* males (among 80 F₁ total) died before eclosion and had unrotated or absent combs (4.1 \pm 3.4 teeth, vs. 11.9 \pm 0.9 in controls; n = 10 each). Suppression of sex combs by *Ubx-GOF* has been reported previously (Shroff *et al.*, 2007).

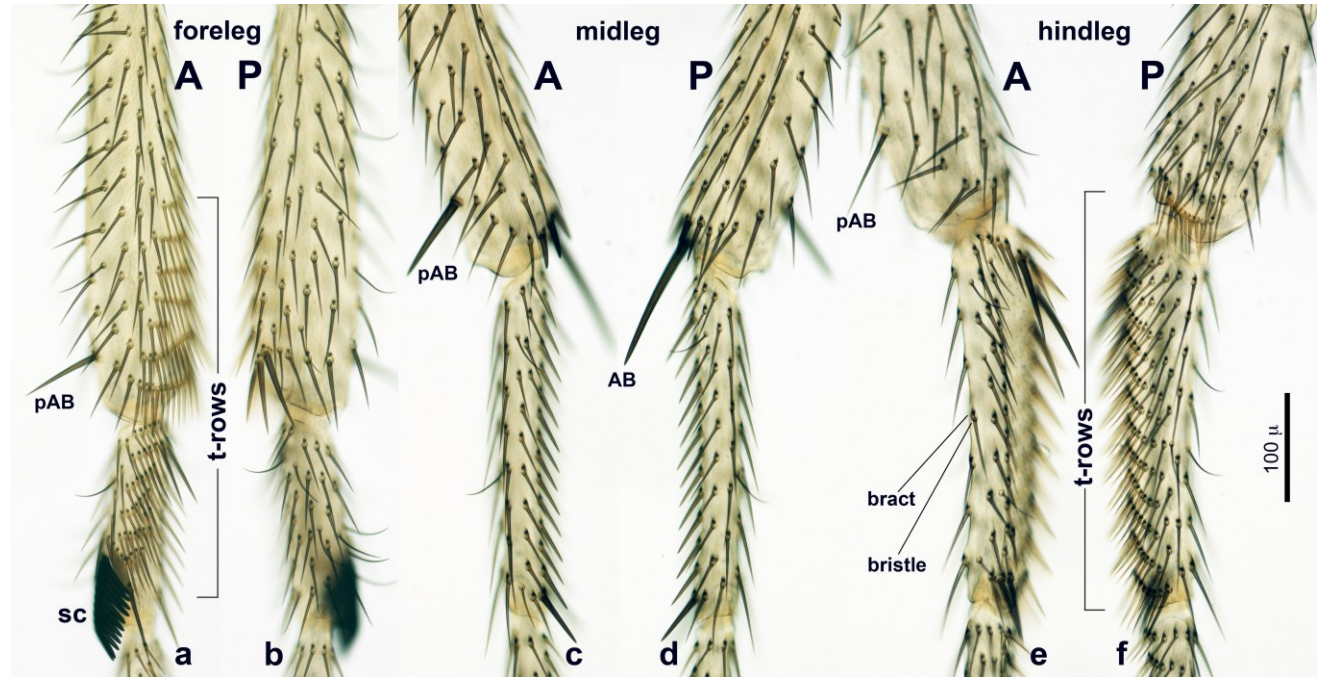


Figure 1. Foreleg (a, b), midleg (c, d), and hindleg (e, f) anatomy in *D. melanogaster* (Hannah-Alava, 1958; Shroff *et al.*, 2007; Schubiger *et al.*, 2012). These legs are from control *Dll-Gal4/+; UAS-UbxWT/tub-Gal80^{ts}* males raised at 18°C—a temperature that lets *Gal80^{ts}* block *Gal4*'s activation of *UAS-UbxWT*, leading to a wild-type phenotype. Two segments are shown per leg: the basitarsus (below) and the distal portion of the tibia (above). Each tibia has a pre-apical bristle (pAB) on its dorsal side, but the midleg pAB is thicker, darker, and blunter. Only the midleg has an apical bristle (AB). Above the AB is an arc of ~6 “spur” bristles (c and d) that are shorter, blunter, and darker than nearby bristles. Parallel rows of transversely aligned (t-row) bristles decorate the anterior (A) side of the foreleg (a) and the posterior (P) side of the hindleg (f), but are lacking from the midleg. Flies use these rows as brushes to remove dust: forelegs bend forward to clean the eyes and hindlegs bend backwards to clean the wings (Szebenyi, 1969; Vandervorst and Ghysen, 1980). The sex comb (sc) develops from a t-row that rotates ~90° (Held *et al.*, 2004; Atallah *et al.*, 2009). Comb bristles are dark, thick, and blunt, while t-row bristles are yellow and tapered. Most tibial and tarsal bristles have a thorn-like protrusion (“bract”) above their socket (e), though most t-row bristles on the tibia lack bracts. All photos are at the same magnification (scale bar in f). A and P images of the left foreleg were flipped horizontally for ease of comparison with the right midleg and hindleg. Some bristles were deflected due to being sandwiched between cover slips.

In the 12-24 h BPF cohort, we recovered 15 *Dll>UbxWT* males (among 83 F₁ total) with unrotated or missing combs. These pharate males fell into two groups based on their tarsi: (1) “mild” pupae whose tarsi had all 5 segments (6 flies) and (2) “severe” pupae whose tarsi had only ~3 segments (9 flies), with the extent of T1→T3 and T2→T3 homeosis being greater in the severe subgroup (Figure 2). This mild/severe distinction

proved to be age-related: when we repeated the experiment using 6-h (vs. 12-h) collection intervals, all of the *Dll>Ubx*WT males (8/8) in the older (12-18 h BPF) cohort displayed the mild syndrome, whereas the majority (9/16) of dead pupae in the younger (18-24 BPF) cohort displayed the severe one. The greater severity of the latter group makes sense because those larvae were exposed to *Ubx* for a longer period.

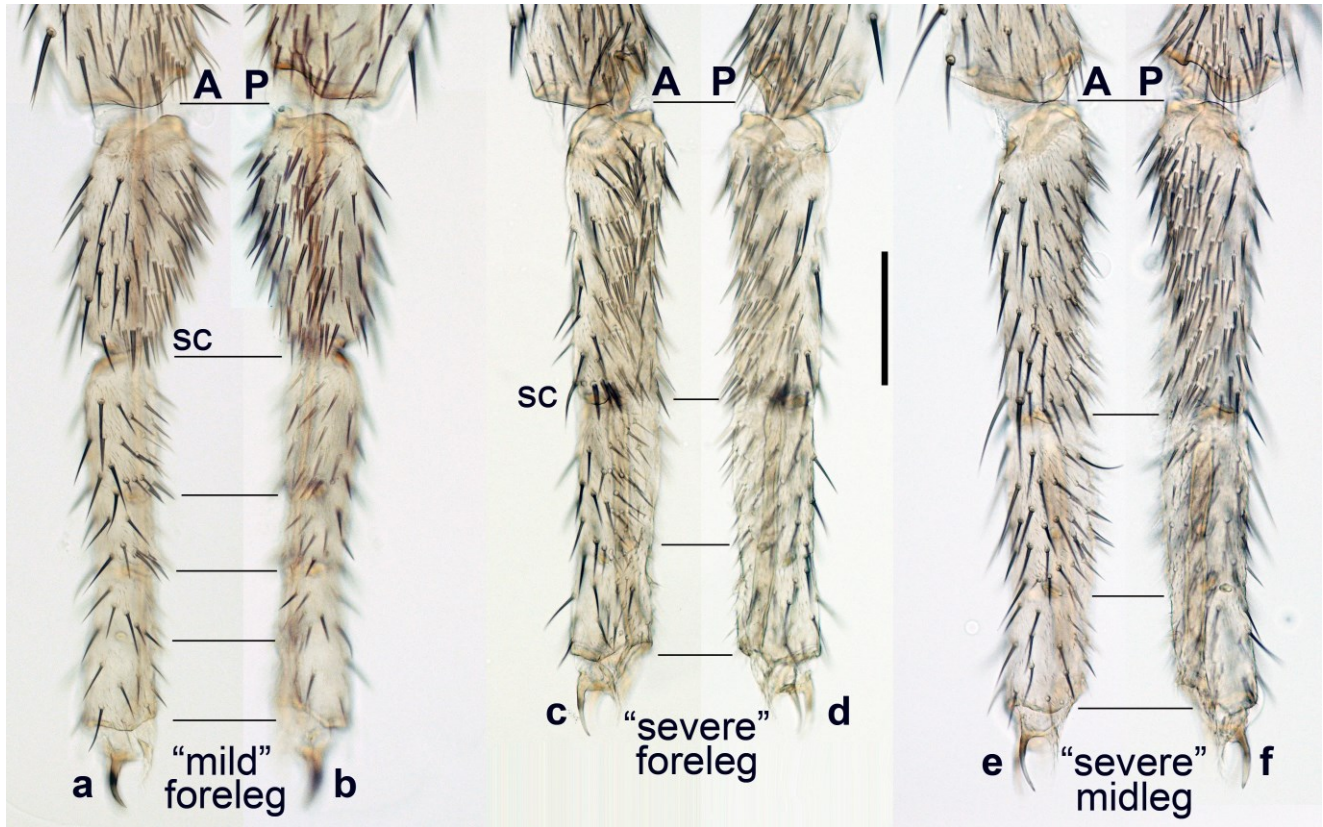


Figure 2. Alternative phenotypes among 15 *Dll-Gal4/+; UAS-UbxWT/tub-Gal80^S* males recovered as dead pupae from temperature shifts at 12-24 h BPF (see Materials and Methods). **a**, **b**. “Mild” phenotype observed for 6 sex-comb-deficient males whose tarsi were stunted but still had 5 segments (= wild-type number). Horizontal lines mark segment boundaries. Flies in this subgroup displayed moderate T1→T3 homeosis, with vestigial (as here) or missing combs and fewer t-row bristles on the tibia and basitarsus. **c**, **d**. “Severe” phenotype for 9 sex-comb-deficient males whose tarsi had only ~3 segments. Flies in this subgroup had more t-row bristles on the P side of the foreleg on average than the mild subgroup (Figure 3), indicating stronger T1→T3 homeosis. **e**, **f**. Midleg tarsus of a “severe” fly. T-rows are visible on the P side of the basitarsus and, to a lesser extent, on the A side as well. Unlike *Scr-GOF* (Held *et al.*, 2017), *Ubx-GOF* does not elicit t-rows on ta2-ta5. Basitarsal shapes (**a-f**) are hindleg-like (*c.f.*, Figure 1). Other anomalies in the mild and severe subgroups included: (1) wider tarsi, (2) fused segments, (3) smaller bristles, (4) lighter pigmentation, (5) missing bracts, and (6) excess trichomes. The latter trait was surprising, given the ability of *Ubx* to suppress trichomes on the femur (Stern, 1998; Kittelmann *et al.*, 2018). Also, most pupae had a wider “apodeme” (Mirth and Akam, 2002; Soler *et al.*, 2004), which appears here as a hollow internal tube, and apodemes were shorter in “severe” pupae—extending from the claws up to ta1 (8/30 legs), ta2 (10), ta3 (5), or absent (7)—vs. “mild” pupae—extending to tibia (6/30 legs), ta1 (8), ta2 (11), ta3 (2), ta5 (2), or absent (1). Images of the left leg in **c** and **d** were flipped for ease of comparison with right legs in other panels. All images are at the same magnification; scale bar in **d** = 100 microns.

With even longer exposure to Ubx (24-36 h BPF cohort), the majority (19/24) of *Dll>UbxWT* pupae had legs that were truncated at the level of the tibia—precluding any assessment of effects on sex combs or tarsal t-rows. Similar truncations also occurred when *Dll-Gal4* was combined with *UAS-ScrWT* instead of *UAS-UbxWT* (Held, 2010), so this stunting could be due to a “flooding” of leg cells with exogenous transcription factors during growth of the imaginal discs. Indeed, we found that *Dll-Gal4* alone (sans *UAS*) can curtail tarsal length by 30% when larvae are exposed to 30°C throughout the third instar, as well as reducing the number of tarsal segments to three. Transcription factor “pollution” of this kind might therefore explain why “severe” pupae have fewer tarsal segments than “mild” pupae (12-24 h BPF cohort; Figure 2).

Figure 3 plots the number of laterally adjacent (“t-row”) bristles as a function of larval age at the time of the upshift. Neither wild-type flies nor *Dll>UbxWT* controls raised at 18°C have t-rows on the P side of their foreleg basitarsi (Figure 1), but *Dll>UbxWT* males that are shifted to 30°C as larvae do display t-rows there, and the number of t-row bristles increases from 16.7 (0-12 h BPF) to 24.0 (12-18 h BPF) to 49.0 (18-24 h BPF; n = 10 each) with the duration of Ubx exposure. Indeed, the maximum (49.0) exceeds the number of t-row bristles on the A side of the same legs (39.5), and it approaches the level on the P side of hindlegs (55.1) in the same cohort. This P-side phenotype was expected for T1→T3 homeosis based on previous reports (Shroff *et al.*, 2007), as was the A-side loss of comb teeth (see above), but the number of t-row bristles on the A side stayed constant instead of vanishing. The endurance of the foreleg’s A-side t-rows may be due to persistence of *Scr* expression there despite the imposition of exogenous Ubx.

In contrast to the forelegs, the midleg and hindleg phenotypes that we observed defy a simple explanation based on homeosis alone, because *Ubx-GOF* induces t-rows on the A side. The numbers of t-row bristles evoked on the A side of midleg basitarsi were 20.2, 22.0, and 37.2 (for 0-12, 12-18, and 18-24 h BPF shift times, respectively), and the numbers on hindleg basitarsi were 21.4, 26.0, and 33.2 for the same cohorts. *Ubx-GOF* is evidently capable of initiating t-row development directly, rather than indirectly (via its orthodox role in enforcing leg identity).

Our previous analysis of *Scr-GOF* (Held *et al.*, 2017) led to the same conclusion about *Scr* as we reached here about *Ubx*—namely, that it can induce t-rows on either the A or P side of any basitarsus (fore-, mid-, or hindleg) with one exception. The ability of excess *Scr* to elicit t-rows on the P side of the foreleg is minimal. Conceivably, *Scr* might be suppressed there by *engrailed* (*en*)—the selector gene for P compartments (Morata and Lawrence, 1975; Lawrence, 1984)—though *En*’s inhibition would probably be post-transcriptional, because neither the *Dll-Gal4* driver nor the *UAS-ScrWT* construct are likely to have *en*-dependent enhancers. *Ubx* is inhibited by *en* in the wing (Emerald and Shashidhara, 2000), but not in the hindleg where *Ubx* is heavily expressed on the P side. If *Ubx* (unlike *Scr*) can evade suppression by *en* on the foreleg as well, then that immunity could explain why *Ubx-GOF* induces four times more t-row bristles on the P side of the foreleg (49.0 at 18-24 h BPF) as *Scr-GOF* (12.9 at 20 h BPF; Figure 3).

As in our earlier *Scr-GOF* study, we used a second *Gal4* driver in addition to *Dll-Gal4*. The *scabrous* gene (*sca*) is expressed in proneural clusters, which are groups of epidermal cells from which bristle cell progenitors are selected. They precede bristles and cover a larger area (Held, 2002). Milder effects were expected for *sca>UbxWT* than for *Dll>UbxWT* because *sca* is expressed just before bristle differentiation, which leaves the epidermal cells only a few hours to switch their identities from T1 or T2 to T3. No *sca>UbxWT* flies were obtained from the 36-48 h BPF shift, so we focused on *sca>UbxWT* males from the 24-36 h BPF cohort.

All of the 13 *sca>UbxWT* males that we recovered (among 104 F₁ total) in the 24-36 h BPF cohort died as pharate pupae, and their second legs all lacked apical bristles, which was consistent with a T2→T3 transformation, as has been reported before (Rozowski and Akam, 2002). Sex combs persisted on all of their forelegs (defying any T1→T3 conversion), but all of the sex comb teeth therein were yellow, thin, pointed, and shriveled. Indeed, all of the bristles on all six legs were yellow, thin, and reduced in length. Transverse rows also persisted on the foreleg basitarsus and tibia (also defying a T1→T3 conversion), but the alignment of the bristles therein was commonly disrupted, often resulting in clumping of bristle sockets along the rows.

Ectopic “t-row” bristles (*i.e.*, extra laterally-adjacent bristles) were commonly seen on *sca>UbxWT* forelegs, midlegs, and hindlegs—albeit far fewer than on the *Dll>UbxWT* legs described above. Foreleg basitarsi had an average of 3.3 ectopic t-row bristles on their P side (osculating with row-1 bristles; n = 20 legs), while hindleg basitarsi had an average of 1.5 ectopic t-row bristles on their A side (osculating with row-

8 bristles; n = 13 legs). Midleg basitarsi had an average of 3.1 ectopic t-row bristles on their P side (osculating with row-1 bristles; n = 20 legs) but only 0.3 ectopic t-row bristles on their A side (osculating with row-8 bristles; n = 20 legs). This 10-fold A/P asymmetry (0.3/3.1) was surprising given the symmetry (4.3/4.6) that we previously witnessed for ectopic t-row bristles on *sca>ScrWT* midlegs (Held *et al.*, 2017).

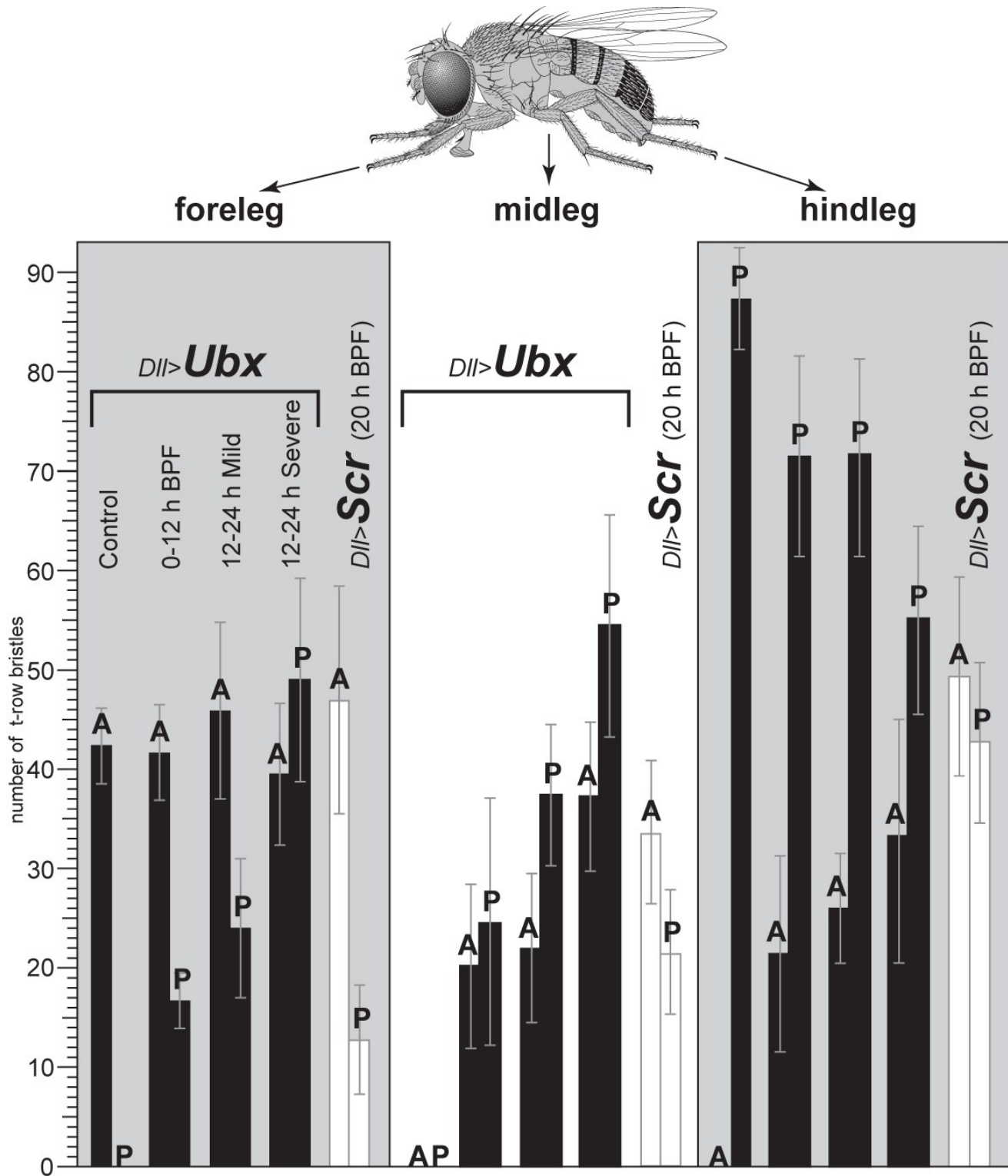


Figure 3. Mean numbers of laterally adjacent (“t-row”) bristles (+/- standard deviation) on the anterior (A) and posterior (P) sides of foreleg, midleg, and hindleg basitarsi from *Dll>Ubx*WT males (N = 10 legs/bar) shifted to 30°C at different times before puparium formation (0-12 h BPF or 12-24 h BPF). All of the *Ubx*-GOF individuals analyzed here died as pharate pupae before eclosion. Pupae with all 5 tarsal segments were assigned to a “mild” group, while those with truncated tarsi (only 2 or 3 segments remaining) were pooled into a separate “severe” group. The mild group was traced to 12-18 h BPF, while the severe group was traced to 18-24 h BPF (see text). The left-most pair of histograms for each leg type pertain to control flies (same genotype as experimental flies) raised entirely at the permissive temperature for *Gal80^{ts}* (18°C). The rightmost pair of histograms for each leg type (white bars) gives comparative data for *Dll>Scr* males shifted to 30°C at 20 h BPF (Held *et al.*, 2017). When legs are sandwiched between cover slips, they tend to orient their A and P sides facing up or down, but sometimes they rotate, and the merging of t-rows can obscure the A/P boundary. In those cases we used the medial sensillum campaniformia as a marker for the ventral midline (see Materials and Methods).

Conclusions

Hox genes are famous for subdividing the bilaterian head-tail axis into metameres (Angelini and Kaufman, 2005; Held, 2017), but within insects they have, over the eons, insinuated themselves into the circuitry of segmental patterning at lower echelons as well (Weatherbee *et al.*, 1998; Pavlopoulos and Akam, 2011), all the way down to the level of bristles (Rozowski and Akam, 2002) and hairs (Stern, 1998; Kittelmann *et al.*, 2018). The data presented here (based upon two different *Gal4* drivers) show that *Ubx*, like *Scr* (Held *et al.*, 2017), can create t-rows in regions beyond its normal jurisdiction. Our results therefore affirm that *Hox* genes are micromanagers, in addition to serving as chief executive officers (Akam, 1998). However, we cannot fully decipher the nature of the link to t-rows until we know much more about how t-rows arise in normal development (Held, 2002).

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Mating latency and mating duration in *Drosophila melanogaster* strains maintained over 400 generations on four types of food.

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Sexual behaviour of *Drosophila* represents series of behavioral steps, expressed by both sexes that culminate in copulation: data concerning their description up to complex genetic backgrounds are widely available in literature (Hall, 1994; Greenspan and Ferveur, 2000; Beaver and Giebultowicz, 2004; Edward *et al.*, 2014). Variation in male nutrition could be important for reproductive behavior, since the nutritional value of food has effects on the properties mediated by the accessory gland proteins (Fricke *et al.*, 2008). In females, protein/sugar ratio in food affects fecundity and lifespan (Lee *et al.*, 2008; Fanson *et al.*, 2009; Rodrigues *et al.*, 2015). Nutrition is also related with morphological traits, such as body size, as well as with physiological abilities that could be linked with mating. In this work, we have examined two components of *Drosophila* mating behavior and fitness, mating latency (ML) and mating duration (MD), in strains that have been cultivated over the years in various nutritive conditions. Mating latency is related to male age (Eastwood and Burnet, 1977), body size (DeBelle *et al.*, 2016) and vigor, and represents an important component of male competitive success (Bacigalupe *et al.*, 2007). It is also referred to as a trait correlated with fecundity, fertility, and longevity (Hegde and Krishna, 1999). In females, ML is influenced by physiological state (Eastwood and Burnet, 1977) and related with their mate preference (Bacigalupe *et al.*, 2007). Mating duration is correlated with female remating (Gilchrist and Partridge, 2000; Bretman *et al.*, 2013) and sperm transfer (Yamamoto *et al.*, 1997). It is determined by both sexes and tested in various experimental designs investigating complex genetic background (Mackay *et al.*, 2005), sexual conflict (Edward *et al.*, 2014), the effects of previous mating experience (Pavković-Lučić *et al.*, 2014), social environments (Taylor *et al.*, 2013), and so forth.

Previous studies that manipulated with environmental factors have revealed considerable plasticity in courtship/mating traits in *Drosophila* (see, for example, Bretman *et al.*, 2009). Since the influence of environmental (nutritional) variation on ML and MD is insufficiently known, the aim of this study was to explore aforementioned behavioral traits in four *Drosophila melanogaster* strains after long-term laboratory growing on different diets. Previously, we have observed that these diets differ in protein content and C/N ratio, which was reflected on mating success and several fitness components (Trajković *et al.*, 2017a; Trajković *et al.*, 2017b).

D. melanogaster strains used in this experiment were maintained for more than 400 generations on four different diets (for recipes see Kekić and Pavković-Lučić, 2003): standard cornmeal diet (“St” strain), and diets that contain tomato (“T” strain), banana (“B” strain), and carrot (“C” strain) under conditions optimal for the species (temperature of 25°C, relative humidity of 60%, 300 lux of illumination, 12 h:12 h light/dark cycle). Once the hatching starts, virgin flies were separated by sex and strain every 8 hours. Females were kept in groups (5 *per* group), while males were housed separately, in order to prevent homosexual behavior (Napolitano and Tompkins, 1989). Separated flies of both sexes and strains were kept under optimal laboratory conditions until they were 4-5 days old, when mating assays were performed.

ML and MD were monitored in four experimental groups. Namely, crosses were allowed between flies within the same strain ($\text{♂St} \times \text{♀St}$, $\text{♂T} \times \text{♀T}$, $\text{♂B} \times \text{♀B}$, $\text{♂C} \times \text{♀C}$). In total, 960 individuals, *i.e.* 240 individuals *per* experimental group, were tested (twelve replicates *per* experimental group, 10 females + 10 males *per* replica). Behavioral traits were quantified as follows: ML was calculated as the time which elapsed from the introducing of flies into a mating vial until copulation started, while MD was scored as the time from the beginning to the end of mating. Since obtained numerical data did not have a normal distribution, a non-parametric Kruskal-Wallis test, with multiple comparisons, was performed.

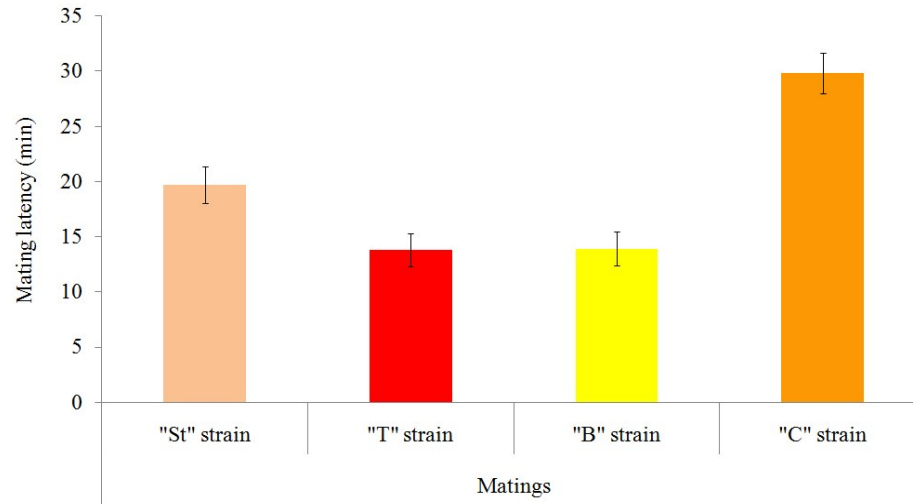


Figure 1. Mean values (\pm S.E.) of mating latency in four *D. melanogaster* strains. Abbreviations: "St"- flies maintained on standard cornmeal substrate, "T"- flies maintained on tomato substrate, "B"- flies maintained on banana substrate, "C"- flies maintained on carrot substrate.

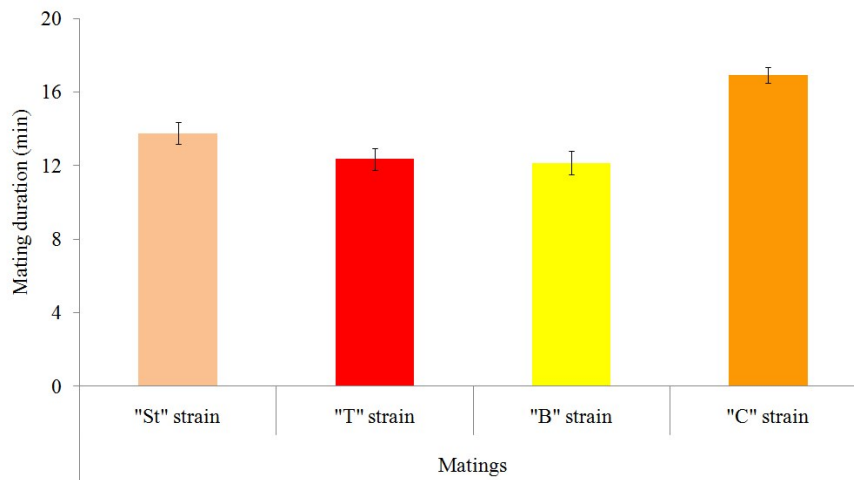


Figure 2. Mean values (\pm S.E.) of mating duration in four *D. melanogaster* strains. Abbreviations: as in Figure 1.

Mean values (\pm S.E.) of ML and MD are presented in the Figures 1 and 2, respectively. Kruskal-Wallis test revealed significant difference in both behavioral traits among strains (mating latency: $H = 52.414$, $df = 3$, $p < 0.001$; mating duration: $H = 48.643$, $df = 3$, $p < 0.001$). *Post hoc* analysis determined that flies

maintained on the carrot diet had the longest mating latency ($p < 0.001$) and mating duration ($p < 0.001$) in comparison with flies from all other strains. Differences in ML and MD were not detected among St, T, and B strains.

In our earlier studies, conducted after more than a year of maintaining of flies on standard cornmeal and fruit/vegetable substrates, differences in ML and MD were not observed neither in female choice tests (Pavković-Lučić and Kekić, 2010), nor in MD in multiple choice tests (Pavković-Lučić *et al.*, 2010). Later, we have determined that flies maintained on the carrot diet were less successful in mating in comparison with flies reared on standard cornmeal diet, and diets containing tomato and banana (Trajković *et al.*, 2017a). It is possible that males from strain maintained on the carrot diet may compensate, in some way, low mating success with prolonged copulation, and, in that way, prevent subsequent female mating. Since, in competitive conditions, males can gain significant fitness benefits from extended mating duration (Bretman *et al.*, 2013), further examination of our assumptions is needed.

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First registry of *Paracoxenus guttatus* (Diptera, Drosophilidae) at neotropical region.

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Abstract

The present study reports the first record of the drosophilid *Paracoxenus guttatus* in a tropical region, more specifically in the south of Brazil. Samples were captured using entomological net when flying

over banana baits, near to a guava tree with rust fungus. Morphological characters identified three males, including male terminalia analysis using scanning transmission electron microscopy. The presence of this species has been associated with rust fungi of the Order Pucciniales. This register expands widely the occurrence area of this species. Key-words: Steganinae, Atlantic Forest, South of Brazil.

Introduction

Amongst Diptera, Drosophilidae is one of the largest families including almost 3000 species (Bachli, 2018). The genus *Paracacoxenus* belongs to the Subfamily Steganinae (Diptera, Drosophilidae) and little is known about ecological, genetic, and developmental aspects. Species from this genus are found using fungi as substrates for feeding and oviposition (Hunt, 1984). Mycophagia within the Drosophilidae family occurs in different genera of the Steganinae and Drosophilinae subfamilies. Therefore, in a same fungus different Drosophilidae species can be found, as well as other species of Diptera (Courtney *et al.*, 1990). *Paracacoxenus guttatus* is highly attracted by the fungus *Cronartium ribicola* Fisch, known as the "pest" of pinus in the United States, and has also been found in another rust fungus, *Cronartium comandrae*. This species, along with *Megaselia* (Phoridae), has been implicated as being the main responsible for the cross fertilization of the fungus *Cronartium ribicola* (Hunt, 1984). This fungus is considered a pathogen that can lead to death of host trees (Newcomb *et al.*, 2010). The occurrence of the *Paracacoxenus guttatus* has never been recorded in the Neotropics. Previous studies indicate that the extension of its distribution is limited only to northern hemisphere, in U.S.A. and Canada (Hunt, 1984; Powell, 1971). The present study provides a first registry of this drosophilid in a region of Atlantic Forest of the south of Brazil.



Figure 1. (A) Santa Catarina State (south region of Brazil); (B) The collecting point is represented here by a red dot at the south of Florianópolis City.

Material and Methods

Sample collections

Drosophilids were collected in the fall of 2015 using an entomological net while flying over fermented banana baits. Collections were made at Parque Estadual da Serra do Tabuleiro (coordinates: $27^{\circ}48'20''$ S;

48°33'50'' W), an Atlantic Rainforest region, in Florianópolis city (an island of Santa Catarina State), south of Brazil (Figure 1).

Species were identified based on morphological characters using an identification key to drosophilid genera (McAlpine, 1968).

Analysis of male terminalia

To the scanning transmission electron microscopy (STEM) analysis of the male terminalia the samples were previously washed with distilled water to remove impurities deposited on the surface. Afterwards they were fixed in 2.5% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.2) and 0.2 M sucrose for 4h, followed by washings in 0.1 M cacodylate buffer (pH 7.2). Material was dehydrated in an aqueous ethanol series of increasing concentrations, with the last exchanges being in 100% ethanol. After drying, the samples were glued to the aluminum bracket and metallised (Blatec Metallizer, CED 030). Samples were observed and photographed in a scanning microscope model Jeol 6390 LV at the Central Laboratory of Electronic Microscopy - LCME of Universidade Federal de Santa Catarina.

Results and Discussion

Only three males were captured when flying over the baits placed near to a guava tree with rust fungus and identified as *Paracacoxenus guttatus* (Figure 2), based on morphological characters, including the male terminalia. This guava tree with the rust fungus was located near the edge of the forest, and it was probably the presence of this rust fungus that attracted these individuals. *Paracacoxenus guttatus* has been reported to be mycophagous of rust fungi, of the Order Pucciniales, and many fungal species of this group are plant parasites (Hunt, 1984; Newcomb *et al.*, 2010). Also were found *Megaselia spp.*, a known genre associated to rust fungus, and some commonly found species of *Drosophila* in that region, like *Drosophila polymorpha*, *Drosophila cardinoides*, *Drosophila capricorni*, *Drosophila willistoni* and species of the Tripunctata group.

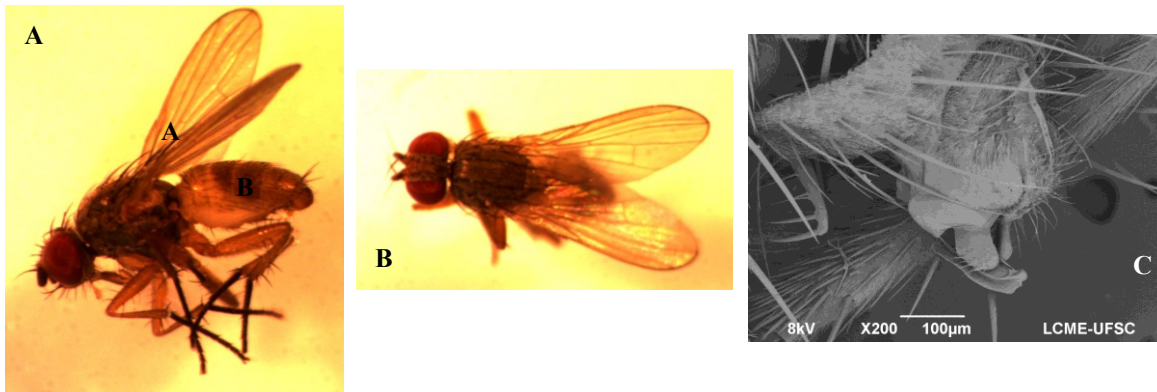


Figure 2. (A) Lateral view and (B) dorsal view of a *P. guttatus* male. In (C) a lateral view of male genitalia by STEM.

Conclusion

This is the first neotropical record of *P. guttatus*, which expands widely the occurrence area of this species. Further studies must be done about *P. guttatus* dispersion, such as resources used, its relation with rust fungi, and specific aspects of its morphology, including internal genitalia as well, which can provide information on ecological characteristics and colonization strategies.

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First occurrence of the tropical vinegar fly *Drosophila vulcana* Graber in West Asia and Iranian Baluchestan region.

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Abstract

The drosophiline species *Drosophila vulcana* Graber is recorded here from the Iranian side of the Baluchestan region and West Asia for the first time. Previous to this discovery, *D. vulcana* was known only from Africa and tropical Asia. The images of the male genitalia are provided.

Introduction

The family Drosophilidae consists of about 4000 species, of which 1500 species belong to the genus *Drosophila* Fallén. The family is divided into two subfamilies (Drosophilinae and Steganinae) and includes predators, plant-feeders, and yeast-grazers (Marshall, 2012). The species *Drosophila vulcana* was originally described from Uganda (Graber, 1957) and later found to occur in West Malaysia and Thailand (Takada and Momma, 1975), India (Singh and Negi, 1992), Kenya (Takada *et al.*, 1990; Andersson Escher *et al.*, 1997), Congo, Ivory Coast, Tanzania, and Zimbabwe (Brake and Bächli, 2008; Bächli, 2018). *D. vulcana* belongs in the relatively large subgenus *Sophophora* Sturtevant and *melanogaster* group (Bächli *et al.*, 2004). The Baluchestan region of Iran is located in the southeast of the country where the three Afrotropical, Oriental, and Palaearctic regions converge to shape its spectacular insect fauna. Our recent discoveries of fly species (Parchami-Araghi *et al.*, 2015, 2016, 2017, 2018) indicate that Baluchestan is home to a number of undescribed or exotic vinegar fly species that necessitates conducting extensive expeditions throughout the region.

Material and Methods

Both Malaise traps and hand-made drosophilid traps were employed during the course of this study. We turned the Malaise traps (Figure 1) into drosophilid traps by dumping plenty of rotting fruit underneath them. We also made specially-designed drosophilid traps out of plastic yogurt buckets to hang them up on the date palm trees (Figure 2). Each bucket contained a mixture of saturated saltwater (as preservative) and a few drops of dish soap (as surface tension breaker) as well as a cup of rotting fruit, which was fixed on top of the liquid surface. Drosophilids, which were attracted to the bait, entered the bucket through the small holes on the upper sides of the container and drowned. They were later recovered from the liquid and transferred to 75% ethanol. The baits were refreshed once a week to maintain the efficiency of the traps. To examine the male genitalia, we gently detached the abdomen from the rest of the body and heated it in 10% KOH and placed in glacial acetic acid to neutralize the base. The collection site is as follows: IRAN: 2♂, Sistan-Baluchestan province, Bampur, Agricultural and Natural Resources Research Center, 525 m, 27°11'56"N

60°29'52"E, 15.iii–20.vi.2016, rotting fruit, leg. F. Basavand. The specimens are preserved at the Hayk Mirzayans Insect Museum (HMIM), Tehran, Iran.

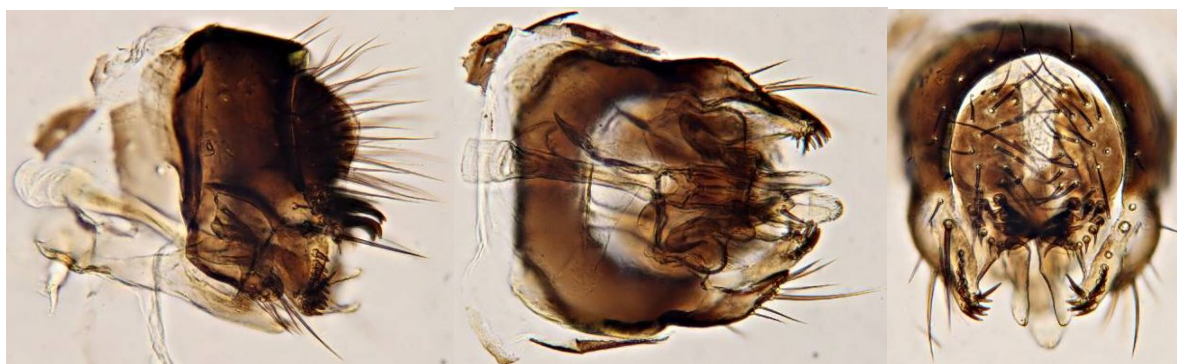


Figures 1–2. General views of the collecting site and traps, Bampour, Baluchestan. 1, Malaise trap in the grounds of the Agricultural and Natural Resources Research Center; 2, bait trap on a date palm tree (photos by F. Basavand).

Results and Conclusion

The species *D. vulcana* is newly recorded here from West Asia and the Iranian Baluchestan region. The male specimen of *D. vulcana* is distinguished from other members of the *melanogaster* group by a combination of the following characters: longitudinal sex combs on tarsomeres 1-2 of protarsus, 3 strong apicoventrally curved spines on cercus and the shapes of aedeagus and decasternum (Figures 3-5). There is nothing known about the biology of *D. vulcana* other than its original collecting site that was reportedly in the flowers of the cucurbit *Adenopus abyssinicus* (Bock and Wheeler, 1972). The Baluchestan region is located in the subtropical zone and its climate is characterized by hot summers and mild winters. The natural habitats of the Baluchestan's region have been under enormous destructive pressure on account of unsustainable policies and mismanagement. Our faunistic study of the drosophilid fauna of the region, which has been continuing over the past years, aims to highlight the fragility of the Baluchestan's natural ecosystems, especially the Jazmourian Wetland.

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Figures 3–5. Male genitalia of *Drosophila vulcana*. 3, lateral view; 4, ventral view; 5. posterior view (photos by E. Gilasian).

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Morphometry of male accessory glands in natural populations of *D. bipectinata* and *D. malerkotliana*.

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Abstract

Morphometric studies of accessory glands in *D. bipectinata* and *D. malerkotliana* collected from Kalghatgi, Yellapur, and laboratory populations were measured. The size of the gland varies between localities within species and between species of the same locality. The size of the gland is larger in the Kalghatgi population compared to other two populations.

Introduction

Morphometry refers to the quantitative analysis of form that includes size and shape of an animal or organ. Morphometric studies are very important in taxonomy to identify species and in analysing the variation among the individuals of same species, between species of a population or between populations, and between different geographical regions (Hegde *et al.*, 2000; Vishalakshi and Singh, 2008).

In *Drosophila*, the accessory gland appears only during adult stage of male and is a protein secretory tissue of the reproductive system that is species specific. The secretory protein later plays an important role in reproduction. These secretory proteins assure reproductive success by reducing the female's receptivity to mating with other males and enhancing both fecundity and productivity (Raviram and Ramesh, 2003;

Suhasini, 2014). Morphometry of accessory glands, qualitative and quantitative analysis of secretory proteins of *Drosophila* of laboratory populations with respect to age of the flies has been studied (Shivanna and Ramesh, 1995a; Hiremani and Shivanna, 2010). However, there are no reports on morphometry of accessory glands of *Drosophila* in the natural populations. Hence, the present study on accessory glands of *ananassae* subgroup species, *D. bipectinata* and *D. malerkotliana*, were selected as these species are easily available in and surrounding localities of Dharwad (Srinath and Shivanna, 2014).

Materials and Methods

D. bipectinata and *D. malerkotliana* were collected from Kalghatgi and Yellapur, Karnataka, India during August 2016 to March 2017, and laboratory stocks were obtained from *Drosophila* Stock Centre, University of Mysore, Mysore. The flies were cultured in wheat cream agar medium (Shivanna *et al.*, 1996).

The male flies were identified to their respective groups by referring to several keys (Parshad and Paika, 1964; Bock and Wheeler, 1972; Markow and O' Grady, 2006) and directly used for morphometric analysis. Length (from junction of the tube to the tip of the gland) and width (at the larger size / middle of the gland) of right and left sides of accessory glands were measured using ProgRes C3 camera attached to stereomicroscope. The mean of right and left side glands were calculated based on 100 samples of each locality of both the species. The size of the gland (mm^2) was calculated according to Hiremani and Shivanna (2010).

The data were analysed by One-way ANOVA to know if there is there any relation between the gland size within species, between localities, and Students *t*- test was applied to check variations of different species from the same locality.

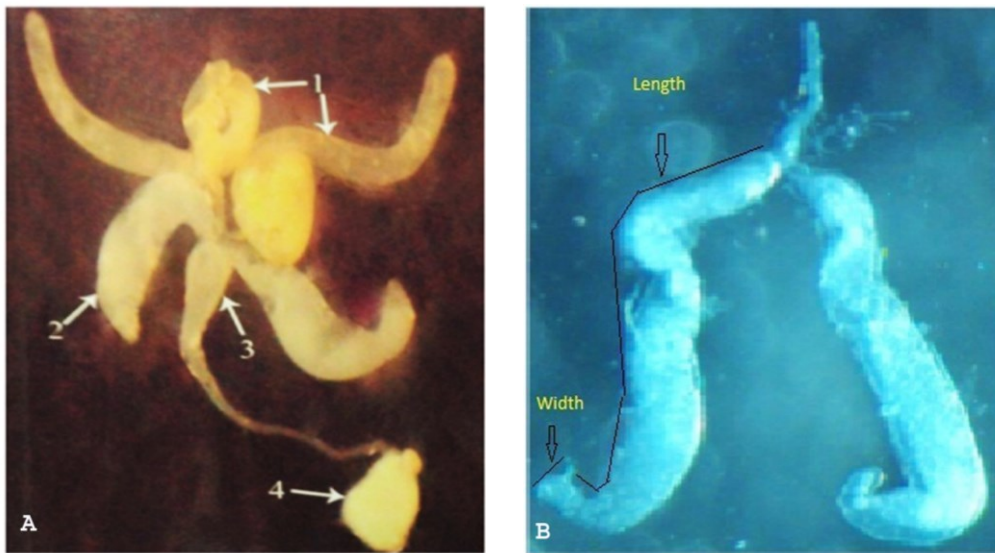


Figure 1. A: Male reproductive system; 1, testis; 2, accessory gland; 3, ejaculatory duct; 4, ejaculatory bulb; B: Accessory gland.

Results and Discussion

Figures 1A and 1B show the reproductive system of male *Drosophila* and the isolated accessory gland. From the present study, it reveals that the size of accessory gland varies between species, within species, and varies from population to population. The mean size of accessory gland of *D. bipectinata* and *D. malerkotliana* of Kalghatgi was recorded (0.0538 ± 0.053 mm and 0.0841 ± 0.110 mm) followed by laboratory population (0.0506 ± 0.0322 mm and 0.0679 ± 0.070 mm) and Yellapur (0.0030 ± 0.00508 mm and 0.0056 ± 0.009 mm), respectively. The one-way ANOVA shows the size of accessory gland of *D. bipectinata* and *D. malerkotliana* shows significant variations between localities ($F = 61.147$; $df_1 = 2$, $df_2 = 297$ and $F = 30.059$; $df_1 = 2$, $df_2 = 297$), respectively. It reveals that the gland size of *D. bipectinata* and *D. malerkotliana* is greater in the Kalghatgi population compared to the other two populations. It suggested that populations of Kalghatgi

might have maximum protein secretions when compared to other populations, as the size of the gland correlated to the fitness of the species (Sahasini, 2014).

The *t*-test revealed that the Kalghatgi and laboratory populations of *D. bipectinata* and *D. malerkotliana* showed significant variations in their gland size ($t = 2.239$; $F = 1.085$; $df = 198$) suggesting that the populations from three localities might vary in their fitness. These results are on par with the earlier studies of Throckmorton (1962), Bairati (1968), and Chen (1984). *Drosophila* has been proved in several instances as a good model, because of its robust nature and highly specific in orientation, morphological, and fitness analysis. The morphology of *Drosophila* male accessory glands with respect to length and width varies from species to species (Chen, 1984).

It is a preliminary step to analyse the size of accessory gland from natural populations, which is first of its kind. Earlier studies on the morphometry of accessory gland were only from the genetic, biochemical, and developmental aspects (Chen, 1984; Shivanna and Ramesh, 1995; Raviram and Ramesh, 2001, 2002; Hiremani and Shivanna, 2010; Suhasini, 2014). The present study on male accessory glands can be helpful in analysing the comparative fitness of the species especially in the natural populations.

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Preliminary observations of colored light effects on *Drosophila virilis* phenotypic traits.

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Introduction

Drosophila has been proven to be a very influential tool in studying genetics and environmental experiments leading to evolution. To better understand the effects of light exposure in living organisms, this study aims to investigate the effects of three different colored lights on phenotypic traits. The research was guided by the following research question: Is there an effect of three different color lights on phenotypic traits in *Drosophila virilis*? The difference of average weight among three lights was investigated.

Materials and Methods

To study the effects of three different lights (red, blue, and white) as an environment, a preliminary observation on *Drosophila virilis* average weight was performed. The experimental procedure was to grow

from two vials of *Drosophila virilis* purchased from Carolina Biological Supply (CBSC) during the summer, 2018. This experiment took place in a laboratory setting where the fruit flies resided under three different colored lights (red, white, and blue; each 25 Watts) as an environment. The males and females were counted and the average weight per male and per female was calculated for each colored light. An Excel spreadsheet was used to calculate the mean and standard deviation for each light in units of micrograms.

Results

For **Red light** a total of 97 males and 95 females with an average temperature of 23.3 degrees centigrade. The **male average weight** was 10688 micrograms with a standard deviation (s.d.) of 6740 micrograms. The **female average weight** produced was 11250 micrograms and the s.d. for females under red light was 8113 micrograms.

For **White light** which had an average temperature of 22.6 degrees centigrade and produced 35 males and 56 females. The **male average weight** was 8700 micrograms and **the female average weight** was 11000 micrograms. The male s.d. was 10935 micrograms and the female s.d. was 10144 micrograms.

For **Blue light**, which had an average temperature of 22.57 degrees centigrade, produced a total of 30 males and 32 females. The **male average weight** was 9500 micrograms with an s.d. of 4323 micrograms, and **the female average weight** was 7817 micrograms with an s.d. of 4517 micrograms.

Discussion

Results show a variation on male and female weight between the three different lights (red, white, and blue). This provides the evidence that the effect of colored lights as an environment has an effect on weight as a phenotypic trait. These results differ from Bangura *et al.* (2011) and Berry-Wingfield *et al.* (2010), where they did not observe effects using three different lights (red, white, and blue).

Acknowledgments: Gratitude expressed to Dr. Antonio Henley and Jonathan Smalls for providing the opportunity and fellowship of the McNair Scholars program.

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***Drosophila suzukii* occurrence in a native forest fragment in São Paulo state, Brazil.**

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Abstract

Considering all species of the *Drosophila* group, only two are pests, *Drosophila suzukii* and *Zaprionus indianus*. The former, commonly called Spotted-Wing *Drosophila*, is an Asian species and currently has been causing serious concern in different parts of the world because of its ability to damage fruits. Due to a serrated ovipositor, the species attacks different fruits, causing significant economic issues, in some cases reaching more than 80% of losses in orchards. The first record in Brazil was in 2013, in the southern region, and since then it has been reported from different Brazilian cities and states. In São Paulo state, this species was found in 2014, in berries from market. We provide a new record of this insect, occasionally found in ethanol traps in the municipality of Piracicaba, São Paulo in 2016, in a forest fragment close to an urban area. This record shows that *D. suzukii* can occupy different environments and this could be an important source of risk for properties of berries in the whole southeast and also other Brazilian regions. Considering the high economic

losses they can cause, and this quick dispersion, the records of this pest are essential. Key Words: Spotted wing *Drosophila*; Native forest; Biological invasion; Insect pest.

Drosophila suzukii (Matsumura, 1931; Diptera: Drosophilidae), also known as spotted wing *Drosophila* (SWD), is native to Southeast Asia and dispersed to Europe and North America in 2008 (Bolda *et al.*, 2010). In Brazil, the fly was first recorded in 2013 in the southern region (Deprá *et al.*, 2014), followed by records from the southeastern and midwestern regions (Bitner-Mathé *et al.*, 2014; Paula *et al.*, 2014; Vilela and Mori, 2014; Andrezza *et al.*, 2016).

This species causes significant damage to different fruits (Santos, 2014), because of its ability to make a hole in these fruits with a serrated ovipositor to lay the eggs. The insect damages mainly thin-skinned fruits such as strawberries, raspberries, and blackberries, causing crop losses of up to 80% in the USA (Walsh *et al.*, 2011) and in strawberry crops in France, and 30-40% in Italy (Santos *et al.*, 2016). In Brazil, although the fly has been found in many fruit crops (Geisler *et al.*, 2015; Silveira *et al.*, 2015; Santos *et al.*, 2016), recorded losses were 30% and only in strawberries (Santos, 2014).

Due to this economic damage, it is important to report the presence of this species in other regions and in the range of environments where it occurs, to better understand its dispersal and try to avoid future economic losses. Andrezza *et al.* (2017) discuss based on the records of this species in the whole Neotropical region that this pest can quickly spread even active or passive dispersion.

This new record of *D. suzukii* in São Paulo state shows the presence of this fly in a fragment of natural forest, an environment never recorded before at least specifically in this Brazilian state. Besides, another notification of SWD's presence in this kind of environment is indeed an alert that this fly has been maintained and can continue to disperse.

Ethanol traps were installed at 16 points at ESALQ (Escola Superior de Agricultura Luiz de Queiroz) in Piracicaba municipality, São Paulo in 2016, for a study of the diversity and distribution of Cerambycidae; and the presence of *Drosophila suzukii* was recorded. Subsequently, species-specific traps were installed with bait made from a yeast-sugar mixture, weekly at two points at ESALQ, 22°42'20.30"S 47°37'36.71"W (Point 1) and 22°42'29.70"S 47°38'4.64"W (Point 2), from August to December 2017.

Flies found in the traps were identified as SWD based on external morphology (Vlach, 2013). In addition, a list of plant species near the traps in the forest fragment is provided.

Two male specimens of *D. suzukii* (Figure 1c) were found in a trap located at 22°42'29.70"S 47°38'4.64"W, in 2016 December. The insects were captured in a fragment of native forest. In additional traps over the entire collection period, 23 specimens of *D. suzukii* were captured at point 1, and nine at point 2 (Figure 1). The insects were deposited in the museum of Entomology, ESALQ/USP (Code Number 6920).

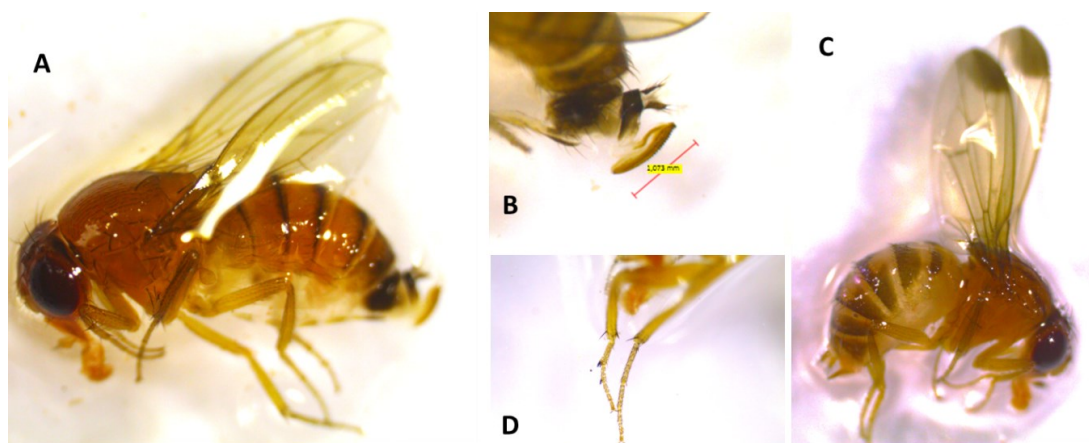


Figure 1. Specimens of *Drosophila suzukii* found in traps in a fragment of native forest. A- Female; B- enlarged photo showing ovipositor, length 1.073 mm; C- Male of *D. suzukii* with spotted wings; D- The two combs on the first and second segments of the male. Photographs by IBS.

Table 1. Numbers of *Drosophila suzukii* collected in 2017 at two points in a natural forest fragment.

Mean Temperature (°C)	Month of collection	No. Individuals collected	
		Point 1	Point 2
18.28	August	0	0
23.48	September	2	0
23.74	October	2	6
22.50	November	19	3
24.56	December	0	0
		23	9

The highest numbers of *D. suzukii* were collected in November (Table 1). This result accords with previous information about the population dynamics, since this fly is found mainly in moderate temperatures (Walsh *et al.*, 2011). About the relation with the plant species that exists in the fragments where *D. suzukii* was collected were *Esenbeckia leiocarpa* (Sapindales: Rutaceae), *Tipuana tipu* (Fabales: Fabaceae), *Anadenanthera sp.* (Fabales: Mimosaceae), *Ceiba speciose* (Malvales: Malvaceae), and *Anadenanthera macrocarpa* (Fabales: Mimosaceae), all of them with dry fruits. There is no evidence of association between *D. suzukii* and these plants as new host, although continuous monitoring is in progress to

attempt to document the dynamics of the species and its associations with plant species.

In other Brazilian states, SWD has been reported from the Atlantic Rainforest in Rio de Janeiro state, in low number (Deprá *et al.*, 2014; Bitner-Mathé *et al.*, 2014), fruit crops (Geisler *et al.*, 2015; Silveira *et al.*, 2015; Santos *et al.*, 2016) and the central-western savanna (Paula *et al.*, 2014). This note reinforces the ability of *D. suzukii* to occupy different environments and helps to trace the occupation and dispersal of this fly.

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The *cinnabar* gene and 3-hydroxy-kynurenine define a pivotal position in eye pigmentation pathways.

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Abstract

Drosophila melanogaster's brick-red eye color is the result of two distinct sets of pigment molecules. The ommochromes are brownish and made from tryptophan, while the pteridines are reddish and made from

GTP. Vermillion and Cinnabar are enzymes in the ommochrome pathway and mutant alleles in either gene result in bright orange to red-orange eyes. Here we show that supplementing fly food with 3-hydroxykynurenine (3-HK) changes the eye color of *vermillion* and *cinnabar* mutants to wild type color. This occurs in adult parents and their progeny. Importantly, this effect does not occur in *scarlet* mutant eyes, which is phenotypically the same as *vermillion* and *cinnabar*, but not in the ommochrome biosynthetic pathway. Further, we tested the effect of 3-HK on flies with mutant alleles in both *cinnabar* and *brown*, which interact epistatically to have white eyes. At low concentrations of 3-HK, *cinnabar*, *brown* flies have apricot-colored eyes, while at higher concentrations they have brown eyes. This effect does not occur in *white* flies nor *brown*; *scarlet* double mutant flies, which are also phenotypically white. Finally, we constructed several fly stocks with *cinnabar* and mutant alleles in the *white* gene that cause brownish eye color. Eyes in flies with *cinnabar* and *white*^{Brownex} were nearly white, while *cinnabar* and *white*^{coffee} were closer to a deep burgundy. Our results suggest that the *cinnabar* gene and gene product can be easily manipulated to alter eye color with its enzyme product 3-HK or in combination with the ABC transporters White and Brown.

Introduction

Eye pigmentation plays an important role in vision. In *Drosophila*, the brick-red color of their eyes is the result of two distinct sets of pigment molecules. The ommochromes are brownish and tryptophan derivatives (Figon and Casas, 2018), while the pteridines are reddish and made from the guanine ring of GTP (Kim *et al.*, 2013). Both of these contribute to the screening of stray light to help form a vision field for the fly, because they provide optical insulation. In the absence of these pigments, such as in the *white* mutant fly, visual acuity and responsiveness to light are reduced (Kain *et al.*, 2012). Optical insulation is necessary so that temporary blindness is not induced at high levels of light exposure. The compound eye has two layers of optical insulation: the pigment rim and the sub retinal pigment layer. These layers of pigment ensure that the light that enters the ommatidium stays in the ommatidium to prevent the light from transferring laterally in the eye. Furthermore, ommochromes are responsible for reacting to bright light. These pigments will migrate to intense areas of light and reduce light absorption in that area (Tomlinson, 2012).

Fruit flies with mutations in the gene *cinnabar* are deficient in 3-hydroxykynurenine (3-HK), a necessary enzyme in the synthesis of ommochromes. Metabolites of tryptophan degradation have been linked to neurodegenerative diseases such as Huntington's disease, Alzheimer's disease, and Parkinson's disease in humans (Breda *et al.*, 2016; Campesan *et al.*, 2011; Giorgini *et al.*, 2013). Tryptophan metabolites also eventually lead to ommochromes in *Drosophila melanogaster* (Campesan *et al.*, 2011). Involved in this biosynthetic pathway are tryptophan-2,3-dioxygenase (TDO, the *vermillion* gene) and kynurenine 3-monooxygenase (KMO, the *cinnabar* gene). Inhibiting TDO and KMO protect against neurodegeneration (Campesan *et al.*, 2011).

In this report we show that supplementing fly food with 3-hydroxykynurenine (3-HK) changes the eye color of both parents and progeny in *vermillion* and *cinnabar* mutants to wild type color. Further we show that at low concentrations, 3-HK gives *cinnabar*, *brown* flies apricot-colored eyes, while at higher concentrations they have brown eyes. The effect is stable for essentially the lifetime of the fly. Finally, double mutants between *cinnabar* and several alleles of the *white* gene result in intermediate and epistatic phenotypes. Overall, the results suggest that Cinnabar as a kynurenine hydroxylase and its product, 3-HK, can be manipulated biochemically and genetically to affect eye color.

Materials and Methods

All fly stocks were obtained from either the Bloomington Stock Center at Indiana University except *cinnabar*¹ mutants, which were purchased from Carolina Biological Supply, Inc. 3-hydroxykynurenine (3-HK) was purchased from Sigma-Aldrich and dissolved in 1 N HCl at 100 μ M, then diluted to working concentrations in deionized water. Flies were grown at 26°C on a pre-formulated dry food, 4-24 Blue (Carolina Biological) and mixed 1:1 with water or various concentrations of 3-HK. Polystyrene vials 28 \times 95 mm were used to culture flies or 25 \times 95 mm for 3-HK treatments. A Canon EOS Rebel 18 megapixel camera was used to capture images of fly eyes using combined fiber optic and LED illumination. Flies were either

frozen and captured within one week or anesthetized using a Genesee Scientific flypad with carbon dioxide gas. For some images, a Z-series was manually acquired with 6-12 different focal planes and Helicon Focus software was used to render the images in a composite focus. Adobe Photoshop or GIMP image-editing software was used to adjust levels, normalize the background, and sharpen all digital photographs. Double mutant flies were made by crossing *cinnabar*¹ virgin females with males from either *white*^{Brownex}, *white*^{satsuma}, *white*^{coffee}, or *carnation*¹ males. The resulting F1 generation was wild-type in eye color and the F2 showed the double mutant only in males, which made it easier to score.

Results

3-HK changes the color in *vermillion*, *cinnabar*, but not *scarlet* mutant eyes.

cinnabar mutant flies respond to food supplements with 3-HK to show wild type eye color in a model for Huntington's disease (Campesan *et al.*, 2011). We extended this observation to flies containing mutant alleles in either *vermillion* or *scarlet*. *cinnabar*, *vermillion*, and *scarlet* mutants were phenotypically indistinguishable with a bright red-orange eye color and faint pseudopupil (Figure 1, 0 μ M 3-HK). When these fly mutants were fed food supplemented with 1 or 3 μ M 3-HK, the first generation had noticeable changes in eye color. Flies with a *cinnabar* mutant allele were nearly identical to wild type (Oregon R) eye color at 1 μ M and 3 μ M 3-HK (Figure 1). A similar effect was observed in flies with a *vermillion* mutant allele (Figure 1). However, flies with a mutation in the *scarlet* gene showed no effect when grown in the presence of 3-HK and remained bright red-orange (Figure 1). This suggested that blocks in the ommochrome biosynthetic pathway could be bypassed with the intermediate, 3-HK and restore normal pigmentation in the eye. However, 3-HK cannot correct the defect in transporting ommochromes into the storage organelles of pigment cells, which is the function of Scarlet (Borycz *et al.*, 2008).

The change in eye color of adult flies is also affected with 3-HK. We examined adult flies (the parental generation) after feeding them food supplemented with 1 μ M 3-HK. Both *vermillion* and *cinnabar* mutant eyes gradually changed to wild-type to near wild-type with the first week of feeding (Figure 2). This suggested that the phenotypic correction of 3-HK did not require development from embryo to adult.

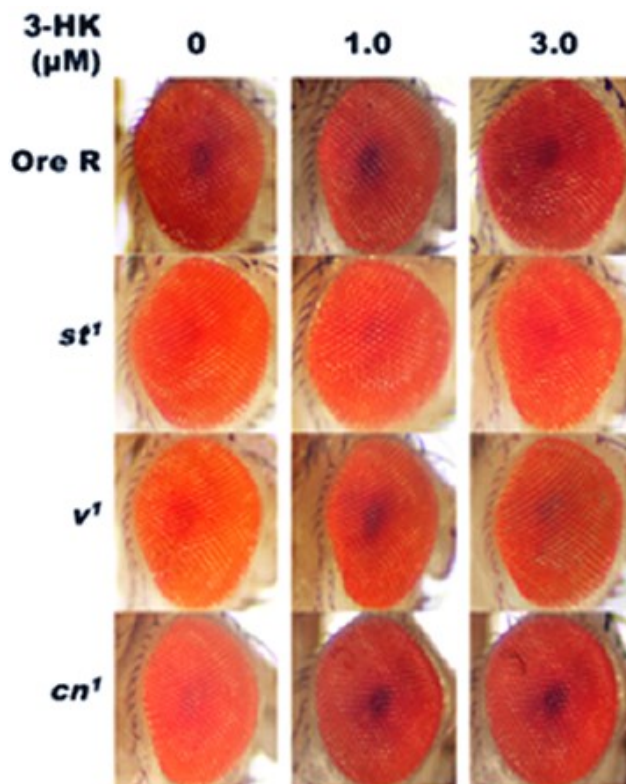


Figure 1. 3-hydroxy-kynurenine (3-HK) produces wild-type eye color in *vermillion* and *cinnabar* mutant alleles but not *scarlet*. Flies were cultured for one generation on the indicated amounts of 3-HK (0, 1.0, or 3.0 μ M). The Oregon R (Ore R) wild-type stock displays the brick-red color and a distinct pseudo pupil and remains unchanged with 3-HK. The *scarlet* mutant (*st*¹) shows a bright red-orange color with faint pseudo pupil and also remains unchanged with 3-HK. *vermillion* (*v*¹) and *cinnabar* (*cn*¹) mutant alleles also appear bright red-orange, but then become “wild-type” in color after one generation on 1.0 or 3.0 μ M 3-HK. The pseudo pupil is also much more prominent in *v*¹ and *cn*¹ mutant flies after 3-HK treatment. The *cn*¹ mutant appears most like Oregon R after 3-HK treatment.

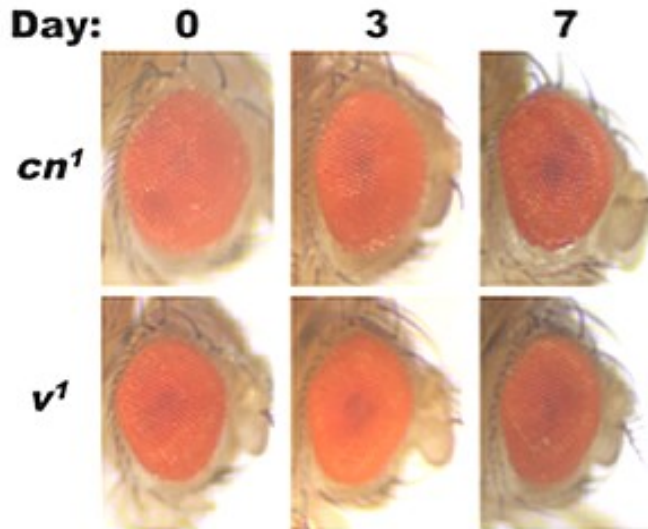


Figure 2. The eye color of adult *cinnabar* (cn^1) and *vermillion* (v^1) mutants changes after 7 days on food containing 3-hydroxy-kynurenine (3-HK). Flies were subcultured on food made with water containing 1.0 μ M 3-HK. One or two flies were removed daily for 1 week to examine their eye color. In general, after 3 days the color was intermediate between the mutant bright red-orange and brick red wild type color. After 7 days the color was nearly the same as wild-type with a distinct pseudo pupil (compare to Oregon R flies in Figure 1)

3-HK has intermediate effects in eye color with *cinnabar*, *brown* double mutants.

We next examined if 3-HK could correct the eye color in a double mutant with white eyes. Flies with mutations in *cinnabar* and *brown* genes have white eyes (Figure 3). In the presence of food with 0.3 μ M 3-HK, the progeny of the parent flies have an eye color resembling apricot (Figure 3). In the presence of food with 1.0 μ M 3-HK, the progeny of the parent flies have an eye color that is brown (Figure 3). The specificity for the *cinnabar* mutant allele was indicated, because no 3-HK effect was observed in *white*¹ mutant flies or a *brown*¹; *scarlet*¹ double mutant, which both have white eyes before and after treatment (Figure 3). Remarkably, the change in eye color was stable for up to 36 days and did not fade or revert to white with the absence of 3-HK in the food (Figure 3). This suggested that the turnover of pigment containing melanosomes was very slow. These results suggested that Cinnabar and 3-HK are located in the ommochrome pathway at a position that made changes in eye color accessible with chemical supplementation.

To test if this was true from a genetic approach, we made double mutants with *cinnabar* and several alleles of the white gene that affect eye color toward brown to burgundy. *white*^{Brownex}, *white*^{satsuma}, and *white*^{coffee} all appear to lower the pteridine levels in eyes, because they are shifted to ommochrome-enriched appearances. *carnation* was also used, because it too has a similar eye color spectrum, yet is involved in vesicular trafficking to the storage melanosomes. This approach also allowed us to ask if the two genes had allele-specific genetic interactions based on the eye color phenotypes. We predicted that such double mutants might appear white in eye color. This was the result with *cinnabar*; *white*^{Brownex} as it appeared nearly white (Figure 4) like the *cinnabar*, *brown* double mutant (Figure 3). However, the other *white* allele, *white*^{satsuma}, and *carnation*¹ allele appeared to be a blend in color between their eye color phenotypes and *cinnabar* (Figure 4). This suggests that *cinnabar* and *white*^{Brownex} genetically interact, but not *cinnabar* and *white*^{satsuma} or *carnation*¹. The *cinnabar*; *white*^{coffee} double mutant appeared more like *white*^{coffee}, which suggested that the *cinnabar* phenotype was recessive to the *white*^{coffee} phenotype (Figure 4).

We attempted to change the eye color of *sepia*, *purple*, and *rosy* mutant alleles in the pteridine pathway (Kim *et al.*, 2013), but did not observe any effects. We supplemented food with biopterin, sepiapterin, xanthopterin, and isoxanthopterin and the eye colors remained unchanged (data not shown). This was also observed with *cinnabar*, *brown* double mutants.

Discussion

The ommochrome and pteridine biosynthetic pathways collaborate to produce molecules that serve as pigments to give *Drosophila melanogaster* eyes their brick-red color (Navrotskaya *et al.*, 2018; Kim *et al.*, 2013). The endosome-lysosome vesicular traffic system along with the ABC transporters White, Brown, and Scarlet also contribute to eye pigmentation (Mackenzie *et al.*, 1999). The ability to affect eye color with

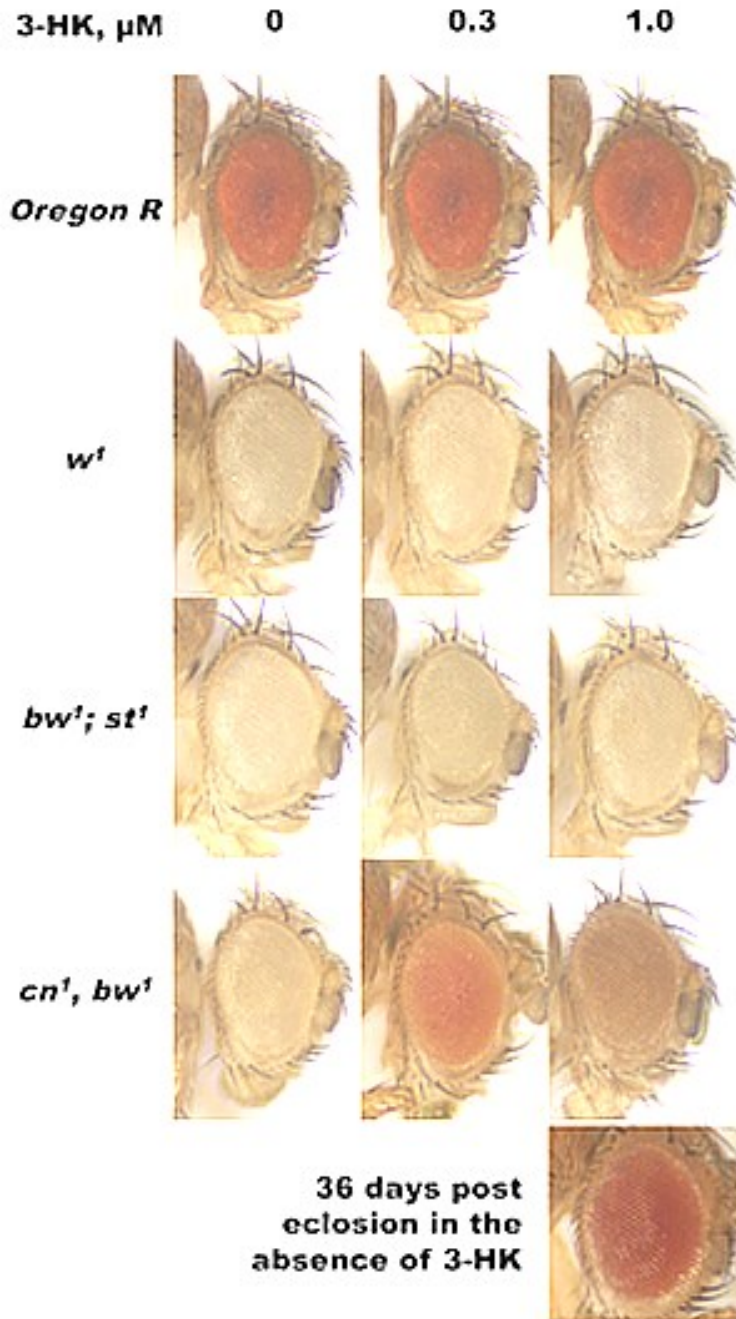


Figure 3. 3-hydroxy-kynurenine (3-HK) shows specificity for *cinnabar* mutant alleles in double mutants that display a white-eyed phenotype. Flies were grown on food containing 0.3 and 1.0 μM , as indicated, and are about 5-7 days old (except where indicated). Wild-type flies (*Oregon R*) showed no change in eye color in the presence of 3-HK. No change in eye color was also observed in *white¹* (*w¹*) and *brown¹*; *scarlet¹* (*bw¹*; *st¹*) double mutant flies. The *cinnabar*, *brown* double mutant (*cn¹*, *bw¹*), while phenotypically white in eye color, showed an apricot-like and brown eye color in 0.3 μM and 1.0 μM , respectively. As indicated, the *cn¹*, *bw¹* flies remained brown and even intensified in color 36 days post eclosion, suggesting the eye color change is very stable.

3-hydroxy kynurenine (3-HK) suggests that this molecule is able to either 1) enter flies through ingestion or absorption and become transported into the pigment cell melanosomes of ommatidia or 2) serve as a precursor to make other pigment molecules. Studies in the silkworm, *Bombyx mori*, suggest that 3-HK is directly transported into pigment granules in eggs (Osanai-Futahashi *et al.*, 2012). This argues that direct transport of 3-HK into melanosomes is affecting the eye color change. The genetic interactions between *cinnabar¹* and various *white* alleles also reflect this view. In particular, the *white^{Brownex}* produces a white-eyed fly similar to a *brown*, *cinnabar* double mutant (Gramates *et al.*, 2017). White and Brown are ABC membrane transporters and most likely heterodimerize (Borycz *et al.*, 2008; Grubbs *et al.*, 2015) to transport 3-HK into the melanosomes. The inability to change the eye color in mutant alleles of the pteridine pathway with several pteridine pigment molecules suggests that 3-HK and *cinnabar* mutants are specific to the biochemical complementation of the ommochrome pathway.

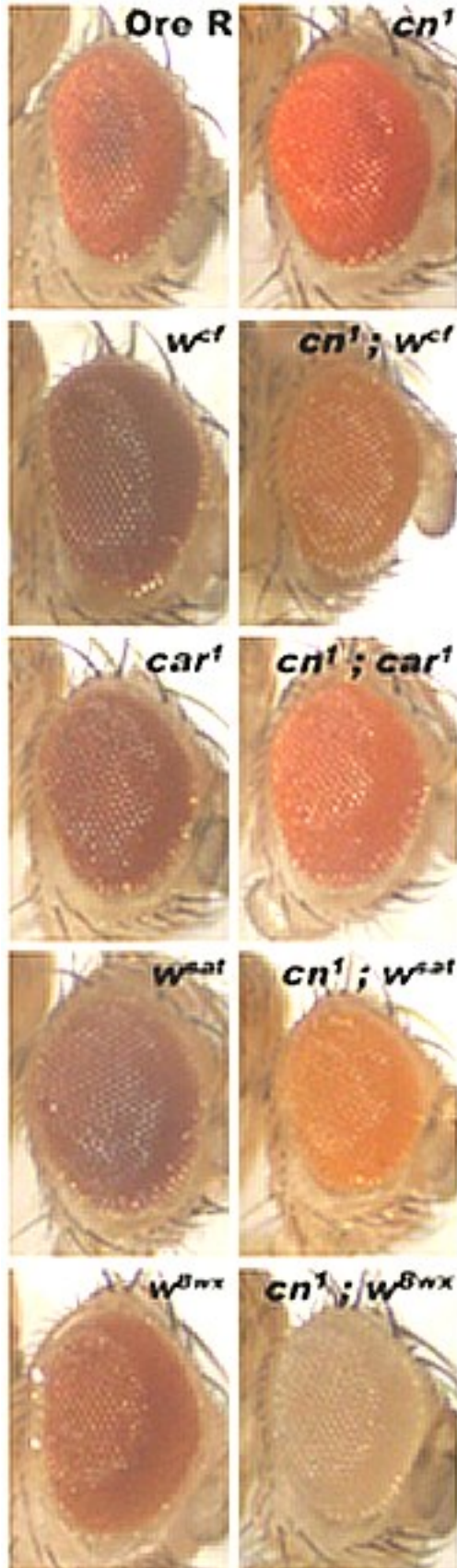


Figure 4. Double mutant flies with *cinnabar* display a range of eye colors. A series of mutants that affect eye color toward the dark brown to burgundy range and located on chromosome 1 were chosen. This helped in detecting the double mutant phenotype exclusively in F2 males. *cinnabar* virgin females were mated with males from *white^{coffee}* (*w^{cf}*), *carnation¹* (*car¹*), *white^{satsuma}* (*w^{sat}*), or *white^{Brownex}* (*w^{Bwx}*) mutant stocks. The resulting F1 were all wild-type for eye color. A sibling mating was performed to produce an F2 and males were scored for a difference in eye color from wild type versus *cinnabar* or the brown-burgundy phenotype. The double mutants (as indicated) range from dark orange to yellow orange to white. Wild type (Oregon R, Ore R) and *cinnabar* (*cn¹*) flies are shown for reference. All flies were males.

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The variable and flexible behavior of larval *Drosophila* in nature.

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In patchy, changing and variable environments as *Drosophila* breeding sites, space available, and food sources vary in time, so does their suitability with consequences for biological fitness (Carson, 1971). Such ecological successional changes are usually rapid, occurring on a scale of days (or even hours) rather than years (Soto-Yéber *et al.*, 2018). In agreement with those ecological features, the behavior of larval *Drosophila* shows flexibility and plasticity (Del Pino *et al.*, 2015). In this context, it is surprising that little research has been reported on routines of larval *Drosophila* in the wild. For example, associations with conspecifics and aliens have rarely been studied in *Drosophila* natural populations by observation and experiment. In fact, responses to the odors emitted by *Drosophila* larvae are critical for the identification of conspecifics and aliens influencing selection of pupation sites (Beltramí *et al.*, 2010). Such an omission necessarily hinders and weakens comprehension of the role of larval behavior in the evolution of species in the genus *Drosophila* (Godoy-Herrera and Connolly, 2008). Larvae have a remarkable variety of receptors and brain structures that process miscellaneous stimuli (Takeshi, Hitoshi and Vosshall, 2010), suggesting an inherent flexibility to adapt to a wide range of complex environmental situations.

In the last years we have put our attention on sensory signals to which larval *Drosophila* responds in its breeding sites. By combining such observations with experiments in the laboratory, we found that the larvae react to substrate vibrations by contracting the body forming a U, and/or stopping for a few seconds. Then, the larvae begin to tunnel into the substrate. Additionally, we have detected *Drosophila* pupae joined to a variety of substrates in the wild as peel of fruits, dry twigs and herbs, on and buried in the ground (Godoy-Herrera, Santander and Figueroa, 1994, and manuscript in preparation), suggesting that tactile receptors could participate in selection of pupation sites. These types of receptors could provide information to the larvae on the type of substrate, rough or smooth, hard or soft, on which it moves.

Tactile receptors are also important in larval recognition of conspecifics and strangers. One bizarre behavior of larval *Drosophila* is touching other larvae in the breeding sites. Mutations that interfere the development of tactile receptors also obstruct touching between conspecific and strange larvae (manuscript in preparation). Summarizing, tactile signals are involved in (i) selection of pupation sites, (ii) recognition of conspecifics and strangers, and (iii) digging of the substrates as different as decaying fruits and the solid surface of the earth. We think that range of different behaviors of larval *Drosophila* in the wild exceeds that of the adult. This should not surprise us. By comparison to the imagoes, larval *Drosophila* mobility is extremely limited. This means that the larva has to remain and adapt to environments that change their ecological conditions in a short period of time. Our observations in the wild and our experiments in the laboratory are in agreement with such a proposition. That is, larval *Drosophila* exhibit a variety of behaviors prone to change, reflecting a variable and flexible nervous system appropriate to adapt the larva to changing, variable environments.

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Sex-comb variation and female abdominal pigment polymorphism in a natural population of *Drosophila jambulina* from North India.

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Abstract

Speciation is an evolutionary process driven by several forces, one of which is the mating preference by the sexes. *Drosophila* is an evolutionary model organism, and many species of this genus have been used in classical genetics and developmental studies to understand genotype-environment-phenotype relationships. Several morphological traits, e.g., sexual dimorphism and sex comb variations, are seen to play a key role during mate-choice in *Drosophila*. Variations in these features drive sexual selection and eventually lead to fixation of the most adaptive traits. In the present study, we have collected *Drosophila jambulina* from Pantnagar, Uttarakhand in India and analysed the observed variations in the female abdominal pigmentation and male sex-comb bristle number using 10 iso-female lines. The results highlight the intra-species variations and provide a basis for further analysis of reproductive fitness of each of the trait. Keywords: *Drosophila*, pigmentation, sex-comb bristles, intra-species variations, India.

Introduction

Drosophila is widely known as a model organism and helps in evolutionary studies. It belongs to Drosophilidae family in arthropods and as many as 1500 species of *Drosophila* are reported worldwide (Singh, 2015). 10% of these species are reported from India, highlighting the rich biodiversity of the genus in the country. The similarity with the human genome accompanied with easy handling has made *Drosophila* a valuable organism in the field of experimental biology. Several studies have been carried out to study the role of morphological traits and secondary sexual characters in various evolutionary events of adaptation and speciation (Snook *et al.*, 2013; Ng *et al.*, 2008). Variation in the sex-comb number and abdominal pigmentation are two of these traits assisting the different species to cohabit and yet maintain their unique integrity. Body pigmentation is known to play a significant role in different physiological as well as physical features of the fly (Ng *et al.*, 2008; Kopp *et al.*, 2000). Pigmentation polymorphism has also been correlated with latitudinal and temperature variations and helps in adaptation (Das *et al.*, 1994; Davis and Moyle, 2018; Telonis-Scott *et al.*, 2011). Different gene loci were found to be associated with such body pigmentation variations in different *Drosophila* species and are reported to be under polygenic control (Wittkopp *et al.*, 2002; Rogers *et al.*, 2013). Sex comb is a secondary sexual character present on the foreleg of male *Drosophila* and assists in the process of copulation (Ng and Kopp, 2008). It is the genetic identity of a species and is, thus, used as a species identification marker. Sex-comb in *Drosophila* species has been widely studied for its pattern variation as *Drosophila* species possess different morphology of the sex-comb. Variation in the number of sex-comb as well as in the bristles of sex-comb has been linked to sexual selection by females making it a significant trait for reproductive fitness of the male fly (Ahuja and Singh, 2008; Ng and Kopp, 2008).

Drosophila jambulina belongs to the montium subgroup, where variations in abdominal pigmentation have already been reported (Schiffer and McEvey, 2006; Watanabe *et al.*, 1982; Ohnishi and Watanabe, 1985; Parkash *et al.*, 2009). This species was firstly reported by Parshad and Paika in 1964 from the northern parts of India. In the present study, we have studied the variations in female abdominal pigmentation and male sex comb bristles of *D. jambulina* collected from North India.

Material and Methods

Flies collection, species identification, and iso-female line establishment

Drosophila flies were collected from Pantnagar, Uttarakhand (28.97°N 79.41°E) from fruit orchards using net sweeping method during morning hours in June 2016. After collection, single inseminated female fly was used to set up iso-female lines and used for species identification. 10 iso-female lines of *D. jambulina* (named as D.Jam_Pant 1-D.Jam_Pant10) were set and reared on standard yeast-molasses media, maintained in BOD incubator at 25°C with 70-80% humidity with 12 hours day-light cycle.

Experimental setup

15 young females each from 10 iso-female (isogenic) lines were etherised and visualised under dissecting microscope. The pigmentation pattern of each female fly was noted and image was captured. The males are found to be free from any pigmentation. For sex-comb bristle number variation, 8 males from each iso-female line were etherised. The foreleg was dissected and the number of bristles in both sex combs (first and second tarsal segments) was counted and noted separately. Both the observations were done immediately after establishing the iso-female lines to avoid bottlenecking of the traits. The variations in sex comb bristle number were visualised in Box plot using XL stat.

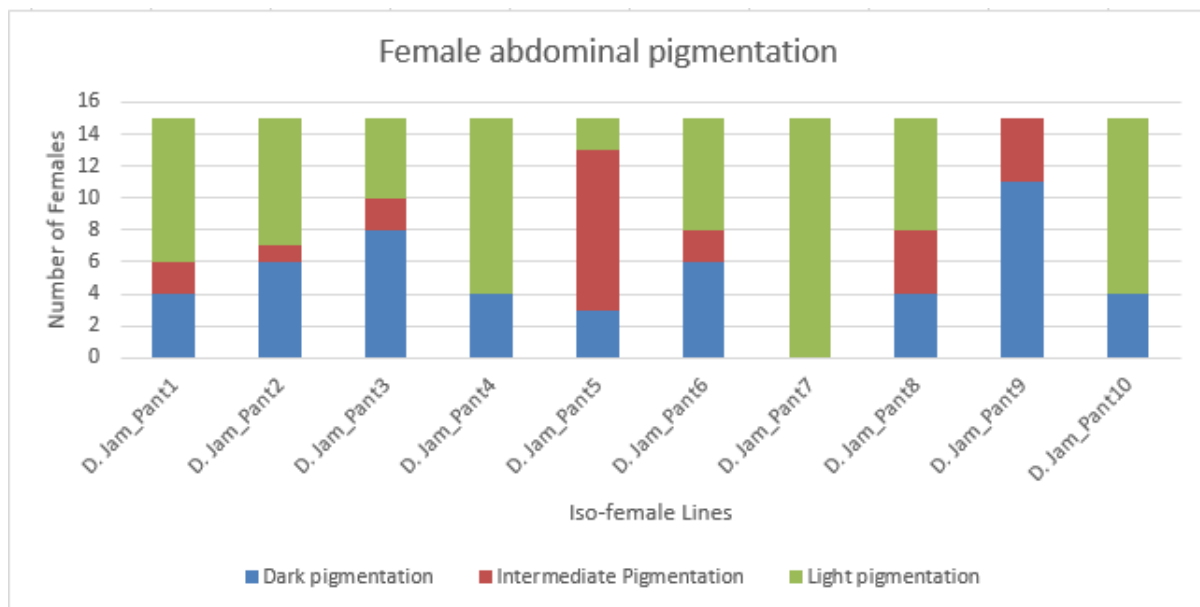


Figure 1. Stacked chart representation showing variation in the female abdominal pigmentation in 10 iso-female lines of *D. jambulina*.

Results and Discussion

Female abdominal pigmentation polymorphism

A total of 150 *D. jambulina* female flies (15 from each line) were analysed for abdominal pigmentation. The last two abdominal segments of the female flies showed variations in pigmentation. Although earlier studies have reported color dimorphism in females of *D. jambulina* with only light or dark, we found three types of morphs in the studied population, *i.e.*, light, intermediate, and dark pigmentation (Ohnishi and Watanabe, 1985; Parkash and Sharma, 1978). These observed variations were represented in the form of a stacked chart (Figure 1). Out of the 10 isofemale lines, one line was found to be monomorphic with all females having light pigmentation (D.jam_Pant7). Also, three of the iso-female lines were observed as dimorphic, *i.e.*, D.jam_Pant 9 females showed either intermediate or dark, whereas D.jam_Pant4, 10 females were of light or intermediate pigmentation (Figure 1). The rest of the six lines show all three kinds of morphs.

The overall pattern of the polymorphism can be summed as light pigmentation > dark pigmentation > intermediate pigmentation (Figure 2). Contrasting results on the dominance of light or dark pigments have been reported earlier with one group reporting light as the dominant phenotype while the other reported dark as dominant (Watanabe *et al.*, 1982; Parkash and Sharma, 1978).

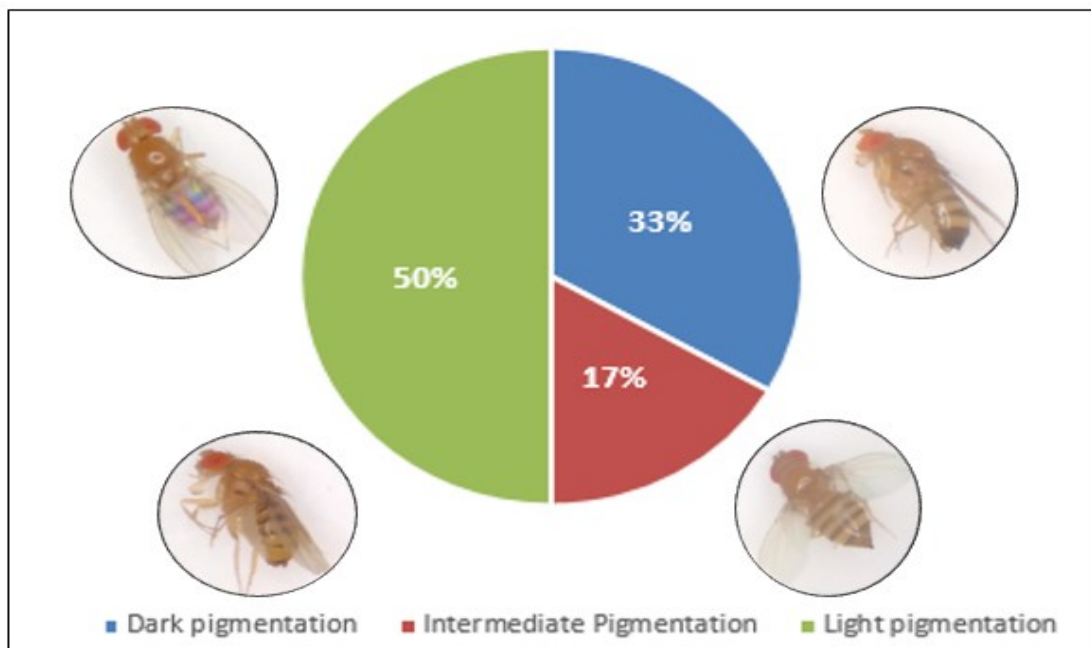


Figure 2. Overall percentage of pigmentation variation in *D. jambulina* females depicted using Pie chart with images of nature caught female flies.

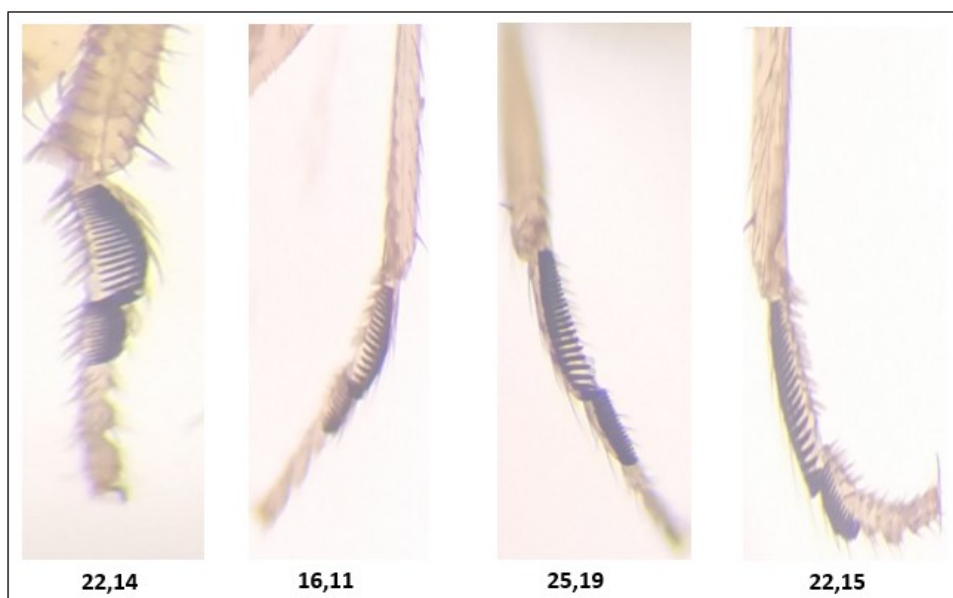


Figure 3. Variation in sex-comb bristle number in *D. jambulina* males collected from Pantnagar, Uttarakhand.

Male sex comb variation

A total of 80 males (from 10 iso-female lines) were used for studying sex-comb variations. *D. jambulina* males have two sex combs, one on each first and second tarsal (Figure 3). The minimum and maximum number of bristles for first and second tarsal segment was 16, 25, and 11, 19, respectively. The box plot shows the range of variations in the number of bristles and the median bristles number for first and second tarsal segment is almost 20 and 15 (Figure 4). Earlier reports in *D. jambulina* show the number to be 24 and 15, whereas in the case of *D. kikkawai*, a close relative of the study species, the number was reported to vary from 26-33,19-23 for first and second tarsal segment, respectively (Parshad and Paika, 1964; Gulati and Mohanty 2013). Our results clearly show the random pattern of variation, while for some iso-female lines the number of bristles in first tarsal segment comb vary by one or two units (*D. jam_Pant* 1,2,4,6,9,10), but for four *D. jam_Pant* 3,5,7,8 the variation is quite high. However, in the case of second tarsal segment, high variation was observed only in one of the lines (*D.jam_Pant*4). All these intra-species variations play significant role in adaptation and highlight the genetic uniqueness of the iso-female lines. Literature suggests that number of bristles in sex-comb is correlated with mating success in opposite directions and sexual selection drives these inter and intra-species variations (Snook *et al.*, 2013; Ahuja and Singh, 2008; Ng and Kopp, 2008).

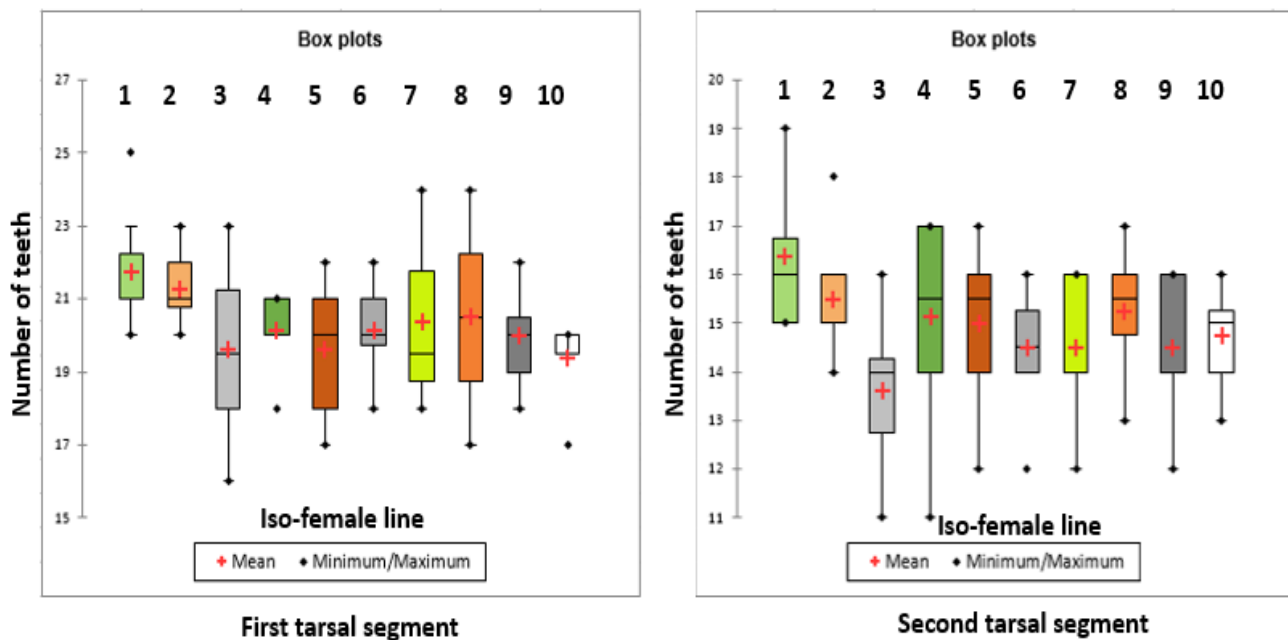


Figure 4. Box-plot showing range of bristle number variation in the sex-comb present in first and second tarsal segment for 10 iso-female lines of natural population of *D. jambulina*.

Conclusion

The variations in the pigmentation pattern of the female and sex comb bristle number in the male of different iso-female lines of *D. jambulina* highlight the intra-species variations in the species. The intra-species variations are a symbol of evolution and also forms the basis of genetic uniqueness of the iso-female lines. These secondary sexual traits are crucial in the mating preference for both sexes and play a significant role in speciation. The reproductive fitness of both male and female flies can be further evaluated to establish a correlation of these traits with fitness of the fly. The molecular mechanism underlying the variations will provide insights into the involvement of different genes and regulatory proteins and how these variations pave the way of selecting other traits as well and helping in the speciation process.

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Recombination rate between inversions in the distal and proximal regions of the second chromosome of *Drosophila mediopunctata*.

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Introduction

Drosophila mediopunctata is a Neotropical forest dwelling species of the *tripunctata* group (Yotoko *et al.*, 2003; Hatadani *et al.*, 2009). Its second chromosome is highly polymorphic for chromosome inversions. There are eight arrangements in the distal region (*e.g.*, *DA*, *DI*, *DS*, *DV*, *etc.*) and nine arrangements in the proximal region (*PA0*, *PB0*, *PC0*, *etc.*). Between these two regions, very seldom rare inversions occur in heterozygosity; thus, crossing over is possible and, in principle, 72 different combinations of distal and proximal inversions, haplotypes, can be found. However, in flies from a natural population, Peixoto and Klaczko (1991) found only 31 out of the 72 possible haplotypes in a sample of 2130 chromosomes. This is a consequence of the strong Linkage Disequilibrium (**LD**) due to a preferential association of inversions in these two regions, such as, *DA-PA0*, *DI-PB0*, *DV-PC0*, *DS-PC0*. For photographs of various haplotypes see Ananina *et al.* (2002).

A critical parameter to understand the evolution of **LD** is the rate of recombination between the genes or genetic elements associated. In our case, the recombination rate between inversions from the distal and proximal regions of the second chromosome is a fundamental factor determining the evolution of the observed **LD**.

Material, Methods, and Results

Here, we report a series of experiments to assess the recombination rates in individuals carrying nonoverlapping paracentric inversions. All the tests were carried out using *Drosophila mediopunctata* flies.

To estimate the recombination rates, we used three visible mutations, all located on the second chromosome: *Delta* (Δ – a dominant wing mutation and recessive lethal), located in the distal region; and *Antennapedia* (*Ant* – a dominant antenna mutation and recessive lethal) and *merlot* (*mt* – a recessive eye color mutation), both located in the proximal part of the second chromosome. Descriptions of the mutants and strains used can be found in Marques *et al.* (1991) and Hatadani *et al.* (2004).

We kept the bottles at a constant temperature of 18°C throughout the development period. The flies were transferred to new fresh vials seven days after the beginning of the experiment, and then every other day

up to 6 or 7 transfers. Figures 1, 2, and 3 show simplified schemes of the crosses performed. Due to lack of recombination in males of *D. mediopunctata*, we expect recombinant individuals to be generated exclusively by heterozygous females (Cavasini *et al.*, 2010). The confidence intervals for recombination rates were obtained at the Sample Size Calculators site of University of California San Francisco (Kohn, 2018).

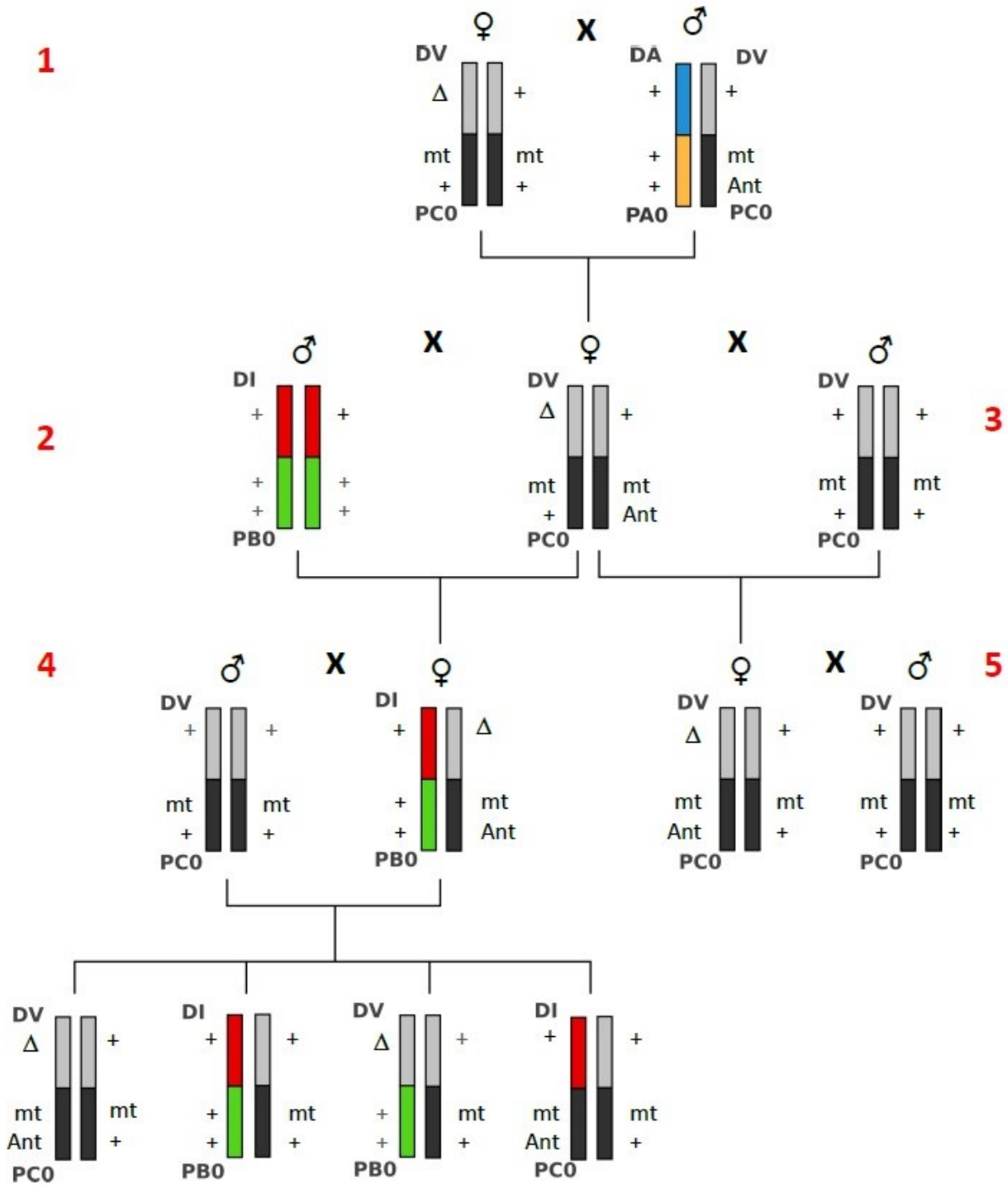


Figure 1. Crosses 1 to 5. Chromosomal arrangements are represented by colored bars (DV, gray; DA, blue; DI, red; PA0, orange; PC0, black; PB0, green).

Table 1. Recombination rates (and 95% confidence interval) between *Antennapedia* (*Ant*) and *Delta* (Δ) in homokaryotypic females (*DV-PC0/DV-PC0*) from three different crosses.

phenotype	sex	cross			total
		2	3	5	
+ +	Male	98	71	78	247
	female	76	69	73	218
Δ +	Male	95	57	60	212
	female	73	72	60	205
+ Ant	Male	90	78	75	243
	female	85	86	61	232
Δ Ant	Male	76	72	60	205
	female	75	70	75	220
total		668	575	561	1794
recombinants		325	282	256	863
<i>r</i> (Δ -Ant)-%		48.7%	49.0%	45.6%	48.1%
		(44.8-52.5%)	(44.9-53.2%)	(41.5-49.8%)	(45.8-50.4%)

Cross 2. ♀ [Δ mt + (*DV-PC0*) / + mt Ant (*DV-PC0*)] x ♂ [+ + + (*DI-PB0*) / + + + (*DI-PB0*)]

Cross 3. ♀ [Δ mt + (*DV-PC0*) / + mt Ant (*DV-PC0*)] x ♂ [+ mt + (*DV-PC0*) / + mt + (*DV-PC0*)]

Cross 5. ♀ [Δ Ant mt (*DV-PC0*) / + mt + (*DV-PC0*)] x ♂ [+ mt + (*DV-PC0*) / + mt + (*DV-PC0*)]

Recombination rate between *Delta* (Δ) and *Antennapedia* (*Ant*) in homokaryotypic females

We estimated the recombination rate between *Delta* (Δ) and *Antennapedia* (*Ant*) in homokaryotypic females (*DV-PC0/DV-PC0*) from crosses 2, 3, and 5 (see Figure 1 and Table 1). The average recombination rate across all of the crosses is 48.1% with a confidence interval at 95% of 45.8 to 50.4%.

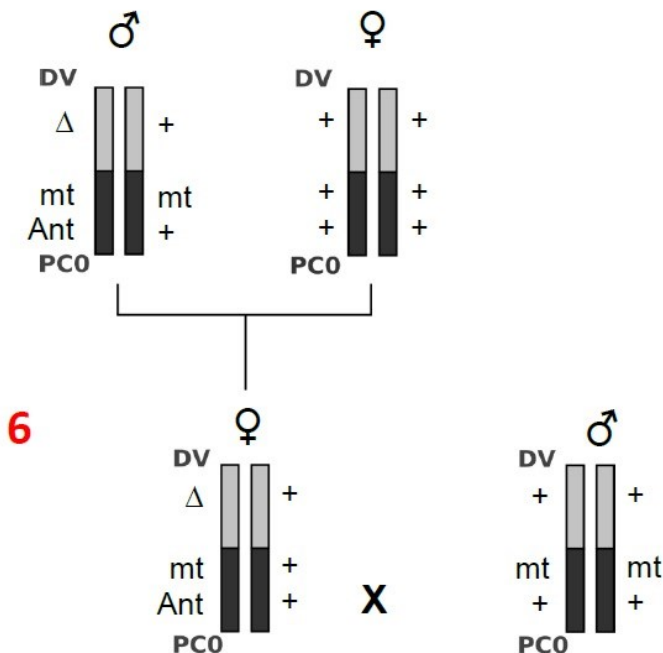


Figure 2. Cross 6. Inversions are represented by colored bars (DV, gray; PC0, black).

Table 2. Recombination rates (95% confidence interval) between *Antennapedia* (*Ant*), *merlot* (*mt*) and *Delta* (Δ) in homokaryotypic females (cross 6).

Phenotype	Sex	n	chrom. type
+ + +	male	39	parental
	female	59	parental
Δ + +	male	40	recombinant ¹
	female	48	recombinant ¹
+ + <i>Ant</i>	male	-	recombinant ²
	female	-	recombinant ²
Δ + <i>Ant</i>	male	-	recombinant ²
	female	-	recombinant ²
+ <i>mt</i> +	male	-	recombinant ²
	female	-	recombinant ²
Δ <i>mt</i> +	male	-	recombinant ²
	female	-	recombinant ²
+ <i>mt Ant</i>	male	30	recombinant ¹
	female	41	recombinant ¹
Δ <i>mt Ant</i>	male	34	parental
	female	33	parental
Total		324	
recombinants ¹		159	
<i>r</i> (Δ - <i>Ant</i>)		49.1% (43.5-54.7%)	
<i>r</i> (<i>Ant</i> - <i>mt</i>)		0% (0.0 – 1.1%)	
<i>r</i> (Δ - <i>mt</i>)		49.1% (43.5-54.7%)	

¹ Phenotypes produced by recombination between loci in distal and proximal regions

² Phenotypes produced by recombination between loci within the proximal region

Recombination rate between *Antennapedia* (*Ant*) and *merlot* (*mt*)

We estimated the recombination rate between *merlot* (*mt*) and *Antennapedia* (*Ant*) in homokaryotypic females (DV-PC0/DV-PC0) from cross 6 (see Figure 2 and Table 2). The females (Δ *mt Ant* / + *mt* +) were obtained from cross 5, shown in Figure 1.

We found no recombinant for the *Ant* and *mt* loci in a progeny of 324 individuals, indicating that these two loci are closely located in the second chromosome. We found the recombination rate between *Delta* (Δ) and *Antennapedia* (*Ant*) to be approximately 49%, which is similar to the observed data from crossings 2, 3, and 5 for the same loci.

Recombination rates in females heterozygous for distal inversions

We estimated the recombination rates in females heterozygous for distal inversions and homozygous for proximal arrangements (DV-PC0/DS-PC0) from cross 7 (see Figure 3 and Table 3).

Consistently with the data obtained from crossing 6, we observed a deficient production of recombinants for the *Ant* and *mt* loci, which suggests that recombination rates between these loci are in fact meager. On the other hand, the estimated recombination rate between *Delta* (Δ) and *Antennapedia* (*Ant*) is approximately 18%, a lower rate than that observed for females homozygous for both distal and proximal inversions (see crossings 2, 3, 5, and 6). Therefore, heterozygosity for distal inversions can lead to a sizable reduction of recombination between distal and proximal regions.

Recombination rate between distal and proximal inversions

Finally, we estimated the recombination rate between distal and proximal inversions in females heterokaryotypic for both distal and proximal regions (double heterozygous; *DI-PB0/DV-PC0*) from cross 4 (Figure 1). We used visual mutant markers *Delta* (Δ) marking the distal inversion *DV*, and *Antennapedia* (*Ant*) and *merlot* (*mt*) marking the proximal inversion *PC0*. The wild alleles marked the haplotype *DI-PB0*. These females were mated to homozygous males for haplotypes *DV-PC0/DV-PC0*; they carried for the mutations the genotype + *mt* +/+ *mt* + to allow inferring the genetic structure of the female gamete that gave rise to the offspring.

Table 4 shows the number of recombinant individuals observed in the offspring. Overall 1439 specimens were examined and five recombinants were found. Two animals had a *Delta* phenotype, implying a genotype of Δ + +/+ *mt* + (where Δ + + came from the mother recombinant gamete). Symmetrically, three flies had *merlot Antennapedia* phenotype, revealing a + *mt Ant* recombinant gamete from the mother. Overall the recombination rate between distal and proximal inversions is 5/1439, which is equal to *r* = 0.3% with a 95% confidence interval of 0.1 to 0.8%.

Table 3. Recombination rates (95% confidence interval) between *Antennapedia* (*Ant*), *merlot* (*mt*) and *Delta* (Δ) in heterozygous females for distal inversions (cross 7).

Phenotype	Sex	n	chrom. type
+++	male	91	parental
	female	93	parental
Δ ++	male	20	recombinant ¹
	female	15	recombinant ¹
++ Ant	male	-	recombinant ²
	female	1	recombinant ²
Δ + Ant	male	-	recombinant ²
	female	-	recombinant ²
+ mt +	male	-	recombinant ²
	female	1	recombinant ²
Δ mt +	male	-	recombinant ²
	female	-	recombinant ²
+ mt Ant	male	18	recombinant ¹
	female	19	recombinant ¹
Δ mt Ant	male	74	parental
	female	78	parental
Total		410	
recombinants ¹		72	
recombinants ²		2	
r (Δ -Ant):		17.6%	(14.0-21.6%)
r (Ant-mt)		0.5%	(0.1-1.8%)
r (Δ -mt):		17.6%	(14.0-21.6%)

¹ Phenotypes produced by recombination between loci in distal and proximal regions.

² Phenotypes produced by recombination between loci within the proximal region.

Conclusions

The recombination rate between *Delta* (Δ) and *Antennapedia* (*Ant*) in homokaryotypic females (double homozygous; DV-PC0/DV-PC0) is on the order of 48% but drops sharply as the heterozygosity for inversions increases. Actually, in heterozygous females for distal inversions (DV-PC0/DS-PC0), this recombination rate is reduced to 18%. In double heterokaryotypic females (double heterozygous) DV-PC0/DI-PB0, the recombination rate estimate is 0.3% with a 95% confidence interval of 0.1-0.8%.

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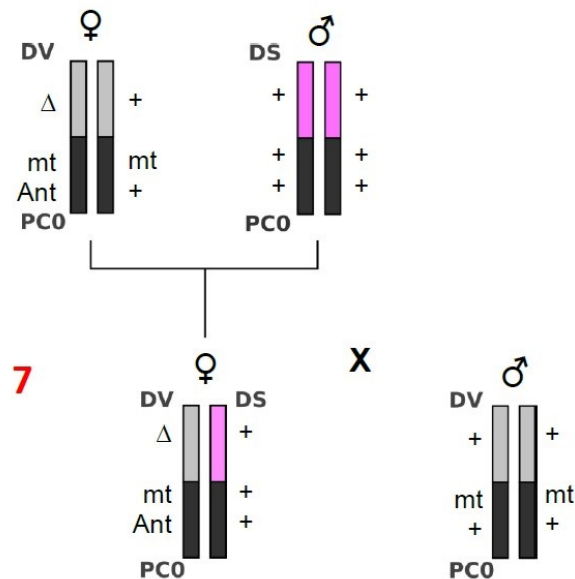


Figure 3. Cross 7. Inversions represented by colored bars (DV, gray; DS, pink; PC0, black).

Table 4. Recombination rate between distal and proximal inversions (cross 4, figure 1)

Phenotype	Sex	n	chrom. type
+++	male	396	parental
	female	404	parental
Δ++	male	1	recombinant ¹
	female	1	recombinant ¹
++ Ant	male	-	recombinant ²
	female	-	recombinant ²
Δ+ Ant	male	-	recombinant ²
	female	-	recombinant ²
+ mt +	male	-	recombinant ²
	female	-	recombinant ²
Δ mt +	male	-	recombinant ²
	female	-	recombinant ²
+ mt Ant	male	2	recombinant ¹
	female	1	recombinant ¹
Δ mt Ant	male	305	parental
	female	329	parental
Total		1439	
recombinants ¹		5	
r (distal-proximal inv.)		0.3% (0.1 – 0.8%)	

¹ Phenotypes produced by recombination between distal and proximal inversions.

² Phenotypes produced by recombination within proximal inversions.

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Naturally occurring recessive lethal alleles in a natural population of *Drosophila melanogaster* appear to result from single locus loss of function effects.

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Introduction

Researchers spent decades attempting to explain why so many individuals in natural populations carry chromosomes with recessive lethal effects. Chromosomes bearing recessive lethal alleles persist at measurable frequencies in numerous outbreeding species, including humans and *Drosophila*. These lethal mutations are often assumed to be caused by single loci rather than summative effects from multiple interacting loci (Lewontin, 1974), but this assumption has never been directly tested.

Lewontin (1974) suggested that mapping is the best way to directly test the single locus model. To the best of our knowledge, only one study has mapped segregating lethal alleles in *Drosophila*, but the mapped locations were coarse (*e.g.*, to one third of a chromosome) (Ives and Band, 1986). By crossing lines that contain chromosomes bearing recessive lethal alleles to lines bearing chromosomal deficiencies, the location of lethal alleles can be narrowed to relatively small regions of the chromosome. Subsequent crosses to knock-out lines may be used to pinpoint specific genes. The successful mapping of recessive lethal alleles to a much finer scale using deficiencies relies on the assumption that the lethal phenotype is caused by single locus loss of function alleles. As proof of principle, we selected a sample of previously established lethal isolines of wild-caught *Drosophila melanogaster* and crossed them to deficiency stocks that cover approximately 24% of the second chromosome.

Materials and Methods

As part of an ongoing project, we collected wild-caught *Drosophila melanogaster* from Durham, NC. Utilizing the second chromosome balancer *CyO*, we performed a series of crosses to isogenize the lines and to identify wild flies with a recessive lethal allele on the second chromosome. We only used wild-caught females in our crossing scheme (Figure 1) to circumvent *P*-element hybrid dysgenesis. *P*-elements invaded wild *Drosophila melanogaster* populations post-1940, and researchers discovered that hybrid dysgenesis results specifically from matings between males with *P*-elements and females without *P*-elements (Kidwell, 1983; David and Capy, 1988). For this reason, we used wild-caught females, which carry *P*-elements, in our crosses to laboratory stocks, which do not carry *P*-elements.

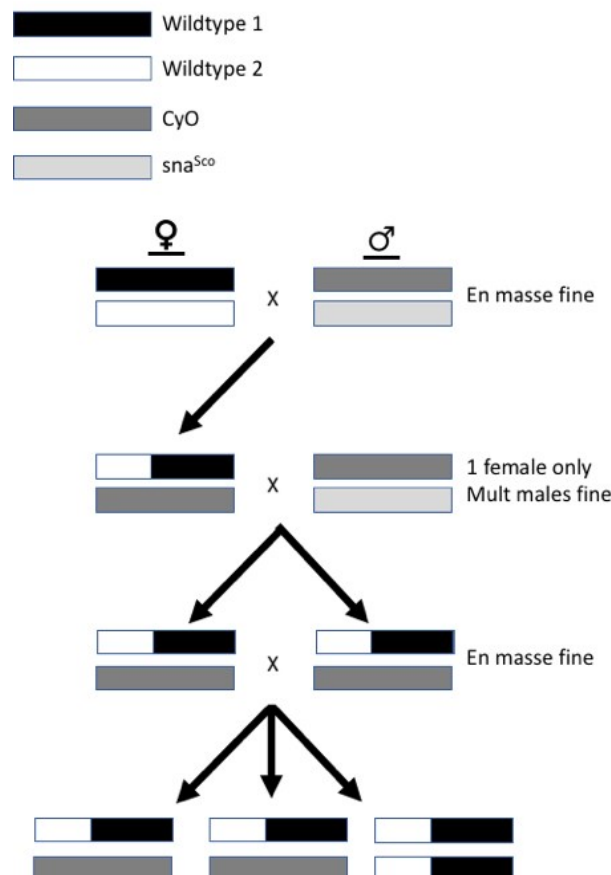


Figure 1. Crossing scheme utilizing balancer chromosome *CyO* to identify wild-caught flies that carry a recessive lethal allele.

We crossed wild-caught female flies with males from a stock heterozygous for two dominant but homozygous-lethal visible marker mutations: "Curly" (*Cy*) (on the *CyO* balancer) and "Scutoid" (*sna^{ScO}*). *Cy/+* females were selected from the first generation and crossed *en masse* to *Cy/sna^{ScO}* males. We crossed individual *Cy/+* females from the second generation to *Cy/sna^{ScO}* males. All *Cy/+* individuals in the third generation from a single cross necessarily carried the same wild second chromosome. We selected *Cy/+* females and males from the third generation and mated the siblings to form an isolate. If no fitness difference existed between *Cy/+* and homozygous +, then 2/3 of the fourth generation would have the curly-winged phenotype and 1/3 would be wild type. To be considered "lethal", the relative fitness of the homozygous wild chromosome must be less than 10% of the fitness of the balancer heterozygote (Simmons and Crow, 1977). We scored at least 35 progeny for all of the isolines and found no wild type progeny in any of the ten lethal isolines used in this study.

We selected seventeen deficiency stock lines from the Bloomington Stock Center with approximately 24% coverage of the second chromosome among them. Each deficiency stock consisted of individuals heterozygous for a second chromosome deletion and a balancer chromosome containing the *Cy* mutation. We crossed three females from each of the 10 lethal isolines with three males from each of the 17 deficiency lines and scored the offspring for wing phenotype (either curly or straight). The null expectation (if the lethal allele was *not* contained within the deficiency) was 2:1 curly to straight wings in the offspring, because the dominant *Cy* marker is homozygous lethal. Consistent with previous usage, we defined lethality as less than 10% fitness of the heterozygote for *Cy*.

Results

Of the 10 lethal lines, 8 complemented with the 17 deficiencies (as observed through numerous straight-winged offspring) and 2 lethal lines failed to complement with a deficiency (a different deficiency for each). One of the lethal lines failed to complement with deficiency Df(2R)ED3791, with 59 *Cy* offspring and 1 wild type. The deficiency is a 552,570-base region on the right arm. The base pair coordinates for the breakpoints are 20,698,411 to 21,250,981 (Grammates, L.S., *et al.*, 2017, FB2018_06, www.flybase.org). The other lethal line that failed to complement mapped to the deficiency Df(2L)H20, with 136 *Cy* offspring and 0 wild type. The deficiency is an approximately 1.7 megabase region on the left arm. The base pair coordinates of the breakpoints have not yet been molecularly characterized, but they range between 16,692,000 to 18,359,026 at the smallest and 16,680,000 to 18,393,441 at the largest (Grammates, L.S., *et al.*, 2017, FB2018_06, www.flybase.org).

Discussion

If lethal alleles are single loci, loss of function, and evenly dispersed, and given the 24% coverage of the deficiencies used, we would expect to successfully map ~2.4 of the lethal lines in this study. Our findings (2/10) are consistent with this expectation. Given the current size of the regions to which we have mapped lethal effects (approximately 0.55 Mb and 1.7 Mb), we still cannot completely exclude the possibility that there are multiple loci affecting the lethal phenotype within these regions, but loss of function seems probable. The results from this study are nonetheless consistent with the previously untested assumption that homozygous lethality is caused by alleles at single loci and encourage further investigation into mapping more segregating natural lethal alleles and their precise gene locations and functions.

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Social effects on patterns of movement and body size and weight of larval *Drosophila melanogaster*.

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Positive ecological interactions as facilitation are important in stressed and resource limited-environments (Berness and Callaway, 1994). In agreement with these authors (*op.cit.*), in the wild we have observed positive behavioral interactions between conspecific larvae related to space allocation. So, larval *Drosophila* pupate near individuals of the species (Del Pino *et al.*, 2014, 2015). On the other hand, in the case of a number of *Drosophila* species breeding sites, ecological conditions change in a short period of time that can go from days to hours. Therefore, such breeding sites are an example of ephemeral environment with limited ecological resources (Powell, 1997).

Here we report that third instar larvae of *Drosophila melanogaster*, the Canton-S strain, reared in isolation of conspecifics of the same strain exhibit: (i) a body size and weight smaller than larvae of the same strain reared with conspecifics, and (ii) movement patterns with less number of turns than those shown by larvae reared with conspecifics, suggesting a social influence on those fitness components. These findings indicate that the presence of conspecifics has a number of consequences for individual life of *Drosophila* larvae. For example, changes in physical features of substrate originated by activity of larvae could facilitate access to food sources.

To test this conjecture, we sowed an egg on culture medium whose surface had previously been raked with a needle, and the larva was weighed and measured at 96 hours old. We also examined patterns of movement of such individuals by counting the number of the body contractions and turns made during two min, N = 30 individuals. As control, we deposited individual eggs, N = 30, on non-raked culture medium; the 96-hours-old larvae were measured and weighed, and their patterns of movement examined as described above.

Canton-S third instar larvae reared individually on raked medium increased three times their size and weight by comparison with conspecifics reared also individually on non-raked medium. In other treatment, we deposited N = 30 eggs on raked and non-raked culture medium, N = 4 repetitions per treatment. We found that third instar larvae reared on raked medium with conspecifics increased 2.30 times the body size and weight by comparison with larvae of the same strain also reared in groups but on non-raked medium. We also discovered that larvae reared on raked medium with conspecifics increased significantly in size and weight by comparison with larvae reared on raked medium without conspecifics (ANOVA, $F_{1,156} = 129$; $P < 0.0001$). These findings show that physical characteristics of medium such as a rough (or smooth), and hard (or soft) surface and the presence/absence of conspecifics affect fitness components as body size and weight.

Patterns of movement: locomotion

On non-raked medium larvae reared in isolation and those reared in the company of conspecifics showed a similar locomotion rate, as measured by number of body contractions in two min. By contrast, on raked medium 96-hours-old larvae reared with conspecifics increased significantly locomotion by comparison with that of larvae of the same age reared on raked medium but without conspecifics (ANOVA, $F_{1,156} = 262$; $P < 0.0001$). Interestingly, larvae reared on raked medium in the presence and in the absence of conspecifics increased locomotion in 64.00% compared with larvae reared on non-raked medium with or without conspecifics.

Patterns of movement: turns

On non-raked culture medium larvae reared with conspecifics showed a number of turns higher than those reared in this same culture medium but in isolation of conspecifics ($F_{1,36} = 23.83$; $P < 0.0001$). On raked

culture medium, larvae reared with congeners increased numbers of turns in 63.00% by comparison with larvae reared with conspecifics but in non-raked culture medium. Similar results were also obtained with larvae reared individually in raked and non-raked culture medium. That is, in raked medium larvae reared in isolation from conspecifics made a 43.00% more turns than those reared in non-raked culture medium. We conclude that the type of medium, raked and non-raked, on which the larvae are reared affects in different ways locomotion and turns made by the larva while moving on the substrate.

In short, our findings show that in larvae of *D. melanogaster*, features of substrate and the presence of conspecifics affect expression of a number of traits that link with morphology, physiology, and functioning of the nervous system.

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Phenotypic plasticity of *Drosophila mercatorum* under different temperatures and microbiota.

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Introduction

The ontogenetic development of organisms responds to various environmental conditions (Stearns, 1989), enabling the production of different phenotypes (Bradshaw, 1965; Pigliucci, 2001). Among the factors capable of altering the development, the availability of food resources, pathogens, and temperature can be emphasized (Huston, 1994). The latter is one of the conditions that most influence the distribution and abundance of organisms, especially of ectodermal animals, such as drosophilids (Precht *et al.*, 1973; Karan and David, 1999).

The composition of microorganisms in the food substrate is also a determining factor in the development of flies. Nipagin[®] (methylparaben) is an antifungal agent traditionally added to *Drosophila* culture medium in the laboratory because it prevents the presence of mold that decreases the viability of flies (Child, 1939). However, little is known about the effect of the fungus-contaminated culture medium on the morphology of these insects.

Drosophilid wings are used for flying and also for courting, in the case of males (Spieth, 1974). In addition, wings can respond to environmental changes in an adaptive (Huey *et al.*, 2000; Hoffmann and Shirriffs, 2002) and plastic (Debat *et al.*, 2003) way. In this study, we investigated whether the wing morphology of *D. mercatorum* responds to environmental variations associated with temperature and presence of fungi in the culture medium.

Material and Methods

Drosophila mercatorum Patterson & Wheeler is a generalist species (Pereira *et al.*, 1983) widely distributed in South America (Vilela *et al.*, 1983), which also colonized part of North America, Europe, Africa, and Oceania (TaxoDros, 2018). In this study, two isofemale lines (L1 and L2) of *D. mercatorum* were established from females collected in natural populations near the city of Brasília, capital of Brazil. Each line originated six replicates (10 couples each), which were transferred to different combinations of temperature (17°C, 21°C, and 25°C) and treatment with the fungicide Nipagin[®] (presence or absence). Thus, each replicate

was submitted to an experimental condition (17^+ , 17^- , 21^+ , 21^- , 25^+ , 25^-). The emerged flies were preserved in 70% alcohol and stored in the Drosophilid Collection of the Laboratory of Evolutionary Biology at Universidade de Brasília.

From each experimental group, the right wings of 50 females (totaling 300 wings per line) were removed, mounted on slides, and photographed under a Leica MZ16 stereomicroscope. Image libraries were created using the TpsUtil software and imported into tpsDig 2.30 (Rohlf, 2010), where 13 landmarks were placed according to the intersections and endings of the veins (Figure 1). The landmark configurations were analyzed by geometric morphometrics using the software MorphoJ 1.06d (Klingenberg, 2011). The Procrustes superimposition, a procedure based on translation, scaling, and rotation of the landmarks, was applied to isolate the shape of other variables (Bookstein, 1991). Wing morphology variation was explored by the Principal Component Analysis (PCA) based on the matrix of covariance.

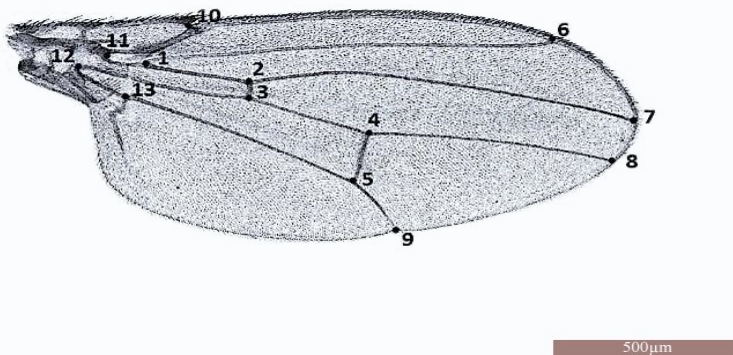


Figure 1. *Drosophila mercatorum* right wing, showing the 13 landmarks evaluated in this study (bar = 500 μ m).

Results and Discussion

In the culture medium vials treated with Nipagin[®], no fungi developed. In the absence of this inhibitor, however, we observed colonies of filamentous fungi at the three temperatures tested.

The morphological differences among the flies reared under different experimental conditions are shown in Figure 2. Visual inspection of the samples along the PC1 axis suggests that wing shape responded to the medium with mold only in the individuals that developed at 17°C. At temperatures of 21°C and 25°C, no differences were detected between flies reared in culture medium with or without mold. These results show that, under certain conditions, drosophilids can reflect the stress associated to the presence of mold.

It is widely known that most *Drosophila* species feed on microbiota decomposing fruits or other substrates (Begon, 1982). However, at the same time that flies use part of the microbiota (mainly yeasts and bacteria) as a nutritional resource, they also compete with some mold fungi species for space- and food-limited patches of organic matter (Rohlf and Hoffmeister, 2005). The coexistence of *D. melanogaster* and the filamentous fungus *Aspergillus*, for example, reduce the fitness of each other influencing both fly development and fungal growth (Hodge *et al.*, 1999). There is also evidence that the viability of *D. melanogaster* is more related to competition than to pathogenic effects of *Aspergillus* (Trienens *et al.*, 2010). Moreover, the appropriate use of mold inhibitors optimizes the survival of drosophilids but, in high concentrations, they can reduce their intestinal microbiota by eliminating important fungi (Téfit *et al.*, 2018). The yeast *Issatchenkia orientalis*, for instance, is essential for protein metabolism and the digestive process of flies (Yamada *et al.*, 2015; Obadia *et al.*, 2018).

Although the effect of mold has been tested at all temperatures, the wing morphology responded clearly to stress only at 17°C. As the strains used here were collected from the Brazilian Savanna, where the mean temperature during the coldest month is higher than 18°C (Silva *et al.*, 2008), these flies should be better adapted to warmer environments. Accordingly, the results obtained here suggest that the phenotypic plasticity in *D. mercatorum* wings is sensitive to stress.

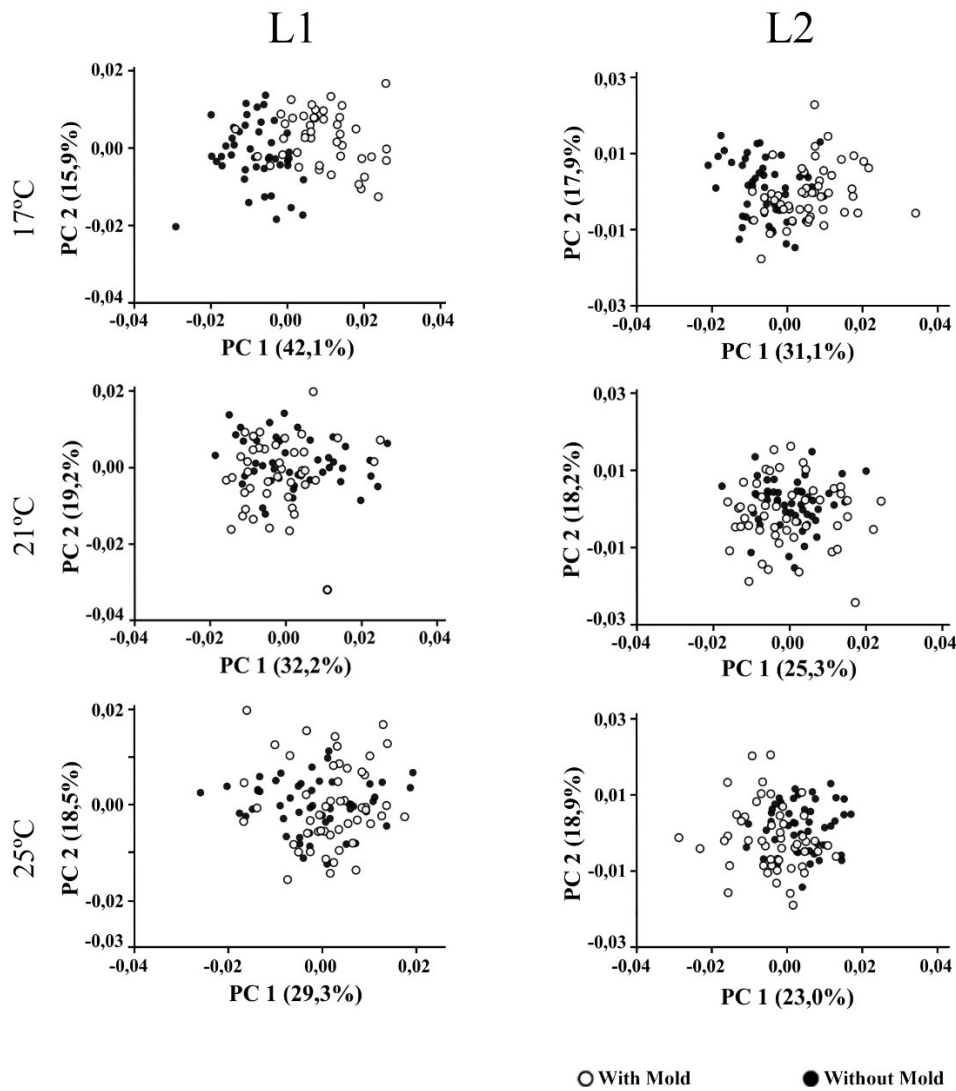


Figure 2. Principal Component Analysis based on the wing morphology of two *Drosophila mercatorum* isofemale lines (L1 and L2) under three different temperatures. The percentage of variance explained by each PC is described in parentheses.

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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 Biology, University of Oklahoma, Norman, OK 73019. Telephone (405)-325-2001; email jthompson@ou.edu; FAX (405)-325-7560.

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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.

Technique Notes



A high-throughput spectrophotometric assay of adult size in *Drosophila* that facilitates microbial and biochemical content analysis.

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Abstract

Large-scale laboratory experiments often necessitate the processing of numerous samples in little time, their long-time storage and the joint analysis of morphological, biochemical, and microbiological features. Combining different types of assays is often not compatible with classical methods to estimate size in adult *Drosophila* flies. We therefore designed a new spectrophotometric assay for the high-throughput estimation of adult size in *Drosophila* that facilitates microbial and biochemical content analysis. The new method uses optical density at 202 nm of single fly homogenates as size proxy. We tested the method in a variety of *Drosophila* populations - including wild caught flies - and compared its explanatory power with two classical size estimates: wet-weight and wing-length. It was also used to control for size when comparing the fat content of different fly populations. Results show fly homogenate optical density is a powerful size proxy that may be used for both male and female flies.

Insect size is a phenotype that responds to genetic and environmental factors and affects important life-history traits and, therefore, fitness (Partridge, *et al.*, 1987). In adult *Drosophila* flies, size can be assayed by several methods; the full body can be weighed wet or dried, its size estimated by dissecting and measuring wings, thorax, or leg segments (David, *et al.*, 1994; Partridge, *et al.*, 1994). Each of these methods has practical limitations. The fresh (*i.e.*, wet) weight of animals changes according to recent food and water intake as well as egg and feces production. Besides obtaining fresh weight implies the prompt manipulation of assayed individuals, which can be too time-consuming when many insects must be processed simultaneously. Measuring dry weight is freed from water intake variations but prevents the study of insect microbial content as the drying process is lethal to numerous species of the microbiota. Fly biologists have a long tradition of measuring wing-length as a proxy of insect size. This is convenient as it does not alter body content (*i.e.*, does not harm microbial symbionts). However, dissection, like wet weighing, can take too long to process numerous individuals in a given timeframe. Besides, environmental factors such as temperature during development can alter the relative sizes of wings and body (David, *et al.*, 1997; Partridge, *et al.*, 1994).

For an experiment on the symbiosis between microbes and flies we had to simultaneously estimate the size of adult flies, count the number of live bacteria and yeast cells they contained, and assay biochemical content (*e.g.*, triglycerides content). None of the methods listed above enabled processing a number of flies that sufficed to keep track with the work-load of an experiment that involved hundreds of flies. We therefore designed an alternative sizing method based on spectrophotometry. In brief, the method consists in homogenizing adult flies in a liquid, split the sample in several sub-samples, some of them with glycerol, and store at -80°C. It is later possible to thaw each sub-sample and measure its optical density (*i.e.*, our size-proxy), plate and count the number of live microbes (sub-samples frozen with cold-protecting glycerol), and perform biochemical analyses.

In the process of designing this protocol, we identified key steps to ensure size estimates were meaningful. First, we had to identify a wavelength where the relationship between sample concentration and optical density was linear. Second, we validated the measure in a variety of *D. melanogaster* samples, from the lab and from the wild. Eventually, we tested the method with a mock study on the relationship between size (*i.e.*, homogenate optical density) and fat reserves.

Choice of wave-length for fly homogenate assay

The first step of our procedure was to identify a wave length at which optical density varies linearly with sample concentration. To this aim we prepared 10 fly homogenates in PBS in 1.5 mL centrifugation tubes. Flies originated from a stock-culture of the Oregon-R strain. Each of the 10 samples of adult flies was homogenized with a pestle and serially diluted so that we could measure Optical Density (OD) pure, or at a concentration of 0.5, 0.25, and 0.125. The diluted sub-samples were then separated in four: two sub-samples were analyzed immediately while two others were analyzed 4 hours later (samples were kept at room temperature $\pm 21^\circ\text{C}$). This step was necessary to ensure delays between sample preparation and analysis did not alter measures.

A volume of 2 μl of each sub-sample was placed in one of sixteen measure locations of a μdrop plate (Thermo Scientific, # N12391) and its optical density read in a Multiskan GO spectrophotometer (Thermo Scientific, # N10588). The μdrop device enables analysis of smaller samples than traditional 96 or 384 well plates and is often used for nucleic-acids quantification. We tested optical densities at ten different wavelengths: 200, 202, 204, 206, 208, 210, 215, 220, 225, and 230 nm.

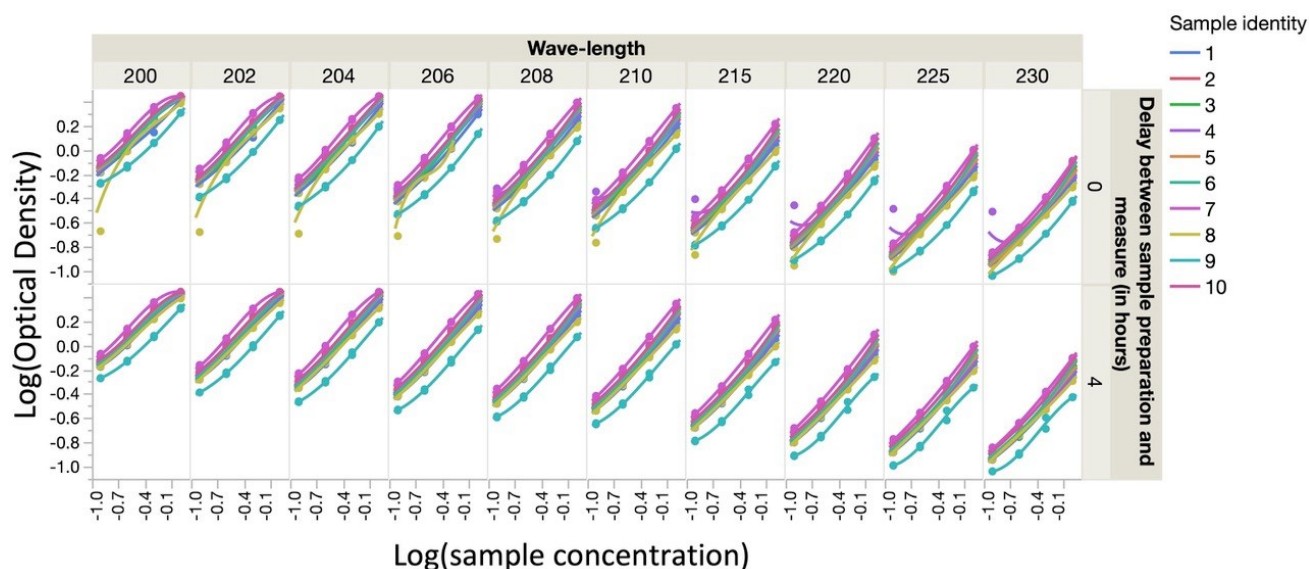


Figure 1. Relationship between sample concentration and homogenate optical density at several wave-lengths. Two series of measurements were run 4 h apart.

Visual exploration (Figure 1) of the data revealed the relationship between sample concentration and optical density was somewhat linear when both variables were log-transformed. In order to choose the wavelength at which linearity was best and test for the effect of time between sample preparation and optical density measurement we used linear models. Models had $\text{Log}(\text{Optical Density})$ as response variable and $\text{Log}(\text{concentration})$ and Sample identity , as well as their interaction, as factors. We fitted separate models for each combination and time between sample preparation and assay (*i.e.*, 0 h or 4 h). The only two factors to be significant in all models were Sample identity and $\text{Log}(\text{Concentration})$ (all $P < 0.0001$); but the interaction $\text{Log}(\text{Concentration}) * \text{Sample identity}$ was significant at wave-length superior to 206 nm, suggesting short wavelengths were better candidates. Similarly, models of data acquired 4 h post sample preparation consistently had slightly better R^2 than when samples were analyzed right after preparation (*e.g.*, at 202 nm, after 0 h $R^2 = 97.8$ while after 4 h $R^2 = 99.6$). In order to choose the best wavelength among those that were visually satisfying, we analyzed the residuals of the models' linear fits. For each wavelength, we saved the residuals of the models with Sample identity and $\text{Log}(\text{Concentration})$. We reasoned the best wavelengths would be the ones which residuals would have the least standard-deviation. The two wavelengths that fitted

this criterion best were 202 and 204 nm (s.d. = 0.0157 and s.d. = 0.0165, respectively, while all other wavelength had standard-deviations above 0.019). From thereon, we chose to work exclusively at 202 nm.

Table 1. Fly samples used for the comparison of homogenate OD with wet weight, wing length and triglycerides content.

Sample name	Description
Oregon-R old	Flies from the Oregon-R strain. Old individuals from vial set up more than 2 weeks before collection.
Oregon-R young	Flies from the Oregon-R strain. Young individuals from vial set up less than 2 weeks before collection.
Wild caught	Wild-caught flies from Southern France.
Wild-type 1	Wild-type population of unknown origin. Adult flies of unknown age.
Hemiclone-type	Mix of adult flies of unknown age from lines used for hemiclone analyses.

Comparison of homogenate OD with wet weight and wing length

The second step of protocol design was to compare how the new proxy compared to classical size estimates, namely wet weight and wing length. To this aim we assayed individual flies, both males and females, from various age and origin (Table 1). In particular we were cautious to include wild-caught adult flies, lab-reared flies from distinct genetic backgrounds, and flies of different ages but from the same background. Males and females were treated separately.

Individuals were anaesthetized with CO₂, individually weighed, and had their wings removed and placed on a microscope slide. Wing length was measured with a stereo-microscope. The average of the two wings was used for further analysis. Right after the wings were removed, individuals were frozen at -80°C in PBS. Several days after freezing the samples were thawed and homogenized in a Tissue Lyser II (Qiagen, #85300) for 30 s at 30 Hz with Ø3 mm glass balls, centrifuged for 30 s at 2000G. Optical density of 2 µL of supernatant was then read on spectrophotometer µDrop device.

Our first approach was to neglect population differences and relate each of our two size predictors (*i.e.*, wing length and OD) to wet-weight. In other words, we assumed wet weight was an adequate size estimator and compared the performance of two size proxies. To this aim we used linear models with *Log(wet weight)* as response variable and the size proxies as explanatory factors. We found that both wing length and homogenate OD were good predictors of wet weight variation and revealed differences among sexes. In males, homogenate OD explained 71.5% and wing length 57% of *Log(wet weight)* variance. In females, homogenate OD explained 39.2% and wing length 51.3% *Log(wet weight)* variance. Linear models explaining *Log(wet weight)* with both OD and wing length as factors showed each metric conveys different information as proportion of explained variance was improved for both males and females (R^2 males = 77.1%; R^2 females = 68.9%; in both cases the two factors were highly significant).

In a second stage, we investigated differences among fly populations and sex. Response variable remained *Log(wet weight)*, factors were *Sex*, *Population*, and either *Wing length* or *Log(OD)*, as well as all possible interactions between the 3 factors the initial model contained. After a step-wise deletion of non-significant terms, models based on wing length or homogenate OD provided similar information (Table 2) and explained a similar proportion of wet weight variance: 82% with *Log(OD)* and 83.6% with wing length. We note none of the interactions comprising sex and OD or sex and wing length were significant - and even though lack of significant difference must not be interpreted as proof of similarity - the above result suggests that the general relationship between the two size estimates and wet weight may be robust relative to fly sex. More importantly, interactions between population and the two size estimates were both significant (Table 2). In both cases the interaction was driven by the steeper slope of the *Hemiclone-type* population (wing length $t = 2.68$, $P = 0.009$; OD $t = 2.32$, $P = 0.023$) and the flatter slope of the *Wild-type 1* population in the case of wing length ($t = -2.52$, $P = 0.014$). Size estimate*population interactions suggest estimates may provide unreliable data when comparing different populations. It is however not possible to know whether population differences

are due to genetic or environmental factors (flies from the same populations may have been sampled in separate vials; however, this information was lost). Comparisons based on wing length and homogenate OD are therefore more accurate for insects from the same populations and reared in similar conditions than for samples from very different origins.

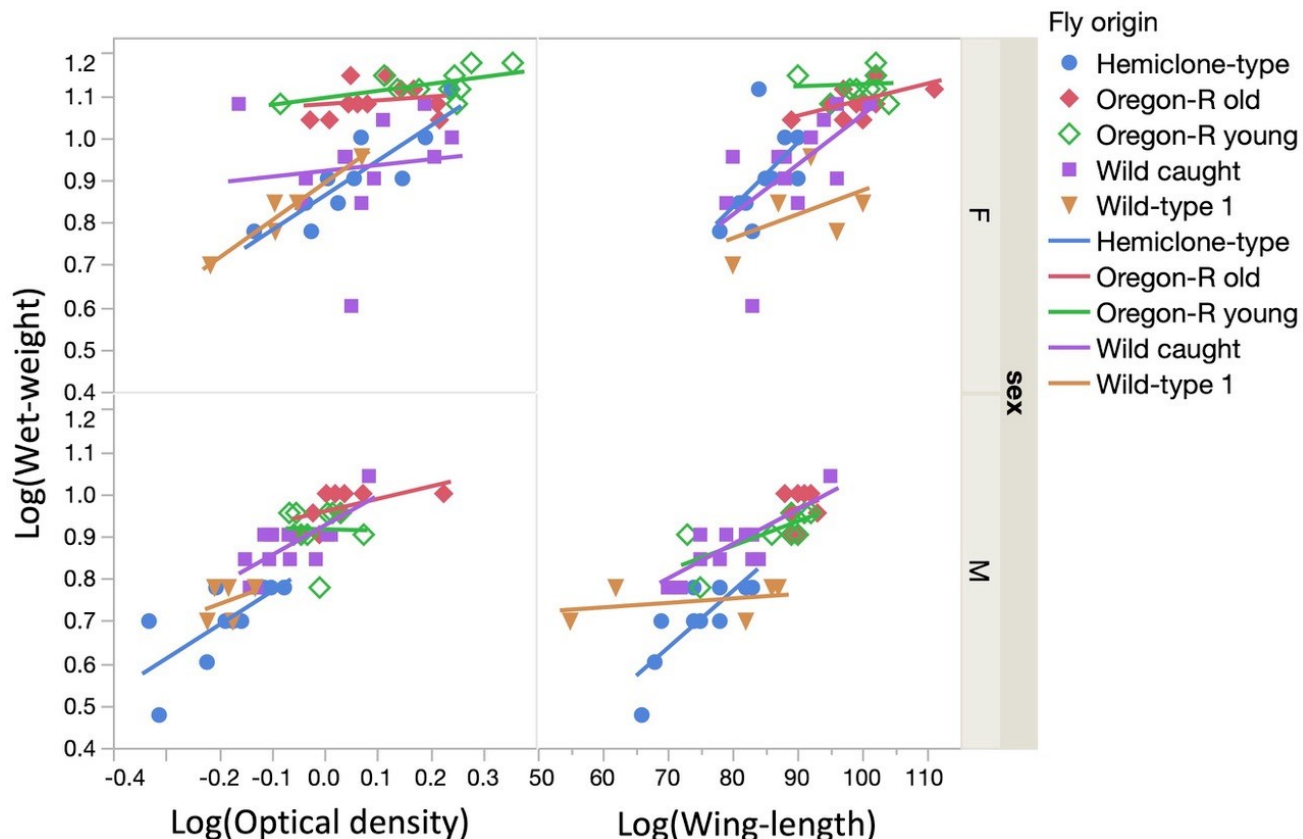


Figure 2. Relationship between either homogenate OD or wing length and wet weight.

Table 2. Statistical models explaining Log (wet weight) as a function of fly sex, population and either Log (homogenate optical density) or wing length.

Size proxy	Factor	DF	F Ratio	P > F
Adult fly homogenate Optical Density Log-transformed)				
AIC= -221.1 R ² = 0.820	Population	4.80	12.7	< 0.0001
	Sex	1.80	24.4	< 0.0001
	Population * Sex	4.80	3.18	0.0177
	Log (OD)	1.80	9.34	0.0030
	Population * Log (OD)	4.80	3.32	0.0142
Wing length				
AIC= -229.7 R ² = 0.836	Population	4.80	12.9	< 0.0001
	Sex	1.80	9.81	0.0024
	Population * Sex	4.80	3.77	0.0073
	Wing length	1.80	32.1	< 0.0001
	Wing length * Population	4.80	3.89	0.0062

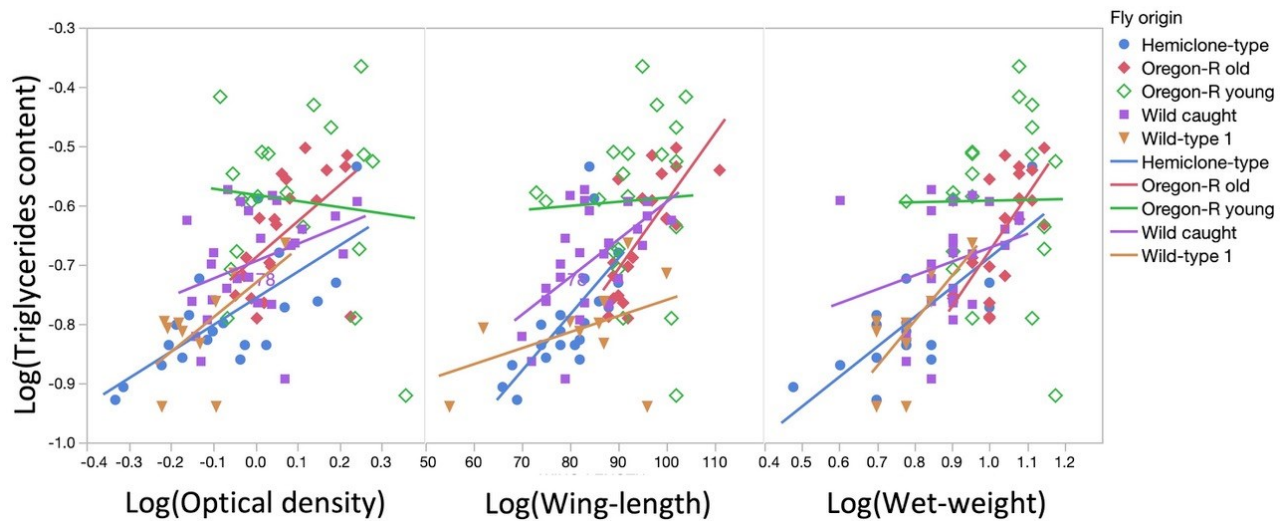


Figure 3. Relationship between either homogenate OD, wing length or wet weight and triglyceride content.

Table 3. statistical models explaining triglycerides content (log-transformed) as a function of fly sex, population and one of three estimators.

Size proxy	Factor	DF	F Ratio	P > F
Adult fly homogenate Optical Density (Log-transformed)				
AIC= -167.8	Population	4.85	8.35	< 0.0001
R ² = 0.527	Sex	1.85	6.50	0.0126
	Log (OD)	1.85	2.50	0.1171
	Population * Log (OD)	4.85	2.73	0.0339
Wing length				
AIC= -169.4	Population	1.85	7.52	< 0.0001
R ² = 0.534	Sex	1.85	1.46	0.2297
	Wing length	4.85	8.18	0.0053
	Wing length * Population	4.85	2.19	0.0766
Wet weight (Log-transformed)				
AIC= -165.8	Population	4.84	4.91	0.0013
R ² = 0.532	Sex	1.84	3.56	0.0623
	Log (wet weight)	1.84	4.66	0.0336
	Population * Log (wet weight)	4.84	2.28	0.0668

Case study: explaining adult fly fat content with size-estimates

The protocol evaluated in this manuscript was developed to enable the high-throughput processing of samples and the streamlining of size, microbial, and biochemical assays. In order to evaluate the relevance of homogenate OD as size proxy in this context, we analyzed the triglyceride content of the fly samples described above (Table 1). Triglycerides are the main form of fat storage in insects. Triglyceride concentration was measured following a classical biochemical method initially described by Clark and Gellman (1985). In brief, homogenate was incubated with Triglycerides reagent (Sigma Aldrich, #T2449) for 20 minutes at 25°C, centrifuged to clear-off particles, incubated again with Free Glycerol Reagent (Sigma Aldrich, #F6428) for 20

more minutes at 25°C, and read at 540 nm in 96-wells flat-bottom microplates (Thermo Scientific, #269620). We then used linear models to model triglyceride content variation in response to size, sex, and population. We compared three types of models with either $\text{Log}(OD)$, *Wing length*, and $\text{Log}(\text{wet weight})$ as size estimate, *Sex* and *Population* as discrete factors, and all second order interactions.

All three size proxies produced similar results (Figure 3). Concentration in triglycerides was explained by size, sex, population and a close to 5% p-value interaction between size and population (Table 3). Comparison of models AIC and R^2 suggest using wing-length as size proxy may give best results, but differences are minimal. We also note that with all three size proxies the interaction term was driven by the flatter slope of the *Oregon-R young adults* population ($t < -2.43$, $P < 0.017$ in three cases).

Conclusions

Homogenate OD appeared a size proxy of similar explanatory power as wing-length and wet-weight. A caveat of this new method is that the nature of the molecules quantified at 202 nm is unknown. However, all other size measurements available convey incomplete information as the concept of size is in itself the simplification of a multi-dimensional phenomenon (e.g., water content may or may not be a relevant parameter, morphology may change independent of volume). Depending on intended use, size may be best estimated by one of the many proxy available. The method we propose here is best suitable when many samples need to be processed at once, and microbial or biochemical content must be analyzed, too.

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References: Clark, A.G., and W. Gellman 1985, *Dros. Inf. Serv.* 61: 190; David, J. *et al.*, 1994, *Genetics Selection Evolution* 26: 229; David, J.R. *et al.*, 1997, *Journal of Thermal Biology* 22: 441-451; Partridge, L. *et al.*, 1994, *Evolution* 48: 1269-1276; Partridge, L. *et al.*, 1987, *Animal Behaviour* 35: 468-476.

Service Announcement:

The Elgin Lab fly room will be closed May 31, 2019, with the flies being euthanized. Over the years we have generated many unique *Drosophila melanogaster* lines. Most of these contain P-element constructs with a visible reporter of Position Effect Variegation (PEV), most often an *hsp70*-driven *white* gene, designed to report on the local chromatin environment. Lines currently in our collection are listed on our webpage – <https://sites.wustl.edu/elginlab> – under stocks, in association with the paper that describes the generation and characterization of the particular lines in greater detail (usually including determining the insertion site of the P-element reporter). PEV lines in the collection include those with insertions into pericentric heterochromatin, telomere-associated regions, the fourth chromosome, and the Y chromosome. **All lines are available now through May 2019**, when the Elgin *Drosophila* lab will be closed. Please direct requests to Jo Wuller at wuller@wustl.edu, with a cc to Sarah Elgin (selgin@wustl.edu).

Mutation Notes

**First record of Curly mutant wing in *Zaprionus indianus* (Diptera Drosophilidae).**

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Drosophila mutants are an excellent model to study genetics and evolution (Li *et al.*, 2011). There are different morphological mutants in *Drosophila*, but the most commonly used as a dominant marker for the second chromosome in *Drosophila melanogaster* is the Curly one (Lindsley and Zimm, 1992). Curly flies show a variable curvature degree of wings, and they were first described by Ward (1923). Despite intensive employment of these mutants in studies of Drosophilidae genetics, the Curly mutation had not been identified in the *Zaprionus* genus until now.

Zaprionus indianus was first detected in South America in 1999 (Vilela, 1999) and since that it spread out for all the American Continent over about ten years (Commar *et al.*, 2012; Leão *et al.*, 2017); however, none of the classical phenotypic mutations in *Drosophila* were described in this species. Regarding the aim of this work, it is to report for the first time the Curly mutant in *Zaprionus indianus*.

The flies used in this report came from laboratory F1 and F2. Parental flies were collected by banana-yeast baits and they are from Uberlândia (MG) - 18°55'08'' S e 48°16'37'' - in December 2016. The original collected flies showed the wild phenotype. After 21 days from parental ovopositions, there emerged five Curly mutant adults, three females and two males. These Curly mutants in *Zaprionus indianus* (Figure 1) are being kept at 25°C until next offspring. So far, the F2 generation has produced 15 flies, 12 Curly mutant and 3 wild phenotypes. It was recorded for F2 Curly mutants a male/female proportion of 6/6 (1:1).

This is the first record of a *Drosophila* phenotypic mutant in *Z. indianus*. These Curly mutants will be used in insecticide resistance and other genetic and evolution studies.

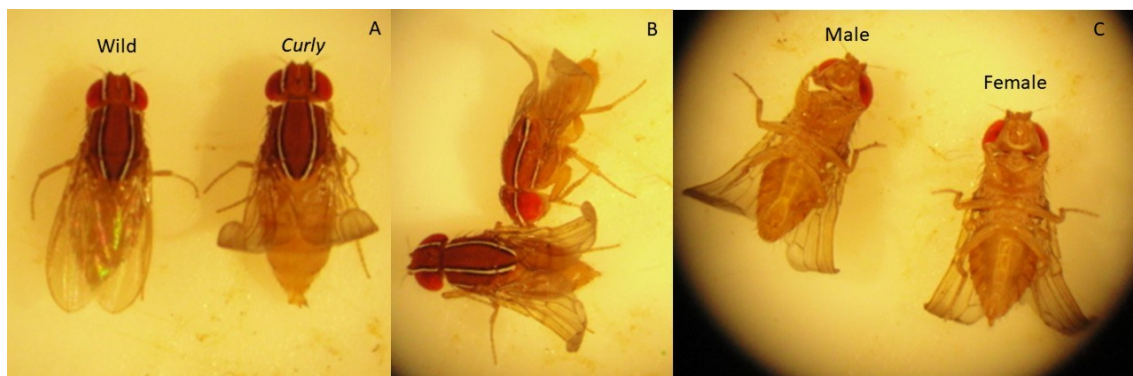


Figure 1. A) Wild and *Curly* phenotypes of *Z. indianus*; B) *Curly* phenotypes in *Z. indianus*; C) Male (left) and female (right) flies of *Z. indianus* with *Curly* phenotypes.

References: Commar, L.S., L.G. da C. Galego, C.R. Ceron, and C.M.A. Carareto 2012, Genet. Mol. Biol. 35(2): 395-4-6; Leão, B.F.D., F. Roque, P.H.M. Deus, and R. Tidon 2017, Dros. Inf. Serv. 100: 65-69; Li, H., L. Lian; C. Zhao, and C. Wu 2011, Asian J. of Animal and Vet. Advances 6(4): 391-396; Lindsley, D.L., and G.G. Zimm 1992, *The Genome of Drosophila melanogaster*. Academic Press; Vilela, C.R., 1999, Dros. Inf. Serv. 82: 37-39; Ward, L., 1923, Genetics 8: 276-300.



Characterization of the new *bithorax* allele *Ubx^{bx-Basel}*.

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We have isolated a new *bithorax* (*bx*) allele called *Ubx^{bx-Basel}*. It appeared in stock *S110501{w+}/TM6C* that we obtained many years ago from the Szeged Stock Center. In this report, we describe the phenotypes and the molecular lesion of *Ubx^{bx-Basel}*. We show that it is a new *gypsy*-based allele and not a re-isolate of a classical *bx* allele.

In *Drosophila*, the *Ultrabithorax* (*Ubx*) gene is required to determine the segmental identity of segments T2 (posterior), T3 and A1 (anterior), which correspond to embryonic parasegments PS5 and PS6. Lewis and others have shown that parasegment-specific control regions exist that independently determine *Ubx* expression levels in these two parasegments: in PS5, *Ubx* is controlled by regulatory elements *abx/bx*; in PS6, *Ubx* is controlled by *bxl/pbx* (see Figure 2A; Lewis, 1978; Bender *et al.*, 1983; White and Wilcox, 1985; White and Akam, 1985; Beachy *et al.*, 1985; reviewed in Peifer *et al.*, 1987; Duncan, 1987).

The *bithorax* region of the *Ultrabithorax* gene is genetically well-characterized. In brief, it is defined by a collection of clustered lesions spanning a ~11 kb interval in the middle of the large *Ubx* transcription unit. Most of them are caused by mobile genetic elements. Hemizygous *bx* flies are viable and display characteristic homeotic phenotypes (Lewis, 1954; Peifer and Bender, 1986). Thereby, the anterior part of T3 is transformed towards the anterior part of T2. In adult flies carrying strong *bx* alleles, this translates into several prominent morphological changes. The most obvious are:

- the anterior part of the haltere acquires wing identity.
- the almost inexistent dorsal tissue of T3 is transformed into a copy of the T2 notum, referred to as metanotum.
- the hypopleural plate just above the T3 leg is replaced by the sternopleural plate of T2 with its characteristic sternopleural bristles.

bx-Basel was picked up from a cross yielding *S110501{w+}/TM3, Sb bx-34e* progeny. A few of these flies showed phenotypes reminiscent to those described above, suggesting that the *S110501{w+}* chromosome could carry a mutation somewhere in *Ubx*. A balanced stock was established with a chromosome that had lost the *mini-white* marker associated with *S110501{w+}* by meiotic recombination. Homozygous *bx-Basel* flies emerging from this stock are viable and can be kept as a stock. Based on its phenotypes, *bx-Basel* can be classified as a strong *bx* allele. Homozygotes show clear dorsal T3 to metanotum, hypopleural to sternopleural plate, and haltere to wing transformations (see Figures 1A, C, and E). The notum and haltere phenotypes are significantly enhanced in hemizygous flies (see Figures 1B, D, and F), indicating that *bx-Basel* is not a null for *bx*-activity.

To map *bx-Basel* and compare its position relative to other *bx* alleles, a series of overlapping PCR primer pairs for the *bx* region were designed. A discontinuity could be detected within one primer pair, which failed to amplify the expected PCR product. The discontinuity within this interval was further narrowed down with a second set of PCR primers (information on diagnostic PCR primer pairs for all *bx* alleles used in this study is presented in Table 1). Then, inverse PCR was applied to obtain sequence information for both ends of the expected insertion. BLAST searches with both sequences identified the same genomic break point and revealed that *bx-Basel* is associated with an insertion of a *gypsy* retrotransposon that locates within the 6982 bp interval defined by the *gypsy* alleles *bx-34e* and *bx-3* (Freund and Meselson, 1984; this study; see Figures 2B and C). Only one other *gypsy* allele resides within these ~7 kb: *bx-83Ka*, one of the strongest *bx* alleles known (Peifer and Bender, 1986). It had been noted that some of the classical *bx* alleles were re-isolates of other *bx* alleles (Peifer and Bender, 1986). In order to rule out the possibility that *bx-Basel* is a re-isolate of *bx-83Ka*, we determined the insertion site of *bx-83Ka*. It maps 316 bp proximal to *bx-Basel* (see Figures 2B and C).

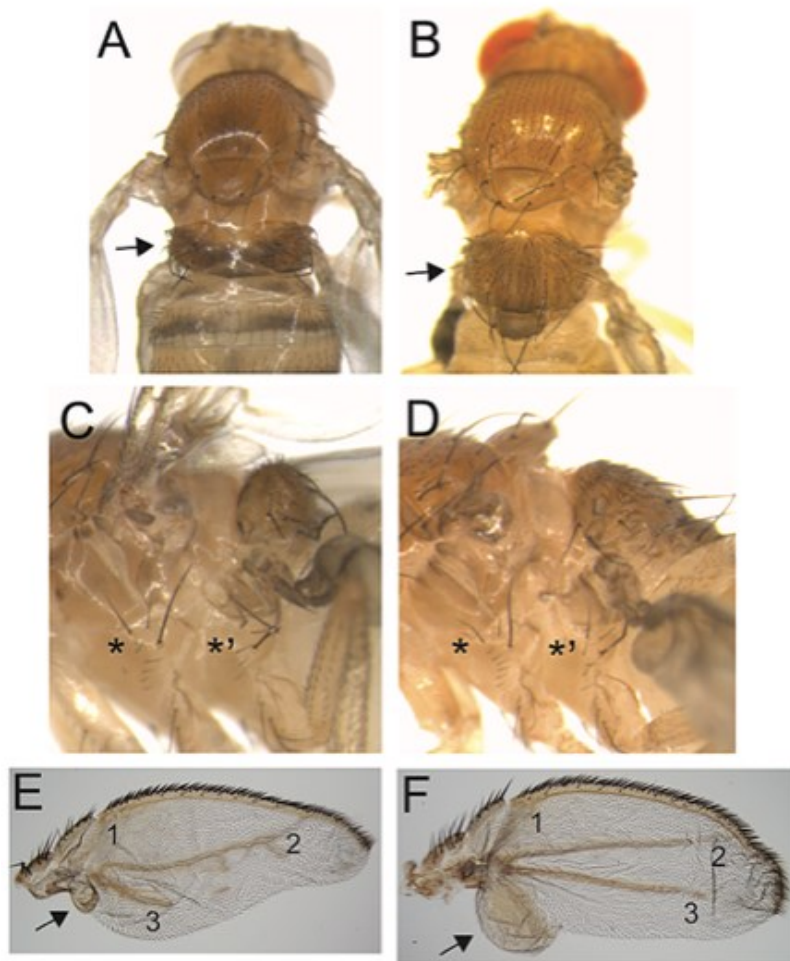


Figure 1. Prominent phenotypes in homo- and hemizygous *bx-Basel* flies. (A, C, F) homozygous *bx-Basel*. (B, D, F) *bx-Basel/Df(3R)P10*. *Df(3R)P10* deletes all relevant DNA for *Ubx* function (Karch *et al.*, 1985). (A, B) Dorsal view of adult females. The arrow points to the prominent T3 to metanotum transformation. Note that it is more complete in hemizygotes. (C, D) Lateral view of adult females. * indicates the sternopleural plate in T2 while *' labels the hypopleural to sternopleural plate transformation just above the T3 leg. (E, F) The haltere to wing transformation is depicted. Note that the anterior part of the haltere acquires anterior wing fate. It can easily be recognized by the characteristic anterior margin bristles. 1, 2, and 3 label wing veins one to three located in the anterior wing compartment. The homeotic transformation is more complete in hemizygous flies. The arrow points to the posterior haltere compartment.

Over the last 30 years, the mutagenic agent within *gypsy* has been extensively studied (Geyer *et al.*, 1986, 1988; Peifer and Bender, 1986, 1988; Dorsett, 1993). It could be pinpointed to its ~370 bp Su(Hw) protein binding region (Spana *et al.*, 1988). This DNA fragment acts as an enhancer blocker. When inserted between an enhancer and its promoter, it interferes with transcriptional activation (Geyer and Corces, 1992; Hogga *et al.*, 2001). While acquiring sequence information for the four *bx* alleles shown in Figure 2, we realized that there was some heterogeneity within the Su(Hw)-binding region located near the 5' end of the *gypsy* retrotransposon. It had been previously noted that compared to an intact *gypsy* element, *bx-34e* carried a 109 bp deletion, which removes 4 of the 12 Su(Hw) consensus binding sites (Peifer and Bender, 1988; this study). While *bx-3* carries an intact Su(Hw)-binding region, we find that the two other *bx* alleles are also associated with lesions within this region (see Figure 2D). *bx-83Ka* carries a 27 bp deletion that removes one of the Su(Hw) consensus sites. *bx-Basel* contains 2 deletions: the first is 27 bp in size and locates right next to the one found in *bx-83Ka*; the other, 82 bp in size, lies 83 bp more 3'. Altogether, this allele is left with only 8 Su(Hw) consensus sites. Previous studies have suggested that fewer consensus sites correlate with reduced phenotypic consequences (Hagstrom *et al.*, 1996). However, we note that *bx-83Ka* and *bx-Basel* are at least as strong as *bx-3*, probably even stronger with respect to the T3 to metanotum transformation.

Searches for enhancer-like elements within the *bx* region have identified a ~500 bp fragment (referred to as BRE (*bx* region enhancer)). Within an 8.8 kb fragment originating from the *bx* region, BRE is the only sub-fragment, which can mediate transcriptional activation of a LacZ-reporter gene (see Figure 2B; Qian *et al.*, 1991, 1993; Starr *et al.*, 2011). This feature classifies it as an initiator element, similar to those identified within each PS-specific control element of the *Abd-B* part of the bithorax complex (reviewed in Maeda and Karch, 2006, 2015). Initiator elements integrate positional information deposited by the early acting segmentation genes and mediate appropriate activation of PS-specific cis-regulatory modules in time and

space. Curiously, the BRE initiator localizes just a bit distal to *bx-34e* and three other *gypsy*-induced alleles. Hence, their enhancer blocker module is not located in between the BRE and the *Ubx* promoter and, therefore, based on the “enhancer blocker dogma”, these *gypsy*-based alleles shouldn’t cause a *bx* phenotype.

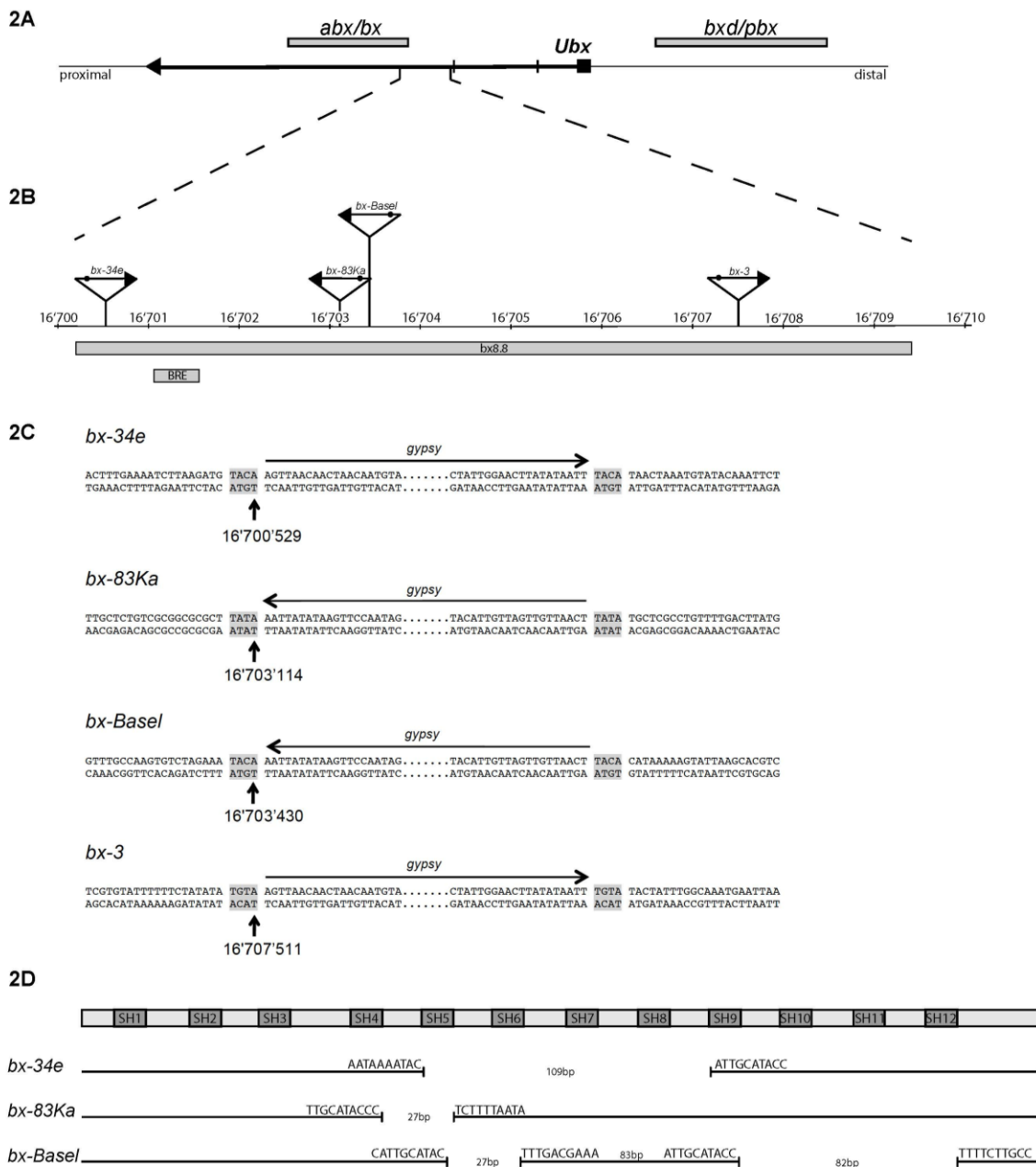


Figure 2. Molecular characterization of *bx-Basel*. (A) Diagrammatic representation of the *Ubx* locus. It spans roughly 120 kbs and is transcribed from distal to proximal relative to the centromere. The thick black line represents the longest transcript. The two grey bars above represent the approximate extent of the *abx/bx* and *bxd/pbx* *cis*-regulatory regions. (B) Zoom-in of *bx* region indicating the relative positions (triangles) and orientations (arrows) of four *gypsy* insertions. The black dot at the 5’ end of *gypsy* represents the location of the Su(Hw) binding region. Exact position and orientation of *bx-34e* and *bx-3* have previously been analyzed by Freund and Meselson (1984). Orientation of *bx-83Ka* was first determined by Peifer and Bender (1986). (continued next page)

Figure 2 (cont.). Our study confirms all previous mappings and adds the exact position of *bx-83Ka* and *bx-Basel*. At the bottom of the panel, the extent of two fragments tested in LacZ-reporter assays are indicated: the 8.8 kb *bx* fragment (*bx8.8*) and the ~500 bp BRE initiator (Qian *et al.*, 1991, 1993). (C) Sequences at the proximal and distal insertion breaks are shown. The 4-bp target site duplication is marked with a grey box. The position of the last base proximal to *gypsy* is indicated below the sequence according to Genome Release R6.23. (D) At the top of the panel, the grey bar represents the 363 bp DNA fragment within *gypsy* containing 12 consensus binding sites for the Su(Hw) protein (darker grey boxes numbered SH1 to 12). The 12 consensus sites were indicated according to Spana *et al.* (1988). Below, the extent of the deletions associated with *bx-34e*, *bx-83Ka* and *bx-Basel* are indicated together with 10 bases flanking each break point. The internal deletion in *bx-34e* has previously been described (Peifer and Bender, 1988). Stocks used in this study were obtained from the following sources: *Ubx^{bx-34e}/TMI* (Bloomington #3437); *Ubx^{bx-83Ka}/TMI* (Kyoto #101-577); *Ubx^{bx-3}/T(2;3)ap^{Xa}* (Bloomington #3419); *Df(3R)P10/TMI* (François Karch, University of Geneva, Switzerland).

Table 1. Diagnostic primer pairs for *bx* alleles.

<i>bx</i> allele	breakpoint	primer pair	fragment size
<i>bx-34e</i>	proximal	bx-34e proximal Gypsy 5' out	1486bp
	distal	bx-34e distal Gypsy 3' out	874bp
<i>bx-83Ka</i>	proximal	bx-83Ka proximal Gypsy 3' out	1369bp
	distal	bx-83Ka distal Gypsy 5' out	1425bp
<i>bx-Basel</i>	proximal	bx-Basel proximal Gypsy 3' out	944bp
	distal	bx-Basel distal Gypsy 5' out	1226bp
<i>bx-3</i>	proximal	bx-3 proximal Gypsy 5' out	1199bp
	distal	bx-3 distal Gypsy 3' out	901bp
		primer name	primer sequence
		Gypsy 3' out	5'-GTATACCTCTGCTACACCGG-3'
		Gypsy 5' out	5'-CTCATTGGTTGTTGGTTGGC-3'
		bx-34e proximal	5'-TCTCGCCATTCTCCGTTCTCC-3'
		bx-34e distal	5'-CCTGCTAAATAAACAGCGACC-3'
		bx-83Ka proximal	5'-GTTTCTTACCCTTATTCGGC-3'
		bx-83Ka distal	5'-ATTCTACATAAATCGCGCAGC-3'
		bx-Basel proximal	5'-CTCACTCTCTCGTTTCTCGC-3'
		bx-Basel distal	5'-AAACATGCGACTATTGCAGC-3'
		bx-3 proximal	5'-AGAGAATGGATCTTGTTTCAGCG-3'
		bx-3 distal	5'-GCAAATTAGTTAATTGGCGGCC-3'

Arguments to accommodate the available phenotypic data and explain the apparent dilemma could go as follows:

- *gypsy* insertions proximal to BRE by and large give rise to weaker phenotypes than those located more distally. It seems possible that this is because they fail to block BRE-*Ubx* promoter interactions.

- PS-specific initiator elements like BRE are thought to activate other tissue-specific enhancers located in its vicinity. Qian *et al.* (1993) have noted that DNA elements within the 8.8 kb *bx* fragment and flanking BRE not only boost reporter gene transcription but that they also lead to its expression in other germ layers. These changes might well be explained by the presence of as yet unmapped tissue-specific enhancers. In fact, instances of homeotic mutations in which the relevant PS-specific initiator is left intact have been reported (Iampietro *et al.*, 2010). Hence, it is conceivable that further cis-regulatory elements also exist proximal to BRE and, in particular, proximal to the *gypsy* alleles in that region. Blocking of those enhancers could have milder phenotypic consequences.

- Finally, all *gypsy*-based *bx* alleles should affect the interaction between the more proximal *abx* enhancers and the *Ubx* promoter (Simon *et al.*, 1990). The phenotypes of *abx* alleles are similar to those of *bx* (Lindsley and Zimm, 1992). The phenotype of *gypsy*-based *bx* alleles located proximal to BRE might, therefore, be a composite of stronger *abx* and weaker *bx* contributions.

In summary, our study characterizes the new *gypsy*-induced *bx-Basel* allele. We present an accurate and comprehensive map of the *bx* region, which indicates the position of *bx-Basel* relative to three classical *bx* alleles and the BRE initiator element. It will serve as a stepping stone for more detailed studies on the *bx* region.

Acknowledgments: We are indebted to the Kyoto Stock Center (DGRC) at Kyoto Institute of Technology and the Bloomington Drosophila Stock Center (NIH P40OD018537) for faithfully maintaining many of the classical *bx*-alleles. We thank François Karch for stocks and critically reading of the manuscript.

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Erratum: Manzano-Winkler, B., 2015, Suppressed double crossovers in *D. pseudoobscura* inversion heterozygotes. *Dros. Inf. Serv.* 98: 57-59.

I erroneously reported “Marker C” as being located on *Drosophila pseudoobscura* Chromosome 3, base position 9.1 Megabase when it is actually located at base position 2.06 Megabase on Chromosome 3 of the genome assembly (Gramates *et al.*, 2017). This location lies outside the ST-PP inversion focus of this study (Fuller *et al.*, 2017) and is, therefore, not informative for this research. The other three marker locations have been confirmed (FlyBase BLAST version FB2018_02, released Apr 3, 2018): Marker A (base position 4.59 Mb), Marker B (position 8.67 Mb), and Marker D (position 9.19 Mb) are all located within the ~12 Megabase ST-PP inversion. Despite the error, and removal of Marker C data, there is no change to the conclusions of this study, as the genotype data from the other three markers still confirm suppression of double crossovers by the chromosomal inversion.

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Teaching Notes



Identification of the phenotype and genotype of an unknown dominant X-linked female sterile mutation in *Drosophila melanogaster*.

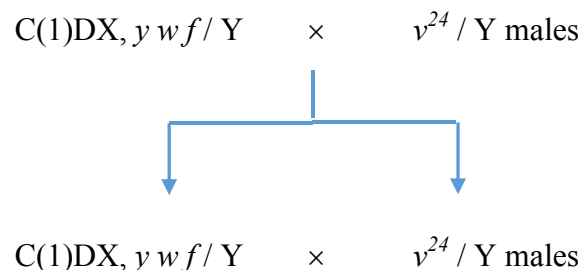
Schwartz, Kayla Christina, Griffith M. Saunders, Cameron Drew Friedman, Nathaniel P. Locke, Rachel Ann Crowl, Michael A. Balinski, and R.C. Woodruff. Department of

Biological Sciences, Bowling Green State University, Bowling Green, Ohio 43403.

In this teaching exercise, undergraduate students (Schwartz, Saunders, Friedman, Locke, and Crowl) were given a stock of C(1)DX, *y w f* females and *ovd^{D1} v²⁴* males (Indiana University stock number 1309) and were asked to determine the phenotype and genotype of the unknown *ovd^{D1}* dominant female sterile mutation.

First, students were asked to describe the phenotype of the above unknown stock. They quickly noticed that females had yellow-colored bodies and wings, white eyes, and small bristles. They were informed that these phenotypes were due to the recessive mutations yellow (*y*), white (*w*), and forked (*f*). Using *The Genome of Drosophila melanogaster* by Lindsley and Zimm (1992) and the *Atlas of Drosophila Morphology* by Chyb and Gompel (2013), they determined that these three mutations were recessive and sex-linked (located on the X chromosome). In a comparison to wild-type (Canton-S) flies, they also noticed that males of the unknown stock had bright red eyes, especially as young adults. They were told that this mutation was vermilion (*v²⁴*), and they determined from the literature and FlyBase (flybase.org) that it was also sex-linked and recessive. They did not understand, however, how over generations the *y*, *w* and *f* mutations and phenotypes stayed in females, while *v²⁴* stayed in males.

The students were then asked to read page 657 of *Drosophila: A Laboratory Handbook* by Ashburner (1989), which describes the C(1)DX compound chromosome. This chromosome has two X chromosomes attached to a single centromere and in the unknown stock carries the *y*, *w* and *f* mutations on each X chromosome. After some discussion, the students were told that the females also carried a Y chromosome (C(1)DX, *y w f* / Y) and that XXY flies are females and XY flies are males, according to *D. melanogaster* sex determination principles. Putting this information together, the students understood that the unknown stock was:



Subsequent generations would show the same pattern of inheritance, producing females that were always *y*, *w* and *f* (had yellow bodies and wings, white eyes, and forked bristles) and males that were always *v²⁴* (had bright red eyes).

With an understanding of the genetics and phenotypes of the stock (except for the still unknown *ovo^{D1}*), the students were asked to mate Canton-S (CS) virgin females with *v²⁴* males (as shown below), then mate the F1 offspring females (*v²⁴ / CS*), as virgins, with Canton-S males from a laboratory stock, and finally, mate the F1 Canton-S males with virgin C(1)DX, *y f* females from a laboratory stock.

the scalloped (*sd*) wing mutation (Polaczyk *et al.*, 1998; Dworkin *et al.*, 2009; Chari and Dworkin, 2013; Chandler *et al.*, 2017). Genetic backgrounds are also known to influence the expression of more complex genetic elements, including the dominance of transposable-DNA-element insertion mutant alleles in *D. melanogaster* (Guio and Gonzalez, 2015), the stage of lethality of a mutation in the epidermal growth factor receptor of mice (Threadgill *et al.*, 1995), and the expression of mutant genes that cause diseases in humans (Chow, 2016; Kammenga, 2017).

The dominant second chromosome *sna^{Sco}* mutation, which is called *Sco*, reduces the number of the four scutellar bristles on the thorax of *D. melanogaster* (Lindsley and Zimm, 1992; Chyb and Gompel, 2013). In this study it is our hypothesis that different genetic backgrounds can lead to significant differences in the expression of the *Sco* mutation in *D. melanogaster*, modifying the number of bristles on the thorax.

To test this hypothesis, we compared the number of bristles in *Sco* flies with different genetic backgrounds. First, we counted the number of scutellar bristles in two stocks that contained the *Sco* mutation (*Sco*/CyO: Indiana University stock number 2555 and *Sco*/CyO;TM6/*Sb*: Indiana University stock number 2551). We observed that the distribution of bristle numbers was significantly different ($P < 0.0001$) for the two stocks, as shown in Table 1 and Figure 1.

Table 1. Number of Scutellar Bristles.

Stock	Zero Scutellar Bristles	One Scutellar Bristles	Two Scutellar Bristles
<i>Sco</i> /CyO	299	168	33
<i>Sco</i> /CyO; TM6/ <i>Sb</i>	156	247	145

$P < 0.0001$

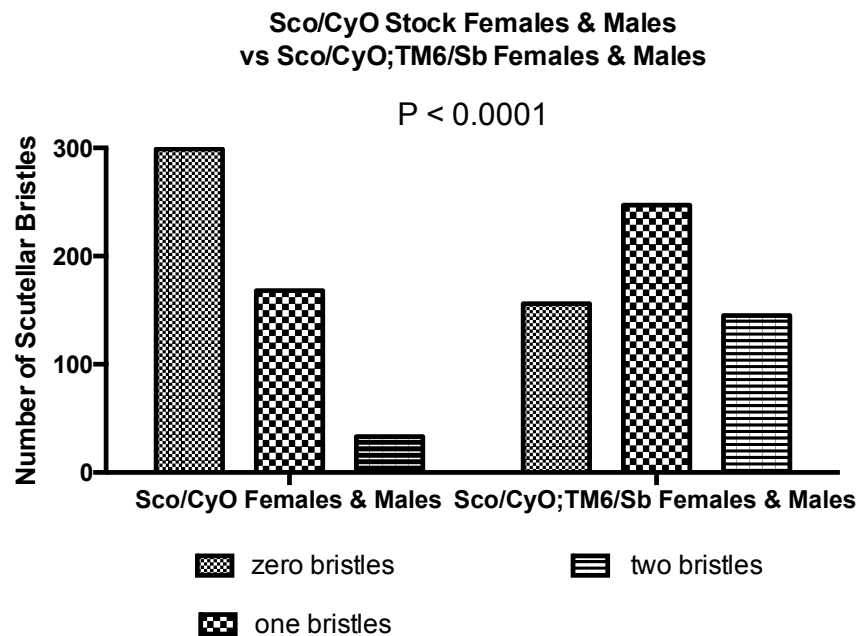


Figure 1. Number of scutellar bristles in *Sco*/CyO and *Sco*/CyO;TM6/*Sb* flies.

In our second comparison, we counted the number of bristles in the *Sco*/CyO stock and compared it with the progeny of the *Sco*/CyO stock that had been outcrossed with the Canton-S, wild-type, stock (*Sco*; CS).

We observed that the distribution of bristle numbers was significantly different ($P < 0.0001$) between the two stocks, as shown in Table 2 and Figure 2.

Table 2. Number of Scutellar Bristles.

Stock	Zero Scutellar Bristles	One Scutellar Bristles	Two Scutellar Bristles
<i>Sco/CyO</i>	299	168	33
<i>Sco; CS</i>	266	59	3

$P < 0.0001$

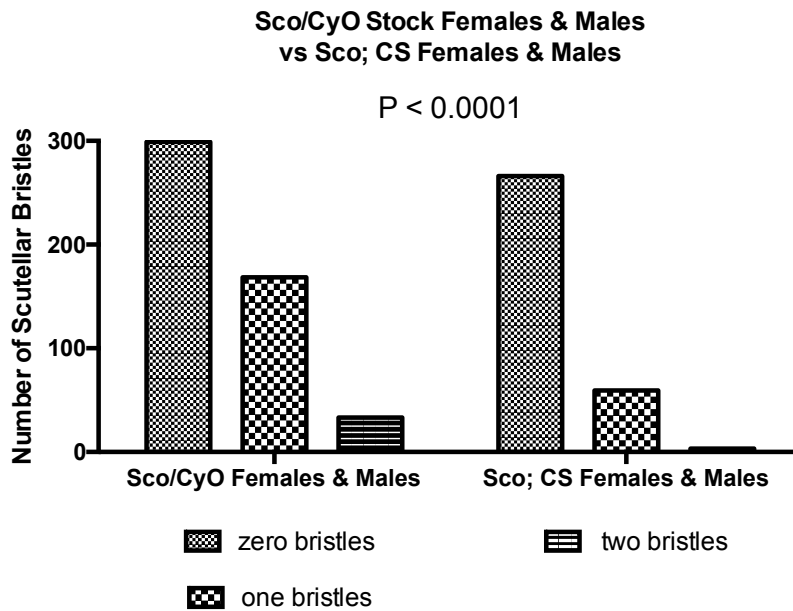


Figure 2. Number of scutellar bristles in *Sco/CyO* flies and in *Sco;CS* flies from an outcross of the *Sco/CyO* stock and the Canton-S wild-type stock.

In the final comparison, we counted the number of bristles in the *Sco/CyO* stock and compared it to the *Sco/CyO* stock that had been outcrossed with a Perrysburg, Ohio (*Per*) wild-type stock (*Sco;Per*). We observed that the distribution of bristle numbers was significantly different ($P < 0.0001$) between the two stocks, as shown in Table 3 and Figure 3.

Table 3. Number of Scutellar Bristles.

Stock	Zero Scutellar Bristles	One Scutellar Bristles	Two Scutellar Bristles
<i>Sco/CyO</i>	299	168	33
<i>Sco; Per</i>	300	35	5

$P < 0.0001$

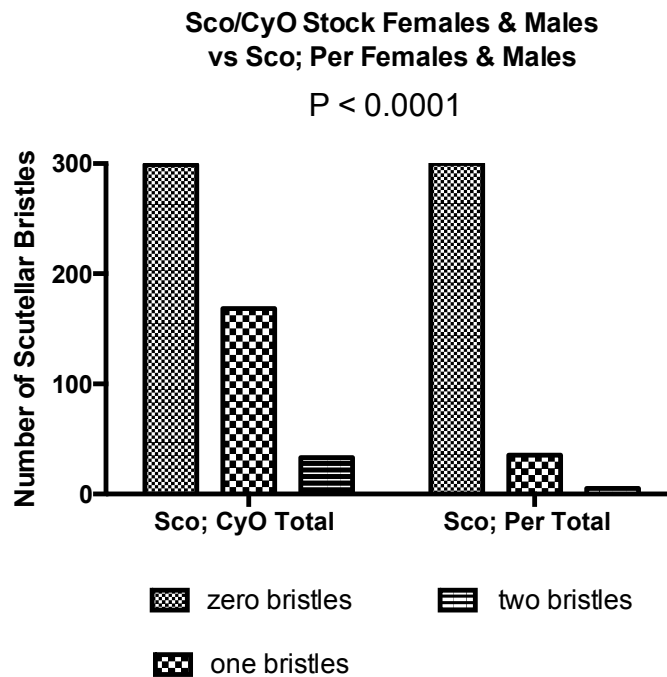


Figure 3. Number of scutellar bristles in *Sco*/CyO flies and in *Sco*;Per flies from an outcross of the *Sco*/CyO stock with a Perrysburg, Ohio wild-type stock (Per).

In summary, the genetic background has a significant influence on expression of the *Sco* mutation in *D. melanogaster*, with each experiment showing significantly different *Sco* bristle expression patterns. These results have important implications, as they support the observation that mutant genes are not islands that act alone, but interact with other genes to give the final mutant phenotype. These results could be important for the expression of mutant genes in all organisms, including those that cause diseases in humans, such as cystic fibrosis, breast and ovarian cancer, and retinal degeneration (Chow, 2016; Kammenga, 2017).

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Hands-on *Drosophila* genetics for primary school children (9-12 year olds).

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Curiosity, motivation, critical thinking, and initiative are some of the skills that should be promoted by science education in primary schools. The project described in this paper has the aim to approach scientific

research and methods to primary school children. This was achieved by inviting a group of students to visit a University science center where they had the opportunity to take part in some activities in the laboratory.

1. Introduction

An important part of science education in schools is to stimulate curiosity and scientific interest in students. Practical classes, field activities, and school trips provide good opportunities for students to learn in a more dynamic and entertaining way than they usually do in an everyday lesson, enriching their education by promoting basic but essential scientific skills such as initiative, motivation, and critical thinking.

The purpose of this work was to approach scientific research to primary school education by inviting a group of students from 9 to 12 years old to visit the facilities of the Department of Genetics at the Faculty of Biology of the University of Barcelona and participate in some activities related to genetics and developmental biology.

In these activities, students had the opportunity to learn some basics about *Drosophila melanogaster*, one of the main model organisms used by research groups in the department.

2. Taking a close look at *Drosophila melanogaster*

Drosophila melanogaster is one of the main model organisms used in developmental biology and genetics, for all its advantages. It has a relatively short life-cycle and females lay eggs at a very high rate, so large amounts of embryos can be obtained to carry out different experimental approaches (Jennings, 2011). Moreover, its genome is completely sequenced, numerous genetic modification techniques are available, and there is a very significant degree of evolutionary conservation between *Drosophila* and humans, which makes the fly a very useful tool for the study of the pathogenic mechanisms of diseases (Ugur *et al.*, 2016).

To introduce the fruit fly, the students were first shown some images and received a brief explanation of its morphological and biological characteristics. Afterwards, in a laboratory, they learned how to use the equipment for working with *Drosophila* and were encouraged to use it themselves to observe living flies.

2.1 Laboratory set-up for *Drosophila* work

We first introduced the equipment required for working with *Drosophila*:

- Stereomicroscope and light source: to observe flies at low magnification.
- Porous pad connected to CO₂: CO₂ that comes out of the pad anesthetizes the flies preventing them to fly away, still keeping them alive.
- Paint brush: to gently manipulate flies when anesthetized.
- Fly morgue (RIP): a bottle containing ethanol to discard flies.



Figure 1. Equipment for working with *Drosophila*.

2.2 Life cycle of *Drosophila*

We then gave them a short presentation with interactive slides to explain the life-cycle. Fruit flies, as all holometabolous insects, undergo a four-stage life cycle: egg, larva, pupa, and adult (Jennings, 2011). After fertilization, the embryo develops for 22-24 hours and then turns into a first instar larva. After one day, it develops into a second instar larva, and after another day it turns

into a third instar larva. This larva develops for approximately 30 hours and then turns into a pupa, which undergoes a metamorphosis process for about 4 days, and after this time, the adult fly emerges. So, the whole process from embryo to mature adult takes about 10 days (at 25°C) (Tyler, 2000).

Afterwards, students were given plates with embryos, larvae, and pupae so they could observe and identify the different stages of the *Drosophila* life cycle.

2.3 Differentiation between males and females

We then explained that many experimental approaches with fruit flies, such as genetic crossings, require distinguishing between male and female flies. In this activity, students learned the differences between males and females by observing basic morphological characteristics, such as size, sexual organs, body shape, or pigmentation.



Figure 2. Lateral view of a male (left) and a female (right).

2.4 Observation of mutants

One of the main advantages of working with *Drosophila* as a model organism is its genetic tractability and the many tools available for its genetic modification (Ugur *et al.*,

2016). As a result, numerous mutant stocks of flies are at one's disposal.

In this activity, students observed five different mutant flies:

- Curly: flies present curled wings
- White: flies present white eyes
- Ebony: flies present a darker body color than the wild type
- Eyes absent: flies have no eyes
- Vestigial: flies with very small wings

When looking at them for the first time, students were not told which characteristic was affected in each mutant. They were asked to compare them to wild type flies and try to identify the differences. We set this up as a quiz, which increased motivation and allowed for a more active participation.

Acknowledgments: We would like to thank the Department of Genetics, Microbiology and Statistics of the Faculty of Biology for letting us use one of the labs to carry out the activities. We also thank all the children for their active participation.

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Printed copies of *Drosophila* Information Service
are available from *lulu.com*

Mather, 1939; Grell, 1966, 1978; Grell and Chandley, 1965; Ashburner, 1989). It should be pointed out that we did not previously observe a significant increase in recombination in *D. melanogaster* raised at 30°C (Dollard *et al.*, 2016).

The frequency of recombination for the *w* and *sn*³ interval was compared between the control crosses and the crosses treated with high temperature or NaCl by using the chi-square test (Whitlock and Schluter, 2009). The expected frequency of recombination between the *white* locus and the *singed* locus in untreated flies is 19.5 percent (Lindsley and Zimm, 1992).

It is our hypothesis that high temperature (32°C) and sodium chloride will increase the frequency of recombination in *D. melanogaster*, suggesting that organisms can respond to an environmental temperature or chemical stressor and produce new genetic variation by recombination. In fact, we observed that recombination frequencies between the *white* gene and the *singed* gene were not significantly changed with exposure to high temperature ($P = 0.50$) or to sodium chloride ($P = 0.72$) (see Figures 1 and 2).

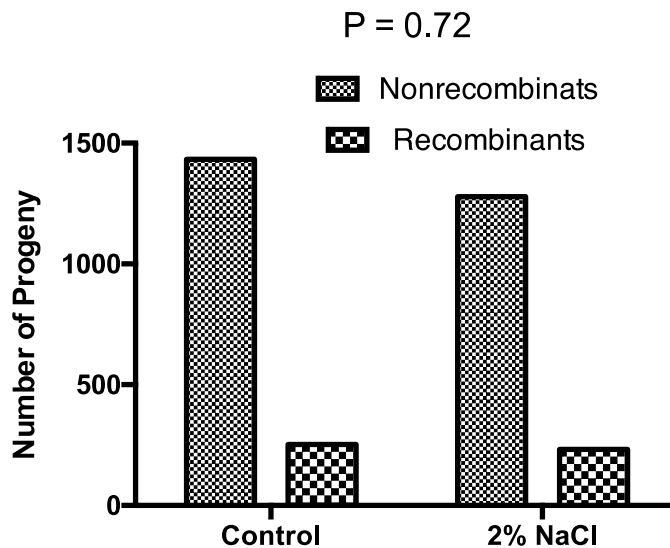


Figure 1. Frequencies of recombination in flies raised at room temperature (21°C to 23°C) compared to flies raised at 32°C.

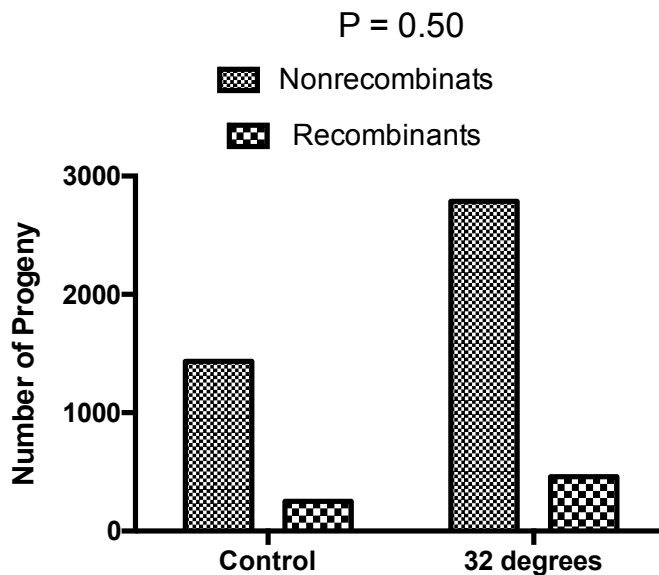


Figure 2. Frequencies of recombination in flies raised on normal *Drosophila* food compared to flies raised in food supplemented with two percent sodium chloride.

The frequency of recombination in the control crosses was 250 recombinants vs. 1433 nonrecombinants ($250/1683 = 14.85\%$), in the 32°C temperature was 459 recombinants vs. 2787 nonrecombinants ($459/3246 = 14.14\%$), and in the two percent NaCl treatment was 231 recombinants vs. 1278 nonrecombinants ($231/1509 = 15.31\%$).

These nonsignificant results were not expected, because the high temperature caused a marked decrease in F2 adult size and the NaCl treatment caused an extended development time for the F2 flies, both indications of stress.

It would be of interest to raise the two parental stocks (w^{sn^3} and Canton-S) for multiple generations on two percent sodium chloride and to then test for an increase in recombination using the methodology of this study. Multiple generations may give the stocks time to evolve resistance to two percent sodium chloride in their diet due to new genetic variation from increased recombination.

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A practical class using *Drosophila melanogaster* as a didactic model for teaching sexual selection.

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Introduction

In a globalized and dynamic society, information is available via different sources. Then, educators are in constant need of developing didactic alternatives that enable their students effective learning conditions. In this context, the investigative aspect of practical classes constitute a powerful resource in the classroom, thus, increasing the probability of participation of all students. This participation eventually leads to the exercise of sociability, creativity, use of the scientific method, and development of student's self-esteem and self-confidence (Martins, 2002). Additionally, teachers can overcome archaic pedagogical practices and offer to the students a chance to learn neglected or misinterpreted concepts such as Biological Evolution (BE) (Tidon and Lewontin, 2004).

Teaching BE has been an arduous task, because of its high level of abstraction, controversies, and misconceptions by both students and teachers on the subject. Another complicating factor is the inadequacy of materials and didactic strategies available on the matter (Tidon and Lewontin, 2004). Thus, many students and teachers do not understand the science behind BE.

In BE, sexual selection is considered one important evolutionary mechanism. It implies that organisms with mating success produce more offspring in future generations promoting changes in the population. Although conceptually identical to natural selection, it arises from differences in mating success, whereas natural selection is due to variance in all other fitness components. As any evolutionary mechanism, its comprehension is still problematic among teachers, which, consequently, results in the dissemination of conceptual errors (Tidon and Lewontin, 2004). However, the development of strategies to facilitate the proper learning of this important concept is crucial.

Drosophila melanogaster has been used as a model in research for nearly one century and can also be a powerful didactic tool (Intra and Pasini, 2016). This is because of the fly's short life cycle, abundant offspring, ease of collection and handling, small size and low maintenance costs in laboratories. Such characteristics enable didactic experiments throughout the school year. Moreover, these insects are especially

useful to BE because, among other reasons, the phenomenon of sexual selection is easily observed and well documented (Ewing and Bennet-Clark, 1968, Tompkins *et al.*, 1983).

Individuals of *Drosophila melanogaster* exhibit sexual dimorphism. Additionally, males perform a variety of mating behaviors that include touching and rubbing the legs and licking or circulating around the females. They also produce a specific mating sound due to the specific vibration of one of their wings before attempting to mount and copulate with the female. If the mating ritual has been properly executed, females will open their wings and thereby allow copulation. Otherwise, they will implement a rejection behavior through kicks, flight to other places, and raising or lowering the abdomen (Ewing and Bennet-Clark, 1968, Tompkins *et al.*, 1983). Therefore, *D. melanogaster* allows teachers to establish a connection between the abstract concepts of their disciplines and reality.

In the educational context, *Drosophila melanogaster* has been an effective didactic tool in genetics (Sepel and Loreto, 2010), but there is no knowledge of its practical application in BE. Then, this study developed a practical class based on *D. melanogaster* as a tool to teach BE by sexual selection, as well as contribute to avoiding conceptual errors related to this neglected matter. The practical class consists of the following materials and procedures.

Proposed practical class

For the preparation of the practical class, vials containing culture medium previously prepared with biological yeast, agar, nipagin, and edible dye, in addition to sterilized cotton, will be used. For manipulation and analysis of the flies, tweezers, paintbrushes, masks, glasses, gloves, ethyl ether, pens, notebooks, and manual magnifiers will also be required.

The time of practice development and the adequacy of the suggested materials are at the discretion of the instructor. However, for didactic purposes, we suggest the division of the class into four steps (described below) to be developed in two sessions of 50 minutes each.

STEP 1 – Division of the students into groups according to material availability;

STEP 2 – Establishment of the artificial population of *Drosophila melanogaster* (one female and five males) from existing stocks. Initially, the insects should be anesthetized with ethyl ether and divided into six groups. Females should be isolated in a standard culture medium. The males, in turn, should be divided into five groups. Each of these groups should be kept in a culture medium specifically colored with edible aniline (red, black, blue, pink or green) previously prepared (Figure 1A and B). With this procedure, it is intended to color the abdomen of the males while they feed for 20 minutes (Figure 1C and D). Such mark will allow students to control the copula pattern during observations. After this interval, an artificial population composed of one female and one male of each colored culture medium must be established. For this, the insects should be reanesthetized and placed in a terrarium without culture medium.

STEP 3 – Once all flies have awakened, students should begin observations for 20 minutes and simultaneously record the copulation pattern. At this point, the objective of each group is to produce quantitative data to characterize their *Drosophila melanogaster* as "under sexual selection" (mating occurs between the female and only one male) or "without sexual selection" (mating occurs randomly);

STEP 4 – Analysis of data obtained by all groups for joint discussion. Teacher and students should discuss the causal mechanisms of the observed pattern, as well as possible improvements of the practice.

During the observations, students should verify if males of *Drosophila melanogaster* perform some mating behavior that includes, among others:

- A. Males rub their legs, lick or touch the females, or circulate around them;
- B. Males vibrate their wings before copulating with the female;
- C. Females open their wings before mating;
- D. Females reject males by kicking, flying and raising or lowering the abdomen.

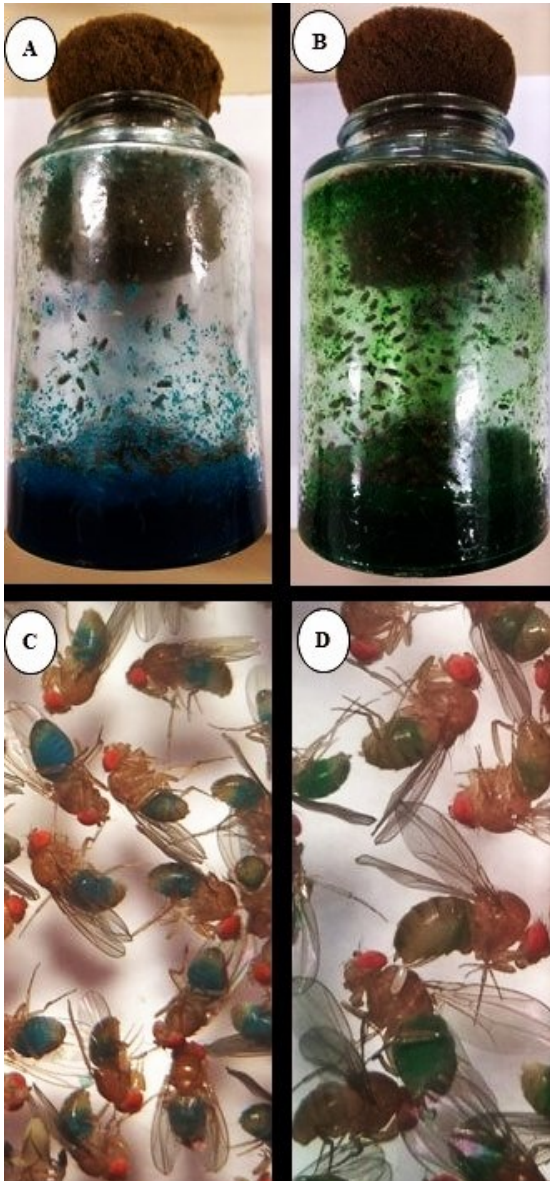


Figure 1. Colored culture medium with edible aniline (A and B) and pigmented flies in culture medium (C and D).

Table 1. Matings and total encounters among individuals of *Drosophila melanogaster*.

Matings	Total encounters
Female and blue male	
Female and green male	
Female and black male	
Female and red male	
Female and pink male	
Others (up to two males)	
Others (up to three males)	
No matings	
Promiscuity	

In the case of mating, students should record in the table below the total mating to describe the reproductive pattern of individuals within their artificial population. With these data, students should discuss the existence or non-existence of sexual selection and, consequently, natural selection, and its evolutionary implications.

Conclusions

Although the effectiveness of the practice proposed needs to be measured, it is concluded that actively teaching BE may be the first step to avoiding misconceptions on the topic among teachers and students. Therefore, it is recommended to use the developed practical class to avoid conceptual errors related to BE.

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Questions and reflections on H.J. Muller's Nobel Prize winning research.

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Priority in scientific discovery traditionally has been determined by dates of article submission, therefore many journals provide an article's submission date at the time of publication. Often a submitted paper requires revisions and the date of publication may be a year or so after the first date of submission. On occasion, a new discovery might be first presented and recognized by an abstract given at a conference; the full details later presented in a longer and more detailed publication elsewhere. Today one might also provide the date of first online appearance prior to the official date of publication.

Recent publications by Calabrese (2018a, c), questioning the review process, priority and results for H. J. Muller's 1946 Nobel Prize winning research, "discovery of the production of mutations by means of X-ray irradiation," has inspired this communication to the *DIS*.

Calabrese's article (2018a) questioning the review process was mentioned as in press, in a paper published in October 2018 (Calabrese 2018b) attempting to show how Muller's research findings had been misinterpreted thus leading to the wrong evaluation of the Linear No-threshold Theory (LNT) of radiation exposure for cancer risk assessment originated due to (1) a critical mistake by Muller that he had discovered X-ray induced "gene" mutation (see also Calabrese 2011, 2017a-d, critical analysis regarding Muller and LNT).

In this report I attempt to provide a different view from Calabrese by I) reminding readers of Muller's early publications and announcements that led to his 1946 Nobel Prize award for the discovery of X-radiation effects and induced mutations, and bringing the reader's attention to the limitations of the Nobel award process; II) briefly discussing the process of publications and review during the early years of the 21st century; and III) offering commentary on how the definition of mutation has changed over time, and citing a few reports showing that Muller's hypothesis that X-rays lead to "point mutations" may have been documented and his thoughts on low-dose threshold remain controversial.

Ia. Muller's 1927/1928 announcements on X-radiation and mutation

On July 22, 1927, Hermann J. Muller, then at the University of Texas, published in the journal *Science* a brief four page paper titled "X-ray Transmutation of the Gene" (Muller 1927a; Muller 1962: 245-251). Not quite two months later, on 15 September at the 5th International Congress of Genetics (5th ICG) in Berlin (11-18 Sept.), as an invited speaker (Anonymous 1927a), Muller presented his data as evidence, and his new discovery was immediately heralded around the world (Serebrovsky 1927, 11 Sept.; Anonymous 1927b, 16 Sept.; Gates 1927, 1 Oct.). Muller's extensive report, including methods and data tables, was published the following year (March 1928) in the 5th ICG *Proceedings* (Muller 1928b; Muller 1962: 252-276). Curt Stern (1974: 29) recalled that Muller arrived in Berlin with only a very rough draft of his congress paper, which was typed up only after he arrived. Stern also recalled that he made the diagrams of the crosses that were presented by Muller at the Congress (Stern was in charge of photographs and "episcopic projections" for presentations; Nachtsheim 1928, p. 8). Yet others besides Muller also brought only "drafts" of their papers, for which editor Hans Nachtsheim (1928) offered grateful thanks in his forward (*Vorwort*) to the published Congress *Proceedings*. Contributors had been requested to submit their manuscripts "to the Secretary, Dr. H. Nachtsheim, before the opening of the Congress"; the ultimate date for receipt of manuscripts was September 17 (see "The Fifth International Genetics Congress" 1927, p. 210). Each lecturer was asked also to "prepare separately a short abstract of his paper and hand it to the Secretary before or at the opening of the Congress."

Hans Nachtsheim, was more than qualified to review drafts and edit the *Proceedings* for the 5th ICG. He was affiliated with Erwin Baur's Institute for Heredity at the Agricultural College in Berlin (Nachtsheim 1928b), and had spent the academic year 1926-1927 in T. H. Morgan's lab at Columbia University on a

fellowship from the Rockefeller Foundation (Deichmann 1996, p. 232). Stern too was well qualified to review Muller's paper after spending two years (1924-1926), in Morgan's lab learning *Drosophila* genetics (Stern 1974). We can expect that they would have had no difficulties accepting, rejecting, or suggesting revisions to Muller's draft manuscript.

Additionally, Nachtsheim (1921) had earlier published a German translation of Morgan's (1919) *Physical Basis of Heredity* (Deichmann 1996 p. 231). [Morgan had sent a copy signed by him inscribed to R. A. Emerson, Chair of Plant Breeding at Cornell; Dr. R. P. Murphy and I sent the inscribed copy to the Cornell Archives for their history of science collection.]

One might inquire why Lewis Stadler, then at the University of Missouri, did not attend the 1927 International Congress of Genetics. He was scheduled to present a paper (Anonymous 1927a), but his name does not appear on the list of attendees in the 5th ICG *Proceedings* (Nachtsheim 1928). Had he been there and given a paper would his discovery of X-rays and mutation in barley and maize, simultaneously and independently of Muller, have been remembered and noted by a Nobel committee 19 years later?

Before Muller's paper appeared in the 1928 Congress proceedings, however, he gave a presentation and published an abstract on his X-ray work at the American Association for the Advancement of Science (AAAS) meeting, in the Joint Genetics Section of the American Society of Zoologists and The Botanical Society of America in Nashville, TN, on Wed, 28 Dec 1927 (Muller 1927b) [This is actually Muller's 3rd presentation on X-ray findings during 1927; First in July, *Science*; second at ICG Berlin, September 1927, and subsequently published in March 1928].

For his contribution "read" at the 1927 Nashville meeting, Muller was awarded the annual prize of the AAAS for his paper titled, "Effects of X-irradiation on Genes and Chromosomes," read before the Joint Genetics Sections and announced in the January 27th 1928 issue of *Science* (Livingston 1928a). Muller's more detailed invited abstract, dated January 8, 1928, was published in the same *Science* issue to accompany the announcement of the Annual AAAS Prize, the fifth to be awarded by the American Association for the Advancement of Science (see Muller 1928a). I quote details of the award here:

"The American Association prize of \$1,000 is awarded annually to the author of a notable contribution to the advancement of science given at the annual meeting. The funds are generously supplied by a member who wishes his name withheld. Nominations for the Nashville prize were received from the secretaries of the sections and societies and the award was made by the committee on prize award and announced through the news service Friday evening.

The winner of the prize is this year Dr. H. J. Muller, professor of zoology in the University of Texas, for his outstanding contribution entitled "Effects of X-Radiation on Genes and Chromosomes," which was presented before the Joint Genetics Section of the American Society of Zoologists and the Botanical Society of America, in the Wednesday-morning session. The following abstract of Dr. Muller's paper has been contributed by him, having been sent from Austin, Texas, by air mail" (Livingston, B. E. 1928a: 81-82).

Muller's "more detailed" abstract immediately follows this announcement. It is reprinted in Muller (1962: 276-277, 593) with the date January 8, 1928 at the bottom of the abstract opposite his full name, exactly as it appeared in *Science*.

Names of the awarding committee are listed below Muller's 1928 *Science* abstract; Two were members of the National Academy of Sciences [NAS], William Duane Prof. of Biophysics, Harvard [NAS elected 1920], and Charles Schuchert, Professor of Paleontology, Yale University [NAS elected 1910]. Three other committee members were Robert J. Terry (Chair) Professor of Anatomy, Washington University, St. Louis; L. J. Cole, Professor of Genetics, University of Wisconsin, and G. Canby Robinson Professor of Paleontology, Yale University, New Haven, Connecticut.

Three months later, at the end of April 1928, Muller (1928c) presented his research to a meeting of the National Academy of Sciences in Washington, DC. His 12 page paper, including references, was titled, "The Production of Mutations by X-Rays." It provided the details of the report for which he won the AAAS award in Nashville, in addition to more recent results, and was published five months later in the Academy *Proceedings*.

Since Muller was not yet a member of the NAS, his paper had first to be reviewed by members of the Academy (see Part II, below), which may have delayed immediate publication. I could find no documentation of who invited Muller to present his work to the NAS, but it may have been T. H. Morgan (NAS elected 1909), with whom Muller studied for his Ph.D. between 1912 and 1915 (degree awarded 1916) at Columbia University (*Catalogue 1916-1917*, p. 281). Or possibly, one of the NAS members, who was on the committee that awarded the AAAS Prize at the 1927 Nashville meeting, had offered the invitation.

Muller's (1928c, p.722) *PNAS* report clearly recognized that he, in addition to others, had been conducting research on the "effectiveness of X-rays in producing both gene mutation and chromosome reorganization in *Drosophila*." L. J. Stadler, in particular was lauded for similar studies in plants:

"Stadler, working on barley and maize, has conclusively demonstrated, by means of ingenious methods, both gene mutations and chromosome aberrations ... to be produced in plants by x-rays. It should be stated, in this connection, that his work was carried on simultaneously with and independently of that of the present writer" (Muller 1928c, p.722).

Muller lists publications by Stadler, and others, to support his accounts, although he does not cite specific papers within this text by author and date, as one commonly does in scientific publications today. Only two of three Stadler publications, listed by Muller, can be found in the literature (1928a, *PNAS* Vol. 14 [Jan., submitted Nov. 1927], and 1928b, *Science* Vol. 68 [August]). One paper that Muller misdated as "Stadler, L. J. 1928a" and titled "Genetic Effects of X-Rays in Maize and Barley," is reported as an "address to Genetics Section, A.A.A.S., Dec., 1927; title in *Anat. Rec.*, 37 [December], 176" [see Stadler unpublished abstract, 1927]. Muller also misdated his own abstract and address [presentation] as "1928a", for his contribution published for that same Dec. 1927 meeting (see Muller 1927b) – the meeting in which he was awarded the AAAS annual prize (see above).

I searched all the titles and abstracts in *The Anatomical Record* Vol. 37, December 1927, and found Muller's abstract, #134, on page 174 (see Muller 1927c). But I could neither locate a title, nor an abstract for Stadler on page 176 (or elsewhere), as listed by Muller in his 1928 *PNAS* paper. I began to think that Muller might be mistaken, when my colleague from University of Missouri brought to my attention that Stadler (1928b) began his August *Science* article on mutation in barley by reminding readers of his contribution at the AAAS meeting in Nashville [Dec. of 1927]: "At the Nashville meeting of the American Association last December I reported the occurrence of mutations in barley following X-ray treatment." And that Marcus Rhoades (1956) had also reported on Stadler's participation at that event. Stadler (1928b) cited no abstract for his participation at the AAAS meeting, but his presentation is mentioned in a National Academy memoir by Rhoades (1957). No abstract dated December 1927, however, accompanies the list of publications enumerated by Rhoades. Confirmation of Stadler's presentation, however, can be found in the report of the Secretary for the Joint Genetics Sections held at the AAAS meetings in Nashville, December 1927, and published in *Science*, February 3, 1928 (Dunn 1928, p. 125; Livingston 1928b). The report summarizes papers given at the Joint Genetics Sections at the Nashville meeting of the AAAS, and mentioned the contributions by both Muller and Stadler:

"Forty contributions were offered, twenty-four of them being read at the formal sessions –Five of the papers read, one by demonstrations, and one of the papers given by title only, dealt with the effect of X-rays on plants and animals. Chief interest centered in the recent attempts to alter the course of inheritance and the frequency of mutation by treatment with X-rays. The most extensive experiments on the question were reported in detail by H. J. Muller, whose paper (for which the American Association Prize was awarded this year) is abstracted in the section on the Prize. By use of a special technique for measuring the frequency of mutations in *Drosophila melanogaster* he obtained results indicating that the application of sublethal doses of X-rays to sperm was followed by a large increase in the mutation rate of treated as compared to control, cultures. The mutation rate in some treated cultures was estimated at 15,000 times the normal rate. ... From the botanical side L. J. Stadler reported on the occurrence of new endosperm characters that apparently had arisen by

mutation in maize ears X-rayed at the time of fertilization. The same investigator presented evidence for the occurrence of mutations in seedlings from treated barley seeds.”

Secretary Dunn’s report certainly confirms both Stadler’s recollection and Muller’s citing [misdated] of Stadler’s presentation at the AAAS meeting in December 1927. Also of note is Dunn’s final entry on the election of officers to the Joint Genetics Section for 1928; H. J. Muller was elected Chairman [succeeding R. A. Emerson].

Worth mentioning is that Stadler announced his X-ray results, not unlike Muller, as (1) a contributed paper to the Joint Genetics Section of the AAAS in December 1927, (2) a publication in *PNAS* (Jan. 1928, submitted in Nov. 1927), and (3) a paper in *Science* (August 1928). The argument made by Calabrese (2018a) regarding Muller publishing in these venues to avoid peer review could also be applied to Stadler (and others). But as I will demonstrate below (Part II), Muller’s reports on X-rays and mutation *were* reviewed by his peers, and his first announcement was the appropriate venue for the time.

Continuing with this topic through 1928, both Stadler and Muller & Altenburg presented papers and abstracts in the Joint Genetics Section, AAAS meeting held in New York City, 28 Dec. 1928 (*The Anatomical Record* 1928: 88, 97, 100). The session was chaired by Muller and included Stadler (1928c, abstract #12, p. 88) who reported that the “rate of mutation ... varies in direct proportion to the intensity of irradiation.” Muller & Altenburg (1928, abstract #19, p. 100) reported that “frequency of translocations... produced by X-rays... was found to arise with nearly the frequency of detectable gene mutations.”

Clearly they were both continuing to study “gene” mutation and chromosomal rearrangements induced by X-rays.

Ib. Limitations of the Nobel Prize awards process

Many would agree that both Muller and Stadler should have shared the Nobel Prize for the “discovery of the production of mutations by means of X-ray irradiation.” Note that the term mutation has changed over time (see below). But many people who should have shared the prize have not—it is influenced by many factors and in some cases this award is quite political, as was shown by Istvan Hargittai in his 2002 book titled *Road to Stockholm*. The decisions are dependent on the nominations (and nominators—see Hargittai 2002, p. 22, for the six groups who may nominate for physiology or medicine prize), and the members of the Swedish Academy who review them. Hargittai (2002 chaps. 3 & 12) elucidates why some received the prize and others have not. He explains that the review process is a considerable task that is bound to produce mistakes, since the committees are not and have not been the most informed on the weight of discoveries in subfields in which they may have no extensive experience. They very much rely on the information submitted by the nominators.

Although Hargittai makes no mention of Stadler with respect to Muller’s unshared 1946 Nobel Prize, we know that Stadler was using X-rays for studies of plant mutations as early as 1926 (or earlier), as was Muller in *Drosophila*, and Muller gave him credit for such in his 1928c *PNAS* article *and* years later in his Nobel Prize Lecture (Muller 1946):

“And Stadler, in his great work on the production of mutations in cereals, started independently of our own, has obtained evidence that in this material X-radiation in the doses used is unable to produce a sensible rise in the gene mutation frequency, though numerous chromosome breakages do arise, leading to both gross and minute rearrangements of chromosome parts. Either the genes are more resistant in this material to permanent changes by X-rays, as compared with their responsiveness to thermal agitation, or a break or loss must usually be produced by X-rays along with the gene change.”

Clearly here Muller was recalling Stadler’s later studies on X-rays inducing chromosomal rearrangements, and not his earliest results, seemingly similar to Muller’s own, on gene mutation.

Muller's understanding of a gene was technically a genetic locus and his point mutation was a place on that locus. Carlson (1991), Muller's former student and his biographer, explained that the definition of the gene [and by extension a gene mutation] was an evolving concept (see part IIIa. below).

Since in 1946 no one really knew what a gene was at that time, and since Muller (1928c) also included translocations, inversions etc. in the "production of mutations by X-rays," the sharing of the prize with Stadler might have been appropriate.

Another view of the politics of the Nobel Prize is offered in a book by Friedman (2001). Even though it focuses on the prizes in chemistry and physics, it depicts how the members of the committees make decisions on who gets a Nobel Prize, and how many of those judgements included political, national, self-interest, and resentful, negotiated, and closed-minded agendas. It shows extensive interpersonal clashes between committee members and how downplaying of important nominees and their works was a frequent practice.

The files on Nobel Prize winners are closed for 50 years (Tønnesson 1999; Hargittai 2002, p. 16), and Muller's would have been available to researchers in 2006. Perhaps answers to some of these procedures clarifying the committee's decision for an unshared prize in Physiology or Medicine for 1946 will be found therein?

II. Process of publications and review during the early years of the 21st century

Publishing in *Science*—speedy publication:

It has been argued (Calabrese 2018a) that Muller circumvented the review process in order to claim priority for his discovery of mutations induced by X-irradiation. As mentioned above, Muller (1928c) promptly and directly credited Stadler for simultaneously and independently conducting similar studies in plants.

Brief announcements of new discoveries were routinely published in *Science* and in *Nature* with an expectation that details would be forthcoming elsewhere. An historical view of this protocol is well presented by Baldwin (2014, 2015) in a paper published by the *Royal Society of London*.

Baldwin (2014) elucidates that, "many of the most influential texts in the history of science were never put through the peer review process, including Isaac Newton's 1687 *Principia Mathematica*, Albert Einstein's 1905 paper on relativity, and James Watson and Francis Crick's 1953 *Nature* paper on the structure of DNA." And academic journals trusted ... "Prominent scientists on their editorial boards to make decisions about which papers to print. The term "peer review" she informs, originated after World War II. Using examples from *Nature*, Baldwin explains that the scientific weekly *Nature*, did not consult referees for every paper it printed until 1973.

Specifically, she uses the example of Watson and Crick's 1953 paper, announcing the structure of DNA and for which they ultimately won a Nobel Prize. Their paper, she explains (Baldwin 2015), serves as a useful illustration of two important features of publications in *Nature*: first, its reputation for relatively speedy publication, and second, the extent to which the editors relied on prominent scientists, particularly British ones, to recommend content. *Nature* was "known as a venue for the fast publication of new results in the early twentieth century," and both Watson and Crick wanted a note that could be published quickly.

I believe that in the United States, an analogy could be made for the journal *Science*. By examining the list of papers provided in the *Biographical Memoirs* of the National Academy of Science, one can see new and exciting "firsts" published in *Science* by its membership. For examples, in addition to Muller and Stadler, one will find papers published in *Science* by Morgan and by McClintock to quickly announce new and exciting research during the 1920s and earlier (see Sturtevant 1959, and Kass 2013). Furthermore, only one month after Watson and Crick had announced their DNA structure in *Nature*, Stanley Miller—then a graduate student of 1934 Nobel Laureate Harold Urey—announced in a two page technical paper in *Science* his famous experiment producing organic molecules in an atmosphere replicating that of primitive Earth (Miller 1953, Bada & Lazcano 2007). Arguably, *Science* had continued to be the best venue in the United States to announce new discoveries.

Publishing in *PNAS*—guidelines for review:

As I mentioned above, Muller's presentation to the 5th ICG in Berlin was in all probability reviewed and edited by Hans Nachtsheim (1928). Furthermore, as a nonmember of the Academy, Muller's 1928c *PNAS* paper, presented in April and published in September *required* review, as per NAS Constitution and Bylaws (Cochrane 1978a):

“OF SCIENTIFIC COMMUNICATIONS, PUBLICATIONS AND REPORTS

XV. Papers from persons not members, read before the Academy, Classes or Sections, and intended for publication, shall be referred at the meeting at which they are read, to a Committee of members competent to judge whether the paper is worthy of publication. Such Committees shall report to the Academy as early as practicable, and not later than the next stated session. If they do not then report, they shall be discharged, and the paper referred to another Committee” (Cochrane 1978b, p. 612).

Muller's (1928c) paper was not published immediately following his presentation. Most likely it had been reviewed by Morgan, who became NAS President in 1927, among others of the NAS section to which his paper was presented in April of 1928.

Similarly, Stadler's (1928a) *PNAS* paper, published in January, had been communicated to the NAS the previous November (1927), most likely by E. M. East (NAS elected 1925), and also would have required review. Stadler, who worked with East as a National Research Council (NRC) Fellow at Harvard during 1925-1926, was not a NAS member (until elected in 1938). Emerson, too, likely reviewed Stadler's (1928, Jan.) paper, and perhaps Muller's as well, being elected to the NAS in 1927. And perhaps, as Chair, Emerson had invited Stadler to present his X-ray work at the Joint Genetics Section in Nashville. (It is not clear why Stadler's abstract was not included in their records). Emerson was well acquainted with Stadler's research, as the latter did part of his NRC fellowship in Emerson's department at Cornell during 1925-1926 (Kass 2005; Synapsis Records 1925-1926, Cornell Archives; Rèdei 1971: 6).

Coe and Kass (2005) reminded readers that *PNAS* was a forum to get new results published quickly and was not supposed to include all details of the investigations. Articles were originally limited to 6 pages and occasionally went over this limit when funds were available; the editors wanted some evidence of how the work was obtained but specifically said that elaborate tables and graphs and the description of details should not be permitted, but exceptions could be made. The editor explained that around 1925-1926, a special grant became available to enlarge the size of the *Proceedings* and take some articles in excess of 6 pages provided they were still short. By 1933 the funds had been spent and they had to return to the old rule of the *Proceedings* to limit articles to 6 pages (which continues today). Indeed, Creighton and McClintock's famous report on crossing over in maize was broken up into two papers (McClintock 1931, Creighton and McClintock 1931) following sequentially in the journal, and issued as one reprint (Coe & Kass 2005; Kass 2013-, pp. 1.123-1.145).

Stadler's (1928a) *PNAS* paper was limited to 6 pages, but Muller's (1928c) paper was twice that and possibly required special permission for the excess pages (No tables or graphs are included, but he did list a paper he submitted in October of 1927, and published in the July issue of *Genetics*, volume 13, 1928—known at the time to be a refereed journal, and then published bi-monthly).

Papers by nonmembers of the NAS, who published in *PNAS*, nevertheless, were definitely reviewed prior to their publication, as per NAS Constitution and By-laws.

IIIa. Definitions of genes and mutation have changed over time

Calabrese's (2018a) argument that Muller had not found that X-rays affect point-mutation seems unrealistic when one did not know what a point-mutation was in 1946.

Carlson (1991) provides a summary and excellent chronological table showing how the term gene and gene mutation has changed over time. Overlooked by Carlson, but credited in *The search for the gene*, published the following year by the *Drosophila* geneticist and NAS member Bruce Wallace (1992), are the contributions of Al Hershey and Martha Chase to the confirmation of the gene as DNA. These researchers

working in the Department of Genetics, Carnegie Institution of Washington, at Cold Spring Harbor, Long Island, New York, “are often credited with having performed the experiment that finally located the gene, thus ending the long search” (Wallace 1992: 115). This was of course seven years after Avery et al. in 1944, demonstrated that genes (the heredity material) were DNA. Max Delbrück, Alfred D. Hershey and Salvador E. Luria shared The Nobel Prize in Physiology or Medicine 1969 “for their discoveries concerning the replication mechanism and the genetic structure of viruses”; Oswald Avery, who died in 1955, was no longer eligible to be considered for the award (Hargittai 2002, p. 226).

Of course knowing that the hereditary material was DNA still did not permit knowledge of the physical limitation of the gene on the chromosome. DNA studies led to the knowledge that point mutations are changes in the sequence of DNA bases, and include substitutions, insertions, and deletions of one or more nucleic acid bases. Many of these were shown to be caused by chemical mutagenic agents, but ionizing radiation was also believed to play a part in such phenomena (Lehninger 1975, p. 881).

When Muller studied genes and mutations, “mutation” was understood to be a sudden, hereditary change in the genetic makeup of an organism. Simply defined, the term mutation can be of two types: gene mutations or point mutations, and chromosomal mutations (Gleason 2017; see also any modern textbook of Genetics). Gene mutations include local changes in the structure or composition of genes whereas chromosomal mutations or chromosomal aberrations involve large changes in the structure (e.g., inversions and translocations) or number of chromosomes.

In 1946, Muller’s contribution to understanding genes and mutations was limited by our knowledge of the gene at that time.

Modern technology has permitted more refinement of how structural and functional genes are studied (see below).

IIIb. Muller’s hypothesis that X-rays may lead to “point mutations”

After the initial work, Stadler continued studies of mutations expanding to work with induced and spontaneous mutations. Stadler eventually argued that X-rays were removing genes rather than changing genes. Stadler and Muller debated this issue (cordially) for some years.

As mentioned above, mutations can be defined as either point mutations or chromosomal mutations. Part of the argument in favor of X-rays causing point mutations was that reverse mutations could be caused by X-rays.

A popular textbook of the time classified mutations as changes in the chromosome and as changes in the composition of individual genes (Sturtevant & Beadle 1939, p. 206). By 1939, doubts raised that “x-rays do not induce gene mutations, but only cause breakage and reunion of chromosomes,” was believed to have been resolved, by producing reverse mutations (wildtype to mutant, and mutant back to wildtype) by X-rays in the *forked* locus in *Drosophila*. By citing work of drosophila geneticists (Muller, Patterson and Timofeeff-Ressofsky), Sturtevant and Beadle (1939, p. 215) reported that this result “leaves little doubt that actual gene changes are concerned” [but see my annotation for this volume]. This was still the prevailing belief when Muller was awarded his Nobel Prize in 1946 (see Sinnott, et al. 1950, p. 257, 291). [See also Srb et al. 1965, section on induced mutation, p. 244ff, for a consideration of ionizing radiation, and chromosomal vs. gene mutations.]

Years later, after insertions of transposable elements (transposons) were recognized to change the action of genes, and long after Barbara McClintock was awarded the 1983 unshared Nobel Prize “for her discovery of mobile genetic elements,” it was shown that the *forked* allele is due to insertion of *gypsy*, an LTR-retrotransposon (long terminal repeat transposon, Kuzin et al. 1994). This means that the reversion in *forked* could be due to a small deletion of part or the entire element, rather than a point mutation (J. Birchler, pers. com, 11 Dec. 2018).

More elaborate results on mutation frequency were show by Timofeeff-Ressovsky in the 1930’s with alleles of the *white* locus in *Drosophila*—presenting data of x-ray dosage with increased gene mutation at the *white* locus, his results showed that “different genes, even different alleles at a single locus, may have quite different mutation rates” (Sturtevant & Beadle 1939, pp. 215-217). Mel Green conducted X-ray reversion studies on alleles of *white*, *yellow* and *scute* (Green 1961), and also found some were capable of being reverted

by X-rays while others were not. There is a perfect correlation of those revertible alleles being retrotransposon insertions and those that were refractory to reversion, having no major restriction fragment alterations. This result is consistent with X-rays causing small deletions (of the retrotransposon to restore lost gene function).

More recent studies of the “white gene” in *Drosophila* (Mackenzie et al. 1999) apparently used an X-ray induced allele reported in 1960 w^{cf} , and a spontaneous mutation first reported by Bridges 1935 w^{sa} , to study partially pigmented eye color mutant strains of *D. melanogaster*. The study reports DNA sequence results on “the nature and location of the *point mutations* which identify functionally important regions of the *D. melanogaster* guanine and tryptophan ABC transporters.” The case of w^{cf} might be a bona fide case of a point mutation induced by X-rays.

Jim Birchler (U of Missouri, pers. com. 30 Nov. 2018) wrote me that he has worked with *white-coffee* (and *satsuma*) of the *white* locus. It could well be a point mutation generated by X-rays. However, he also noted he had recovered transposon induced mutations from [chemical mutagen] EMS treatments, and he noted ... it is always the case that, when you are looking for something, whatever you find may or may not be caused by the applied agent but instead be “spontaneous”. Indeed, the case of *satsuma* illustrates that “point mutations” can be spontaneous.

I fully concur with Jim’s comment, “Whether X-rays mainly delete genes or change genes or both does not detract from the fact that the ability to produce alterations of genes rather than relying on spontaneous mutations was a milestone in the history of the field of genetics.”

IIIc. Is Muller’s correlation of no threshold for cancer risk still controversial?

In a workshop on Radiation and Cancer, Muller’s early research on the danger of low-dose X-radiation was reviewed, along with the correlation that ionizing radiations may also have a carcinogenic potential. Citing the work of Calabrese (2011), which questions Muller’s low-dose radiation studies with respect to cancer, Magrini (2015) summarized epidemiologic studies in Japan on the effects in humans exposed to the atomic bomb. He stated, “It is not possible to distinguish the tumors possibly caused by radiation, morphologically, from “naturally” occurring cancers. ... The dose effect curve generally rises more steeply with high-LET [Linear Energy Transfer] radiation than with low-LET, especially at low dose rates (the reduction in radiation dose-response at high doses is consistent with a cell killing effect).”

Magrini explains that study of cancers caused by radiation exposure has been the “subject of a vast amount of scientific contributions ... Yet, the issue is largely an unresolved one ... and it is charged with a strong ‘emotional’ content. The consequences of under-or overestimating this problem may be equally dangerous and costly.”

Regarding the potential risks of “low radiation doses,” he continues, “... the theory holds that excess cancer risks related to low-dose radiation are directly proportional to the dose. Despite some controversy over the excess cancer risk of low-dose radiation, the linear no-threshold theory [LNT] is widely used because an alternative method for assessing the potential risks of low-dose radiation is lacking. Some [researchers] question the validity of the linear no-threshold theory and think that below a certain threshold carcinogenesis ceases to be a concern” [no citations given, but see Calabrese 2017a-d].

He cautions, “Although the estimated risks from low levels of radiation of a single CT (computer assisted tomography) exam are uncertain, it is prudent to minimize the dose from CT by applying common sense solutions and using other simple strategies as well as exploiting technologic innovations. These efforts will enable us to take advantage of all the clinical benefits of CT while minimizing the likelihood of harm to patients.”

Do recent studies of correlations of low dose radiation and cancer uphold Muller’s warnings?

The U.S. Department of Health and Human Services, National Institutes of Health, National Cancer Institute, has reported on a recent study demonstrating that low dose radiation has been linked to leukemia. Investigators in their Radiation Epidemiology Branch and colleagues from other institutions, led by senior investigator Mark Little, Ph.D. were able to quantify—for the first time—excess risk for leukemia and other myeloid malignancies following low-dose exposure to ionizing radiation in childhood. The findings were

published online July 16, 2018 in *Lancet Haematology* (<https://dceg.cancer.gov/news-events/research-news-highlights/2018/low-dose-rad-leukemia>).

A second report, published online August 27, 2018 in the *European Journal of Epidemiology*, identified a significant exposure-response between low-dose cumulative occupational radiation exposure to the eye lens and risk of cataracts, based on self-reported history (Little et al. 2018, August; <https://dceg.cancer.gov/news-events/research-news-highlights/2018/cataract>).

As emphasized by Haynes (1998) Muller's discovery sensitized him immediately to the health hazards of the careless or excessive use of diagnostic X-rays in medical practice, and later of radioactive fallout from nuclear weapons tests. It may have taken a long time to prove, but the studies cited above may help resolve the controversy regarding the dangers of low, in addition to, high dose exposure to X-rays over time.

CONCLUSIONS

The answer to Calabrese's (2018a) query, "Was Muller's 1946 Nobel Prize research for radiation-induced gene mutations peer reviewed?": 1) May not be an appropriate question to ask for the time period, but 2) Yes, as per the accounts and documents presented here.

Historical perspective demands that we interpret reviews of Muller's Noble prize publications in the context of the times, and not in light of current academic guidelines (see Kass 2003: 1255). Additionally, as geneticists and historian of science Bentley Glass (1990) reminded us, "Let us grant that personal emotions and failures of memory may obtrude in such documents" as testimonies written years after the events. "It is of course the historian's mission to check all conflicting evidence against other records, and so attempt to ferret out the truth." Reading the memories that accompany Glass's (1990) article, certainly demonstrates that recollections must be documented by contemporaneous records in order for truth to emerge.

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“Lunch at Oskar’s 5/29/05,” A Reunion of Dobzhansky’s students, post-docs and friends.

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On October 28th 2015, Ross MacIntyre and I got together for a chat about Bruce Wallace’s former students. Ross suggested that I try to locate an audio tape recording that was made of memories by former students of T. Dobzhansky, who had been a Professor of Genetics at Columbia University. Their recollections were recorded at a reunion in New York City organized by Bruce Wallace. Even though only Ross remembered that the reunion discussions were recorded, and others were sure it was not, we now have documentation that the lunchtime reunion discussions were indeed recorded. The audio tape of the reunion made by Bruce Wallace, assisted by Ross MacIntyre, at Oscar’s restaurant, the Waldorf Astoria, in New York City, on May 29th 2005, has been found. Below is the story of its unearthing.

Bruce Wallace sent an audio tape to Dr. R.P. Murphy (Murph), former Professor at Cornell University, who apparently gave it to me, probably for the archives. Bruce knew that Murph and I had published on the history of Cornell's Plant Breeding Department (Murphy and Kass, 2007, 2011). And the two had been close friends since Murph, as Department Chair, had hired Bruce into his department at Cornell in 1958 (MacIntyre *et al.*, 2016). The envelope was post marked June 21, '05, and along with the tape were pages from the *Roanoke Times*, dated Sunday, July 10, 2005, pp. 1A-8A. Neither Murph nor I knew what this was about since there is no other information in the envelope with the tape and newspaper. I have no recollection of receiving this tape, but I recently found it in a file I kept on Dr. Murphy. The tiny Sony tape was in an envelope, which I had not previously looked at. The tape itself is only labeled by a small piece of paper "lunch at Oscars, 5/29/05", scotch-taped to the audio tape cassette box, so I did not know its significance until Ross suggested I try to find the reunion tape. I had no idea where or what Oscar's was, but I subsequently discovered it had been a restaurant in the Waldorf Astoria. Bruce's daughter Roberta Wallace recalled a reunion lunch at the Waldorf, and Lee Ehrman recalled the "Waldorf Astoria Café," and that the "parking was costly," so that helped me discover the lunch venue. Jeff Powell had an entry in his appointment book for a tentative Sunday, New York Lunch, May 29th 2005, but he did not recall what it was about.

Since neither Murph nor I had a tape recorder that could play this tiny tape, we never listened to it. A librarian at West Virginia University, Martin Dunlap, recently was able to transfer the tape into a digital recording, which I obtained on Thursday, September 20th 2018. I am most grateful to Martin for his help. The recordings are of two types and can be opened with Windows Media Player: 1) Wave sound format 708 MB and 2) MP3 format 40.3 MB.

The recording is difficult to hear because of background noise. Some people are easier to hear than others. The tape begins with Bruce toasting Doby, aka Dodik, Prof. Dobzhansky; Natasha (Doby's wife), Miriam (Wallace, who had passed in 2003), and to kind and generous fellowship. Lee Ehrman had recalled a toast to Miriam.

In addition to **Bruce Wallace**, who organized the reunion, other persons attending, who had been students of Dobzhansky, were **Lee Ehrman**, **Jeff Powell**, **David Weisbrot**, **Lou Levine** (The latter two, since deceased); Doby's post-doc **Chana Malogolowkin** (Chana Malogolowkin-Cohen). Because Chana was visiting NYC from Israel, Bruce thought it would be a good idea to get former Doby students together for a reunion. In addition were family or friends of Doby's students: **Richard Ehrman** (deceased; husband of Lee), **Roberta Wallace** (daughter of Bruce), **Reba Mirsky Goodman** (friend of Bruce, and a Columbia University graduate student), **Gabriela DeBeer** (emeritus Professor, Department of Classical and Modern Languages and Literatures, CUNY; wife of Lou Levine); and **Ross MacIntyre** (former post-doc of Bruce), who Bruce had asked to supervise the taping. Ross is now Emeritus Professor at Cornell. I was also invited to the reunion, but I don't recall why I declined the invitation.

Eleven people attended the reunion. Only Ross MacIntyre recalled that it had been taped. Four attendees that I had contacted via email had absolutely no recollection that they were being taped, and a few swore that it was not. Their other recollections, however, as mentioned above, also helped to confirm the tape. It is not clear if Bruce had intended for the tape recording to be discreet in order to permit recollections to flow freely, but it is clear that Bruce wished the event to be documented.

Copies of the tape can be obtained by contacting Professor Lee B. Kass, lbk7@cornell.edu.

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***Drosophila* for drug discovery: useful tool or wishful thinking?**

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Introduction

In the quest for new effective drugs there is the push for faster ways to identify active lead compounds to develop and validate in drug discovery pipelines. Cell culture-based screens are easy to run and can be both standardized and automated to perform efficient high-throughput screens. Combined with the large available compound libraries, they can be useful to identify leads. However, many of such leads have been found to fail in the subsequent validation steps, where system complexity increases, with many compounds revealing as toxic to a multicellular organism or unable to penetrate the target organs.

Flies are increasingly and successfully being used for pharmacological studies (Freires *et al.*, 2016) as demonstrated by the highly attended Fly Pharmacology workshop at the 2018 *Drosophila* Research Conference in Philadelphia earlier this year. In this crowded venue, it emerged that there appears to be a substantial bias against using flies for drug discovery, which is manifested in frequent proposal rejections and caustic comments from reviewers. Here I will forego extensive discussion of the worth of *Drosophila* studies, as this community is well-aware of the strength of this model. However, I want to reflect on some of the common concerns and share our experience in hopes to encourage the use of flies in drug discovery and ideally to start a discussion or reflections on this topic that may help to strengthen grant proposals and build strategies for the success of drug discovery research in *Drosophila*.

Whole-Animal Screens in Drug Discovery and Development

Whole-animal screens can efficiently reveal toxicity and can thus eliminate many false positives at an early stage, saving time and expenses, remaining aware that, depending on animal model, screening and protocol administration used for some active compounds may be missed because of improper absorption and permeability issues. Concerns have been raised regarding the real significance of the results in model organisms and how they may translate to humans. This is particularly true of the invertebrate disease models *Coenorhabditis elegans* and *Drosophila melanogaster* that have, however, demonstrated remarkable successes in pharmacological assays. Key to these achievements were the careful use of the disease model and proper definition of the experimental question, which are paramount in comparative and model organism studies. In fact, mammalian models, particularly mice where a large statistic is available, have yielded results that do not always translate to humans. Thus, warnings about overreliance on mice models have been voiced (Strange, 2016). Vertebrate models demand high maintenance costs ranging from about one thousand dollars per year for one mouse and 2500 dollars for a rat, to approximately 40,000 dollars for one monkey, and are subjected to ethical concerns. Moreover, both disease mechanism and drug's mechanism of action are often largely unknown and relatively intractable in mammalian models, reducing experimental power to little more than careful description of the processes. While important, descriptive knowledge scarcely contributes to mechanistic knowledge of the disease and drug action. In search for productive ways forward, *Drosophila* appears to offer key advantages as a drug discovery model. For example, *Drosophila* has excellent genetic conservation, the equivalent of most human organs (unlike *C. elegans*), and has sophisticated genetic tools that can be used for mechanistic studies. Both disease mechanism and drug mechanism of action can be probed in the fly. In comparison, zebrafish, a popular vertebrate model for pharmacology, has much less developed genetics and is aquatic, which may impact certain studies (*e.g.*, kidney pharmacology). *Drosophila*'s short life span and culture economy lends itself well to longevity studies and to monitoring the consequences of prolonged drug administration, potentially decreasing costs of the drug discovery pipeline that may become prohibitive in other animals. While long-term drug response monitoring in complex vertebrates could in principle be implemented by investing with bold funding programs, current high-throughput pharmacological screens often remain brute-force approaches with low success rates. Therefore, in the long term it seems

prudent to work towards improving efficiency of the drug pipeline, ideally by integrating data from interdisciplinary research on multiple systems to accelerate discovery. The resulting improved success rates would free up resources to tackle more diseases. Because these efforts have been embraced by the scientific community, there is the need for dialog and sharing thoughts and experiences to fully comprehend this new field with its obvious strengths and yet-to-be-defined limitations.

Common Approaches and Concerns for Fly-Centered Drug Discovery

Drug development requires structure-activity relationship studies, which involves experimental iterations to determine which positions in the molecule can be modified to optimize drug properties. For expedience, analytical amounts of synthetic compounds should ideally be used in this phase, which can be conveniently achieved in whole-animal models using flies.

Chronic disease appears to pose a particularly complex problem for pharmacology. Compared to forms of aggressive cancer, for example, chronic disease may take longer to become life-threatening and may require maintenance regimes with long-term drug administration. In these conditions, even slight toxicity may become a serious concern which reduces effective drug options, highlighting the need for long-term toxicological studies.

When using flies in drug discovery, one important concern regards possible laborious drug administration. The insect cuticle may present a barrier to administration of certain drugs, and microinjection has been used successfully in the quest for drugs targeting the nervous system. While conveniently done, microinjection is laborious and may limit high- and medium-throughput drug-screening efforts. Other forms of administration such as spraying are also possible and can be automated (Pandey and Nichols, 2011). Oral administration, on the other hand, can be easily and qualitatively monitored by mixing the drug with food and coloring agents, the ingestion of which can be seen through the semi-transparent cuticle of both adults and larvae. If deemed important, a fluorescent compound can be added instead and used as a proxy to quantify ingested amounts. It is often feared that flies will reject certain drugs, but that may not be a frequent occurrence. In our experience, we have administered 24 different compounds of four different chemical families including peptides, peptide derivatives, and different small-molecule drugs, used both alone and in combination. We never found an instance of drug rejection as determined with food-coloring-spiked mixes of food and drugs. Speaking with other colleagues, we can report anecdotally that drug ingestion seems to be a common occurrence, suggesting that drug rejection may be infrequent and possibly limited to some pungently smelling molecules.

It is likely that oral administration may require higher compound dosage compared to microinjection, due to in-animal drug processing and may even result in either inactivation of certain drugs (*e.g.*, unmodified peptides) or even drug uptake by the yeast in which the drug is often mixed prior to being fed to the flies. While remedial use of yeast extract instead of whole cells may be attempted in the latter case, the ease of oral administration and the small scale of fly-based drug assays make the use of possibly higher doses of compounds much more attractive than microinjection. We found that dose-response of rapamycin administration to a fly model of polycystic kidney disease indicated an effective dose of 12.5 μM , which is nine to ten times higher than those injected into mice models (Gamberi *et al.*, 2017). Albeit this particular proof-of-principle experiment only represents a single instance, we noticed that this concentration of orally-administered drug was within one order of magnitude of the doses injected into mice, which may optimistically be considered as suggestive of possible similar range of activity in the two systems, at least for rapamycin. Future investigations will likely provide more information to evaluate this possibility.

In our experience it has always been useful to perform dose-response assays in the fly to ensure drug activity. Precise drug dosage on the other hand is regarded as largely species-specific due to the exact aspects of physiology typical of each species. Thus, model organisms, including *Drosophila*, may occasionally guide drug dosage range, but are not considered valid guidelines *a priori*. In exciting new developments, flies have shown conservation of certain drug-binding sites (Ziehm *et al.*, 2017) and of toxicological pathways (Zhou *et al.*, 2017) corroborating the accumulating evidence for *Drosophila* being a *bona fide* model for drug discovery in which toxicological studies are also possible. Signs of activation of conserved toxicological pathways can be monitored over time via -omics approaches that can also simultaneously enable the basic study of how

cellular pathways respond and adapt to drug treatment, doubling the return on investment of these types of studies.

Conclusions

We are in the pioneering days for fly pharmacology in which healthy skepticism abounds, yet the accumulating evidence suggests that flies can be useful models in which to accelerate drug discovery, identify good-quality lead compounds, and ultimately provide indications of drug efficacy for specific conditions. The research frontline recognizes the need to invest into validating the use of invertebrate models in drug discovery. Studies of global responses to chemical treatments in different genetic backgrounds will help to precisely define the boundaries of what is possible to achieve using fly models combined with clever choices of experimental questions based on basic comparative knowledge. Considering the current trend of successes, fly pharmacology promises to be here to stay.

References: Freires, I.A., J. De Cassia Orlandi Sardi, and R. Dias De Castro 2016, *Pharm. Res.* 34(4): 681-686. doi: 10.1007/s11095-016-2069-z; Gamberi, C. (corr.), D.R. Hipfner, M. Trudel, and W.D. Lubell 2017, *PLoS Genet.* 13(4): e1006694. doi: 10.1371/journal.pgen.1006694; Pandey, U.B., and C.D. Nichols 2011, *Pharmacol. Rev.* 63: 411–436; Strange, K., 2016, *ILAR Journal* 57(2): 133–143; Zhou, S., S.E. Luoma, G.E. St Armour, E. Thakkar, T.F.C. Mackay, and R.R.H. Anholt 2017, *PLoS Genet.* 13(7): e1006907. doi: 10.1371/journal.pgen.1006907; Ziehm, M., S. Kaur, D.K. Ivanov, P.J. Ballester, D. Marcus, L. Partridge, and J.M. Thornton 2017, *Aging Cell*, doi: 10.1111/ace1.12626.

59th Annual *Drosophila* Research Conference

The 59th Annual *Drosophila* Research Conference was held on 11-15 April 2018 at the Philadelphia Marriott Downtown. The Conference Organizers were Tin Tin Su (Chair), Gio Bosco, Pamela Geyer, and Noah Whiteman. The conference was sponsored by The *Drosophila* Board in association with the Genetics Society of America, 9650 Rockville Pike, Bethesda, MD 20814-3998.

Opening Remarks

Tin Tin Su: Welcome
Debbie Andrew: State of the Fly Community
Lynn Cooley: GSA Awards
Kim McCall: Larry Sandler Award Presentation

Keynote Address

Terry Orr-Weaver: Research taking flight from foundational biology.

Plenary Lectures (in presentation order)

Tatsushi Igaki. Mechanisms and roles of tumor-suppressive cell competition.

Daniela Zarnescu. Lost in translation – RNA processing defects impact synaptic metabolism in neurodegeneration.

Anandasankar Ray. Mechanisms of odor-coding and its manipulation to alter behavior.

Cassandra Extavour. Reproductive capacity evolves in response to ecology through common developmental mechanisms.

Amir Yassin. The genomic basis of adaptation in *Drosophila*: sex, poison and other dramas.

Yashi Ahmed. The guts of Wnt signal transduction.

Michael O'Connor. Non-conventional autophagy in the prothoracic gland mediates a larval nutritional checkpoint through alteration of cholesterol trafficking.

Leonie Moyle. Sexual interactions and the evolution of species isolating barriers.

Benjamin Ohlstein. Regulation of stem cell number in the intestine.

Irene Chiolo. Highways for repair: nuclear actin filaments and myosins relocalize heterochromatic DNA breaks to the nuclear periphery.

Julien Royet. Effects of the gut microbiota on host behavior and homeostasis.

Chaoting Wu. Looking at chromosomes.

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. The three most recent Presidents continue participation on the Board as Past-President. In addition, the Board has *ex officio* members who represent *Drosophila* community resources or centers. For more information about the Board and the summaries of the annual Board meetings, see: the FlyBase web site.

***Drosophila* Board Membership 2017 - 2018**

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Susan Celniker (BDGP; Lawrence Berkeley National Laboratory, Berkeley)
Kevin Cook (Bloomington Stock Center & Nomenclature Committee; Indiana University)
Patrick O'Grady (Drosophila Species Stock Center; Cornell University)
Jim Thompson (Drosophila Information Service; University of Oklahoma)
Liz Perkins (Harvard TRiP; Harvard University)
Hugo Bellen (Bloomington Stock Center Advisory Committee & P Element Project; Baylor College of Medicine)
Allan Spradling (P-Element Project; HHMI/Carnegie Institute)
Stephanie Mohr (Harvard DRSC; Harvard University)
Scott Hawley (Nomenclature Committee; Stowers Institute for Medical Research)
Lisa Meadows (VDRC; Vienna, Austria)
Masanobu Itoh (DGRC, Kyoto; Kyoto, Japan)
Toshiyuki Takano-Shimizu (DGRC, Kyoto; Kyoto, Japan)
Chuck Langley (At-large; University of California, Davis)
Brian Oliver (FlyBase Advisory Board; NIH)

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