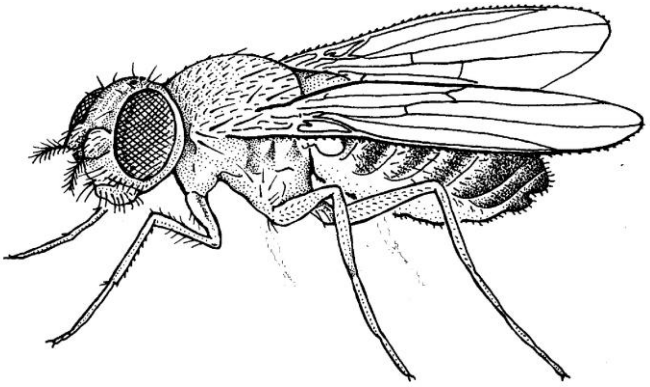
Drosophila Information Service



Number 96

December 2013

Prepared at the
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Norman, OK 73019 U.S.A.

Preface

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

The production of DIS volume 96 could not have been completed without the generous efforts of many people. Robbie Stinchcomb, Carol Baylor, and Clay Hallman maintained key records and helped distribute copies and respond to questions. Thanks to the efforts of Clay Hallman and Jenna Hellack, all issues, except the special issues that contained mutant and stock information now provided in detail by FlyBase and similar material in the annual volumes, are now freely-accessible from our web site: www.ou.edu/journals/dis.

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

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Research Notes

Persistence of an inversion in laboratory stocks of *Drosophila bipectinata.*

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In *Drosophila*, chromosomal polymorphism due to paracentric inversions is very common (Dobzhansky, 1951; Da Cunha, 1960; Sperlich and Pfriem, 1986; Singh, 1994, 1998; Singh and Singh, 2008). There are inter- and intraspecific variations in the degree and pattern of inversion polymorphism (Powell, 1997; Singh, 2001). Further, inversion polymorphism in *Drosophila* is an adaptive trait and is balanced owing to adaptive superiority of inversion heterozygotes. According to the ecological niche hypothesis of Dobzhansky and coworkers (Dobzhansky *et al.*, 1950; Da Cunha *et al*., 1950; Da Cunha and Dobzhansky, 1954), inversion polymorphism in *Drosophila* is a device to cope with the diversity of environments. A large number of *Drosophila* species have been investigated for inversion polymorphism (Singh, 1994, 2001; Powell, 1997). A number of *Drosophila* species found in India have also been studied for inversion polymorphism (Singh, 1998, 2001, 2013).

Table 1. Details of the laboratory stocks of *D. bipectinata.*

Mass culture stocks:		
Name of the stocks	Place of collection	Year of collection
Mysore (Mys-88)	Mysore (Karnataka)	1988
Alipurduar (AD-93)	Alipurduar (West Bengal)	1993
Siliguri (SL-93)	Siliguri (West Bengal)	1993
Trivandrum (TD-93)	Trivandrum (Kerala)	1993
Arumanai(AR-96)	Arumanai (Tamil Nadu)	1996
Pune (PN-99)	Pune (Maharashtra)	1999
Bodh Gaya (B.Gaya-02)	Bodh Gaya (Bihar)	2002
Raebareli (RB-03)	Raebareli (Uttar Pradesh)	2003
Navsori (NV-03)	Navsori (Gujarat)	2003
Nilgiri (NG-03)	Nilgiri (Tamil Nadu)	2003
Isofemale lines:		
Name of the stocks	Place of collection	Year of collection
Siliguri (SL-01)	Siliguri (West Bengal, India)	2001
Akola (Akl-12)	Akola (Maharashtra, India)	2012
IROT 8	Iriomote (Japan)	-
K-aaj 072 146	Nairobi (Africa)	-
K-aaj 078 174	Kola Kinabala (Malaysia)	-

The *Drosophila bipectinata* species complex is comprised of four closely related species: *D. bipectinata*, *D. parabipectinata*, *D. malerkotliana*, and *D. pseudoobscura*. Females of all the four species are indistinguishable, but males can be differentiated by their abdominal tip coloration and sex comb pattern. A number of studies have been documented with regard to interspecific hybridization, phylogenetic relationships, inversion polymorphism, degree of crossability, mechanisms underlying hybrid sterility, sexual isolation, and behavior genetics (Bock, 1971, 1978; Jha and Rahman, 1972; Hegde and Krishnamurthy, 1979; Sisodia and Singh, 1996; Banerjee and Singh, 1996; Tomimura *et al.*, 2005; Kopp *et al.*, 2005; Mishra and Singh, 2007; Banerjee and Singh, 2012; Singh and Singh, 2013). *Drosophila bipectinata* is the most widely distributed and genetically most variable among the four species. Inversion polymorphism has been studied in Indian populations of *D. bipectinata* (Gupta and Panigrahy, 1990; Singh and Banerjee, 1995, 1997; Banerjee and Singh, 1996; Singh and Das, 1991; Das and Singh, 1992). A number of chromosome inversions are known to occur in this species, but there is no interpopulation variation in the frequencies of inversions, which suggests that inversion polymorphism in this species is rigid (Banerjee and Singh, 1996). Further, certain inversions persist in laboratory stocks owing to heterotic buffering associated with the inversions (Singh and Das, 1991; Das and Singh, 1992; Singh and Banerjee, 1997).

Table 2. Presence (+) and absence (-) of In 2R C in fifteen laboratory stocks of *D. bipectinata.*

Name of the stock	In 2R C
Mysore (Mys-88)	–
Alipurduar (AD-93)	–
Siliguri (SL-93)	+
Trivandrum (TD-93)	–
Arumanai (AR-96)	+
Pune (PN-99)	+
Siliguri (SL-01)	+
Bodh Gaya (B.Gaya-02)	+
Raebareli (RB-03)	–
Navsori (NV-03)	–
Nilgiri (NG-03)	–
Akola (Akl-12)	–
IROT 8	–
K-aaj 072 146	+
K-aaj 078 174	+

Table 3. Number of larvae analyzed and inversion heterozygotes detected in seven laboratory stocks of *D. bipectinata*. Frequency (in %) of inversion heterozygotes are given in parenthesis.

Name of the stock	Number of larvae analyzed	Number of heterozygotes
Siliguri (SL-93)	20	04 (20.00)
Arumanai (AR-96)	16	02 (12.50)
Pune (PN-99)	20	02 (10.00)
Siliguri (SL-01)	24	08 (33.33)
Bodh Gaya (B.Gaya-02)	20	01 (05.00)
K-aaj 072 146	08	02 (25.00)
K-aaj 078 174	09	05 (55.55)

Fifteen laboratory stocks of *D. bipectinata* were analysed chromosomally by squashing a large number of third instar larvae with the help of the lacto-aceto-orcein method. Both types of stocks, mass culture and isofemale lines, were used. Details of the stocks are shown in Table 1. In seven out of fifteen stocks analysed, only one inversion, *i.e.*, In 2R C was detected (Figures 1 and 2). This is a large paracentric inversion occurring in the right arm of the second chromosome. The presence (+) and absence (-) of heterozygous inversion in different stocks are shown in Table 2. In Table 3, the number of larvae analysed and inversion heterozygotes detected in seven stocks (with percentage of heterozygotes) are given.

Most of the stocks have been maintained in the laboratory for a large number of generations, and the presence of In 2R C inversion in seven stocks clearly demonstrates that heterotic buffering is associated with this inversion as demonstrated earlier (Singh and Das 1991; Das and Singh, 1992; Singh and Banerjee, 1997). The absence of this inversion in eight stocks might be due to the fact that

either it was not present in the founding individuals or it might have been eliminated due to random genetic drift.

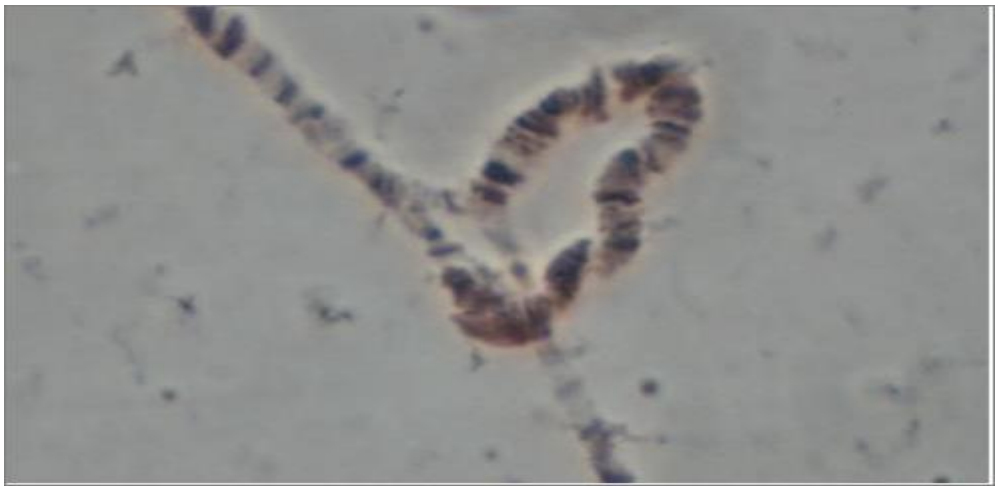

Figure 1. Microphotograph of In 2RC heterozygous inversion (100× magnification).

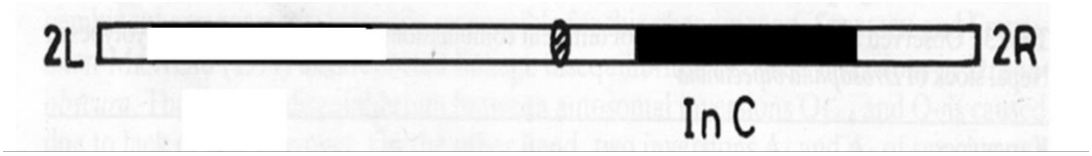

Figure 2. Location of In C inversion in the right arm of the second chromosome of *D. bipectinata.*

Acknowledgments: BNS and PB thank the University Grants Commission, New Delhi for the UGC-BSR Faculty Fellowship Award and Meritorious Fellowship, respectively. We also thank Dr. M. Matsuda for providing stocks of *D. bipectinata* from Japan.

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volume 3e, Academic Press, New York; Tomimura, Y., M. Matsuda, and Y.N. Tobari 2005, Genome 48: 487-502.

Mating frequency of *Drosophila melanogaster* strains reared on carrot and banana diets.

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Mating in *Drosophila* is probably one of the most studied behaviors. Different aspects of courtship, quality, and quantity of stimuli exchanged, as well as various components of reproductive behavior (mating latency, mating speed, mating success, copulation duration, sexual rejection, remating time, and frequency, female benefits of remating, sperm competition, and so forth) were studied in many *Drosophila* species, using diverse experimental designs and techniques (for reviews see Hall, 1994; Greenspan and Ferveur, 2000; Markow, 2002; Singh *et al*., 2002; Markow and O' Grady, 2005; Lasbleiz *et al*., 2006). The complex genetic basis involved in determination of mating behavior was thoroughly studied over decades, starting from using mutant strains and gynandromorphs (for review see Yamamoto *et al*., 1997), up to modern researches of underlying neural, neurochemical, and molecular mechanisms (Mackay *et al*., 2005; Zhou *et al*., 2012). Beside genetic influences, environmental conditions may also contribute to some components of mating behavior *via* their impact on traits involved in sexual selection. Among them, nutrition is one of the most important factors that may influence both morphological and/or physiological characteristics, and, indirectly, behavioral ones.

In this work, number of matings of two *Drosophila melanogaster* strains maintained for twelve years on different substrates was estimated. These substrates are modified standard *Drosophila* laboratory food, prepared by adding fruit (banana, flies designated as "B strain") and vegetable (carrot, flies designated as "C strain"), without sugar and yeast (Kekić and Pavković-Lučić, 2003). Two diets differ in chemical composition, odor, taste, and color.

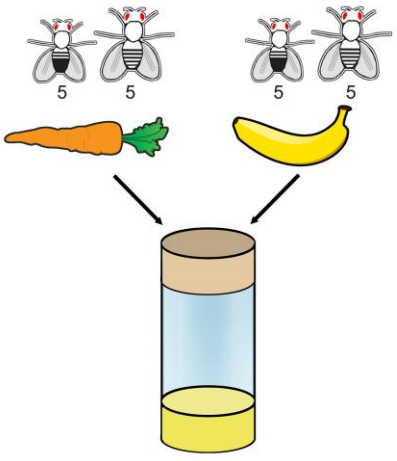

Figure 1. Mating scheme *per* replica.

"Multiple choice" behavioral assays were composed of replicates in which twenty flies (*per* replicate) were introduced into a mating vial containing standard cornmeal substrate. Flies were crossed following mating scheme (Figure 1): 5 males reared on carrot substrate (C males) + 5 females reared on carrot substrate (C females) + 5 males reared on banana substrate (B males) + 5 females reared on banana substrate (B females). Thirty-seven replicates were run, *i.e*., a total of 740 flies participated. Mating observations lasted 90 minutes and every single pair was removed into the new separate vial. Flies of both sexes and strains were scored for number of matings during each of three separate, consecutive 30 minutes periods. Flies of different strains were later identified using UV lamp, since they were alternatively marked with fluorescent powder 24 hours before mating. Using UV dust

does not influence mating frequency in this species (Terzić *et al*., 1994). Significant differences in number of matings achieved by flies of both sexes and strains were tested using Z-test.

Out of a possible 370 matings, 205 matings occurred (55.4%). During the first 30 minutes, 56 matings were observed (27.3%); during the next 30 minutes, 105 matings were observed (51.2%), and within the last 30 minutes, 44 matings were recorded (21.5%). Most of the copulations occurred within one hour observing period (78.5%).

Carrot-fed males were more successful in mating (58.54%) than males developed on banana diet (41.46%). During the first 30 minutes, C males realized 51.79% matings, while B males took part in 48.21% of the matings, and this difference is not significant ($Z = 0.535$, $p > 0.05$). Over the next 30 minutes, C males achieved 20% more matings than B males ($Z = 4.099$, $p < 0.01$). The same situation was observed in the last 30 minutes when C males realized 63.64% matings, which is significantly more ($Z = 3.618$, $p < 0.01$) than B males (36.36 %) (Figure 2). When cumulative data were used (Figure 3), C males outperformed B males during 60 minutes observing period ($Z = 3.625$, $p < 0.01$), as well as within 90 minutes observing period ($Z = 4.889$, $p < 0.01$).

Females developed on carrot diet were also more successful in mating. They achieved significantly more matings (78.57% and 56.19%) than banana-fed females (21.43% and 43.81%) during the first 30 minutes ($Z = 8.552$, $p < 0.01$), as well as during the next 30 minutes ($Z = 2.537$, $p < 0.05$), respectively. C and B females achieved the same number of matings in the last 30 minutes (Figure 2). Using cumulative data (Figure 3), C females were more successful in mating during 1 hour observing period ($Z = 7.093$, $p < 0.01$), as well as within whole 90 minutes ($Z = 6.286$, $p < 0.01$).

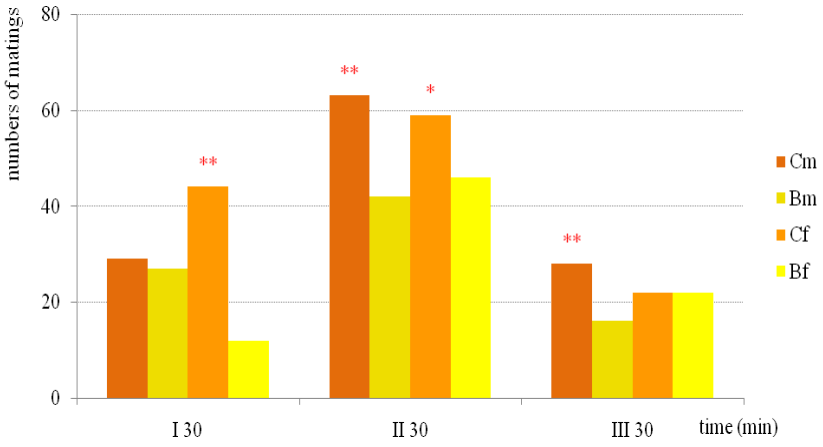

Figure 2. Number of matings achieved by both sexes of two *D. melanogaster* strains during every 30 minutes of observing period (I, II, and III). Abbreviations: B – flies reared on banana substrate, C - flies reared on carrot substrate, m – males, f - females.

*p < 0.05; **p < 0.01

Comparing to some other experimental designs, when sex ratio is biased toward one sex, in "multiple choice" design, which was used in this experiment, sex ratio was equal (F:M = 1:1). Therefore, competitive interactions were less pronounced, since every single fly had a chance to find a mate. Additionally, prolongation of the time of mating observations led to a greater number of recorded copulations. According to results presented here, carrot-fed flies achieved significantly more matings than B flies. Subsequent morphometric analysis showed that B flies were significantly larger than C flies, but body size *per se* did not provide them higher success in mating (Trajković *et al*., 2012). It seems that some other traits (physiological and/or behavioral) contributed to higher mating success of C flies.

Fruit flies use their olfactory and gustatory systems for evaluating smell and taste of food as well as odor and taste stimuli exchanged with mates (Ferveur, 2005; Laissue and Vosshall, 2008; Ruebenbauer *et al*., 2008; Everaerts *et al*., 2010; Lebreton *et al*., 2012; Rohwedder *et al*., 2012).

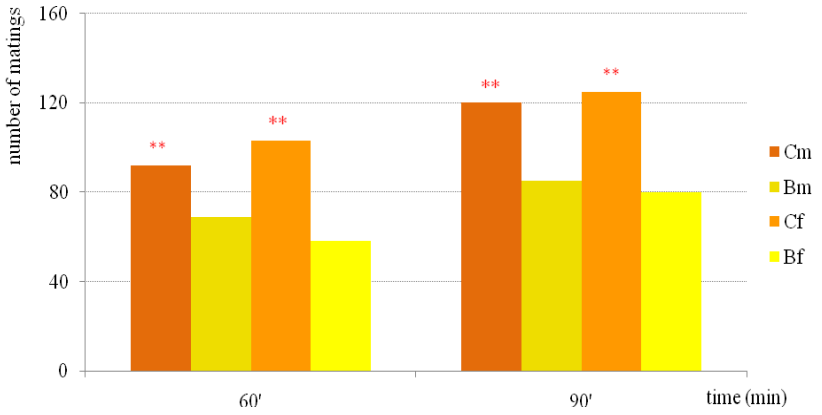

Figure 3. Cumulative number of matings achieved by both sexes of two *D. melanogaster* strains within 60 minutes and 90 minutes observing period. Abbreviations: as in Figure 1.

*p < 0.05; **p < 0.01

For *D. melanogaster*, banana is one of the favorite diets (Shorrocks, 1974), and their larvae were less-stressed during development on banana than on standard laboratory food (Carsten *et al*., 2005). However, when competed with carrot-fed flies, they were less successful in mating. Contrary to information available for banana as a *Drosophila* feeding substrate, less is known about the effect of carrot diet, especially long-lasting, on fruit fly mating performances. It is possible that carotenoids may have some impact on flies' physiological state, through their influence on vision and production of mating signals, as precursors to pheromones, which was observed for some insects (Heath *et al*., 2013). Precise roles of carotenoids in diet on insect behavior, including *Drosophila* mating performances, are insufficiently known and further researches are demanded in this area.

Acknowledgment: This work was supported by Serbian Ministry of Education, Science and Technological Development (Grant 173012). We are very grateful to Dr. Vladimir Tomić and Vesna Đoković for technical assistance.

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Developmental adaptive strategies for water balance mechanisms mediates range shift in *Drosophila* species of the *takahashii* subgroup from the western Himalayas.

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Abstract

Physiological limits determine susceptibility to environmental changes and can be assessed at the individual, population, or species/lineage levels. *Drosophila nepalensis* could serve as an indicator species for analyzing range changes under changing climatic conditions. Ectothermic Drosophilids are profoundly affected by thermal selection (*i.e.*, genetic effects) or through induced effects on phenotypes (*i.e.*, plastic effects). Climatic data for the last fifty years involves a significant change in average temperature (T_{ave}) of Western Himalayas, which has affected the distribution and boundaries of various Drosophilids in this region. There is a significant decline in the number of *D. nepalensis* from lower ranges. *D. nepalensis* is more abundant under colder and drier montane habitats in the western Himalayas, but the mechanistic basis of such a climatic adaptation is largely unknown. Here we discuss the physiological levels in *D. nepalensis* and consider implications for determining species susceptibility to climate change. Thus, temperature-specific divergence in water-balance-related traits in this species is consistent with its adaptations to cold and dry habitats of Western Himalayas. Our results suggest that *D. nepalensis* from lowland localities seems vulnerable due to acclimation potential in the context of global climate change in the Western Himalaya. Finally, this is the first report on higher desiccation resistance of *D. nepalensis* due to developmental plasticity of cuticular melanisation when grown at 15°C, which is consistent with its abundance in temperate regions. Abbreviations: 'D', Desiccation resistant strains; I.F., Isofemale; J/mg, Joules/mg; RWL, Rate of water loss.

Introduction

Physiological limits occur when the abiotic conditions of an organism's environment become stressful, reducing fitness to survive, potentially leading to the extinction of populations and even species (Hoffmann and Parsons, 1991). Physiological limits can influence the susceptibility of organisms to climate change. Due to climate warming, many insect species have shifted their ranges to higher latitudes and altitudes (Hill *et al.*, 2011; Parmesan *et al.*, 1999; Parmesan and Yohe, 2003). Insects, especially those species that have narrow thermal tolerances (Addo-Bediako *et al.*, 2000; Deutsch *et al.*, 2008) are particularly sensitive to temperature changes. There is a significant decline in the number of *Drosophila nepalensis* from lower ranges, and climate warming mediates its range shift in the Western Himalayas (Parkash *et al.*, 2013; Singh, 2012).

D. nepalensis was first described from collections made from Nepal by Okada (Okada, 1955). Subsequently, Parshad and Paika (1964) reported that abundance of *D. nepalensis* (70–80%) was associated with lower temperature (T_{ave}=18-20°C) as well as humidity (~43–45% relative humidity) at Manali (2050 m). Thus, we expect that this species will differ in its desiccation-related traits for

different quantitative traits conferring adaptation to harsh climatic conditions in the Himalayas. Therefore; we compared *D. nepalensis* for evolved physiological mechanisms that may affect its adaptations to drier climatic conditions. *D. nepalensis* might employ phenotypic plasticity as a strategy to cope with colder and drier conditions in temperate regions, but this aspect has not been tested so far.

Water conservation is crucial to the ecological success of diverse insect taxa as well as terrestrial arthropods (Edney, 1977; Hadley, 1994). The ability to maintain water balance is associated with species distribution patterns (Willmer *et al.,* 2000). Several studies have shown substantial variation in desiccation resistance of *Drosophila* species living in different habitats (Gibbs and Matzkin, 2001; Gibbs *et al.,* 2003). Insects can increase their desiccation resistance through three different avenues of water balance: (1) higher bulk water, (2) reduced rate of water loss, and (3) greater dehydration tolerance (Hadley, 1994; Gibbs *et al.*, 1997). Insects with higher initial body water content can survive longer under arid conditions, *e.g*. laboratory-selected desiccation-resistant strains of *D. melanogaster* have shown a 300% increase in hemolymph water content compared with control strains (Folk *et al.,* 2001). If laboratory selection responses result in increases in the bulk water content, then it would be interesting to compare the water budget of *Drosophila* species differing in their desiccation resistance levels. Further, water balance related traits have been investigated in Indian populations of *D. nepalensis* reared at 21°C, but not at 15 and 25°C (Parkash *et al.,* 2012). Thus, the plasticity for evolved physiological mechanisms for water balance in *D. nepalensis* is largely unknown.

For ectothermic insects, more than 80% of body water loss occurs through the cuticle. Reduction in cuticular permeability has been associated with changes in the amount or composition of surface lipids in several large sized insect taxa such as scorpions and tenebrionid beetles (Hadley, 1977; Toolsons and Hadley, 1979; Hadley, 1994). In contrast, cuticular lipid amount did not vary between xeric and mesic *Drosophila* species; and therefore showed no correlation with habitats or water loss rate (Gibbs *et al.,* 2003). Further, similar relationships are evident between laboratory selected desiccation resistance and control strains of *D. melanogaster* (Gibbs *et al.,* 1997). However, it is not clear whether *D. nepalensis* grown at different growth temperatures have evolved changes in the amount of cuticular lipids to confer greater desiccation resistance. Further, association between cuticular permeability and quantity of cuticular lipids can be demonstrated through treatment of cuticular surfaces in over-etherised or dead insects with organic solvents such as hexane or chloroform: methanol (Hadley, 1989; Hadley and Quinlan, 1989; Hadley, 1994). This approach can be helpful in distinguishing cuticular lipids as water proofing barrier or not. There is evidence of acquisition of carbohydrates as energy reserves to alleviate the consequences of desiccation stress in laboratory selected desiccation resistant strains of *D. melanogaster* (Graves *et al.*, 1992; Gibbs *et al.*, 1997; Chippindale *et al.*, 1998; Djawdan *et al.*, 1998; Folk *et al.*, 2001; Folk and Bradley, 2005). In contrast, higher percentage of body lipid content has conferred greater survival under desiccation stress in a new set of laboratory selected desiccation resistant lines (Telonis-Scott *et al.*, 2006). It is likely that *Drosophila* species of the subgenus *Sophophora* might store and utilize similar or varying levels of energy metabolites to cope with desiccation stress.

Acclimation to desiccation stress has received lesser attention (Hoffmann, 1990, 1991; Bazinet *et al.*, 2010). It would be interesting to assess acclimation potential of *D. nepalensis* from the Western Himalayas. In the present study, we examined relative abundance of *D. nepalensis* as a function of changes in relative humidity along an altitudinal gradient in the Western Himalayas. We analyzed *D. nepalensis* for desiccation-related traits and investigated effects of developmental phenotypic plasticity (15° *versus* 25°C growth temperatures) on desiccation-related traits as well as energy metabolites. We tested three different routes of water balance that may result in differences of desiccation resistance. We assessed whether higher desiccation potential is associated with greater

storage of energy metabolites. Finally, we examined the desiccation acclimation potential for evolved physiological mechanisms that may affect its adaptations to drier climatic conditions.

Materials and Methods

Collections and cultures

D. nepalensis (n = 150–300) were collected in a single trip during autumn in October, 2008 from five altitudinal localities of the western Himalayas (Figure. 1). Wild-caught individuals of a midland locality (Solan, 1440 m; 30.55°N) were used to initiate 20 isofemale lines (for all analyses, 20 lines with 10 replicates each were used unless otherwise indicated). All cultures were maintained at low density (60–70 eggs per vial; egg vials were 40 × 100 mm in size) on cornmeal-yeast-agar medium at 15°, 21°, and 25°C and 65 ± 1% relative humidity in a temperature- and humidity-controlled incubator for five generations before experimental analysis. All assays were performed on 7-day-old female flies, because the trait values did not vary as a function of age between 6 and 21 days (Gibbs and Matzkin, 2001; Parkash *et al.,* 2008a). Climatic data for thermal variables and relative humidity were obtained from the Indian Meteorological Department, Government of India, New Delhi. Percent abundance was estimated as the number of individuals of a particular *Drosophila* species divided by the total number of individuals of all the different *Drosophila* species in the samples collected from a given locality.

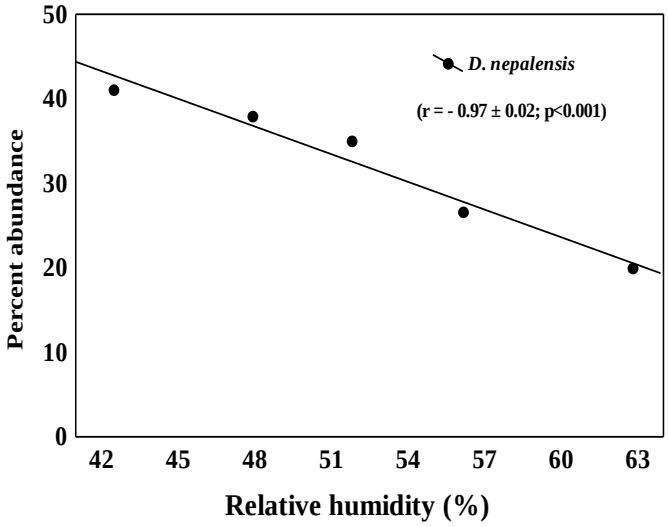

Figure 1. Regression analysis of percent abundance as a function of relative humidity (RH%) of the origin of five altitudinal populations of *D. nepalensis*. Populations include (altitude; RH%): (1) Parwanoo (512m; 62.8%); (2) Bhuntar: (1096m, 56.2%); (3) Solan (1440m, 51.8%); (4) Barog: (1680m, 47.9%); (5) Kasuali (1951m, 42.5 %).

Trait analysis

We used 10 individuals of each replicate (10 replicates × 20 isofemale lines each) to quantify body melanisation, epicuticular lipid mass, desiccation resistance, multiple measures of water balance, and levels of energy metabolites. For flies grown at 15°, 21°, and 25°C, we tested desiccation-related traits at their respective growth temperature, *i.e*., at 15°, 21°, and 25°C, respectively. Therefore, growth temperature and experimental temperature was same in our experimental setup.

Analysis of body melanisation

The progeny of each isofemale line was examined for differences in body melanisation patterns on the abdominal segments. Body melanisation of individual female flies (n = 20 I.F. lines × 10 replicates per isofemale line) was visually scored with Olympus stereo zoom microscope SZ-61 (www.olympus.com). It was estimated from dorsal as well as lateral views of the female abdomen giving values ranging from 0 (no melanisation) to 10 (complete melanisation) for each of the six

abdominal segments (2^{nd} to 7^{th}). Further, the relative size of each abdominal segment was calculated in proportion of the largest 4^{th} abdominal segment, which was assigned the value of 1.0. Since the abdominal segments differ in size, these relative sizes (*i.e.*, 0.86, 0.94, 1.0, 0.88, 0.67, and 0.38 for 2^{nd} to 7^{th} segments, respectively) were multiplied with segment-wise melanisation scores. Data on percent melanisation were calculated as (Σ observed weighted melanisation scores of abdominal segments per fly / Σ relative size of each abdominal segment ×10 per fly) ×100 (Parkash *et al.*, 2008).

Assessment of cuticular lipid mass

We assessed cuticular lipid mass in individual adult (20 I.F lines × 10 replicates each) reared at 15°, 21°, and 25°C. Each individual was dried overnight at 60°C to get dry mass, *i.e.*, devoid of body water. Each dried individual was kept in HPLC-grade hexane in 2 ml eppendorf tube (www.tarsons.in) for 3 minutes and, thereafter, it was removed from the solvent and was again dried at room temperature and finally reweighed on a Sartorius microbalance (Model-CPA26P; 0.001 mg precision; www.sartorious.com). Cuticular lipid mass per cm^2 was calculated as the difference in mass following solute extraction divided by surface area (cm^2).

Desiccation resistance

Desiccation resistance was measured as the time to lethal dehydration (LT_{100}) effect under dry air. Seven day old flies were separated out and placed individually in dry plastic vials (40 × 100 mm) in which the open end was covered with muslin cloth. These vials were kept on top of another vial containing 2 g of silica gel at the bottom. Finally, this apparatus was made airtight with Parafilm and kept in the desiccator chamber (Secador electronic desiccator cabinet; www.tarsons.in), which maintained 0–5% relative humidity. Number of immobile flies was counted after every one hour interval, and LT_{100} values in dry air were recorded.

Basic measures of water balance

To estimate total body water content and dehydration tolerance (%), 10 flies of each isofemale line (20 I.F lines × 10 replicates each) were used. First, individual flies were weighed on Sartorius microbalance (Model-CPA26P; 0.001 mg precision) and then reweighed after drying overnight at 60°C. Total body water content was estimated as the difference between mass before and after drying at 60ºC. Further, after mild anesthesia (one minute) with solvent ether, flies were weighed on a Sartorius microbalance both before and after desiccation stress until death. Dehydration tolerance was estimated as the percentage of total body water lost until death due to desiccation and was calculated by formula (wet body mass − body mass at death)/(wet body mass − dry body mass) ×100 (Gibbs *et al.*, 1997). For calculation of the rate of water loss, we followed Wharton's method (1985). Total body water content (m) was calculated as the difference between wet (f) and dry mass (d), *i.e.*, $m = f - d$. Individual flies were weighed and placed at 0-5% relative humidity for a specified time at one hour intervals (1 to 8 h) and reweighed. The rate of water loss was derived from the slope of regression line on a plot of $ln(m_t / m_0)$ against time according to Wharton's exponential equation (Wharton, 1985) $m_t = m_0 e^{-k_t}$, where m_t is the water mass at time t, and m_0 is the initial water content. Rate (k_t) is the slope of the regression line and was expressed as % per hour.

Assessment of extractable hemolymph content

An individual adult fly was placed on a paper towel and cleaned with distilled water followed by air drying for 2 minutes. The dry individual was carefully pinned to a microdissection dish at its anterior and posterior ends with microdissection pins, and a narrow incision was made through the cuticle with a third pin while observing through a stereo-zoom microscope (SZ-61; www.olympus.com). The leaking extractable hemolymph was absorbed with an absorbent tissue

moistened with an isotonic saline solution (Folk *et al.*, 2001). Hemolymph content was estimated as reduction in mass following hemolymph blotting (Cohen *et al*., 1986; Hadley, 1994). Tissue water was estimated after subtracting exsanguinated mass before and after drying. From the same data, we also calculated hemolymph water content by subtracting tissue water from total body water content.

Assessment of desiccation acclimation responses

To measure pretreatment duration, 10 adult individuals of each replicate (20 I.F lines × 10 replicates each) were subjected to desiccation stress at ~0-5% relative humidity. The initial body water content in each replicate group was recorded. The time period in which flies lost ~15–17% body water was considered as the pre-treatment time duration. Further, for the recovery period, individuals were placed on laboratory food till the original mass was regained. Such individuals were subjected to desiccation stress until death in order to test the increased desiccation resistance due to acclimation. Thus, absolute acclimation capacity (increased desiccation survival hours) was calculated by subtracting the desiccation resistance (h) of non-acclimated (control) from desiccation resistance (h) of acclimated individuals. Control and treatment experiments were run simultaneously under identical experimental conditions.

Analysis of body lipid content

Individual adult flies were dried in 2 ml eppendorf tubes (www.tarsons.in) at 60°C for 48 h and then weighed on Sartorius microbalance (Model-CPA26P; 0.001 mg precision; www.sartorious.com). Thereafter, 1.5 ml di-ethyl ether was added in each eppendorf tube and kept for 24 h under continuous shaking (200 rpm) at 37°C. Finally, the solvent was removed and individuals were again dried at 60°C for 24 h and reweighed. Lipid content was calculated per individual by subtracting the lipid free dry mass from initial dry mass per fly.

Estimation of trehalose and glycogen

For trehalose and glycogen content estimation, 10 adult flies of each isofemale line were homogenized in a homogenizer (Labsonic@ M; www.sartorious.com) with 300 µl Na_2CO_3 and incubated at 95ºC for 2 hours to denature proteins. An aqueous solution of 150 µl acetic acid (1M) and 600 µl sodium acetate (0.2M) was mixed with the homogenate.

Thereafter, the homogenate was centrifuged (Fresco 21, Thermo-Fisher Scientific, Pittsburgh, USA) at 12000 rpm (9660 × *g*) for 10 minutes. This homogenate was used for independent estimations of trehalose and glycogen as given below. For trehalose estimation, aliquots (200 µl) were placed in two different tubes; one was taken as a blank whereas the other was digested with trehalase at 37°C using the Megazyme trehalose assay kit (K-Treh 10/10, www.megazyme.com). In this assay, released D-glucose was phosphorylated by hexokinase and ATP to glucose-6-phosphate and ADP, which was further, coupled with glucose-6-phosphate dehydrogenase and resulted in the reduction of nicotinamide adenine dinucleotide (NAD). The absorbance by NADH was measured at 340 nm (UV-2450-VIS, Shimadzu Scientific Instruments, Columbia, USA). The pre-existing glucose level in the sample was determined in a control reaction lacking trehalase and subtracted from total glucose concentration. For estimation of glycogen, a 50 µl aliquot was incubated with 500 µl *Aspergillus niger* glucoamylase solution (8.7 U/ml in 200 mM of acetate buffer) for 2 hours at 40ºC with constant agitation, and the suspension was centrifuged at 4000 rpm (1073 × *g*) for 5 minutes. It mainly hydrolyzed alpha-(1,4) and alpha-(1,6) glycosyl linkages and was suited for breakdown of glycogen. Glucose concentration was determined with 20 µl of supernatant from the suspension and added with 170 µl of a mixture of G6-DPH (0.9 U/ml); ATP (1.6mM); and NADP (1.25mM) in triethanolamine hydrochloride buffer (380mM TEA–HCl and 5.5mM of MgSO4)} and 10 µl of

Hexokinase solution (32.5 U/ml in 3.2M ammonium sulphate buffer), and absorbance was measured at 340nm.

Protein assay

Protein levels were determined by using the bicinchoninic acid method as followed by Gibbs and coworkers (Marron *et al.,* 2003). For protein assay, 10 female flies per isofemale line (n = 10 replicates × 20 I.F. lines of each species) were homogenized in 3 ml distilled water and centrifuged at 10000 rpm for 5 minutes. Further, 50 µl of aliquot was taken from supernatant and treated with 2 ml of Sigma BCA reagent and incubated at 25°C for 12 hours. Absorbance was recorded at 562 nm, and protein concentration was determined by comparing with standard curve.

Energy metabolites and energy budget

We measured each energy metabolite (carbohydrates, body lipids, or proteins) in multiple replicate sets of isofemale lines (20 I.F lines × 10 replicates each) in *D. nepalensis* reared at 15°, 21°, and 25°C. Total energy budget was calculated using standard conversion factors following Schmidt-Nielsen (1990).

Statistical analyses

For each trait, mean values (± S.E.; 20 isofemale lines, 10 replicate each) were used for illustrations and tables. Effects of developmental temperatures (15°, 21°, and 25°C) on desiccation-related traits, energy metabolites, body weight, basic measures of water balance, and dehydration tolerance were compared with ANOVA. Pearson's correlation coefficients were calculated on the basis of isofemale line data (10 I.F lines × 10 replicates each). For multiple comparisons, alpha value was adjusted with Bonferoni corrections. Energy contents due to carbohydrates, lipids, and proteins of adults were calculated using standard conversion factors (Schmidt-Nielsen, 1990; Marron *et al*., 2003). Statistica (Statsoft Inc., Release 5.0, Tulsa, OK, USA) was used for calculations as well as illustrations.

Results

Data on percent abundance of wild-caught flies of *D. nepalensis* from five altitudinal localities (512–1951 m) as a function of relative humidity of origin of populations are shown (Figure 1). *D. nepalensis* is more abundant (~42.5%) in highland localities but occurs less frequently in lowland localities (~21%). The highland localities are moderately colder and drier (T_{ave} = 15.2°C; RH = 42.5%), while lowland localities are warm and less desiccating (T_{ave} = 27.6°C; RH = 62.8%). Therefore, significant reduction in T_{ave} (~2ºC per 200 m) as well as relative humidity (~3.9% per 200 m) along an elevational gradient may act as selection factors for affecting its relative abundance. Thus, *D. nepalensis* is better adapted under colder and drier conditions in highland localities.

Comparison of plastic effects for desiccation-related traits data on desiccation resistance and energy metabolites due to growth temperatures, *i.e*., (15°, 21°, and 25°C) in *D. nepalensis* are shown (Table 1). Body melanisation increased ~1.5-fold at 15°C and ~15-fold at 25°C from mid thermal range temperature (Figure 2a), but no change in epicuticular mass due to thermal plastic effects (melanisation: $p < 0.001$; epicuticular lipids: 15°C = 22.10 ± 0.37 µg cm^{-2}; 25°C = 22.00 ± 0.27 µg cm^{-2}) in *D. nepalensis*. We observed ~1.5-fold higher desiccation resistance at 15°C and ~3-fold lower desiccation resistance at 25°C from mid thermal range temperature, *i.e*., 21°C (Table 1 and Figure 2b). Further, trehalose content was significantly higher (1.45-fold) at 15°C (0.131 ± 0.004 mg) than 21°C (0.090 ± 0.002 mg) and significantly lower (1.42-fold) at 25°C (0.063 ± 0.004 mg)

than 21°C (0.090 ± 0.002 mg) ($F_{2, 18}$ = 195.43; $p < 0.001$). In contrast, our results did not evidence significant changes in the levels of proteins but in glycogen content (Table 2). Thus, plastic responses for desiccation related traits differ in *D. nepalensis*.

Table 1. Data (mean ± S.E.) on cuticular components(cuticular melanisation and cuticular lipids), desiccation resistance hours, water balance related traits, and dehydration tolerance in adult flies (n = 20 I.F lines × 10 replicates) of *D. nepalensis* grown at 15°C, 21°C, and 25°C (plastic effects). Trait values for each species grown at 15°C, 21°C, and 25°C were compared as ratio (fold-differences) and with ANOVA (*F* – values).

Traits	15°C	21°C	25°C	Ratio of 15°C vs. 21°C	Ratio of 21°C vs. 25°C	$F_{2, 18}$
1. Melanisation (%)	90.00 ± 2.01	60.00 ± 1.08	4 .02 ± 0.51	1.50	14.93	789.89***
2. Epicuticular lipids (µg cm $^{-2}$)	22.10 ± 0.37	22.05 ± 0.30	22.00 ± 0.27	1.00	1.00	0.32ns
3. Desiccation hours	55.11 ± 1.78	40.07 ± 1.58	14.01 ± 0.29	1.37	2.86	392.55***
4. Wet weight (mg fly $^{-1}$)	2.265 ± 0.03	1.516 ± 0.05	1.075 ± 0.02	1.494	1.410	254.63***
5. Dry weight (mg fly $^{-1}$)	0.679 ± 0.006	0.455 ± 0.004	0.322 ± 0.003	1.492	1.413	199.50***
6. Total water content (mg fly $^{-1}$)	1.586 ± 0.01	1.061 ± 0.005	0.753 ± 0.008	1.494	1.409	230.47***
7. Hemolymph content (mg fly $^{-1}$)	0.747 ± 0.006	0.501 ± 0.008	0.355 ± 0.003	1.491	1.411	165.98***
8. Hemolymph water content (mg fly $^{-1}$)	0.523 ± 0.004	0.350 ± 0.001	0.249 ± 0.002	1.494	1.405	286.33***
9. Tissue water content (mg fly $^{-1}$)	1.063 ± 0.003	0.711 ± 0.003	0.504 ± 0.003	1.495	1.410	268.94***
10. Dehydration tolerance (%)	82.09 ± 0.05	82.18 ± 0.02	82.07 ± 0.04	1.001	1.001	1.86ns

Data were arcsin transformed for ANOVA.
ns – nonsignificant, ****P* < 0.001.

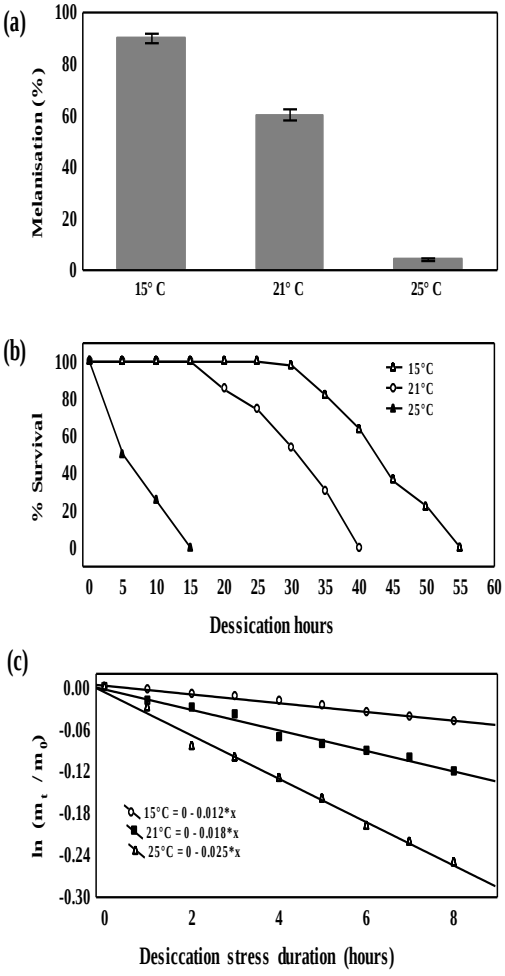

Figure 2. A comparison of melanisation, desiccation hours, and water loss rate (according to Wharton's method) in adult flies of *D. nepalensis* grown at 15, 21, and 25°C. The water loss rate was derived from the slope (b) of $\ln(m_t / m_0)$ as a function of different durations of desiccation stress at < 5% relative humidity. Slope values for rate of water loss vary significantly between species when grown at 15, 21, and 25°C.

Analysis of trait variability

We used ANOVA for partitioning %variance in three desiccation-related traits (desiccation resistance, cuticular lipid mass, and total carbohydrate content) in 20 isofemale lines (20 I.F lines × 10 replicates) in *D. nepalensis* grown at 15°, 21°, and 25°C (Table 3). Interestingly, the results of ANOVA for all the three desiccation-related traits showed similar levels of variability in 7 day old adult flies. The percent variance for desiccation resistance and carbohydrate content were 72.90% and 60.12% due to growth temperatures, isofemale lines, and interaction effects, respectively. However, for cuticular lipid mass, adult flies have shown 0.01% and 0.02% non significant variability due to growth

temperatures, isofemale lines, and their interactions, respectively. Thus, we found major differences in desiccation-related traits due to growth temperatures (Table 3).

Table 2. Comparison of energy budget at 15, 21, and 25°C in *D. nepalensis*. Data are from 20 isofemale lines, 10 replicates each.

	Metabolites	15°C	21°C	25°C	Fold difference due to 15°C vs. 21°C	Fold difference due to 2°C vs. 25°C
1.	Carbohydrates	3.537	2.411	1.689	1.47	1.43
2.	Lipids	3.615	2.397	1.689	1.51	1.42
3.	Proteins	0.925	0.925	0.925	1.00	1.00
4.	Total	8.077	5.733	4.303	1.41	1.33

Conversion factors: 17.6 Jmg^{-1} for carbohydrates, 39.3 Jmg^{-1} for lipids and 17.8 Jmg^{-1} for proteins. (Schmidt and Nielsen, 1990; Marron et al., 2003).

Table 3. Analysis of variance (n = 20 I.F × 10 replicates each) for explaining trait variability due to growth temperatures (T), isofemale lines and their interactions in *D. nepalensis*.

	df	Temperature (T) 2	IF lines 19	IF × T 38	Error 540
1. Desiccation resistance	MS	130068.18	372.33	101.41	0.32
	F	1282.59	1163.53	316.91	
	% Var	72.90***	7.93***	2.16***	0.13
2. Cuticular lipid mass	MS	16.38	7.74	9.64	0.95
	F	6.04	2.85	1.94	
	% Var	0.01ns	0.02ns	0.01ns	0.94
3. Carbohydrate content	MS	104573.32	587.73	137.77	2.01
	F	759.04	292.40	68.54	
	% Var	60.12***	12.89***	3.01***	0.87

ns = nonsignificant; ***$P < 0.001$.

Comparison of rate of water loss

We used nine sets of independent experiments to determine changes in the rate of body water loss in control *versus* flies exposed to different durations (1 to 8 h) of desiccation stress in *D. nepalensis* reared at 15°, 21°, and 25°C; and the data are shown in Figure 2b. Comparison of slope values (Wharton's method) has shown a significant increase in rate of water loss in adult flies (Figure 2c) grown at 25°C than 21° and 15°C. These observations on rate of water loss suggest greater desiccation resistance of *D. nepalensis* when grown at 15°C.

Effects of growth temperature on acclimation potential

We tested whether *D. nepalensis* show similar or different desiccation acclimation responses to prior treatment of desiccation stress when compared across three growth temperatures (15°, 21°, and 25°C). Interestingly, similar trends for acclimation effects at 15° and 21°C but varied in trait values. *D. nepalensis*, reared at 15°C showed significant increase in desiccation resistance (net

increase in desiccation resistance due to acclimation: 8.48 ± 0.38 h) and corresponding values at 21°C were significant (6.00 ± 0.05 h; Figure 3a), *i.e*., *D. nepalensis* showed higher acclimation response at both 15°C and 21°C (Figure 3), and at 25°C it shows negligible response. However, we did not find any significant change in the cuticular lipid mass as a consequence of desiccation acclimation across three growth temperatures (Figure 3b). Interestingly, we observed a non-significant reduction in rate of water loss due to acclimation across the growth temperatures (Figure 3c). Thus, our results suggest that *D. nepalensis* responds to acclimation for desiccation stress at lower growth temperature.

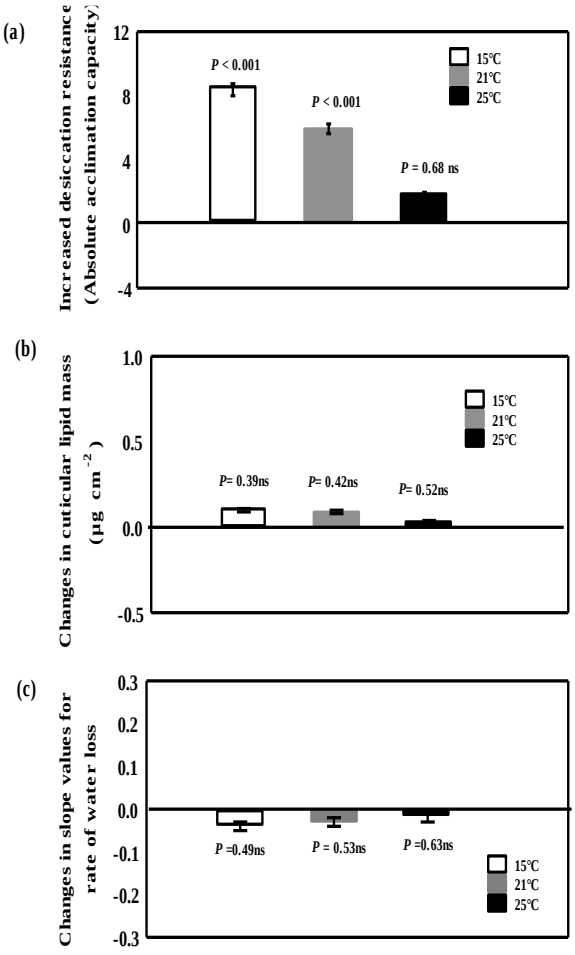

Figure 3. Changes in desiccation related traits due to acclimation of adult flies of *D. nepalensis* grown at 15, 21, and 25ºC. Changes in trait values are shown for desiccation resistance (a), cuticular lipid mass (b), and rate of water loss (c). For (a), absolute acclimation capacity = desiccation hours of acclimated – non-acclimated adults.

Differences in basic measures of water balance and dehydration tolerance

A comparative analysis of body mass has shown consistent differences in multiple measures of body weight as well as body water content in *D. nepalensis* (wet mass: $F_{2, 18} = 254.63$; $p < 0.001$; dry mass: $F_{2, 18} = 199.50$; $p < 0.001$; body water content: $F_{2, 18} = 230.47$; $p < 0.001$) when grown at three different temperatures, *i.e*., 15°, 21°, and 25°C. Hemolymph content and hemolymph water content have shown ~2-fold increase and decrease at 15°C than 25°C, respectively, as compared to 21°C (Figure 4). We observed ~2 fold increase in tissue water due to variation in growth temperatures ($F_{2, 18} = 268.94$, $p < 0.001$). However, dehydration tolerance did not change significantly due to variation in growth temperatures at 15°C and 25°C as compared with 21°C ($F_{2, 18} = 1.86$, ns).

Correlation between desiccation resistance and energy budget

Data on differences in the storage of desiccation resistance and energy budget in *D. nepalensis* when reared at 15°, 21°, and 25°C are shown (Tables 1 and 2), respectively. We calculated energy budget due to carbohydrates (which are actually consumed under desiccation stress) by using standard conversion factors (Schmidt-Nielsen, 1990), and the data for adult flies are shown (Figure 5c). The energy budget due to stored carbohydrates is about 30-50% higher at 15°C than 21°C (Table 2). Further, storage levels of carbohydrates are about 30-50% lower at 25°C than 21°C. We found significant correlations between carbohydrates energy budget (J/mg) and desiccation resistance (Figure 5c) of *D. nepalensis* grown at 15°, 21°, and 25°C. Thus, there are significant correlations between carbohydrate energy budget and desiccation resistance.

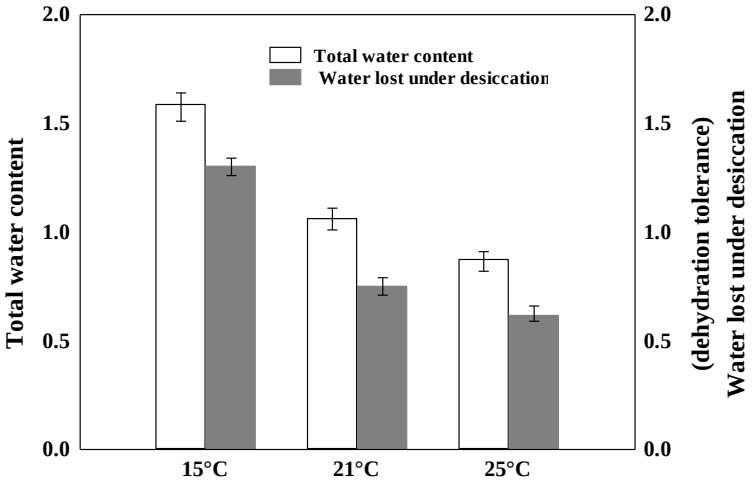

Figure 4. Bars (mean ± SE) represent differences in total body water content and dehydration tolerance in *D. nepalensis* reared at 15, 21, and 25°C. There is higher total water content as well as dehydration tolerance in *D. neplansis* at colder growth temperature as compared to warmer growth temperature.

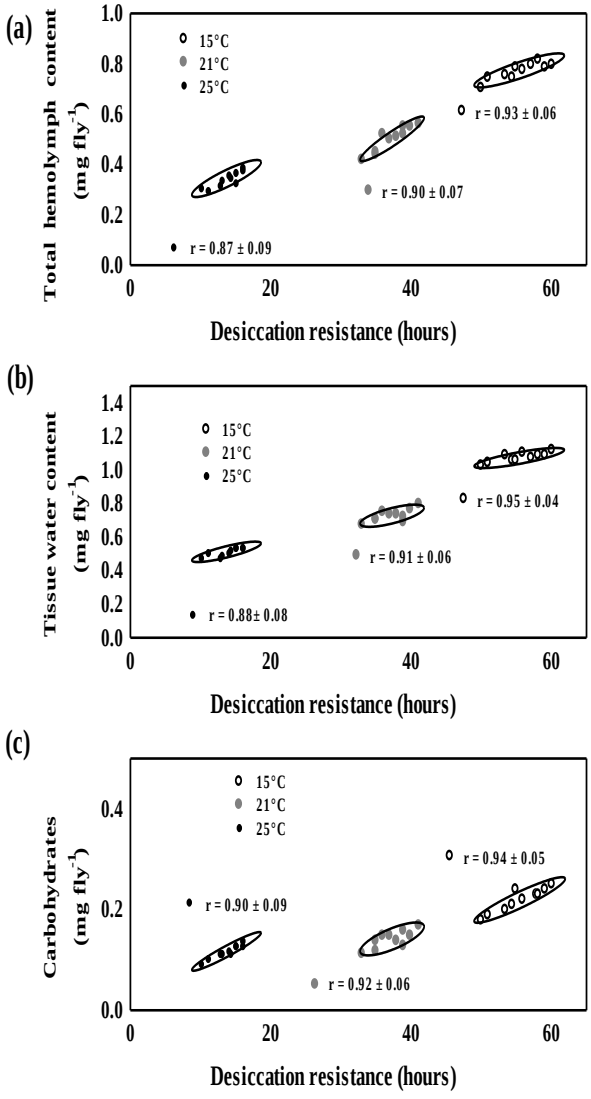

Figure 5. Trait correlations are based on 10 isofemales lines (10 replicates) of *D. nepalensis.* Desiccation resistance is positively correlated with Hemolymph (a), Tissue water content (b), and Carbohydrate (c). Correlation coefficients are shown ± s.e.m.

Discussion

In the present study, we found significant differences in desiccation-related traits in *D. nepalensis* across three growth temperatures that differ in their abundance under field conditions. Interestingly, there are developmental plastic effects for body melanisation. For *D. nepalensis*, plastic response for body melanisation is consistent with higher desiccation potential. Further, differences in desiccation resistance due to developmental plasticity match significant increase in the body mass (wet and dry mass), body water content, and hemolymph water when reared at 15°C as compared to 21°C. We also observed a significant effect of growth temperatures on the storage of carbohydrates. For example, there was a lower storage level of carbohydrates when grown at 25°C, whereas the reverse trend was evident at 15°C. Finally, we found that absolute desiccation

acclimation capacity was quite low in *D. nepalensis* reared at 25°C, and this might reflect its future vulnerability under global climate warming.

Role of cuticular lipids

Insect cuticle is a complex structure and its components might vary between species and populations (Willmer *et al.*, 2000). Several studies have shown variable cuticular permeability due to changes in the composition or amount of cuticular lipids in diverse insect taxa (Edney, 1977; Toolson, 1984; Hadley, 1994; Rourke, 2000). However, no previous study has examined development plastic effects (due to growth temperatures) on cuticular lipid mass. In the present work, we observed negligible increase in the cuticular lipid mass of adults reared at 15°, 21°, and 25°C. In contrast, developmental plastic effects for cuticular melanisation are evident. Thus, we found a decrease in cuticular permeability due to single component (cuticular melanisation). As evident (Figure 4b), we may argue that cuticular lipids are not contributing to the total desiccation survival hours.

Plastic changes for hemolymph content and dehydration tolerance

In insects, hemolymph is a major source for changes in higher level of body water to support longer survival under dehydration stress (Hadley, 1994; Chown and Nicolson, 2004; Folk *et al.*, 2001; Folk and Bradley, 2005). In contrast, several studies on wild populations of various *Drosophila* species have not considered changes in hemolymph content to enhance survival under desiccation stress (Gibbs and Matzkin, 2001; Gibbs *et al.*, 2003; Parkash *et al.*, 2010). In the present study, we found changes in hemolymph content as a consequence of developmental plastic effects (*i.e.*, 15°, 21°, and 25°C). *D. nepalensis* has shown changes in hemolymph content consistent with their different levels of desiccation resistance potential. Further, most arthropods can tolerate ~30-50% loss of body water, but some taxa adapted to drier habitats have evidenced higher dehydration tolerance (Hadley, 1994; Willmer *et al.*, 2000; Benoit *et al.*, 2005). In the present study, we found increased dehydration tolerance at 15°C as compared with 21°C, which is consistent with differences in desiccation resistance. Therefore, dehydration tolerance has evolved as a common physiological mechanism to support survival under desiccation stress in *D. nepalensis*.

Differences in the storage of energy metabolites

The acquisition of greater energy reserves has been associated with increased survival under dehydration stress (Gibbs 2002; Chown and Nicolson 2004). Laboratory selected desiccation resistant lines (D) have shown higher storage of carbohydrates as compared with control (Graves *et al.*, 1992; Gibbs *et al.*, 1997; Djawdan *et al.*, 1998; Chippindale *et al.*, 1998; Folk *et al.*, 2001; Folk and Bradley, 2005). In contrast, a new set of laboratory selected desiccation resistant lines has shown increased lipid content in selected (D) lines when compared with control (Telonis-Scott *et al.*, 2006). Therefore, results of laboratory selection experiments are not consistent whether carbohydrates or lipids support survival under desiccation stress. Further, wild *Drosophila* species from xeric and mesic habitats vary in desiccation resistance, despite lack of differences in the storage of energy metabolites (Marron *et al.*, 2003). Further, no previous study has examined changes in the storage levels of energy metabolites due to thermal plastic effects. In the present study, we observed higher levels of carbohydrates when *D. nepalensis* reared at 15° than 21°C and lower levels of carbohydrates when *D. nepalensis* reared at 25° than 21°C, which is in agreement with differences in their desiccation resistance at different growth temperatures. Thus, *D. nepalensis* have stored higher levels of carbohydrates (15°C) to alleviate the effects of desiccation stress. In contrast, low storage

level of carbohydrates at 25°C is consistent with significantly lower desiccation resistance. Our results suggest that storage of energy metabolites is constrained by growth temperatures.

Acclimation potential

Ectothermic organisms are capable of increasing their stress resistance level due to prior exposure of few or more bouts of thermal stresses (Bale, 2002; Hoffmann *et al.,* 2003). In *Drosophila* species, there are a few studies which have shown increase in desiccation resistance due to prior treatment of non-lethal level of desiccation stress in two Australian populations of *D. melanogaster* and *D. simulans* (Hoffmann, 1991) and in one Canadian population of *D. melanogaster* (Bazinet *et al.*, 2010). Two pairs of sibling species grown at 25°C (*D. serrata versus D. birchii*; and *D. melanogaster versus D. simulans* from Australia) have shown species-specific differences in the acclimation to desiccation stress (Hoffmann, 1991). In that study, both *D. melanogaster* and *D. simulans* from Cairns (Australia) showed increased desiccation resistance due to acclimation to desiccation stress. Thus, higher acclimation capacity was evident in adults of *D. simulans* as compared with *D. melanogaster* (Hoffmann, 1991). However, the effects of acclimation at ecologically relevant growth temperatures have not been considered in any *Drosophila* species so far. In the present study, we found higher acclimation capacity in *D. nepalensis* (~8.4 h) and (~6 h) when grown at 15° and 21°C, respectively. In contrast, at 25°C, it showed negligible acclimation response. Our results suggest that contrasting levels of acclimation capacity are constrained by their basal levels of desiccation resistance at different growth temperatures. *D. nepalensis* at warmer growth temperature have shown lower desiccation potential as well as lower acclimation response as compared with colder growth temperatures. Thus, *D. nepalensis* can be vulnerable under global climate warming. Therefore, acclimation to drought conditions is adaptive for *D. nepalensis* only with its potential varies according to growth temperatures. *D. nepalensis* is a stenothermal species and physiologically adapted to a narrow range of developmental temperatures. Thermal sensitivity has the potential to influence ecology and fitness of a species. Several investigations have shown the role of thermal plastic effects on quantitative traits for adaptations to temporal and spatial changes in climatic conditions (Willott and Hassall, 1998; Mousseau *et al.,* 2000). In the present study, *D. nepalensis* has shown significant plastic effects for traits. The evolutionary changes in these traits can be explained on the basis of climatic selection of trait variability. In *D. nepalensis*, trait values are significantly reduced at 25°C, which can limit its occurrence at higher temperatures. Thus, due to global warming, the cold adapted species *D. nepalensis* has retracted from low to midland localities as a consequence of plasticity. In conclusion, the observed range changes of *D. nepalensis* have resulted as a consequence of plasticity in traits under global warming effects at the northern border limits.

Temperature differences are sufficiently pronounced in various geographical regions. Hence, we might predict a positive correlation between various traits and adaptation to different geographical regions. Phenotypic plasticity in Drosophilids is still insufficiently investigated but is relevant to understanding natural population ecology. Temperate and tropical species differ significantly in the thermal range at which they can develop under laboratory conditions. The associations of various traits with the environments suggest that such traits may present the adaptive characteristics underlying the diversification and distribution of *Drosophila* species. The present study suggests that *Drosophila* species respond to climate change by shifting their distribution range, changing in abundance and physiology.

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Divergent strategies for stress adaptations in *D. punjabiensis* in context of global warming.

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Abstract

In subtropical parts of the Indian subcontinent, autumn is a cold and dry season, while spring is wet and humid, and ectothermic *drosophilids* are expected to evolve desiccation resistance to cope with drier climatic conditions. Previous studies have described that in tropical populations of *D. punjabiensis*, body color polymorphism is maintained through humidity changes as opposed to thermal melanism, and seasonal changes in the frequency of body color morphs in this tropical species supports the melanism-desiccation hypothesis. But the mechanistic bases of such climatic adaptations in two body color morphs of *D. punjabiensis* are largely unknown. We tested the hypothesis that divergence in the physiological basis of desiccation-related traits is consistent with body color morph-specific adaptations to climatic conditions, for which we examined the response of water balance to relative humidity (RH), temperature, and their interaction in *D. punjabiensis*, using two body color morphs that had been allowed to rear at low or high humidity at 17°C and 25°C. We found, at low RH, dark body color morph had significantly greater trait values than light body color morph at both the temperatures. A comparative analysis of water budget of the two body color morphs showed that higher water content, reduced rate of water loss, and greater dehydration tolerance confer higher desiccation resistance in dark morph of *D. punjabiensis* at low RH. We found that carbohydrates act as metabolic fuel during desiccation stress in both the morphs, but a higher level of stored carbohydrates was evident in dark morph at low humidity. Further, total energy budget differs significantly between these two body color morphs at two humidities. Thus, body color morph-specific divergence in water-balance-related traits in *D. punjabiensis* is consistent with their adaptations to wet and dry habitats.

Introduction

Seasonal variations in quantitative traits are generally assessed by differences in thermal conditions (Kingsolver and Wiernasz, 1991; Holloway *et al*., 1997; Lee and Denlinger, 1991; Loeschcke *et al.*, 1994; Addo-Bediako *et al*., 2000; Hoffmann *et al.*, 2003; Overgaard and Sorensen, 2008), but evolutionary responses due to other environmental factors have received lesser attention (Tauber *et al*., 1998; Chown and Nicolson, 2004; Danks, 2007; Brakefield *et al*., 2007). Tropics experience relatively consistent temperatures, but there are significant seasonal variations in precipitation. Seasonal variations have been reported for body size in different *Drosophila* species (Tantawy, 1965; Kari and Huey, 2000) and for desiccation resistance in a single population of *D. simulans* (Mckenzie and Parsons, 1974). In tropical species of butterflies, wet season forms (wsf) are lighter, while dry season forms (dsf) are darker and such differences correspond with local climatic adaptations (Brakefield and Reitsma, 1991; Roskam and Brakefield, 1999). However, there are limited data on seasonal adaptations in different insect species (Majerus, 1990; Osawa and Nishida, 1992; Aldridge *et al*., 1993; Kari and Huey, 2000; Torres and Madi-Ravazzi, 2006).

Heritable changes in body melanisation with possible fitness consequences constitute a suitable model for phenotypic evolution in different insect species (Majerus, 1998; Hollocher *et al*., 2000a, b; Llopart *et al*., 2002; True, 2003; Wittkopp *et al*., 2003; Dombeck and Jaenike, 2004). In different insect taxa, there are diverse patterns of body melanisation, *i.e*. (a) several black species of Collembola occur in temperate regions, *i.e*., Pyrenees, Swiss Alps, and Himalayas (Mani, 1968; Rapoport, 1969); (b) in *D. melanogaster*, a cosmopolitan species, the extent of melanism varies with geographical location (Pool and Aquadro, 2007; Parkash *et al*., 2008a, b); (c) discrete melanic and non-melanic morphs occur as genetic polymorphism in species of montium species subgroup (Ohnishi and Watanabe, 1985). Such diversity of melanisation patterns might correspond with contrasting changes in thermal and/or humidity conditions in the temperate *vs*. tropical regions. In temperate localities, melanics can warm up faster and become active as compared with non-melanics (Watt, 1968; Brakefield and Willmer, 1985; Berry and Willmer, 1986; True, 2003). Thus, the increase in the frequency of melanic morph in temperate regions is in agreement with thermal melanism hypothesis.

Besides thermal effects, body melanisation can control water balance in ectothermic insect species (Rajpurohit *et al*., 2008). The role of body melanisation in conferring desiccation resistance has been shown in a generalist species – *D. melanogaster* (Parkash *et al*., 2008b), in a cold-adapted species – *D. immigrans* (Parkash *et al*., 2008c), and a tropical species – *D. polymorpha* (Brisson *et al*., 2005). These studies have shown that assorted darker and lighter flies from a given population differ in their desiccation resistance level and show differential rates of cuticular water loss, which are negatively correlated with body melanisation (Parkash *et al*., 2008a, b, c). In order to test which of the climatic variables could be a selective agent in the tropics, we may raise the following hypothesis: (a) changes in phenotypic frequencies of dark and light morphs may occur through thermal effects and/or humidity changes; (b) humidity changes (dry *vs*. wet) may impact water balance for different body color morphs; (c) if body melanisation confers desiccation resistance, the dark morph is expected to prevail under dry seasons, while the reverse may occur for light morph under humid conditions.

Seasonal climatic changes might be mild or harsh in different geographical regions. This is supported by significantly steeper clines for desiccation resistance in various *Drosophila* species on the Indian subcontinent (Parkash and Munjal, 1999; Parkash *et al*., 2005), but a lack of clinal variation on the Australian continent (Hoffmann and Harshman, 1999; Hoffmann and Weeks, 2007). Subtropical countries usually receive significant rainfall during only some part of the year leading to substantial variation in the level of humidity in many parts of the tropics. So the variation in temperature and humidity has profound effect on the properties of water balance. Therefore, water balance in tropical climate needs to be given special attention to incorporate the variation in its properties.

D. punjabiensis belongs to the montium species subgroup of *melanogaster* group and has been reported to be widely spread in India as well as south-east Asian regions (Parshad and Paika, 1964). This species exhibits color dimorphism for the last two abdominal segments in females, and light morph is dominant over dark morph (Ohnishi and Watanabe, 1985; Parkash *et al.*, 2009). On the Indian subcontinent, geographical populations of *Drosophila* species of the subgenus Sophophora and mesic have been investigated for water conservation (Parkash *et al.*, 2008a, b, 2010, 2012), but there are no studies on montium species of subgenus *Drosophila* despite their abundance on the Indian subcontinent.

It would be interesting to find the significance of light and dark morph in context or varying climatic condition in India. Seasonally varying environments impose strong selection and can cause rapid phenotypic changes in quantitative traits (Shaipro, 1976; Tauber, 1981), but there is a dearth of

field data in *D. punjabiensis*, which is widely spread in the Indian subcontinent.

Water availability is an important determinant of the range position of many species and is also a significant correlate of species richness in many areas. Several comparative studies of water balance in *Drosophila* have been performed (Hoffmann and Harshman, 1999); unfortunately, *D. punjabiensis* and its body color morphs are not yet explored in the Indian subcontinent. Water can be lost through the spiracles during respiration, by transpiration through the cuticle, or by excretion from the mouthparts or feces. Reductions in any or all of these routes could be responsible for lower overall water-loss rates. Excretory water loss accounts for a small fraction, no more than 6%, of total losses in either laboratory or natural populations (Gibbs *et al.*, 1997). This leaves cuticular component water losses as the main sites of water conservation. This indicates towards the vital role of cuticular lipid in terms of water balance related traits. It would be amusing to relate phenotypic plasticity with energy metabolite and how it contributes to the spread of *D. punjabiensis* in extensive space.

We find *D. punjabiensis* behavior very engrossing and one of the untouched species of the Indian subcontinent. Thus in this study we will strive to find solutions to the following questions. (i) Whether seasonal variation in frequency of body color morphs results due to thermal and/or humidity changes? (ii) Whether body color polymorphism in *D. punjabiensis* is ecologically relevant in tropics? (iii) Whether there is variation in water conservation strategy of two body color morphs and, if any, which morph performs better in which conditions? (iv) Does melanisation derive the morphological traits (water balance) on dominance of light morph over dark commands irrespective of the climate and environmental conditions?

Materials and Methods

Collections and cultures

Wild individuals of *D. punjabiensis* (n = 100–120) were collected from six latitudinally (11°02', 12°59', 17°27', 27°09', 28°35', 31°06') varying localities from tropical and subtropical parts of the Indian subcontinent. The collections were made in spring (July–August) and in autumn (October–November) seasons with net sweeping and bait traps from fruit markets and godowns (Table 1). Wild-caught females were used to initiate isofemale lines (20 lines per population). All cultures were maintained at low density (60–70 eggs per vial of 40 mm × 100 mm size) on cornmeal-yeast-agar medium at 17°C-40% RH, 17°C-80% RH and 25°C-40% RH, 25°C-80% RH. All experiments were performed with G_6 and G_7 generations on six days old mated female flies. Climatic data for thermal variables of origin of populations were obtained from Indian Institute of Tropical Meteorology (IITM; www.tropmet.res.in), but data on relative humidity were obtained from 'Climatological Tables' published by the Indian Meteorological Department, Govt. of India, New Delhi.

Trait analysis

We used 10 individuals of each replicate (10 replicates × 20 isofemale line each) of *D. jambulina* to quantify body melanisation, epicuticular lipid mass, desiccation resistance, multiple measures of water balance, and levels of energy metabolites. For flies grown at low and high humidity each of low and high temperature, we tested desiccation related traits at their respectively growth conditions, *i.e.*, 17°C-40% RH, 17°C-80% RH and 25°C-40% RH, 25°C-80% RH. Therefore, growth condition and experimental conditions were the same in our experimental setup. For analysis we use the mean of ten replicates for each isofemale line of *D. punjabiensis.*

Table 1. Geographical and seasonal climatic data of the sites of origin of *Drosophila punjabiensis* populations.

Populations	Lat.(°N)	Spring season				Autumn season			
		T_{min}(°C)	T_{max}(°C)	T_{ave}(°C)	RH(%)	T_{min}(°C)	T_{max}(°C)	T_{ave}(°C)	RH(%)
Simla	31º06'	14.8	21	17.9	85	10.4	17.2	13.8	42
Dehi	28º35'	26.8	34	30.97	58.16	20	30	24.88	21.54
Agra	27º09'	22.6	31.4	27	79	15.8	30.6	23.2	60
Hyderabad	17º27'	22.6	31.4	27	70	19.7	30	24.8	62
Banglore	12º59'	19.3	28.2	23.7	79	18.4	27.6	23	75
Coinbatore	11º02'	21.8	31.5	26.6	77	21.4	31.1	26.2	74

For each season, T_{min}, mean monthly minimum temperature; T_{max}, mean monthly maximum temperature; T_{ave}, mean monthly average temperature; RH (%), relative humidity

Analysis of body melanisation

Body melanisation of individual female flies was visually scored with an Olympus stereo-zoom microscope SZ-61 (www.olympus.com) from the dorsal and lateral views of the female abdomen, giving values ranging from 0 (no melanisation) to 10 (complete melanisation) for six abdominal segments. Further, the relative size of each abdominal segment was calculated in proportion to the largest (fourth) abdominal segment, which was assigned a value of 1.0. Because the abdominal segments differ in size, these relative sizes (*i.e*., 0.86, 0.78, 0.92) were multiplied with segment-wise melanisation scores. Data on percent melanisation were calculated as (Σ observed weighted melanisation scores of abdominal segments per fly/Σ relative size of each abdominal segment × 10 per fly) × 100 (parkash *et al.*, 2008a).

Desiccation and starvation resistance

Desiccation resistance was measured as the function of time to lethal dehydration (LT 100) under dry air. Ten batches of 10 female individual were isolated in individual dry plastic vials (40 mm × 100 mm) with 2 g silica gel at the bottom and were covered with a foam disc. The vials were then placed in a respective growth condition (humidity chambers). The numerical value for desiccation and starvation is obtained by the ratio of water left after death to the difference of initial weight to weight of dried fly after dehydration protocol. A fly is announced dead when it does not show any motion. We pooled data on isofemale lines for survival curve analysis. Similarly, starvation stress was given in the same set up except silica gel is replaced with cotton ball dipped in 2 ml of distilled water to provide moisture.

Basic measures of water balance

To estimate total body water content and dehydration tolerance (%), 10 flies of each isofemale line were used. First, individual flies were weighed on Sartorius microbalance (model CPA26P, 0.001 mg precision) and then reweighed after drying at 60°C overnight. Total body water content was estimated as the difference between masses before and after drying at 60°C. Further, after mild anesthesia (1 min) with solvent ether, flies were weighed on a Sartorius microbalance both before and after desiccation stress until death.

Dehydration tolerance was estimated as the percentage of total body water lost until death due to desiccation and was calculated by the formula: (wet body mass – body mass at death) / (wet body mass – dry body mass) × 100 (Gibbs *et al.*, 1997). For calculation of the rate of water loss in *D. punjabiensis*, we followed the method of Wharton (1985), modified by Benoit *et al.* (2005) and

Yoder *et al.* (2009). Total body water content (*m*) was calculated as the difference between wet or fresh (*f*) and dry mass (*d*), *i.e.*, $m = f - d$. Individual flies were weighed and placed at ave (average percent relative humidity/100) for a specified time at 1 h intervals (1 to 8 h) and reweighed. The rate of water loss was derived from the slope of regression line on a plot of $\ln(m_t/m_0)$ against time according to Wharton's exponential equation (1985) $m_t = m_0 e^{-kt}$, where m_t is the water lost at time *t*, and m_0 is the initial water content. Rate (k_t) is the slope of the regression line and is expressed as % per hour. To verify comparison between control and organic solvent treated flies, students *t* test is applied, which gives significant difference between them.

Assessment of extractable hemolymph content

Individual flies were carefully pinned to a microdissection dish at its anterior and posterior ends with microdissection pins, and a narrow incision was made through the cuticle with a third pin while visually observing through a stereo-zoom microscope. The leaking extractable hemolymph was absorbed with an absorbent tissue moistened with an isotonic saline solution (Folk *et al.*, 2001). Extractable hemolymph content was estimated as reduction in mass following hemolymph blotting (Cohen *et al.*, 1986; Hadley, 1994). Further, tissue water was estimated after subtracting exsanguinated mass before and after drying. From the same data, we also calculated hemolymph water content by subtracting tissue water from total body water content.

Lipid content

Individual 8 day old mated female flies were dried in 2 ml Eppendorf tubes (www.tarson.in) at 60°C for 48 h and then weighed on a Sartorius microbalance (model CPA26P, 0.001 mg precision). Thereafter, 1.5 ml di-ethyl ether was added in each eppendorf tube and kept for 24 h under continuous shaking (200 r/min.) at 37°C. Finally, the solvent was removed and flies were again dried at 60°C for 24 h and reweighed. Lipid content was calculated per individual fly by subtracting the lipid free dry mass from initial dry mass per fly (Hoffmann *et al.*, 2001).

Assay sensitivity for cuticular lipids and total body lipids

We tested the assay sensitivity by measuring cuticular lipids as well as total body lipids in the replicate samples. We followed 1 h treatment with hexane without shaking for cuticular lipids. However, for total body lipids, we first removed cuticular lipids followed by 24 h treatment with di-ethyl ether with continuous shaking at 200 r/min. We did not find a difference in the estimates of either cuticular lipids or total body lipids under our assay conditions.

Trehalose and glycogen estimation

For trehalose and glycogen content estimation, 10 flies of each isofemale line were homogenized in a homogenizer (Labsonic M, www.sartorius.com) with 300 µL Na_2CO_3 and incubated at 95°C for 2 h to denature proteins. An aqueous solution of 150 µL acetic acid (1 mol/L) and 600 µL sodium acetate (0.2 mol/L) was mixed with the homogenate. The homogenate was subsequently centrifuged (Fresco 21, ThermoFisher Scientific, USA) at 12,000 r/min for 10 min. This homogenate was used for independent estimations of trehalose and glycogen as given below.

For trehalose estimation, aliquots (200 µL) were placed in two different tubes; one was taken as a blank whereas the other was digested with trehalase at 37°C using the Megazyme trehalose assay kit (K-Treh 10/10, www.megazyme.com). In this assay, released D-glucose was phosphorylated by hexokinase and ATP to glucose-6- phosphate and ADP, which was further coupled with glucose-6-phosphate dehydrogenase and resulted in the reduction of nicotinamide adenine dinucleotide (NAD). The absorbance by NADH was measured at 340 nm (UV-2450-VIS, USA). The pre-existing glucose level in the sample was determined in a control reaction lacking trehalase and subtracted from total

glucose concentration. For estimation of glycogen, a quantity of 50 µL aliquots were incubated with 500 µL *Aspergillus niger* glucoamylase solution (8.7 U/mL in 200 mmol/L of acetate buffer) for 2 h at 40°C with constant agitation, and the suspension was centrifuged at 4000 r/min for 5 min. It mainly hydrolyzed alpha-(1,4) and alpha-(1,6) glycosyl linkages and was suited for breakdown of glycogen. Glucose concentration was determined with 20 µL of supernatant from the suspension and added to 170 µL of a mixture of G6-DPH (0.9 U/mL), ATP (1.6 mmol/L), and NADP (1.25 mmol/L) in triethanolamine hydrochloride buffer (380 mmol/L TEA–HCl and 5.5 mmol/L of MgSO4) and 10 µL of hexokinase solution (32.5 U/mL in 3.2 mol/L ammonium sulphate buffer), and absorbance was measured at 340 nm.

Protein assay

Protein levels were determined using the bicinchoninic acid (BCA) method as followed by Marron and coworkers (Marron *et al.*, 2003). For the protein assay, 10 mated female flies per isofemale line were homogenized in 3 mL distilled water and centrifuged at 10,000 r/min for 5 minutes. Further, a 50 µL aliquot was taken from the supernatant and treated with 2 mL BCA reagent (Sigma-Aldrich,) and incubated at 25°C for 12 h. Absorbance was recorded at 562 nm, and protein concentration was determined by comparison with a standard curve.

Energy metabolites

We measured each metabolite (carbohydrates, lipids, or proteins) in 10 replicate sets of 20 isofemale lines. Total energy budget was calculated using standard conversion factors following Schmidt- Nielsen (Schmidt-Nielsen, 1990).

Statistical analysis

For each trait, population means (20 isofemale lines, 10 replicates each) are presented ± s.e.m. Effects of morph on desiccation-related traits, energy metabolites, body weight, basic measures of water balance, and dehydration and starvation effect were compared with mixed model ANOVA (morph and humidity: fixed effect; temperature: random effect) in *D. punjabiensis*. Effects of different organic solvents on rate of water loss was compared with student's *t* test (Zar, 1999). Total energy budget in *D. punjabiensis* due to differential storage of energy metabolites was calculated using standard conversion factors (Schmidt-Nielsen, 1990; Marron *et al.*, 2003). For multiple comparison, we adjusted the alpha value at 0.05 significance level; asterisks denote a significant difference (*$P < 0.1$; **$P < 0.01$; ***$P < 0.001$). Statistica (release 5.0, Statsoft Inc., Tulsa, OK, USA) was used for calculations as well as figures.

Results

Humidity of the sites selected for collection varies according to the seasonal change. Autumn is characterized by more dry conditions and spring describes more humid conditions. Such variation in the field condition facilitates governance of light and dark body color morph. As shown in Figure 1A, dark body color flies more profoundly appeared in autumn, and the reverse happens in spring with more light body colored flies captured while collecting.

Effect of seasonal changes in humidity on body color polymorphism of *D. punjabiensis*

Data on percent abundance of wild caught flies of *D. punjabiensis* from 6 different sites as a function of relative humidity of origin of population are shown in Figure 1. *Drosophila punjabiensis* dark body color morph is more abundant in autumn season, whereas the lighter morph of *D.*

punjabiensis is more abundant in rainy/spring season of the same site (Figure 1A). The autumn season of India is characterized by cold and dry conditions (dark: $y = 61.60 + 0.26x$; and light: $y = 38.40 - 0.26x$; Figure 1B), while the monsoon put forth comparatively hot and humid climatic conditions (dark: $y = 72.21 - 0.54x$; and light: $y = 27.78 + 0.54x$; Figure 1C). Therefore, significant reduction in T_{ave} as well as relative humidity along an elevation gradient may act as selection factors for abundance of dark morph. Thus, darker morph of *D. punjabiensis* is better adapted to cold and dry climatic condition as compared to lighter morph.

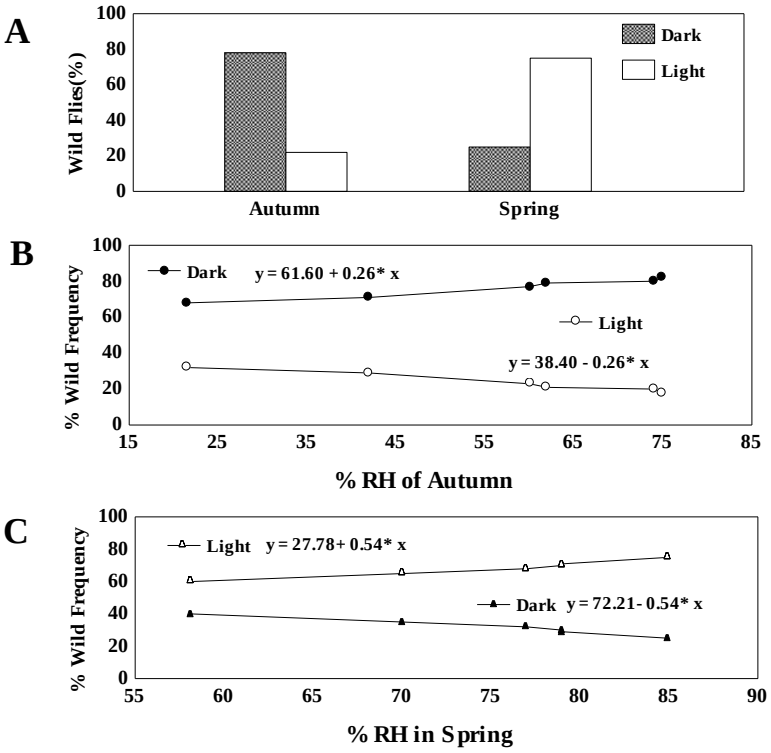

Figure 1. Field data on seasonal changes in the % frequency of dark and light morphs in (A) autumn & rainy population; comparison of light and dark morph frequency in humidity of the six sites selected across the latitude (B & C) in autumn and rainy season, respectively, of *D. punjabiensis*. The number of wild-caught individuals was 200–250 for each season as well as locality.

Body melanisation

We observed melanisation in relation with the humidity condition on the growth temperature (Figure 2). It was startling to find constant melanisation (74 ± 1.23: mean ± S.E) score of dark morph at both the humidity condition of low temperature (17°C) (Figure 2E), but decreases at high humidity (80% RH) of high temperature (25°C), *i.e.*, 60 ± 1.45 in comparison to low humidity (40% RH) of high temperature (25°C), which is 62.4 ± 1.53 (Figure 2F), whereas melanisation scores of light morph is approximately equal irrespective of the humidity and growth temperature. Melanisation at low and high humidity at both temperatures 17°C and 25°C is ~32% (Figure 2E, F).

Desiccation and starvation resistance

We found significant differences in desiccation survival hours of dark morph of *D. punjabiensis* at both the temperatures of low humidity (40% RH), ($F = 10.28$, $P < 0.001$); Figure 2C, Table 2, in comparison both temperature of high humidity (80% RH) ($F = 19.01$; $P < 0.001$) (Figure 2D, Table 2). In contrast, we found rather different results in starvation resistance, here light morph produced a better result and survived more hours in comparison to dark morph at low temperature of high humidity, *i.e.*, ~158.88 hrs at 17°C of 80% RH ($F = 331600$; $P < 0.01$, Table 2).

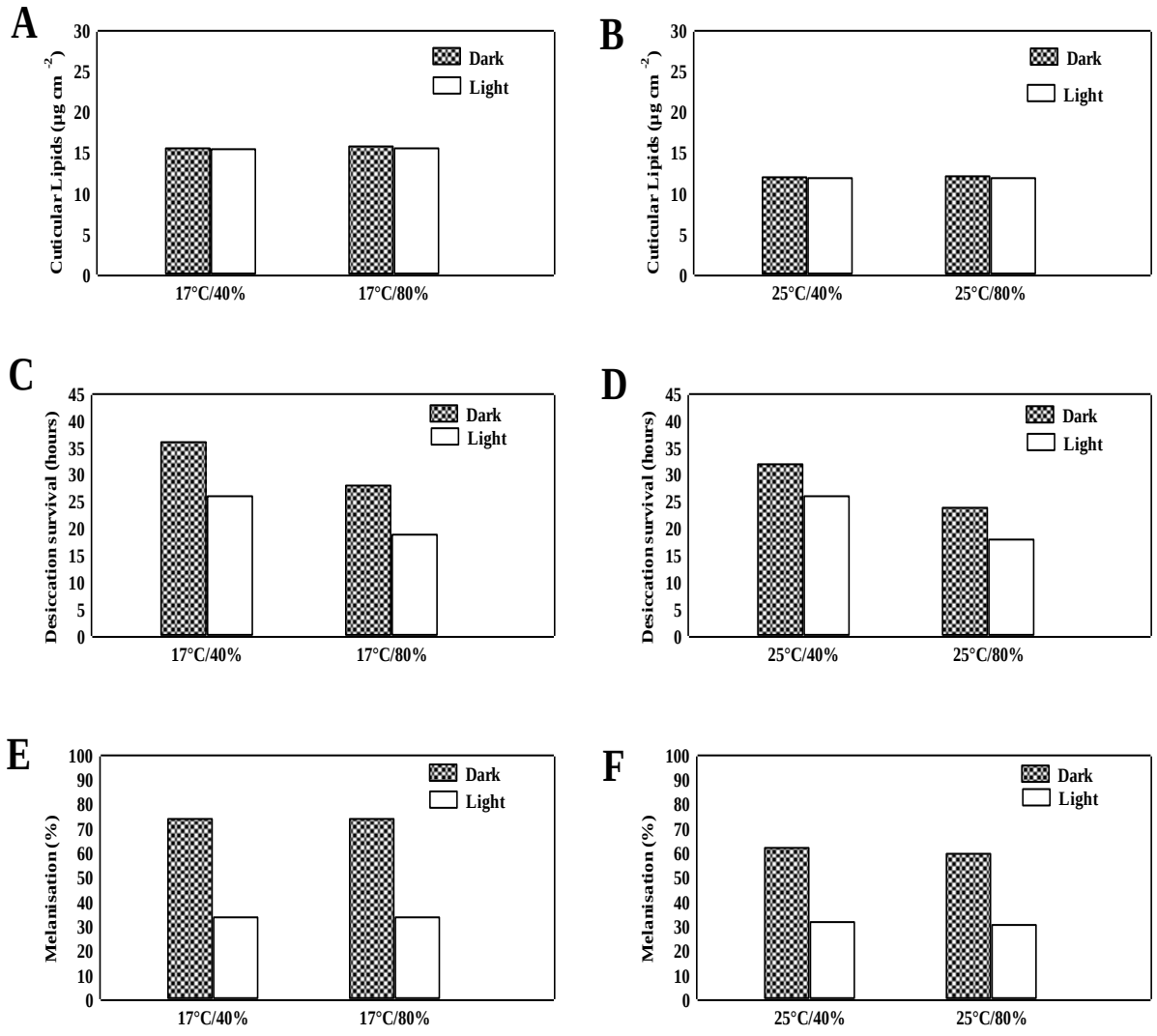

Figure 2. Mean ± s.e. bars of cuticular components (% melanisation E, F, and cuticular lipids A, B) along with desiccation survival hours (C, D) for low (40% RH) and high humidity (80% RH) maintained in a cold (17ºC) and warm temperature (25ºC), respectively.

Dehydration tolerance

Dark morph of *D. punjabiensis* happens to be more tolerant to dehydration conditions at high as well as low humidity at both growth temperatures in comparison with light morph, thus lost more water in the process (Table 2).

Basic measures of water balance and tissue water

Data on morph-specific differences in water balance traits, desiccation hours, and starvation hours due to high and low relative humidity at two different growth temperatures (17°C and 25°C) in adult stage of *D. punjabiensis* are shown in Table 2, Figure 3A, and Figure 3B. In flies reared at low humidity of 17°C, we observed a significant increase in wet and dry mass as well as total body water

content and tissue water content (~1.5 fold) of dark morph in comparison to light morph (wet mass: $F = 168.2$, $p < 0.01$; dry mass: $F = 3411$, $p < 0.01$; total body content: $F = 3572$, $p < 0.01$; tissue water content: $F = 1851$, $p < 0.01$; Table 2). Similar inclination was observed with dark morph reared at different temperatures of high humidity 80% (wet mass: $F = 554$, $p < 0.01$; dry mass: $F = 6004$, $p < 0.01$; total body content: $F = 7336$, $p < 0.01$; tissue water content: $F = 2178$, $p < 0.01$; Table 2).

Table 2. Data on trait values (mean and ANOVA, *F**) of adult flies (6 days post eclosion) for basic measures of water balance (a), dehydration tolerance (b), desiccation (c), starvation (d) in dark and light body color morph of *D. punjabiensis* for low (40%RH) and high (80%RH) humidity maintained at cold (17°C) and warm (25°C) temperature.

Traits	Low humidity				F value	High humidity				F value
	17°C		25°C			17°C		25°C		
	Dark	Light	Dark	Light		Dark	Light	Dark	Light	
a) Basic measures of hemolymph and tissue water										
Wet mass (mg fly $^{-1}$)	1.83	1.28	1.05	0.70	168.2**	1.60	1.06	0.97	0.65	554.0**
Dry mass (mg fly $^{-1}$)	0.549	0.384	0.32	0.21	3411**	0.48	0.32	0.29	0.20	6004**
Total water content (mg fly $^{-1}$)	1.281	0.896	0.73	0.49	3572**	1.12	0.74	0.68	0.45	7336**
Hemolymph content (mg fly $^{-1}$)	0.46	0.19	0.26	0.11	3078**	0.40	0.17	0.24	0.10	3690**
Wet mass after hemolymph removal (mg fly $^{-1}$)	1.37	1.09	0.79	0.59	5228**	1.20	0.89	0.73	0.55	7748**
Hemolymph water content (mg fly $^{-1}$)	0.322	0.133	0.177	0.077	4212**	0.28	0.21	0.17	0.07	2064**
Tissue water content (mg fly $^{-1}$)	0.959	0.763	0.553	0.413	1851**	0.84	0.53	0.51	0.38	2178**
b) Dehydration tolerance (%)	98.59	82.77	96	78	212510**	91.93	70.78	76	59	3170**
c) Desiccation survival (hours)	36	26	28	19	10.28***	32	26	24	18	19.01***
d) Starvation (hours)	78.42	116.12	60.25	89.25	3010**	118.12	158.88	87.70	122.6	331600**

*F** value here is the interaction of morph and humidity conditions; **p< 0.01; ***p<0.001

Hemolymph content

Hemolymph content was found to be higher in dark morph at both humidities in comparison to light morph (~2 fold, Table 2). Dark morph of both low and high growth temperature at low humidity showed 58% more hemolymph content than light morph, whereas light morph of both low and high growth temperature at high humidity showed 42% less hemolymph then dark morph (Table 2).

Analysis of energy budget (J/mg) of carbohydrates, lipids, and proteins of adult

Analysis of energy budget (J/mg) of carbohydrates, lipids, and proteins of adult flies (6 days post eclosion) in dark and light body color morph of *D. punjabiensis* for low (40%) and high (80%) humidity maintained at cold (17°C) and warm (25°C) temperature is shown in Table 3. The storage level of proteins was similar in both the morphs (0.712 J/mg). Based on standard conversion factors (Schmidt-Nielsen, 1990; Marron *et al.*, 2003), we compared the energy budget due to each metabolite (carbohydrates or lipids or proteins) in both body color morphs of *D. punjabiensis* (Table 3). Dark morph has greater carbohydrate storage level, while light morph has greater triglyceride level for both humidities and at both temperatures (Figures 3,C, D, respectively).

Discussion

For ectothermic insects, spatial variations in quantitative traits are well documented, but temporal variations have received lesser attention (Bijlsma and Loeschcke, 1997; Hoffmann and

Table 3. Analysis of energy budget (J/mg) of carbohydrates, lipids and proteins of adult flies (6 days post eclosion) in dark and light body color morph of *D. punjabiensis* for low and high humidity at 17°C and 25°C.

Metabolites	Low humidity				High humidity			
	17°C		25°C		17°C		25°C	
	Dark	Light	Dark	Light	Dark	Light	Dark	Light
Carbohydrates	9.28	5.62	7.10	4.32	8.92	5.60	6.82	4.30
Lipids	5.84	7.68	4.36	5.58	5.99	9.05	4.44	6.70
Proteins	0.712	0.712	0.712	0.712	0.712	0.712	0.712	0.712
Total	15.83	13.42	12.17	10.61	15.62	15.36	18.38	11.71

Conversion factors: 17.6 J/mg for carbohydrates; 39.3 J/mg for lipids; and 17.8 J/mg for proteins. (Schmidt-Nielsen 1990; Marron *et al*., 2003

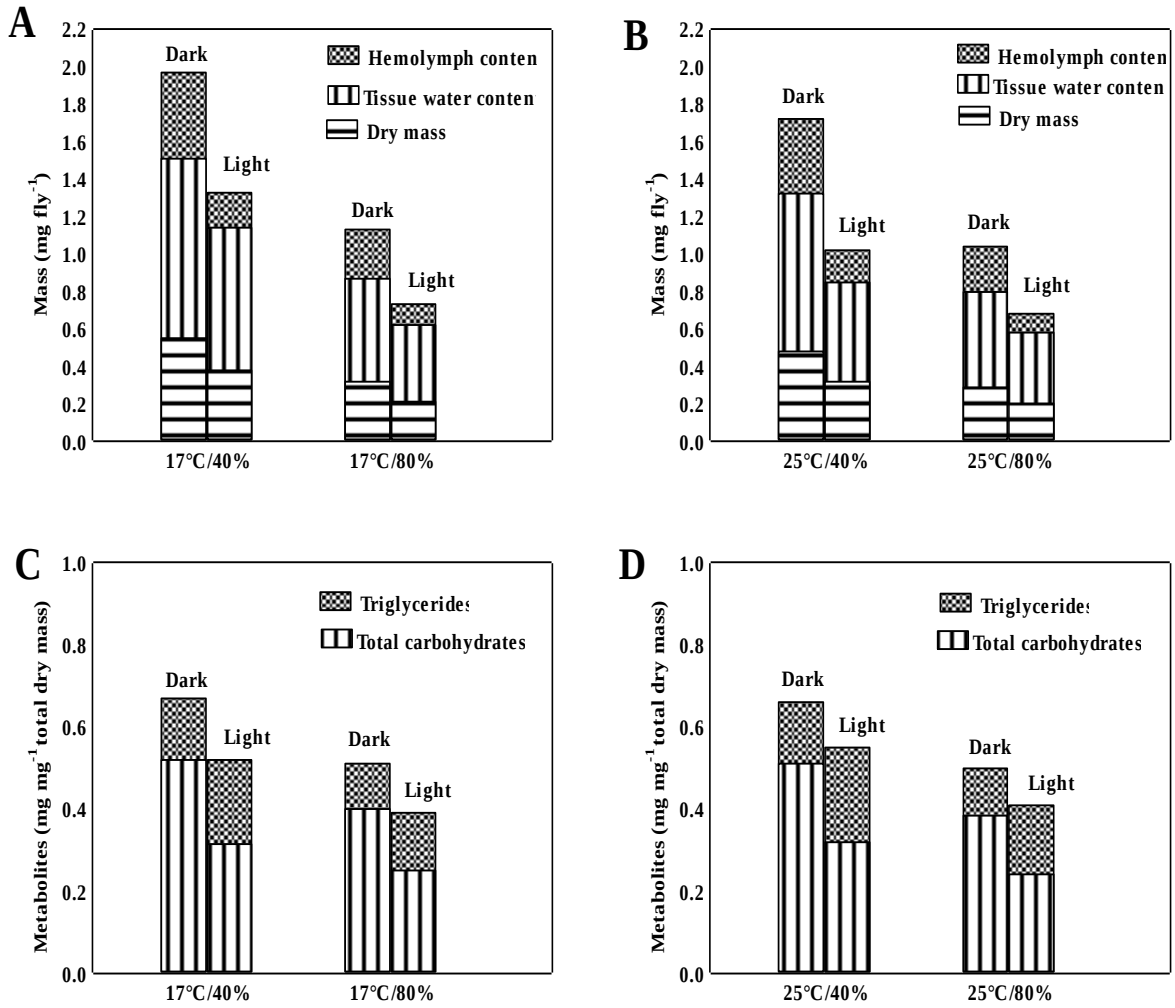

Figure 3. Water balance related traits of dark and light body color morphs at variable humidity condition of 17ºC and 25ºC. Comparison of hemolymph content, tissue water content, and dry mass of dark and light body color morph (A & B, respectively) and triglycerides and total carbohydrates of dark and light morph (C & D, respectively) for low and high humidity at 17°C and 25°C.

Weeks, 2007). In temperate regions, several studies have shown huge variations in body size for different *Drosophila* species. By contrast, there are few studies on the analysis of desiccation related traits (which show high heritability) in wild populations living under seasonally varying conditions. A single study on Australian populations of *D. melanogaster* has shown lack of temporal variation in desiccation resistance (Mckenzie and Parsons, 1974). However, on the Indian subcontinent, there are significant seasonal variations in the montane localities (Table 1). Our data have evidenced considerable changes in body melanisation and desiccation related traits across two seasons (autumn and spring). Further, there is lack of changes in cuticular lipids which do not account for variations in desiccation resistance across seasons. To the best of our knowledge, adaptive significance of body color plasticity to cope with seasonally varying desiccation stress has not been previously analyzed.

D. punjabiensis falls under the cosmopolitan category, which simply means it is accessible throughout the year at various sites. Our collection along a constant longitude and constantly variable latitude rightfully signifies the survival of this very species at varying climatic condition. Melanism is common in insect taxa, but its evolutionary causes are quite diverse (True, 2003; Wittkopp *et al*., 2003). Ohnishi and Watanabe in 1985 reported the presence of body color polymorphism in *D. punjabiensis.* As the Figure 1 implies, availability of the species is constant all over the year but the frequency of the body color morph varies according to season. We discovered that the frequency of the morph is subject to temperature and humidity conditions, in which survival of darker morph is enhanced in autumn conditions and monsoon/ rainy is suitable for lighter morph. Thus the seasonally varying environment puts a selection within the species. The case seems to be similar to the study of pepper moths (Tutt, 1896; Kettlewell, 1955, 1956). To examine the significance of melanisation or any other mechanism, we tried to mimic the nature's condition in laboratory scale and performed the earlier described test along with help of various literatures.

On the Indian subcontinent, T_{ave} does not vary along latitude. There is lack of correlation between T_{ave} and latitude ($r = 0.19 \pm 0.44$; ns). By contrast, humidity changes are significant along latitude ($r = 0.92 \pm 0.10$). Field data have shown significant seasonal variation in the percent frequency of dark and light morphs, *i.e*., greater frequency of melanic morph in autumn as compared to rainy/monsoon season (Figure 1). Data on varying humidity conditions under laboratory set-up have also shown changes in the frequencies of dark and light morphs. Thus, seasonally varying humidity seems to be the principle selective agent for adaptive changes in the frequencies of dark and light body color morphs.

Our study comes up with some new and interesting nitty-gritty about *D. punjabiensis*. Talking in terms of dominance, Watanabe (1985) and Parkash *et al.* (2009) have already proved that light morph is prevailing over dark body color morph, but high frequency of dark morph in the autumn season indicates different selection criteria. Our study signifies that dark morph flourish better at low humidity and low temperature, whereas high temperature and high humidity condition suits better to light morph. This is in relation to circumstances found in nature. Genetically light morph dominates over dark, but when it comes to survival at low temperature and humidity condition, dark morph performs better. Melanisation provides the required resistance and hence makes it more adaptive. This is perfectly shown by constant melanisation score of dark morph at variable humidity (74 ± 1.23: mean ± S.E at both 40% and 80% RH of low temperature - 17°C). In conclusion, melanisation derives the elevated morphometric traits (water balance) in the dark body color morph, irrespective of it being recessive, than light morph. Water balance traits and performance in extreme condition are not the deciding factor for dominance. It settles on the appearance of a particular trait in the subsequent progeny, when a cross is made between two extremely contrasting traits.

Unlike temperature, the role of humidity as a selection agent has not been considered for different quantitative traits in various *Drosophila* species so far. A single study has used laboratory

selection to determine the evolutionary effects of relative humidity, temperature, and their interactions on adult wing area (body size) in *D. melanogaster* (Kennington *et al*., 2003). In this study, after 20 weeks of laboratory selection, low RH lines had significantly greater wing area than high RH lines. Thus, body size in *D. melanogaster* can evolve rapidly in response to humidity selection. Thus, it may be argued that evolutionary responses for desiccation stress related traits might result due to humidity selection. Based on evidence in the present studies, we find that changes in the frequencies of dark and light morphs correspond with humidity selection under field conditions.

Possible role of body melanisation in conferring desiccation resistance was initially demonstrated for ebony mutant strains of *D. melanogaster* (Kalmus, 1941). Subsequently, darker and lighter laboratory strains of *D. polymorpha* were shown to differ in desiccation resistance (Brisson *et al*., 2005). For altitudinal populations of *D. melanogaster*, assorted dark and light flies exhibited significant differences in desiccation resistance and cuticular water loss (Parkash *et al*., 2008b). In correlation with the previous study, we also come up with the same result that dark morph has higher survival under desiccation condition which indicates towards melanisation as the underlying mechanism of resistance towards water loss.

Insects can enhance their desiccation resistance by increasing their total body water content, reducing the rate of body water loss, and tolerating a larger proportion of overall water loss from the body (Hadley, 1994; Gibbs *et al.*, 1997). Dark body color morph of *D. punjabiensis* contains the highest body water content at low humidity and temperature condition, thus showing higher desiccation resistance. At the same condition on the other hand lighter morph does not show any such relation with the temperature and humidity conditions suitable to it. This makes us think the requirement of desiccation resistance when a species is enjoying better temperature and humidity conditions. This indicates lighter morph of *D. punjabiensis* does not require any such resistance mechanism thus did not perform in desiccating conditions.

In the present study, we tested the role of cuticular lipids to support desiccation resistance, as expected amount of cuticular lipid and survival in desiccation stress are highest for dark morph in low humidity and low temperature condition. On the other hand such mechanism seems to be missing in light morph; therefore, no better performance at favorable conditions but slight hint of developing such mechanism at adverse condition is foreseen. This is advent with significantly better survival of light morph at low humidity and temperature condition, which is certainly not suitable for their survival in nature.

In the present study seasonal data on wild living flies have shown frequency changes of dark and light morphs in *D. punjabiensis*, which correspond with varying humidity levels at six different latitudinal sites. Change in humidity level is the principle agent of natural selection, which modifies the frequencies of dark and light morphs. The melanism-desiccation hypothesis finds support from studies on body color mutant strains (Kalmus, 1941), use of assorted dark and light individuals from a given population (Parkash *et al*., 2008b). In tropical regions, seasonal changes in precipitation cause desiccation stress in autumn. *D. punjabiensis* has adapted to wet (spring season) and dry (autumn) seasons by modifying the frequencies of color morphs through assortative matings. Laboratory data on *D. punjabiensis* show evidence in favor of melanism-desiccation hypothesis. Like *D. punjabiensis*, other species of montium species subgroup are expected to provide similar evidence. Thus, seasonally varying humidity conditions in the tropics can maintain body color polymorphism and desiccation resistance in *D. punjabiensis.*

Acknowledgment: Financial assistance from University Grants Commission, New Delhi (F 41-823/2012/SR) is gratefully acknowledged.

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Sexual selection in search of good genes: analysis of mate choice experiments and ecophysiological stress tolerance in *Drosophila biarmipes*.

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Abstract

Female preference for male ornament is favored by sexual selection. It is not clear whether the preference is limited to male ornament only or it is actually for genes that affect fitness of the upcoming progeny. In mating systems in which males provide direct benefits of fitness to the female or their offspring, the answer seems straightforward – females should prefer to mate with males that are able to provide more resources. We tested the hypothesis whether spotted and spotless males and progeny from sexually preferred males of *Drosophila biarmipes* vary in their levels of environmental stress tolerances. Our results showed that male flies with dimorphism for wing spot varied in their mating success and stress tolerance. Data have shown significant differences in mated pairs with spotted and spotless males. Spotted and spotless males also differ significantly in ecophysiological stress tolerance. Finally, females mating with a high-quality male (spotted wing) results in offspring with high performance. We found significant effects on fecundity of mated females and egg-to-adult viability of their progeny. Our results are consistent with good genes sexual selection and suggest that mate choice could provide indirect benefits to females. To the best of our knowledge, this is the first report on the ecological significance of wing color dimorphism in a species – *Drosophila biarmipes.* **Keywords:** wing spot dimorphism, *D. biarmipes*, sexual selection, good gene.

Introduction

Evolutionary and behavioral ecologists have long been interested in, and puzzled by, mate choice. In many species, females are highly selective when it comes to mating (Darwin, 1871; Bateson, 1983; Andersson, 1994; Kokko *et al*., 2003). In some of these species, females are congruent in their mate preference for a particular male, while in other species, females are incongruent in their preference, with each preferring a different male. The least controversial models of female mate choice emerged from resource-based mating systems in which males provide resources directly to females or offspring. These resources obviously could have a profound impact on female fitness. Furthermore, researchers realized that natural selection could lead to the evolution of male indicator traits that facilitated mate choice by advertising the quality or quantity of a male's resources (Møller and Jennions, 2001). Several experimental studies on captive domestic or laboratory species reported indirect benefits (Welch *et al*., 1998; Moller and Alatalo, 1999; Petrie, 1994; Evans *et al*., 2004; Drickamer *et al*., 2000; Bluhm and Gowaty, 2004), but a demonstration that the free choices of females in nature influence offspring survival by means of a sire effect. Good genes models of sexual selection predict the evolution of female mate choice based on preferences for male traits associated with additive genetic variance in fitness (Jennions and Petrie, 2000; Iwasa *et al*., 1991; Houle and Kodrashov, 2002). The strength of the association between secondary sexual characters and offspring survival suggests that viability selection may render small but significant

increases in fitness (Moller and Alatalo, 1999), but some studies suggest otherwise (Qvarnstrom *et al*., 2006), and the subject of indirect selection on female mate choice or mating behavior remains a contentious issue in the study of sexual selection (Cameron *et al*.. 2003).

Wing melanisation patterns in insects are highly diversified and have played an important role in thermoregulation, mate choice, defense against predators and mimicry in various species of butterflies (Watt, 1968; Roland, 1982; Kingsolver, 1987; Wiernasz, 1989; Ellers and Boggs, 2002, 2003). In alpine (*Colias* species) as well as copper butterflies, wing melanisation has been associated with increased flight ability under colder environmental conditions (Watt, 1969; Roland, 1982; Ellers and Boggs, 2004; Karl *et al.*, 2009). Some studies have shown the role of wing patterns in mate recognition in butterflies (Wiernasz and Kingsolver, 1992; Jiggins *et al.*, 2001); Calypterygid damselfly (Siva-Jothy, 1999), whereas quite a few studies have shown the direct effect of wing spot on mated pairs in two *Drosophila species – D. suzukii* (Fuyama, 1979) and *D. biarmipes* (Singh and Chatterjee, 1987). However, for *Drosophila* species the ecological significance of wing spot dimorphism and sexual preference for a particular morph remains largely unknown.

Drosophila biarmipes, a warm adapted tropical species, belongs to Oriental region (eastern part of Asia), and in India, except northern region, it is found in all the parts (Markow, 2006). Based on morphological features, *D. biarmipes* was assigned to the suzukii subgroup of the melanogaster species group. In *D. biarmipes*, melanic spots always appear in roughly the same area of the wing, and the melanin pattern takes the form of an intense spot centered on the distal L-2 vein (Kopp and True, 2002). This pattern is limited to males, although very slight melanization is occasionally seen in females of this species. However, it is intriguing that this species is spread in the south to north Indian localities, which encounter seasonally varying climatic conditions. On the Indian subcontinent, $T_{ave.}$ is poorly correlated with latitude ($r = -0.54 \pm 0.30$), but seasonal variations increase with latitude. For example, seasonal variations (calculated as coefficient of variation in mean monthly temperature or humidity changes) have shown lower values in the south ($T_{cv} = 3.14\%$ and $RH_{cv} = 5.39\%$) as compared with north ($T_{cv} = 29.6\%$ and $RH_{cv} = 26.6\%$). Further, rainy and autumn seasons differ significantly in thermal as well as humidity conditions in the north. Thus, we may expect evolutionary responses to natural selection on traits related to desiccation and cold stress in subtropical populations of *D. biarmipes*.

We considered *D. biarmipes* suitable to find answers to the following questions: (i) whether there is plasticity for wing spot? (ii) whether there is any role of wing spot in mating success (mating latency, copulation duration)? (iii) do the spotted and spotless males differ in desiccation stress, corresponding rate of water loss and cold tolerance? (iv) Finally, we tested whether there is significant variation in two important components of fitness (fecundity and egg-to-adult viability) in *D. biarmipes*. Present work on impact of wing spot on mating success is interesting in several respects. (a) in *D. biarmipes*, there is no plasticity for wing spot. (b) a significant difference in mating propensity and copulation duration of spotted males and spotless males. Spotted males were desiccation resistant and have lower rates of water loss and are more tolerant towards cold stress. We have performed a quantitative genetic analysis of egg-to-adult viability and adult survival to sexual maturity. We found significant levels of differences in offspring fitness, supporting a picture of good genes sexual selection in this species.

Material and Methods

Collections and cultures

Wild *D. biarmipes* (n = 180-200 per population) were collected from four lowland localities (~ > 200 m; Rohtak, Chandigarh, Mandi and Kalka) by net sweeping method. Wild caught females

were used to initiate isofemale lines (20 lines per population). All cultures were initiated with 6-8 hour egg laying period and maintained at low density (60-70 eggs per vial of 37 × 100 mm size) on cornmeal yeast-agar medium at 21°C. All experiments were initiated soon after collections and performed with G_1 and G_2 (Generations 1 and 2) in order to avoid possible effects of laboratory adaptation. All assays were performed on 7 day old flies (sexed soon after eclosion). For each isofemale line as well as wild caught individuals of both the species, we first analyzed wing spot, and this was followed by mating latency (ML), copulation duration (CD), and desiccation resistance and rate of water loss. Males of *D. biarmipes* show wing spot dimorphism, whereas females lack wing spot.

For investigating developmental plastic effects of wing spot, 25 to 30 pairs of each isofemale line were allowed to lay eggs at 21ºC in 20 replicate vials. Five such vials were then transferred to each of 19º, 21º, 25º, and 28ºC growth temperatures for *D. biarmipes*. Thus, we checked plastic effects for wing spot area at these growth temperatures.

Since ANOVA showed non-significant F-values between wing morphs isolated from different populations, the data were pooled for spotted males. Likewise, data on spotless males were also pooled. For each trait, 10 isofemale lines per population were used and 10 randomly chosen individuals per strain were investigated. Flies were sexed soon after eclosion and were maintained as virgins on cornmeal medium seeded with live yeast. All flies were 6 to 8 days post-eclosion at the start of desiccation stress assays. For measuring desiccation stress, ten virgin males of each morph were isolated in a dry plastic vial, which contained 2 g of silica gel at the bottom and were covered with a disc of foam piece. Such vials with foam plugs were placed in a desiccation chamber (Secador electronic desiccator cabinet), which maintains 1-2% relative humidity. Mortality due to desiccation stress was inspected every hour until half the flies (LT_{50}) died, and thereafter observations were made every half an hour. Desiccation survival curves were drawn as a function of time of desiccation stress.

Rate of water loss (mg hr^{-1}) due to short-term desiccation (8 hr) was estimated in groups of five flies. Both before and after desiccation, flies were weighed on a Sartorius microbalance and water loss/hr was calculated as: (initial body weight – body weight after 8 hour desiccation stress)/ initial body weight × 8. Total body water (%) was estimated as the difference between wet and dry weight / initial wet weight × 100.

To evaluate cold hardiness or chill coma recovery, spotted and spotless males were placed in groups of ten each in 10 ml glass vials, which were submerged in a 10% glycol solution cooled to 0ºC. The vials were removed after varying duration of stress (1 – 10 hours) and recovery time was scored. The flies were considered recovered when they were able to stand up on their legs.

Mating propensity experiments on laboratory reared virgin females and males (spotted and spotless) were performed. In each mating chamber, 10 virgin females and 5 virgin males, spotted as well as spotless, were placed and observations on 10 such pairs were made for 60 minutes under female choice condition. For all the observed matings, % mated pairs (MP); mating latency (ML; the time from introduction of flies to initiation of copulation time); copulation duration (CD; from initiation to detachment of mated pairs); were recorded. In this way, matings were observed for 10 lines × 10 replicates each.

Fecundity of mated females and egg-to-adult viability of progeny

For estimating fecundity, each mated pair was aspirated and placed in an oviposition chamber for 24 hours and thereafter the male was removed. The eggs laid on the food placed at the replaceable bottom plate of the oviposition chamber were counted daily. The flies were transferred to fresh food vials every day, and the number of eggs laid during 24 hours by each female was recorded.

This was followed for 15 successive days (7^{th} to 31^{st}) as this period coincided with maximum egg production, and the data were shown as daily fecundity.

Given that each individual replicate (n = 20 lines × 10 replicates, for each of spotted and non-spotted male) contains a single offspring, we were able to measure egg-to-adult viability by monitoring adult emergence from the eggs. Egg-to-adult viability can be affected by mortality during embryonic, larval, or pupal stages and their transitions. Emergence of adults occurred over the period of two weeks. On emergence, flies undergo a period of maturation feeding, during which males mature their testes and begin to produce sperm and females mature their ovaries and develop eggs. We adult survival to sexual maturity.

Statistical analyses

Means (n = 10 lines × 10 replicates × 10 individuals) along with standard deviation (SD) for all traits were used for illustrations and tabular data. Possible variations between homozygous spotted and spotless males and significant levels of other assays were checked on the basis of ANOVA. The Statistica package (Statsoft Inc., Release 5.0, Tulsa, OK, USA) was used for statistical calculations as well as illustrations.

Table 1. Analysis of nested ANCOVA (body mass as a covariate) to compare the ecophysiological traits for wing color morphs in D. biarmipes (20 lines × 10 replicates each; lines were nested into morphs). df – degree of freedom; MS – means square; ns = nonsignificant, $^{**}P < 0.01$; $^{***}P < 0.001$; Percent data were arcsine transformed for ANCOVA.

Traits		Morphs	Lines	Error
	df	1	38	359
1. Wing Melanisation Area (%)	MS	126.48	1.34	2.64
	F	23.84***	0.41ns	
2. Cuticular lipid mass (µg cm $^{-2}$)	MS	3.31	2.24	12.10
	F	0.52 ns	1.07 ns	
3. Desiccation resistance (hours)	MS	1275.90	244.53	1.37
	F	945.17***	48.43***	
4. Rate of Water loss (%)	MS	91189.54	329.14	7.37
	F	5931.35***	49.20**	
5. Cold Survival (%)	MS	49912.01	387.21	5.21
	F	9150.04***	29.44**	

Results

We found lack of plastic effects due to three growth temperature (20°, 25°, and 30°C) for spotted and spotless wing males. Thus, the data across growth temperature were pooled for three sets of traits (body melanisation, stress related traits, and mating propensity), and results for ANCOVA on various traits between spotted and spotless males are given in Table 1. Cuticular lipid content did not vary between the two males ($F = 0.52$ ns; Table 1). Spotted and spotless males evidenced significant differences in desiccation survival curves, *i.e.*, LT_{100} values are 17.12 and 6.40 hours, respectively (Figure 1B). Spotted males exhibits significant lower rates of water loss as compared with spotless males. Since changes in body size may impact desiccation resistance, we checked vial effects on body size due to possible differences either in nutrition or density. ANOVA showed no variation due to within as well as between vial effects for populations and morphs (data not shown). Thus, the

observed differences in desiccation resistance in *D. biarmipes* cannot be attributed to body size. Interestingly, this warm adapted species lacks thermal selection effects for body size in geographical populations as well as between morphs. Further, spotted males showed significantly higher desiccation resistance as well as cold stress survival (Figure 1A, B), higher mated pair frequency, longer copulation duration, and higher fecundity per day (Figure 2). In contrast, the light morph is characterized by higher values of cuticular water loss, percent mortality, as well as longer recovery time due to cold stress (Figure 1B), and a much longer mating latency (Figure 2B). For all the traits, differences were highly significant ($p < 0.001$) on the basis of F- values from ANCOVA analysis.

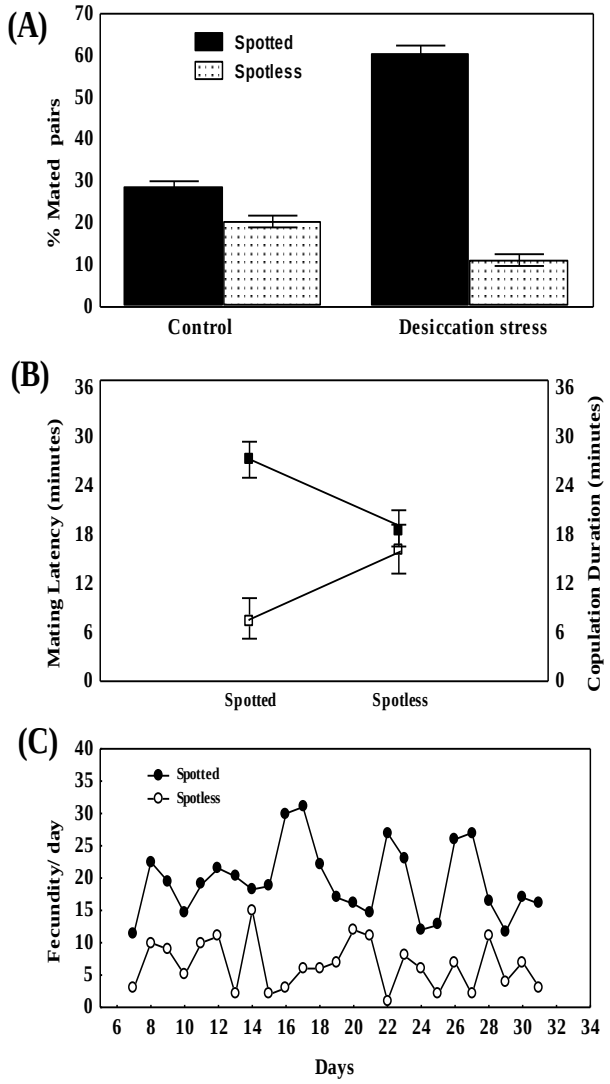

Figure 1. Comparison of fitness traits in *D. biarmipes.* (A) Percent mated pairs under control and desiccating conditions, (B) mating latency and copulation duration, (C) fecundity of spotted and spotless males (means ± s.e.m. of 20 isofemale lines grown at 21°C).

In order to test for mating preferences of spotted and spotless males with females in *D. biarmipes*, we attempted no choice mating experiments, and the data on observed matings are given in Figure 2. For all types of matings spotted males showed higher percent mated pairs (MP) as compared to spotless males (Figure 2A; Table 2). Based on male-choice mating experiments, we tested mating propensity of spotted and spotless wing males under control *vs.* stress conditions (due to desiccation or cold) and the data are shown in Table 2 and Figure 2A. As we found no obvious differences under desiccation and cold stress, we used data from desiccation stress for analysis. Mating preferences or propensity were estimated on the basis of two components of mating process (mating latency and copulation duration), and significant differences were found under control and desiccation conditions (Table 2). Finally there are significant differences in daily fecundity for all possible matings under control conditions ($F = 7897.36$, $p \leq 0.001$; Table 3; Figure 1C) in agreement with sexual selection for fitness benefits. Egg-to-adult viability exhibited significant differences in offspring from both types of matings. Progeny from spotted males had significantly higher egg- adult as compared spotless male progeny ($F = 3279.14$, $p \leq 0.001$; Table 3). Genetic correlations between fecundity and egg-to-adult viability were not significantly different ($p = 0.12$ ns) suggesting that these components of fitness are likely to be independent.

Table 2. Data on the basis of no choice method for frequency of mated pairs (MP), mean ± S.E. for mating latency (ML), copulation duration (CD) and fecundity under control and stressful conditions for *D. biarmipes.* For each experiment, there were twenty replicates. *** p < 0.001, ns = nonsignificant.

Experiment		Observed Mated pairs	MP (%)	ML (min) m ± SE	CD (min) m ± SE	Fecundity m ± SE
Contingency χ^2		-----	***	***	***	***
Control	1.	♀ X S ♂	28.66	5.40 ± 1.75	29.40 ± 2.10	24.10 ± 2.33
	2.	♀ X SL ♂	19.50	15.10 ± 2.16	17.54 ± 2.43	16.40 ± 2.50
Contingency χ^2		-----	***	***	***	***
Desiccation stress	1.	♀ X S ♂	61.58	6.37 ± 1.15	30.21 ± 2.00	23.15 ± 2.42
	2.	♀ X SL ♂	11.15	10.21 ± 2.04	15.00 ± 1.70	17.00 ± 2.61
Contingency χ^2		-----	***	***	***	***

Table 3. Results of nested ANOVA for fecundity and egg to adult viability (E- A Viability) of progeny of females mated to spotted and spotless males of *D. biarmipes*. ns = nonsignificant, **P < 0.001.

		Fecundity		E- A Viability	
Source	df	MS	*F*	MS	*F*
Morph	1	24527.21	7897.36***	30198.41	3279.14***
Lines	38	109.71	32.26**	121.98	14.97**
Error	360	3.23		9.14	

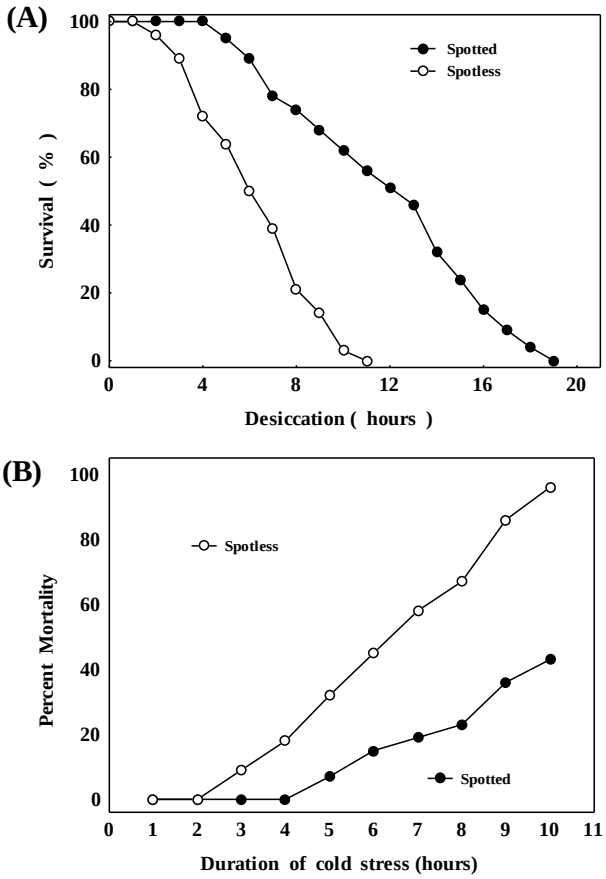

Figure 2. Survival (%) of spotted and spotless males with varying stress durations under desiccating conditions. (A) and during cold stress, (B) in males of *D. biarmipes*.

Discussion

A large number of studies have shown the role of abdominal melanisation in ecology of diverse insect taxa (Wittkopp *et al*., 2003). However, very few studies have directly tested the role of wing pigmentation. Interactions between an organism's morphology and its behavior are crucial to its fitness. Majerus and co-workers have done extensive studies on mating preference of color morphs of two-spot ladybirds (Majerus, 1994). Selection experiments for increased or decreased level of mating preference showed that this trait is genetically

controlled in two-spot ladybirds (Majerus, 1986). However, it has been shown that the maintenance of melanic polymorphism in two-spot ladybird is unrelated to any environmental factor (O'Donald and Muggleton, 1979). By contrast, in *Harmonia axyridis,* mating preference varied with season, *i.e*., in spring, both melanic and non -melanic preferred to mate with non-melanic males, while in the summer, melanics were over-represented in matings (Osawa and Nishida, 1992). The present study shows the impact of male wing spot dimorphism on mating success in warm adapted (*D. biarmipes*) species.

Fitness, a measure of relative performance of different genotypes at a locus, is generally assessed indirectly in terms of life history traits (Roff and Mousseau, 1987). Fitness consequences as survival under semi-field conditions for dark and light morphs of hoverflies (due to phenotypic plasticity) have shown clear adaptive differences, *i.e*., higher survival of lighter flies under summer conditions (Ottenheim *et al*., 1999). Further, two body color phenotypes (due to genetic polymorphism) of *Harmonia axyridis* differ significantly in development time and consumption rate (Soares *et al*., 2001). Thus, morphs resulting due to plastic or genetic effects show fitness consequences. In the present study, we have shown adaptive differences between spotted and spotless wing morphs of *D. biarmipes* for various ecophysiological traits. In the laboratory, male-choice mating experiments on spotted and spotless wing morphs showed the occurrence of assortative matings among spotted wing morphs.

Choosy females can increase the genetic quality of their offspring by mating only with males that will contribute good genes or compatible genes to their offspring. Good genes can be conveyed by condition-dependent traits (Rowe and Houle, 1996). For example, we have discussed that female guppies prefer to mate with longer males and peahens prefer to mate with males with larger eye-spots, because these males pass on to the offspring good genes that increase their fitness (Reynolds and Gross, 1992; Petrie, 1994). Mate choice also can be used to select males with compatible genes. Higher fitness of spotted wing males over spotless males is evident from measures of cold mortality as a function of duration of cold stress. Spotted and spotless males differ significantly in mortality due to cold stress. Finally, for desiccation resistance, there was lack of changes in the amount of cuticular lipids in spotted and spotless males. Thus, cuticular lipids cannot account for desiccation resistance in *D. biarmipes*. However, we observed significantly lower rates of water loss in spotted males and its impact on desiccation resistance. A major conclusion is that a spotted male is positively correlated with resistance to cold and desiccation stress. Thus, there are significant differences for various ecophysiological traits in spotted and spotless males of *D. biarmipes*.

Male vigor is measured by the number of females inseminated by a male in a given unit of time and·number of progeny produced from their insemined females. In this experiment, the males with patch inseminated more females and produced more progeny than males without patch, indicating that males with patch have higher vigor than males without patch. Thus, these studies have suggested that, in *D. biarmipes*, males with patch, fast mating abilities, and greater courtship activity convinced the females faster during both competitive and non-competitive situations. With these qualities, they are also able to mate quickly and produce more offspring. We have found significant effects on fecundity and egg-to-adult viability as well as moderate to high coefficients of genetic variation for these traits in *D. biarmipes*. These results indicate that there is genotypic variation among males that translates into variation in offspring viability. This variation provides the raw material for the accruement of indirect benefits resulting from female choice.

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In vivo and *in vitro* genotoxicity analysis of silver nitrate.

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Abstract

Silver nitrate is an inorganic compound which is toxic and corrosive. This heavy metal, when overdosed, leads to skin disease, blindness, and organ damage. The present study investigated the genotoxicity of silver nitrate in *Drosophila melanogaster* and human peripheral blood lymphocytes. Canton flies were exposed to 0.1M, 0.01M, and 0.001M of silver nitrate. Phenotypic analysis revealed discoloration of head and thorax in the treated flies and their progeny. The DNA from both parent and F1 was subject to Fragmentation assay to study the damages induced by the heavy metal, and the analysis showed significant shearing with fragmentation in the parent DNA. However, the F1 DNA depicted only shearing. To understand the type of mutation induced, Wing Somatic Mutation and Recombination test (SMART) was performed using trans-heterozygous larvae of *mwh/flr3* cross-over, exposed to the different concentrations of silver nitrate. Analysis of wings obtained from the emerging flies revealed spot formation characteristic of both the recessive markers. *In vitro* analysis

by Chromosomal Aberration (CA) assay was performed by exposing human peripheral blood lymphocytes to varying concentrations of silver nitrate for 24 hours and 48 hours. CA assay demonstrated absence of aberrations in the chromosomes of peripheral blood lymphocytes after 24 hours and 48 hours of exposure. KEY WORDS: *In vitro, in vivo,* silver nitrate, chromosomal aberration, fragmentation, genotoxicity.

Introduction

D. melanogaster is one of the most extensively studied organisms for genetic research as about 75% of recognizable human disease genes have been matched with them (1,2). They have been used as experimental models to study the role of genes for several diseases such as neurodegenerative disease, cancer, diabetes, and many more (3). The advantages of using these fruit flies as model organisms are that they are small and easily grown as they have a short generation time of 10 days and have high breeding efficiency (about 100 eggs/day) (4).

Genotoxicity is the property of a substance which makes it harmful to the genetic information of an organism. Substances which exhibit genotoxicity are known as genotoxins. These genotoxins can be carcinogens, mutagens, or teratogens. There are several ways by which genotoxicity can affect the genetic information. One such common mechanism of action is by forming chemical bonds between the genotoxin and the molecules which carry the genetic information, such as DNA or RNA, therefore affecting its integrity. Genotoxins can be of any type – chemical compounds and radiation. Examples of such chemical compounds include silver nitrate, benzaldehyde, ethyl methane sulfonate, and so on. Therefore, it is necessary to determine the genotoxic level of every chemical compound (5).

Silver nitrate, a salt of silver, is an inorganic compound which is generally used for the prevention of gonococcal ophthalmia neonatorum, cauterization (burning) of wounds and sluggish ulcers, removal of granulation tissue and warts, and aseptic prophylaxis of burns. Initially silver nitrate was used in newborns where a drop of the silver nitrate solution is applied in the eyes of the baby to clear gonococcal infection. But excess of the same can cause blindness and other infections such as blue-grey stain on skin (skin pigmentation), ulcerations, diarrhoea, shock, coma, convulsions, and methemoglobinemia. Fatal dose of silver nitrate is as low as 2 g. The mechanism of action of silver nitrate is by coagulating cellular proteins to form an eschar. Eschar is dead tissue that sheds from a healthy skin (5, 6).

The Wing Somatic Mutation and Recombination test (SMART) is a gold-standard technique, employed for the purpose of assessing the degree of genotoxicity, that is, exhibited by the mechanism of induction of loss of heterozygosity (LOH) resulting from incidents like gene mutation, chromosome break, and chromosomal rearrangement. In transheterozygous *mwh+/flr3* flies, multiple wing hairs (*mwh*) and flare (*flr3*) are employed as wing- cell recessive markers in SMART. A mutation in the mitotic cells of wing disc gives rise to a clone of *mwh* and *flr3* cells. This expresses as spots on the wings of adult fly. The spots may appear singly or as twins. Single spots indicate the occurrence of a point mutation, a chromosomal alteration, or mitotic recombination. Twin spots indicate the occurrence of mitotic recombination (7).

Chromosomal aberrations result from changes in the structure or number of chromosome. The damage that occurs in a cell gives rise to abnormalities in chromosome. These defects are assessed by a technique called Chromosomal aberration (CA) assay (8). CA assay is often performed on human peripheral blood lymphocytes. As lymphocytes are in the G0 (resting) stage of cell cycle, they are stimulated to divide by an antigen like Phytohemagglutinin (PHA). A spindle inhibitor, Colcemid, is added at the end of 46.5 hour to arrest the cells in the metaphase of first mitosis. The

culture is then harvested after 48 hours of incubation. In the present study, the blood culture is exposed to silver nitrate for 24 hours and 48 hours (9,10).

Materials and Methods

Exposure of Canton flies and DNA fragmentation assay

Flies were bred in the ratio of 1:3 (males:females) and were cultured in corn meal agar and incubated at 25°C during the day and 19°C at night. Test concentrations of silver nitrate solution of 0.1M, 0.01M, and 0.001M were prepared. Instant food (4-24 formula plain – Carolina Biologicals) was mixed with the above concentrations of silver nitrate, and the food was allowed to set for 3 hours. About 30 males were isolated added to the above and exposed for 24 hours and 48 hours at 25°C. After 48 hours, the surviving male flies were transferred (by etherization) to fresh corn meal medium, and 90 virgin female flies were added to each of the vials to check the breeding efficiency. All the vials were maintained in duplicates. The control used had instant food mixed with sterile distilled water. DNA extraction from both treated and control flies was performed by Phenol Chloroform method (PCI), and the quality of the DNA was checked using Nanodrop. Good quality DNA was run on 3% agarose gel for DNA fragmentation assay to assess the damage induced.

Wing spot assay

Wing Spot assay was performed by exposing 60 *mwh/flr*^{3} larvae to the test concentrations of silver nitrate. The larvae were monitored till the emergence, and the emerged flies were dissected for their wings and analysis was performed on the wings for spots.

Chromosomal aberration assay

For Chromosomal aberrations (CA) assay, culture was set up for exposure to silver nitrate for 24 hours and 48 hours. The procedure involves 48 hours culture setup and harvesting at 47th hour. Two sets of culture were set up by adding 8 ml of RPMI-1640 and 2 ml of Fetal Bovine serum (FBS) to 1 ml of human peripheral blood. 400 µl of phytohemagglutinin was added to all the culture vials. To the first set of cultures, test concentrations of silver nitrate were added along with a control culture and incubated for 46 hours. The second set of cultures was incubated for 24 hours after which the test concentrations were added to the respective vials along with a control and incubated further till 46 hours. At the end of 46 hours, 10 µl of Colcemid was added to all the vials and incubated for 1 hour. At the 47th hour, all the cultures were harvested by adding 8 ml of hypotonic solution (KCl prewarmed to 37°C) and incubated at 37°C for 20 minutes. The contents were then centrifuged at 1000 rpm for 10 minutes, and the pellet was resuspended in 8 ml of prechilled Carnoy's fixative (methanol : acetic acid – 3:1) and incubated overnight at 4°C. The cultures were again centrifuged and the pellet was suspended in a small amount of the fixative and casted on slide held at an angle of 45°. The slides were then allowed to dry and observed under the microscope for any chromosomal aberrations.

Results

Phenotypic Changes

Adult male flies exposed to test concentrations of silver nitrate survived for 24 hours and 48 hours. To check the breeding efficiency, 90 female flies were added to each vial. The flies bred well.

The females laid their eggs in the untreated medium. The bottles were observed carefully each day for the emergence of adult flies. The adult flies emerged 19 days after the eggs were laid. The progeny were screened for pupal lethality, adult emergence, and adult mutant phenotypes. The F1 observed under the microscope showed discoloration of head and thorax as shown in Figure 1. Survival rates of both exposed flies and the F1 progeny were 100% in all the test concentrations of silver nitrate (Table 1).

Figure 1. Phenotypic changes observed following exposure to silver nitrate. Picture shows discoloration of head and thorax observed in flies (parent and F1). This change was seen at all concentrations.

Table 1. Results of phenotypic changes observed in exposed flies and F1 progeny.

Experiment	Concentration of Silver Nitrate	Survival Rate	Changes Observed
1	0.1M	100%	Discoloration of head and thorax
2	0.01M	100%	Discoloration of head and thorax
3	0.001M	100%	Discoloration of head and thorax
F1 analysis	All three concentrations	100%	Discoloration of head and thorax

Table 2. Results of wing spot assay.

Concentration of Silver Nitrate (µL)	*flr3*	*mwh*	*mwh/flr3*	Observations
Control	-	-	-	Absence of spots
0.1M	+	+	+	Presence of large single spots-*flr3*; cluster of multiple trichomes per cell-*mwh*
0.01M	+	+	+	Single large spots-*flr3*; multiple trichomes and 2 hair per cell-*mwh*
0.001M	+	+	-	Multiple trichomes per cell-*mwh*; multiple single spots-*flr3*

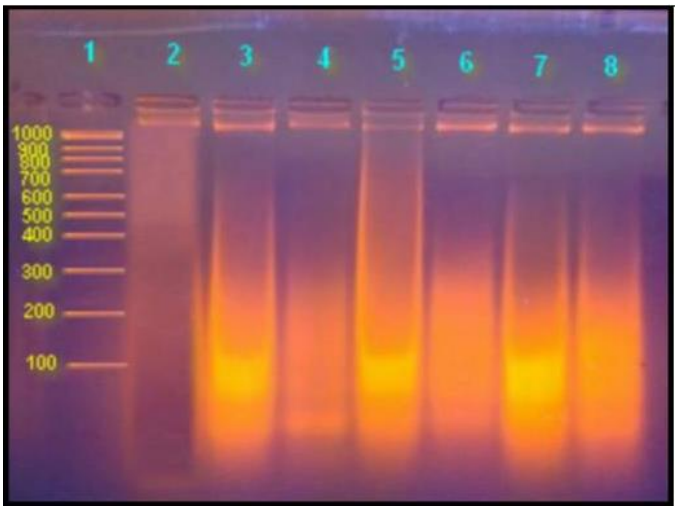

Figure 2. Agarose gel electrophoresis showing DNA fragmentation of Parent DNA samples. Description: Well 1 – 100 BP Ladder; 2 - Control; 3,4 – 0.1M silver nitrate for 24 hrs and 48 hrs; 5,6 – 0.01M silver nitrate for 24 hrs and 48 hrs; 7,8 – 0.001M silver nitrate for 24 hrs and 48 hrs.

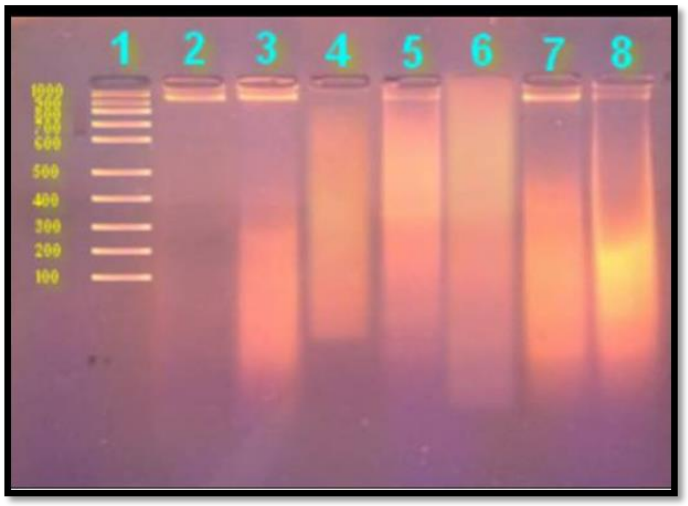

Figure 3. Agarose gel electrophoresis showing DNA fragmentation of F1 DNA samples. Description: Well 1 – 100 BP Ladder; 2 - Control; 3,4 – 0.1M silver nitrate for 24 hrs and 48 hrs; 5,6 – 0.01M silver nitrate for 24 hrs and 48 hrs; 7,8 – 0.001M silver nitrate for 24 hrs and 48 hrs.

DNA Fragmentation assay

DNA was isolated from Control, exposed flies, and F1 progeny by Phenol chloroform method, and the quality of the DNA was checked using Nanodrop. The Nanodrop results showed that the DNA was found to be of good quality. Good quality DNA was subjected to Fragmentation assay on 3% agarose gel to detect the damage. The results obtained were documented (Figures 2 and 3). Distinct shearing with fragments was observed in all the concentrations (after 24 and 48 hours exposure) of exposed sample (Parent), whereas DNA from the F1 sample showed patterns of shearing.

Wing Spot Assay

Analysis of patterns on the dissected wings of both control and exposed flies emerged from the trans-heterozygous larvae of the *mwh/flr3* cross-over clearly showed the genotoxic potential of silver nitrate (Table 2). No spots were observed in the control wing. In the wings of the exposed flies, spot formation was clearly seen. Single large spots indicative of *flr3* marker and multiple trichomes with two hair cells specific to *mwh* marker were both present (Figure 4).

Chromosomal Aberrations (CA) assay

The *in vitro* chromosomal aberrations assay performed on human peripheral blood lymphocytes demonstrated absence of any aberrations in all the concentrations (Figure 5).

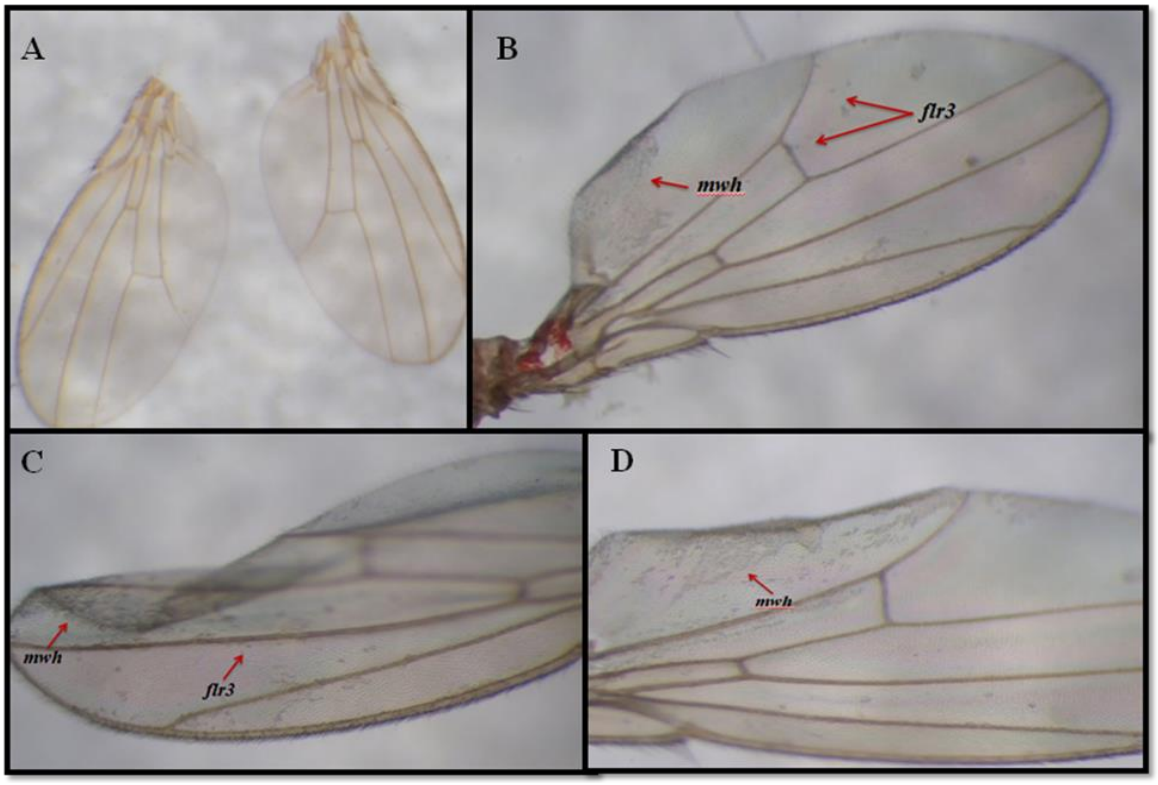

Figure 4. Wing spot assay analysis after exposure to silver nitrate. A - Control showing absence of spots; B – 0.1M - presence of large single spots - *flr3*; cluster of multiple trichomes per cell - *mwh*; C – 0.01M - single large spots-*flr3*; multiple trichomes and two hair per cell-*mwh*; D – 0.001M - multiple trichomes per cell-*mwh*; multiple single spots-*flr3*.

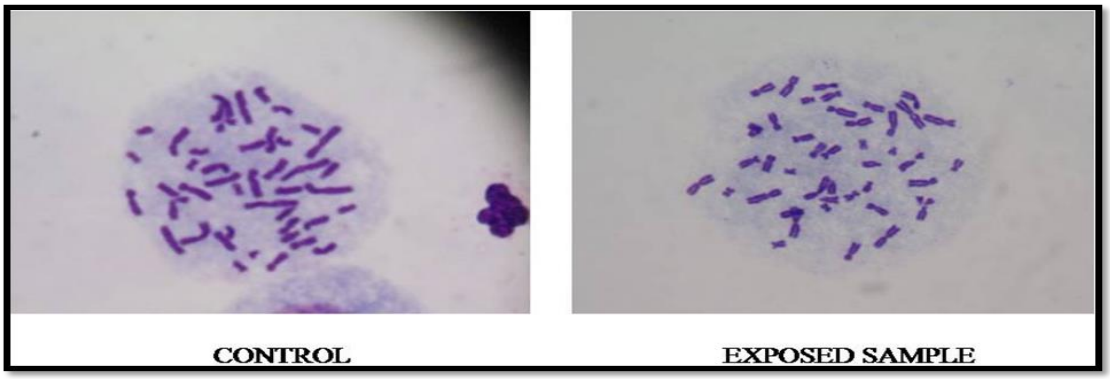

Figure 5. Chromosomal aberration assay. The picture shows chromosomes of control blood sample and blood sample exposed to test concentrations of silver nitrate. The results observed were the same in all the three concentrations.

Discussion

The present study evaluated the genotoxicity of three concentrations of Silver nitrate using *in vitro* and *in vivo* methods. In the *in vitro* chromosome aberration assay performed on human peripheral blood lymphocytes, no significant chromosomal aberrations were observed in all the three concentrations indicating that all the concentrations were not genotoxic at the cytogenetic level. However, the *in vivo* assessment of the same concentrations on *Drosophila melanogaster* was performed by evaluating the phenotypic changes and employing DNA fragmentation assay on the DNA isolated from the exposed flies and F1 generation.

The phenotypic changes observed in the exposed flies included mild discoloration of the head and thorax in all the three concentrations. Also, DNA isolated from both exposed and F1 was quantified by nanodrop and run on 3% agarose. The results of the parent DNA revealed significant shearing and defined fragments in all the three concentrations at both 24 and 48 hours of exposure. However, the DNA obtained from F1 demonstrated only shearing, and no fragments were observed. Shearing is indicative of extensive damage and hence both parent and F1 population have faced pronounced genotoxicity on exposure to the above said concentrations of silver nitrate. Once again the Wing spot assay performed on the third instar trans-heterozygous larvae of the *mwh/flr3* cross-over showed and confirmed the recombinagenic action of the heavy metal. Single large spots indicative of *flr3* marker and multiple trichomes with two hair cells specific to *mwh* marker were both present. In comparison with the control wing, the extent of damage induced in the exposed larvae was well understood. From the *in vivo* studies conducted it was evident that silver nitrate was genotoxic on both wild type and mutant flies.

Understanding the mechanism of genotoxicity and type of genetic damage is, however, beyond the scope of this study, and specific tools have to be employed to evaluate the same. The results of the *in vitro* and *in vivo* are not consistent, indicating the profound expression of genotoxicity at *in vivo* stages, and *in vitro* tests may be expanded on cell lines to validate their genotoxic effect. Further higher and lower concentrations of silver nitrate may be evaluated to arrive at the threshold concentration at which genotoxicity is defined and below which no genotoxicity is observed.

Acknowledgments: The authors sincerely thank the management of Sri Ramachandra University for the Intramural grant for research. The authors also thank the Head of the Department, Department of Biomedical Sciences, for permitting us to conduct the research.

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Orthologs of the eggshell gene *Vml* contain a diverse number of coding tandem repeats.

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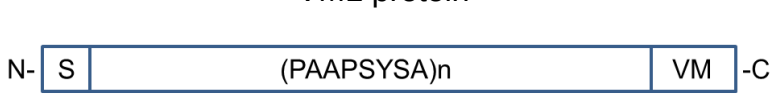
Gene *Vml* encodes a structural protein of the *Drosophila* eggshell vitelline membrane in *D. melanogaster* (Alatortsev, 2006) and participates in the establishment of the dorsal/ventral axis of the *Drosophila* embryo (Zhang *et al*., 2009). Protein VML contains an unusually large array of perfect and imperfect repeats PAAPSYSA in the central part (Alatortsev, 2006). Through genomes of 12 *Drosophila* species that were sequenced (Clark *et al*., 2007), orthologs of the *D. melanogaster* gene *Vml* were found only in closely related species *D. sechellia*, *D. simulans*, and *D. erecta* (FlyBase release FB2013_04, http://flybase.org).

For searching *Vml* orthologs in the rest of sequenced *Drosophila* species, fragments of the *Vml* gene and the FlyBase BLAST Service (http://flybase.net/blast) were used. It was found that orthologs in five *Drosophila* species (*ananassae*, *pseudoobscura*, *persimilis*, *mojavensis*, and *grimshawi*) correspond to the registered genes with unknown function (Clark *et al*., 2007). Unpredicted earlier genes were found in three species (*D. yakuba*, *D. willistoni*, and *D. virilis*). Possibly, these genes were not found earlier due to the strong searching parameters in gene finding programs used for genome annotations. All *Vml* orthologs have similar one exon gene structure and lie in synthenic regions between the corresponding orthologs of the *CG2918* and *CG2865* genes. Orthologous proteins have signal sequence for secretion at the N-end and cysteine-rich domain specific for vitelline membrane proteins (Scherer *et al*., 1988) at the C-end (Figure 1A,B). Lengths of orthologous proteins vary from 457 to 738 amino acids due to the presence of the different number of 8 aa repeats PAAPSYSA.

A



B

Species\Gene	VM domain
Dmel\Vml	**-SLPSPPCPKNYVFSCSSVFTPAPCSQGYGY**
Dsim\GD16605	**-SLPAPPCPKNYVFSCSSVFTPAPCSQGYGY**
Dsec\GM19220	**-SLPSPPCPKSYVFSCSSVFTPAPCSQGYGY**
Dere\GG12928	**-SLPSPPCPKNYVFSCSSVFTPAPCSQGYGY**
Dyak\Vml **X:6,157,204..6,158,844 [-]**	**-SVPSPSCPKNYVFSCSSVFTPAPCSQGYGY**
Dana\GF22208	**-SVPAPPCPKSYVFSCQSVFSPAPCSQGSAY**
Dpse\GA22863	**-SIPAQPCPKNYVFSCLGVFQKAPCSQGLQY**
Dper\GL13336	**-SIPAQPCPKNYVFSCQGVFQKAPCSQGLQY**
Dwil\Vml **scf2_1100000004401:330,889..332259 [+]**	**-SIPAPPCPTNYVFSCNSVFKPAPCSQGSAY**
Dmoj\GI21801	**-SIPAPPCPANYVLSCKSVFTPAPCSQGAAY**
Dvir\Vml **scf_12928:5,684,456..5,686,129 [+]**	**-KIAAPACPTSYLFSCNSVFTPAPCNQGAAY**
Dgri\GH24704	**-SIPAPPCPTTYLFSCNSVFTPAPCSQCSKY**

C

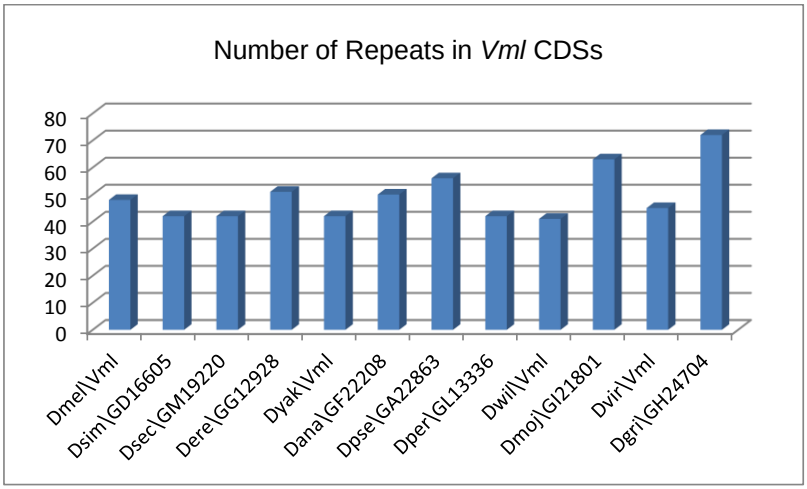


Figure 1. Structures of the VML orthologs. A, Domain structure of the VML protein: S, signal peptide; VM, domain specific for vitelline membrane proteins. B, Alignment of VM domain amino acid sequences of 12 orthologous VMLs. Orthologs found in this work are highlighted. Genome positions of orthologous genes in *D. yakuba*, *D. willistoni*, and *D. virilis* are given according to corresponding genome maps (FlyBase release FB2013_04). The VM domain sequence logo was created by WebLogo (Crooks *et al*., 2004). C, Number of 24 bp DNA tandem repeats in orthologous *Vml* coding sequences.

Quantity of 24 bp tandem repeats in orthologous genes was determined by the TRF program (Benson, 1999). It was found that orthologs contain a diverse number of coding tandem repeats (Figure 1C). Thus, *Vml* orthologs in closely related species *D. pseudoobscura* and *D. persimilis* have considerable differences in repeat number, 56 and 42 copies, accordingly. A functional consequence of this diversity between species remains unclear. Possibly, changes in repeat number participate in species-specific changes of eggshell morphology.

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Comparative analysis of the fragment of the Y chromosome gene *kl-2 1-beta dynein heavy chain* in *Drosophila virilis* species group (Diptera: Drosophilidae).

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Introduction

Drosophila virilis species group is one of the best studied models of speciation and microevolution (Morales-Hojas *et al*., 2011). We analysed Y chromosome DNA sequence variation

among all 12 *Drosophila* species of the *virilis* group for the first time in order to resolve uncertain aspects of the group phylogenesis. Sequence variation of genes locating on the Y chromosome provides a unique opportunity to elucidate the order of phylogenetic diversity in the group of closely related species due to the non-recombining nature of the Y chromosome.

Gene *kl-2 1-beta dynein heavy chain* was acquired by the Y chromosome of the ancestral *Drosophila* species before the split of the *Drosophila* and *Sophophora* subgenera between 260 and 63 Myr ago (Koerich *et al.*, 2008). *kl-2* gene remains Y-linked in all sequenced *Drosophila* species with the exception of *D. pseudoobscura* (Koerich *et al.*, 2008). In the case of *D. pseudoobscura* the ancestral Y chromosome became part of an autosome (Carvalho *et al.*, 2005).

In this study we present comparative analysis of the PCR fragment of the gene *kl-2* in all 12 species of the *virilis* species group.

Materials and Methods

Fly strains: All strains used in this work are from the collection of the Laboratory of Genetics, Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences and National *Drosophila* Species Resource Center (Bowling Green, United States): *D. americana americana* (Spencer) 405, *D. americana texana* (Stone, Griffen, and Patterson) 423, *D. ezoana* (Takada and Okada) 572, *D. kanekoi* (Watabe and Higuchi) 1540, *D. lacicola* (Patterson) 0991.13, *D. littoralis* (Meigen) 06-17a - North population and AB54 – South population, *D. montana* (Patterson, Stone, and Griffen) 1021.13, *D. novamexicana* (Patterson) 424, *D. flavomontana* (Patterson) 0981.0, *D. lummei* (Hackman) 200, *D. borealis* (Patterson) 0961.00 East population, *D. virilis* (Sturtevant) B9. Each strain was founded by a single female fertilized in nature and maintained as a mass culture in vials on a standard *Drosophila* medium. The PCR amplification has been performed on the matrix of total DNA that was isolated from individual imago.

The primers used to amplify the *kl-2* sequences were as follows: the Dv-dy-f direct primer - 5' - GCTGCAGGCGGTAATAGAAG - 3' and the Dv-dy-r reverse primer - 5' - TTGCATTTGCGGATCAATAA - 3'. The length of the amplified fragment is 429 bp. A fragment of a genomic DNA was amplified by PCR in the volume of 25 µL using an Applied Biosystems PCR amplificator. The conditions of PCR were as follows: initial DNA denaturation at 94°C for 5 min; then, 35 cycles were performed as follows: denaturation at 94°C for 30 s, annealing at 60°C for 30 s, DNA synthesis at 72°C for 60 s, and finishing synthesis at 72°C for 7 min. The products of the amplification were separated by electrophoresis in 1.5% agarose gel. The obtained PCR fragments were sequenced without cloning.

Results and Discussion

Within the set of newly obtained sequences the number of nucleotide substitutions per site varies ten fold from 0.005 in the pair of *D. americana americana* and *D. americana texana* to 0.051 in the pair of *D. americana americana* and *D. borealis.* The overall mean distance for the whole group is 0.036. Based on these data we constructed median network of Y chromosome haplotypes of *Drosophila* of the *virilis* group with the TCS software (Figure 1). TCS is a program that implements the estimation of gene genealogies from polymorphic DNA sequences using statistical parsimony (Clement *et al.*, 2000). Results are, in general, consistent with previous studies based on the multilocus data (Morales-Hojas *et al.*, 2011) in recovering the four major lineages of the group: *D. virilis* phylada, *D. montana* phylada, *D. kanekoi* phylada and *D. littoralis* phylada. The most

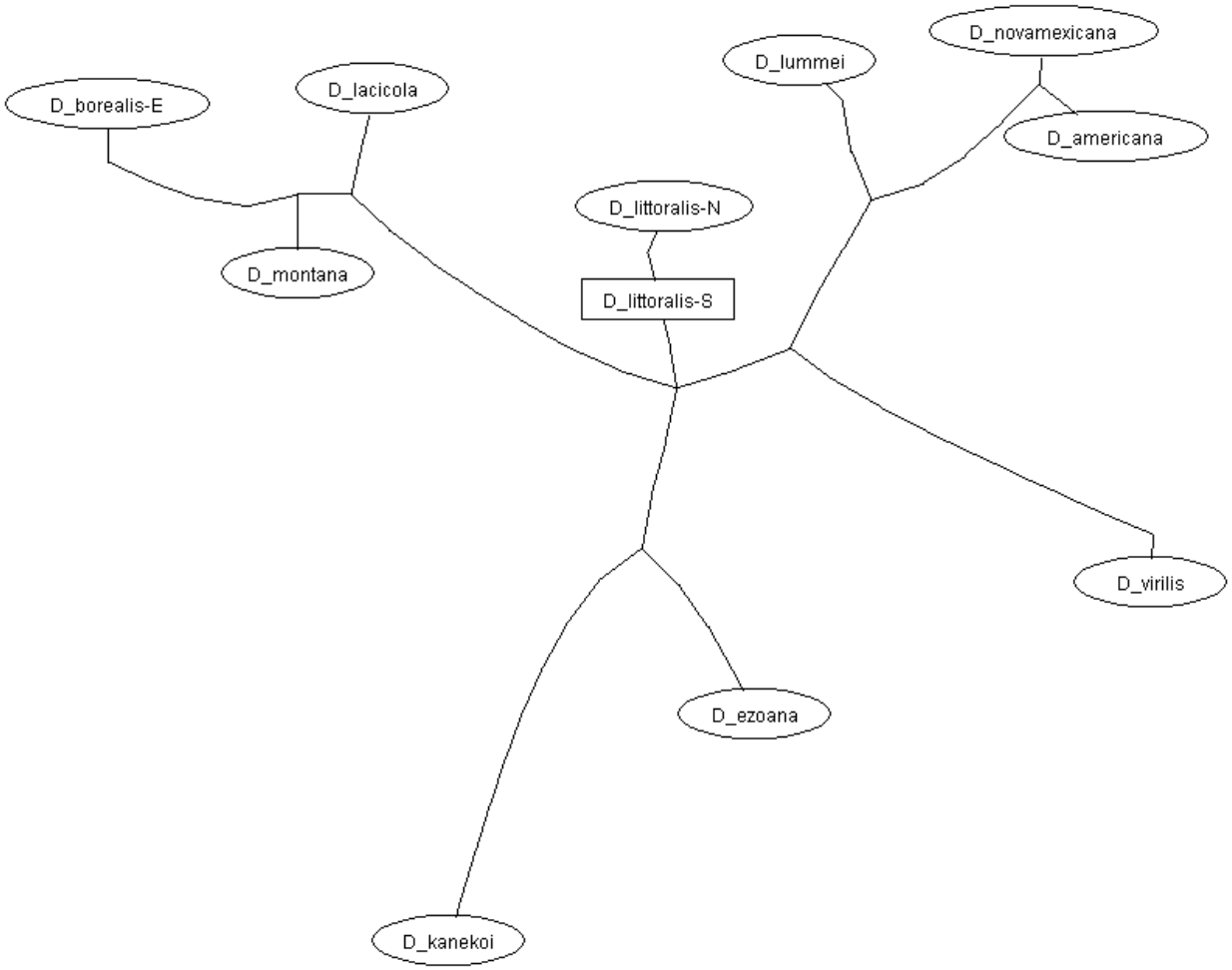

Figure 1. The median network of Y chromosome haplotypes of the *Drosophila virilis* group constructed using the TCS software (Clement *et al.*, 2000). The reconstruction is based on the nucleotide polymorphism of *kl-2* gene fragment (GenBank ID: KF600714 - KF600726). The lengths of the brunches are proportional to the minimum number of nucleotide substitutions that are necessary for transformation of one haplotype into another. If there are synonymous haplotypes, only one synonym is indicated.

interesting new point concerns the order of phyladas filiation. Multilocus analysis based on six nuclear genes and two mitochondrial genes revealed that the last common ancestor of the group had a Holarctic distribution from which the North American and the Eurasian lineages evolved from 7.5 to 8.9 Mya. Two competing evolutionary hypotheses have been proposed and compared (Morales-Hojas *et al.*, 2011). First, simple consensus tree model for analysing multilocus data leads to the conclusion that *D. kanekoi* and *D. ezoana* form one linage, and *D. littoralis* has a more ancient origin. More sophisticated method, using multispecies coalescent model (BEST), leads to the alternative conclusion. *D. kanekoi* was the first species to divide from the common ancestor of these three species. These conclusions are in agreement with localization of an ancient retrotransposon old *bilbo*-like non-LTR retroelement, which is present in *D. littoralis* and *D.virilis*, but absent in *D.*

kanekoi (Reis *et al.*, 2008). Our data strongly support the first simple model and indicate that *D. kanekoi* and *D. ezoana* form one linage. Besides, it is clear that the speeds of nucleotide fixations in different species vary greatly. This may be due to the differences in the population size. Small island populations of *D. kanekoi* from Japan evolve much more rapidly than large continental populations of *D. littoralis*.

We found no difference between *kl-2* sequences of different laboratory lines of *D. virilis.* It is not surprising because *D. virilis* is a nearly monomorphic species (Mirol *et al.*, 2008). On the contrary *kl-2* sequences of two lines of *D. littoralis* from the South Caucasian population and from North European population (Andrianov *et al.*, 2010) are different. South population is ancestral in relation to North population. These data support a previously made proposal that South population of *D. littoralis* is a separate species.

We found no difference between *kl-2* sequences of Eastern *D. borealis* and *D. flavomontana*. Fly lines from Western population of *D. borealis* were unavailable to us. *D. borealis* comprises two different species, Eastern and Western. Eastern *D. borealis* is much more closely related to *D. flavomontana* (Morales-Hojas *et al.*, 2011). Our data support the hypothesis of hybrid origin of Eastern *D. borealis.* It may originate from hybridisation of *D. flavomontana* male with *D. borealis* female.

Acknowledgments: This research was supported by grants from the Russian Foundation for Basic Researches, Projects: 13-04-01250-a, 12-04-00926-a, 11-04-01630-a, and 13-04-90621.

References: Andrianov B.V., I.I. Goryacheva, N.S. Mugue, S. Yu Sorokina, T.V. Gorelova, and V.G. Mitrofanov 2010, Trends in Evolutionary Biology 2: e4.; Carvalho, A.B., and A.G. Clark 2005, Science 307: 108–110; Clement, M., D. Posada, and K.A. Crandall 2000, Mol. Ecol. 9: 1657–1659; Koerich, L.B., X. Wang, A.G. Clark, and A.B. Carvalho 2008, Nature 456: 949-951; Mirol, P.M., J. Routtu, A. Hoikkala, and R.K. Butlin 2008, BMC Evolutionary Biology 8: 59; Morales-Hojas, R., M. Reis, C.P. Vieira, and J. Vieira 2011, Molecular Phylogenetics and Evolution 60: 249–258; Reis, M., C.P. Vieira, R. Morales-Hojas, and J. Vieira 2008, Gene 425: 48–55.

A preliminary report and the frequency distribution of *Drosophila* species of Rabwah, Pakistan.

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To study the frequency distribution of some *Drosophila* species, a study was carried out in Rabwah, Pakistan (31° 32’ 59”N, 74° 20’ 37” E). Survey was made for the first time in this area and revealed seven species, which were already found before in Lahore, Pakistan. The collections were made twice, once during March-April and then during the month of September, 1992. It was observed that two *Drosophila* species, *D. immigrans* and *D. nepalensis*, were found only during the month of April. This observation is similar to the observation of Gupta (1973), who, while studying the fauna of Manipur, India, observed that these two species can be obtained only in the month of February. These collection data also reveal that all of these species are members of *melanogaster* and *immigrans* species groups of subgenera *Sophophora* and *Drosophila*, thus supporting the view of Bock and Wheeler (1973), who proposed that South East Asia is a fertile region for the rapid diversification and speciation of the members of the *immigrans* and the *melanogaster* species groups of *Drosophila*.

Table 1. Frequency distribution of the species.

Species	Subgenus	No. ♂	No. ♀
D. melanogaster	*Sophophora*	262	198
D. jambulina	〃	9	7
D. malerkotliana	〃	1	-
D. ananassae	〃	1	-
D. takahashii	〃	72	52
D. nepalensis	〃	8	-
D. immigrans	*Drosophila*	39	77

Acknowledgment: The author is grateful to the Chairman of Biology Dept., Quaid-i-Azam University, Islamabad, and her supervisor, Dr. Mahmud Ahmad, to provide facility for the study.

References: Bock, I.R., and M.R. Wheeler 1972, Univ. Texas Publ. 7213: 1-102; Gupta, J.P., 1973, Dros. Inf. Serv. 50: 112.

Intra-chromosomal association between allozyme loci in *Drosophila ananassae.*

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Non-random association between gene arrangements was referred to as linkage disequilibrium by Lewontin and Kojima (1960) and according to them this happens due to linkage between such arrangements. Chromosomal polymorphism in association with allozymes has been extensively studied in *Drosophila* (Rodriguez-Trelles, 2003; Iriarte *et al.*, 2002; Rodriguez *et al.*, 2000; Kamping and Delden, 1999; Zapata and Alvarez, 1992). Gametic disequilibrium studies have been done between second chromosome polymorphic arrangements and seven linked loci, in seven populations of *D. buzzatii* from Argentina, and a significant and consistent association for *Est-1*, *Est-2*, *Aldox* and *Xdh* has been reported (Rodriguez *et al.*, 2001). They explained that restriction of recombination in heterokaryotypes seems to be the best explanation for the linkage disequilibrium between inversion and enzyme loci located inside the rearranged segments.

Extensive work on allozyme-allozyme linkage disequilibrium in natural populations of *D. melanogaster* has been done involving 36 allozyme pairs (Langley *et al.*, 1974). Among all those, only three showed significant deviation from expectation and only one of them (*Odh-Ao*) could be established to show non-random association. In another study, Langley *et al*. (1977) found no linkage disequilibrium among even tightly linked loci which were nearly 3 cM distant from each other in natural populations of *D. melanogaster*.

D. ananassae, first described by Doleschall (1858), belongs to the *ananassae* species complex of the ananassae subgroup in the melanogaster species group of the subgenus Sophophora and is one of eight cosmopolitan species (Bock and Wheeler, 1972; Tobari, 1993). It occupies a unique status in genus *Drosophila* due to its several genetic peculiarities (Singh, 2010). One of its peculiar features is that of spontaneous crossing over in males, although at much lower frequencies than observed in females (Tobari, 1993; Singh and Singh, 1988). Recently we have started working on the enzyme polymorphism in this species to see genetic diversity in different natural populations derived from various parts of India (Kumar and Singh, 2012; Singh *et al.*, 2013). While studying enzyme polymorphism, we also planned to see association between those enzyme loci which are situated on the same chromosome. In this note we wish to report intra-chromosomal association among three

enzyme loci, *i.e*., *Xdh*, *Acph 1*, and *Acph 2*, present on 2L chromosome arm of *Drosophila ananassae*. The locations of these enzyme loci on polytene chromosomes have been known by sequence analysis from *D. melanogaster* by using BLAST with *D. ananassae* genome sequence (Altschul *et al.*, 1997). The polytene chromosome position of *Xdh* is 28A and *Acph* is 37B on 2L arm (Stephen *et al.*, 2008). The two loci, *i.e*., *Xdh* and *Acph* are approximately 11.5 Mb apart from each other. *Acph 1* and *Acph 2* are clustered together owing to gene duplication during the course of evolution.

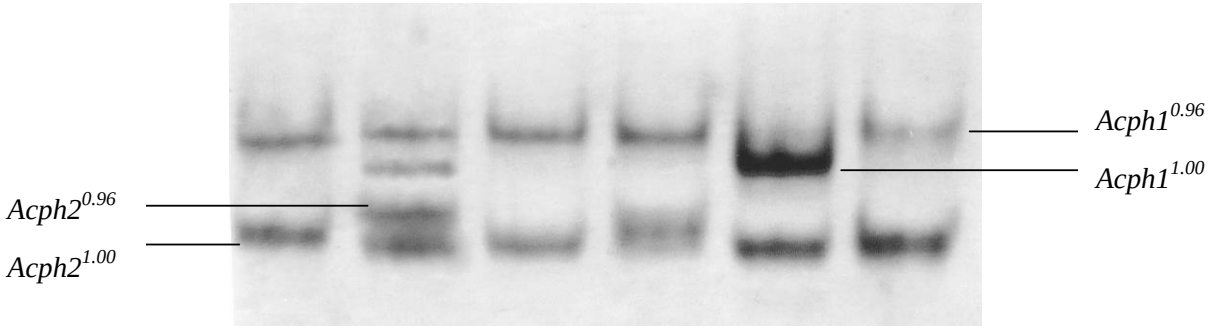

Figure 1. Native polyacrylamide gel of Acid phosphatase enzyme showing two isozyme loci.

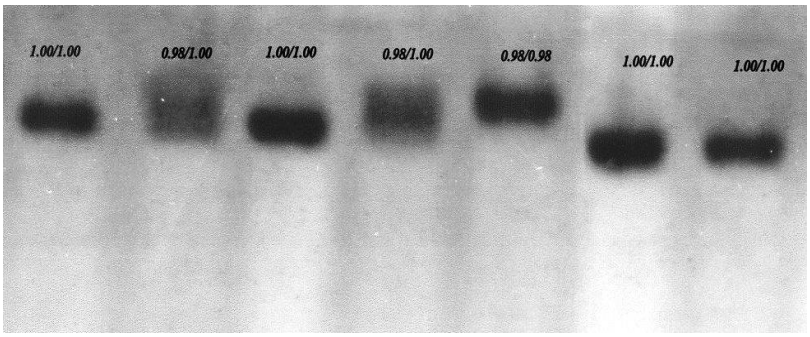

Figure 2. Native polyacrylamide gel showing homozygous $Xdh^{1.00}$, $Xdh^{0.98}$, and heterozygous $Xdh^{0.98/1.00}$ bands for Xanthine dehydrogenase locus.

We are able to record three genotypes for each of the three enzyme loci studied. Since these loci are linked on a single chromosome, intra-chromosomal associations among these three loci can be looked into by seeing the association of genotypes related with two enzyme loci. Due to occurrence of three genotypes for each of the three loci, nine combinations between *Xdh-Acph 1*, *Xdh-Acph 2*, and *Acph1-Acph2* could be ascertained. So far, we have analyzed more than 200 individuals derived from five different natural populations of *D. ananassae* and the trend indicates that there is absence of linkage disequilibrium in all the cases observed so far. The random occurrence of different possible combinations may be due to ample rate of recombination between the enzyme loci and selection playing no role to favor specific association.

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

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Esterase-4 **locus comprises active and null alleles in** ***Drosophila ananassae*****.**

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Genetic variations in natural populations of *Drosophila* have been studied extensively for many enzymes using electrophoretic mobility (Hubby and Lewontin, 1966; Lewontin and Hubby, 1966; Morton *et al*. 2004). Esterase is one of the most studied enzyme systems in *Drosophila*. However, there is very little literature pertaining to Esterase polymorphism in *D. ananassae* (Johnson, 1971). *D. ananassae* Doleschall (1858) is a cosmopolitan and domestic species. The extent and pattern of inversion polymorphism is well documented in *D. ananassae* (Singh, 1998, 2001; Singh and Singh, 2007). Enzyme polymorphism in *D. ananassae* has also been studied to some extent. Kumar and Singh (2012) observed Xanthine dehydrogenase polymorphism in this species and reported four alleles represented by the *Xdh* locus. Alcohol dehydrogenase (*Adh*) locus has also been shown to be polymorphic in this species (Singh, Kumar and Bhumika, 2013), and on the basis of electrophoretic mobility, two variants, *i.e*., slow and fast, have been found. Esterases (3.1.1.1) are classified as hydrolases, a large and diverse group of enzymes that catalyze the hydrolysis of a wide range of aliphatic and aromatic esters, choline esters, and organophosphorous compounds (Dauterman, 1985). Esterases act on molecules that are completely dissolved in water, hydrolyzing carboxylesterases into alcohol and carboxylate. Over thirty carboxylester hydrolases have been identified in *D. melanogaster* and most are identified as acetyl carboxyl or cholinesterases. About 22 soluble esterase isozymes have been detected by native polyacryamide gel electrophoresis, and more could be resolved if 2-D gel electrophoresis was applied (Oakeshott *et al.*, 1993).

Esterases are highly polymorphic in the genus *Drosophila* (Powell, 1975; Oakeshott *et al.*, 1993). In the present study, Esterase banding pattern of *D. ananassae* was identified using native polyacrylamide gel electrophoresis. In native PAGE α-naphthylacetate is used as substrate and fast blue RR as staining reagent. The *D. ananassae* stock used in this study is GT-ST, a mass culture stock having standard gene arrangement in all the chromosomes derived from flies collected from

Gangtok (India). We have identified *Est-2*, *Est-3*, *Est-4*, and *Est-5* as polymorphic loci. *Est-4* comprises two alleles: active and null.

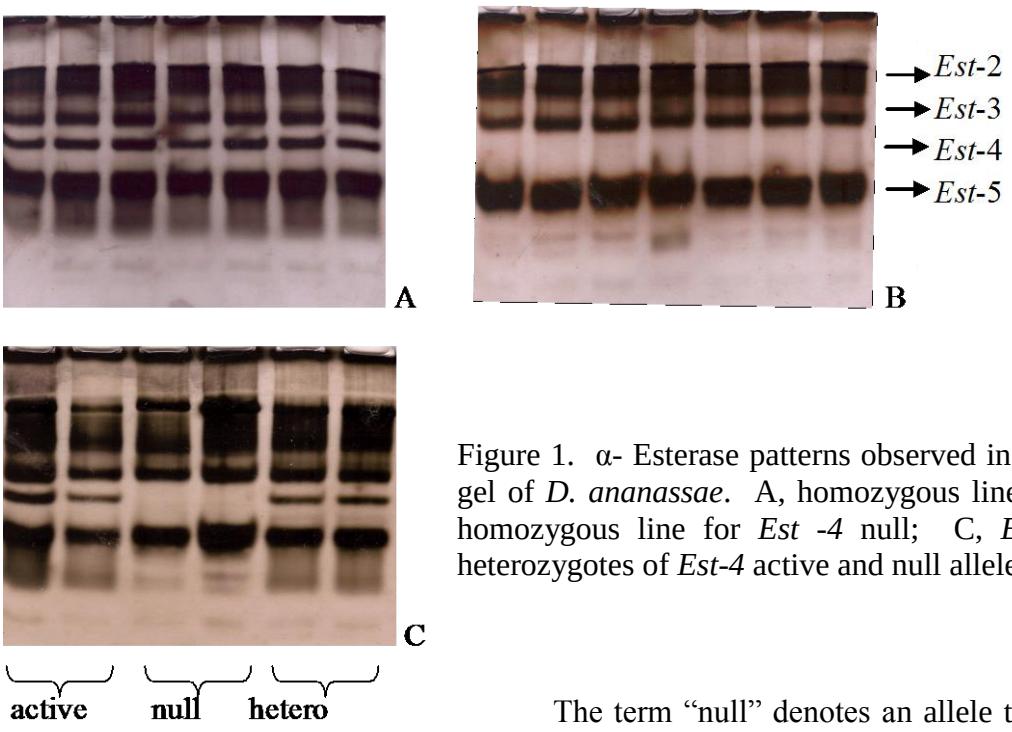

Figure 1. α- Esterase patterns observed in native polyacrylamide gel of *D. ananassae*. A, homozygous line for *Est -4* active; B, homozygous line for *Est -4* null; C, *Est-4* active, null, and heterozygotes of *Est-4* active and null alleles.

The term "null" denotes an allele that specifies a product that shows no catalytic activity as a monomer or heterodimer (depending on the locus) in our *in vitro* gel staining assay.

Survey of electrophoretic variation provided little information about the frequency of the null allele in natural populations. In most surveys null alleles go undetected, and a null-active heterozygote would be indistinguishable from a homozygote for an active allele (Voelker *et al*., 1980). Here, *Est-4* was detected as a polymorphic locus consisting of active and null alleles. Enzyme activity variation is likely to be of more significance as regards fitness than mobility variation, since changes in the activity of enzyme directly affects the biological function of enzymes. We have been able to prepare two separate lines from the GT-ST Stock, one homozygous for *Est-4* active and the other homozygous for *Est-4* null. The two stocks when crossed with each other showed enzyme activity as all the members were of *Est-4* active variant. The two homozygous lines thus prepared and the heterozygous forms derived from their crosses are being analyzed for their role in different aspects of behavior and fitness traits.

Acknowledgment: We extend our thanks to Mr. Sanjay Kumar for his help during this study.

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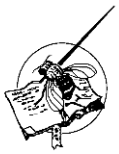

A preliminary report on *Drosophila* fauna of Lahore, Pakistan.

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Despite the fact that luxuriant flora and suitable climatic conditions exist in Pakistan for propagation of *Drosophila* species, little is known about *Drosophila* fauna of Pakistan. A few people have initiated the work in this field in Pakistan.

With a view to surveying *Drosophila* fauna of whole Pakistan, a project was begun during 1990-1992 in the Department of Biological Sciences, Quaid-e-Azam University, Islamabad, under the supervision of Dr. Mahmud Ahmad. As a part of that project, Din and Mazhar conducted a survey of Islamabad (33° 43′N, 73° 04′E) and identified ten *Drosophila* species. Another attempt was made by Shahjehan and Iqbal in NWFP (34.00°N 71.32°E) and, as a result, nine species had been reported.

Another attempt was made during 1990 to 1991 to explore *Drosophila* fauna of Lahore, Pakistan (31° 32’ 59”N, 74° 20’ 37”E). Baits were tried with different fruits such as banana, apple, guava, plum, muskmelon, orange, and lemon. Of all these fruits, fermenting bananas with a little yeast extract were found to be most effective bait. Net sweeping over bins containing peeling and decaying fruits also proved quite useful to collect large numbers of flies. It was found that drosophila flies were most abundant during April, September, and October. Cultures were made by using single female trapped from the wild, and their progeny was used to identify species. As a result ten species were identified on the basis of morphological criteria, which are as follows: *D. busckii, D. setaria, D. immigrans, D. melanogaster, D. takahashii, D. nepalensis, D. malerkotliana, D. ananassae, D. jambulina*, and *D. brevis.* The first three of these belong to subgenera *Dorsilopha*, *Pholadoris*, and *Drosophila*, respectively, and each of the remaining seven belongs to *melanogaster* species group of subgenus *Sophophora*. In order to verify identification further, seven species were studied for their reproductive isolation and mitotic as well as salivary gland chromosome complements. The chromosome number and morphology of each of these species were found to be in full conformity with already published literature on chromosomes of these species.

Acknowledgments: The author would like to thank her supervisor Dr. Mahmud Ahmad and Mr. Sayedul Haque of Quaid-i-Azam University, Islamabad, for the facilities and support which made this research possible.

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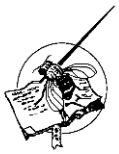

Sucrose improves sexual performance in the male fruit fly.

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We are interested in the regulation of sexual behavior in *Drosophila* (Terhzaz *et al*., 2007) and would like the males we use in our experiments to be in good physiological shape. From the extensive work by Dethier (1976), we know that male blow flies feed essentially on sugar water and

unlike females do not take extensive protein meals. It seems plausible that the same is the case for male fruit flies. As our standard fly food is made up of corn meal and yeast extract without any added sugars we wondered whether giving males access to sucrose water might enhance their sexual prowess. We tested this by keeping Canton males after emergence in tubes containing either our standard food, or in addition a small reservoir (Sellami *et al*., 2010) filled with 2 Molar sucrose and 1% E133 Brilliant Blue FCF. Both the fecal materials and the crop content turn blue, thus confirming that the flies have taken sucrose. Sexual performance was then tested by exposing a 4- to 7 day old virgin with two males of the same age, one of which had, and one which had not, been given access to sucrose. Courtship was followed for twenty minutes and the outcomes scored as to which male succeeded in copulating with the female and how long it took for it to achieve copulation. The sucrose-fed male was easily recognized by the brilliant blue in its crop.

Table 1. Outcome of sexual competition for a single virgin between two males of the same age, one which has had access to sucrose and one that did not. n, number of males that copulated with the female and either had (+) or had not (-) access to sucrose; none, refers to the number of assays in which neither male achieved copulation within 20 minutes. Number refers to the total number of tests performed and % to the percentage of tests in which the female copulated with either male within 20 minutes. P, statistical significance between the copulation success rates of the two types of flies (Wilcoxon sign test, one-sided). Time refers to the time from start to copulation, values are means ± SEM. There are no significant differences (NS) in the success rates for the different males at ages of 1 and 5 days, but the difference at 3 days is statistically significant, while the differences at 2 and 4 days are not statistically significant but might have become had more tests been performed (in both cases 0.12 > P > 0.10).

	Age of male Flies									
sucrose	1 day		2 days		3 days		4 days		5 days	
	n	time	n	time	n	time	n	time	n	time
+	8	6'03" ± 1'57"	42	7'02" ± 0'47"	45	5'33" ± 0'50"	32	4'23" ± 0'45"	18	4'08" ± 0'36"
-	12	9'31 ± 1'27"	32	7'57" ± 1'13"	23	3'26" ± 0'23"	22	3'24" ± 0'22"	16	3'40" ± 0'48"
none	20	-	31	-	5	-	9	-	0	-
number	40		105		73		65		34	
%	50		70.5		93.5		86.2		100	
P	NS		NS		P < 0.01		NS		NS	

If one lumps the data (Table 1) of all five age groups together, the results are statistically significant ($P < 0.01$), but if one looks at the different ages, it is clear that the difference is most pronounced for 3-day old males, with a tendency for 2- and 4-day old flies. Energy stores after the adult moult can be expected to be low, and a likely explanation of the results is that sucrose provides an easily metabolized energy source, which allows the recently-emerged males to compete more efficiently than the males which did not have access to sucrose. As has been shown for flight in *Drosophila*, sucrose and other sugars can immediately restore low energy stores (Wigglesworth, 1949). Higher energy levels are also suggested by the shorter time needed for 1 day-old flies to achieve copulation when they have access to sugar (Table 1). Presumably with time flies that do not have access to sucrose accumulate sufficient glycogen from the digestion of the starch in the standard food to allow efficient competition with males having access to sucrose. In conclusion, sugar consumption at an early age significantly enhances sexual performance of young male flies, and it may be useful to supply sucrose water to male flies if one intends to use them for courtship behavior.

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New distribution records of four *Drosophila* species (Diptera, Drosophilidae) in Mato Grosso, Brazil.

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Introduction

Mato Grosso is the third largest state in Brazil and encompasses three biomes: Amazonas, Cerrado and Pantanal. The Cerrado is the most widely distributed and threatened biome of the state and is considered one of the 25 biodiversity hotspots for conservation in the world (Myers 2000). In 2008, just 30 species of Drosophilidae were recorded in the state, a very low number compared to the number of species recorded in the states of São Paulo, Santa Catarina, and Rio Grande do Sul (review in Gottschalk *et al*., 2008). These data suggest that few studies of the family Drosophilidae have been conducted in this vast Brazilian region. In 2007, our group conducted a taxonomic survey of the geographical distribution of fifteen species of Drosophilidae in Brazil, which included common species such as *Drosophila melanogaster*, *D. hydei*, and *D. mercatorum* (Blauth and Gottschalk, 2007). In the present study, we documented the first observations of four species in a forested fragment of urbanized perimeter in Mato Grosso, Brazil.

Material and Methods

In June 2008, samples were collected in Parque Natural Ilto Ferreira Coutinho, or Bosque Municipal, a highly anthropized, forested area of 11.77 ha, in Tangará da Serra, Mato Grosso (14°37'39.61"S, 57°29'35.29"W) (Figure 1). Samples were collected in 10 traps constructed according to Tidon and Sene (1988) and baited with banana and commercial yeast. The traps were suspended at a height of 1.5 m, placed in a line, distanced 30 m apart, and kept in the field for three days. The identification of specimens was based on external morphology, and sibling males were identified by genitalia and were then dissected and prepared according to Bächli *et al*. (2004).

Results

A total of 497 individuals of 16 species were collected (Table 1). *Drosophila malerkotliana* was the dominant species collected, followed by *Z. indianus*; both are considered exotic species in the Neotropical Region. Each of the remaining 14 species had an absolute abundance lower than 20 individuals, amounting to a total of 103 individuals.

Table 1. Absolute abundance of Drosophilidae sampled at Parque Natural Ilto Ferreira Coutinho in Tangará da Serra, Mato Grosso, Brazil.

Genus	Group	Species	males	females	Total
Drosophila	*annulimana*	[1]*D. annulimana* Duda, 1927	1	2	3
	cardini	*D. cardini* Sturtevant, 1916	5	12	17
		D. cardinoides Dobzhansky & Pavan, 1943	3	0	3
	melanogaster	*D. malerkotliana* Parshad & Paika, 1964	173	141	314
		D. melanogaster Meigen, 1830	0	1	1
		D. simulans Sturtevant, 1919	9	11	20
	repleta	[1,2]*D. mapiriensis* Vilela & Bächli, 1990	1	0	1
		D. mercatorum Patterson & Wheeler, 1942	1	1	2
		[1] *D. papei* Bächli & Vilela, 2002	1	0	1
		[1,2]*D. querubimae* Vilela, 1983	1	0	1
	saltans	*D. prosaltans* Duda, 1927	4	7	11
		D. sturtevanti Duda, 1927	12	7	19
	willistoni	*D. nebulosa* Sturtevant, 1916	1	1	2
		willistoni subgroup	7	5	12
Scaptodrosophila	*latifasciaeformis*	*S. latifasciaeformis* Duda, 1940	6	4	10
Zaprionus	*armatus*	*Z. indianus* Gupta, 1970	25	55	80
Total			250	247	497

[1] First record in Mato Grosso, Brazil. [2] First record in the Cerrado biome.

Figure 1. Map of South America highlighting in dark gray the map of Brazil. The map indicates the first records of *D. annulimana* (*Da*), *D. mapiriensis* (*Dm*), *D. papei* (*Dp*), and *D. querubimae* (*Dq*) in Tangará da Serra, Mato Grosso (MT). Previous records of these species in Brazilian states and neighboring countries are indicated: (AM) state of Amazonas, (TO) state of Tocantins, (MG) state of Minas Gerais, (SP) state of São Paulo, (SC) state of Santa Catarina, and (RS) state of Rio Grande do Sul.

Four of these species are rare and newly recorded in Mato Grosso: *D. annulimana*, *D. mapiriensis*, *D. papei*, and *D. querubimae* (Figure 1). We also documented the first observations of *D. mapiriensis* and *D. querubimae* in the Cerrado biome.

Discussion

Despite being newly recorded in Mato Grosso, *D. annulimana* is amply distributed in Brazil and has been recorded in Amazonas, Minas Gerais, Santa Catarina, São Paulo (review in Gottschalk *et al*., 2008), and Rio Grande do Sul (Garcia *et al*., 2008; Garcia *et al*., 2009; Garcia *et al*., 2012). This species also occurs in Bolivia, Colombia, and Peru (Hunter, 1964; Vilela and Bächli, 1990; Villamizar and Álvarez, 2010).

Drosophila papei was previously recorded in Santa Catarina, Atlantic Forest biome (Gottschalk *et al.*, 2006), and in the state of Tocantins, Cerrado biome (Mata *et al.*, 2008). In each of the previous studies, only one individual was sampled. Three individuals were sampled in Rio Grande do Sul, a transitional area of the Atlantic Forest and Pampa biomes. Despite this species low abundance and rarity (six individuals sampled in Brazil until now), it seems that the species were widely distributed in Brazil, reaching Venezuela as the northern limit (Vilela and Bächli, 2002).

Drosophila querubimae was previously recorded in São Paulo (Vilela, 1983; Medeiros and Klaczko, 2004), and Santa Catarina (Gottschalk *et al.*, 2006), both in the Atlantic Forest biome, while *D. mapiriensis* was previously recorded in Santa Catarina (Gottschalk *et al.*, 2006; Dogë *et al.*, 2008; Bizzo *et al.*, 2012). These species were more abundant in urban areas of Santa Catarina than in forested areas (Gottschalk *et al.*, 2006).

Despite the few records of these species in the literature, *D. papei*, *D. querubimae*, and *D. mapiriensis* were not recorded in other sampling events carried out in Mato Grosso by our research group (unpublished data; Blauth and Gottschalk, 2007), which included pasture forest fragments and crop matrices.

This study contributes important information on the geographic range and distribution of Neotropical Drosophilidae species, furthering our understanding of the habitat of these species.

Acknowledgments: We are thankful to the administrator of Parque Natural Ilto Ferreira Coutinho for the permission to conduct our study in the Park.

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Fluctuation of *Phorticella striata* in mango plantations of Dharwad District.

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Abstract

Seasonal variation of *P. striata* was studied from May 2010 to April 2013 in five mango plantation sites of Dharwad District. It revealed that there was an increase in population with respect to the fruiting season of mango (May and June), and it decreased after the fruiting season. The fluctuation is due to the changes in the microhabitat of the collection sites.

Introduction

Drosophilidae are considered as model organisms in ecological studies, because they show variations in their population sizes and they have diverse species distribution. They also possess an enormous amount of intra- and interspecific variation which enables to study the evolutionary relationship of this group of insects (Parsons, 1973). Studies on the community composition of the species over time have now become an important criteria for ecologists because of widespread changes in climate that are now taking place (Markow and O' Grady, 2006). In most of the habitats, plant communities provide the physical structure of the environment, and, hence, it plays a considerable role on the distribution and interactions of animal species (Tews *et al*., 2004).

Drosophila flies are generally attracted to fruits especially fallen fruits under natural conditions. *Phorticella striata* is a non-drosophilid genus of the family Drosophilidae which is endemic to South India. They are large yellow flies with two distinct longitudinal chalky white striations extending from the base of antennae to tip of scutellum (Sajjan and Krishnamurthy, 1975). Seasonal changes in *P. striata* populations have been studied with reference to altitude, rainfall, and temperature in the forests of South Western Ghats and Mysore region. It was found to be one of the dominating species at all the seasons (Guruprasad and Hegde, 2006; Prakash and Ramachandra, 2008; Pranesh and Harini, 2012).

Dharwad district lies in the north western sector of Karnataka state with varied climate consisting of rugged foothills (part of Western Ghats) and plain lands (maidan). It lies between the latitudinal parallels of 15° 15' and 15° 35' North and longitudes of 75° 00' and 75° 20' East. There are no reports on *P. striata* population fluctuation or variation with respect to a particular fruit plantation at different seasons. In view of this, it is planned to study the populations of *P. striata* in different mango plantations.

Materials and Methods

Collection: *Drosophila* flies were collected in five different sites of mango plantations of Dharwad district from May 2010 to May 2013 using bottle trapping and net sweeping methods. These sites were named as site 1, site 2, site 3, site 4, and site 5. Site 1and site 2 are considered under western ghat belt which receive more rainfall (more than 713 mm), whereas site 3, 4, and 5 come under the plain lands, which receive scanty rainfall (less than 713 mm). The distance from site 1 to

site 2 is about 30 km west, site 3 is 20 km south, site 4 is 45 km east, and site 5 is 45 km north. In trapping method, 250 ml capacity bottles containing about 1 cm of smashed over-ripened banana fruit sprayed with yeast were tied to the twigs of mango trees about 2½ feet above the ground. Bottles were collected after 48 hours by plugging the mouth with cotton and brought to the laboratory. Net sweeping was also done for collecting the flies using rotting banana fruits, which were placed beneath the shaded areas of mango trees. The flies attracted to the fruits were swept using insect collecting net in the early morning after two days and were transferred to fresh media bottles. The flies collected were transferred to the fresh bottles containing wheat cream agar medium prepared as per the procedure of Shivanna *et al*. (1996). They were identified according to their characters as described by Bock and Wheeler (1972), Sajjan and Krishnamurthy (1975), and Markow and O' Grady (2006).

Results

Figure 1 depicts the seasonal variation of *P. striata* of five sites in different months. Average population size calculated for three years showed maximum number of flies during the mango harvesting season (May and June), whereas there was decline in its population after the month of June and by the time of winter (January and February) it reaches zero. But again from the month of April, there is an increase in its population, which reaches its maximum level during May and June. Figure 1 also revealed the variations of average population size among five different sites. Sites 1 and 2, on an average, receive more rainfall (more than 713 mm) and had maximum (392.1 and 160) flies, whereas sites 3, 4, and 5 receive lesser rainfall (less than 713 mm) and had maximum (34.6, 92, and 69.7) flies. There was a steep decrease in population size of *P. striata* after the month of June in case of site 1, whereas in site 2 there was a gradual decrease in population size during monsoon, and it was more when compared to site 1. In sites 3, 4, and 5 similar observations were made as that of site 1, but there was a difference in the number of flies obtained.

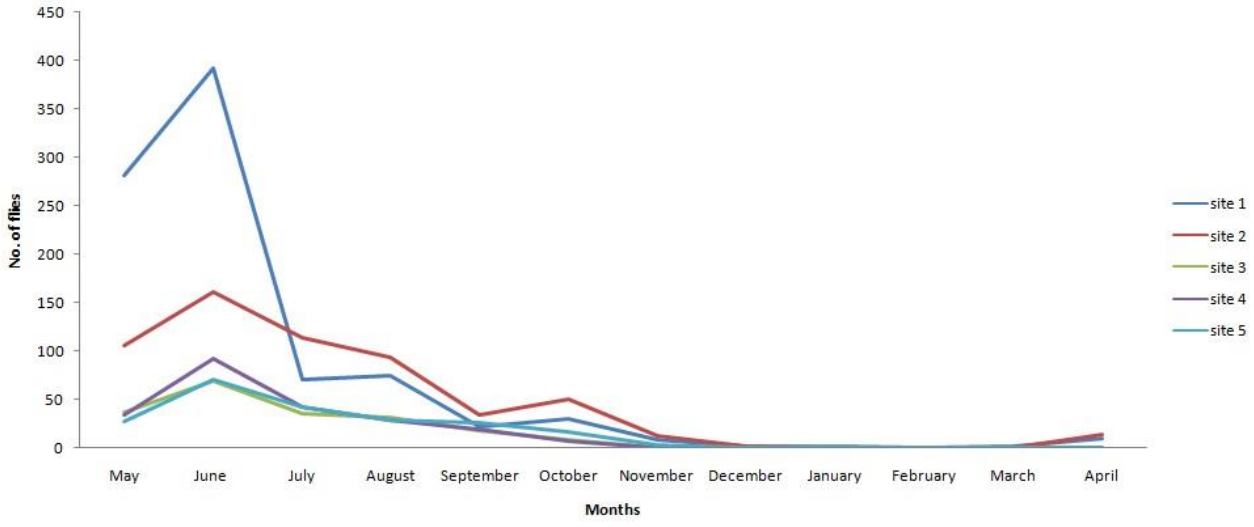

Figure 1. Average population size of *P. striata* in five different sites of mango plantations from May 2010 to April 2013.

Discussion

Mangifera indica, which is the major horticultural crop in Dharwad district, is grown in an approximately 3136 ha area with 5450 tonnes of yield. May and June is the mango fruit harvesting season; during this period maximum yield is obtained. The review of Parsons (1973) states that population expansion of *D. ananassae*, *D. hypocausta*, and *D. anuda* in a tropical wet climate (Moen Island, Truk, eastern Caroline Islands) is due to the presence of fruit-trees. The range of species is determined by the availability of its host plant range (Barker *et al*., 2005). The present study reveals that *P. striata* predominantly depends/ is found in mango fruiting season (Figure 1). Population variation of *P. striata* between sites is also because of their physiographical conditions. Sites 1 and 2 are located at the edge of hilly regions of Western Ghats and receive more rainfall, whereas sites 3, 4, and 5 come under the arid zones, which are plain lands receiving less rainfall. Sites 1 and 2 are known as vibrant mango growth regions with higher yield, site 3 comes under medium mango growing regions, whereas sites 4 and 5 are low mango growing regions (Dharwad District Profile, 2011). Species belonging to the genus *Drosophila* were found to be abundant during monsoon and post-monsoon seasons and were found less during winter and summer (Prakash and Reddy, 1978; Prakash and Ramachandra, 2008; Guruprasad *et al*., 2010). Monsoon season provides sufficient rainfall, which also provides moisture to the soils, and in post-monsoon season this moisture is retained till winter and hence provides enough resources for *Drosophila* species to survive (Hegde *et al*., 2001). The present study on *P. striata* contradicts the earlier studies on *Drosophila* variation during different seasons. Even though *P. striata* was predominantly found in mango plantations during its fruiting season, it is not considered as a pest of mango as they are not primary infectors like other fruit flies (*Bactrocera dorsalis*, *B. correcta*, and *B. zonata*) in India (Verghese *et al*., 2006). There are no reports of *P. striata* species being considered as pest of mango fruit. During most of the seasons, *P. striata* species were found to be feeding on fallen fruits. Hence, it cannot be considered as a pest of the mango fruit.

The comparison of the population showed that in site 1 the number of flies is more (392) than at other sites, wherein site 3 has the lowest of 69 flies. This shows that there is variation in the number of flies among the sites; it is due to physiogeographic variations. During harvesting season of mango the flies are more frequent in all the sites. After the season in some sites it drastically drops in number, whereas in other sites it gradually decreases. The variations/ fluctuations in number of flies are due to variations in the surrounding microhabitats of the plantations. Further studies on micro habitats may provide information on complexity of species interaction and biodiversity of Drosophilids.

Acknowledgment: The authors are thankful to the chairperson, Dept. of Zoology, Karnatak University, Dharwad, for providing necessary facilities. S.B.S. is thankful to UGC for award of Rajiv Gandhi National Fellowship and N.S to UGC MRP F. NO. 37/-241/2009 (SR) for financial assistance.

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Sex comb size in *Drosophila melanogaster* males maintained on carrot and banana substrates.

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Sex comb in *Drosophila melanogaster* male represents one of the secondary sexual traits. It is located on prothoracic legs and is composed of dark, strong bristles, usually referred to in the literature as "teeth" (Figure 1). This species possesses one row of sex combs, with an important role during courtship and copulation (Spieth, 1952). The relationship between size and symmetry of this trait and male mating success was reported for different sophophorans (Markow *et al.*, 1996; Polak *et al.*, 2004; Ng and Kopp, 2008; Pavković-Lučić and Kekić, 2011; Vishalakshi, 2011; Pavković-Lučić *et al.*, 2013a). Sex comb differs in the number of rows, bristle orientation, their color, size, and shape; these are of taxonomic importance (Mishra and Singh, 2006; Tanaka *et al.*, 2009). Besides, there is variability in teeth number in different strains of the same species. Phenotypic and genotypic variation underlying sex comb teeth number were thoroughly studied (Ahuja *et al.*, 2011), as this quantitative trait is determined by both genes (see for review Nuzhdin and Reiwitch, 2000; Kopp, 2011) and environmental factors during development.

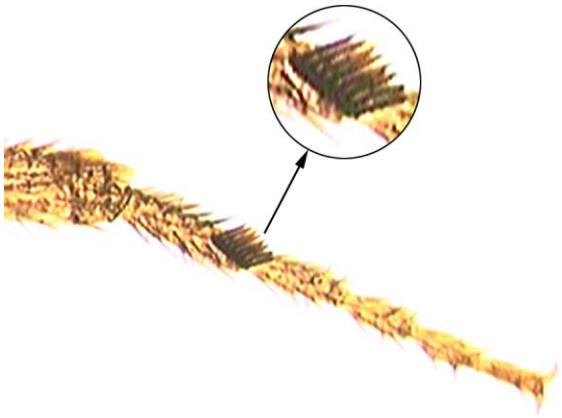

Figure 1. Foreleg of *Drosophila melanogaster* male with sex comb.

In this note, we investigated effects of long-term maintenance of flies on two substrates on male sex comb teeth number. Flies were reared more than twelve years, over 300 generations, on two substrates prepared with carrot and banana (for recipes see Kekić and Pavković-Lučić, 2003), in laboratory conditions optimal for the species (at a temperature of approx. 25°C, relative humidity of 60%, 300 lux of illumination, and 12 h:12 h light/dark cycle). Fifty males from both substrates were randomly taken and their front legs were carefully removed

from the body and put on microscope slides. The number of sex comb teeth was counted under a Leica MZ16 microscope at a magnification of 115×. Sex comb size was scored by counting the teeth number in left (L) and right (R) legs. Mean number of sex comb teeth was estimated as (L+R)/2, while means between two "nutritive strains" was tested using t-test. Results showed that mean number of sex comb bristles significantly differs between males developed on two substrates, with higher average value in males reared on banana substrate (Table 1). This result is opposite to results obtained for mean sex comb bristle number compared among three strains maintained more than one year (over 35 generations) on banana, tomato, and standard cornmeal-sugar-agar-yeast substrates, when significant differences were not observed (Pavković-Lučić *et al.*, 2013a). However, significant differences in sex comb size and length were recorded in males reared under poor and rich diets, with higher values of those traits in rich diet (Ahuja *et al.*, 2011).

Table 1. Mean ± SE and range of sex comb teeth number in males maintained on banana and carrot substrates.

Male type	N	Mean ± SE (range)	t	d.f.	p
banana substrate	50	9.11 ± 0.101(6-11)	-3.091	98	< 0.01
carrot substrate	50	8.65 ± 0.109 (7-11)			

It is possible that rearing of flies on substrates with different nutritive values for such a long period led to significant differences in their body sizes, also reflected in divergence in sex comb size. Since males maintained on banana medium possess longer wings than males maintained on carrot medium (Trajković *et al.*, 2013), our result is consistent with expectation that larger individuals would express more bristles in the combs, because sex comb is body size dependent trait (Polak and Tomkins, 2012), as is wing length (Partridge *et al.*, 1987). Beside morphological traits, long-term maintenance and development of flies on these substrates influenced also behavioral characteristics (Pavković-Lučić *et al.*, 2013b; Trajković *et al.*, 2013) and some fitness components (Filipović *et al.*, *in preparation*).

Acknowledgment: This work was funded by Serbian Ministry of Education, Science, and Technological Development (Grant 173012).

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Cytotype repression of hybrid dysgenesis in *D. melanogaster*: Limited synergism between a telomeric *P* element and individual non-telomeric *P* elements implies a failure in Ping-pong amplification of regulatory piRNAs.

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Transposable *P* elements are the cause of hybrid dysgenesis, a syndrome of germ-line abnormalities that includes frequent mutation, chromosome breakage, and sterility. This syndrome is repressed by the P cytotype, a maternally inherited state that is mediated by small RNAs generated by *P* elements inserted in the Telomere Associated Sequences (TAS) at the left end of the X chromosome (Brennecke *et al.*, 2008). Because these RNAs associate with the Piwi class of proteins, they are called Piwi-interacting, or "pi," RNAs. Cytotype regulation anchored in X-linked telomeric *P* elements is enhanced by *P* elements situated at non-telomeric loci, even though these other elements have no repression ability of their own (Simmons *et al.*, 2007, 2012). The enhanced regulation is thought to reflect a process that is fed by primary piRNAs from the telomeric *P* element and sense RNAs from the other *P* elements. With repetition, this process, called the ping-pong cycle (Aravin *et al.*, 2007; Gunawardane *et al.*, 2007), can amplify the population of *P*-specific piRNAs significantly.

Studies with the telomeric elements *TP5* and *TP6* have shown that all but the smallest non-telomeric *P* element can bring about synergistic repression of hybrid dysgenesis (Simmons *et al.*, 2012). These studies employed a set of *hobo* transgenes that contained different *P* elements (see Figure 1 in Simmons *et al.*, 2012): *SP* (naturally 0.5 kb long), *TP5* (1.8 kb long), *TP6* (1.9 kb long), *CP* (the complete, transposase-encoding *P* element, 2.9 kb long), and *P** (a frameshifted *P* coding sequence lacking the *P* promoter and all the *P* introns, 2.2 kb long). Each of these elements was terminally truncated and placed downstream of an *hsp70* promoter in a *hobo* transposon marked with the mini-*white* gene; the *SP*, *TP5*, *TP6*, and *CP* elements also possessed the natural *P*-element promoter. None of these *H*(*hsp*/*P*) transgenes have any intrinsic ability to repress GD, and none are located at a telomere (Jensen *et al.*, 2008; Simmons *et al.*, 2002, 2012).

We used the *H*(*hsp*/*P*) transgenes to determine what kinds of *P* elements could enhance cytotype regulation anchored in the telomeric *P* element *NA*-*P*(*1A*), which is deficient for the first 871 base pairs of the canonical *P*-element sequence (Marin *et al.*, 2000); hereafter, this telomeric *P* element is denoted simply as *NA*. Reciprocal crosses between an *NA* w^{sp} strain and each of several (*y*) *w*; *H*(*hsp*/*P*) strains were carried out; in cross A, the *NA* element was derived maternally and in cross B it was derived paternally. The *NA* w^{sp}/*w*; *H*(*hsp*/*P*)/+ F_1 daughters from these reciprocal crosses were then mated to Harwich *y w* males, which are powerful inducers of hybrid dysgenesis. The F_2 females from the F_1 matings were scored for dysgenic sterility (gonadal dysgenesis, or GD) according to standard procedures (Simmons *et al.*, 2012), but without regard to whether or not they carried *NA* or the *H*(*hsp*/*P*) transgene (Table 1).

In the absence of any transgene, the frequency of GD was 47.9% in cross A and 99.1% in cross B. Thus, by itself, the *NA* element was a strong repressor of GD, but only when it was inherited maternally by the F_1 females. Only one of the *NA*-*H*(*hsp*/*P*) transgene combinations—the one with *H*(*hsp*/*TP5*)*D*—produced significantly lower frequencies of GD than the controls; in cross A the frequency of GD was 2.7% and in cross B it was 84.2%. Thus, only the transgenic *TP5* element enhanced *NA*'s intrinsic ability to repress dysgenesis.

We performed two more experiments to test for synergism between *NA* and the transgenic *P* elements. They began with reciprocal crosses between flies from an *NA* y^+ *w*/*FM7*, sn^{x2} *B* strain and flies from different *y w*; *H*(*hsp*/*P*) transgenic strains. In cross A, *NA* y^+ *w*/*FM7* sn^{x2} *B* females were mated to *y w*; *H*(*hsp*/*P*) males and in cross B, *NA* y^+ *w* males were mated to *y w*; *H*(*hsp*/*P*) females. The *NA* y^+ *w*/*y w*; *H*(*hsp*/*P*)/+ F_1 females from both sets of crosses were mated to Harwich *y w* males to induce GD in their daughters, which were sorted by genotype and scored (Table 2); a diagram of a mating scheme similar to the one used in these experiments is presented in Figure 1 of Merriman and Simmons (2013).

By itself, *NA* was a very weak repressor of dysgenesis in the F_2 females from cross A in both experiments (94-97% GD), and it did not repress at all in the F_2 females from cross B (99.5-100% GD). The weakened repression ability in these controls may be due to the single dosage of *NA* in the females that were used to set up cross A. In the results reported in Table 1, where the control level of repression was stronger, cross A was initiated with females that had a double dose of *NA*.

In the first experiment we tested four different *H*(*hsp*/*P*) transgenes for interactions with *NA*. Only one of them, *H*(*hsp/TP5*)*D*, significantly enhanced repression in the F_2 females of cross A. The frequency of GD was roughly the same for all four classes of F_2 females from this cross (55-58%), and the F_2 flies that inherited neither *NA* nor *H*(*hsp*/*TP5*)*D* repressed GD as effectively as the flies that inherited both of these factors. Thus, the enhanced capacity for repression was transmitted independently of either the *TP* or the transgenic *P* element—that is, it operated as a strictly maternal effect. Only two of the transgenes, *H*(*hsp*/*CP*)*2* and *H*(*hsp*/*TP5*)*D*, were tested for synergism with *NA* in cross B; in these tests the frequency of GD was 98-100%. Thus, unlike the results obtained previously (Table 1), there was no evidence for synergism between *NA* and *H*(*hsp*/*TP5*)*D* in cross B, probably because the intrinsic level of repression by an *NA* element derived from a "single dose" *NA* stock is so low.

Table 1. Synergism between the telomeric *NA* element and various *H*(*hsp*/*P*) transgenes assessed in the daughters of *NA* w^{sp}/*w*; *H*(*hsp*/*P*)/+ F_1 females from reciprocal crosses between *NA* w^{sp} and (*y*) *w*; *H*(*hsp*/*P*) strains.

Transgene	Cross	No of vials	No of flies	%GD ± SE[a]
None	A	25	499	47.9 ± 4.8
H(*hsp*/*SP*)*A*	A	25	426	73.1 ± 4.2
H(*hsp*/*CP*)*2*	A	25	500	44.4 ± 2.0
H(*hsp*/*TP5*)*D*	A	21	413	2.7 ± 1.1
H(*hsp*/*TP6*)*C*	A	25	479	57.1 ± 4.6
H(*hsp*/*P**)*B*	A	25	470	36.9 ± 3.1
None	B	25	457	99.1 ± 0.4
H(*hsp*/*SP*)*A*	B	25	393	99.0 ± 0.6
H(*hsp*/*CP*)*2*	B	25	497	99.4 ± 0.3
H(*hsp*/*TP5*)*D*	B	25	493	84.2 ± 2.8
H(*hsp*/*TP6*)*C*	B	25	487	99.6 ± 0.3
H(*hsp*/*P**)*B*	B	25	498	98.8 ± 0.4

[a] Unweighted average percentage GD ± standard error. The standard error was computed from the empirical variance among replicate cultures.

In the second experiment we determined if other insertions of the *H*(*hsp*/*TP5*) transgene could interact synergistically with the telomeric element *NA*. In these tests, significant synergistic repression of GD was seen in the F_2 females from cross A, the strongest being with *H*(*hsp*/*TP5*)*B* (30% GD compared to 70-73% with insertions *A*, *C* and *D*), and in all cases, the synergism between *NA* and the *H*(*hsp*/*TP5*) transgenes involved a strictly maternal effect. Only the *H*(*hsp*/*TP5*)*B* insertion was tested for synergism with *NA* in cross B, and none was observed.

Ping-pong cycling has been invoked to explain the synergistic repression of hybrid dysgenesis by telomeric and transgenic *P* elements (Simmons *et al.*, 2012). For this cycling to occur, the telomeric and transgenic *P* elements must be at least partially homologous. Cytotype regulation anchored in *NA* was enhanced by the small transgenic element *TP5*, but not the slightly larger transgenic element *TP6*. This difference might be due to greater homology (293 nucleotides) between *NA* and *TP5* than between *NA* and *TP6*.

However, increased homology is not a guarantee that a transgenic *P* element will enhance cytotype regulation, because the transgenic elements *CP* and *P**, which are substantially more homologous to *NA* than *TP5* is, did not increase *NA*'s regulatory power. This failure suggests that the transcripts of these two elements are unable to feed a ping-pong cycle anchored in the *NA* element. However, *CP* and *P** do robustly enhance cytotype regulation anchored in the telomeric elements *TP5* and *TP6* (Merriman and Simmons, 2013; Simmons *et al.*, 2012). *CP* and *P** sense RNAs are, therefore, able to feed a ping-pong cycle initiated by primary piRNAs from either of these telomeric *P* elements.

Table 2. Synergism between the telomeric *NA* element and various *H*(*hsp*/*P*) transgenes assessed in the daughters of *NA* y^+ *w*/*y w*; *H*(*hsp*/*P*)/+ females from reciprocal crosses between *NA*-bearing and *H*(*hsp*/*P*)-bearing strains.

Transgene	Cross	No. of vials	Neither		Transgene only		*NA* only		Both		Pooled overall	
			No. of flies	%GD ± SE[a]	No. of flies	%GD ± SE[a]	No. of flies	%GD ± SE[a]	No. of flies	%GD ± SE[a]	No. of flies	%GD ± SE[a]
None	A	38	631	97.7 ± 1.0			612	96.7 ± 1.7			1243	97.2 ± 1.2
H(*hsp/SP*)*A*	A	35	288	96.7 ± 2.9	295	97.1 ± 2.9	326	96.3 ± 2.9	300	95.1 ± 3.0	1209	96.3 ± 2.9
H(*hsp/CP*)*2*	A	44	333	93.3 ± 1.6	352	92.1 ± 1.9	330	94.8 ± 1.6	328	91.1 ± 1.8	1343	92.7 ± 1.3
H(*hsp/TP5*)*D*	A	41	322	58.0 ± 5.2	312	55.5 ± 5.4	284	57.1 ± 5.2	349	56.9 ± 5.3	1267	57.7 ± 4.8
H(*hsp/TP6*)*C*	A	37	267	98.6 ± 0.7	298	96.9 ± 1.5	287	98.5 ± 0.8	274	97.8 ± 1.0	1126	97.8 ± 0.5
None	B	28	524	100 ± 0			511	100 ± 0			1035	100 ± 0
H(*hsp/CP*)*2*	B	26	293	100 ± 0	311	99.5 ± 0.4	290	100 ± 0	286	99.7 ± 0.3	1180	99.8 ± 0.1
H(*hsp/TP5*)*D*	B	26	296	98.2 ± 1.3	311	98.3 ± 0.7	307	98.3 ± 1.0	315	97.6 ± 0.9	1229	98.2 ± 0.5
None	A	17	193	93.9 ± 3.1			210	94.0 ± 3.2			403	94.2 ± 3.1
H(hsp/TP5)A	A	34	327	68.5 ± 5.8	300	71.9 ± 5.8	355	71.1 ± 5.7	358	70.2 ± 5.6	1340	70.9 ± 5.6
H(hsp/TP5)B	A	21	140	34.0 ± 7.5	144	30.1 ± 7.1	189	24.4 ± 5.7	169	31.8 ± 6.9	642	30.2 ± 6.2
H(hsp/TP5)C	A	30	219	71.5 ± 6.3	239	68.8 ± 6.4	205	71.1 ± 6.8	226	65.2 ± 6.5	889	69.6 ± 6.1
H(hsp/TP5)D	A	21	191	73.5 ± 5.9	170	76.7 ± 6.5	185	73.4 ± 6.7	188	67.4 ± 7.1	734	72.8 ± 5.9
None	B	17	298	99.7 ± 0.3			269	99.2 ± 0.6			567	99.5 ± 0.3
H(hsp/TP5)B	B	12	130	90.7 ± 3.9	118	97.1 ± 1.5	144	96.5 ± 1.6	106	97.9 ± 1.6	498	94.9 ± 1.6

In cross A, *NA* y^+ *w*/*FM7*, sn^{X2} *B* females were mated to *y w*; *H*(*hsp*/*P*) males, and in cross B, *NA* y^+ *w* males were mated to *y w*; *H*(*hsp*/*P*) females. The F_1 females resulting from these two sets of crosses were then mated to Harwich *y w* males to induce GD in their daughters, which segregated into four genotypic classes—carrying *NA* or not, and carrying the *H*(*hsp*/*P*) transgene or not. The data in the upper part of the table were collected from an experiment to survey different types of transgenes for interactions with the *NA* element; those in the lower part were collected from another experiment to test for synergism between *NA* and different insertions of the *H*(*hsp*/*TP5*) transgene.

[a] Unweighted average percentage GD ± standard error. The standard error was computed from the empirical variance among replicate cultures.

Why do *CP* and *P** enhance regulation anchored in *TP5* or *TP6*, but not in *NA*? One possibility is that with *NA* as the anchoring element, the longer RNAs from *CP* and *P** actually get in the way of ping-pong cycling. These sense RNAs might base pair with antisense transcripts from *NA* to form comparatively long double-stranded molecules that are diverted from the ping-pong cycle into an RNA interference pathway, where they generate siRNAs instead of piRNAs. Greater unbroken homology between telomeric and transgenic *P* RNAs may, therefore, prevent the production of enough piRNAs to repress hybrid dysgenesis strongly. The absence of synergism between the telomeric element *NA* and the transgenic elements *CP* and *P** implies that RNAs from these combinations of *P* elements interact in ways that keep them from entering the ping-pong cycle.

Acknowledgments: Stéphane Ronsseray kindly provided a strain with the telomeric *NA* element, and the Department of Genetics, Cell Biology and Development of the University of Minnesota provided financial support.

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Repression of hybrid dysgenesis in *D. melanogaster* males by the X-linked telomeric *P* element *NA-P(1A)*.

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Repression of hybrid dysgenesis is anchored in *P* elements that have inserted in the Telomere Associated Sequences (TAS) at the left end of the X chromosome. These telomeric *P* elements confer the P cytotype, a regulatory state that is mediated by small RNAs that interact with the Piwi class of proteins; the RNAs are, therefore, called Piwi-interacting, or "pi", RNAs. *NA-P*(*1A*) is a telomeric *P* element that produces *P*-specific piRNAs (Brennecke *et al.*, 2008). This element, hereafter denoted simply as *NA*, is inserted at the junction of the distal retrotransposon array and the TAS of chromosome XL (Marin *et al.*, 2000). The *NA* element is structurally incomplete, lacking the first 871 base pairs of the canonical *P*-element sequence, including the *P* promoter, the first *P* exon, the first *P* intron, and half of the second *P* exon; consequently, it cannot encode the transposase that catalyzes *P*-element activity or a truncated polypeptide that might interfere with this activity. The discovery that this element represses hybrid dysgenesis was a strong indication that cytotype regulation does not involve *P*-encoded repressor polypeptides.

Marin *et al.* (2000) demonstrated that *NA* represses hybrid dysgenesis in females. To determine if it also represses dysgenesis in males, we used a genetic test that detects excisions of the *P* elements inserted in sn^w, a weak mutant allele of the X-linked *singed* bristle locus (Engels, 1979). Two incomplete *P* elements are inserted in the 5' untranslated region of sn^w (Roiha *et al.*, 1988). Excision of the upstream element converts sn^w into sn^e, an allele with an extreme mutant phenotype. Excision of the downstream element converts sn^w into $sn^{(+)}$, a pseudo-wild allele. The extreme mutant and pseudo-wild phenotypes are easily distinguished from the weak mutant phenotype. We screened for *P*-element excisions from sn^w that occurred in the germ lines of males carrying sn^w and $H(w^+, \Delta 2\text{-}3)6$, a hobo transgene that produces the P transposase (Merriman and Simmons, 2013). These males were crossed to females with attached-X chromosomes so that sn^w or its sn^e or $sn^{(+)}$ derivatives would be inherited patroclinously. The sons of these crosses were then scored on days 14 and 17 for the three bristle phenotypes (weak, extreme, and pseudo-wild), and the frequency of the extreme and pseudo-wild sons was used to estimate the germ-line sn^w excision rate. A reduced rate indicates that *P* excisions have been repressed.

The $H(w^+, \Delta 2\text{-}3)6$ transgene contains a terminally truncated *P* element that lacks the last intron of the transposase gene—the one between exons 2 and 3 in a complete *P* element; hence its designation as $\Delta 2\text{-}3$. This transgene, inserted on chromosome 3, produces the P transposase in the soma as well as in the germ line. Genetic analyses have shown that like the widely used P transposase source $P(ry^+, \Delta 2\text{-}3)99B$ (Robertson *et al.*, 1988), $H(w^+, \Delta 2\text{-}3)6$ does not transmit

transposase activity through the egg independently of the element itself—that is, *H*(w^+, Δ*2-3*)*6* does not induce *P*-element excisions through a strictly maternal effect (Merriman and Simmons, 2013). The absence of such an effect allowed us to ascertain if *NA*-mediated regulation of *P* activity has a maternal component.

The experiment was similar to one that studied the regulatory abilities of *TP5* and *TP6*, two other X-linked telomeric *P* elements (Simmons *et al.*, 2004). It consisted of "reciprocal" crosses between *y w* flies heterozygous for the *H*(w^+, Δ*2-3*)*6* transgene and flies hemizygous or homozygous for an X chromosome carrying *NA* and the markers *w* and sn^w. The *NA w* sn^w; *H*(w^+, Δ*2-3*)*6*/+ F_1 males from these crosses were mated individually to *C*(1)*DX*, *y f* females with attached-X chromosomes. Due to somatic production of the P transposase by the *H*(w^+, Δ*2-3*)*6* transgene, these F_1 males were all bristle mosaics; sn^w and $sn^{(+)}$ or sn^e bristles were present on every fly. The *NA* element—like other telomeric *P* elements—therefore does not repress transposase activity in the somatic tissues. The sons of these F_1 males were scored for their bristle phenotype to estimate the frequency of *P*-element excisions from the sn^w allele that had occurred in each F_1 male's germ line. To permit these sons to be scored unambiguously, the somatic mosaicism caused by the segregating *H*(w^+, Δ*2-3*)*6* transgene had to be repressed by using attached-X females from a special strain in the F_1 matings (Robertson and Engels, 1989; Simmons *et al.*, 2004); this strain carries *P* elements that produce polypeptide repressors of somatic *P* activity.

Table 1. *NA*-mediated repression of *P* excisions from the sn^w allele in the male germ line.

Stock	Cross A: *NA* inherited maternally			Cross B: *NA* inherited paternally		
	No. vials	No. flies	Excision rate [a]	No. vials	No. flies	Excision rate [a]
w sn^w	32	928	0.632 ± 0.029	32	1063	0.470 ± 0.032
NA w sn^w #1	32	1163	0.250 ± 0.038	32	1210	0.498 ± 0.029
NA w sn^w #2	31	1117	0.209 ± 0.033	32	1111	0.482 ± 0.032
NA w sn^w #3	31	949	0.194 ± 0.028	32	1158	0.471 ± 0.026

To obtain these data, (*NA*) *w* sn^w; *H*(w^+, Δ2-3)*6*/+ F_1 males were mated at 25°C to *C*(1)*DX*, *y f* females from a somatic *P* repressor strain and their sons were scored for the three bristle phenotypes—sn^w, $sn^{(+)}$, and sn^e. The F_1 males were obtained from "reciprocal" crosses at 18 °C; cross A was (*NA*) *w* sn^w females x *y w*; *H*(w^+, Δ2-3)*6*/+ males and cross B was (*NA*) *w* sn^w males x *C*(1)*DX*, *y w f*; *H*(w^+, Δ2-3)*6*/+ females. The *H*(w^+, Δ2-3)*6*-bearing flies for crosses A and B were obtained by mating *C*(1)*DX*, *y w f* females to *y w*; *H*(w^+, Δ2-3)*6* males at 25 °C. The three *NA w* sn^w stocks that were tested had been independently generated by recombining an *NA* w^{sp} X chromosome with a *y w* sn^w X chromosome and then making each *NA w* sn^w recombinant chromosome homozygous.

[a] Unweighted excision rate, computed by averaging the proportion of $sn^{(+)}$ and sn^e males among all the males in each culture; the standard error was calculated from the empirical variance among cultures.

Three independently generated stocks with the *NA w* sn^w X chromosome were tested along with a *w* sn^w control stock that did not carry *NA*. In cross A, the *NA* element was inherited maternally and the *H*(w^+, Δ*2-3*)*6* transgene was inherited paternally. In cross B, the derivations of *NA* and *H*(w^+, Δ*2-3*)*6* were reversed. The results of the experiment are summarized in Table 1. For the control stock, the excision rate was 0.632 in cross A and 0.470 in cross B. These rates indicate that *H*(w^+, Δ*2-3*)*6* induces a high frequency of germ-line *P*-element excisions in the male germ line. We do not

know why the excision rate in cross A is significantly higher than in cross B. For the three *NA* stocks, the excision rates in cross A were significantly lower than the corresponding control rate (and also significantly lower than the control rate in cross B); however, the excision rates for the *NA* stocks in cross B were not lower than the rate for the cross B control. These observations indicate that *P*-element excisions in the male germ line are repressed, but only when the *NA* element is inherited maternally. Thus, regulation of *P* activity by *NA*, as by other telomeric *P* elements, involves a maternal effect, and an *NA* element that passes patroclinously from father to son loses its ability to repress hybrid dysgenesis.

Acknowledgments: Stéphane Ronsseray kindly provided a stock carrying the *NA* element, and the Department of Genetics, Cell Biology and Development of the University of Minnesota provided financial support.

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Influence of age on mating and fitness of *Drosophila melanogaster*.

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Abstract

The effect of age on mating time, remating duration, quantity of ACP transferred, fecundity, and productivity was studied within and between 7 and 26 days old *Drosophila melanogaster*. Mating time, accessory gland secretory protein transferred, fecundity, and productivity of 1st mating is more than the 2nd mating irrespective of age. The remating duration among young and old flies is almost similar, whereas it is more in different aged flies mating. Mating time, fecundity, and productivity are more in young flies compared to other combinations. In old flies mating time, fecundity, and productivity are more than the different aged flies. The fecundity is more if the female is young and productivity is more if the male is young in different aged flies mating. Key words: *Drosophila*, mating behavior, fecundity, productivity, age.

Introduction

Sexual reproduction occurs in a wide range of organisms. Reproductive capacity is particularly a good index of fitness in organisms that go through repeated cycles of rapid population growth, and it has evolved as a way for species to maximize their potential of survival. Mating is the most important and fundamental process of reproduction. Male fitness depends largely on the

number of matings and the average number of progeny sired from each mating; female fitness depends on the number of progeny production (Bateman, 1948; Singh and Singh, 2000, 2004).

Ageing is a multifaceted phenomenon that occurs in most of the species. In sexual reproduction, age is regarded as one of the most important factors for mate choice. It depends on genetic quality in sexual reproduction of insects. The effect of age has been studied on fitness parameters, such as mating choice, mating time, duration between matings, fecundity, productivity, and viability in different species of *Drosophila* (Moore and Moore, 2001; Anderson *et al*., 1973). *Drosophila* is proving to be a powerful system for understanding the age dependent mating behavior.

Mating time and duration between matings are important components of *Drosophila* mating behavior. Mating time is the time from initiation of copulation till the time of release or departures of males and females. Mating time was observed in many species of *Drosophila.* It varies from 25 seconds (*D. polychaeta*) to 137 minutes (*D. acanthoptera*) (Markow, 1996). In *Drosophila* multiple mating is common in both natural and laboratory conditions. First and second mating has been studied. Whereas the male and female after 1st mating take some time to remate (2nd), it is called duration between matings. Male takes less time to remate than female in all the species. Remating has been studied in different species of *Drosophila.* It ranges between 1 hour to 5 days in some females, and some females (*D. differens, D. teteroneura, D. subobscura, D. sylvestris, D. acanthopera*) rarely or never remate (Markow, 1996; Singh and Singh, 2004).

Accessory gland secretory proteins (ACPs) are transferred from male to female genital track during mating. It induces morphological and behavioral changes in mated females. ACPs are major components of seminal fluid; it contains about 80 proteins and peptides in *D. melanogaster* (Chen, 1984; Chapman and Davies, 2004). The synthesis of ACP increases up to 7 days. The quantity of secretion has been estimated in few species of *Drosophila*. It varies from 50 to 84% in *D. rubida* and *D. ananassae*, respectively, and 1/3 amount of the quantity is released during the 1st mating (Chen, 1984; Shivanna and Ramesh, 1995; Ravi Ram and Ramesh, 2001; Hiremani and Shivanna 2010).

Fecundity is the number of eggs laid per female after mating. It has been studied in different species of *Drosophila* on different aspects and it varies from 98 to 333 (Ashadevi and Ramesh, 1999; Harini and Ramachandra, 2007; Harini, 2010; Rezaei, 2012; Lushchak *et al.*, 2013; Pavkovic and Kekic 2013). Productivity is the number of progeny produced by each mated female. It has been observed in many species of *Drosophila* on different aspects. It varies from 55 to 500 in *D. hydei* and *D. melanogaster*, respectively (Markow, 1996; Ashadevi and Ramesh, 1999; Roopashree *et al.*, 2001).

Most of the studies are made in different species and various aspects. The relationship between mating activities, quantity of ACP transferred during mating, and effect of age on fitness has not been analyzed. In view of this, the present work has been taken to study the relationships between above aspects in *D. melanogaster*.

Materials and Methods:

Drosophila melanogaster was obtained from *Drosophila* stock center, Department of Zoology, University of Mysore, Mysore. The flies were cultured in a standard wheat cream agar medium, prepared as per the procedure described by Shivanna *et al*. (1996) and maintained at a constant temperature of 22 ± 1ºC. The virgin males and females were aged up to 7 (young) and 26 (old) days and used for experiments.

Male and female flies were allowed to mate in a vial (9 × 2.5 cm). After initiation of mating, the 1st mating time was recorded. After 1st mating, the female was removed and another virgin

female was introduced. Time required for remating and 2nd mating time were noted as per the procedure described by Singh and Singh (2000, 2004). The mated and unmated males were dissected in an insect saline, accessory glands were isolated and fixed in 95% ethanol, and then the glands were washed with methanol and chloroform (1:1) and dried at 37°C in oven. The glands (5 pair) were dissolved in 40µl of sample buffer. Samples were prepared as per the procedure described by Shivanna and Ramesh (1995). Quantity of protein present in the samples was estimated using the procedure of Bradford (1976). Optical density of the solution was measured using a spectrophotometer at 595 nm. The quantity of protein present in the sample was calculated by extrapolation with BSA as the standard. The quantity of protein ejected during mating was calculated by subtracting the amount of protein of mated male from the amount of protein of unmated male.

The mated females were kept in separate vials; eggs were counted for a period of 30 days. Yeast was added to the vials containing larvae for feeding. After pupation of the larvae, the pupae were counted to know the productivity. All the experiments were conducted within young age, old age, and between young and old aged flies.

Table 1. Mean ± SD of mating time, duration between mating, quantity of protein transferred, fecundity and productivity in *D. melanogaster* (♂- Male, ♀- Female).

Age of flies ♂ x ♀	Number of mating	Mating time (min)	Duration between mating (min)	% of Protein transferred	Fecundity	Productivity
7 × 7	I	20.4 ± 1.21	27.4 ± 10.88	42.67	429.0 ± 32.80	261.4 ± 21.41
	II	18.4 ± 1.60		11.11	293.2 ± 29.25	205.0 ± 26.95
26 × 26	I	23.6 ± 1.54	29.6 ± 8.78	36.0	245.4 ± 37.04	96.4 ± 20.68
	II	16.6 ± 1.33		19.0	214.0 ± 28.30	65.6 ± 10.31
7 × 26	I	18.6 ± 1.46	50.2 ± 11.07	39.12	188.0 ± 25.5	56.4 ± 13.01
	II	11.4 ± 2.89		24.50	127.8 ± 5.60	27.0 ± 6.51
26 × 7	I	15.4 ± 2.94	43.8 ± 12.60	10.0	256.8 ± 36.22	48.4 ± 10.70
	II	14.2 ± 1.74		6.0	206.0 ± 16.99	18.0 ± 6.28

Results

Table 1 reveals that the mating time of young flies is more than other combinations except 1st mating of old flies. First mating time is more than second mating time in all the combinations of mating. The remating duration of young flies and old flies is almost similar, whereas it is half of the duration of old and young flies mating. The quantity of ACP transferred in first time mating of young flies is four times more than the second time mating. Protein quantity of first mating is double that of the second mating when old flies were crossed. When old and young flies were crossed, the quantity of secretion transferred in second mating is slightly more than half the quantity of the first mating. The number of eggs laid by the female mated with second time mated male produced 2/3 of the female mated with bachelor male in all the crosses. Productivity/pupa produced by the female mated with second time male is almost 3/4 of the first female mated with bachelor male in young flies and old flies. When young male mated with old female, the pupa produced is 1/2 in the second mating than the first mating. Whereas old male mated with young female the pupa produced is less than 1/2 in the second mating than the first mating. The difference in number of egg and pupa of 1st and 2nd mating is highly significant (egg of 1st mating: $F = 9.718$, 2nd mating: $F = 6.6$. pupa of 1st mating $F = 10.73$, 2nd mating $F = 19.961$ $\{df_1 = 3, df_2 = 116\}$).

Discussion

D. melanogaster is a domestic, cosmopolitan and most extensively characterized multi cellular organism used over more than 100 years for research. Since then it has been used for behavior, reproductive and developmental biology, ecology, evolution, population biology, genetics, and other studies.

Present study reveals that in *D. melanogaster* the first mating time is 2 minutes more than 2nd mating of 7 days old flies. In 26 days old flies it is 7 minutes more than 2nd mating, which is similar when young males mate with old females. It is 1 minute more in 1st mating than 2nd mating when young females mate with old males. These results are par with Pavkovic and Kekic (2009). Singh and Singh (2004) reported the contrasting result in *D. melanogaster*. Comparison of mating time of different age crosses showed significant variation. *Drosophila* females take more time to remate when compared to males (Markow 1996; Singh and Singh 2004). In the present study males have been given the chance to remate; it showed significant difference in remating duration when crossed with same and different aged females.

Accessory gland proteins contribute to important reproductive processes that lead to fertilization in species. Maximum accumulation of accessory gland takes place at 7 days in different species of *Drosophila,* such as in *D. melanogaster* (80.0%), *D. n. nasuta* (72.0%), *D. s. neonasuta* (70.0%), *D. rubida* (50.0%), and *D. pararubida* (54.20%) of *D. immigrans* group. *D. ananassae* and *D. varians* are about 84.84% and 58.13%, respectively (Chen, 1984; Ravi Ram and Ramesh, 2001; Hiremani and Shivanna, 2010). In the present study we selected 7 day old flies as the young age and 26 day old flies as the old age. The difference in transfer of protein in different crosses varies significantly with age of the flies.

Fecundity and productivities are the important components of fitness treaties and are increased with remating (Singh and Singh, 2004). Fecundity is usually considered as a female fitness component. It is affected by the presence of males and other factors, including male accessory gland protein in *D. melanogaster, D. ananassae, D. nasuta, D. albomicans* (Serradilla and Ayala, 1983; Chapman, 2001; Roopashree *et al.,* 2001; Harini, 2010; Rezaei, 2012; Lushchak *et al.,* 2013; Pavkovic and Kekic, 2013). To overcome the effects of different factors in the present study, standard conditions were maintained. Table 1 reveals that fecundity and productivity of young flies is more compared to other crosses. The fecundity and productivity of old flies is more than mixed fly crosses and less than young flies. The fecundity and productivity is more in 1st mating than 2nd mating in all the combinations, irrespective of age.

If same-aged flies mate with each other, mating time, fecundity, and productivity are more and remating duration is less compared to mixed flies. Mating time, duration between mating, quantity of ACP transferred, fecundity, and productivity are affected by the age of the mates.

Acknowledgment: Thanks are due to *Drosophila* Stock Centre Department of Zoology, University of Mysore, 'Manasagangotri' Mysore, for *Drosophila* stocks, UGC- SAP and UGC- MRP F No.37-241/2009 for financial assistance to Dr. N. Shivanna.

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Influence of extremely low frequency magnetic field (50 Hz, 0.5 mT) exposure on fitness components of *Drosophila subobscura.*

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Summary

In addition to naturally occurring radiation, magnetic fields which are introduced by a man with the advanced development of industry and technology present an additional factor in environment that could have a significant influence on living systems. Impact of magnetic fields at different developmental stages of biological systems might induce changes on different organizational levels. In this study are presented developmental time, developmental dynamics, and viability of *Drosophila subobscura* after exposure of egg-first instar larvae developmental stage to extremely low frequency magnetic field (50 Hz, 0.5 mT). Exposure for 48 h at egg-first instar larvae developmental stage significantly shortens developmental time and increases viability of *D. subobscura.*

Introduction

Extremely low frequency magnetic fields (ELF-MFs, ≤ 300 Hz) derived from power lines as well as from the majority of household electrical appliances, represent one of the most important scopes for researching in magneto biology (Gandhi *et al*., 2001; Gauger, 1985). ELF-MFs are in interaction with biological systems' tissues inducing electric fields and currents in them (Mathie *et al*., 2003). Mostly, studies about effects of ELF-MFs on *Drosophila* are dealing with influence on reproductive behavior and fitness components. Ramirez *et al*. (1983) show decreased oviposition after exposure to pulsated ELF (100 Hz, 1.76 mT) and sinusoidal fields (50 Hz, 1 mT). In addition, there are found increased egg mortality and diminished adults' viability. *D. melanogaster* females and progeny exposed to ELF-MF show weakened oviposition in their subsequent generations (Gonet *et al*., 2009). Also, Mirabolghasemi and Azarnia (2002) show a significant increase in the number of

abnormal adult flies from the exposed larvae at different stages of development, contrary to groups raised from the exposed eggs. If exposure to ELF-MF at any developmental stage makes changes on biological systems, it might be expected that the duration of ELF-MF exposure could evoke different levels of influence and, therefore, also a different response of the biological system. The aim of this research was to determine effects of exposure for 48 h of ELF-MF (50 Hz, 0.5 mT) on the following components of fitness: developmental time, developmental dynamics as a number of eclosed flies in every successive day, and viability of *Drosophila subobscura*.

Materials and Methods

Drosophila stock

D. subobscura was collected from a beech forest on Serbian mountain Goč and formed isofemale (IF) lines which were maintained in five full-sib inbreeding generations at temperature 19 ± 1°C, humidity 60%, in a 12h:12h light:dark cycle and at 300 lux illumination on standard corn meal medium (9% sugar, 10% cornmeal, 2% agar, 2% yeast, nipagin dissolved in 96% ethanol).

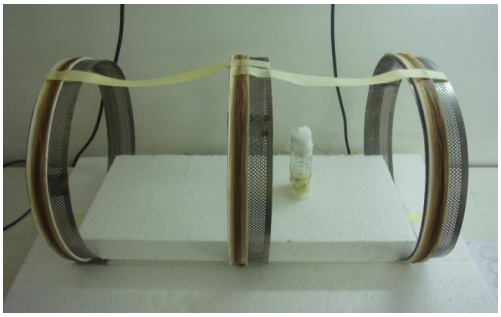

Figure 1. ELF-MF apparatus.

ELF-MF apparatus

The ELF-MF was obtained by an electromagnet that consisted of three circular coils (37 cm in diameter) of insulated copper wire (0.75 mm in diameter). The coils were set at 23 cm distance from each other, which produced homogeneous magnetic field in a horizontal direction (Figure 1). The 50 Hz current was taken from local 220 V power network via an adjustable transformer. The electromagnet was supplied by a current of 2.8 A, producing uniform 50 Hz magnetic field without any observable temperature fluctuation or vibrations. Within the coils where the samples were placed, magnetic field was 0.5 ± 0.01 mT. Magnetic field was measured using a Hirst GM05 Gaussmeter (probe PT 2837, Hirst Magnetic Instruments LTD, Cornwall, UK).

Experimental procedure

Randomly collected 30 non-virgin 3-8 days old females were put in a 200 cm^3 vials, covered and pasted with Petri dish which contains standard corn meal medium. Vials were placed upside-down (Figure 2) and maintained for 24 h to lay eggs on standard corn meal medium for *Drosophila*. The following three groups of eggs were made:

1. intact (20 replicas) – egg-first instar larvae were out of ELF-MF apparatus,
2. *sham* (15 replicas) – egg-first instar larvae were in ELF-MF apparatus turned off, and
3. ELF-MF (35 replicas) – egg-first instar larvae were in ELF-MF apparatus turned on.

Since intact and *sham* group did not significantly differ they were cumulated and expressed as one control group.

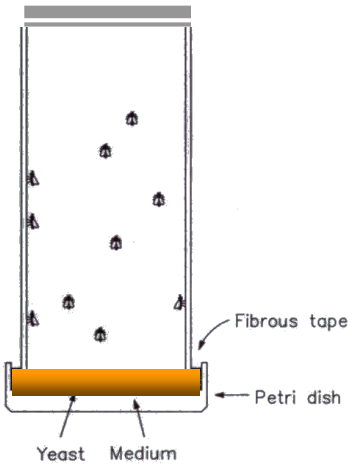

Figure 2. Vials with females laying eggs.

The following components of fitness were analyzed: developmental time, developmental dynamics as a number of eclosed flies in every successive day, and viability.

Collected eggs (75 per vial) were transferred to a thin film of standard corn meal medium in Petri dishes and exposed, or not, to the ELF magnetic field. After 48 h thin films were placed in 60 cm^3 vials with standard corn meal medium for completing egg-adult development and maintained at optimal laboratory conditions (19 ± 1°C, 60% humidity, 300 lux illumination and 12h day/12h night regime). In order to measure fitness components, eclosed flies were counted for each replica, every day at the same time.

Statistical analysis

The Kolmogorov–Smirnov test was used to confirm data normality. The normality of data was found in all treatments. Z test was used for testing intact and *sham* groups. The mean values of developmental time and viability were analyzed by one-way ANOVA. Embryonic and post-embryonic developmental time was measured in days once all the adults had emerged. Egg-to-adult viability was calculated as the ratio of the emerged adults to the number of collected eggs. All analyses were performed using Statistica 6.0 for Windows (StatSoft Inc., Aurora, CO, USA).

Results and Discussion

It is well known that insects have the ability to perceive external magnetic fields. There are many literature data concerning magnetic field influences on genetic, development, growth, viability, reproduction, and orientation in insects (Mirabolghasemi and Azarnia, 2002; Graham *et al*., 2000). Also, proliferating and less differentiated cells are more sensitive and vulnerable to electromagnetic radiation than non-proliferating and more differentiated ones (Prasad, 1995). One of the goals of this study was to show how exposure to ELF-MF (50 Hz, 0.5 mT) affects embryonic and post-embryonic developmental time of *D. subobscura*. Results are presented in Table 1. One-way ANOVA showed significantly shorter developmental time in flies from ELF-MF exposed groups ($F = 11.45$; $p < 0.01$).

Table 1. Embryonic and post-embryonic developmental time of *D. subobscura* in control conditions and after exposure to ELF-MF (50 Hz, 0.5 mT). Each value represents mean ± SEM. Significance was tested with one-way ANOVA.

	Developmental time
Control	20.329 ± 0.035
ELF-MF	19.845 ± 0.041**

**p < 0.01

In terms of the developmental dynamics of the formation of adults, hatching in control group started the 18th day. Eclosing of adults from ELF-MF exposed groups started earlier, between 17th and 18th day (Figure 3). Peak of eclosing in ELF-MF exposed groups was a day earlier (the 19th day, 39.81 %) comparing to control groups (the 20th day, 45.56 %). Likewise, adults from ELF-MF exposed groups finished eclosing one day earlier (the 25th day) comparing to control groups.

In groups exposed to the ELF-MF, 50% of individuals were eclosed by about the 19th. Eclosion of *D. subobscura* adults in control group was started after time point of 19 days.

Viability of *D. subobscura* flies was higher after the ELF-MF exposure compared to control (Figure 4). Mean values of viability of control flies was 51.2 ± 2.3 % and 57.2 ± 2.9 % for ELF-MF exposed flies. One-way ANOVA showed significantly higher viability of flies from ELF-MF exposed groups ($F = 4.191$; $p < 0.05$).

Results obtained for exposure of egg and the first instar larvae developmental stages of *D. subobscura* to ELF-MF show significant influence on developmental time, developmental dynamics, and viability. Results in this study revealed that exposure to ELF-MF (50 Hz, 0.5 mT) at egg-first

instar larvae developmental stage shortens developmental time and increases viability of *D. subobscura*. Due to literature data in which it is shown that insects are more resistant to different types of electromagnetic radiation than mammals (Koval *et al*., 1977, 1979; Koval and Kazmar, 1988), it should be considered that effects of ELF-MF obtained on *Drosophila* can be significant in human protection.

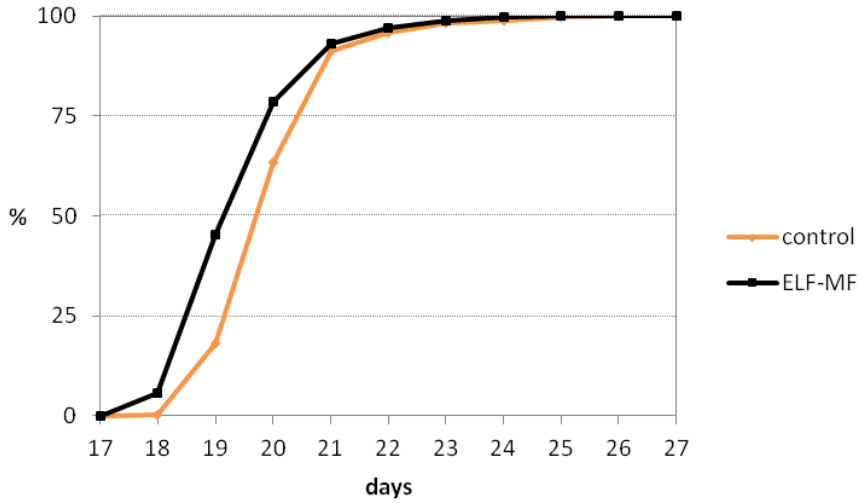

Figure 3. Developmental dynamics of *D. subobscura* after exposure of egg-first instar larvae developmental stage to ELF-MF (50 Hz, 0.5 mT).

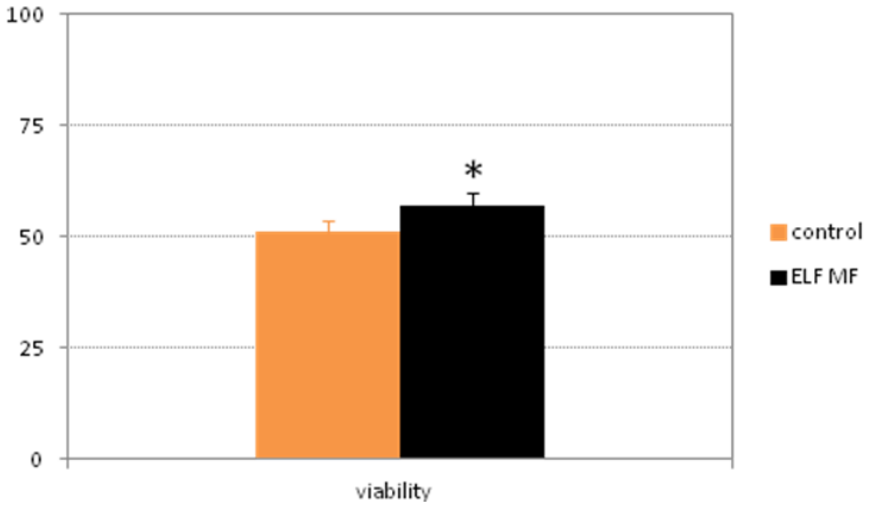

Figure 4. Viability of *D. subobscura* after exposure of egg-first instar larvae developmental stage to ELF-MF (50 Hz, 0.5 mT). Significance was tested with one-way ANOVA (*$p < 0.05$).

Acknowledgment**:** This work was funded by Serbian Ministry of Education, Science and Technological Development (Grant 173012).

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Grygiel 2009, Bioelectromagnetics 30: 687-689; Graham, J.H., D. Fletcher, J. Tigue, and M. McDonald 2000, Bioelectromagnetics 21: 465-472; Koval, T.M., R.W. Hart, W.C. Myser, and W.F. Hink 1977, Genetics 87: 513-518; Koval, T.M., R.W. Hart, W.C. Myser, and W.F. Hink 1979, International Journal of Radiation Biology & Related Studies in Physics, Chemistry & Medicine 35: 183-188; Koval, T.M., and E.R. Kazmar 1988, Radiation Research 113: 268-277; Mathie, A., L.E. Kennard, and E.L. Veale 2003, Radiation Protection Dosimetry 106: 311-316; Mirabolghasemi, G., and M. Azarnia 2002, Bioelectromagnetics 23: 416-420; Prasad, K.N., 1995, *Handbook of Radiobiology*, 2nd ed., CRC Press; Ramírez, E., J.L. Monteagudo, M. García-Gracia, and J.M. Delgado 1983, Bioelectromagnetics 4: 315-326.

Check-list of drosophilid species so far described and recorded from Uttarakhand state, India.

Sati, Pradeep Chandra[1], Rajendra Singh Fartyal*[1], Manisha Sarswat[1], Saurabh Dewan[1], Mukul Kandpal[2], Kanita[1], Manoj Kumar Patel[1], and Sushmika Pradhan[3,4]. [1]UGC-SAP Department of Zoology & Biotechnology, HNB Garhwal University, Chauras Campus, Srinagar Garhwal, UK, India, [2]Cytogenetics Laboratory, Department of Zoology, Kumaun University, Nainital, Uttarakhand, India, [3]P.G. Department of Zoology, Darjeeling Government College, Darjeeling, West Bengal, [4]Genetics Research Unit, Department of Zoology, University of Calcutta, West Bengal, India; *author for correspondence Email: fartyalrs@gmail.com / rs.fartyal@hnbgu.ac.in

Systematic position:

PHYLUM: ARTHROPODA
CLASS: INSECTA
SUBCLASS: PTERYGOTA
DIVISION: ENDOPTERYGOTA
ORDER: DIPTERA
SUBORDER: BRACHYCERA
SUPER FAMILY: EPHYDROIDEA
FAMILY: DROSOPHILIDAE

Subfamily Steganinae

1. Genus *Gitona* Meigen

1. *Gitona distigma* Meigen, 1830

2. Genus *Phortica* Schiner
Subgenus *Phortica*

2. *Phortica* (*Phortica*) *bandes* (Singh and Negi, 1992)
3. *Phortica* (*Phortica*) *biprotrusa* (Chen and Toda, 1998)
4. *Phortica* (*Phortica*) *pseudotau* (Toda and Peng, 1990)

3. Genus *Leucophenga* Mik

5. *Leucophenga albiceps* (de Meijere, 1914)

6. *Leucophenga angulata* Singh, Dash and Fartyal, 2000
7. *Leucophenga angusta* Okada, 1956
8. *Leucophenga argentata* de Meijere 1914
9. *Leucophenga bellula* (Bergroth, 1894)
10. *Leucophenga champawatensis* Fartyal and Singh in Fartyal *et al*., 2005
11. *Leucophenga chaubattiaensis* Fartyal and Toda in Fartyal *et al*., 2005
12. *Leucophenga clubiata* Singh, Dash and Fartyal, 2000
13. *Leucophenga confluens* Duda, 1923 **new record**
14. *Leucophenga kumaonensis* Fartyal and Singh in Fartyal *et al*., 2005
15. *Leucophenga nainae* Fartyal and Singh in Fartyal *et al*., 2005
16. *Leucophenga neoangusta* Godbole and Vaidya in Vaidya and Godbole, 1976
17. *Leucophenga neointerrupta* Fartyal and Toda in Fartyal *et al*., 2005
18. *Leucophenga neolacteusa* Singh and Bhatt, 1988
19. *Leucophenga okhalkandensis* Singh, Dash and Fartyal, 2000
20. *Leucophenga ornata* Wheeler, 1959
21. *Leucophenga quadripunctata* (de Meijere, 1908)
22. *Leucophenga regina* Mollach, 1935
23. *Leucophenga subpollinosa* (de Meijere, 1914)
24. *Leucophenga trispina* Upadhyay and Singh, 2007

4. Genus *Paraleucophenga* Hendel

25. *Paraleucophenga neojavanaii* Singh and Negi, 1992
26. *Paraleucophenga todai* Fartyal and Singh, 2004

5. Genus *Stegana* Meigen

Subgenus *Steganina* Wheeler

27. *Stegana* (*Steganina*) *nainitalensis* Singh and Fartyal, 2002

Subfamily Drosophilinae

6. Genus *Dettopsomyia* Lamb

28. *Dettopsomyia nigrovittata* (Malloch, 1924)

7. Genus *Drosophila* Fallén

i. Subgenus *Dorsilopha* Sturtevant

29. *Drosophila* (*Dorsilopha*) *busckii* Coquillett, 1901

ii. Subgenus *Drosophila*

30. *Drosophila* (*Drosophila*) *analspina* Singh and Negi, 1995
31. *Drosophila* (*Drosophila*) *bageshwarensis* Singh, Dash and Fartyal, 2004
32. *Drosophila* (*Drosophila*) *bishtii* Singh and Negi, 1995

33. *Drosophila* (*Drosophila*) *bizonata* Kikkawa and Peng, 1938
34. *Drosophila* (*Drosophila*) *dwarahatensis* Upadhayay and Singh, 2006
35. *Drosophila* (*Drosophila*) *hexaspina* Singh, Dash and Fartyal, 2004
36. *Drosophila* (*Drosophila*) *immigrans* Sturtevant, 1921
37. *Drosophila* (*Drosophila*) *khansuensis* Singh, Dash and Fartyal, 2004
38. *Drosophila* (*Drosophila*) *kulouriensis* Fartyal and Singh in Brake and Bächli, 2008
39. *Drosophila* (*Drosophila*) *nainitalensis* Singh and Bhatt, 1988
40. *Drosophila* (*Drosophila*) *nasuta* Lamb, 1914
41. *Drosophila* (*Drosophila*) *paharpaniensis* Singh, Dash and Fartyal, 2004
42. *Drosophila* (*Drosophila*) *painii* Singh and Negi, 1995
43. *Drosophila* (*Drosophila*) *paraimmigrans* Gai and Krishnamurthy, 1986
44. *Drosophila* (*Drosophila*) *paralongifera* Gupta and Singh, 1981
45. *Drosophila* (*Drosophila*) *paramarginata* Singh, Dash and Fartyal, 2004
46. *Drosophila* (*Drosophila*) *paunii* Singh and Negi, 1989
47. *Drosophila* (*Drosophila*) *sattalensis* Fartyal and Singh in Brake and Bachli, 2008
48. *Drosophila* (*Drosophila*) *sikkimensis* Gupta and Gupta, 1991
49. *Drosophila* (*Drosophila*) *sulfurigaster* (Duda, 1923)
50. *Drosophila* (*Drosophila*) *surangensis* Singh, Dash and Fartyal, 2004
51. *Drosophila* (*Drosophila*) *tetradentata* Singh & Gupta 1980
52. *Drosophila* (*Drosophila*) *trisetosa* Okada, 1966
53. *Drosophila* (*Drosophila*) *trizonata* Okada, 1966

iii. Subgenus *Siphlodora* Patterson and Mainland

54. *Drosophila* (*Siphlodora*) *hydei* Sturtevant, 1921
55. *Drosophila* (*Siphlodora*) *lacertosa* Okada, 1956
56. *Drosophila* (*Siphlodora*) *repleta* Wollaston, 1858

iv. Subgenus *Sophophora* Sturtevant

57. *Drosophila* (*Sophophora*) *bifasciata* Pomini, 1940
58. *Drosophila* (*Sophophora*) *gangotrii* Muniyappa and Reddy, 1981
59. *Drosophila* (*Sophophora*) *hubeiensis* Sperlich and Watabe in Watabe & Sperlich, 1997
60. *Drosophila* (*Sophophora*) *jambulina* Parshad and Paika, 1964
61. *Drosophila* (*Sophophora*) *kikkawai* Burla, 1954
62. *Drosophila* (*Sophophora*) *malerkotliana* Parshad and Paika, 1964
63. *Drosophila* (*Sophophora*) *melanogaster* Meigen, 1830
64. *Drosophila* (*Sophophora*) *neobaimai* Singh and Dash, 1998
65. *Drosophila* (*Sophophora*) *neokhaoyana* Singh and Dash, 1998
66. *Drosophila* (*Sophophora*) *nepalensis* Okada, 1955
67. *Drosophila* (*Sophophora*) *pulchrella* Tan, Hsu and Sheng, 1949
68. *Drosophila* (*Sophophora*) *punjabiensis* Parshad and Paika, 1964
69. *Drosophila* (*Sophophora*) *saraswati* Singh and Dash, 1998
70. *Drosophila* (*Sophophora*) *sargakhetensis* Joshi, Fartyal and Singh, 2005
71. *Drosophila* (*Sophophora*) *suzukii indica* Parshad and Paika, 1964
72. *Drosophila* (*Sophophora*) *takahashii* Sturtevant, 1927
73. *Drosophila* (*Sophophora*) *trapezifrons* Okada, 1966

v. *Incertae sedis*

74. *Drosophila mukteshwarensis* Joshi, Fartyal and Singh, 2005
75. *Drosophila notostriata* Okada, 1966
76. *Drosophila repletoides* Hsu, 1943

8. Genus *Hirtodrosophila* Duda

77. *Hirtodrosophila hexaspina* Fartyal and Singh, 2002
78. *Hirtodrosophila quadrivittata* (Okada, 1956)
79. *Hirtodrosophila seminigra* (Duda, 1926) **new record**
80. *Hirtodrosophila sexvittata* (Okada, 1956) **new record**

9. Genus *Liodrosophila* Duda

81. *Liodrosophila nitida* Duda, 1922 **new record**

10. Genus *Lordiphosa* Basden

82. *Lordiphosa antillaria* (Okada, 1984)
83. *Lordiphosa tripartita* (Okada, 1966)

11. Genus *Microdrosophila* Malloch

Subgenus *Microdrosophila*

84. *Microdrosophila* (*Microdrosophila*) *bamanpuriensis* Upadhyay and Singh, 2007

12. Genus *Mulgravea* Bock

85. *Mulgravea parasiatica* (Takada and Momma, 1975)

13. Genus *Mycodrosophila* Oldenberg

Subgenus *Mycodrosophila*

86. *Mycodrosophila* (*Mycodrosophila*) *gratiosa* (de Meijere, 1911) **new record**

14. Genus *Scaptodrosophila* Duda

87. *Scaptodrosophila chandraprabhiana* (Gupta and Ray chaudhuri, 1970)
88. *Scaptodrosophila coracina* (Kikkawa and Peng, 1938)
89. *Scaptodrosophila hirsuata* Singh and Dash, 1998

15. Genus *Scaptomyza* Hardy

i. Subgenus *Parascaptomyza* Duda

90. *Scaptomyza* (*Parascaptomyza*) *elmoi* Takada, 1970
91. *Scaptomyza* (*Parascaptomyza*) *himalayana* Takada, 1970

ii. Subgenus *Scaptomyza*

92. *Scaptomyza* (*Scaptomyza*) *tistai* Kumar and Gupta, 1992

16. Genus *Zaprionus* Coquillett

i. Subgenus *Anaprionus* Okada

93. *Zaprionus* (*Anaprionus*) *grandis* (Kikkawa and Peng, 1938)

ii. Subgenus *Zaprionus*

94. *Zaprionus* (*Zaprionus*) *indianus* Gupta, 1970

Acknowledgments: This work is financially supported by University Grants Commission, New Delhi, India as Major Research Project to R.S. Fartyal, Ref. No. UGC Project F. No. 37-198/2009 (SR). We especially thank Emeritus Prof. M.J. Toda of Hokkaido University Museum, Sapporo, Japan for giving us valuable expert comments on an early draft of this check list. The authors also thank the Director ZSI and Dr. Dhriti Banerjee in charge Diptera section Zoological Society Kolkata for their cooperation in unknown sample keeping her ZSI Museum and also acknowledge National Biodiversity Authority (NBA), Chennai, India.

Guide to Authors

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Temporal pattern of *Drosophila subobscura* locomotor activity after exposure to extremely low frequency magnetic field (50 Hz, 0.5 mT).

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Summary

The aim of this study was to determine whether exposure for 48 h to extremely low frequency magnetic field – ELF-MF (50 Hz, 0.5 mT) at different developmental stages of *Drosophila subobscura* (egg-first instar larvae and just eclosed adult) changes locomotor activity recorded for 30 min. Linear regression analysis, performed with values of successive 10-min time intervals, showed significant differences in travelled distance and mobility in flies from all experimental groups. No matter which developmental stage was exposed, ELF-MF decreased locomotor activity of *D. subobscura* adults.

Introduction

All biological systems are constantly exposed to natural magnetic fields, but with intensive technological progress during the last 30 years, there are additional sources of electromagnetic fields. Many studies deal with magnetic field effects on different biological levels (Balcavage *et al.*, 1996; Santini *et al.*, 2009; Ivancsits *et al.*, 2003; Neumann, 2000; Rollwitz *et al.*, 2004). Effects of extremely low frequency magnetic field (ELF-MF, < 300 Hz) on nervous system are still undetermined, but many studies are researching it on different behavioral levels. Locomotor activity of animals is implicated in their everyday activities and as a complex type of behavior it is important to be analyzed through different parameters (distance travelled, mobility, speed, and so forth). Central neurotransmitter systems which play important roles in motor behavior expression might be modulated with applied ELF-MF. Therefore, these modulations might induce alternated motor behavior (Osborne, 1996). It has already been shown that in *Drosophila* larvae after exposure to ELF-MF there is decreased ability in digging of substrate (Ho *et al.*, 1992). Mostly in everyday life at different developmental stages humans are exposed to ELF-MF derived from power lines and almost all household electrical appliances. Therefore, ELF-MF presents an important ecological factor. The aim of this study was to detect changes in locomotor activity of *Drosophila subobscura* adults, after 48 h exposure of different developmental stages to ELF-MF (50 Hz, 0.5 mT).

Materials and Methods

Drosophila stock

D. subobscura were collected from beech a forest on Serbian mountain Goč and formed into isofemale (IF) lines. They were maintained in five full-sib inbreeding generations in controlled laboratory conditions at temperature 19°C, with humidity 60%, on standard cornmeal medium, which

consists of 9% sugar, 10% cornmeal, 2% agar, 2% yeast, and nipagin dissolved in 96% ethanol, in a 12 h:12 h light:dark cycle - lights turned on at 6:00 AM, at 300 lux illumination.

Experimental procedure

Two types of experiments were performed. The first one was exposure of *D. subobscura* egg-first instar larvae developmental stage for 48 h to ELF-MF (50 Hz, 0.5 mT). After that their development was completed out of the ELF-MF apparatus. The second one was exposure of just eclosed *D. subobscura* adults for 48 h to ELF-MF (50 Hz, 0.5 mT). In both experiments when eclosing started males and females were separated under CO_2 anesthesia and placed in vials with standard medium for *Drosophila*.

Figure 1. ELF-MF apparatus (left), camera (middle) above Petri dishes, and computer (right) for locomotor activity recording.

Locomotor activity monitoring

There were three experimental groups:

- intact (egg-first instar larvae and just eclosed flies were out of ELF-MF apparatus),
- *sham* (egg-first instar larvae and just eclosed flies were in turned off ELF-MF apparatus) and
- ELF-MF (egg-first instar larvae and just eclosed flies were in turned on ELF-MF apparatus).

Since between intact and *sham* group there was not detected statistically significant differences, those groups were pooled in one control group. Single 3 days old *D. subobscura* naive males and virgin females were separately released to move in the empty plastic Petri dish – "open field" arena (35 mm diameter, 10 mm high to maximally limit vertical and fly movement) immediately before recording (Figure 1, middle). In our previous research we found that locomotor activity of *D. subobscura* flies was the highest in the morning time interval from 8:00 AM to 9:00 AM (Dimitrijević *et al.*, 2013). In this study was used this morning time interval for monitoring locomotor activity for 30 min. Seven *Drosophila* flies were simultaneously videotaped by camera (Microsoft LifeCam VX600) positioned above the dishes (Figure 1, middle). All experiments were performed at optimal laboratory conditions for *D. subobscura* (temperature 19°C, humidity 60%, and 300 lux illumination). ANY-maze software (v.4.73, Stoelting Co., Wood Dale, Illinois, USA) was used to analyze two parameters:

- distance travelled (the total distance in meters that fly travelled during the recording time) and
- mobility (the amount of time in seconds that fly was mobile during the recording time).

ELF-MF apparatus

The ELF-MF was obtained by an electromagnet that consisted of three circular coils of insulated copper wire (0.75 mm in diameter). The coils are 37 cm in diameter and are set at 23 cm distance from each other (Figure 1, left). The 50 Hz current was taken from local 220 V power network via an adjustable transformer. The electromagnet was supplied by a current of 2.8 A, producing uniform 50 Hz magnetic field without any observable temperature fluctuation or vibrations. Within the coils where the samples were placed, magnetic field was 0.5 ± 0.01 mT, measured using a Hirst GM05 Gaussmeter (probe PT 2837, Hirst Magnetic Instruments LTD, Cornwall, UK).

Statistical analysis

Linear regression analysis was used to detect changes in locomotor activity parameters during 30 min, in 10-min intervals. Since data did not show normal distribution (Shapiro–Wilk test), non-parametric Spearman Rank Order Correlations test was used for testing differences between regression slopes of locomotor activity parameters of control and ELF-MF exposed *Drosophila*. All analyses were conducted using Statistica 5.0 for Windows (StatSoft Inc., Aurora, CO, USA).

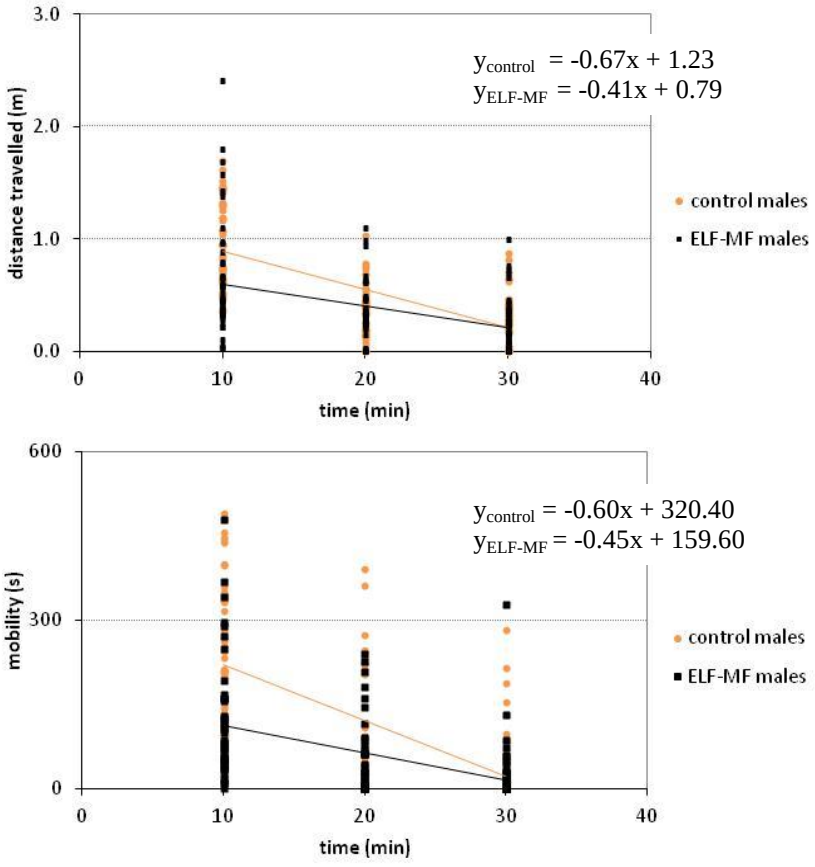

Figure 2. Linear regression lines and equations of both parameters of locomotor activity (distance travelled and mobility) for 3 days old *D. subobscura* males exposed to ELF-MF (50 Hz, 0.5 mT) at egg-first instar larvae developmental stage; $n_{control} = n_{ELF-MF} = 45$.

Results

Linear regression analysis of locomotor activity of 3 day old *D. subobscura* flies exposed to ELF-MF at egg-first instar larvae developmental stage showed significant reduction during recording (Figures 2 and 3, Table 1A).

The highest values of the examined parameters for males and females from both experimental groups were observed in the first 10 min. The slopes of linear regressions from control and ELF-MF exposed groups indicated that the mean travelled distance by males significantly decreased by 0.67 m and 0.41 m, respectively, in each 10-min time interval. The mean mobility by males from control and ELF-MF exposed group significantly decreased by 0.60 s and 0.45 s, respectively, in each successive 10-min time interval. In females from control and ELF-MF exposed group the mean travelled distance and mobility significantly decreased in each successive 10-min time interval by 0.41 m and 0.45 m, and 0.49 s and 0.46 s, respectively.

Table 1. Linear regression analysis for both parameters of locomotor activity (distance travelled and mobility) of 3 days old *D. subobscura* males and females, previously exposed to ELF-MF (50 Hz, 0.5 mT) at egg-first instar larvae developmental stage (A) and at just eclosed adult developmental stage (B), during 30 min of recording.

A	egg-fist instar larvae	df	R^2	F	
distance travelled	control males	133	0.442	107.14	***
	ELF-MF males	133	0.168	26.78	***
	control females	133	0.366	76.87	***
	ELF-MF females	133	0.203	33.83	***
mobility	control males	133	0.359	74.47	***
	ELF-MF males	133	0.199	33.23	***
	control females	133	0.236	41.15	***
	ELF-MF females	133	0.213	35.97	***

B	just eclosed adults	df	R^2	F	
distance travelled	control males	223	0.619	361.92	***
	ELF-MF males	223	0.389	141.95	***
	control females	223	0.624	369.70	***
	ELF-MF females	223	0.424	163.87	***
mobility	control males	223	0.622	367.63	***
	ELF-MF males	223	0.414	157.45	***
	control females	223	0.580	308.41	***
	ELF-MF females	223	0.435	171.6	***

*** $p < 0.001$

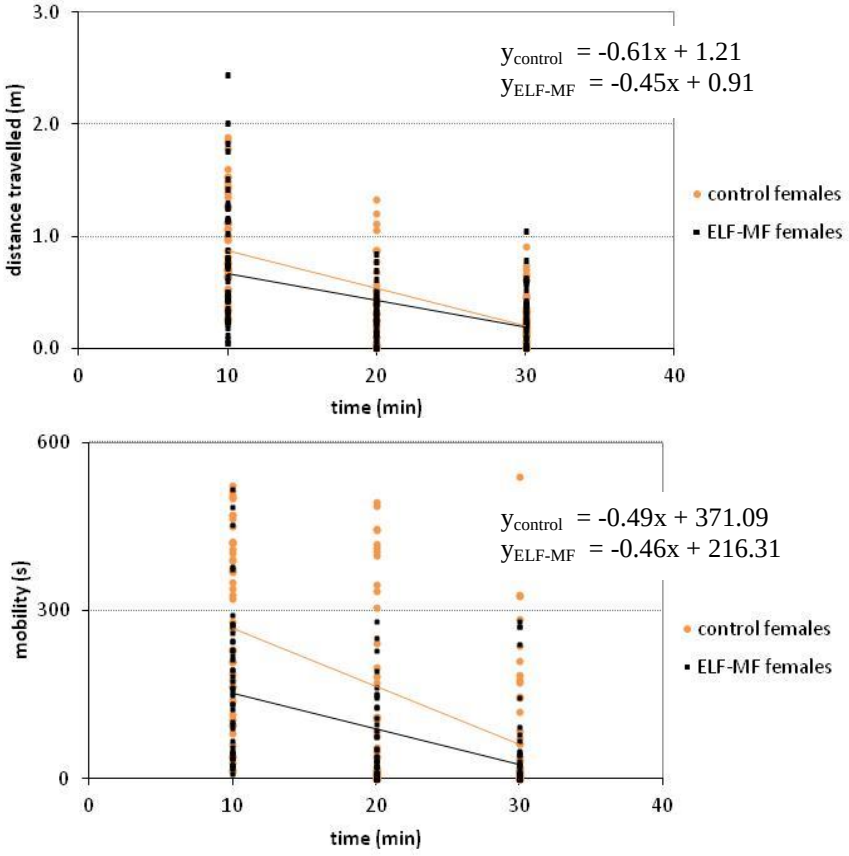

Figure 3. Linear regression lines and equations of both parameters of locomotor activity (distance travelled and mobility) for 3 days old *D. subobscura* females exposed to ELF-MF (50 Hz, 0.5 mT) at egg-first instar larvae developmental stage; $n_{control} = n_{ELF-MF} = 45$.

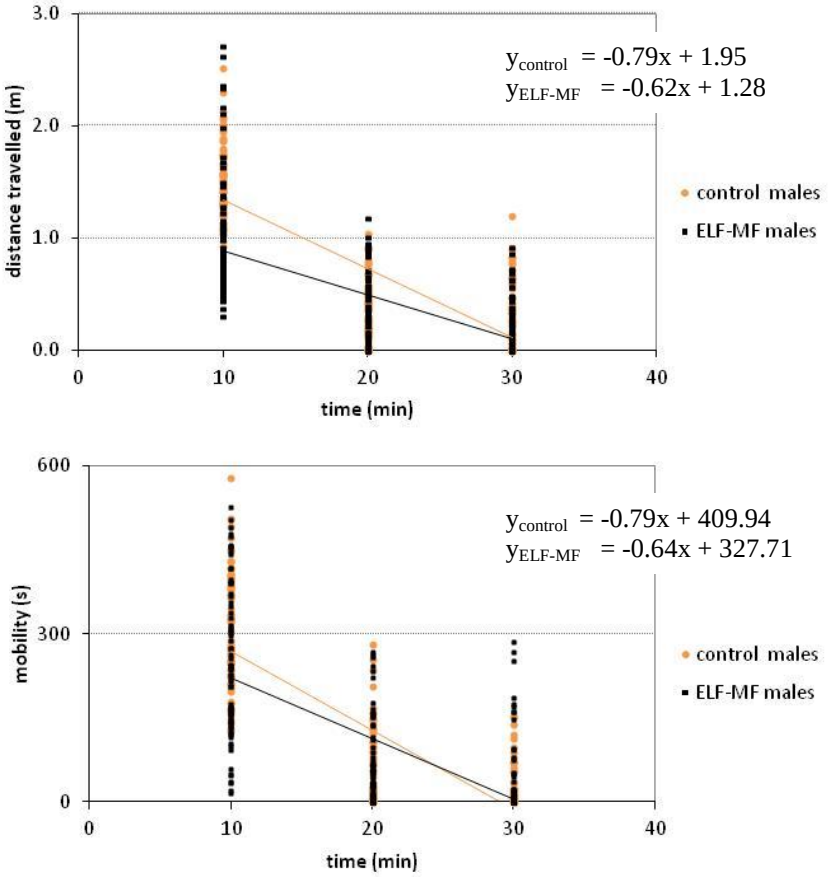

Figure 4. Linear regression lines and equations of both parameters of locomotor activity (distance travelled and mobility) for 3 days old *D. subobscura* males exposed to ELF-MF (50 Hz, 0.5 mT) for 48 h at just-eclosed adult developmental stage; $n_{control}$ = n_{ELF-MF} = 75.

The percentage of the total variation in distance travelled and mobility, that is measured and shown by the fitted regression, was defined with a coefficient of determination (R^2), which represents a measure of the strength of the straight line relationship. Results in Table 1A for groups of egg-first instar larvae developmental stage show that total variation in travelled distance and mobility of males from control and ELF-MF group was 44% and 17%, and 36% and 20%, respectively. For females it was 37% and 20%, and 24% and 21%, respectively. These results showed that travelled distance and mobility of each sex from both experimental groups were more variable after exposure to ELF-MF.

Spearman Rank Order Correlations test showed that linear regression slopes of males and females distance travelled and mobility after exposure of egg-first instar larvae developmental stage to ELF-MF (50 Hz, 0.5 mT) were significantly lower compared to control groups (Spearman $Rho_{males,DT}$ = 0.21; Spearman $Rho_{females,DT}$ = 0.13; Spearman $Rho_{males,M}$ = 0.21; Spearman $Rho_{females,M}$ = 0.18; $p < 0.05$).

Analyzing locomotor activity of 3 day old *D. subobscura* males and females after exposure to ELF-MF (50 Hz, 0.5 mT) for 48 h of just eclosed adults, linear regression analysis showed significant reduction during recording (Figures 4 and 5, Table 1B). As it was seen from results of exposed egg-first instar larvae developmental stage, in this experiment the highest values of the examined parameters for males and females from both control and ELF-MF exposed group were observed in the first 10 min.

Results in Table 1B for just eclosed adult developmental stage showed that total variation in travelled distance and mobility of males from control and ELF-MF group was 62% and 39%, and 62% and 41%, respectively. For females it was 62% and 42%, and 58% and 43%, respectively. These results showed that travelled distance and mobility of each sex from both experimental groups

were more variable after exposure to ELF-MF. It was shown that regression slopes of males and females distance travelled and mobility after exposure of just eclosed adults to ELF-MF (50 Hz, 0.5 mT) for 48 h were significantly lower compared to control groups. The slopes from control and ELF-MF exposed groups indicated that the mean travelled distance by males significantly decreased by 0.79 m and 0.62 m in each 10-min time interval. The mean mobility by males from control and ELF-MF exposed groups significantly decreased by 0.79 s and 0.64 s in each successive 10-min time interval. In females from control and ELF-MF exposed groups, the mean travelled distance and mobility significantly decreased in each successive 10-min time interval by 0.79 m and 0.65 m, and 0.76 s and 0.66 s, respectively.

Testing slopes of linear regressions for both parameters of locomotor activity using Spearman Rank Order Correlations test, a significant decrease was found in travelled distance (Spearman $Rho_{males,DT}$ = -0.18; Spearman $Rho_{females,DT}$ = -0.70; $p<0.05$) of males and females, which were exposed to ELF-MF (50 Hz, 0.5 mT) for 48 h at just eclosed adult developmental stage, compared to control ones. On the other hand, there were no significant differences in regression slopes of either males or females for mobility.

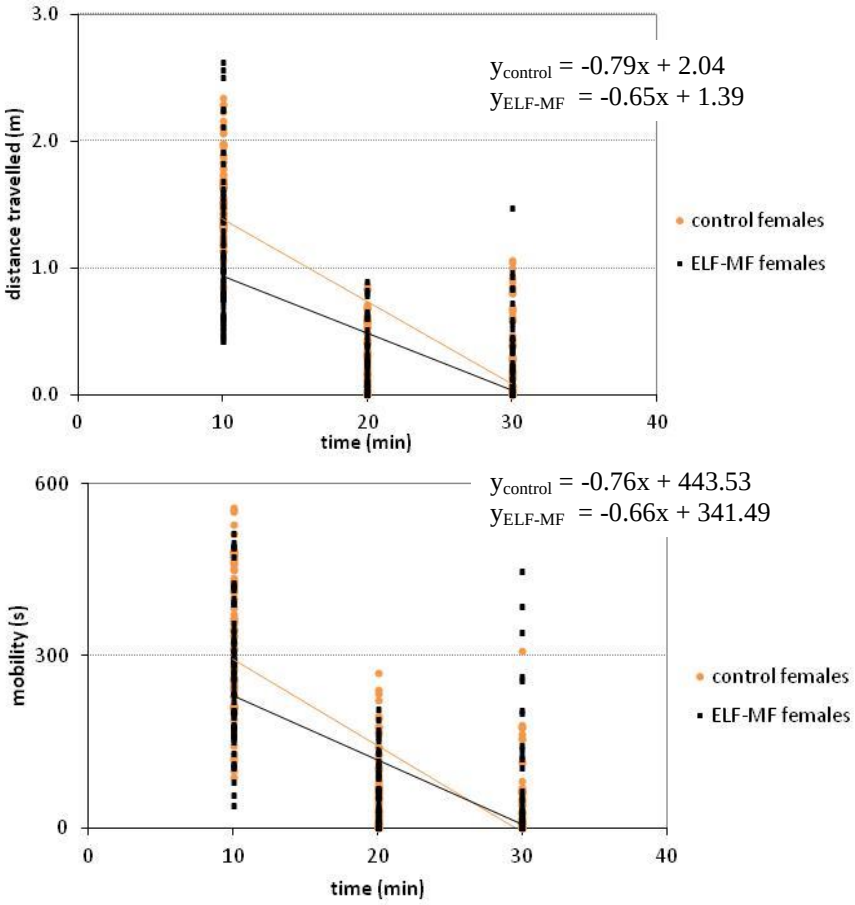

Figure 5. Linear regression lines and equations of both parameters of locomotor activity (distance travelled and mobility) for 3 days *D. subobscura* females exposed to ELF-MF (50 Hz, 0.5 mT) for 48 h at just-eclosed adult developmental stage; $n_{control}$ = n_{ELF-MF} = 75.

Discussion

In changing environments organisms use sensory systems to respond appropriately to different cues and they also might develop mechanisms to adapt (Mayr, 1974; Seligman, 1970; Domjan, 2005). The adaptive advantage of organisms in that kind of environment depends on many functions, but one of the important ones is locomotor activity, because it facilitates animals to disperse, to find food, to mate, and to respond to different stress situations. It is shown that neurotransmitter serotonin has a role in response to stimuli from the environment (Water-house *et al.*, 2004). Moreover, serotonergic neurons are sensitive to changes in behavioral activation (Jacobs and Fornal, 1999; Portas *et al.*, 2000). This study demonstrated that changes in adults' locomotor activity after exposure to ELF-MF (50 Hz, 0.5 mT) at egg-first instar larvae developmental stage were detected even though other developmental stages (2nd, 3rd instar larvae, pupa, and adult) were

not exposed to ELF-MF. Moreover, exposure of just eclosed adults to ELF-MF also alters locomotor activity. According to all these results we can propose that the effect of the applied magnetic field might be on the serotonergic transmission. In the case of exposure of egg-first larvae developmental stage to ELF-MF, changes expressed at adult stage indicate that egg and the first instar larvae developmental stage are very sensitive to ELF-MF.

In conclusion, observed significant reduction of locomotor activity during recording in males and females of *D. subobscura* which were exposed to ELF-MF for 48 h at egg-first instar larvae and just eclosed adult developmental stage could point out the consequences of exposure to ELF-MF (50 Hz, 0.5 mT) which might be extrapolated to humans.

Acknowledgments: This work was funded by Serbian Ministry of Education, Science and Technological Development (Grant No 173012 and 173027).

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Mating frequency of *Drosophila subobscura* from two populations.

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Mating is a fundamental process for animals with sexual reproduction. Mating success and sexual selection are poorly investigated in *Drosophila subobscura*, a species with complex courtship behavior represented by: orientation, "wing dance", jump (attempting copulation), forelegs posture, vibration, rowing, and copulation (Milani, 1956; Brown, 1965). The specific nature of behavior during courtship and mating, as well as mate choice, are genetically and environmentally determined (Terzić *et al*., 1996; Jennions and Petrie, 1997; O'Dell, 2003). Signals for mating, which are reciprocally exchanged between the sexes, allow identification of pairs. Mating choice largely depends on sexual traits and preferences for them (Servedio and Saetre, 2003). Adaptation to different environmental conditions can lead to modification of these signals and recognition systems (Coyne and Orr, 1998).

The aim of this study was to examine the intra- and interspecific variability in the number of achived matings of both sexes, as well as mate choice in *D. subobscura* sampled from two populations from ecologically different habitats. Considering that significant chromosomal inversion polymorphism between these two populations exists (Andjelković *et al*., 2003), as well as phenotypic variances in body size (Stamenković-Radak *et al*., 2008), which are at some degree related to the variable environmental factors, possible sexual behavior divergence between these populations is expected.

Mating success of *D. subobscura* flies was estimated in the "multiple choice" test. Flies were sampled on the mountain Goč (Serbia), from oak and beech forests, which are located at a distance of approximately 6.9 km and at an altitude of 787 m and 875 m, respectively. The oak forest with E exposure (*Fraxineto-Quercetum*, N 5 43°32'57.38" and E 20°40'2.32") is a light and changeable habitat, while beech forest on exposure NE (*Abieto-Fagetum*, N 43º 33' 28.43" and E 20º45'10.96") is dark forest with small daily and intraseasonal fluctuations of environmental factors. Flies used in the experiment were maintained on standard cornmeal substrate for *Drosophila* in full-sib lines through four generations under optimal laboratory condition for this species (temperature ~ 19°C, relative humidity of 60%, under 300 lux of light and 12 h: 12 h light: dark regime).

"Multiple choice" test was done in eighteen replicates. Flies were placed in the vials containing standard cornmeal substrate according to the following scheme: 5 males from oak (O_m) + 5 females from oak (O_f) + 5 males from beech (B_m) + 5 females from beech (B_f). Flies from different populations were alternatively marked with fluorescent dust 24 hours before mating observation. Mating was observed during 90 minutes *per* replica, and flies of both sexes and populations were scored for number of matings during each of three separate, consecutive 30 minute periods. After copulation started, mated individuals were transferred into separate vials, and their population origin identified later under the UV lamp. Using UV dust does not influence mate choice in *Drosophila* (Terzić *et al*., 1994). Differences in percents of realized matings were tested with Z - test (Zar, 1984).

During 90 minutes of observation, 40 out of possible 180 matings were realized (22.2%). Within the first hour of observation 31 copulations occurred (82.5%). During the first 30 minutes 19 matings were observed (47.5%), while 14 matings (35.0%) were observed during the second 30 minutes, and 7 matings (17.5%) within the last 30 minutes.

In relation to the total number of realized matings, the males from oak forest realized 38.46% of matings during the first 30 minutes, 46.15% within the next 30 minutes, and in the last period O_m achieved 15.38% of matings. Females from oak forest achieved 50% of matings during the first period, 35% in the next 30 minutes and 15% of matings within the last period. Males from beech forest realized 51.85% of the total number of matings during the first 30 minutes, 29.63% during the second period, and 18.52% within the last 30 minutes. Within the first period of observation B_f realized 45% of the total number of matings, 35% during the second and 20% of matings in the last 30 minutes. Males from beech forest were more successful in mating (67.50%) than males from oak forest (32.50%). During the first 30 minutes, B_m realized significantly more matings then O_m ($Z = -4.129$, $p < 0.01$), while in the second period, O_m and B_m achieved approximately equal number of matings ($Z = -1.069$, $p > 0.05$). Within the last 30 minutes, B_m realized significantly more matings than O_m ($Z = -2.268$, $p < 0.05$) (Figure 1). If data for 60 and 90 minutes of observation periods are taken cumulatively (Figure 2), B_m out-performed O_m ($Z = -3.830$, $p < 0.01$; $Z = -4.427$, $p < 0.01$, respectively). However, females from both populations were equally successful in mating if each observation period is compared, as well as cumulatively (Figure 1 and Figure 2).

Number of realized heterogamic matings ($O_m \times B_f$ and $B_m \times O_f$) was significantly higher (62.50%) than the number of realized homogamic matings ($O_m \times O_f$ and $B_m \times B_f$) ($Z = -3.162$, $p < 0.01$). Individuals from the oak forest realized more heterogamic (86.21%) than homogamic matings

(13.79%). Flies from the beech forest also realized more heterogamic than homogamic matings, but with smaller ratio (69.44% and 30.56%, respectively).

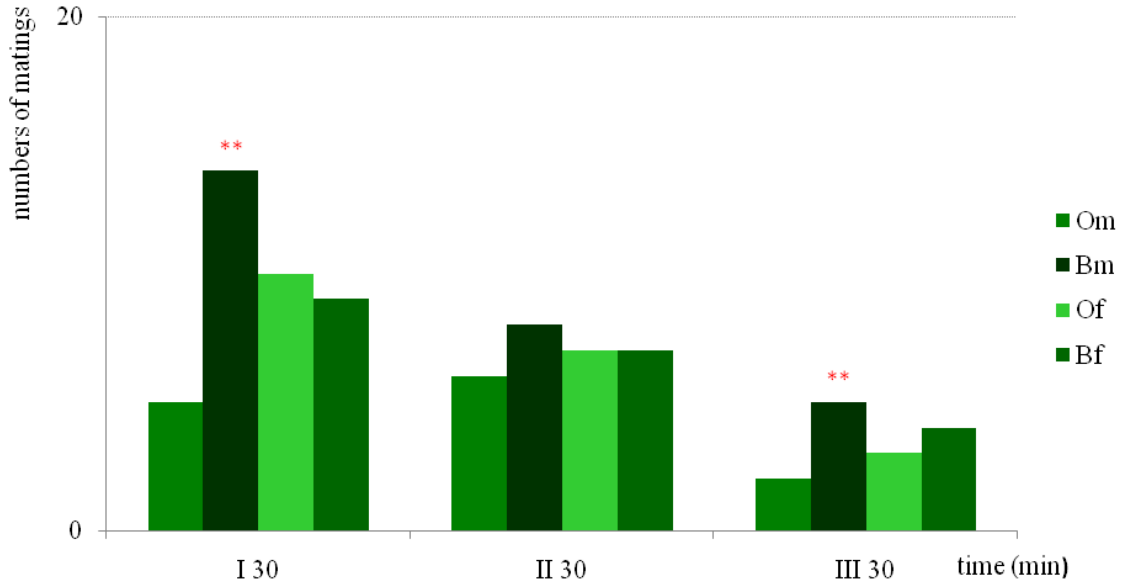

Figure 1. Number of matings achieved by both sexes in *D. subobscura* from two populations during every 30 minutes of the observation (I, II, and III). Abbreviations: O – flies from oak forest, B - flies from beech forest, m – males, f - females. **p < 0.01

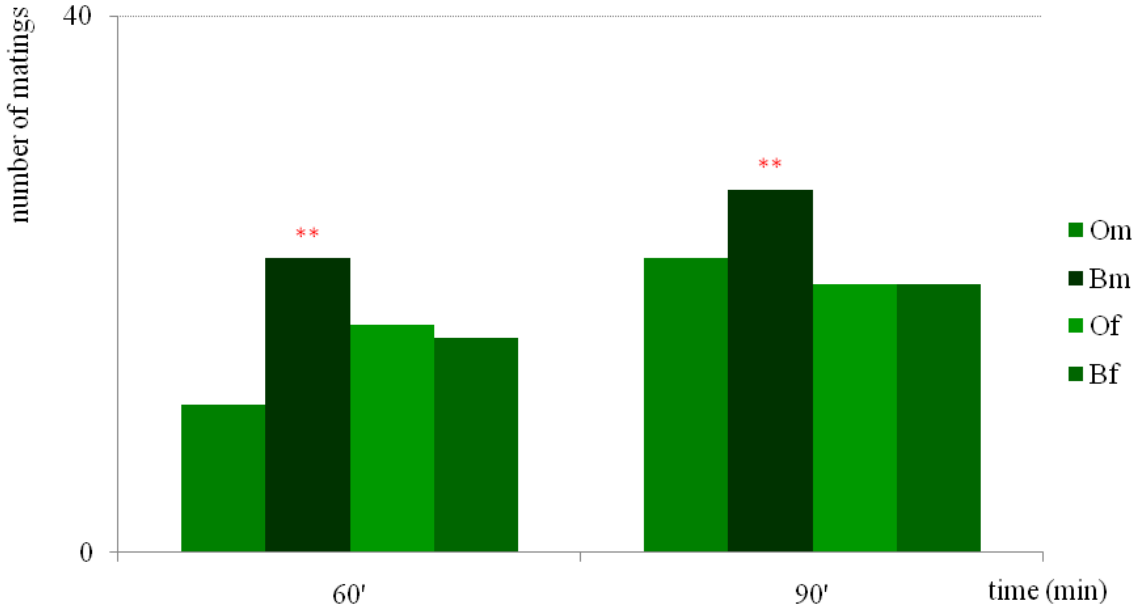

Figure 2. Cumulative number of matings within 60 and 90 minutes observing periods achieved by both sexes in *D. subobscura* from two populations. Abbreviations as in Figure 1.

A significant difference in the number of homogamic matings of B flies compared to the O flies was observed during the first and last 30 minutes of observation period, as well as during 60 minutes and the whole observation period of 90 minutes. The O_m mated more often with B_f than with females from the same population (O_f) during the last 30 minutes, and in the whole 60 and 90 minutes observation periods. The O_f achieved more heterogamic matings with B_m during each observation period, as well as in cumulative instance. The B_f mated equally with both B and O males in each period of observation, except during the first 30 minutes when more homogamic matings were realized. Results showed that no difference was observed in number of homo- and heterogamic matings in which B_m took part. The O_f realized more heterogamic matings than B_f during the first 30 minutes, and cumulatively, during 60 and 90 minutes. Results of Z - test for achieved homogamic and heterogamic matings are presented in Table 1.

Table 1. The comparison of the obtained homogamic and heterogamic matings; Z-test values. Abbreviations: as in Figure 1.

	I 30		II 30		III 30		60'		90'	
$O_m \times O_f$ / $B_m \times B_f$	-2.828	**	-0.894		-2.828	**	-4.221	**	-3.614	**
$O_m \times O_f$ / $O_m \times B_f$	-0.894		-1.633		-2.828	**	-3.333	**	-2.774	**
$O_m \times O_f$ / $B_m \times O_f$	-3.795	**	-2.268	*	-3.464	**	-5.680	**	-5.367	**
$B_m \times B_f$ / $O_m \times B_f$	2	*	-0.756		0		1		0.894	
$B_m \times B_f$ / $B_m \times O_f$	-1.069		-1.414		-0.894		-1.705		-1.925	
$O_m \times B_f$ / $B_m \times O_f$	-3.015	**	-0.667		-0.894		-2.683	**	-2.8	**

*p < 0.05; **p < 0.01

In this experiment, "multiple choice" design was used, where sex ratio was equal (F: M = 1:1), so competitive interactions were less pronounced. Prolonged time of mating observation led to the greater number of recorded copulations. Still, the most of copulations occurred within one hour observing period.

Higher number of heterogamic compared to homogamic matings indicated that there was negative assortative mating, and flies did not discriminate each other. Mating preference, propensity to mate with a certain phenotype, is variable even in females from the same population, which may be the result of genetic differences, developmental trajectories, or due to environmental factors (Jennions and Petrie, 1997). Traits that are sexually selected can be reliable indicators of mating benefits, and females can have substantial benefits from choosing males with larger values of these characteristics in different environments (Kokko, 2003; Schwartz and Hendry, 2006).

However, according to our results, males from beech forest achieved significantly more matings than those from the oak. It seems that some traits (morphological, physiological, and/or behavioral) contributed to higher mating success of B_m which will be the subject of further research. *D. subobscura* may be a reliable model system for mating experiments, including studying of different effects of environmental conditions on this behavior.

Acknowledgment: This work was supported by Serbian Ministry of Education, Science and Technological Development (Grant 173012).

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Effects of fungicide Dithane M 45 in *Drosophila melanogaster* on courtship behavior.

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Introduction

The dithiocarbamate pesticide Dithane M 45 is being used extensively to kill fungi. Although its primary function is to increase crop yield and food production, researchers showed that it has toxic effects and effect on genetic recombination in *Drosophila melanogaster* (Vasudev and Krishnamurthy, 1976, 1979, 1982), chromotoxic effects in plants (Pandey *et al.*, 1994), toxic effects on alga, *Stichococcus bacillaris* (Marton, 1974), embryo mortality in chickens (Keseru *et al.*, 2003), and non-clastogenic in mice (Vasudev and Krishnamurthy, 1994).

D. melanogaster has been proved beyond doubt as the best available sub-mammalian test system to screen genotoxic effects of environmental pollutants (Vogel and Sobels 1976; Sobels 1974; Vasudev, 1980; Wurgler *et al.*, 1985; Siddique *et al.*, 2005). Even though protocols of *D. melanogaster* have been validated for genotoxicity studies, time and again attempts are being made to introduce inexpensive, short duration and efficient parameters. Hence, in this direction, we presume that courtship behavior may be used as a parameter to understand the effects of environmental pollutants. It is pertinent to mention here that *D. melanogaster* with its well established series of sequential stereotyped elements of courtship behavior (Spieth, 1974, 1983; Bastock and Manning, 1955; Guruprasad *et al.*, 2010), an attempt has been made to use this protocol to understand the genetic effects of environmental pollutants. Nonetheless, Yamamoto and Koganezawa (2013), Dauwalder (2011), and Latham *et al.* (2013) have demonstrated that *fruitless* and *doublesex* genes are involved in courtship behavior. Furthermore, until now as far as we are aware there are no reports on the effects of environmental pollutants on courtship behavior. Therefore, the present work has been undertaken to understand the effects of a fungicide Dithane M 45 on courtship behavior of *D. melanogaster* and to authenticate this protocol for genotoxicity studies.

Materials and Methods

Dithane M 45, a zinc ion manganese ethylene bisdithiocarbamate, where 2% zinc, 16% manganese, and 62% ethylene bisdithiocarbamate obtained from Indofil chemicals Ltd, Mumbai, India and *D. melanogaster* Oregon-K strain were used for the present studies.

Wheat cream agar medium containing sub-lethal concentrations of Dithane M 45 (50, 100, and 150 ppm) were prepared and distributed to food vials (Vasudev and Krishnamurthy, 1979). Normal medium was used as control. 25 eggs per vial were collected following Delcour (1969) technique, and newly-hatched larvae were continuously fed on the above food media, *i.e*., larval feeding method was used. When adults emerged, virgin females and bachelors were isolated within four hours of eclosion and maintained separately in normal media for five days.

The following combination of crosses were made for observing the effect of Dithane M 45 on courtship behavior:

(A) Control crosses (untreated males × untreated females)

(B) Male treated crosses (treated males × untreated females)

(C) Female treated crosses (treated females × untreated males)

(D) Both treated crosses (treated males × treated females)

Single virgin female was aspirated out gently and introduced into an Elens-Wattiaux mating chamber (Elens and Wattiaux, 1964). A bachelor male was added to it and allowed to acclimatize to the chamber for 30 seconds. The details of courtship behavior were directly observed through hand lens of 10× magnification. All the experiments were conducted during morning (7-10 A.M.) in a room with a temperature of 24 ± 1˚C under normal laboratory light condition. Orientation, tapping, wing vibration, licking, and copulation duration were recorded separately and simultaneously by two observers in the control and treated group. From these data, courtship latency and copulation latency were analyzed and tabulated. Means and standard errors were calculated. Two-way ANOVA using DMRT statistical test was carried out.

Results and Discussion

In both treated and control groups, it has been observed that male engages in a series of actions, which include orientation towards females, tapping her with his fore legs, courtship song by expanding and vibrating his wings, licking the female's genitalia, curling his abdomen to attempt copulation, and lastly mounting the female by holding with first forelegs. Thus, in the present study, qualitative data are similar to that described by Bastock and Manning (1955) and Spieth (1974). However, the results in Tables 1, 2, and 3 revealed that there are quantitative differences between control and different treated groups. It is clear from these tables that courtship elements such as orientation, tapping, wing vibration, and licking are significantly increased in dose dependent manner. Further, values of higher doses (100 and 150 ppm) of different groups are highly significant compared to control ($p < 0.05$). Thus, higher doses of Dithane M 45 are effective in altering courtship behavior of *D. melanogaster*. Nontheless, Dithane M 45 induced toxicity in the form of increased rate of development and reduced viability (Vasudev and Krishnamurthy, 1979). It has also been demonstrated that Dithane M 45 significantly reduced the morphological characters such as body length, wing length, and size of the pupae above 50 ppm compared to controls. Hegde and Krishna (1999) showed that bigger the better in courtship behavior of *D. malerkotliana.* Thus, it can forward the opinion that reduced body size and wing size of *D. melanogaster* due to Dithane M 45 results in altered courtship behavior, which is a disadvantage for the flies.

Courtship latency is the time lag before performance of the first courtship behavior (orientation) after pairing (Ejima and Griffith, 2007) or it is the period during which the pairs acclimatize in the mating chamber and then start courtship activities. It actually indicates the vigor of the male (Markow, 1985). The courtship latency is shown to be significantly longer in treated groups

($p < 0.05$, Tables 1, 2, and 3) compared to control. Thus, it can be said that males require longer duration to attract the females.

Table1. Effect of Dithane M 45 on courtship elements of *D. melanogaster* male.

Behavioral elements	Control	50 ppm	100 ppm*	150 ppm*
Orientation	25.4 ± 0.72	26.4 ± 0.76	32.7 ± 0.65	42.03 ± 0.66
Tapping	3.7 ± 0.56	4.01 ± 0.60	5.2 ± 0.59	7.02 ± 0.62
Wing vibration	4.6 ± 0.4	5.2 ± 0.49	7.06 ± 0.84	12.07 ± 0.65
Licking	2.9 ± 0.07	3.01 ± 0.48	5.01 ± 0.36	8.95 ± 0.50
Courtship latency (sec)	10.9 ± 0.32	15.7 ± 0.72*	17.8 ± 0.75	19.6 ± 0.78
Copulation latency (mins)	9.1 ± 0.89	12.3 ± 0.25*	14.5 ± 0.67	22.5 ± 0.49
Copulation duration (mins)	16.3 ± 0.87	13.4 ± 0.28*	14.2 ± 0.36	13.3 ± 0.61

*P < 0.05

Table 2. Effect of Dithane M 45 on courtship elements of *D. melanogaster* female.

Behavioral elements	Control	50 ppm	100 ppm*	150 ppm*
Orientation	25.4 ± 0.72	27.4 ± 0.60	34.6 ± 0.59	45.3 ± 0.66
Tapping	3.7 ± 0.56	4.75 ± 0.40	6.2 ± 0.48	8.1 ± 0.78
Wing vibration	4.6 ± 0.4	5.75 ± 0.72	8.01 ± 0.36	13.07 ± 0.65
Licking	2.9 ± 0.07	3.25 ± 0.21	6.02 ± 0.18	9.25 ± 0.11
Courtship latency (sec)	10.9 ± 0.32	12.25 ± 0.23	18.02 ± 0.19	20.1 ± 0.25
Copulation latency (mins)	9.1 ± 0.89	13.02 ± 0.40*	15.03 ± 0.39	24.75 ± 0.28
Copulation duration (mins)	16.3 ± 0.87	14.01 ± 0.68*	13.2 ± 0.61	14.2 ± 0.62

*P < 0.05

Table 3. Effect of Dithane M 45 on courtship elements of *D. melanogaster* in both male and female.

Behavioral elements	Control	50 ppm	100 ppm*	150 ppm*
Orientation	25.4 ± 0.72	26.75 ± 0.05	34.15 ± 0.08	44.15 ± 0.12
Tapping	3.7± 0.56	4.25 ± 0.15	5.75 ± 0.17	8.25 ± 0.18
Wing vibration	4.6 ± 0.4	5.75 ± 0.05	8.01 ± 0.12	15.15 ± 0.21
Licking	2.9 ± 0.07	3.25 ± 0.03	6.02 ± 0.14	9.25 ± 0.18
Courtship latency (sec)	10.9 ± 0.32	16.25 ± 0.06*	18.75 ± 0.18	22.85 ± 0.22
Copulation latency (mins)	9.1 ± 0.89	13.25 ± 0.09*	15.2 ± 0.29	24.0 ± 0.26
Copulation duration (mins)	16.3 ± 0.87	12.0 ± 0.16*	14.0 ± 0.21	12.75 ± 0.28

*P < 0.05

Copulation latency is measured as the time taken for the male to orient towards female until initiation of copulation (Markow, 1985) or it is the time lag before successful mounting after pairing (Ejima and Griffith, 2007). It actually indicates the vigor of male (Hegde and Krishnamurthy, 1979; Mansoor and Hedge, 2006). A male with high vigor reacts quickly in the presence of a female, whereas a male with less vigor reacts slowly. On the other hand, it also reflects the receptivity of females. Obviously, longer copulation latency indicates lesser vigor of males and also non-receptivity of females. The longer copulation latency was notified in experimental treated groups suggesting that the females are non-receptive and males are less vigorous. Thus, this element not

only indicates the vigor of males but also receptivity of females. A male with high vigor has to perform the same courtship act many times to a non-receptive female than to a receptive female and vice versa. It is clear from the results that the treated males could not maintain high vigor and treated females are less receptive.

Courtship activity of the male or female culminates in copulation as suggested by Spiess (1970). 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 animal's life. According to Guruprasad *et al.* (2008) longer duration of copulation permits the transfer of more sperm by males, thus enhancing the fitness of males. It also enhances the fitness of the females, because the sperm received by a female can fertilize more eggs. In the present study in the treated groups, the copulation duration is significantly less compare to control. This proves that both treated male and treated female are less fit compared to non-treated ones (Table 3). On par with this, treated flies are less fecund than controls (Vasudev and Krishnamurthy, 1982). It is also demonstrated that mean daily egg production is reduced with reduced size of imago (Vasudev and Krishnamurthy, 1982). When the data are pooled together it is interesting to note the toxicity in terms of less viability, less fecund and reduced size of imago on one hand and effect on courtship behavior leading to less vigor and less receptivity on the other. All these factors are coinciding, resulting in the effect of Dithane M 45 on fitness of *D. melanogaster*.

Conclusion

The advantages of *D. melanogaster* to be used as a model organism in courtship behavior are: 1) they are small but not minute organisms (2.7mm); 2) short adult life span; 3) sexual dimorphism; 4) single pair mating can be easily achieved; 5) elaborate courtship behaviors; 6) known pedigree can be studied; 7) courtship behaviors can be analyzed qualitatively and quantitatively. These are similar under natural and field conditions and 8) many behavioral mutants are available. Hence, it is the strong opinion of the authors that *D. melanogaster* is an appropriate model to test the effects of sub-lethal concentrations of environmental pollutants using different courtship activities within short period of time.

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Enriched nutrient diet shortens the developmental time –A transgenerational effect in *Drosophila sulfurigaster sulfurigaster.*

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Abstract

Developmental time has a great relevance to fitness in all organisms. We set out to investigate the effect of parental larval diet on offspring development time, with relatively low amounts of sugar as a carbohydrate source and different concentrations of protein to assess the role of macronutrient balance on developmental time in *Drosophila sulfurigaster sulfurigaster* species. In the current study, the influence of larval diet experienced during just one generation extends into the next generation. Offspring reared on high protein and relative sugar concentration underwent metamorphosis significantly faster compared to the offspring of adults from low protein diet relative to sugar diets. A transgenerational effect of parental diet on offspring was found. Developmental time recorded was shortest in the case of offspring when compared to parents fed with high protein diet.

Introduction

Deficiency or imbalance of fat, carbohydrate, or protein can affect characters such as growth and reproduction. Protein deficiency reduces fecundity and growth in *Drosophila melanogaster* (Wang *et al.*,1995), and in fruit-feeders protein is often limiting macronutrients (Markow *et al*., 2001). Many organisms face a challenge of meeting their optional nutritional requirement for somatic and reproductive growth under natural conditions (Raubenheimer *et al.*, 1991). During development, body tissues constantly require a specific quantity and proportion of nutrients in order to attain optimal growth and performance (Bauerfeind *et al.*, 2005). In contrast diet restriction on mild starvation can increase longevity as well as tolerance to stressors such as heat stress (Wenzel and Smith, 2006) demonstrating the complexity of organismal nutrient acquisition and utilization. A variety of factors may affect organismal stress tolerance. These include physiological as well as behavioral changes. The bulk of studies on physiological and evolutionary responses to nutrient deficiencies focus on reproduction and fecundity (Naya, 2007). On the other hand, parents may also respond to environmental cues in ways that enhance offspring performance under particular

environmental circumstances. Under this scenario, offspring will do best in an environment similar to that experienced by their parents (Cruz-Neto *et al.*, 2007).

Developmental time, a very important life history trait, is largely affected by environmental conditions (James and Partridge, 1995). Nutritional manipulation is one of the most used ways to expose the effects of food as an environmental variable on aging and development of the organisms. *Drosophila* is being increasingly used as a laboratory model for life history evolution (Powell, 1997). *Drosophila* is an organism that breeds and feeds in ephemeral substrates; therefore, the larval developmental time is a very important trait (Chippindale *et al.*, 1997; Folguera *et al*., 2008). Important levels of genetic variation in developmental time occur in natural populations (Cortese *et al*., 2002; Fanara *et al*., 2006).

The most obvious way by which environmental variation may influence body condition and fecundity is via nutritional effects resulting from variability in food type availability. In general terms, diet effect can be classified as either quantitative (*i.e*., food availability) or qualitative (*i.e*., food composition). The quantitative effects are evident, since animals obtain energy and other nutritional requirements from food. Thus, under a natural range of conditions there is a positive correlation between food availability and body condition or fecundity. Qualitative effects often are divided into two categories: namely, nutritional deficiencies and inhibitory metabolites.

Parental genotype and environment often influence offspring fitness through non-genetically transmitted parental effects. Such effects may be maladaptive, *e.g*., malnourished parents may produce offspring of poorer quality (parental stress hypothesis). However, parents may also respond to environmental cues in ways that enhance offspring fitness. In particular, if the nutritional conditions experienced by the mother and offspring are positively correlated, mothers subject to nutritional stress would be favored to induce plastic changes in the offspring that make the latter more tolerant to nutritional stress. This adaptive hypothesis thus predicts that fitness of offspring on poor diet would be enhanced if their parents also experienced poor diet (Mousseau and Fox, 1998; Badyaev and Uller 2009). One potential mechanism of such adaptive parental effects involves adjustment of investment per offspring, which in organisms lacking parental care can be approximated by egg or newborn size (Azevedo *et al*., 1997). Life history theory predicts that under adverse conditions the optimal trade-off between offspring size and number is expected to shift towards fewer but better provisioned offspring (Roff, 1992).

The *Drosophila nasuta* subgroup, belonging to the *Drosophila immigrans* species group, includes more than ten species which are morphologically similar distributed in Pacific-Australasian and the pan-Indian Ocean areas. A number of these populations in different continental areas and islands of the Pacific were demonstrated to have diverged to the point of being separate sibling species (Wheeler and Takada 1964; Kitagawa *et al*., 1982). One of the species, *Drosophila sulfurigaster*, has the largest distribution among the *Drosophila nasuta* subgroup and consists of four subspecies. In light of the above investigation, a transgenerational effect has been sensed in the offspring fed with enriched nutrient diet with reference to developmental time in *Drosophila sulfurigaster sulfurigaster*.

Materials and Methods

Drosophila sulfurigaster sulfurigaster stock was obtained from the *Drosophila* stock center, University of Mysore, Mysore, India. The stocks were maintained in an uncrowded culture condition at 22 ± 1˚C, 70% humidity, and 12h:12h light and dark cycles in standard wheat cream agar medium. From this stock about 200-250 eggs were collected and placed in culture bottles (about 10 to 50 eggs/bottle). Further, the eggs laid were recorded for hatchability and adult eclosion (Harini, 2011).

About 30 males and females were separated by gender and were transferred to the fresh media vials containing control and variable diet composition of proteins and carbohydrate. Two days after eclosion the adults (F1) from each of the standard wheat cream agar medium and experimental diet were collected to record the effect of nutrition on developmental time. Developmental time was recorded daily from the egg to adult eclosion. The said experiments were carried out by feeding different concentrations of protein (Brewer's yeast), *i.e.*, 5g/L, 15g/L, 25g/L and glucose (30g/L), through the food media along with the control.

Statistical Analysis

Mean egg to adult development time (egg, larval hatchability, pupation, and adult emergence) were subjected to one-way ANOVA, Tukey's HSD by using SPSS 20.0.

Table 1. One way-Analysis of variance for developmental time testing for differences between groups of parental and F1 generation.

Generations	Stages	df	Mean Square between groups	F	P-Value
Parental	Egg	4	.057	.515	.725
	Larvae	4	5.340	10.890	.990
	Pupae	4	4.283	2.217	.050
	Adult	4	2.224	6.143	.000
F1	Egg	4	.057	.515	.725
	Larvae	4	5.640	12.170	.990
	Pupae	4	8.717	5.799	.000
	Adult	4	61.067	30.474	.000
Error		145			
Total		149			

(Note: P < 0.05 significance values)

Results

Both egg to pupation and from pupa to adult eclosion (metamorphosis) time is considered for developmental time, but from pupa to adult eclosion showed a difference and was, therefore, analyzed further. Flies whose parents developed on the high protein diet had metamorphosed early. The data observed have shown that pupal to adult eclosion time lasted for 4 days and 4.50 days with low protein diet and only carbohydrate, when compared to control 5.50 days. The differences were insignificant from egg to larval hatchability, while it was significant from pupa to adult eclosion ($P < 0.05$) with that of control (Figure 1). The analysis of variance (Table 1) indicates significant difference for all the concentrations of protein and carbohydrate with that of control ($P < 0.000$), respectively. In addition to this the developmental time in F1 generation has significantly increased compare to parental the generation on exposure to high protein concentration (25 g Brewer'syeast) than in control and in low protein (5 g/L, 15 g/L) and only carbohydrate (30 g/L) fed flies, *i.e.*, the pupal to adult eclosion time lasted for 2 days and 3.50 days low protein diet and only carbohydrate with that of the control, which lasted for 5.50 days in the parent generation as shown in Figure 2. Thus the overall data reveal that the higher the concentration of protein and low carbohydrate has shortened the developmental time in both parental and as well in F1 generation. Interestingly, developmental time (in days) has still more reduced in F1 generation than the parental stock. Thus the enriched media with protein and carbohydrate have a positive effect by reducing the egg to adult eclosion time significantly, which depicts the transgenerational effect found in *Drosophila sulfurigaster sulfurigaster*.

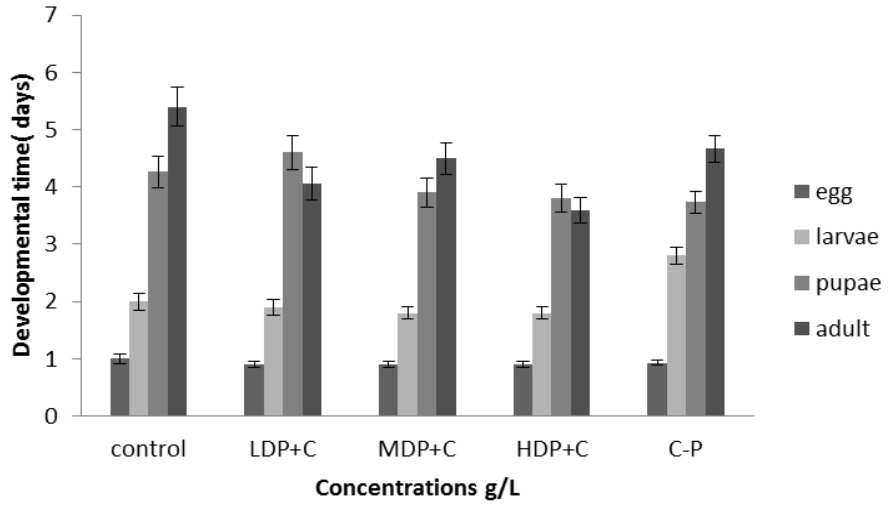

Figure 1. Mean developmental time (in days) of *Drosophila sulfurigaster sulfurigaster* parental stock (F0) fed with different concentrations of protein (Brewer's yeast) and carbohydrate (Glucose) diet. (Note: LDP = Low dose protein + Carbohydrate, MDP + C = Mid dose protein + Carbohydrate, HDP + C = High dose protein + Carbohydrate, C-P = Carbohydrate-Protein).

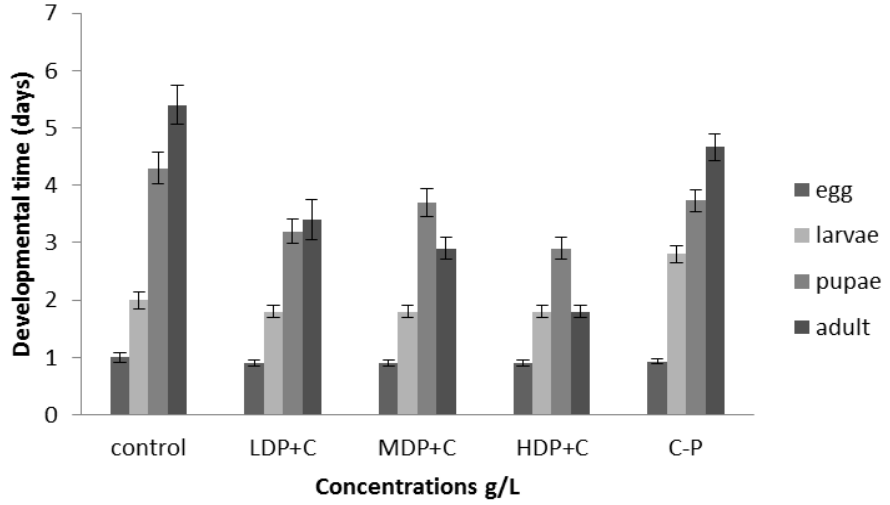

Figure 2. Mean developmental time (in days) of *Drosophila sulfurigaster sulfurigaster* offspring (F1) fed with different concentrations of protein (Brewer's yeast) and carbohydrate (Glucose) diet. (Note: LDP = Low dose protein + Carbohydrate, MDP + C = Mid dose protein + Carbohydrate, HDP + C = High dose protein + Carbohydrate, C-P = Carbohydrate-Protein).

Discussion

According to life-history theory, natural selection could be expected to favor parents that produce fewer but better provisioned offspring in response to cues indicative that offspring will experience nutritional stress (Smith *et al.*,1974; Fox *et al.*,2000). The effects of DR have been investigated for more than 70 years in various organisms. Although DR is known to extend the lifespan of a wide range of organisms, species-specific effects of DR restriction have also been recorded (*e.g.*, Mockett *et al*., 2006). There are various DR studies that were focused on the adult stage of *Drosophila*, but only a few studies were conducted to investigate the effects of DR on juvenile stages (Tu and Tatar, 2003). The quality of the larval medium is very important with respect to developmental time (Chippindale *et al*., 1997; Soto *et al*., 2006; Folguera *et al*., 2008), as larvae with limited dispersal ability should complete their development in the poor medium conditions. The

developmental rate of *Drosophila* is a function of numerous metabolic and developmental processes (Church and Robertson, 1966). By subjecting a wild-type population to fast and slow developmental rate selection, Robertson (1964) has shown that genetic variation exists in natural populations of *Drosophila melanogaster* for developmental rate. A significant interaction between the parental diets indicates that a parent's dietary effect on offspring development time was dependent upon the dietary source. The hatchability was significantly different ($P < 0.05$) between the groups of different concentrations in comparison to control, and insignificant between 5 g/L, 15 g/L of the protein concentration with ($P > 0.990$) and glucose (30 g/L). Experimental and controlled flies have not shown differences in all concentrations with that of control $P < 0.05$ and insignificant between 15 g/L and 25 g/L ($P > 0.755$) with respect to Table 1. Dietary restriction (DR) in *Drosophila* is often achieved by dilution of the food medium, and complete records of food intake are needed to determine if flies compensate for the reduced nutritional content of food by increasing the total amount of food they consume. Yeast has been shown as the most important compound of the food medium in *Drosophila* studies by several researchers. Life history traits like ageing, fecundity, viability, and development are directly affected by the levels of yeast used in the food medium. We emphasize the importance of carbohydrate and protein intake for developmental time. By comparing development times of offspring of parents with those of offspring parents it would appear that parents transferred their condition to their offspring. However, because the shortest development times were found among offspring of parent and F1 generation one can conclude that the complex interplay between nutrient balance and development time highlights the necessity of accurately measuring food intake when conducting development studies.

Acknowledgment: The authors thank DST-SERC and UGC-RGNF New Delhi, India, for providing financial assistance and also Department of Zoology, Bangalore University, Bangalore, to carry out the above work.

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Intra-specific cytogenetic variations among three Drosophilid species, *viz*., *Drosophila melanogaster*, *Drosophila repleta*, and *Zaprionus indianus* collected along an altitudinal gradient in Garhwal region, India.

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Abstract

Garhwal region of Central Himalaya is bestowed with highly varied ecological habitats ranging from near tropic to cool-temperate regions, ascertaining its status as a true Drosophilid biodiversity hotspot. The researchers until now have worked extensively towards exploration and taxonomic data-basing of Drosophilid diversity of this region. However, keeping in view diverse ecological conditions prevalent here, it could well be an arena for other research implications like cytogenetics and molecular systematics. Thus, Drosophilid biodiversity was assessed in Garhwal region along an altitudinal gradient and three most abundant cosmopolitan species, *viz*., *Drosophila melanogaster*, *Drosophila replete*, and *Zaprionus indianus* were analyzed cytogenetically (for localization of nucleolus organizer region-NOR and variation in Nucleolar chromatin threads-NCTs).

Introduction

Despite the major impact of *Drosophila* cytogenetics in scientific studies, however, cytogenetic studies concerning Drosophilid species inhabiting biodiversity rich Uttarakhand region are still scanty. Considering their wide distribution in the region over such varying ecological habitats, it was interesting to analyze the individuals for intra-specific cytogenetic variations. Thus, sampling surveys were carried out especially in Garhwal region to assess Drosophilid diversity along an altitudinal gradient. Though some species were found be specialists occupying particular type of habitats, few were cosmopolitan inhabiting highly varied ecological habitats at different altitudes with highly varied temperature regimes and other climatic conditions. Such cosmopolitan species were analyzed cytogenetically (localization of nucleolus organizer region-NOR and variation in Nucleolar chromatin threads-NCTs).

An appealing observation about these threads in different Drosophilid species was that the pattern of the thread-like connection in the matrix of the nucleolus was not constant. The pattern of NCTs varied between species (Barr and Plaut, 1966) but, even between same species collected from different altitudes with highly varied climatic conditions, there was a considerable degree of variation in the morphological configuration of the threads, providing a persuasive tool for future research on evolutionary biology.

Materials and Methods

For the present study different Drosophilid species were collected along an altitudinal transect in Garhwal region especially from Srinagar Garhwal - SG (550m asl, 30° 22´ N and 78° 78´ E,

District-Pauri), Upper Chamoli - UC (1150m asl, 30° 24´ N and 79° 21´ E, District-Chamoli), Mandal - MD (1600m asl, 30° 46´ N and 79° 26´ E, District-Chamoli), Kanchula Kharak - KK (2100m asl, 28° 43´ N and 77º 34´ E, District-Chamoli) and Chopta - CP (2700m asl, 30° 29´ N and 79° 10´ E, District- Rudraprayag).

The flies were collected using the net sweeping method and exposing fermenting fruit as baits or directly through aspirator. These flies thus trapped were sorted out and identified under stereo-zoom trinocular microscope. Further, the stock culture from single female, assuming its natural insemination was established in the laboratory. The slides of salivary gland chromosome were prepared by the usual squash method as suggested by Ashburner (1970), analysed and observed directly by captured pictures through dino-lite digital microscope camera for localization of nucleolus organizer region-NOR and variation in Nucleolar chromatin threads-NCTs.

Results and Discussion

The data obtained through sampling were pooled to furnish a spatial distribution pattern of Drosophilid species inhabiting along different altitudes in Garhwal region. The most abundant cosmopolitan species, *viz*., *Drosophila melanogaster*, *Drosophila replete*, and *Zaprionus indianus*, were analyzed cytogenetically. Observations on localization of nucleolus organizer region-NOR and variation in Nucleolar chromatin threads-NCTs in these three species are as follows-

Drosophila melanogaster

The salivary gland polytene chromosome preparation of *Drosophila melanogaster*, depicted that Y Short arm and the proximal part of the X both possess a nucleolus organizer region (NOR). Moreover, the pattern of Nucleolar chromatin threads (NCTs) varied among individuals collected from different altitudes.

Four types of Nucleolar Chromatin Threads were observed in *Drosophila melanogaster* collected from different altitude:

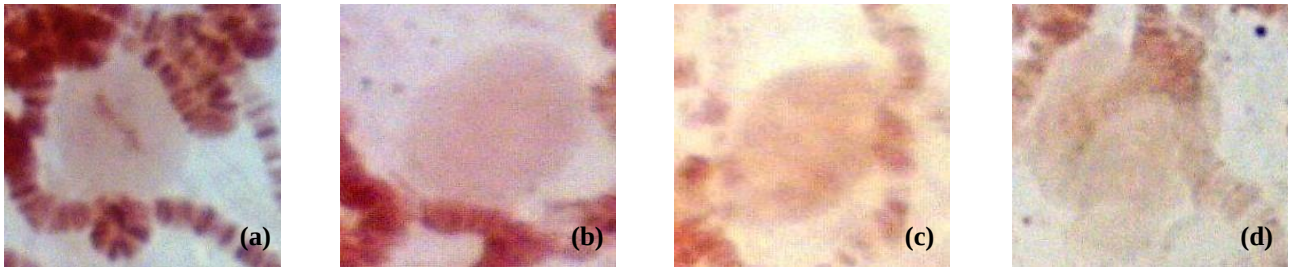

Figure 1. (a) - Chromatin threads not clearly visible highly branched and scattered throughout the Nucleolar mass with a darkly stained body. Sampled at SG and UC; (b) - More than one thread, ramified and with less condensed granules. Sampled at MD; (c) - The threads more condensed, positively stained with many darkly stained granules concentrated towards the periphery of the nucleolus. Sampled at KK; (d) - Many thread, ramified with more condensed granules concentrated at the point of origin. Sampled at CP.

Drosophila repleta

The Nuclear Organizer Regions (NOR) in *D. repleta* was present in both X and microchromosomes. Five different patterns of Nucleolar Chromatin Threads were observed in *Drosophila repleta* collected along altitudinal transect:

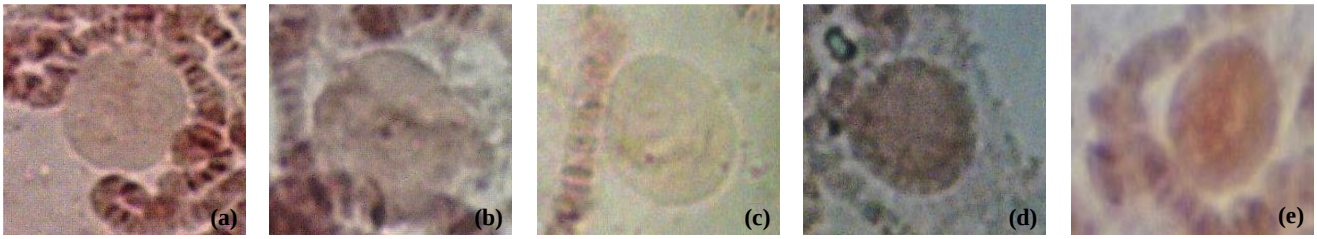

Figure 2. (a) - Nuclear Chromatin Threads thin and scattered throughout the nucleolus with small light granules. Sampled at SG; (b) - Threads branched and scattered throughout the mass but more condensed and prominent in the centre. Sampled at UC; (c) - Many threads, ramified and with less condensed granules. Sampled at MD; (d) – More condensed threads with positive stain and number of darkly stained granules near the periphery. Sampled at KK; (e) - Threads most prominent and more condensed scattered throughout the nuclear mass. Sampled at CP.

Zaprionus indianus

Cytogenetic studies of the polytene chromosomes in the species *Zaprionus indianus* showed localization of the nucleolar organizer region in the X chromosome and in the dot pair. Furthermore, four different types of Nucleolar Chromatin Threads were observed in *Zaprionus indianus* collected at different altitudes:

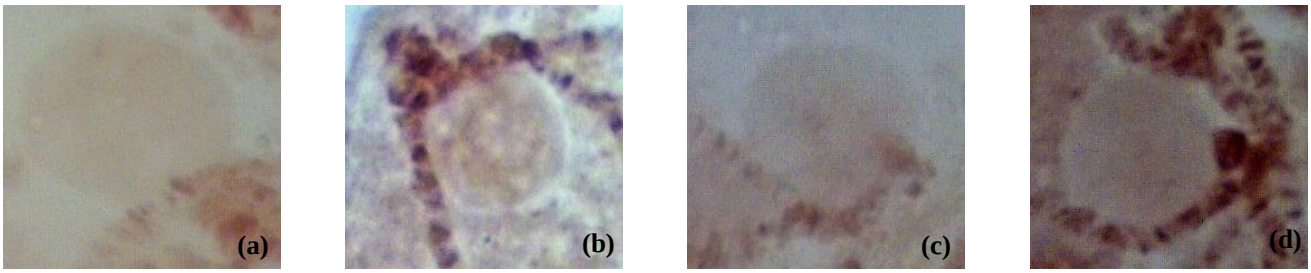

Figure 3. (a) - Thin chromatin threads, scattered throughout the nucleolus with some light granules. Sampled at SG; (b) - Threads condensed with positive stain and several darkly stained granules around the periphery of the nucleolus. Sampled at UC; (c) - Thread not visible, light granules forming a semilunar structure. Sampled at MD; (d) - Thread small with dark stain and a round chromatin mass near the point of origin. Sampled at KK and CP.

Thus, these intra-specific patterns of NCTs among individual flies of the same species collected from different altitudes with highly varied climatic conditions depict a considerable degree of variation in the morphological configuration of the threads, providing a persuasive tool for future research on evolutionary biology.

Acknowledgments: This work is financially supported by University Grants Commission, New Delhi, India as Major Research Project to R.S. Fartyal, Ref. No. UGC Project F. No. 37-198/2009 (SR), and thanks for UGC-BSR fellowship to M. Sarswat. And we also acknowledge National Biodiversity Authority (NBA), Chennai, India.

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Inversion and isozyme variation, mating behavior, and fitness in *Drosophila ananassae*.

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Abstract

Natural populations are endowed with a large amount of chromosomal and genetic variation. In the present study, we have tried to find out whether any correlation between different polymorphisms exists. For this purpose, **i**nversion frequencies and α-esterase variation were studied in four different populations of *D. ananassae* collected from Dharwad, Bellur, Krishnarajanagar and Mysore, India. Present study has demonstrated high polymorphism of both inversions and α-esterase. The role of inversions and α-esterase phenotypes on mating behavior and fitness has also been studied. Three different strains of *D. ananassae* carrying 2LA, 3LA and 2LA+3LA inversions and one inversion free strain were built up in the laboratory using the female flies collected from natural habitat at Mysore, India. Mating behaviors, such as courtship latency, mating latency, and copulation duration, and fitness characters, such as fecundity, viability, and fertility, were studied in these inversion and inversion-free stocks using no choice experiment. The mating behaviors and isozyme pattern in three different inversions and inversion-free strains of *D. ananassae* were quantified and compared between different strains. The carrier of two inversions (2LA+3LA) took more time to copulate but had higher fitness than inversion-free stock or stock carrying single inversion. The concept of inversion heterokaryotype superiority is confirmed with reference to both inversion and α-esterase polymorphism.

Introduction

One of the most important aspects of evolutionary biology is the study of how natural selection modifies the genetic structure of populations. For this to happen, populations must encompass some degree of chromosomal or genetic variation or other kinds of modifications in the gene pool. Analysis of inversion polymorphism is one of the strategies to know the extent of genetic variation between populations. Such an analysis has been made extensively in many species of *Drosophila* (Krimbas and Powell, 1993). The early studies on inversion polymorphism have also demonstrated the superiority of inversion heterokaryotypes over homokaryotypes (Singh and Chatterjee, 1986). Genetic variation at allozyme loci has also been studied in *Drosophila* (Barker, 1981). A combination of these two kinds of analyses has demonstrated the association between inversion and allozyme polymorphism (David, 1982). Non-random association between allozymes and inversions in natural populations of *D. melanogaster* has been demonstrated by Langley *et al.* (1977). Over dominance at different isozyme loci and linkage disequilibrium among multiple neutral alleles was also demonstrated (Hill, 1975). Barker and Mulley (1976) have demonstrated that out of six allozyme loci which are consistently polymorphic in Australian populations of *D. buzzatii*, three (esterase-1, esterase-2, and aldehyde oxidase) are linked with chromosomal inversions. Knibb *et al.* (1987) also found that esterase locus is tightly linked to an inversion complex in *D. buzzatii*. Thus the available literature amply supports the existence of association between inversion polymorphism and allozyme polymorphism.

Studies on different aspects of sexual, non-sexual behavior, and fitness in various species of *Drosophila* have also been well documented (Smith, 1956; Sisodia and Singh, 2005). Mating behavior of *Drosophila* consists of specific actions which are accompanied by orientation movements. Such actions referred to as courtship displays are made up of several signals which are performed sequentially. Mating occurs only if the female responds by performing acceptance signals. Since sexual behavior of males and females affects and modifies the contribution of different genotypes to the gene pool of succeeding generations, it becomes an important component of fitness. In *Drosophila*, successful mating depends on male activity and female receptivity because usually the female is the discriminating partner in the mating act, *i.e*., she actively accepts or rejects a courting male (Bastock, 1956).

Mating speed (or courtship time), the time from the beginning of courtship to copulation (Spieth and Ringo, 1983), is a good estimate of sexual receptivity of females and sexual activity in males. It is known that male activity and female receptivity are the main factors responsible for successful mating in *Drosophila* (Bastock, 1956). A considerable amount of information on genetic determination of sexual behavior in *Drosophila* is available (Singh and Singh, 1999). Investigations on mating propensity in various species of *Drosophila* (Spiess, 1970) have also shown that efficiency of mating varies for different genotypes. The contribution of males to the variation in mating propensity is greater than that of females and thus males are inherently more subject to intrasexual selection (Parsons, 1965; Singh and Chatterjee, 1987). However, Kessler (1968) reported that females contribute more to the variation of mating speed than males of *D. pseudoobscura*. From previous reports on sexual behavior in the genus *Drosophila*, it is clear that the efficiency of mating differs in different genotypes (Smith, 1956; Sisodia and Singh, 2001).

In addition to inversions, isozyme variants also have influence on sexual behavior and fitness. Although vast literature is available, no effort has been made to correlate between each of these parameters. The question is whether inversion polymorphism and / or enzyme polymorphism, sexual behavior and fitness are interrelated or independent of one another? Whether inversion polymorphism has any influence on the expression of allozyme alleles? What is the role of inversion or allozyme polymorphism on these traits? In the present studies, the authors have tried to address the above questions. For this purpose *D. ananassae* has been selected as the experimental model because of its following characteristics. It is a cosmopolitan domestic species belonging to *melanogaster* group of *ananassae* subgroup and *ananassae* species complex (Bock and Wheeler, 1972). This species occupies a unique status in the whole of genus *Drosophila* due to certain peculiarities in its genetical behavior (Singh, 1985). Absence of male crossing over, high level of inversion polymorphism, and high mutability are the features which make it useful for certain genetic studies. Although the species harbors large number of inversions, most of the inversions are found in isolated populations. One interesting feature of inversion polymorphism of this species is that it carries three well-knit co-extensive inversions found in geographic populations. They are 2LA, on the left arm of the 2nd chromosome, 3LA on the left arm of the 3rd chromosome, and 3RA on the right arm of the 3rd chromosome. The frequency of these inversions varies in different geographical populations and, hence, they can be subjected to different types of genetic analysis on inversions.

Materials and Methods

Analysis of inversion frequencies in natural populations:

D. ananassae flies collected from Dharwad, Bellur, Krishnarajanagar and Mysore following the procedure described by Hegde *et al.* (1999) were used for the present study. After the flies were

brought to the laboratory, the females were individually placed in glass vials (2.5 cm × 8.5 cm) containing wheat cream agar medium, and males were used for identification and for analysis of α-esterase polymorphism. The female flies were then maintained at constant temperature of 22 ± 1°C and relative humidity of 70%. When larvae appeared, eight third instar larvae from each isofemale line were used for analysis of inversion frequency and others were allowed to continue their development. The inversion frequency was studied by polytene chromosome preparation following the procedure described by Reddy and Krishnamurthy (1974).

Analysis of α-esterase variation in natural populations:

The same four populations used for analysis of inversion polymorphism were also used for analyzing the α-esterase polymorphism. This was intended to understand the extent of α-esterase variation present in the natural population. For this purpose, the wild caught males and the females of the F_1 progeny of wild caught females of the same four natural populations of *D. ananassae* employed above were used. Single fly homogenates were used as sample and electrophoresed separately. The polyacrylamide gel electrophoretic technique described by Davis (1964) modified for vertical slab gel was used. The gels were stained for α-esterase using the staining procedure described by Hegde and Krishnamurthy (1976).

Calculation of allelic frequencies:

After electrophoresis, the zymograms were drawn for each individual and used for calculation of allelic frequencies. To identify the alleles and to assign the locus to which they belong, a cross was conducted between individuals of F1 progeny of wild caught females and the pattern of segregation of different bands was analyzed. Accordingly three loci were recognized for α- which were designated as α-esterase α-est1, α-est-2, α-est-3. On the basis of mobility two alleles in each locus, *viz*., fast (F) and slow (S) were recognized. When there was a single band at a given locus (either F or S), then the individual was considered as homozygote for that locus and if there were two bands together (both F and S), then it was considered as a heterozygote. To calculate the allelic frequencies – as per the Hardy-Weinberg equilibrium, half of the total number of heterozygotes observed is added to the homozygotes scored and this value is divided by the total number of genomes (N) sampled. Further, Z-Statistics (H) was calculated using the formula $H = 1 - \sum p_i^2$, where p is the frequency of the i'th allele at the given locus and $\sum$ is the summation of the over all F and S alleles.

Establishment of inversion stocks:

To analyze the role of inversions on mating behavior and isozyme pattern, three different strains carrying 2LA, 3LA, and 2LA+3LA inversions and one inversion free strain were built up in the laboratory using the female flies collected from natural habitat at Mysore. For the sake of convenience, these strains were designated as IA, IB, IC, and ID, respectively. IA is monomorphic (inversion free), IB is with 2LA, IC is with 3LA, and ID is with 2LA+3LA strains. These females were individually placed in vials containing wheat cream agar media (isofemale line) and when larvae appeared, eight larvae from each vial were sacrificed to check for presence or absence of inversions in their salivary gland chromosomes. The cytological detection of these inversions was made using the procedure of Rajeshwari and Krishnamurthy (1969). *D. ananassae* populations collected from Mysore carries two common inversions namely, 2LA and 3LA. The wild caught individuals, therefore, would be either without inversion or carry 2LA alone, or 3LA alone, or both 2LA+3LA. When all the eight larvae carried a given inversion, then that individual (their mother)

was designated as the strain carrying that particular inversion. The adult progenies which appeared from such mothers were classified as inversions free, 2LA, 3LA, and 2LA+3LA strains.

These strains were separately maintained for six generations and at each generation, three to five larvae were used to check for the presence or absence of respective inversions. Although in each generation, the polytene chromosomes showed the presence of either inversion loop or absence of loop, because they originate from same isofemale line, all progeny contained only that particular inversion homokaryotype or heterokaryotype. The adults emerged from these strains were used to build up populations for the study of variation in mating behavior, fitness, and isozymes.

Analysis of mating behaviour and fitness among four inversion phenotypes:

To study the mating behavior of the four inversion phenotypes, virgin females and bachelor males from each inversion line were isolated within three hours of eclosion from stocks developed as above and were kept separately for the study of mating behavior. They were aged for 5 days; then a virgin female along with a bachelor male was placed in an Elens-Wattiaux mating chamber (a circular chamber with a diameter of 9 cms). Each pair was observed for 1 hr and if there was no mating, then the pair was discarded. For each mating pair, courtship latency (time between introduction of male and female together into mating chamber until orientation of male towards female - usually measured in seconds), mating latency (time between introduction of male and female together into mating chamber until initiation of copulation of each pair – measured in minutes) and copulation duration (time between the initiation of copulation to termination of copulation of each pair – measured in minutes) were observed following the procedure of Hegde and Krishna (1997). A total of 30 pairs were observed in this way and the means and standard errors were calculated. To analyze fitness, each mated pair was transferred into a vial containing wheat cream agar medium. After 24 hours, the pairs were transferred to fresh food vial, and the eggs laid in the previous vial were counted. This procedure was continued for 15 days, and the total number of eggs laid and the adults emerged from each pair was recorded to determine fecundity, viability, and fertility of these strains. The data were statistically analyzed by One-way ANOVA followed by DMRT.

Analysis of α-esterase variation in the four inversion phenotypes:

The variations of α-est in four strains were studied here by homogenizing the flies individually in 40% sucrose solution and kept separately. Electrophoresis was carried out as per the procedure described above. The zymograms were drawn for each individual for α-est of all the four inversion strains.

Results

Variation inversion and α- esterase isozymes in natural populations:

Table 1 shows percentage of different inversions present in different geographical populations of *D. ananassae.* It was noticed that the frequency of inversions differ in different geographical populations. In all populations, the highest number of individuals carried 3LA inversion while the least number of individuals was inversions free. Figure 1 shows the zymogram of α-esterases found in four different populations of *D. ananassae*. Three loci were identified at α-esterase locus, each with two alleles, F and S. Table 2 shows the allelic frequencies of alpha esterase (α-est) isozymes of four different natural populations. The frequencies of slow moving allele (S) of α-est-1 were less than the frequencies of the corresponding fast moving alleles in all the four populations studied. In the α-est-1 locus, the frequency of slow moving allele of Dharwad population was lowest while that

of Mysore population was highest. The χ^2 values calculated for the percentage of slow moving allele present in different populations show that the allelic frequencies between populations were significant. Correspondingly, the frequency of fast moving allele of Mysore population was highest and of Dharwad population was lowest. Further, the H value calculated on the basis of Z-statistics showed that Mysore population is more polymorphic than others.

Table 1. Inversion frequency (%) in different geographic populations of *D. ananassae.*

Strains	Inversion frequency (%)				
	N	2LA	3LA	2LA+3LA	Inversion free (Monomorphic)
Bellur	32	16.7	40.0	26.7	16.6
Dharwad	38	20.0	50.0	20.0	10.0
Krishnarajanagar	42	36.7	53.3	06.7	03.3
Mysore	40	33.3	43.4	10.0	13.3

Table 2. Allelic frequencies at α-est loci in different populations of *D. ananassae.*

Locus	Allele	Dharwad	Krishnarajanagar	Mysore	Bellur	χ^2-value
α-est-1	Slow	0.19	0.32	0.33	0.29	20.84*
	Fast	0.81	0.68	0.67	0.71	13.07*
H		0.31	0.43	0.44	0.41	
N		38	42	40	32	
α-est-2	Slow	0.95	0.81	0.84	0.85	11.33*
	Fast	0.05	0.19	0.14	0.15	28.11*
H		0.95	0.30	0.27	0.25	
N		38	42	40	32	
α-est-3	Slow	0.84	0.76	0.72	0.80	10.09*
	Fast	0.16	0.24	0.28	0.20	19.05*
H		0.26	0.52	0.40	0.32	
N		38	42	40	32	

*χ^2 values are significant at 0.05 levels.

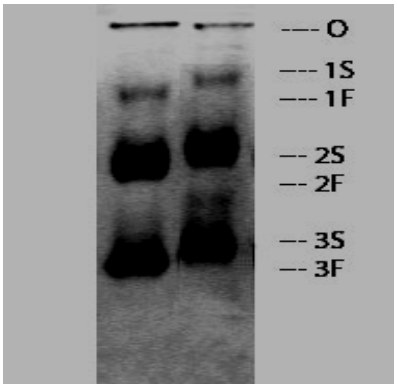

Figure 1. Showing the α-est loci found in the natural populations of *D. ananassae.* (O = Origin, F and S are the alleles of the three loci).

Contrary to the α-est-1 locus, the slow moving alleles in α-est-2 and α-est-3 were found to be more than the fast moving allele. The frequency of the slow moving allele of α-est-2 locus ranged from 0.81 in Krishnarajanagar population to 0.95 in Dharwad population. Similarly the frequency of fast moving allele ranged from 0.05 in Dharwad to 0.19 in Krishnarajanagar population.

The χ^2 values calculated for these allelic frequencies showed significant differences between different populations at 0.05 levels. The H value showed that even with regard to α-est-2 locus all the four populations analyzed were polymorphic. In α-est-3 locus, the slow moving allele had a frequency ranging from 0.72 in Mysore to 0.84 in Dharwad population. On the other hand the fast moving allele of Dharwad was represented with a low frequency of 0.16 and Mysore population had the frequency of 0.28. Corresponding with allelic frequencies, the H value also varied between different populations.

Table 3. Mating behavior, fecundity, viability and fertility of different inversion strains of *D. ananassae* (Values are Mean ± SE).

Strain → ↓Parameters value	IA (Inversion free)	IB (2LA inversion)	IC (3LA inversion)	ID (2LA+3LA Inversion)	F
Courtship latency (in seconds)	50.13 ± 3.44[a]	46.73 ± 4.25[a]	41.66 ± 3.00[a]	69.93 ± 5.78[b]	08.45**
Mating latency (in minutes)	27.92 ± 2.72[a]	24.41 ± 2.99[a]	26.08 ± 3.19[a]	28.60 ± 3.30[a]	11.78**
Copulation duration (in minutes)	3.76 ± 0.14[a]	4.08 ± 0.18[b]	3.60 ± 0.13[a]	4.11 ± 0.10[b]	2.99*
Fecundity (in nos)	45.53 ± 4.81[a]	57.53 ± 5.33[a]	122.06 ± 5.35[b]	131.53 ± 11.95[b]	49.10**
Viability (in nos) (No.of larvae/100 eggs)	42.06 ± 1.19[b]	36.66 ± 0.91[a]	79.10 ± 2.42[c]	94.26 ± 2.00[d]	259.35**
Fertility (in nos)	31.53 ± 4.15[a]	24.26 ± 2.76[a]	69.80 ± 9.93[b]	83.86 ± 11.88[b]	12.68**

IA, IB, IC and ID are α-esterase phenotypes (refer Fig. 2)
Same superscript in each row indicates that the value is nonsignificant by DMRT.
*P< 0.005, **P< 0.001.

Variation in mating behavior and fitness of inversion phenotypes:

Table 3 shows mating behavior, fecundity, viability, and fertility of different strains of *D. ananassae*. It is noticed that courtship latency was highest in the strain with double inversion (ID) while it was lowest in the 3LA (IC) strain. One way ANOVA followed by DMRT applied on mean courtship latency of different strains showed that mean courtship latency varied significantly between them. The mean mating latency of different strains of *D. ananassae* is also provided in Table 3. It is noticed that mating latency was highest in strain 2LA+3LA (ID), while it was lowest in the 2LA (IB) strain. Although the analysis of variance showed significant differences between different strains, by DMRT these differences were found to be non-significant. Mean copulation duration of different inversion strains revealed that mean copulation duration was highest in strain 2LA+3LA (ID) (value significant over all others) while lowest in 3LA (IC) strain. The application of ANOVA followed by DMRT showed that the mean copulation duration of inversion free (IA) strain was significantly less than 2LA (IB) and 2LA+3LA (ID) strains but non-significant with 3LA (IC).

Mean fecundity of different strains is provided in Table 1. It was noticed that fecundity was highest in 2LA+3LA (ID) strain and lowest in inversion free (IA) strain. The data on mean fecundity subjected to one way ANOVA followed by DMRT showed significant variation in fecundity between different strains. DMRT showed that mean fecundity of inversion free strain was significantly less with all other strains. Thus the mean fecundity of different inversion strains in the decreasing order was, inversion free < 2LA < 3LA < 2LA+3LA. It was noticed that viability was highest in 2LA+3LA (ID) strain and lowest in 2LA (IB) strain. The data on mean viability subjected to one way ANOVA followed by DMRT showed significant variation in viability between different strains. DMRT showed that mean viability of 2LA (IB) strain was significantly less with all other strains. Thus the mean viability of different inversion strains in the decreasing order was, 2LA < inversion

free < 3LA < 2LA+3LA. Further, highest fertility was noticed in 2LA+3LA (ID) strain and least in 2LA (IB) strain.

Mean fertility data when subjected to one way ANOVA followed by DMRT showed significant variation in fertility between different strains. DMRT showed that mean fertility of inversion free (IA) strain was significantly less than 3LA (IC) and 2LA+3LA (ID) strains but non-significant with the 2LA (IB) strain. In summary, the mean fertility of different inversion strains in the decreasing order was, 2LA < inversion free < 3LA < 2LA+3LA.

Variation of α-est isozymes in the four inversion phenotypes:

Figure 2 shows the variation of α-est isozymes in the inversion free strain and different inversion strains. The IA (inversion free) strain carried only two bands, representing α-est-2F and α-est-3F. The IB (2LA inversion) strain also showed two bands but representing α-est-2S and α-est-3S. Similarly the IC (3LA inversion) strain had α-est-2S and α-est-3S. The ID (2LA and 3LA inversions) strain had three bands representing α-est-1F, α-est-2S, and α-est-3S.

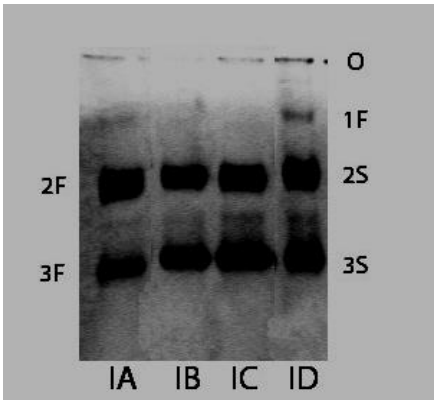

Figure 2. Showing the α-est loci found in different inversion strains of *D. ananassae.* (IA = Inversion free, IB = 2LA inversion, IC = 3LA inversion and ID = 2LA+3LA inversion strains).

Discussion

Variation inversion and α-esterase isozymes:

The study of inversion and α-esterase polymorphism in four natural populations of *D. ananassae* shows high polymorphism of both these parameters (Tables 1 and 2). Both 2LA and 3LA were present in these populations with varying frequencies. This observation agrees with the findings of Reddy and Krishnamurthy (1974), who have also demonstrated variation in the frequencies of 2LA and 3LA inversions in certain South Indian populations of *D. ananassae*. There are three α-esterase loci in the four populations of *D. ananassae* and all these loci were found to be polymorphic (Figure 1). There are a number of such studies, which demonstrate the inversion and isozyme studies (Johnson, 1971; Barker and Mulley, 1976). Our study also confirms the existence of high polymorphism in natural populations of *D. ananassae*.

In the present studies, the authors have tried to correlate the enzyme variation with chromosomal inversions. It is found that the inversion free (IA) strain showed only two esterase (isozymes) forms α-est-2F and α-est-3F (Figure 2). The 2LA (IB) inversion strain also showed two isozymes but representing α-est-2S and α-est-3S. Similarly the strain carrying inversion 3LA (IC) had α-est-2S and α-est-3S. The inversion strain carrying both 2LA and 3LA together (ID) had three isozymes representing α-est-1F, α-est-2S, and α-est-3S. Thus more alleles expressed in the double inversion strain compared to others. This indicates the heterotic effect even with reference to the expression of isozymes in *D. ananassae*. The study thus agrees with those of Zouros and Johnson

(1976) who while demonstrating allozyme variation also found heterotic effect at certain loci in *D. buzzatii*.

In *D. ananassae*, the α-esterase gene complex is located on left arm of the second chromosome in scaffold 13340 in the Muller's element E. The authors have noticed that the inversion phenotypes 2LA and 3LA have the same pattern of expression of α-esterase (see Figure 2). This means carrying these inversions has no effect on the expression of α-esterase alleles.

Based on the distribution, Mettler *et al.* (1977) classified the inversions of *D. melanogaster* as cosmopolitans, which occur in many populations often at frequencies greater than 5%, and rare cosmopolitans, which are present in many populations but at frequencies usually less than 5%. Recurrent endemics are those that occur in only few individuals in the same or adjacent populations, while unique endemics are those recorded only once. Knibb *et al.* (1987) have demonstrated lower esterase activity due to linkage disequilibrium of these loci on the second chromosome of *D. buzzatii* associated with certain cosmopolitan inversions. In *D. ananassae*, the two inversions, 2LA and 3LA, are cosmopolitans because they occur in most of the populations at frequency greater than 5% (Futch, 1966; Reddy and Krishnamurthy, 1974). Even in the present study the four populations in which the inversion frequencies have been estimated, they exist at high frequency (Table 3). If inversion has an effect on the expression of α-esterase there should have been a difference in the expression pattern between the inversion strains. However, no such difference in α-esterase pattern was noticed by the authors between 2LA (IB) and 3LA (IC) strains. Thus the present study demonstrates that these inversions have no effect on the esterase alleles.

Variations in courtship acts and fitness:

In *Drosophila* many adaptive functions have been found to be associated with inversion polymorphism. The present study demonstrates that the courtship acts and fitness are associated with inversion polymorphism. Courtship latency is one of the parameters that indicate vigor of a male (Singh and Singh, 1999; Sisodia and Singh, 2001). A male with high vigor reacts quickly in the presence of female, while a male with less vigor reacts slowly (Markow, 1978). Mating latency indicates both vigor of males and receptivity of females. Higher the vigor of males and receptivity of females, shorter is the mating latency. During this period, courtship acts are performed mostly by males, to increase the receptivity of females and to make her sexually excited (Spieth, 1968). A male with high vigor has to perform the same courtship act more times to a non-receptive female than to a receptive female. Correspondingly when a female is receptive, males' activity may be brief and he may mate her more quickly than a non-receptive female. In the present studies, both courtship and mating latency were highest in the double inversion strain (2LA+3LA) than others. This means carrying two inversions reduces the vigor of males and receptivity of females. However, the copulation duration was highest in the double inversion strain than others. During copulation sperm from the male is transferred to the female reproductive tract and, therefore, the duration of copulation has a lot of significance in an animals' life. Since increased copulation duration increases the number of ejaculations, longer copulation duration is more advantageous at least for animals with limited number of matings during their lifespan. Many workers have demonstrated heterotic effect of inversion polymorphism in both laboratory and natural populations of *Drosophila* (Singh and Chatterjee, 1986; Krimbas, and Powell, 1993) with reference to the fitness. The present study thus demonstrates the heterotic effect with regard to copulation duration also. The purpose of courtship is to transfer maximum number of sperms to the female reproductive tract so that maximum number of eggs is fertilized. Although courtship and mating latencies were longer in the double inversion strain, by increasing copulation duration the males enhance their fitness. Even fecundity, viability, and fertility were higher in the double inversion strain than all others. The result obtained in the present study thus confirms the observation of earlier workers (Bostock, 1956; Singh and Chatterjee, 1986).

The 2LA inversion strain performed less than even the inversion free strain particularly with reference to fecundity and viability. Although inversions have an adaptive function, not all inversions are adaptive at all environments. Perhaps this is the reason for low performance of the inversion 2LA with respect to fertility and viability.

Acknowledgments: The authors are grateful to the Principal, Yuvaraja's College, Department of Zoology, University of Mysore, Mysore -570005 for providing facilities. Authors are also grateful to UGC financial support for Minor research project to Dr. S.C. Jayaramu.

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Inversion polymorphism, sexual behavior, fitness, and morphometric traits in *Drosophila ananassae*.

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Abstract

In the present study experimental stocks of *Drosophila ananassae* carrying 2LA, 3LA, 2LA+3LA inversions and a stock without any inversion were established from wild caught flies collected at Mysore, India. The mating activities (male courtship activities such as tapping, scissoring, vibration, circling, licking; activities of non-receptive females such as ignoring, extruding,

and decamping; and mating behaviors such as courtship latency, mating latency, and copulation duration), fitness characters (fecundity and fertility), and morphometric traits (sternopleural bristles, scutellar bristles, head width, and wing length) were studied in these inversion and inversion free stocks using no choice experiment. The courtship activities and mating behaviors were quantified and compared between different strains. No relation was found between inversion stocks and male courtship activities. The carrier of two inversions (2LA+3LA) took more time to copulate but had higher fitness than inversion free stock or stock carrying single inversion. There was also no clear association between morphometric traits and inversions. The concept of inversion heterokaryotype superiority appears to be valid only to fitness traits and not for others. Key Words: *Drosophila ananassae*, courtship and mating activities, inversion, morphometric traits.

Introduction

Evolutionary response to selection depends on the amount of genetic variation present in the population of a given species. According to White (1977) chromosomal rearrangements – one of the modes of genetic variation – have played a major role in evolution, and the phenomenon has occurred many times in the evolutionary history so as to produce new variants. Inversions, in particular, found in certain species are of most frequent occurrence in *Drosophila.* A large number of investigations have been carried out on inversion polymorphism in many *Drosophila* species, and these studies have demonstrated that chromosomal polymorphism in *Drosophila* is due to paracentric inversions (Dobzhansky, 1951; Da Cunha, 1955; Krimbas and Powell, 1993). The inversions are believed to have adaptive value, and thus many workers have demonstrated superiority of inversion heterokaryotypes over homokaryotypes (Singh, and Mathew, 1993; Singh, 2008). Studies on inversion polymorphism in natural populations and role of inversions on fitness in the laboratory have confirmed the heterokaryotype superiority of inversions (Parsons, 1970; Sperlich and Pfriem, 1986; Singh and Chatterjee, 1986; Singh, 2001). Inversions also have influence on morphological traits such as sternopleural bristle numbers in natural populations (Gracia-Vazuiez *et al*., 1989; Singh and Das, 1991; Das *et al*., 1994; Yadav and Singh, 2006).

Sexual behavior and sexual selection are other important aspects of an organism's activity that have been under direct influence of natural selection. Mating propensity and success are traits that are under quantitative genetic control. Mating behavior of *Drosophila* consists of specific actions which are accompanied by orientation movements (Shorey, 1962; Limatainen, *et al*., 1992; Hegde and Krishna, 1997; Sisodia and Singh, 2005). Like propensity such actions are also genetically controlled and these actions are performed sequentially (Spiess, 1970; Shrimpton and Robertson, 1988). Since sexual behavior of males and females affects and modifies the contribution of different genotypes to the gene pool of succeeding generations, it becomes an important component of fitness. The earlier studies have clearly established the heterokaryotype superiority of inversions in terms of fitness traits such as fecundity, fertility, viability, rate of development, and so forth (Smith, 1956; Singh and Chatterjee, 1986; Singh and Mathew, 1997). These fitness traits have quantitative genetic control (Mather, 1941; Sokoloff, 1966; Sisodia and Singh, 2004). The sexual behavioral traits are also quantitative in inheritance and determine the fitness. This raises the question, whether these inversions have any effect on the sexual behavioral traits. Thus it is hypothesized that the carriers of inversion display better courtships than those of inversion-free individuals. The authors have tried to demonstrate the role of inversions on these behavioral traits and also tried to correlate with a few morphometric traits that have quantitative genetic control using an inversion-free strain and three strains of *Drosophila ananassae* carrying the inversions.

Materials and Methods

To analyze the role of inversions on mating behavior and morphometric traits, three different strains carrying 2LA, 3LA, and 2LA+3LA inversions and one inversion-free strain of *D. ananassae* were built up in the laboratory using the female flies collected from natural habitat at Mysore. These females were individually placed in vials containing wheat cream agar media (isofemale line) and, when larvae appeared, eight larvae from each vial were sacrificed to check for presence or absence of inversions in their salivary gland chromosomes. *D. ananassae* populations collected from Mysore carries two common inversions namely, 2LA and 3LA. Among these two inversions, 2LA is a sub-terminal inversion present of the left arm of second chromosome and 3LA is a terminal inversion on the left arm of the third chromosome. The wild caught individuals, therefore, would be either without inversion, or carry 2LA alone, or 3LA alone, or both 2LA+3LA. When all the eight larvae carried a given inversion, then that individual (their mother) was designated as the strain carrying that particular inversion. The adult progenies which appeared from such mothers were classified as inversions free, 2LA, 3LA, and 2LA+3LA strains. For the sake of convenience, these strains were designated as IA, IB, IC, and ID, respectively. These strains were separately maintained for six generations and at each generation, three to five larvae were used to check for the presence or absence of respective inversions. Although in each generation, the polytene chromosomes showed the presence of either inversion loop or absence of loop, because they originate from same isofemale line all progeny contained only that particular inversion homokaryotype or heterokaryotype. The adults emerged from these strains were used to build up populations for the study of variation in mating behavior and morphometric traits.

Analysis of mating behavior and fitness among four inversion phenotypes:

Virgin and bachelor flies were separated from the above cultures and allowed to age for five days. Then a bachelor male and a virgin female were introduced into an Elens Wattiaux mating chamber and allowed to copulate. The pairs which did not mate within two hours of introduction into the mating chamber were considered as unmated. The quantitative courtship acts, such as tapping, scissoring, wing vibration bouts per minute, licking, circling, ignoring, extruding, and decamping, and also mating behavior, such as courtship latency, mating latency, and copulation duration, of 30 successful pairs from each of the four strains (IA, IB, IC, and ID strains) were recorded following the procedure of Hegde and Krishna (1997).

To analyze fitness each mated pair was transferred into a vial containing wheat cream agar medium. After 24 hours, the pairs were transferred to fresh food vial, and the eggs laid in the previous vial were counted. This procedure was continued for 15 days and the total number of eggs laid and the adults emerged from each pair was recorded to determine fecundity and fertility of these strains. Mean number of eggs laid and the flies emerged per day per female was calculated and these data on the mean courtship traits, fecundity, and fertility were statistically analyzed by One way ANOVA followed by DMRT.

Analysis of morphometric characters among four inversion phenotypes:

To analyze the relation between inversions and morphometric traits, the same four strains developed as above were used. The number of sternopleural bristles, scutellar bristles, of 15 males and 15 females of IA, IB, IC, and ID strains and their head width and wing length were studied using the procedure described by Naseerulla and Hegde (1992).

Result and Discussion

Table 1 shows the mean values of courtship acts exhibited by the males and females of different inversion strains of *D. ananassae*. Tapping was observed to be higher in ID strain while lesser in IA strain. Application of one way ANOVA followed by DMRT showed that males of IA strain tapped significantly less number of times than those strains that carried inversions (Table 1). Similarly the circling activity of the males of inversion free strain was also significantly less than the carriers of inversion. It was noticed that mean wing vibration was highest in IA strain while it was least in ID. One way ANOVA followed by DMRT (Table 1) carried out on mean wing vibration showed that the mean wing vibration of IA strain was significantly greater than IC and ID strains but non-significant with IB strain. Highest scissoring was noticed in males of IC strain while lowest scissoring was found in males of IB strain. The licking activity by males was also highest in IC strain and lowest in IB. The differences of these two parameters between different strains were statistically significant by ANOVA and DMRT. The behavior of non-receptive females is also shown in Table 1. Highest ignoring was noticed in ID strain while it was lowest in IA strain. Similarly extruding by females was also highest in ID strain. Both these traits were significantly different between inversion free strain and the carriers of inversion (by One way ANOVA and DMRT). On the other hand the decamping activity performed by the females of ID female to the courting male was highest while that of the females of IB strain was lowest. These data were also statistically significant.

Table 1. Courtship acts in different inversion strains of *Drosophila ananassae* (Values are Mean ± SE.

Strain → ↓Parameters	IA (Inversion free)	IB (2LA inversion)	IC (3LA inversion)	ID (2LA+3LA Inversion)	F value
Tapping	11.00 ± 0.82^{a}	12.26 ± 1.09^{b}	11.73 ± 1.09^{a}	13.40 ± 1.26^{b}	1.80
Scissoring	13.13 ± 1.08^{b}	10.13 ± 1.30^{a}	16.27 ± 1.41^{c}	11.86 ± 1.31^{a}	8.38**
Vibration	12.46 ± 0.96^{b}	12.13 ± 1.02^{b}	9.60 ± 1.03^{a}	9.13 ± 1.18^{a}	5.23*
Circling	5.20 ± 0.30^{a}	6.53 ± 0.70^{b}	5.80 ± 0.58^{b}	5.33 ± 0.48^{a}	2.06
Licking	8.86 ± 0.76^{a}	8.46 ± 0.80^{a}	12.80 ± 1.05^{c}	10.66 ± 1.04^{b}	9.12**
Ignoring	5.00 ± 1.07^{a}	9.46 ± 1.50^{b}	6.80 ± 0.70^{a}	10.00 ± 1.53^{b}	7.26**
Extruding	1.53 ± 2.55^{a}	2.40 ± 0.49^{b}	2.13 ± 0.37^{b}	2.73 ± 0.45^{b}	3.25*
Decamping	2.66 ± 0.43^{a}	2.40 ± 0.47^{a}	3.40 ± 0.45^{a}	5.06 ± 1.11^{b}	6.42**

Note: Same superscript in each row indicates that the value is non-significant by DMRT.

*P<0.05; **P< 0.001.

In *Drosophila* many adaptive functions have been found to be associated with inversion polymorphism. Morphometric traits, fitness and certain genetic loci are associated with inversion polymorphism (Sisodia and Singh, 2001). The present study demonstrates the absence of any association of male courtship acts such as tapping, scissoring, wing vibration, licking and circling with the strains carrying inversion. Although the mean values of these courtship acts are significantly different between different inversion strains, no specific inversion or inversion-free strain showed higher activity or lower activity (Table 1). The number of times these acts performed depends on 1) the activity or vigor of males and / or 2) the receptivity of females. If the male is more active, then he performs more activity, so as to quickly mate with the female. These acts are performed to make the female sexually excited. Hence if the female is non-receptive, then also these acts are performed more. As there is no distinction between the performances of males of different strains, it seems inversions have no role to play in the performance of these acts. Thus the result indicates the absence of any association between the male courtship acts and inversions.

The behavior of non-receptive females, such as ignoring, extruding, and decamping, showed some association with inversions. Ignoring and extruding were lowest in the inversion free strain (IA) and highest in double inversion strain. Even decamping was highest in the double inversion strain. This shows that the females of inversion free strain are more receptive than the carriers of inversion, and carrying two inversion decreases the receptivity. Therefore this result contradicts the findings of Stalker (1960), Parsons (1970), Singh and Chatterjee (1986, 1987), and Singh (2008) on heterokaryotype superiority.

Table 2. Mating behavior in different inversion strains of *Drosophila ananassae* (Values are Mean ± SE).

Strain → ↓Parameters	IA (Inversion free)	IB (2LA inversion)	IC (3LA inversion)	ID (2LA+3LA Inversion)	F value
Courtship latency	50.13 ± 3.44a	46.73 ± 4.25a	41.66 ± 3.00a	69.93 ± 5.78b	8.45**
Mating latency	27.92 ± 2.72a	24.41 ± 2.99a	26.08 ± 3.19a	28.60 ± 3.30a	11.78**
Copulation duration	3.76 ± 0.14[a]	4.08 ± 0.18[b]	3.60 ± 0.13[a]	4.11 ± 0.10[b]	2.99*
Fecundity No./day/female	45.53 ± 4.81a	57.53 ± 5.33a	122.06 ± 5.35b	131.53 ± 11.95b	49.10**
Fertility No/day/female	31.53 ± 4.15[a]	24.26 ± 2.76[a]	69.80 ± 9.93b	83.86 ± 11.88b	12.68**

Note: 1) Same superscript in each row indicates that the value is non-significant by DMRT. 2) Courtship latency is measured in seconds while mating latency and copulation duration are measured in minutes. 3) *P<0.05; **P< 0.001.

Variation in mating behavior and fitness of inversion strains:

Table 2 shows mean courtship latency, mating latency, copulation duration, fecundity, and fertility performed by the flies of different strains of *D. ananassae*. It is noticed that courtship latency, mating latency, and copulation duration were highest in ID, which is a strain carrying two inversions. The strains carrying only one inversion, *i.e*., either IB or IC, showed the lowest values. One way ANOVA followed by DMRT applied on mean courtship latency of different strains (Table 2) showed that these behavioral traits varied significantly between them. This means the females of strains carrying single inversion are sexually more active and receptive than either inversion free or double inversion strains. The table also shows that fecundity was lowest in IA strain, increased in the single inversion strains of IB and IC and highest in ID. Similar was the observation with reference to fertility except that IB strain has the lowest fertility. Both these data on mean fecundity and fertility subjected to one way ANOVA followed by DMRT showed significant variation.

Courtship latency and mating latency are the indicators of mating speed and copulation duration is the time available for males to transfer the sperms to the female genital tract (Spiess and Langer, 1964; Sisodia and Singh, 1996). It is noticed that courtship latency, mating latency, and copulation duration were highest in ID, which is a strain carrying two inversions. The double inversion strain does not exhibit any superiority over inversion free strain or the strains carrying single inversions. On the other hand strains carrying only one inversion, *i.e*., either IB or IC showed the lowest values (Table 2). This means the females of strains carrying single inversion are sexually more active than either inversion free or double inversion strains. The observation of heterozygote superiority is applicable provided the fly has only single inversion. However this observation contrasts that Stalker (1960) who has demonstrated concentration of inversions on the same chromosome.

Table 2 also shows that fecundity was lowest in IA strain, increased in the single inversion strains of IB and IC and highest in ID. Similar was the observation with reference to fertility except that IB strain has the lowest fertility. Both these data on mean fecundity and fertility subjected to one way ANOVA followed by DMRT showed significant variation. It is evident that although the

carriers of inversions have no heterozygote superiority, with reference to courtship acts, mating speed, they exhibit this phenomenon with reference to fecundity and fertility. This observation supports the work of earlier authors (Singh and Mathew, 1997; Krishna and Hegde, 2003; Sisodia and Singh, 2001, 2005).

Variation in morphometric traits of inversion strains:

The data on morphometric characters such as sternopleural bristles, scutellar bristles, head width, and wing length in IA, IB, IC, and ID strains are provided in Table 3. Mean number of sternopleural bristles of IB strain was highest while, it was lowest in IC. Mean number of scutellar bristles, head width, and wing length was highest in ID strain. However, lowest number of scutellar bristles was noticed in the inversion-free strain. Further the head width and wing length were lowest in the carriers of 2LA inversion. The data on these morphometric traits when subjected to one way ANOVA showed that they are significantly different between different strains.

Table 3. Morphometric traits in different inversion strains of *Drosophila ananassae* (Values are Mean ± SE).

Strain → ↓Parameters	IA (Inversion free)	IB (2LA inversion)	IC (3LA inversion)	ID (2LA+3LA Inversion)	F value
Sternopleural bristles	6.73 ± 0.16[a]	7.03 ± 0.11[b]	6.46 ± 0.09[a]	6.90 ± 0.14[b]	11.31**
Scutellar bristles	4.30 ± 0.08[a]	4.40 ± 0.09[a]	4.50 ± 0.09[a]	4.60 ± 0.09[b]	6.86**
Head width	0.82 ± 0.01[b]	0.76 ± 0.01[a]	0.77 ± 0.01[a]	0.83 ± 0.01[b]	6.96**
Wing length	1.97 ± 0.01[a]	1.95 ± 0.02[a]	2.01 ± 0.02[b]	2.03 ± 0.01[b]	3.06*

Same superscript in each row indicates that the value is non-significant by DMRT.
*P<0.05; **P< 0.001.

Heterokaryotypic superiority of inversions is also evident in the morphometric characters such as sternopleural bristles, scutellar bristles, head width, and wing length (Hegde and Krishna, 1997; Sokoloff, 1966; Shrimpton and Robertson, 1988). Mean number of scutellar bristles, head width, and wing length was highest in ID strain, which has two inversions (Table 3). The strain carrying 3LA (IC strain) also performs well compared to inversion-free strain. Only in 2LA inversion strain mean head width and wing length were less than others. Thus the present study demonstrates the heterokaryotypes superiority of only certain fitness traits and not all. Further no such superiority or association between the presence of inversions and the courtship behavior is noticed in *D. ananassae*.

Acknowledgment: The authors are grateful to Principal, Yuvaraja's College, University of Mysore, for providing facilities, and grateful to University Grants Commission for awarding minor research project.

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The first record of *Zygothrica orbitalis* (Sturtevant, 1916) for the state of Rio Grande do Sul and the southernmost limits for seven species of Drosophilidae (Insecta: Diptera).

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Introduction

Although the state of Rio Grande do Sul (RS) has been one of the most well-surveyed Brazilian states for Drosophilidae, with 86 species records, most of these surveys were conducted in the Atlantic Forest Biome, whereas the Pampa Biome has been less explored in terms of species occurrence (Gottschalk *et al*., 2008; Poppe *et al.*, 2012). Furthermore, most collections were made exclusively using banana-bait traps in the city of Porto Alegre (Silva *et al*., 2005; Garcia *et al*., 2008; Garcia *et al*., 2012) and its surroundings, *e.g*., at Itapuã State Park (Valente and Araújo, 1991) and in fields of native grasses at Guaíba (Saavedra *et al*., 1995). Recently, collections were made using the same bait in a transitional area between the Pampa and Atlantic Forest Biome by Hochmüller *et al.* (2010) and near the northern border of the Pampa Biome by Poppe *et al*. (2012). In this study, we aim to record the Drosophilidae species trapped in McPhail traps with Karo® Syrup bait in the Pampa Biome, southern Brazil. These records show the southern-most limit of distribution for eight species and one new record for RS.

Materials and Methods

In January 2010, flies were collected in a Restinga forest area of 23 ha belonging to the Federal Preservation Unit Horto Botânico Irmão Teodoro Luís (HBITL) (31°48'54"S; 52°25'48"W), southern RS, Brazil (Figure 1).

HBITL is located in the Pampa Biome and has a strong influence of Seasonal Forest Semidecidual Submontane. The climate is Mesothermal Bland Superhumid, without distinct dry

season (IBGE, 1997). According to the Köppen–Geiger climate classification system, this area belongs to the Cfa type. Data provided from Agrometeorological Station of Pelotas (distant 8.7 Km from sample site) showed that the average of the annual temperature is 17.8°C, with a maximum and minimum average of 28.2°C and 8.6°C, respectively. Rainfall is 1367 mm a year, with about 120 rainy days. The relative annual humidity is 80%. HBITL is close to the cultivated area and open environments with grassland vegetation, characteristic of the Pampa Biome.

The sample was collected with two McPhail traps, each containing 200 mL of 10% Karo® Syrup. The traps were placed at a distance of 50 m from each other on the boundary of the forest area and were set for three days. The specimens collected were maintained in 70% ethanol for fixation. External morphology and male terminalia were used to identify the flies. Dissection of male terminalia was performed according to Bächli *et al.* (2004). Two species of *Leucophenga* were characterized by differences in their pigmentation and morphological patterns, but they could not be determined.

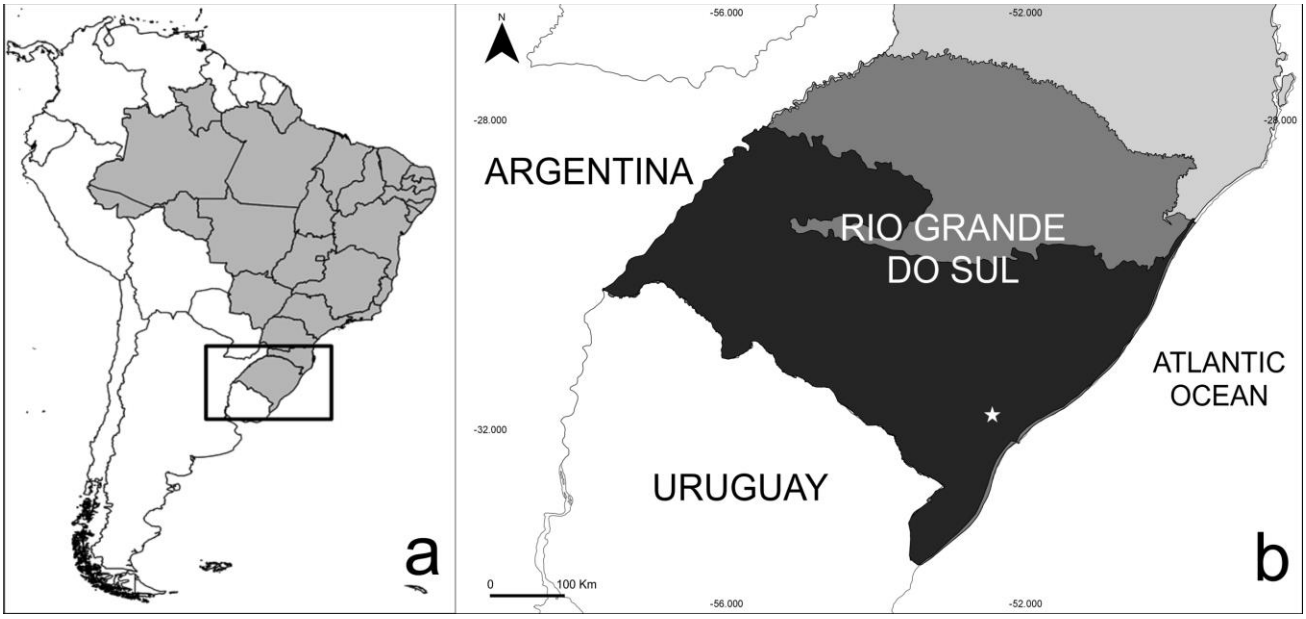

Figure 1. Location of the study area. a) Map of South America, with Brazil in gray and the state of Rio Grande do Sul highlighted. b) Map of Rio Grande do Sul, with the location of the Horto Botânico Irmão Teodoro Luís indicated by a star. Pampa Biome in black; Atlantic Rain Forest Biome in dark gray.

Results

A total of 78 drosophilids were collected. The specimens belonged to four genera of two subfamilies, Drosophilinae and Steganinae (Table 1). *Drosophila* was the genus with the greatest diversity. The most abundant species was *L.* cf. *maculosa*. This study reports the southernmost records for six species and the genus *Amiota*. *Zygothrica orbitalis* is recorded for the first time in the state of RS. *Drosophila* sp. Z2 is the same undescribed species cited by Gottschalk *et al.* (2007) as *Hirtodrosophila* sp. Z2, reclassified in the genus *Drosophila* after further analysis (Gottschalk, M.S. personal communication).

Table 1. Drosophilidae species trapped in the Horto Botânico Irmão Teodoro Luís in January 2010 using McPhail traps.

Taxon	Abundance		Total
	Males	Females	
Amiota sp. [1] Loew 1862	0	1	1
Drosophila bocainensis Pavan and da Cunha 1947	1	4	5
D. cardini Sturtevant 1916	1	2	3
D. flexa[1] Loew 1866	0	6	6
D. fumipennis[1] Duda 1925	0	1	1
D. griseolineata[1] Duda 1927	3	2	5
D. mediopunctata[1] Dobzhansky and Pavan 1943	2	6	8
D. mercatorum Patterson and Wheeler 1942	1	1	2
D. piratininga[1] Ratcov and Vilela 2007	1	1	2
D. polymorpha Dobzhansky and Pavan 1943	1	2	3
D. simulans Sturtevant 1919	5	5	10
Drosophila sp. Z2	2	2	4
Drosophila subgroup *willistoni*	0	3	3
Leucophenga cf. *maculosa* (Coquillett in Johnson 1895)	9	10	19
Leucophenga sp. 1	1	2	3
Leucophenga sp. 2	0	1	1
Zygothrica orbitalis[1]* (Sturtevant 1916)	2	0	2
Total	29	49	78

[1] Southernmost records; *First record for the state of Rio Grande do Sul.

Discussion

The results of the sample obtained with the McPhail traps with Karo® Syrup are in general agreement with the previous data obtained for collections made with banana bait in RS (Saavedra *et al*., 1995; Silva *et al*., 2005; Garcia *et al*., 2008; Hochmüller *et al*., 2010; Garcia *et al*., 2012; Poppe *et al*., 2012), in which *Drosophila* was the most species-rich genus. In these previous studies, *D. simulans* was the most abundant species and showed marked dominance, but this species was surpassed in abundance in our study by *L.* cf. *maculosa*. Other species highly representative of RS, such as *D. willistoni* and *Z. indianus*, were not collected. Interestingly, the use of a different bait, Karo® Syrup, was effective in capturing species such as *D. flexa* and *Leucophenga* spp., poorly represented in many collections for the state.

Drosophila flexa has been considered a rare species and has been absent from many collections, due most likely to biased methods of capture (Schmitz *et al.*, 2004). The presence of *D. flexa* in our collections could be related to maize cultivation near HBITL, the only breeding site previously known for this species (Vilela and Bächli, 2000).

Leucophenga species are primarily mycophagous (Throckmorton, 1975). Presumably for this reason, these flies are neglected in most studies. As a result, the ecology and biogeography of the genus are poorly known. In view of the dominance of *Leucophenga* cf. *maculosa* in the sample, its presence in our collection could not be occasional. We collected this species emerging from fungi fructifications in HBITL. These observations indicate that the species is abundant in this area (unpublished data). However, the species has never been found in flight over the fruiting bodies of

fungi at HBITL. Lachaise and Tsacas (1983) suggest that the adult feeding site of *Leucophenga* may differ from the larval breeding site. This suggestion could explain the abundance of *L.* cf. *maculosa* found by the present study.

According to Ricklefs (1973), certain climatic factors, such as seasonality, average temperature, and annual rainfall show a relatively simple relationship to latitude. And latitude, in turn, is related to diversity. Whereas low-latitude tropical regions have the highest levels of diversity, higher latitudes show a reduction in their diversity indices (Stevens, 1989). This difference could be explained by the simple observation that low-latitude regions include more types of habitats and are thus able to maintain a larger number of species (Pianka, 1966). Eight *taxa* have their southernmost record in HBITL. Thus, this result can be interpreted in the following two ways: (1) because diversity decreases with increasing latitude, this gradient could explain the absence of records of these *taxa* in countries farther south (Goñi *et al.*, 1998; Goñi *et al.*, 2012); and (2) these *taxa* are absent from collections made in areas farther south due to biased sampling methods. Thus, more effort is needed to determine where the species are distributed, both for conservation purposes and to examine their biodiversity.

This study extends previous knowledge of the diversity of Drosophilidae in the Pampa Biome, reporting the first record of *Z. orbitalis* in RS. We also extended the geographical distribution of eight *taxa* southward to latitude 31°48'S, defining the southernmost records for these *taxa* in South America. Furthermore, our results highlight the use of McPhail traps with Karo® Syrup bait as an additional tool for collecting Drosophilidae.

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Post-ecdysis behavior of exarate adults in *Drosophila melanogaster* and *Ceratitis capitata.*

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Introduction

The life cycle (LC) of cyclorrhaphans follows a well-conserved developmental program in which the different instars and stages within instars show a similar sequence of events (Denlinger and Žďárek, 1994). In spite of the evolutionary distance (around 120 MY), the duration of metamorphosis of *Drosophila melanogaster* and *Ceratitis capitata* seems to represent a similar proportion of time of the whole life cycle, *i.e*., 48.1 and 50.1%, respectively (Bainbridge and Bownes, 1981; Rabossi and Quesada-Allué, 1995). The duration of stages within the puparium expressed as percent of total metamorphosis time also seems to be highly conserved between these two cyclorrhaphans, in spite of the respective slow (600 hs) *C. capitata* and rapid (239 hs) *D. melanogaster* LCs. This might also be true for certain evolutionary and ecologically distant flies, like the blood-sucking fly *Haematobia irritans* (Basso *et al*., 2011) and other muscidae (Denlinger and Žďárek, 1994). In cyclorrhaphans, when the pharate adult inside the puparium opens the puparial operculum, a stage of extrication is initiated, ending when the legs support the body and the insect is able to walk (Žďárek and Denlinger, 1986, 1987). In *D. melanogaster* this stage has been described as Stage P15(i+ii) by Bainbridge and Bownes (1981). Then follows a phase in which the exarate imago acquires the final size, shape, and body coloration. This phase has been described in *D. melanogaster* by Bainbridge and Bownes (1981) as Stages A1 to A3. During these first hours as "unfinished" imago the exarate fly undergoes complex behavioral and molecular processes giving rise to final body maturation. In particular, the ptilinium cuticle region retracts and, after muscular pulsations and body expansion, the wings reach their definitive extension (Johnson and Milner, 1987). Then, the final steps of cuticle sclerotization and pigmentation occur, mediated by catecholamine derivatives (Perez *et al.*, 2002; Hopkins and Kramer, 1992), thus attaining the final external phenotype of the imago. Studies on this phase were reported in muscoids like *Sarcophaga crassipalpis* (Žďárek and Denlinger, 1986, 1987) or *Glossina*-Tsetse (Žďárek and Denlinger, 1992). However, as far as we know, no detailed comparison between *D. melanogaster* and Tephritids post-ecdysis behavior has been published.

Materials and Methods

D. melanogaster (Canton S) was reared in Formula 4.24 Instant Drosophila Medium (Carolina Biological Supply). Wild-type medflies, *C. capitata* (Mendoza), were reared in pumpkin-based medium as described by Pujol-Lereis *et al*. (2006). The flies were kept in a Conviron chamber (CMP 3244) at 23ºC, 60% RH, with a photoperiod of 16:8 h (light:dark). To record extrication and the initial behavior of single male exarate adults, the experimental arenas were plastic petri dishes, where a single puparium was glued to the center with a drop of 2% agar. The arena for each one of 12 *D. melanogaster* males was 35 mm diameter and 4 mm high. The arena for the medflies (24 males) was 90 mm diameter and 5 mm high.

Using these dishes, the flight was avoided and the flies could be recorded in 2-D using Logitech-C-625 cameras. Each camera was able to simultaneously record two arenas, using different zooming for the two different sizes. The final setting, at 23ºC and 60% RH, included 9 LED lamps, that gave 1000 Lux through a translucent paper circular sheet. The recorded data from the onset of extricaton to final body characteristics completion, the phase that we named BMP (see below), were registered and partially processed using a Fly-tracker-β1 experimental program from our laboratory. The timing and events of extrication behavior were analyzed separately. After extrication, the timing and length of the pathway followed by the exarate flies was continuosly recorded, including the stops. The initial position of the exarate fly was recorded as t_0 and the final position (final size and coloration) was t_f (see Figure 2 A,B). *In situ* movements like ptilinum pumping, proboscis bobbing, grooming with the legs, abdominal pulses, expansion of the wings, and so forth, that do not involve body displacement were considered for our purposes as equivalent to immobile stationary behavior. These morphogenetic activities were summarized by Žďárek and Denlinger (1992). Time of immobility was proportional to the radii of circumferences in Figure 2 C,D. These were generated using Microsoft Office Excel.

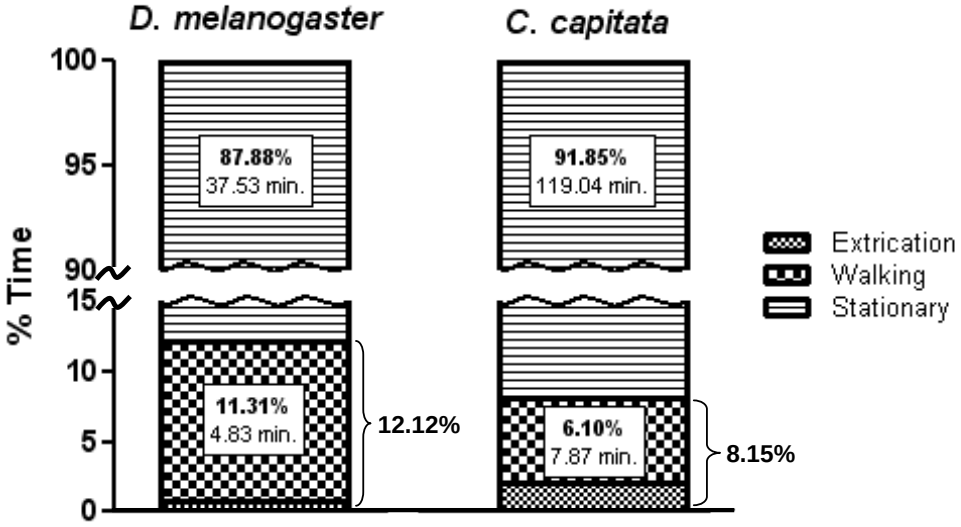

Figure 1. Percentage of time spent in extrication, walking, or stationary behaviors during the total phase BMP of exarate adults. The arrows indicate the percentage of time spent in the active periods: extrication + walking.

Results and Discussion

We have carefully analyzed the stages after ecdysis of male exarate adults of *D. melanogaster* and *C. capitata*, to analyze and quantify the timing of events until full body maturation, when final rigidity and coloration were attained. We defined the total phase of whole body maturation phase (BMP) as starting at the moment of the operculum opening and onset of pharate adult extrication and ending when the definitive features of the body are attained. Thus, this phase of the exarate adult, BMP, represents the transition from the pharate adult to the full imago, in our experimental conditions. In *Drosophila* this roughly corresponds to the P15(ii) and A1-A3 stages described by Bainbridge and Bownes (1981). Extrication in males of *D. melanogaster* required, in average, around 21 seconds (*i.e.*, 0.81% of the BMP), whereas in *C. capitata* it lasted for 2.66 ± 1.03 minutes, *i.e.*, 2.05% of BMP (Figure 1). This difference is probably more related to the anatomy of the puparium than to differences due to insect size or life cycle length. The initial position of the exarate insect standing up on its legs for the first time (t_o, Figure 2 A,B) was the one after complete extrication from the puparium, that was glued to the center of petri dishes used as arenas (see Materials and Methods). From the preliminary results reported in this communication, the total phase BMP including the extrication period required 42.70 ± 11.94 min in *D. melanogaster* and

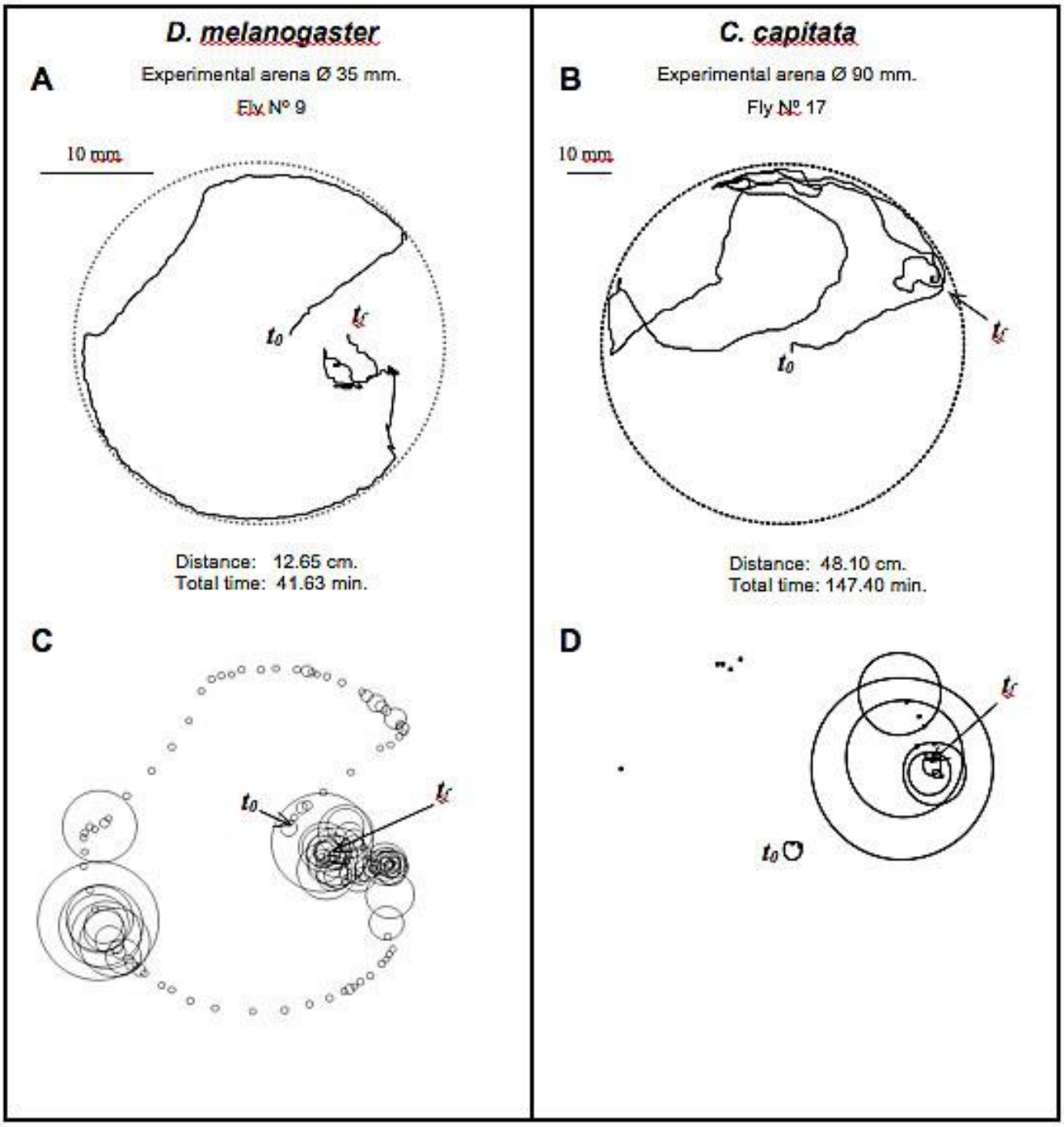

Figure 2. Examples of behavioral patterns in petri-dish arenas. A, B: records of the flies' pathways, starting at extrication point (t_0) and ending when final phenotype is attained (t_f). C, D: diagram of the same paths as in A,B, indicating the position and duration of resting periods. The diameters of the circumferences in the diagrams are proportional to the duration.

126.91 ± 22.60 min in *C. capitata*. The post-extrication behavior of both flies exarate adults was analyzed and compared. Figure 2 A,B show representative examples of the respective pathways followed in a restricted circular arena. During this post-extrication stage *D. melanogaster* shows a walking behavior frequently interrupted by numerous stationary periods of resting (Figure 2 A,C), whereas medflies displayed first a rapid exploratory behavior (on the average 7.86 ± 3.75 min) followed by a single long period of resting (119.04 ± 25.24 min) until the final phenotype of the imago is attained (Figure 2 B,D). The diameters of the circumferences in the diagrams (Figure 2 C,D) are proportional to the duration of the resting time in that position. The length of the path for the examples in Figure 2 was 12.65 cm for *D. melanogaster* and 48.10 cm for *C. capitata*. The average length of the pathways was 11.02 ± 1.91 cm for *D. melanogaster* and 45.40 ± 13.47 cm for *C. capitata*. Total time for *D. melanogaster* periods of walking represented 6.1% of the time of the whole phase and for *C. capitata* the single walking period represented 11.31% of the total BMP (Figure 1). Significantly, when the extrication times were added to the mobility times, total activity time represented 12.12% of the *Drosophila* BMP, whereas the equivalent time for *Ceratitis* represented 8.15% (Figure 1). Comparing these observations with the previously reported equivalent post-ecdysial behavior in other flies like the sarcophagids *Sarcophaga crassipalpis*, *S. bullata*, and *S. argirostoma* (Žďárek and Denlinger, 1986, 1987), all seem to follow a similar pattern to that of *C. capitata*, very different from that of *D. melanogaster* and (probably) other drosophilids. The mobility parameters in our experimental conditions of reduced movement might be proportional to the remainder of the energy resources available for metamorphosis, mainly haemolymph trehalose, muscle glycogen and lipids (Bochicchio, 2012; Nestel *et al.*, 2003) and in this case might be very different in wild conditions. Although behavior heterogeneity among individuals of each species is significant, the post-ecdysial exarate adult behavioral pattern indicates that in both flies around 90% of resting time is required during this period (Figure 1). This seems to indicate that for full completion of exarate adult body features, a similar proportion of resting time is required in both flies. In turn, this suggests that in the wild, a bottleneck for the behavior of cyclorrhaphans during the non-eating BMP might be the availability of energetic reserves to be spent during that phase of the life cycle. This kind of data is also important for the male-sterile programs for pest flies control.

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The effect of pyrogallol on the resistance to starvation in *Drosophila bipectinata.*

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Organisms often face stressful environmental conditions in nature, defined as environmental factors that reduce fitness (Koehn and Bayen 1989). Common environmental stressors, desiccation

and starvation, are the most important sources of natural selection. Development of behavioral and physiological mechanisms by organisms to deal with periods of starvation and desiccation allow them to alleviate the consequences of environmental stress (Hoffmann and Parsons, 1993; Randall *et al*., 1997). Studies carried out on insect groups, such as Lepidoptera, Orthoptera, and Coleoptera, showed that females mate with multiple males during periods of starvation and desiccation, perhaps because males transfer nuptial gifts containing large amounts of water and nutrients that may improve female stress resistance (Boucher and Huignard, 1987; Butlin *et al*., 1987; Ivy *et al*., 1999; Edvardsson, 2006). Also, studies in fruit flies of the genus *Drosophila* inhabiting desertic areas have shown that mated females are more resistant to desiccation than unmated females (Knowles *et al*., 2004, 2005). These physiological responses suggests that male-female may affect ecologically relevant traits and indicates that, in a natural scenario, mating may not be harmful as previously thought (Chapman *et al*., 1995; Wolfner, 1997; Lung *et al*., 2002; Chapman and Davies, 2004).

Apart from the physiological and genetic factors that influence the starvation resistance, the quantity and quality of food also plays an important role in the flies' ability to survive starvation. Studies conducted on the quality of nourishment have shown that a protein enriched diet increases the resistance to starvation more than the diet enriched with carbohydrates (Djawdan *et al*., 1998). Antioxidants, which are a constituent of the flies natural diet in the wild, have, however, so far not been tested to check their effect on resistance to starvation in flies. For this reason, here we carry out the experiments in order to determine if any such relationship exists. Most starvation studies have been focused on *D. melanogaster* (Service *et al*., 1985; Rose *et al*., 1992; Rion and Kawecki, 2007) and hence we use the species *D. bipectinata*, which is a member of the *bipectinata* species complex of the *ananassae* subgroup of the *melanogaster* species group (Bock and Wheeler, 1972), and is of common occurrence in India (Gupta and Panigrahy, 1990).

Materials and Methods

Establishment of stock:

The experimental stock of *Drosophila bipectinata*, was established from progenies of 50 iso-female lines collected from Chamundi Hills at Mysore, Karnataka, using mixed fruit bait. These flies were cultured in a standard wheat cream agar medium and maintained at a constant temperature of 22 ± 1ºC with a relative humidity of 70%. In each generation, the emerged flies were mixed together and redistributed to ten new culture bottles each with 20 flies (10 males + 10 females). This procedure was continued for 3 generations to acclimatize the flies to laboratory conditions. At the fourth generation eggs were collected using Delcour's procedure (1969) and 100 eggs were seeded in new culture bottles. When adults started emerging, virgin females and males were isolated within 3 hours of their eclosion. These flies were aged for 5-6 days to be used in the present experiments.

Preparation of antioxidant media at different concentrations:

The antioxidant pyrogallol was dissolved in water at a concentration of 1000 ppm (*i.e*., 1 g in 100 ml of distilled water), which was maintained as stock solution from which different amounts were taken and mixed in 30 ml of the standard wheat cream agar media in concentrations of 1 ppm, 10 ppm, 15 ppm, 20 ppm, and 25 ppm, respectively, to check the LD_{50} concentration of the compound (that is, the concentration at which 50% of the progeny dies). The LD_{50} was found to be 10 ppm. The flies raised in three sublethal doses of 1 ppm, 3 ppm, and 5 ppm were used for the present experiments.

Effect of pyrogallol on SOD enzyme levels:

Enzyme preparation for Superoxide Dismutase analysis: The flies cultured on different sub-lethal concentrations of 1 ppm, 3 ppm, and 5 ppm of pyrogallol were subjected to assay the effect of pyrogallol on the stress enzyme Superoxide Dismutase (SOD). To assay this, five adult (5 males and 5 females) flies from each concentration were taken along with the untreated (control) in different Eppendorff tubes. 200 microlitres of 50 mM Phosphate buffer of pH 7 for Catalase assay, 200 microlitres of 250 mM Phosphate buffer of pH 7.8 for SOD Assay were added and crushed using tissue homogenizer in ice cold conditions and centrifuged at 8000 rpm for 20 min in a cooling Microfuge. After this, the supernatants were poured into other Eppendorff tubes. 0.1 ml/100 µl of this enzyme extract was used for the assay.

Measurement of Superoxide Dismutase enzyme activity: SOD enzyme was assayed using a modified procedure originally described by Beauchamp and Fridovich (1971). The enzyme extract was added to a mixture of solutions containing 250 mM phosphate buffer (0.8 ml), 100 mM Methionine (1 ml), 100 mM Riboflavin (0.5 ml), 5 mM EDTA (0.1 ML), 750 mM NBT (0.1 ML), the volume of which was made up to 3 ml with distilled water. A mixture without the enzyme and NBT was prepared which served as blank and a control with NBT and no enzyme was prepared. These mixtures are exposed to sunlight or a bright light of about 400 watts; during this reaction NBT gets reduced to formazone, and was read at 560 nm. The total protein content of enzyme was estimated by Lowry's method, and the activity was expressed in units /mg of protein.

Effect of pyrogallol on starvation resistance:

To study the effect of pyrogallol on starvation resistance, 5-6 day old adults, mated and unmated males and females obtained from untreated and pyrogallol treated (1 ppm, 3 ppm, 5 ppm) media. Ten mated/unmated, males/ females from each medium (untreated/pyrogallol treated) were transferred into vials containing non-nutritive agar (12.4 g agar and 2.4 g *p*-hydroxybenzoic acid in 23 ml ethanol per litre). The number of days survived by each mated/unmated, male/ female was recorded by observing the vials every 12-24 hours for mortality, and the assay was continued until all the flies died. A total of four replicates (40 males and 40 females) were run separately for both mated and unmated flies. The results obtained were subjected to one way ANOVA followed by the Tukey's *post hoc* test.

Effect of pyrogallol on the accessory gland proteins:

Unmated males from the untreated and pyrogallol treated groups were obtained to study the effect of pyrogallol on the quantity of Accessory gland proteins.

Sample preparation of unmated males:

Accessory glands of unmated (etherized) males (untreated and treated males) were individually and separately dissected out using insect saline with the help of entomological needles. These glands were fixed in 95% ethanol. Fixed glands were taken on a clean slide and membrane was removed with the help of fine needles and a stereomicroscope. The result secretion alone was washed in a mixture of methanol and chloroform (1:1) and dried at 37°C in an incbator for about 15 minutes. About 100 µl of sample buffer of about 100 µl (0.623 M Tris HCl pH 6.8, 1% Sodium Dodecyl Sulphate, 1% β-mercaptoethanol, 10% glycerol) was added to each sample to dissolve the glands and secretions. 10 pairs of accessory glands from each group were separately collected for quantitative estimation of total accessory gland proteins using Lowry's method (1951).

Sample preparation of mated males:

To obtain mated males, a 5-6 day old virgin female (untreated and treated) and an unmated untreated and treated male were individually aspirated into Elens-Wattiaux mating chamber (1964) and observed for 1 hour (untreated male with untreated female and treated male with treated female of the same concentration). Pairs that remain unmated within 1 hour were discarded. If mating occurs, mating latency (time between introduction of male and female into a mating chamber until initiation of copulation) and the Copulation Duration (time between initiations to termination of copulation of each pair) was recorded. Soon after copulation (within 5 minutes), mated males were etherized and sacrificed to obtain Acps and fixed in 95% ethanol. Ten pairs of accessory glands from each group were collected separately for quantitative estimation of Acps using Lowry's method (1951).

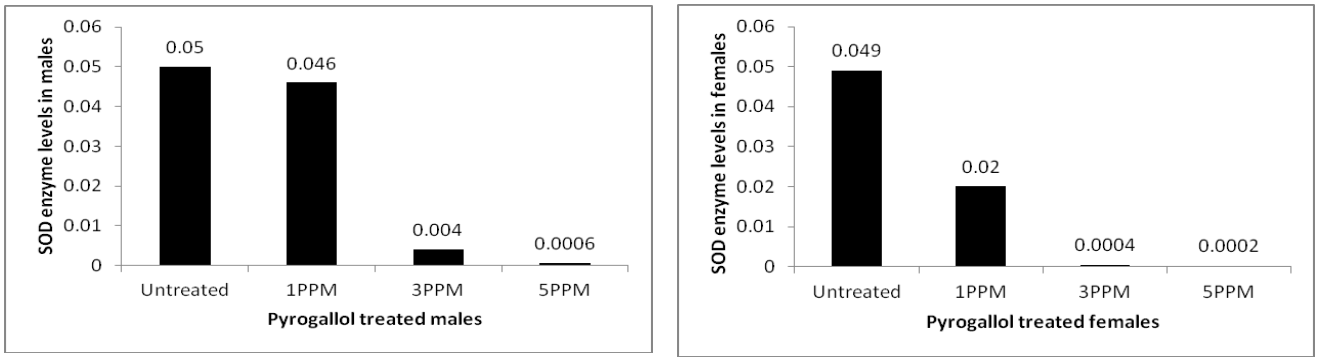

Figure 1a, Left: Effects of pyrogallol on the SOD levels in males of *D. bipectinata.* SOD enzyme levels in µl/fly in untreated and pyrogallol treated flies. Figure 1b, Right: Effects of pyrogallol on the SOD levels in females of *D. bipectinata.*

Results

Effect of pyrogallol on Superoxide Dismutase enzyme activity:

Figure 1a shows the SOD enzyme levels in the pyrogallol treated males of *D. bipectinata.* It was observed that the SOD enzyme levels were found to be higher in the untreated males when compared to the treated males. Among the pyrogallol treated males the levels of SOD were found to have decreased with an increase in the concentration of pyrogallol. The lowest level of the SOD enzyme was found at the 5 ppm concentration and highest was at the 1 ppm concentration. This suggests that in males of *D. bipectinata* SOD activity decreased with increasing pyrogallol concentration.

SOD enzyme levels of untreated and pyrogallol treated females of *D. bipectinata* is depicted in Figure 1b. SOD levels were found to be higher in the untreated when compared to the pyrogallol treated females. Among the pyrogallol treated females SOD activity was the least at 5 ppm and highest at 1 ppm. Futher SOD activity decreased with increase in the concentration of pyrogallol. Between the sexes the SOD enzyme levels in the female flies was lower than that of the males, both in the untreated and the pyrogallol treated groups, suggesting that the females had a lower level of SOD enzyme activity than the males.

Effects of pyrogallol on starvation resistance:

The Kaplan-Meier survival curve of unmated and mated female flies grown in treated and control groups when subjected to starvation are shown in Figures 2a and 2b. The variation in

survival of the flies could be clearly observed between the mated and unmated flies, with the mated flies surviving significantly longer than the mated flies. Also a variation between the control and treated groups could be observed with the treated surviving longer than the control. In the treated groups of 3 ppm and 5 ppm females survived longer than that of the 1 ppm group. Between the sexes, both mated and unmated females survive longer than the males, and the females of 3 ppm and 5 ppm survive longer than that of the control and 1 ppm.

The Kaplan-Meier survival curve of unmated and mated male flies grown in control and treated groups when subjected to starvation are depicted in Figures 2c and 2d. A variation was observed in the survival of the mated and unmated flies where, the survival of the mated flies was higher than that of the unmated flies. Between the control and treated groups, the survival of the flies increased with an increase in the concentration of the antioxidant. Within the treated group, among the unmated males there was an increase in the survival with an increase in the concentration of pyrogallol, whereas, among the mated males the 3 ppm flies survived the longest followed by the 5 ppm and the 1 ppm.

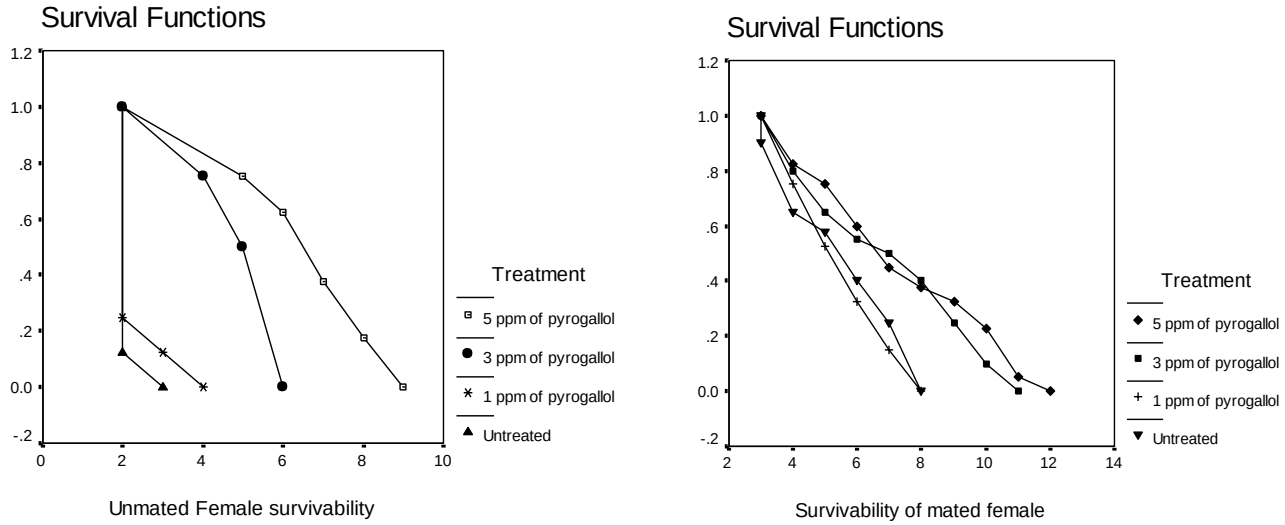

Figure 2. Survival curve of of *D. bipectinata.* (a, Left) unmated females; (b, Right) mated females.

Effect of pyrogallol on the accessory gland proteins:

The mean values of the amount of accessory gland proteins present in unmated and mated males grown in untreated and pyrogallol treated groups, along with the amount transferred, are depicted in Table 3. A slight variation was observed in the concentration of Acps in the untreated and the pyrogallol treated groups, in the unmated males, lower concentrations were observed in the untreated group than the treated. The highest concentration was seen in 5 ppm followed by 1 ppm and the least in 3 ppm concentration, while in the mated males, the least concentration was in the 3 ppm group followed by the 5 ppm and 1 ppm, however the untreated group had a higher concentration of Acps than the 3 ppm group. The amount of Acp transferred between the untreated and the 3 ppm concentration of the pyrogallol treated groups was the same. Among the treated groups there was a difference seen between the three groups, with the highest amount of transfer seen in the 5 ppm concentration and the least seen in 1 ppm concentration of the pyrogallol treated groups.

The one way ANOVA followed by the Tukey's *post hoc* test, when carried out on the data obtained for the unmated and mated males, showed that there was a significant variation between the

untreated and 3 ppm pyrogallol treated group, while within the treated group, significant difference between 1 ppm, 5 ppm, and 3 ppm was seen. However, there was no significant difference seen between the untreated and the 1 ppm and 5 ppm groups. In the case of the amount of Acp transfer, a significant difference was seen between the untreated and the 5 ppm concentration of the pyrogallol treated groups; however, there was no significant difference between the other concentrations with the untreated group. Within the pyrogallol treated group significant difference was seen between the 5 ppm group and the 1 ppm and 3 ppm groups; however, there was no significant difference seen between the 1 ppm and 3 ppm groups by the Tukey's *post hoc* test.

Table 1a. Mean and median of survival time in unmated females of *D. bipectinata.*

Treatment Unmated female)	Mean				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
			lower bound	upper bound			lower bound	upper bound
Untreated	2.13	0.05	2.02	2.23	2.00			
1 ppm	2.38	0.11	2.16	2.59	2.00			
3 ppm	5.25	0.13	4.99	5.51	5.00	0.21	4.59	5.41
5 ppm	6.93	0.23	6.48	7.37	7.00	0.31	6.40	7.60
Overall	4.16	0.18	3.83	4.51	4.00	0.50	3.02	4.98

- Overall comparisons

	Chi Square	df	Sig.
Log Rank (Mantel-Cox)	192.86	3	0.00*
Breslow (Generalized Wilcoxon)	159.07	3	0.00*
Tarone-Ware	174.80	3	0.00*

* Significant at $p < 0.01$

Table 1b. Mean and median of survival time in mated females of *D. bipectinata*

Treatment (mated females)	Mean				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
			lower bound	upper bound			lower bound	upper bound
Untreated	5.78	0.28	5.23	6.33	6.00	0.44	5.13	6.87
1 ppm	5.75	0.22	5.31	6.19	6.00	0.35	5.32	6.68
3 ppm	7.25	0.39	6.46	8.02	7.00	1.05	4.93	9.06
5 ppm	7.60	0.42	6.76	8.42	7.00	0.52	5.97	8.03
Overall	6.59	0.18	6.24	6.95	6.00	0.27	5.46	6.54

- Overall comparisons

	Chi Square	df	Sig.
Log Rank (Mantel-Cox)	28.48	3	0.00*
Breslow (Generalized Wilcoxon)	16.46	3	0.001*
Tarone-Ware	21.64	3	0.00*

* Significant at $p < 0.01$

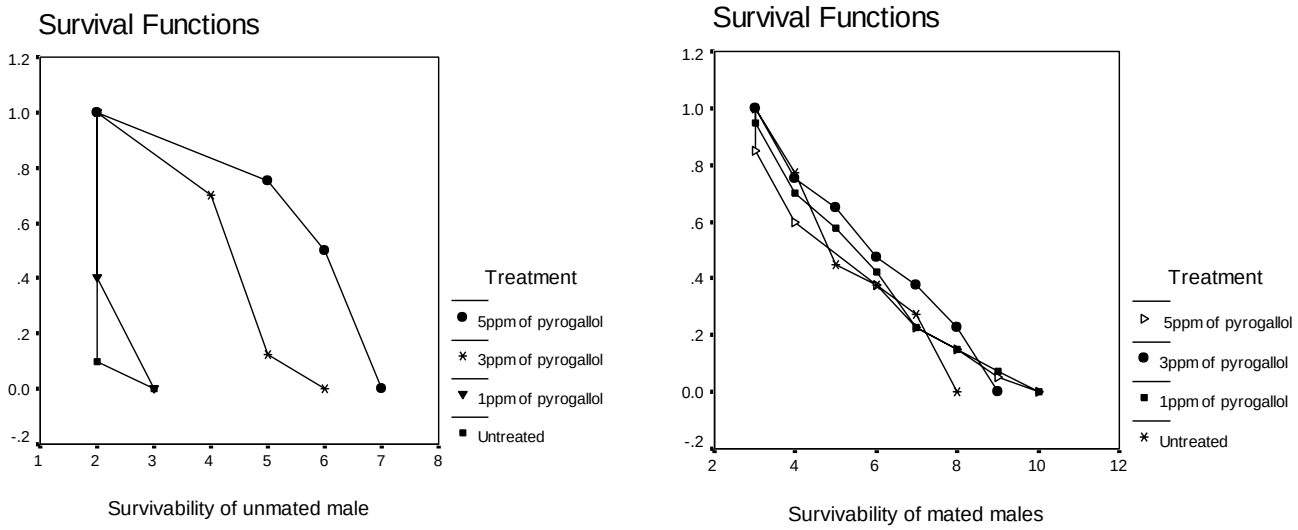

Figure 2. Survival curve of of *D. bipectinata.* (c, Left) unmated males; (d, Right) mated males.

Discussion

The Table 2a and 2b shows that females of *D. bipectinata* had a significantly greater resistance to starvation, when compared to males. This supports the work of Rion and Kawecki (2007), who also found that the females in a species have a greater resistance to starvation than males. This variation can be explained by the energy budget, the major ways of which can be elucidated to increase starvation resistance: (1) sequestering greater energy reserves; (2) reducing the rate at which the reserves are used under starvation conditions; (3) lowering the minimal level of body energy content which allows survival (Rion and Kawecki, 2007). In addition to these, greater resistance to starvation by females could also be due to higher lipid contents than males (Hoffman and Harshman, 1999), because of which females have a higher body weight than males (Chippindale *et al*., 1998). In the present study also, it was observed, that females had a greater body weight than the males of both the untreated and treated groups; therefore, an observed variation in the body weight of males and females flies could be attributed to greater lipid content in females than males of *D. bipectinata*. The SOD levels in the untreated male and female flies (shown in Figures 1a and b) were more or less the same, suggesting that the greater resistance to starvation in females of *D. bipectinata* could be due to the difference in the lipid contents in the body.

Apart from the reserve materials available in the body, other sources of energy comes from the quantity and quality of nutrients available, which are also known to play an important role in overcoming stressful conditions (Djawdan *et al*., 1998). Flies grown on protein rich diet show a significantly greater resistance to starvation in *Drosophila* (Djawdan *et al*., 1998). The tables show that pyrogallol treated males and females had a significantly greater starvation resistance when compared to untreated males and females suggesting the influence of pyrogallol on starvation resistance in *D. bipectinata*. Our study supports the role of antioxidants in resisting environmental stress, thus confirming the results of earlier studies on the role of nutrition in starvation resistance. In the present study the SOD enzyme activity levels were also in the untreated and pyrogallol treated flies, in order to understand the role of the antioxidant pyrogallol on resistance to starvation. It was noticed that the treated flies had lower levels of enzyme activity when compared to the untreated flies, which supports the "free radical theory", as the SOD (Super Oxide Dismutase) enzyme, which is an antioxidant enzyme is used as a defence against free oxygen radicals generated in the cell due to

oxidative stress. Antioxidants are known to reduce the free reactive oxygen species (ROS), which are produced when the cells are subjected to stress or oxidative stress, in turn decreasing the SOD enzyme levels and thus, known to increase the longevity of the flies under starvation resistance. Furthermore this study on *D. bipectinata* showed that an increase in the concentration of pyrogallol there is an increase in the resistance to starvation. Hence, with an increased intake of the antioxidant the flies show higher rates of survival at higher concentrations, which leads us to propose a hypothesis that greater the quantity of antioxidants in the food the higher the resistance to starvation.

Table 2a. Mean and median of survival time in unmated males of *D. bipectinata.*

Treatment (unmated male)	Mean				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
			lower bound	upper bound			lower bound	upper bound
Untreated	2.1	0.05	2.01	2.19	2.00			
1 ppm	2.4	0.08	2.45	2.55	2.00			
3 ppm	4.83	0.1	4.63	5.02	5.00	0.91	4.82	5.18
5 ppm	6.25	0.13	5.99	6.51	6.00	0.21	5.59	6.41
Overall	3.89	0.15	3.61	4.17	3.00	0.39	2.23	3.76

➢ Overall comparisons

	Chi Square	df	Sig.
Log Rank (Mantel-Cox)	200.39	3	0.00 *
Breslow (Generalized Wilcoxon)	164.48	3	0.00 *
Tarone-Ware	181.49	3	0.00 *

* Significant at $p < 0.01$

Table 2b. Mean and median of survival time in mated males of *D.bipectinata.*

Treatment (mated male)	Mean				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
			lower bound	upper bound			lower bound	upper bound
Untreated	5.86	0.25	5.39	6.36	5.00	0.24	4.53	5.47
1 ppm	6.1	0.32	5.48	6.72	6.00	0.52	4.98	7.02
3 ppm	6.48	0.3	5.88	7.07	6.00	0.57	4.87	7.13
5 ppm	5.85	0.34	5.19	6.51	6.00	0.68	4.67	7.33
Overall	6.08	0.15	5.78	6.37	6.00	0.25	5.51	6.49

➢ Overall comparisons

	Chi Square	df	Sig.
Log Rank (Mantel-Cox)	3.07	3	0.38 ^{NS}
Breslow (Generalized Wilcoxon)	2.69	3	0.44 ^{NS}
Tarone-Ware	2.79	3	0.43 ^{NS}

NS- Non- significant at $p < 0.01$ and $p < 0.05$

Table 3. Effects of pyrogallol on the quantity of ACP males of *D. bipectinata.*

Concentration of pyrogallol	Quantity of Acp		
	Unmated male flies	Mated male flies	Transferred (unmated-mated)
Untreated (control)	10.33	7.2	3.13
1 ppm	10.4	7.33	3.07
3 ppm	10.13	7	3.13
5 ppm	10.47	7.2	3.27
F value	8.42*	5.71**	7.55**

* Significant at $p < 0.01$ **Significant at $p < 0.05$

Since the same diet was used, except pyrogallol, the antioxidant at different concentrations added to the culture of *D. bipectinata* suggesting that greater resistance to starvation of treated flies could be attributed to pyrogallol. Our results support the earlier studies on the role of antioxidants in stress management (Tapiwanashe *et al*., 2006). Hence, the increased resistance with an increase in the concentration levels of the antioxidants (known to decrease the oxidative stress) can be inferred as an important factor influencing the resistance to starvation in flies of the treated groups. Stress related studies have been carried out to a large extent in order to study adaptations and counter interactions that may be an effect of changing climatic conditions (Hoffmann and Harshman, 1999) of which starvation resistance is a commonly measured trait (Huey *et al*., 2004), populations of *Drosophila* appear to harbour sufficient genetic variation for starvation resistance, as shown by the quick and high amount of responses to laboratory selection experiments for this trait (Hoffmann and Harshman, 1999; Archer *et al*., 2003; Hoffmann *et al*., 2005).

Studies have shown that mating is not harmful to the female, instead a useful process which could attribute to greater starvation resistance in females (Chapman *et al*., 1995; Wolfner, 1997; Lung *et al*., 2002; Chapman and Davies, 2004). In the present study it was noticed that resistance to starvation was significantly greater in mated females, compared to unmated females in *D. bipectinata* suggesting that females obtained fitness benefits from mating. Our study also supports the work of Brandy *et al.* (2007), where they found that mated females survive starvation longer than unmated ones. This increased resistance to starvation could be attributed to the higher fat reserves stored as triglycerides in mated females to ensure reproductive success, than the leaner unmated ones, with which our results agree. The longer survival in mated females could also be explained by the accessory gland proteins transferred to the female during mating, which can act as a source of protein to survive for longer periods of starvation. Also a decrease in the reproductive stress faced by mated flies can be attributed to the increase in their resistance to starvation in both males and females, hence in this experiment mated females and males from all groups were used to make a comparison between the control and the treated groups in which though there is a reduced reproductive stress in both groups the antioxidants further reduce these stress levels in the treated flies in turn increasing their survival. Uniformity was maintained by choosing both young unmated and mated females and males to rule out the effects of age on the survival of the flies in both groups.

Our results show that the amounts of Acps in the control and the treated groups are slightly similar in both the mated and the unmated flies, with the amount of transfer a little higher in the treated group, showing that the antioxidant has no significant effect on the Acp contents in the fly, as antioxidants are only involved in the oxidative stress reduction. Studies so far have, however, not been focused on studying the effects of antioxidants on proteins or the Acps in the fly. On the other hand new studies now focus on the antioxidant properties of proteins such as albumin (Rafael *et al*., 2010), thus leading us to infer that antioxidants, which are known to lower the oxidative stress and increase the longevity of the fly, also infer an additive property of starvation resistance in flies and increase the ability to survive starvation, when the antioxidant is included in the diet.

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Quantitative assessment of ommatidial distortion in *Drosophila melanogaster.*

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Assessing the structural characteristics of an organism's physical appearance can be an important aspect of evaluating the effect of protein-protein interactions. Many biomedical

applications require the extraction of structural features from images, in order to infer information about the underlying biological processes causing a phenotype. This is typically arduous and subject to human bias. A general methodology for extracting structural information from images using edge detection and boundary-walking algorithms is presented. This yields quantitative descriptors and comparators of disorder, applied to the problem of unambiguous identification of phenotypic alterations induced by *tau*-dependent neurodegeneration in the adult eye of *Drosophila melanogaster*. This method obviates traditional subjective assessment techniques, enabling quantitative tests of the influence of genetic interactions on *Drosophila* phenotypes. Using this framework, we quantify from images of *Drosophila* eyes, a coefficient of distortion for a number of genotypes, and demonstrate that subtle changes in phenotype are detectable, allowing the direct numerical comparison between the extent of distortion.

Introduction

A major challenge in neurodegenerative disease research is obtaining information regarding interactions within cellular signaling networks. Model organisms such as *Drosophila melanogaster* can be used to study these networks, either through the insertion of relevant human genes, or their homologues, into the model organism's genome (Bilen and Bonini, 2005) or the mutation of endogenous genes (St. Johnston, 2002). These genetic alterations can result in changes in the morphology of structures within the organism, which are then used as convenient and experimentally tractable surrogates for manipulations in mammalian models. However, when these morphological changes are captured in images, difficulties arise in identifying and isolating the structural features present in order to quantitatively assess their deviation from the norm.

The *Drosophila* eye is frequently used as a model in developmental and neurodegenerative disease research due to its very regular morphology (Lu and Vogel, 2009). The fly's compound eye is comprised of approximately 700 to 800 individual ommatidia, displaying ordered hexagonal packing, and each containing a cluster of neurons (Moses, 2002). Human Tau protein can be expressed in *Drosophila* in order to study its interaction with other candidate proteins implicated in tauopathies such as Alzheimer's disease. Expression of human *tau* is toxic to developing eye cells, resulting in distortion of the ommatidial lattice (*e.g*., Iijima-Ando and Iijima, 2010) through fusion of ommatidia, loss of regularity of the hexagonal lattice, and a decrease in ommatidia count and eye size (Chatterjee *et al*., 2009).

Currently, *Drosophila* biologists often apply qualitative judgment to describe the extent to which disorder is visible in the eye, with terms such as “better” or “slightly rougher” qualifying any differences between samples; even unofficial grading systems, if present, are purely subjective (Muraro and Moffat, 2006). Recent work has tried to introduce a more quantitative version of visual inspection using rating scales (Pandey *et al*., 2007), but these are also subjective in the weight that is given to each rating. Others have focused on the packing expected in the *Drosophila* pupal eye ommatidia, measuring defects in the expected hexagonal lattice (Johnson and Cagan, 2009). This methodology, however, retains subjective weighting by failing to account for the severity of observed defects and requires the data to be hand-processed, introducing possible human error. Yet another approach focused on calculating the volume occupied by the eye, a method which may overlook the small-scale detail present in the ommatidia (Ambegaokar and Jackson, 2010).

A framework for the extraction of information from biomedical images and for direct comparison between physical attributes is presented, specifically applied to the measurement of the distortion present in the *Drosophila* eye phenotype as a result of Alzheimer's disease-related gene expression, in this case, human *0N4R tau* and *Drosophila shaggy*, a *GSK3-β* homologue. The

methodology is demonstrated to quantify and statistically test the effect of gene interactions within the *tau* regulatory network. This framework is adaptable to other areas of the fly, such as the wing, and indeed other organisms, as in the phenotypic changes seen in Parkinson's disease research in *Caenorhabditis elegans* (Buckingham and Sattelle, 2008) or in cancer screening in human blood samples (Zhang, Wang, and Qi, 2006). The implementation of the algorithm is available freely as source code online, as QED (http://github.com/QCaudron/QED).

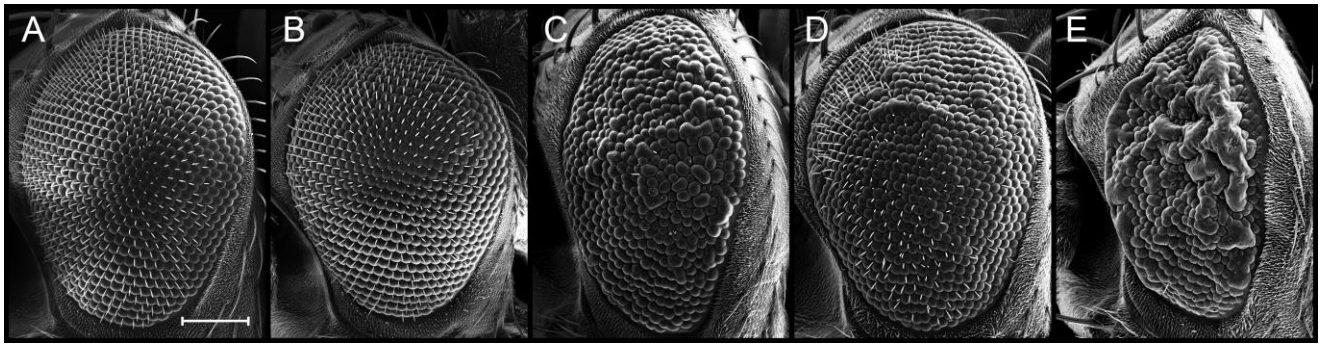

Figure 1. Example images representing the five genotypes compared in this work. Image A shows the wild type CS eye, B shows the GMR::GAL4 control, C shows GMR>*tau*, D shows GMR>*sgg*, and E shows the strain expressing both *tau* and *sgg* transgenes, GMR>tau>sgg. The scale bar, measuring 100 μm, applies to all images.

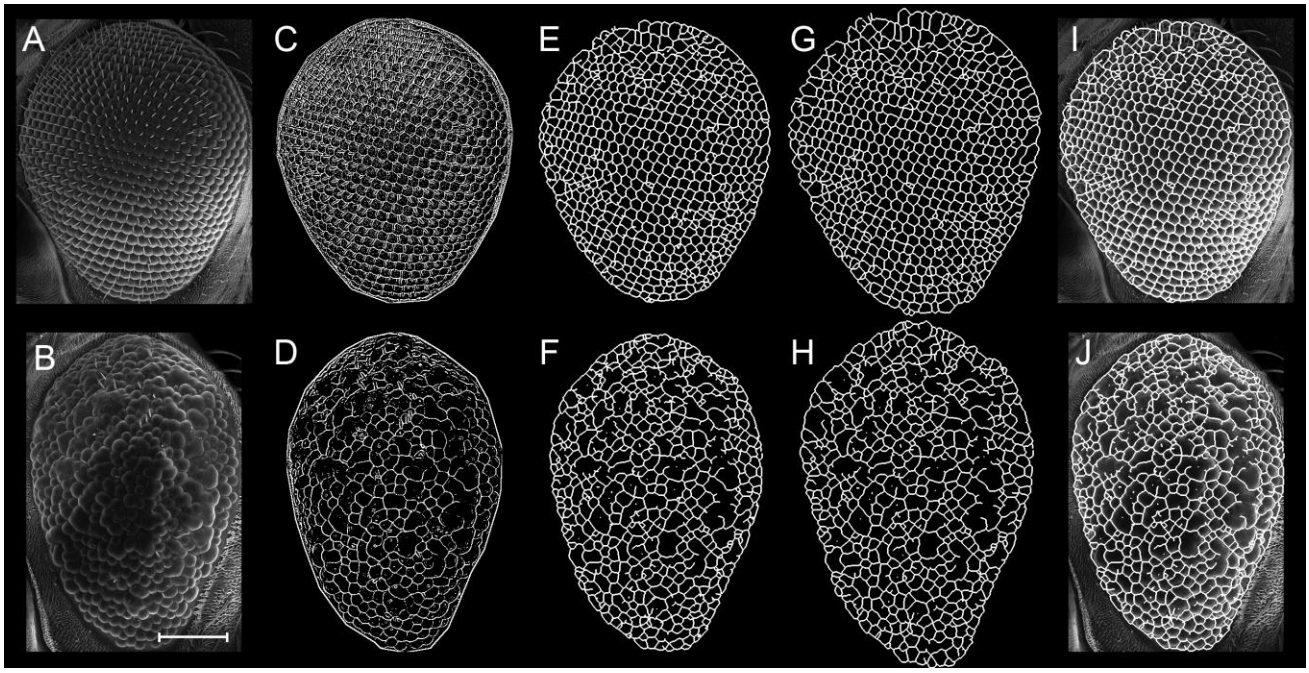

Figure 2. The progression of the edge detection framework on a GMR control eye (top row) and on a GMR>tau eye (bottom row). A-B show the original images; C-D have been masked around the eye and had the *PLUS* edge detection method applied; E-F show the edges after Sobel edge detection and morphological dilation and thinning; G-H show the edgeset after its perspective transform; I-J detail the final edgeset superimposed onto the original image, for comparison. The scale bar applies to all images, and measures 100 μm.

Results

Example images of the five genotypes used can be seen in Figure 1.

Figure 2 shows the QED methodology applied to images of a GMR control eye (2A) and of a *GMR>tau* eye (1B). Results of the first pass of edge detection algorithms are presented in Figure 2C-D, and after final edge detection and edgeset reconstruction in 2E-F. Figure 2G-H show the edgeset after its perspective transform. Here the outer regions of the eye were enlarged to compensate for the curvature of the eye. Figure 2I-J show the edgeset before its perspective transform, superimposed onto the image of the eye.

Figure 3 presents the cumulative distributions of distortion coefficients for the five genotypes, showing all distributions to be distinct. The number of samples, mean roundness coefficients $\bar{\Delta}_R$ and *p*-values for tests against the control genotype are shown in Table 1.

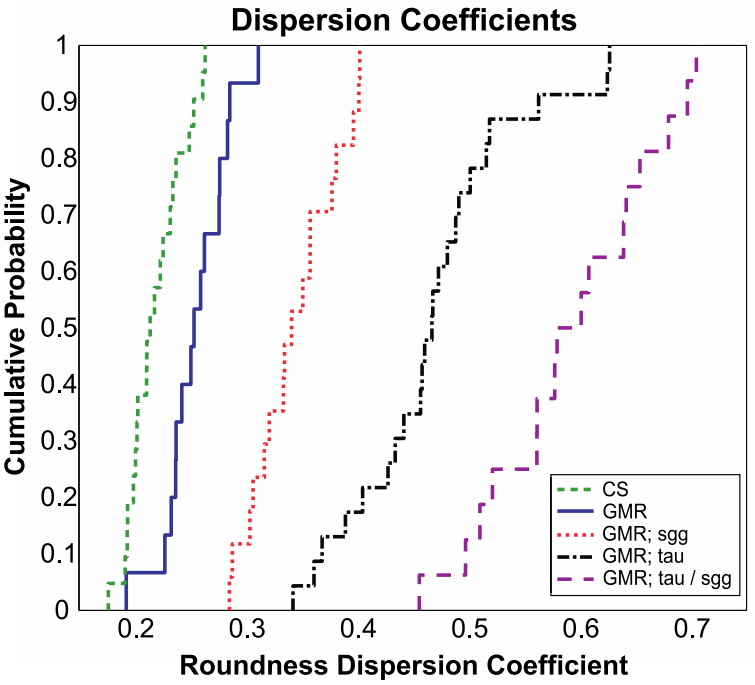

Figure 3. The cumulative distributions of roundness coefficients for all five genotypes. The wild type (CS) flies (green line) have the lowest average roundness coefficient, and hence the least variation in the shape of their ommatidia, followed by the GMR control genotype (blue line). The GMR>*tau*>*sgg* genotype (purple line) shows the most variation in roundness, worse than when tau is expressed alone (black line). The GMR>*sgg* genotype (red line) lies in the middle, showing clear signs of toxicity, but much less than GMR>*tau* or GMR>*tau*>*sgg* flies.

Table 1. Number of images for each genotype *N*; mean roundness distortion coefficient $\bar{\Delta}_R$; and *p* -values of the comparisons between the different genotypes.

Genotype	*N*	$\bar{\Delta}_R$	p_{GMR}	$p_{GMR;tau}$	$p_{GMR;sgg}$
CS	21	0.219	8.47×10^{-4}		
GMR :: GAL4	15	0.255			
GMR > *tau*	23	0.470	2.80×10^{-7}		
GMR > *sgg*	17	0.345	4.08×10^{-6}	9.71×10^{-7}	
GMR > *tau* > *sgg*	16	0.593	2.32×10^{-6}	6.03×10^{-5}	1.06×10^{-6}

Visually, it is clear that the GMR>*tau*>*sgg* eyes were the most distorted, showing very pronounced ommatidial fusion and large-scale warping of the surface of the eye along with complete loss of bristles. The very large mean distortion coefficient $\bar{\Delta}_R$ for this genotype reflects the distortion

present in this genotype. The extent of the distortion in GMR>*tau*>*sgg* was found to be significantly different ($p = 6.03 \times 10^{-5}$ or smaller) to the distortion of other genotypes.

Ommatidial fusion was evident on GMR>*tau* eyes, though on a much smaller scale, and the surface showed significantly less distortion than in GMR>*tau*>*sgg* eyes, as is evident by the mean distortion coefficient $\bar{\Delta}_R = 0.470$ being significantly smaller when compared with those of GMR>*tau*>*sgg* ($\bar{\Delta}_R = 0.593, p = 6.03 \times 10^{-5}$), but significantly larger than those of the control genotype, GMR::GAL4 ($\bar{\Delta}_R = 0.255, p = 2.32 \times 10^{-6}$).

GMR>*sgg* eyes were found to be less distorted than GMR>*tau* or GMR>*tau*>*sgg* eyes, with a mean distortion coefficient of $\bar{\Delta}_R = 0.345$. Visually, the most obvious defects are weak ommatidial fusion and a slight disordering of the hexagonal lattice. The distortion coefficients were noted to be significantly different to those of other genotypes.

GMR::GAL4 eyes could not consistently be distinguished from wild type (CS) eyes by visual inspection. Analysis, however, yields a significant difference ($p = 8.47 \times 10^{-4}$), identifying GMR::GAL4 eyes as being more distorted with a mean distortion coefficient of $\bar{\Delta}_R = 0.255$ compared with $\bar{\Delta}_R = 0.219$ for CS, implying that the GMR::GAL4 control eyes display a weak phenotypic distortion.

Discussion

These results confirm the ability of the QED methodology to statistically quantify the effects of genetic interactions, thereby eliminating subjectivity in the analysis of structural phenotypes. Moreover, it allows subtle changes in phenotype to be unambiguously determined, as evidenced by the comparison of CS and GMR::GAL4 flies. QED also provides quantitative descriptions of the relative phenotypic differences.

The modular approach allows specialized measures of distortion to be constructed to fit a specific function, allowing for a wide scope of operation. By tailoring the measures to the problem, a considerable amount of information can be extracted from images and normalized to allow for comparison by the statistical processes described.

This methodology can be expected to contribute significantly to fields where visual analysis is the current *modus operandi*, such as in neurodegenerative disease research as exemplified here, but also in biomedical scanning procedures such as screening for abnormally-shaped cells in cancer detection or identification of anomalous structural features or growths in tissue.

Materials and Methods

Drosophila melanogaster Stocks and Imaging

The eye-specific glass multiple repeat (GMR::GAL4) driver (Ellis, O'Neil, and Rubin, 1993; Brand and Perrimon, 1993) was used for expression of a constitutively-active *Drosophila shaggy* kinase (UAS-sgg S9A; Bourouis, 2002; Chatterjee *et al*., 2009), the 0N4R isoform of human *tau* (Wittmann *et al*., 2001), or both transgenes in combination. Flies expressing the GMR driver alone were used as controls. All genetic combinations were hemizygous for the transgenes. In addition, wild-type flies of the Canton-S strain were imaged.

Female flies were fixed overnight in 4% paraformaldehyde at 4°C. Flies were then dehydrated using an acetone series (10%, 30%, 50%, 70%, 90%), incubated on a rotator in 1 ml of solution for fifteen minutes at room temperature. Flies were then transferred to 100% acetone stored

over a molecular sieve for at least two days at 4°C. Flies were attached to stubs using double-sided tape, sputter-coated in gold for sixty seconds and imaged using a Zeiss Supra55 VP scanning electron microscope, by secondary electron detection. Each image was taken at 150× magnification at a resolution of 1024 × 768 pixels, with each pixel representing an area of 0.54 μm^2. Images were saved as single-channel, 8-bit, uncompressed TIFF files. Figure 1A shows an example SEM image of the GMR genotype, and Figure 1B shows one from the GMR; tau genotype.

UAS-*sgg* S9A and GMR::GAL4 were obtained from the Bloomington Stock Center (stock numbers 5255 and 1104, respectively) and UAS-0N4R *tau* flies were supplied by Prof. Mel Feany, of Harvard Medical School.

Edge Detection

Images were masked such that only the eye region remained, and all surrounding information had its pixel intensity values set to zero (black). The image was then cropped closely in both *x* and *y* directions to contain the eye region as tightly as possible.

Contrast was enhanced where required by contrast-limited adaptive histogram equalization (Zuiderveld, 1994). The image is partitioned into 8 × 8 tiles, each of which has their histograms adjusted to approximate a uniform distribution. The tiles are then recombined using bilinear interpolation to ensure artificially-induced boundaries are removed.

In gray scale images, the edges of physical objects appear as sharp changes in pixel intensity values. Because edges occur over a wide range of scales, we seek a filter that is band-limited in the frequency domain, thereby also reducing sensitivity to noise. In order to accurately localize the edges in space, our filter should also be applied to a smooth local average of pixels, and so should be band-limited in space. The distribution that minimizes the product of spatial and frequency variances, and hence optimizes these constraints, is the Gaussian distribution (Basu, 2002). Edges can then be sought in the maxima or minima of the derivative of the filtered image, or equivalently, in the zero-crossings of the second derivative. The direction in which the second-derivative is taken affects the measured magnitude of a zero-crossing and will be at a maximum in the direction normal to the edge. The second derivative of the Gaussian-filtered images is, therefore, taken as a combination of the second derivative in the gradient direction (SDGD) and the linear Laplacian, an orientation-independent second derivative operator. The resulting edge detector, *PLUS* (Verbeek and van Vliet, 1994), is more accurate by an order of magnitude than its components, the SDGD and Laplacian filters. A simplification in the implementation of *PLUS* saves on computation time by convolving the image with second derivatives of Gaussians instead of taking the second derivative of the Gaussian-convolved image; this is equivalent by convolution theory.

PLUS edge detection was complemented by convolution with a simple Sobel filter (Sobel and Feldman, 1973), the kernels of which approximate a first derivative of Gaussian independently in both *x* and *y* directions at each pixel. Due to the *PLUS* filtering, edges are now trivial to detect, and a threshold ε easily set, returning a 1-bit (binary) image where each pixel is either black (no edge) or white (edge). The resulting image contains disconnections in the edgeset, but less noise than in the *PLUS* image, and with edges localized on the boundaries of the thicker *PLUS* edges, leading to hollowed edge sections.

A morphological dilation algorithm (Gonzalez and Woods, 1992) was applied to the edgeset image, where all white pixels had their 3 × 3 environment made white. This algorithm was run twice sequentially, effectively reconnecting broken clusters and filling out the hollow edgesets, especially in the bristles. The opposite algorithm, a morphological thinning, was then applied, removing pixels on the outer regions of thick segments until only the central pixel remained. This shrinks the thick

edges to minimally-connected strokes, and strongly reduces the appearance of bristles, both in thickness and in length. The edgeset is now of single-pixel thickness and fully connected.

Scanning electron microscopy images are two-dimensional representations of three-dimensional objects. The curvature of the eye leads to distortion in the information captured by the image, especially visible in the outer regions of the eye, where ommatidia are, pixel for pixel, smaller and less round than those in the center.

It is assumed that the curvature is hemiellipsoidal and hence convex, and that the depth of curvature (*z*-axis) is determined by the (*x*, *y*) dimensions of the eye:

$$z = \left\| \sqrt{c^2 \left[1 - \left(\frac{x}{a}\right)^2 - \left(\frac{y}{a}\right)^2 \right]} \right\|$$

A further assumption is made in relation to the different characteristic curvatures of various eye phenotypes: an approximation was made whereby the curvature of any eye was taken to be similarly-parameterized as that of the wild-type eye. SEM images were taken at various orientations to calculate the average polar radius c of the wild-type eye, in order to compensate for the spatial distortions obtained in SEM images. The curvature was, therefore, parameterized empirically at $c = 0.4865$, and a hemiellipsoidal surface was created for each image with this same radius of curvature. The equatorial radii, a and b, were defined as half of the *x* and *y* dimension of the eye, respectively, leading to a perfect half-ellipsoid.

The image was projected orthographically onto the curved surface (Maynard, 2005). This projection maps each pixel from the planar image onto a corresponding pixel on the curved surface. The projection angle is normal to the surface of the plane at all points, or equivalently, the center of focus lies at infinity.

The curved image, lying on a hemiellipsoidal surface approximating the curvature of the fly eye, was projected back onto a plane azimuthally via a vertical perspective projection. The center of focus is now a finite distance away, and the projection maps each pixel from the hemiellipsoid onto a larger plane. This induces distortions in the form of exaggerated lengths and areas, but preserves angles. Distortions are greatest on the outer regions of the eye, reducing to no exaggeration at the center of the image. This simulates an "unfolding" of the curved surface onto a plane, and compensates for the outer regions of the original image being smaller than those in the center, due to the angle of capture. Extra pixels on the larger surface were given a luminosity value of zero (black), and edgesets were reconnected by applying one morphological dilation and thinning cycle as described above.

Information Extraction

Let O, a family of sets indexed $\{O_j\}_{j=1}^{N}$, describe all of the ommatidia in an image. We define b_i as the ith (background) pixel inside a particular ommatidium. The set $B_j = \{b_1, \ldots, b_{A_j}\}$ is the set of all pixels inside a specific ommatidium O_j, with A_j being the total number of pixels forming the area inside O_j. These pixels appear as black pixels in the binary edgeset image.

We further define f_i as a (foreground) edge pixel, contained in the set of all edge pixels $F_j = \{f_1, \ldots, f_{L_j}\}$ that define ommatidium O_j, where L_j is the number of pixels forming the length of the boundary around O_j.

Each ommatidium consists of the union of its background and foreground pixels, that is, $O_j = B_j \cup F_j$ where $B_j \cap F_j = \emptyset \;\; \forall O_j \in O$.

Boundaries F_j were found using a Moore-neighbor tracing algorithm modified by using Jacob's stopping criterion (Gonzalez, Woods, and Eddins, 2004). Individual ommatidia were isolated, allowing the calculation of their perimeters L_j and areas A_j. Any boundaries with perimeter $L_j > 1000$ pixels were removed, being either open boundaries or the outline of the compound eye. Likewise, any boundaries with perimeter $L_j < 50$ pixels were eliminated as erroneous measurements caused by noise or bisection of ommatidia by bristles.

Even with hexagonal packing, individual ommatidia in the wild-type eye should have regular, mostly-circular contours. A dimensionless measure of roundness of ommatidia was based on the idea that circular objects maximize their area-to-perimeter ratios. This measure is defined for each O_j as

$$R_j = 4\pi \frac{A_j}{L_j^2} \in (0,1),$$

where a higher value of R_j indicates a rounder ommatidial shape.

Distributions of roundness measures for each image were characterized by a percentile coefficient of distortion, defined as the difference between the ninety-fifth and the fifth percentile, normalized by their sum:

$$\Delta = \frac{p_{95} - p_5}{p_{95} + p_5}.$$

This measure of distortion attempts to capture the width of the distribution, and thereby the variability present in each eye, while excluding any outliers caused by errors in edge detection, hence a 90% interval taken. Wild-type eyes should have very well-defined distributions with small widths, whereas genotypes presenting a high degree of distortion, such as those with fused or warped ommatidia, will have wider distributions.

A distortion coefficient Δ_R is calculated for the distribution of roundness for each image. These distortion coefficients are collected into overarching distributions of genotypic variability. In this way, the variation in roundness can be compared between two genotypes by comparing distributions of distortion coefficients between the genotypes.

No parametric form is assumed for the distribution of distortion coefficients. Comparisons between genotypes are made using the Mann-Whitney test (Corder and Foreman, 2009), a non-parametric statistical test with the null hypothesis that the two independent samples being compared come from the same distribution. This significance test allows one to make a qualified judgment as to whether certain differences between genotype are present. In addition, due to the number of gene interactions that were tested, the *p*-value was assessed against a multiple comparison Bonferroni-corrected threshold (Salkind, 2007), where the number of tests was equal to the number of possible pairs of genotypes considered – in this case, four genotypes are tested, totalling seven pairwise comparisons.

The results of these comparisons provide an estimate of dissimilarity between two genotypes. If, instead, one genotype were to be compared with an ideal set of measurements of distances, angles, and roundness measures, this test would provide an assessment of how much the genotype of interest deviates from the norm. By choosing a control genotype, an approximation of the ideal distributions of distortion coefficients, incorporating measurement error, is made, which can be compared against the distortion coefficient distributions from a genotype of interest. It is then possible to obtain a quantitative measure of the distortion of a genotype, if it is statistically significant.

Acknowledgments: This work was supported by the EPSRC (grant EP/E501311/1). JA was supported by the HEFCE / EPSRC CRiSM grant. CL-A was an Alzheimer's Research Trust PhD scholar.

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Neuroprotective activity of Curcumin against paraquat induced oxidative stress markers in *Drosophila melanogaster*.

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Introduction

Neurodegenerative disorders are an important source of morbidity and suffering for mankind. The roles of free-radical-mediated oxidative injury in acute insults to the nervous system including stroke or trauma, as well as in chronic neurodegenerative disorders, are being increasingly recognized. It is known that oxygen is an essential molecule for survival of the majority of living

organisms. Oxidative stress is the harmful condition that occurs when there is an excess of free radicals and/or a decrease in antioxidant levels. The evidence to date for oxidative stress in Parkinson's disease, Schizophrenia, Alzheimer's disease, and other neurodegenerative diseases is strongly persuasive. Clinical studies have shown that a number of events associated with Alzheimer's are capable of stimulating production of free radicals and depletion of antioxidant levels. Tackling of the free radical involvement offers a novel therapeutic target in the study of neurodegenerative disorders. Strategies aimed at limiting free radical production oxidative stress and damage may slow the progression of neurodegenerative diseases (Singh *et al.,* 2004).

Curcumin, commonly called diferuloyl methane, is a hydrophobic polyphenol derived from the rhizome (turmeric) of the herb *Curcuma longa*. It exhibits antioxidant, anti-inflammatory, antimicrobial, and anticarcinogenic activities (Anand *et al.,* 2008). We have tested the antioxidant property of Curcumin in *Drosophila melanogaster.*

Materials and Methods

Culturing of flies

D. melanogaster (Oregon K) adults (8-10 days old) were obtained from *Drosophila* stock centre, Department of Studies in Zoology, University of Mysore, Manasagangotri, Mysore, Karnataka, India. Fly populations were built up and maintained at 22 ± 1°C and 70-80% relative humidity, fed on a standard wheat cream agar medium seeded with yeast.

Safety evaluation of compound

In the present set of experiments, the test flies were fed on a medium containing Curcumin at 100, 200, and 300 µg/ml concentrations of Curcumin dissolved in 0.5% DMSO.

The toxicity of 0.5% DMSO was checked in the medium by culturing the flies in media with and without 0.5% DMSO. Since there was no mortality in the flies reared on medium containing 0.5% DMSO, further studies were carried out to find out whether Curcumin is causing mortality in the experimental batches.

In each culture vial, 4 ml of food with or without Curcumin was added. Lethality due to compounds was monitored by counting dead flies every 24 h up to 7 days in the vials containing Curcumin, and data were expressed in terms of percentage mortality.

Preparation of compound for feeding the flies

Curcumin was dissolved in 0.5% dimethyl sulfoxide (DMSO) and was used as control. The compounds were introduced into the medium in a semisolid state, mixed well, and allowed to solidify. 50 adult flies were introduced into the vials containing media.

Whole body homogenate preparation

0.1M Sodium-phosphate buffer (pH 7.4) was used for preparing whole body homogenates. The flies (30 nos.) from control and tested groups were used for this purpose. After homogenizing, the samples were centrifuged at 2500 ×g for 12 min at 4°C. The supernatant was filtered through nylon mesh (pore size, 10 µm) and used for biochemical assays (Smith *et al.,* 1978).

Paraquat exposure and concentrations

In a preliminary study, flies were exposed to paraquat at concentrations of 20, 25, 30, and 35 mM for 96 h to determine lethality. However, to assess the neuroprotective effects of Curcumin, only

one concentration of Paraquat (25 mM) was employed. For these studies, paraquat exposed flies were supplemented with Curcumin (300 µg/mL) in the diet and were tested for the modulatory effect of Curcumin on paraquat induced lethality, locomotor dysfunctions, and oxidative impairments.

Paraquat resistance test

Three to five days old adult flies were fed with control food or food containing the compounds for a period of 7 days. Then 50 flies were fed with the test compound Curcumin and the control were starved for 6 h to make sure that no food remained in the digestive tract so that none of the compounds would alter the uptake of paraquat. Afterwards, the flies were transferred to vials containing only filter paper soaked with 25 mM paraquat in 5% sucrose solution (Hosamani *et al.*, 2010). This concentration of paraquat was selected, because it enables us to study the flies for a period of 24 h or more after treatment. Survival was determined at 24 h and at 48 h later. Surviving flies were used for preparing samples for biochemical assays. Each assay was repeated thrice (Anand *et al.*, 2008).

Biochemical investigations

Following the exposure to various treatments, the flies were mildly anesthetized using diethyl ether in a small airtight glass container for 1 min. Quantification of oxidative markers *viz*., MDA (Ravikumar and Muralidhara, 2009), GSH (Ernesto *et al.*, 2006) and activities of a few of the antioxidant enzymes were made using the whole body homogenates. Protein estimation was done using samples obtained after homogenization (Wolf, 1994).

Results and Discussion

Our aim was to elucidate antioxidant and neuroprotective efficacy of Curcumin *in vivo*. *Drosophila* is an excellent *in vivo* system for testing the therapeutic compounds due to its relatively short life span wherein the adult flies appear to show many of the manifestations of cellular senescence observed in mammals. Oxidative stress plays an important role in governing the life span of the fly. The results revealed that Curcumin has protective action against paraquat induced oxidative stress in *Drosophila*. There was a significant induction of oxidative stress among flies exposed to paraquat (25 mM), which was evidenced by the marked elevation in MDA and further a significant change in the activities of antioxidant enzymes such as CAT, SOD suggested an increased generation of ROS (Feany *et al.*, 2000).

Ameliorative effects of Curcumin on paraquat induced oxidative stress markers in whole body homogenates of adult flies fed with Curcumin supplemented diet for 7 days showed significant diminution in MDA levels. In Curcumin treated flies homogenate, the level of MDA was found to be 3.47 ± 0.25 nmol malondialdehyde/mg protein (Figure 1) as against the paraquat treated flies that showed 12.34 ± 0.51 nmol malondialdehyde/mg protein. Severe depletion in cellular GSH levels upon paraquat exposure in *Drosophila* adds further evidence that a state of oxidative stress exists *in vivo*, which may lead to mitochondrial damage, increase in free radical generation, and peroxidation of membrane lipids (Barclay *et al.*, 2000). A moderate increase in GSH level was evident among flies treated with Curcumin (40.325 ± 1.78 µg GSH/mg protein), although paraquat exposure caused a significant decrease in GSH level, on co-exposure with Curcumin; flies were able to restore the depleted GSH levels (Figure 2), thus clearly suggesting the ability of Curcumin to up-regulate levels of GSH.

Conclusion

Based on biochemical investigations, we have found that dietary feeding of Curcumin to *Drosophila* for a short duration has the propensity to attenuate paraquat induced oxidative stress owing to its antioxidative nature and its ability to modulate the activities of antioxidant defenses, such as reduced GSH and antioxidant defenses. Additional evidence, *viz*., lower incidence of paraquat induced mortality and higher resistance to paraquat among flies pretreated with Curcumin, clearly support such a mechanism. Further, its antioxidant property was clearly evident by its ability to significantly abrogate paraquat induced oxidative stress, by depleting the lipidperoxidation product malanoldialdehyde.

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***Drosophila polymorpha* life cycle.**

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Introduction

Many studies on the subject of the *Drosophila* life history have revealed that much of the observed interspecific variability can be explained by genetic interaction and ecological traits (Markow and O'Grady, 2006; Prasad and Joshi, 2003). The life cycle is one of the most important factors that determine the *Drosophila* life history.

There is considerable interspecific variation in each of the *Drosophila*'s life cycle stages, making this type of fly a quite versatile model for life history studies (Jennings, 2011). Thorough knowledge of each developmental stage of *Drosophila* could clarify some evolutionary questions, such as the mechanisms underlying morphological differentiation, and also the ecological results during the speciation process. Our study expands upon the *Drosophila polymorpha* life cycle, from egg to adult. Furthermore, a comparison is made with other species of *Drosophila*, an important factor that leads to a better understanding of evolution within the genus.

Increasing numbers of studies on sexual isolation of *Drosophila* have ensured that there are many inter-specific differences in the reproductive biology for this group that contribute to the speciation process (Coyne *et al*., 1994).

The elucidating life cycle of *Drosophila polymorpha,* from egg to adult, and its age of sexual maturity in particular, are important aspects to be explored, once it can be shown that they have valuable roles involving specific ecological traits. The investigation of these topics also incites good maintenance of the flies stock for its studies.

D. polymorpha belongs to the cardini group, and, as for most of Neotropical species, there are not many studies regarding their reproductive biology and life cycle. Therefore, so far, these topics remain poorly understood.

Material and Methods

Seven different samples of *D. polymorpha* were obtained from two conserved areas in the central and southern regions of Rainforest in the state of Santa Catarina, Brazil: the Parque Estadual da Serra do Tabuleiro (27°48’20"S; 48°33’50"O), and the Reserva Biológica Estadual do Aguaí (27º16'49.55"S; 49º8'31.7"O).

In order to guarantee that all flies were raised under identical conditions, the vials containing a potato flake medium (Bizzo *et al*., 2012) were maintained at 22ºC in a 12-hr light/12-hr dark photoperiodic cycle. With the purpose of facilitating observation of both eggs and larvae, the medium was colored with blue food dye. These conditions were maintained throughout the experiment.

The life cycle was tested using 15 females and 5 males, all of the test objects with a lifespan of one week. They were isolated in a vial containing medium for one hour with the aim of achieving copulation and oviposition. Subsequently the adults were removed, thus ensuring that all eggs were laid at a similar time. During the 12 h of light, the vials were inspected under a stereoscopic microscope every three hours, in order to check the larval development. The experiment was repeated four times, and the oviposition action was considered as the starting point of the cycle.

In the second experiment, 20 couples of flies were collected on the day of hatching and individualized in vials containing a culture medium. During the daylight period, the couples were observed every hour for 15 minutes. Male sexual maturity was considered to be reached once 80% of male flies showed courtship behavior or copulation. The evidence of sexual maturity for females was based on either at least 80% of females allowing copulation or the presence of eggs (Markow and O’Grady, 2006).

Results

During the life cycle experiment of *D. polymorpha*, on the first and second day of the experiment, no larval instars were detected; only eggs were observed. On the third day, the first instar larvae were found in all vials. On the fifth day, second instar larvae were observed, and on the sixth day, third instar larvae. On the seventh day the pupal stage took place, and finally, on the 14th day, adult flies hatched. Thus, the *D. polymorpha* developmental time, from egg to adult, is achieved in 14 days. The whole life cycle is represented in Figure 1.

In the second test, only 6 vials containing the fly couples remained alive until the end of the experiment. We observed two males displaying courtship on the fourth day, three on the fifth day, and six on the sixth day. Regarding the females, one showed receptivity on the fifth day and another on the sixth day. Interesting data showed intraspecific variation: At 8:40 am on the fourth day of experiment, the first male demonstrated courtship behavior, and the first copulation was recorded

only on the next day at the same time. Although these are preliminary results, the age of male sexual maturity is clearly reached on the fifth day after hatching, for females on the sixth day.

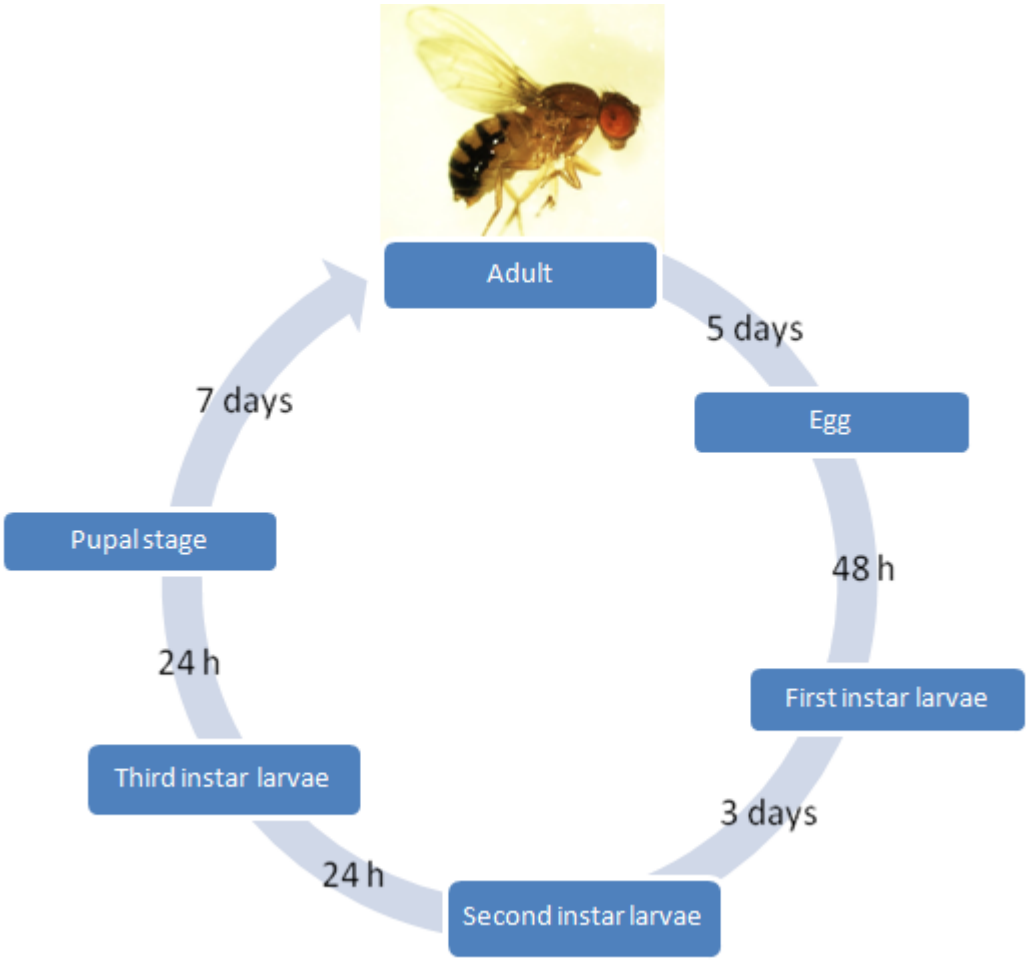

Figure 1. *Drosophila polymorpha* life cycle.

Discussion

The average life cycle of *Drosophila polymorpha* reported on here was 14 days in duration, close to the 15 days of lines at the Tucson *Drosophila* Stock Center under similar temperature conditions (Markow and O'Grady, 2006). It is very difficult to explain variations; however, a number of factors could provide some important insight. The first is culture medium variation, since we use a different recipe compared to Tucson. Bizzo *et al*. (2012) also reported that *D. polymorpha* presented an average 30 days life cycle in corn medium. A second factor is intraspecific genetic variation, since the lines of the Stock Center are from Central America, while ours are from southern South America. A third influential factor is endogamy: Stock Center lines have been maintained for decades in artificial laboratory conditions. The fly lines that were used during this investigation were established in March 2013, which might suggest that the results presented here are in closer resemblance of natural conditions.

Table 1. Egg to adult development time of the *cardini* group at the Tucson *Drosophila* Stock Center.

Species	Days	Temperature (ºC)
subgroup *dunni*		
D. dunni	15	18
D.nigrodunni	15.5	18
subgroup *cardini*		
D. cardinoides	15	18
D. neocardini	15	18
D. parthenogenetica	15	18
D. polymorpha	15	18
D. procardinoides	15	18

Table adapted from Markow & O'Grady, 2006

In any case, the differences were small and were within the expected length for the *cardini* group, as shown in Table 1. The fact that in this experiment the flies were kept at higher temperatures may be the reason for acceleration of the cycle. Also, the San Diego *Drosophila* Stock Center maintains its flies of the *cardini* group at temperatures between 18-25°C, and reports life cycles between 12-16 days, depending on the species and the temperature used. Still, this study enriches the literature, recounting the life cycle in more detail.

For the majority of species, freshly hatched adults are not sexually mature (Markow, 1996), and *D. polymorpha* keep this pattern. In fact, sexual maturity may require up to several weeks, depending on the species (Markow and O'Grady, 2006). While males of some species mature earlier than females, most males mature later than females (Markow and O'Grady, 2008). The results of our work indicate that this species belongs to the first case, similar to *D. melanogaster* that requires 4 days for females to mature and 2 days for males. Equally they are unlike *D. mojavensis* that requires 3 days for females to mature and 7 for males. Furthermore, it can be seen that there is much variation in time between the three species mentioned, a fact that can be justified by the phylogenetic distance between them.

Aiming to map the start of sexual maturity for South Brazilian non-inbreeding lines, more experiments will need to be performed using this species, especially owing to the high mortality rate. Also, the flies' courtship behavior should not necessarily be considered a fully decisive indication of sexual maturity. This is demonstrated by the fact that immature males can achieve copulation without releasing sperm, and females can become sexually receptive before they in fact present mature eggs (Markow, 1996). Furthermore, because metabolic waste from males, present in the culture medium, can change the age at which sexual maturity is reached (Joshi *et al*., 1998), new isolines have been collected in order to reinforce the data obtained from this study.

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Drosophila **collections in the Arc of Deforestation, Brazil.**

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Introduction

The region known as "Arc of Deforestation" covers a massive Amazonian frontier (Figure 1) and shows alarming rates of clearcutting. Close to half of the world's tropical deforestation occurs in

this region (TNC, 2010) due to unplanned clearing for pasture and crops. Therefore, it is a priority area for research and conservation studies.

Although drosophilid field studies have been expanding in Brazil (Gottschalk *et al.*, 2008), the collection sites are distributed unevenly across the country. There is a concentration of efforts near urban centers (Santos *et al.*, 2011), and great areas of gaps. The most critical situations are found in the States of Mato Grosso, Maranhão, Tocantins, and Piaui (Chaves and Tidon, 2008).

This study presents a preliminary evaluation of the drosophilid fauna from two areas in the northeast of Mato Grosso that have never been sampled before. Hence, it fills a gap in the knowledge of drosophilid species distribution in an endangered neotropical region.

Material and Methods

The collections were made in the northeast of the State of Mato Grosso (Figure 1), in a transition area between the Cerrado and Amazon Forest biomes, using banana-baited traps (Roque *et al*., 2011). The first site, Fazenda Tanguro situated in *Querência* County (13°05' S, 52°22'W), was sampled in September 2012 using 16 traps. The second site, Fazenda Destino in *Ribeirão Cascalheira* County (12°52'S, 52°05'W), was sampled in July 2013 using 30 traps. All traps were placed in forest patches.

The specimens were identified to the species level, whenever possible, through external morphology and male terminalia in the case of cryptic species. Vouchers of the captured species were deposited at the Collection of the *Laboratório de Biologia Evolutiva da Universidade de Brasilia*.

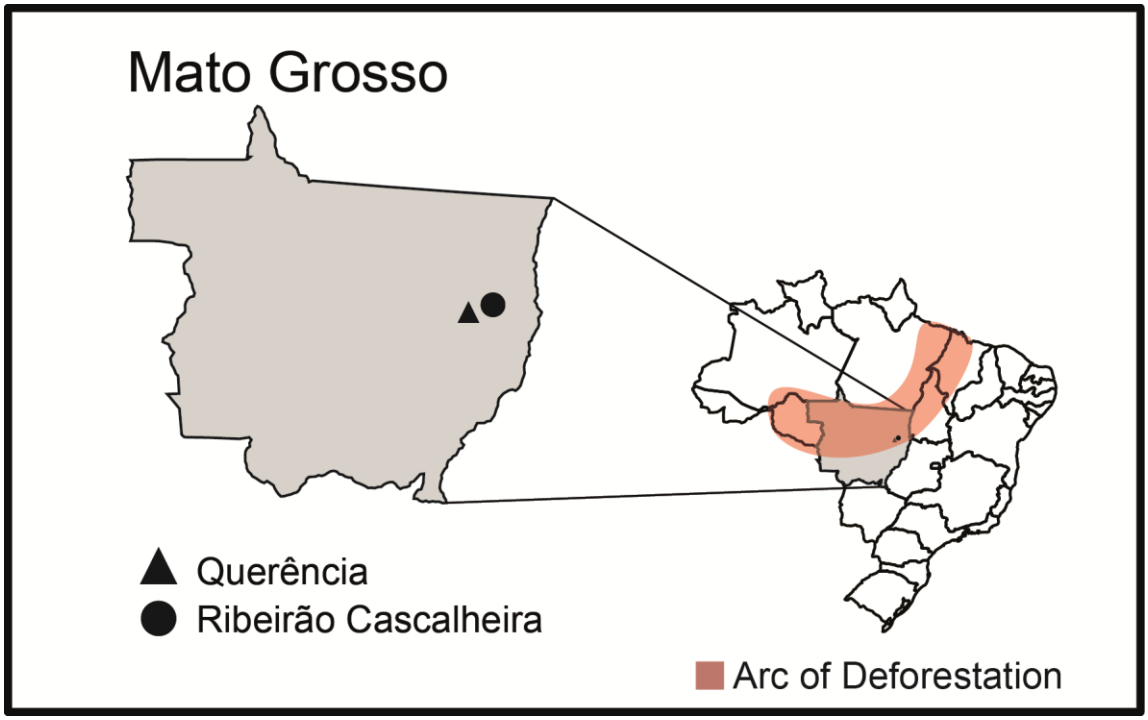

Figure 1. Location of the sampled sites in the Arc of Deforestation, State of Mato Grosso, Brazil.

Table 1. Total and relative abundance (%) of drosophilid species at two sites in the State of Mato Grosso, located in the Brazilian Arc of Deforestation.

Species	*Querência*		*Ribeirão Cascalheira*	
	Total	(%)	Total	(%)
Drosophila annulimana Duda	-		1	(0.04)
D. ararama Pavan & Cunha	-		2	(0.07)
D. cardini Sturtevant	7	(3.80)	1	(0.04)
D. fumipennis Duda	-		7	(0.26)
*D. marlekotliana** Parshad & Paika	-		6	(0.22)
D. mediostriata Duda	-		10	(0.36)
D. mercatorum Patterson & Wheeler	7	(3.80)	-	
D. nebulosa Sturtevant	168	(91.30)	691	(25.10)
D. neocardini Streisinger	-		1	(0.04)
D. nigricruria Patterson & Mainland	1	(0.54)	-	
D. quadrum Wiedemann	-		2	(0.07)
D. sturtevanti Duda	1	(0.54)	12	(0.44)
*Scaptodrosophila latifasciaeformis** (Duda)	-		1	(0.04)
*Zaprionus indianus** Gupta	-		16	(0.58)
Group *D. coffeata*	-		2	(0.07)
Group *D. repleta*	-		4	(0.15)
Group *D. tripunctata*	-		5	(0.18)
Group *D. saltans*	-		4	(0.15)
Subgroup *D. willistoni***	-		1988	(72.20)
Total	184		2753	

* Exotic species.
** *D. willistoni*, *D. paulistorum* and *D. tropicalis*.

Results and Discussion

A total of 2,937 individuals representing 14 nominal species were sampled, besides several unidentified species from the groups *Drosophila coffeata, D. repleta, D. tripunctata, D. saltans,* and the subgroup *D. willistoni* (Table 1). Among them, three species are exotic: *Drosophila malerkotliana, Scaptodrosophila latifasciaeformis,* and *Zaprionus indianus*. All neotropical species are from the *Drosophila* genus.

The two sampled locations revealed singular drosophilid assemblages. The difference in abundance certainly reflects sampling effort, but may also reveal the high spatial and temporal environmental heterogeneity of the biome. The drosophilid community in *Querência,* sampled at the end of the dry season, was poorer in species and strongly dominated by *Drosophila nebulosa,* a well-adapted species to arid areas. *Ribeirão Cascalheira* was richer in neotropical species associated to forests and dominated by the *D. willistoni* subgroup. Several species still need taxonomic determination; some of them can be new species.

This brief catalogue, based on samples collected in an endangered and highly diverse area considered a gap in the knowledge of drosophilid distribution, recorded at least 20 nominal species of drosophilids. We are aware that we have sampled only a fraction of the species that should occur in

this area and that more collections are needed. Therefore, the present study aggregates a basis for future modeling, conservation efforts, and emphasizes the need for a broader sampling.

Acknowledgments: We are grateful to Universidade de Brasília for logistical support, and to CAPES and CNPq for financial support.

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Chronic exposure to tunicamycin during development has little effect upon the eyes of *GMR-Gal4 UAS-lacZ* males.

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Introduction

Endoplasmic reticulum (ER)-stress is caused by the intracellular accumulation of proteins and is implicated in several degenerative disease states (Boyce and Yuan, 2006; Haeri and Knox, 2012). Although characterized as a source of cellular damage, the ER-stress response to mild insult (ER pre-conditioning) has been demonstrated to be protective in *Drosophila* through an autophagy-dependent process (Fouillet *et al.,* 2012). In part, this response was achieved through acute exposure by feeding flies - for only four hours - with a medium containing tunicamycin, an antibiotic that inhibits glycosylation. Although this approach proved quite successful, a set of conditions that allow for chronic exposure to produce a continual level of protection or damage is very desirable.

Several avenues of research into ER-stress in *Drosophila* may depend upon the use of the *UAS/Gal4* system (Brand and Perrimon, 1993) to express various transgenes under conditions of stress. Our laboratory has characterized apoptosis-dependent developmental defects caused by *GMR-Gal4*^{12} (Kramer and Staveley, 2003; unpublished) under conditions of elevated temperatures and increased gene-dosage. As a result, we investigated the possibility that induction of ER-stress by tunicamycin might induce toxic effects when coupled with normally non-detrimental levels of *Gal4* expression controlling a standard *lacZ* transgene.

Materials and Methods

Drosophila *media*

The standard cornmeal-yeast-molasses-agar medium in our laboratory is made with 65 g/L cornmeal, 10 g/L nutritional yeast, and 5.5 g/L agar in water, heated to form a slurry, then cooked by autoclave @ 30 minutes under standard conditions for liquids. This is supplemented with 50 ml/L fancy grade molasses after cooking and with 5 ml of 0.1 g/ml methyl paraben (methyl 4-hydroxybenzoate from Sigma Life Science Research: *www.sigma.com*) in 95% ethanol and 2.5 ml of propionic acid when cooled to 55 to 60°C prior to decanting into standard plastic shell vials. Once

solidified, the medium is stored at 4 to 6°C and warmed to room temperature for use.

To induce an ER-stress response, tunicamycin (BioShop Canada Inc.: *www.bioshopcanada.com*) was dissolved in 95% ethanol to produce a 0.1 mg/ml stock solution and was added to the standard medium to the concentrations of 0.1 mg/L, 0.01 mg/L, and 0.001 mg/L just prior to decanting the media into vials.

Drosophila *stocks and culture*

The *GMR-Gal4*^{12} (Freeman, 1996) and *UAS-lacZ*$^{Bg4-1-2}$ (Brand and Perrimon, 1993) lines were obtained from the Bloomington Drosophila Stock Center at Indiana University. The *GMR-Gal4*^{12} line was originally selected from a collection of fifteen transgenic lines as one of two insertions that did not produce a "rough eye" phenotype as a heterozygote at 25°C but did drive the expression of *lacZ* behind the morphogenetic furrow (Freeman, 1996). Crosses between these parental lines are routinely used to express *lacZ* in the developing eye, in the "*Glass Multiple Reporter* (*GMR*)" pattern, as a benign control for the ectopic expression of transgenes of interest in the eye. As the *GMR-Gal4*^{12} transgene can produce a "rough eye" phenotype at 29°C (Kramer and Staveley, 2003), temperature must be well-controlled.

Due to poor success in preliminary crosses, a non-standard mating regimen was carried out: 1) three *GMR-Gal4* females and three *UAS-lacZ* males were initially mated upon standard media overnight and 2) then the adults were moved to the test vials (containing standard media supplemented with 0.1 mg/L, 0.01 mg/L, and 0.001 mg/L of tunicamycin plus control) for a period of six hours. To encourage oviposition, the vials were freshly "yeasted" with 5 to 10 grains of Fleischmann's "Instant Yeast" (*www.breadworld.com*) prior to introduction of the flies. Afterwards, the recovered mated adults were held on standard medium overnight and re-brooded twice. All incubations were carried out at 25°C.

Biometric analysis of the Drosophila *eye*

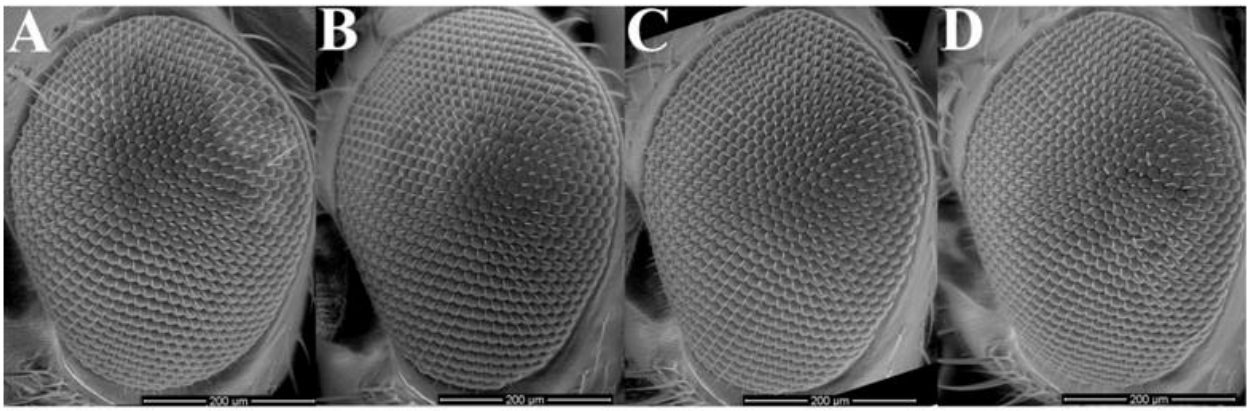
A cohort of critical class males were collected and aged for three to five days on the test medium at 25°C. They were frozen, stored at -80°C, and then mounted on aluminum studs and desiccated for at least 24 hours. Micrographs were taken with the FEI Quanta 400 Environmental SEM at a magnification of 543× and a horizontal field width of 550 μm. Micrographs were analyzed using NIH ImageJ software (Abramoff *et al.*, 2004). For each cross five images were analysed and the mean number of ommatidia and bristles were determined and standard error of the mean was calculated.

Results and Discussion

In *Drosophila melanogaster*, eye development is tightly controlled during the organization of the ommatidial array (reviewed by Cagan, 2009). Expression of the benign *lacZ* gene in the developing eye under the control the transgenic driver *GMR-Gal4* can act as control for the expression of genes of interest under any of a number of given circumstances. Here we demonstrate that our attempts to challenge flies to ER-stress via chronic exposure to tunicamycin does not alter eye development under these conditions very much.

As can be observed by analysis of scanning electron micrographs (Figure 1), the mean number of ommatidia was 724.2 (SEM = 6.47) and the mean number of interommatidial bristles was 599.4 (SEM = 9.99) when *GMR-Gal4/UAS-lacZ* males develop on our standard medium. Supplementation of the media with tunicamycin at the concentrations of 0.001 mg/L, 0.01 mg/L, or

0.1 mg/L does alter the number of ommatidia. However, there is a slight decrease in bristle number in the two treatments of 0.01 mg/L and 0.1 mg/L of tunicamycin. As a consequence, we conclude that experiments that utilise the *GMR-Gal4*^{12} transgene to drive expression in the eye will not be compromised greatly by interactions between tunicamycin and *Gal4*-induced toxicity.



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Tunicamycin (mg/L)	Ommatidia (n = 5)	Bristles (n = 5)
0 mg/L	724.2 (SEM 6.47)	599.4 (SEM 9.99)
0.001 mg/L	718.0 (SEM 4.56)	608.2 (SEM 7.50)
0.01 mg/L	719.6 (SEM 8.87)	561.4 (SEM 8.23)
0.1 mg/L	727.3 (SEM 6.92)	563.4 (SEM 12.4)

Figure 1. Tunicamycin does not greatly influence the eye development of *GMR-Gal4 UAS-lacZ* flies. Scanning electron micrographs of the eyes of *GMR-Gal4/UAS-lacZ* males that have developed upon (A) control medium or in the presence of tunicamycin at concentrations of (B) 0.001 mg/L, (C) 0.01 mg/L, and (D) 0.1 mg/L at 25°C. (E) The number of bristles reveals a very slight decrease at the two highest concentrations of tunicamycin. SEM = standard error of the mean.

Acknowledgments: This research was supported by Memorial University of Newfoundland's Department of Biology Honours Dissertation Research Fund to MKM, the National Sciences and Engineering Council of Canada (NSERC) Undergraduate Student Research Award to ADSG, and by a NSERC Discovery Grant to BES.

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Neurotransmitter enzymes profile in age-related memory impairment in *Drosophila melanogaster.*

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Oxidative stress induces alterations in the structure and functions of cellular macromolecules that result in many pathological conditions including the aging process. According to the free radical theory of aging, oxidative stress is implicated in the age-related functional decline. Brain is more vulnerable to free radical damages due to its higher rate of metabolism and lower capacity for regeneration as compared to the other organs. Therefore, we have previously showed that oxidative stress can be considered as the main causal factor involved in the impairment of cognitive ability (Haddadi *et al.*, 2014). Induction of oxidative stress is accompanied by reduction in acetylcholine levels in neuronal degeneration. The active brain requires the synthesis of acetylcholine as reflected by the activity of choline acetyl transferase (ChAT). On the other hand, neuronal plasticity underlying memory formation needs appropriate activity of Cholinesterase enzymes. Therefore, age-associated alterations in the activity of neurotransmitter enzymes can be considered as a part of age-related memory impairment (AMI). Hence, in the present study, we have made a comparative study on the activity of neurotransmitter enzymes between 5-day and 50-day old flies before and after conducting long olfactory conditioning training paradigm.

Acetyl cholinesterase (AChE) and Butyryl cholinesterase (BChE) activities were determined by the method of Ellman *et al.* (1961). Immunohistochemical localization was conducted using monoclonal anti *Drosophila* choline acetyl transferase (ChAT) antibody, and whole brain samples of flies were observed under a Zeiss LSM710 confocal microscope to evaluate ChAT activity (Seki *et al.*, 2010).

Long training paradigm was performed by employing classical olfactory conditioning, wherein octanol was associated with 12 pulses of 90 V DC electric shocks. Methylcyclohexanol served as a counterpart odor in the absence of electric shock (Yu *et al.*, 2006).

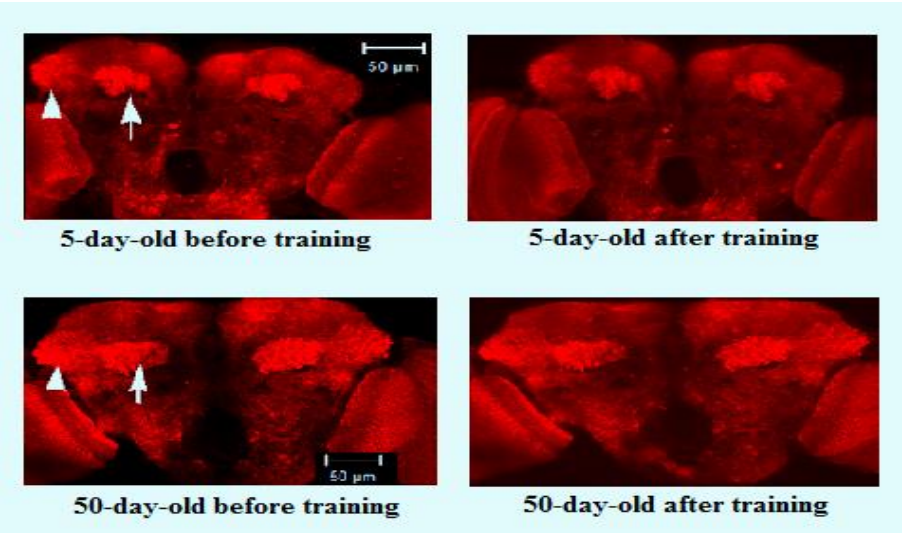

Figure 1. Activity of ChAT.

Our results showed that due to aging, the activity of the enzyme ChAT was decreased, whereas the activity of AchE and BchE enzymes were unchanged. Biochemical investigations carried out after training showed that in mushroom bodies extrinsic neurons

(MBENs) and lateral horn (LH) neurons of the brain of both the age group flies, the activity of ChAT was not affected by training (Figure 1).

In the case of young flies the activity of ChE enzymes was significantly increased in trained flies, but the extent of enhancement was considerably higher in AchE (Figure 2). Surprisingly, 50-day-old trained flies exhibit decreased enzyme activity compared to untrained ones. AChE shows more reduction compared to BChE (Figure 2).

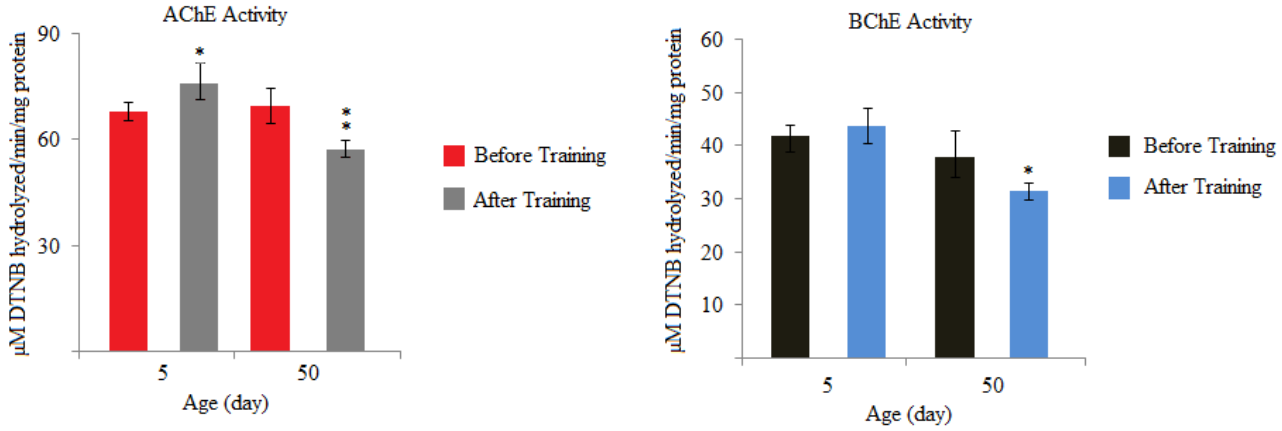

Figure 2. Activity of ChE enzymes.

Age-related decrease in the activity of ChAT accompanied with constant activity of ChE enzymes were observed in older flies. Lack of enhancement in the activity of ChAT and noticeable decrease in ChE enzymes at the time of training in aged flies can induce a process, which could lead to lower levels of acetylcholine, compromising the neuronal plasticity and impaired memory in the aging brain.

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The effect of pyrogallol on the pre-adult fitness of *Drosophila bipectinata.*

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Abstract

The effect of pyrogallol (an antioxidant) on the pre-adult fitness has been studied using an outbred population of *D. bipectinata*. It was noticed that there was a decrease in the egg to larva, larval to pupal, and pupal to adult development and the rate of development in the pyrogallol treated *D. bipectinata* flies, which had significantly lower pre-adult viability and rate of development, when compared to the untreated flies. The SOD levels were significantly greater in untreated flies than the

treated flies. Among the treated flies, SOD levels decreased with an increase in the concentration of pyrogallol in the diet. Thus, these studies suggest that, in *D. bipectinata*, pyrogallol (an antioxidant) has a negative effect on the product viability and the rate of development of the fly. Key Words: Antioxidants, Superoxide Dismutase, Viability, Rate of development

Introduction

The net fitness of an organism is determined by various components which can be divided into two major categories: the pre-adult fitness and the adult fitness (John *et al.*, 1981). Every character may contribute to the total fitness of the organism through one or more of the many biochemical and physiological pathways in the developmental process. An interrelation of these pathways can be deduced, which is complicated but can be categorized distinctly (Yuichiro *et al.*, 1960). The rate of development, along with pre-adult fitness is known to be highly correlated, while the rate of development (time from egg laying to eclosion) is considered as a major component of the pre-adult fitness of an organism. The pre-adult viability is measured by the survival through the pre-adult stages. Therefore, a study of developmental rate also gives information about viability (Bonnier *et al.*, 1959).

Many studies have been carried out in *Drosophila* at the intraspecific level, using quantitative traits to investigate climatic adaptations by comparing flies' performance in laboratory assays, *e.g.*, development time (James *et al.*, 1995; Norry *et al.*,2001; Sgro and Blows, 2003; Griffiths *et al.*, 2005), larval growth efficiency (Robinson and Partridge., 2001), larval survival and pre-adult competitive ability (James and Partridge, 1998), stress resistance (Krebs and Loeschcke, 1995; Karan *et al.*, 1998a; Bubliy *et al.*, 2002; Hoffmann *et al.*, 2002, 2005). Also, the effects of light regimes, temperature along with the region of occurrence (like temperate and tropical areas), and male age on the pre-adult fitness have been studied.

Parental environment and fitness have also been correlated with that of the offspring, thus indicating that an enhancement in the parental environment will lead to enhanced offspring phenotype (Fox *et al.*, 1997; Donohue and Schmitt., 1999). Nutritional enhancements in the diet are known to enhance fitness (Djawdan *et al.*, 1998). Work by Anderson *et al*. (2010), shows that larval nutrition affects a wide range of life history traits, tested by assaying the heat, cold and desiccation tolerance in the larvae raised on carbohydrate and protein enriched medium. They also showed that the increased egg to adult viability in media rich in sucrose along with the positive effects of protein enrichment on the female survival. Thus, indicating that nutrition at the pre-adult stages plays a major role in the overall fitness of the adult. The different levels of food available at these stages also cause the regulation of the resource processing. In addition to this they showed that lower levels of food leads to lower body size (Zoltan and Gerdien, 2003), which is a general phenomenon in ectotherms (Atkinson and Sibly, 1997) and is shown in *Drosophila melanogaster* and other species by Gebhardt and Stearns (1998).

The complete nutrition of an organism in nature consists of carbohydrates, proteins, lipids, fats, and antioxidants at different concentrations. Although many experiments have been done using carbohydrate and protein enrichment in media, antioxidant enrichment and its effects have seldom been carried out. Thus in this experiment the effect of enhanced quantities of antioxidants in the diet are measured on the pre-adult fitness and the rate of development tested in the species *D. bipectinata*, which is a complex of the *ananassae* subgroup of the *melanogaster* species group, a member of the *bipectinata* species (Bock and Wheeler, 1972).

Materials and Methods

Establishment of stock:

The experimental stock of *Drosophila bipectinata* was established from progenies of 50 iso-female lines collected from Chamundi Hills at Mysore, Karnataka, using mixed fruit bait. These flies were cultured in a standard wheat cream agar medium and maintained at a constant temperature of 22 ± 1ºC with a relative humidity of 70%. In each generation, the emerged flies were mixed together and redistributed to ten new culture bottles each with 20 flies (10 males + 10 females). This procedure was continued for 3 generations to acclimatize the flies to laboratory conditions. At the fourth generation eggs were collected using Delcour's procedure (1969) and 100 eggs were seeded in new culture bottles. When adults started emerging, virgin females and males were isolated within 3 hours of their eclosion. These flies were aged for 5-6 days to be used in the present experiments.

Preparation of antioxidant media at different concentrations:

The antioxidant pyrogallol was dissolved in water at a concentration of 1000 ppm (*i.e*., 1 g in 100 ml of distilled water), which was maintained as stock solution, from which different amounts were taken and mixed in 30 ml of the standard wheat cream agar media in concentrations of 1 ppm, 10 ppm, 15 ppm, 20 ppm, and 25 ppm, respectively, to check the LD_{50} concentration of the compound (that is, the concentration at which 50% of the progeny dies). The LD_{50} was found to be 10 ppm. The flies raised in three sublethal doses of 1 ppm, 3 ppm, and 5 ppm were used for the present experiments.

Effect of pyrogallol on SOD enzyme levels:

Enzyme preparation for Superoxide Dismutase analysis: The flies cultured on different sub-lethal concentrations of 1 ppm, 3 ppm, and 5 ppm of pyrogallol were subjected to assay the effect of pyrogallol on the stress enzyme Superoxide Dismutase (SOD). To assay this, five adult (5 males and 5 females) flies from each concentration were taken along with the untreated (control) in different Eppendorff tubes. 200 microlitres of 50 mM Phosphate buffer of pH 7 for Catalase assay, 200 microlitres of 250 mM Phosphate buffer of pH 7.8 for SOD Assay were added and crushed using tissue homogenizer in ice cold conditions and centrifuged at 8000 rpm for 20 min in a cooling Microfuge. After this, the supernatants were poured into other eppendorff tubes. 0.1 ml/100 µl of this enzyme extract was used for the assay.

Measurement of Superoxide Dismutase enzyme activity: SOD enzyme was assayed using a modified procedure originally described by Beauchamp and Fridovich (1971). The enzyme extract was added to a mixture of solutions containing 250 mM phosphate buffer (0.8 ml), 100 mM Methionine (1 ml), 100 mM Riboflavin (0.5 ml), 5mM EDTA (0.1 ml), 750 mM NBT (0.1 ml), the volume of which was made up to 3 ml with distilled water. A mixture without the enzyme and NBT was prepared which served as a blank, and a control with NBT and no enzyme was prepared. These mixtures are exposed to sunlight or a bright light of about 400 watts; during this reaction NBT gets reduced to formazone, and was read at 560 nm. The total protein content of the enzyme was estimated by Lowry's method and the activity was expressed in units /mg of protein.

Effect of pyrogallol on Pre adult fitness parameters:

(a) *Egg to larval hatchability*:

Eggs were collected separately from the cultures of untreated and pyrogallol treated flies (1 ppm, 3 ppm, 5 ppm). Eggs were collected by Delcor's procedure (1969) to study the effect of

pyrogallol on the egg to larval hatchabilty. Eggs (100) were seeded separately into each vial (1 × 3 cm) containing ~ 6 ml of wheat cream agar media (control) and pyrogallol at different sublethal concentrations. The total number of eggs that hatched into larvae was counted using a stereomicroscope at 40× magnification. A total of 10 replicates was run separately for both untreated and pyrogallol treated (1 ppm, 3 ppm, 5 ppm) flies.

(b) Larval to pupal viability:

First instar larvae were collected separately from the cultures of normal and pyrogallol treated flies (1 ppm, 3 ppm, 5 ppm) were used to study the effect of pyrogallol on larval to pupal viability. First instar larvae (100) were seeded separately into each vial (1 × 3 cm) containing ~ 6 ml of wheat cream agar media (control) and pyrogallol at different sublethal concentrations. The total number of pupae formed from these larvae was counted. A total of 10 replicates was run for both untreated and pyrogallol treated (1 ppm, 3 ppm, 5 ppm) flies.

(c) Pupal to adult eclosion:

To study the effect of pyrogallol on the pupa to adult eclosion, pupae were obtained from both untreated and pyrogallol treated cultures (1 ppm, 3 ppm, 5 ppm). Pupae (100) were transferred separately into each vial (1 × 3 cm) containing ~ 6 ml of wheat cream agar media (control) and pyrogallol at different sublethal concentrations. The total number of adults eclosed from the pupae was counted. A total of 10 replicates was run for both untreated and treated (1 ppm, 3 ppm, 5 ppm) flies.

The data obtained from each of the above experiments (egg to larval hachability, larval to pupal viability, pupal to adult eclosion), was subjected to a one-way ANOVA analysis followed by the Tukey's *Post Hoc* test.

Table 1. Effects of pyrogallol on the pre-adult viability of *D.bipectinata.*

Pre-adult viability	Untreated (Control)	Pyrogallol treated flies			F-value
		1 ppm	3 ppm	5 ppm	
Egg to larval hatchability	65.1 ± 4.37^{a}	40.5 ± 1.87^{b}	49.7 ± 2.44^{b}	48.9 ± 2.15^{b}	26.72*
Larval to pupal viabilty	55.1 ± 4.12^{a}	26.1 ± 2.25^{b}	16.1 ± 0.9^{c}	14.6 ± 0.91^{c}	713.39**
Pupal to adult eclosion	39.2 ± 3.32^{a}	11.8 ± 1.13^{b}	8.1 ± 0.62^{b}	8.5 ± 0.92^{b}	141.551***

*Significant at p<0.01; **Significant at p<0.0001; ***Significant at p<0.001;
Different letters in the super script indicate significance at 0.05 levels by Tukey's *post hoc* test.

Effect of pyrogallol on the rate of development of pre-adult stages:

Eggs were collected separately from flies of both untreated and pyrogallol treated (1 ppm, 3 ppm, 5 ppm) media using Delcor's procedure (1969). One hundred eggs/larvae/pupae were transferred separately to vials containing ~ 6 ml of normal and pyrogallol treated (1 ppm, 3 ppm, 5 ppm) media. The number of hours taken by each egg to hatch into larvae was observed. Similarly, the number of days taken by the first instar larva to form pupa and the pupa to eclose to adult were also observed and recorded. One-way ANOVA analysis followed by the Tukey's *post hoc* test was carried out on the data on the rate of development of the pre-adult stages.

Results

Effect of pyrogallol on Superoxide Dismutase enzyme activity:

The Figure 1 shows the SOD enzyme levels in the pyrogallol treated males of *D. bipectinata.* It was observed that the SOD enzyme levels were found to be higher in the untreated males when compared to the treated males. Among the pyrogallol treated males the levels of SOD were found to have decreased with an increase in the concentration of pyrogallol. The lowest level of the SOD enzyme was found at the 5 ppm concentration and the highest was at the 1 ppm concentration. This suggests that in males of *D. bipectinata* SOD activity decreases with increasing pyrogallol concentration.

SOD enzyme levels of untreated and pyrogallol treated females of *D. bipectinata* is depicted in Figure 2. SOD levels were found to be higher in the untreated when compared to the pyrogallol treated females. Among the pyrogallol treated females, SOD activity was the least at 5 ppm and highest at 1 ppm. Further, SOD activity decreased with an increase in the concentration of pyrogallol. Between the sexes the SOD enzyme levels in the female flies were lower than that of the males, both in the untreated and the pyrogallol treated groups, suggesting that the females had a lower level of SOD enzyme activity than the males.

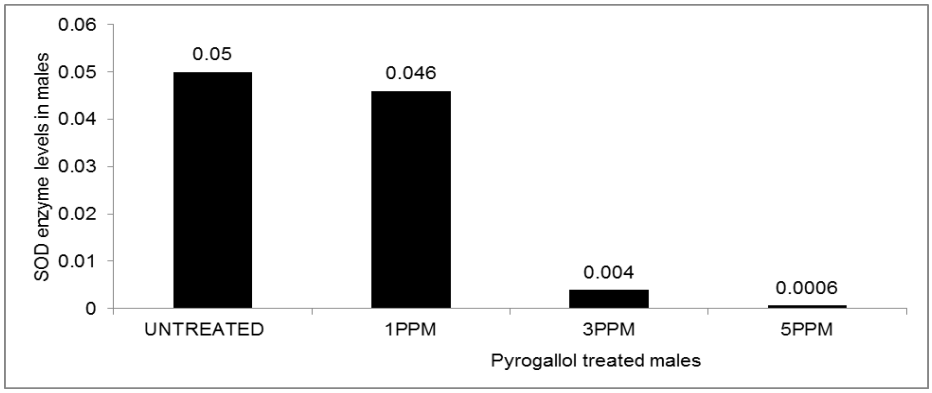

Figure 1. Effects of pyrogallol on the SOD levels in males of *D. bipectinata.*

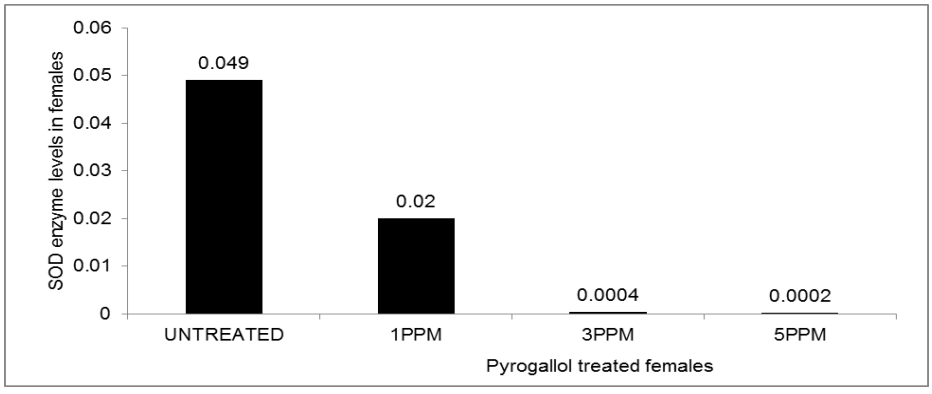

Figure 2. Effects of pyrogallol on the SOD levels in females of *D. bipectinata.*

Effect of pyrogallol on Pre adult fitness parameters:

Table 2 shows the mean values of egg to larval hatchability, larval to pupal viability, and pupal to adult eclosion in untreated and pyrogallol treated groups. The number of eggs that hatched into larvae, larvae that formed pupae, and pupae that eclosed into adults was higher in the untreated when compared to the pyrogallol treated groups. Among the pyrogallol treated group the egg to

larval hatchability also varied, with the least egg to larval hatchability noticed in the sequence of 1 ppm < 5 ppm < 3 ppm, while the larval to pupal viability showed variation with the least viability in the 5 ppm concentration and the highest in 1 ppm. The pupal to adult eclosion showed a decrease in the eclosion with increased concentration of pyrogallol. Flies rared at 1 ppm concentration of pyrogallol had the highest pupal to adult eclosion and the least pupal to adult eclosion was noticed at the 5 ppm concentration.

One-way ANOVA followed by the Tukey's *post hoc* test carried out on the egg to larval hatchability, larval to pupal viability, and pupal to adult eclosion data obtained, showed significant variations between untreated and pyrogallol treated groups. From the Tukey's *post hoc* test it was found that there was a significant difference in the egg to larval hatchability between the untreated and pyrogallol treated groups. And among the pyrogallol treated group, egg to larval hatchability was found insignificant by the Tukey's *post hoc* test. While the larval to pupal viability in 1 ppm was found to significantly lower than the untreated flies, it was significantly higher when compared to 3 ppm and 5 ppm pyrogallol concentration. However, larval to pupal viability was found to be insignificant between 3 ppm and 5 ppm pyrogallol concentrations, and the pupal to adult eclosion showed a significant difference in the eclosion between the untreated and treated groups; among the treated there was no significant variation between 1 ppm and 3 ppm and 5 ppm concentrations.

Table 2. Effects of pyrogallol on the rate of development in *D.bipectinata.*

Rate of development (in hours)	Untreated (Control)	Pyrogallol treated flies			F-value
		1 ppm	3 ppm	5 ppm	
Egg to larava	8.64 ± 0.008^{a}	12.05 ± 0.008^{b}	13.25 ± 0.11^{c}	15.54 ± 0.21^{d}	479.45*
Larval to pupa	60.64 ± 0.26^{a}	72.00 ± 0.31^{b}	95.98 ± 0.19^{c}	120.98 ± 0.34^{d}	8858.63*
Pupal to adult	92.61 ± 0.25^{a}	120.00 ± 0.33^{b}	144.30 ± 0.27^{c}	96.01 ± 0.21^{d}	7929.28*

* Significant at $p < 0.001$;

Different letters in the super script indicate significance at 0.05 levels by Tukey's post hoc test.

Effect of pyrogallol on the rate of development of pre-adult stages:

Table 2 shows the mean values of rate of development from egg to larva, larva to pupa, and pupa to adult. The rate of development was the lowest in the untreated when compared to the pyrogallol treated groups, and it increased with an increase in the concentration of pyrogallol among the treated groups. In contrast to this, the rate of development of the pupa among the pyrogallol treated groups was least in the 5 ppm concentration, followed by 1 ppm and 3 ppm.

The data when subjected to a one-way ANOVA followed by the Tukey's *post hoc* test showed significant variation in the rate of development in egg to larvae, larvae to pupae, and pupae to adult, between the untreated and pyrogallol treated groups. Among the treated groups, in the egg to larval and larval to the pupal rate of development, there was a significant variation between concentrations in the 5 ppm concentration having significantly higher rates of development than the 1 ppm and 3 ppm groups, with significant variations also seen between the 1 ppm and 3 ppm groups by the Tuckey's *post hoc* test. However, in the pupal to adult development there was a significant variation between concentrations in the rate of development in the 3 ppm concentration being significantly higher than the 1 ppm and 5 ppm groups, with significant variations also seen between the 1 ppm and 5 ppm groups by the Tuckey's *post hoc* test. The least developmental rate was seen in the 5 ppm concentration.

Discussion

The developmental theory of ageing suggests a positive correlation between rate of development and adult longevity (Zwaan *et al.,* 1991), although other studies by Lints and Lints (1978) and Lints (1985) have shown that there is a negative correlation between lifespan and developmental rate. Since antioxidants are known to increase longevity by decreasing the oxidative stress in an organism, the effect of the antioxidant pyrogallol was tested in order to determine this correlation. From the results obtained, in the SOD enzyme levels, there was a decrease in the SOD level with an increase in the concentration of pyrogallol, indicating that there is an evident effect of pyrogallol on the fly. Thus testing for the pre-adult fitness along with the rate of development between the untreated (control) and pyrogallol treated groups estimates the effects of antioxidants on the fly.

A variation in the egg to adult hatchability along with the mean values of the rate of development was noticed. The egg to adult hatchability decreased with the increase in concentration of pyrogallol in the media, while the rate of development was higher with an increase in pyrogallol. This comparison can be used to infer that the antioxidant pyrogallol also plays a significant role in the pre-adult fitness as well as the rate of development in the treated flies.

Temparature has been shown to affect the rate of development and the pre-adult survivability, where lower the temperature, slower the development (Santos *et al.,* 2006) and higher the chances of survival (Partridge *et al.,* 1994). Hence, under the controlled conditions of the lab the temperatures are regulated at 22 ± 1°C, to ensure temperature does not act as a trade-off between the effects of the antioxidants on the rate of development and the developmental time.

Studies have also shown that larval crowding can have an effect on the pre-adult growth and rate of development (Santos *et al*., 2006) causing a reduction in growth efficiency; however, it was ensured in our experiments that the larval number per vial was maintained to a maximum number of 20, which rules out the effect of crowding on the results obtained. The male age is another known factor to play an important role on the pre adult fitness, showing that older males are preferred by the female in order to gain better genetic benefits (Prathibha *et al*., 2012); therefore, it was ensured that the males and females were of the same age to ensure that there are no such effects. In addition to this light is known to play a role in the rate of development. Studies have shown that flies exposed to continuous light show faster development rates than the flies exposed to light and dark periods.

Nutrition is known to enhance fitness (Djawdan *et al.,* 1998), agreeing to the work by Anderson *et al.* (2010) that larval nutrition like enrichment of carbohydrate and protein affects a wide range of life history traits, along with the increased egg to adult viability in media rich in sucrose along with the positive effects of protein enrichment on the female survival, indicating that nutrition at the pre-adult stages plays a major role in the overall fitness of the adult. In our results there was a lower viabilty in the larval stages after antioxidant enrichment, indicating the effect of the antioxidant pyrogallol, which was administered through the diet.

Even though antioxidants are known to increase the longevity of flies, studies have shown that there is no correlation of the pre-adult fitness and the longevity a fly (Zwaan *et al.,* 1991). Hence the results obtained certify that the effect of pyrogallol was the sole reason for the results obtained as all the other factors that may have played a role were kept constant. Thus, pyrogallol has a role to play in the rate of development and the survivability of pre adult stages in *D. bipectinata*.

Acknowledgments: We thank the chairman, Department of Studies in Zoology for the facilities.

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Male age influence on mating activities of monomorphic and polymorphic strains of *Drosophila ananassae.*

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Introduction

Females of a species select males to derive either direct or indirect fitness benefits. Therefore, male success in mating depends on his ability to provide material benefits to mating female or genetic benefit to their offspring. Thus, females of a species use a variety of male phenotypes to select the potential mate (Anderson, 1994). Male age is one such trait used as reliable signal to determine male quality as has been shown in earlier studies of age based female mate choice experiments (Avent *et al.*, 2008; Beck and Powell, 2000). A series of verbal models (Trivers, 1972; Manning, 1985, 1989) predict that, given a choice, females should prefer older mates. As old males have proven survival ability, choosy females may gain indirect benefits from their choice of an old mate through the production of higher quality offspring. However, testing the effects of indirect benefit unequivocally requires exploration of the effects of female mating preferences on offspring survival and reproduction.

In species of *Drosophila* inversion karyotypes are known to influence on mating activity, mating advantage, mating speed, and female mate preference (Spiess and Langer, 1964a, b; Day and Butlin, 1987). It was also found that age based female mate preference is in a few species of

Drosophila (Avent *et al.*, 2008). These studies have found females of these species prefer to mate with older of the two competing males, and female mating with older male obtains indirect genetic benefits to their offspring. However, it is not known the role of inversion karyotypes on age based female mate preference.

Therefore, the present study has been undertaken in *D. ananassae,* a cosmopolitan domestic species, having a high degree of inversion polymorphism. The Indian natural populations of this species are genetically at the level of chromosomal polymorphism (Singh 1985a, b). The subterminal (alpha) inversion has become universally established in the species (Singh, 1970). Further it is known that behavioral properties are controlled by chromosomal variants. Therefore, in the present study a polymorphic strain has been established to rule out the role of inversion system in age based female mate preference. Although a number of adaptive functions have been found to be associated with inversion polymorphism, the association of mate choice, male age with inversion polymorphism has not been studied. Therefore, the present study was undertaken in *D. ananassae* to understand the relationship between male age, mate choice, and inversion polymorphism. So in *D. ananassae* we have used both monomorphic and polymorphic strains to study interrelation between male age, inversion system, and male reproductive success.

Materials and Methods

Experimental stock: Population without inversion (monomorphic population) and population with inversion (polymorphic population-2LA, 3LA, and 3RA inversion) of *D. ananassae* were established from progenies of 50 isofemale lines collected at semi domestic localities of Mysore, Karnataka, India (see more details in Prathibha, 2011). These culture bottles were maintained at 21 ± 1°C at a relative humidity of 70% using 12:12 L: D cycle. Virgin female and unmated males were isolated within 3 hrs of their eclosion and were aged individually in a vial containing wheat cream agar medium until they were used in the experiment.

There is a pericentric inversion in *D. ananassae* on the X or A element that converts the normally acrocentric X into a metacentric chromosome. A further remarkable karyotypic change can also be seen on Muller element F of *D. ananassae*. The F element that is normally a small, dot-like chromosome is a large metacentric that is equivalent in size to the X or A element. The large autosomes of *D. ananassae* are products of centromeric fusions between the B and C elements (symbolized as Muller element B_C for chromosome (3LA-3RA) and D_E elements 2LA(Stephen *et al.*, 2008).

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

Mate choice tests: A female along with two competing males from different male age classes (young *v* middle age; young *v* old age; middle *v* old age; N =5 0 dyads per male pairing) were individually transferred into an Elens-Wattiaux mating chamber (1964). The effect of paint was

tested before commencing the experiment by painting one of the two young /middle/old age males and allowing them to mate. This pair was observed for 1 hr. There was no effect of the presence of paint on the probability of mating (all groups $P > 0.50$). When mating occurred, pairs in copulation were aspirated out from the mating chamber. Rejected male in female mate choice experiment was also transferred to new vial. A total 50 trials were made separately for each combination of female mate choice experiment. We also measured wing length of 50 selected and rejected males in each combination of the female mate choice experiment following the procedure of Hegde and Krishna (1997). Separate experiments were conducted for both monomorphic and polymorphic strains.

Evaluation of mating activities in monomorphic and polymorphic strains: We used unmated young, middle and old aged males and 5-6 days old virgin female of monomorphic and polymorphic strains (monomorphic male with monomorphic female; polymorphic male with polymorphic female) to study male age influence on male mating activities. A female along with a male (young/middle/old age) were individually transferred into an Elens-Wattiaux mating chamber and observed for 1hr. A pair unmated within 1 hr was discarded. We recorded mating latency (time between introduction of male and female together into mating chamber until initiation of copulation of each pair) and copulation duration (time between initiation of copulation to termination of copulation of each pair). We also quantified courtship acts such as tapping, scissoring, vibration, licking, circling, ignoring, extruding, and decamping following the procedure of Hegde and Krishna (1997). The behavior of male and female was recorded simultaneously but separately by two observers for 1 hr; the number of pairs mated was also recorded. Soon after copulation mated female was individually aspirated into a new vial containing wheat cream agar medium to check that insemination has occurred or not (by observing larval activity). Mated male was allowed to mate with second female (virgin 5-6 days old). If mating occurred with second female, we allowed the pair to complete copulation and checked for insemination as above. This process was continued and the number of females inseminated by each male in 1 hr was recorded as male mating ability.

Statistical analysis: A total of 50 trials were used separately for each of the three male age classes. Chi-square analysis was carried out on data of female mate choice experiment and paired 't' test on wing length of selected and rejected males in female mate choice experiment. One-way ANOVA followed by Tukey's Honest *post hoc* test (Tukey's test) was carried out on data of mating activities, courtship activities using SPSS 10.0 Programme. In addition Bonferroni correction factor was applied on courtship activity data (tapping, scissoring, vibration, licking, ignoring, extruding, and decamping).

Resuts and Discusion

Mate choice tests: In both monomorphic and polymorphic strains females of *D. ananassae* generally chose to mate with the older of the two competing males of different age classes. Success of old males in crosses involving young and old aged males was 70% in monomorphic strain ($\chi 2 = 8.00$ df = 1; $P < 0.05$; N = 50) and 68% in polymorphic strain ($\chi 2 = 20.48$ df = 1; $P < 0.05$; N = 50). Success of middle aged males involving young and middle aged males was 72% in monomorphic strain ($\chi 2 = 6.48$ df = 1; $P < 0.05$; N = 50) and 73% in polymorphic strain ($\chi 2 = 9.68$ df = 1; $P < 0.05$; N = 50). Old males success in crosses involving middle and old aged males was 64% in monomorphic strain ($\chi 2 = 3.92$ df = 1; $P < 0.05$; N = 50) and 66% in polymorphic strain ($\chi 2 = 5.12$; df = 1; $P < 0.05$; N = 50).

Mean wing length of rejected males was slightly greater than that of mean wing length of selected males in all the combinations of female mate choice experiment in monomorphic and polymorphic strains studied (Table 1). Paired 't' test carried out on mean wing length data of

selected and rejected males showed insignificant variation in both monomorphic and polymorphic strains.

Table 1. Mean wing length (in mm) of selected and rejected males of monomorphic and polymorphic strains of *D. ananassae* in female choice experiment (Values are mean ± SE).

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

NS- Non significant; df – 49.

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

Parameters	Male				F-values
	Strains	Young (2-3days)	Middle (24-25 days)	Old (46-47days)	(df-2,147, 149)
Mating latency (in min)	Mono	24.68 ± .79^{a}	19.90 ± .64^{b}	14.56 ± .51^{c}	58.87**
	Poly	18.86 ± .68^{x}	13.74 ± .52^{y}	10.20 ± .37^{z}	64.27**
Tapping (in no)	Mono	8.92 ± .21^{a}	10.88 ± .28^{b}	11.86 ± .29^{c}	30.67**
	Poly	9.84 ± .34^{x}	11.56 ± .34^{y}	12.88 ± .37^{z}	18.43**
Scissoring (in no)	Mono	9.96 ± .35^{a}	11.08 ± .31^{b}	12.08 ± .36^{c}	9.44**
	Poly	11.56 ± .4^{x}	12.14 ± .38^{y}	13.12 ± .42^{z}	10.12**
Vibration (in no)	Mono	8.50 ± .35^{a}	9.04 ± .39^{a}	11.00 ± .47^{b}	10.37**
	Poly	9.02 ± .41^{x}	10.34 ± .56^{x}	12.60 ± .39^{y}	15.13**
Circling (in no)	Mono	3.40 ± .15^{a}	3.98 ± .14^{b}	4.78 ± .15^{c}	21.06**
	Poly	3.76 ± .18^{x}	5.52 ± .20^{y}	7.08 ± .33^{z}	43.02**
Licking (in no)	Mono	3.08 ± .19^{a}	4.00 ± .21^{b}	4.66 ± .16^{c}	16.83**
	Poly	3.74 ± .26^{x}	4.36 ± .21^{y}	5.24 ± .17^{z}	11.65**
Ignoring (in no)	Mono	5.54 ± .24^{a}	4.32 ± .16^{b}	3.54 ± .20^{c}	26.14**
	Poly	5.04 ± .25^{x}	3.96 ± .17^{y}	2.98 ± .16^{z}	25.94**
Extruding (in no)	Mono	4.94 ± .19^{a}	3.50 ± .14^{b}	2.74 ± .13^{c}	47.56**
	Poly	4.42 ± .20^{x}	3.24 ± .13^{y}	2.32 ± .12^{z}	44.22**
Decamping (in no)	Mono	3.80 ± .16^{a}	3.12± .17^{b}	2.48 ± .11^{c}	18.43**
	Poly	3.46 ± .15^{x}	2.92 ± .16^{y}	2.12 ± .12^{z}	19.95**
Copulation duration (in min)	Mono	3.14 ± .03^{a}	3.64 ± .04^{b}	4.04 ± .04^{c}	87.02**
	Poly	3.74 ± .05^{x}	4.15 ± .04^{y}	4.35 ± .05^{z}	37.40**
Male mating ability (in no)	Mono	1.24 ± .06^{a}	1.46 ± .07^{b}	1.72 ± .07^{c}	12.62**
	Poly	1.66 ± .09^{x}	1.92 ± .08^{y}	2.52 ± .07^{z}	27.82**

**P< 0.001; Note: 1) Different letter (a, b, c for Monomorphic strain; x, y, z for polymorphic strain) in superscript in each row indicates significant by Tukey's test. 2) Mono= Monomorphic strain and Poly= polymorphic strain.

Mating activities: Table 2 shows mean values of courtship and mating activities data of both monomorphic and polymorphic strains. It was noticed that old aged male had lowest time for mating,

copulated longer, performed greater male courtship activities (tapping, scissoring, vibration, licking, circling), and inseminated more females than those of middle aged and young males. Female showed least rejection responses (ignoring, extruding, and decamping) to old aged males, while she showed highest rejection responses to young aged males. One-way ANOVA followed by Tukey's test carried out on mean mating latency, copulation duration, courtship activities, and male mating ability showed significant variation in all the above characters between male age classes, in both monomorphic and polymorphic strains (Table 2). Tukey's test showed that in both monomorphic and polymorphic strains old males had significantly less time for mating, copulation longer, and showed greater mating activities than middle aged and young males. However, wing vibration behavior of young and middle aged males was found to be insignificantly less than old males by Tukey's *post hoc* test. Similarly, middle aged males had significantly less time for mating latency, copulated longer, and showed greater mating activities compared to young males. In turn females significantly showed less rejection behavior to old males compared to middle aged and young males. Polymorphic strains showed significantly greater courtship, mating activities compared to monomorphic strain. In addition to this, principal component analysis has been carried out on courtship activity data Table 3 and Figure 1 (A to D). It was noticed that tapping, scissoring, vibration, and circling were known to influence significantly on male mating success in both monomorphic and polymorphic strains. In turn female rejection responses such as ignoring and extruding have significant influence on the mating success. This result was found to be similar both in monomorphic and polymorphic strains.

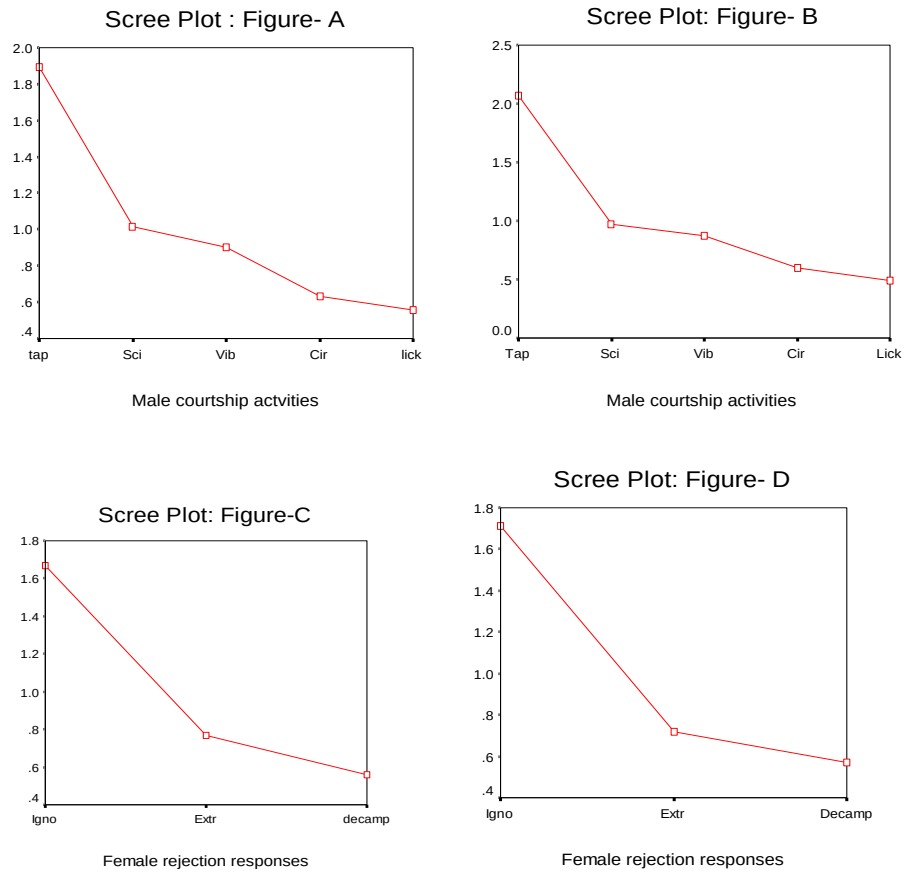

Figure 1. Scree plot of principle component analysis (for male courtship activities: A-Monomorphic, B- polymorphic; female rejection responses: C-Monomorphic, D- polymorphic strains) of *D. ananassae*.

Females of *Drosophila* are able to discriminate males on the basis of size and she preferred larger males more frequently over small males (Patridge *et al.*, 1987; Santos *et al.*, 1992; Hegde and Krishna, 1997; Krishna and Hegde, 2003). As more and more studies have been made, correlation between male size and male mating success was not found to be strong as thought previously (Markow *et al.*, 1996). Apart from male size, male age is another possible cue for female

Table 3. Principle component analysis for male courtship activities and female rejection responses in monomorphic and polymorphic strains of *D. ananassae*.

Male courtship activities		Initial Eigen values			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings		
		Total	% of varience	Cumulative %	Total	% of varience	Cumulative %	Total	% of varience	Cumulative %
Monomorphic strain	Tapping	1.895	37.892	37.892	1.895	37.892	37.892	1.555	31.093	31.093
	Scissoring	1.018	20.350	58.243	1.018	20.350	58.243	1.357	27.150	58.243
	Vibration	0.903	18.055	76.298						
	Circling	0.630	12.598	88.895						
	Licking	0.555	11.105	100.000						
Polymorphic strain	Tapping	2.070	41.401	41.401	2.070	41.401	41.401			
	Scissoring	0.966	19.326	60.727						
	Vibration	0.873	17.455	78.182						
	Circling	0.602	12.049	90.231						
	Licking	0.488	9.769	100.000						
Female rejection responses										
Monomorphic strain	Ignoring	1.670	55.681	55.681	1.670	55.681	55.681			
	Extruding	0.770	25.664	81.345						
	Decamping	0.560	18.655	100.000						
Polymorphic strain	Ignoring	1.710	56.989	56.989	1.710	56.989	56.989			
	Extruding	0.719	23.977	80.966						
	Decamping	0.571	19.034	100.000						

Extraction Method: Principal Component Analysis

preference for male character. In the present study in both monomorphic and polymorphic strains of *D. ananassae* we found that females of *D. ananassae* can discriminate males on the basis of male age, and she preferred old males more frequently than young or middle aged males. This confirms the earlier studies of female discrimination for male age classes in *D. melanogaster* and *D. simulans* (Moulin *et al.*, 2001), in *D. pseudoobscura* (Avent *et al.*, 2008), and in *D. bipectinata* (Somashekar and Krishna, 2011). They found that in these species females preferred old males more than young. These results are also consistent with some previous observations in other insects and birds (Zuk, 1988; Conner, 1989; Hassalquist *et al.*, 1996) and also with the theoretical models of good gene hypothesis (Kokko, 1997, 1998) suggesting that female preference for old aged male can be an evolutionary stable strategy (Avent *et al.*, 2008). In a few of the above studies male age used in their studies did not match with their longevity (Moulin *et al.*, 2001).

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

Insignificant variation was also found in mean wing length of selected and rejected males and even in some combination mean wing length of rejected males were slightly longer than that of selected males but showed insignificant (Table 1). This suggests that observed greater mating success of old aged males was not due to difference in the mean wing length between young, middle and old aged males. Instead it could be attributed to male age itself.

Rearing condition provided for young, middle, and old aged males in the experiment were the same and all these males were unmated; therefore, the observed greater mating success of old aged male cannot be attributed to difference in the rearing condition male experience and male mating

history as found in some previous experiments in insects (Svetec and Ferveur, 2005; Jones and Elgar, 2004).

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

Another potential factor known to influence male mating success in *Drosophila* is male activities and female receptivity during courtship (Bastock, 1956; Hegde and Krishna, 1997). In our study in both monomorphic and polymorphic strains old aged male mated faster (as the time is reverse of the speed), copulated longer, and performed greater courtship activities than young or middle aged males (Table 2). Through these activities old aged male convinced the female faster and had greater mating success, because through these courtship acts male communicates with female through visual, chemical, tactile, and auditory stimuli. As a result he increases the female receptivity (Speith, 1966; Hegde and Krishna, 1997; Tompkin *et al.*, 1982). This confirms the earlier studies of courtship in different species of *Drosophila* suggesting that flies which show greater activities during courtship have greater mating success than males which show less activities (Hegde and Krishna, 1997). Females of *D. ananassae* showed less rejection activities, *i.e*., extruding, decamping, ignoring to old aged males compared to young or middle aged males (Table 2). This suggests influence of male age on female receptivity. This confirms earlier studies of *Drosophila* where females which show less rejection response, have greater receptivity, and have greater mating success than the females which show greater rejection to courting males (Hegde and Krishna, 1997). Studies of sexual behavior in *Drosophila* suggest that courtship activity of male and female culminates in copulation (Spiess, 1970). In our study we noticed that old aged males copulated longer compared to young or middle aged males. This suggests male age influence on copulation duration. This supports the work of Avent *et al*. (2008) who while working in *D. pseudoobscura* has also found longer copulation duration of old age male. It is not known whether the delayed mating could be the reason for older male to copulate longer or not. A strong theoretical reason to expect greater duration of copulation of old age male was that old males have not encountered females for many days to be investigating more resources in the first female he encounters (Wedell *et al.,* 2002) or old age males have lower residual reproduction value and may increase their ejaculate investment per mating with increasing age (Roff, 1992). Also, old males may have accumulated a larger quantity of ejaculate (Sperm and Seminal fluids) that takes a longer time to transfer to the females (Jones *et al.,* 2007). In *D. pseudoobscura* it was found that males kept as virgins for 14 days had high ejaculate quantities (Avent *et al.,* 2008). This suggests that sperm quantity may increase with increasing male age.

The primary function of mating is the transfer of sperm to females, since each mating offers an opportunity for males to produce offspring. Males can generally increase their fitness by mating with many mates, and high mating rates are thus typically associated with high male reproductive success. In our study in both monomorphic and polymorphic strains, old age males had inseminated a greater number of females in a given unit of time than young or middle aged males (Table 2). This suggests that male age has significant influence on male mating ability and old age male had greater male fitness than young male. This supports the earlier studies of male flies which inseminate greater number of females have greater fitness than male flies which inseminate less number of females (Thornhill and Alcock, 1983; Krishna and Hegde, 1997).

In our study we also found that polymorphic strain had greater mating activities over monomorphic strain (Singh and Chatterjee, 1988; Singh, 1989; Singh and Som, 2001).

Thus these studies in *D. ananassae* suggests that females of *D. ananassae* discriminate males on the basis of age and older males had greater reproductive success over young or middle age males, which is found to be independent from influence of inversion system.

Acknowledgments: The authors are grateful to the Professor and Chairman, Department of Studies in Zoology, University of Mysore, for providing facilities and also grateful to the Yuvaraja's College, Mysore. M. Prathibha is also grateful to UGC for awarding teacher fellowship to carry out this work.

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Male age effects on fitness are independent of inversion system in *Drosophila ananassae* (Diptera: Drosophilidae).

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Introduction

Viability indicator models of age-related mate choice predict that females should prefer older males as mates, because they have proven survival ability or because their signals are potentially more revealing (Kokko and Lindstrom, 1996; Kokko 1997). Such models argue that females use age as reliable signal of male quality and that, by mating with older males, they gain indirect benefits through the production of higher-quality offspring. Females in many animal species have mating preferences based on male age. Brooks and Kemp (2001) evaluated the conditions under which preferences for older or younger males are expected to evolve and provided several empirical examples of age-based mate choice across diverse group of taxa. Age based mate choice is often linked to the kinds of reproductive benefits females derive. For example, by choosing males on the basis of age females may obtain direct benefits, such as increased fecundity or hatching success of offspring (Jones and Elgar, 2004; Zuk, 1988), or indirect benefits, such as increased offspring survivorship (Conner, 1989; Jones *et al*., 2007).

Female mate choice is an important feature of most animal mating systems, but the benefits the females derive from their mating preferences often remain obscure (Andersson, 1994). In species in which males provide material benefits to females or their offspring (*e.g*., food, shelter, and parental care), females can obtain direct fitness benefits by preferentially mating with those males most likely to invest materially in females or their offspring.

A series of verbal models (Trivers, 1972; Manning, 1985, 1989) predict that, given a choice, females should prefer older mates. As old males have proven survival ability, choosy females may gain indirect benefits from their choice of an old mate through the production of higher quality offspring. However, testing of indirect benefit must require the next generation and empirical evidence for indirect genetic benefit.

Therefore, the present study has been undertaken in *D. ananassae* which is a cosmopolitan domestic species. Males of *D. ananassae* do not show parental care and only supply sperm during copulation. In addition to this, this species shows a high degree of chromosomal variability (Singh, 1982). It is known that inversion polymorphism in the genus *Drosophila* is of frequent occurrence and has adaptive function (Da Cunha, 1960; Dobzhansky, 1970).

In contrast to direct benefit, whether females also gain a net indirect benefit from that choice of mates is poorly understood. Therefore much attention has been diverted at good genes' indirect benefits, because their magnitude is key to determining the net fitness effects of mate choice, especially in systems in which direct benefits are weak or lacking and indirect costs exist (*e.g*., *D. melanogaster*). Contrasting theoretical analyses have suggested that good genes' indirect benefits are capable of overcoming direct costs of mate choice (Hansen and Price, 1995; Kokko, 1998) or that direct selection on mate choice will commonly overwhelm any indirect benefits (Beck and Powell, 2000). Most studies of age based female mate choice were concentrated on direct benefits, but females also gain indirect benefits from such mating. Therefore, more studies are required to test good gene model of age based female mate choice especially in systems in which direct benefits are weak or lacking and indirect costs exist. Further, studies have also found females of species are selective in their mates even in species in which males provide no material resources to females beyond sperm, suggesting that females derive indirect genetic benefits by mating selectively (Andersson, 1994; Jennions and Petrie, 1997). So we used monomorphic and polymorphic strains of *D. ananassae* in order to study the role of age and inversion on fitness.

Materials and Methods

Experimental stock:

Monomorphic (inversion free) and polymorphic (with inversion) strains of *D. ananassae* were established from already established (Jayaramu, 2009; Prathibha and Krishna 2010) out-bred populations collected at semi-domestic localities of Mysore, Karnataka, India. To study polytene chromosomes and to screen for the presence of inversion, the larvae were dissected in 0.7% sodium chloride solution. These salivary glands were fixed in 1N HCL for five minutes. The stained glands were individually placed on slides with two drops of 45% acetic acid. Then salivary glands were squashed by placing a clean cover glass and by applying uniform pressure. This achieves uniform spreading of the chromosomes. After squashing, edges of the cover slip were sealed with paraffin lanolin mixture. The observation of slides was done under Leitz Ortholux II scientific and clinical microscope both at low (10×) and high (45×) magnification. If inversion loop is present in the larvae then the mother of those larvae was treated as polymorphic. If there is no inversion loop in the larvae then the mother of those larvae was treated as monomorphic. Presence or absence of inversions was checked for five generations. Like this polymorphic and monomorphic strains were established. These experimental stocks were maintained at 21 ± 1°C at relative humidity of 70% using 12:12 L: D cycle. At the 5^{th} generation after testing the inversion, virgin females and bachelor males were isolated within 3 hrs of their eclosion separately from monomorphic and polymorphic strains and aged as required and cultured using same laboratory condition until they were used in the experiments.

Selection of male age classes:

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

Fitness evaluation in monomorphic and polymorphic strains:

We used unmated young, middle, and old aged males and 5-6 day old virgin females of monomorphic and polymorphic strains to study fitness and longevity. A female along with a male (young/middle/old age) were individually transferred into an Elens-Wattiaux mating chamber and observed for 1 hr. Pairs unmated within 1 hr were discarded. All the mated female by each male were individually transferred to a new vial once in 24 hr until their death to study fecundity (number of eggs laid), fertility (progeny production), and longevity. After counting the eggs as above, eggs (100) were taken randomly using spatula to study egg-larval hatchability and were seeded separately for each of the three male age classes in a small petri dish containing wheat cream agar medium. Number of 1^{st} instar larvae emerged from above eggs was counted to account for percentage of egg-larval hatchability. For studying larval-adult viability, 100 1^{st} instar larvae were collected as above and were placed in a petri dish, and the number of adult flies emerged from these larvae were also counted, to account for percentage of larval-adult viability.

Statistical analysis:

A total of 50 trials were used separately for each of the three male age classes. Two-way ANOVA followed by Tukey's Honest *post hoc* test (Tukey's test) was carried out on data of fecundity, fertility, longevity, egg-larval hatchability, larval-adult viability using SPSS 10.0 Programme. Experiments were done separately for both monomorphic and polymorphic strains.

Results

Female fitness:

Females mated to old male had significantly greater number of eggs and progeny number than female mated to young males. These results were found to be similar in both monomorphic and polymorphic strains (Figure 1). These data subjected to one-way ANOVA followed by Tukey's *post hoc* test showed significant variation between male age classes, and Tukey's test found that females mated to old males had significantly greater fecundity, fertility, egg-larval hatchability, and larval-adult viability than those of female mated to either young or middle aged males.

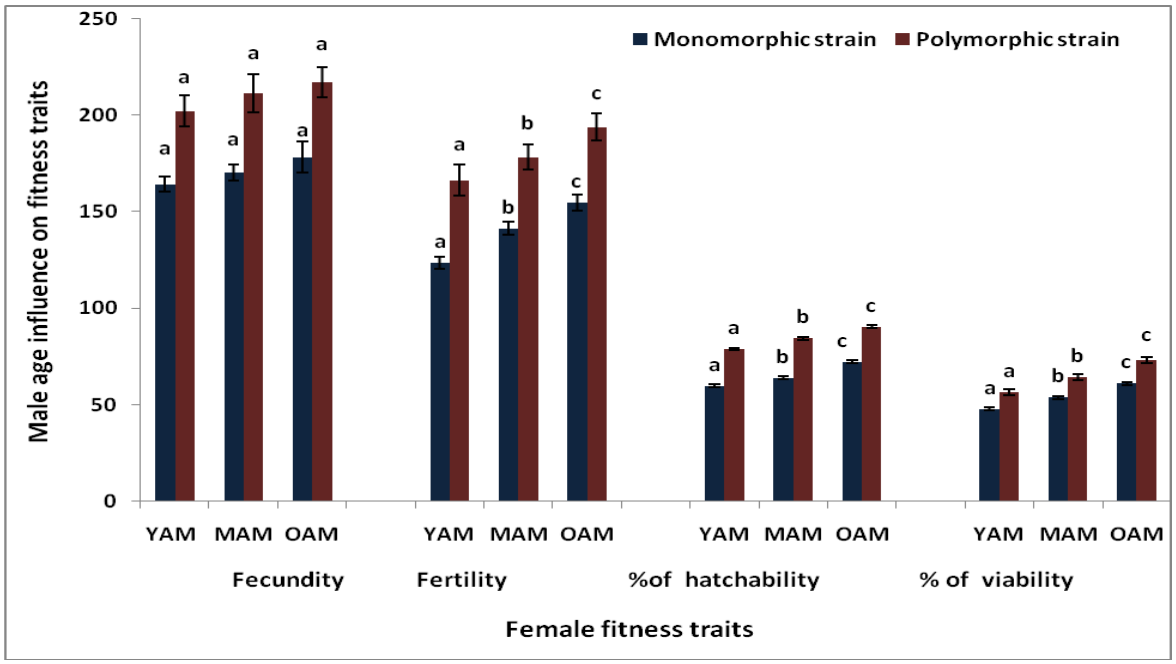

Figure 1. Male age influence on fecundity, fertility, percent of egg hatchability, larva- adult viability in monomorphic and polymorphic strains of *D. ananassae.* (Values are mean ± SE).

YAM-Young age male (2-3 days), MAM- middle age male (24-25 days), OAM-Old age male (46-47 days); Different letter on bar graph indicates significant variation by Tukey's test, df=1, 147. [F-Values of female mated to different male age classes: for fecundity: monomorphic $F=1.51^{NS}$; polymorphic $F=0.76^{NS}$; for fertility: monomorphic F=19.25; P<0.001; polymorphic F=3.67; P<0.05; for % of egg hatchability: monomorphic F=55.18; P<0.001; polymorphic F=57.46; P<0.001 for % of larva-adult viability: monomorphic F=70.97; P<0.001; polymorphic F=34.46, P<0.001].

Figure 1 shows that in *D. ananassae* females mated with old aged males had significantly greater fecundity, fertility, percent of egg-larval hatchability, percent of larval-adult viability, than female mated with young or middle aged males, confirming greater reproductive success of old aged males (Singh 1985a, b; Nicholas *et al.*, 2008a, b; Singh 1989). The result was found to be similar in both monomorphic and polymorphic strains. This could be attributable to longer copulation duration and greater male mating ability. The difference in performance of males of different age classes should be exclusively due to differences in male quality that are attributable to male age. Further experimental design used here eliminated potential influence of female age by mating 5-6 day old female to males of different age classes.

Figure 2. Survival curve of female longevity of *D. ananassae* (A: Monomorphic strain; B: Polymorphic strain).

Table 1. Over all tests of the equality of survival times of monomorphic and polymorphic strains of *D. ananassae.*

Strains	Test Statistics for Equality of Survival Distributions for AGES of Monomorphic and polymorphic strains			
		Statistics	df	Significance
Monomorphic strain	Log Rank	63.37	2	.000
	Breslow	67.36	2	.000
	Tarone-Ware	67.16	2	.000
Polymorphic strain	Log Rank	34.37	2	.000
	Breslow	48.63	2	.000
	Tarone-Ware	42.29	2	.000

Female longevity:

Figure-2 and Table 1 show average longevity of females mated to males of different age classes in both monomorphic and polymorphic strains. It reveals that females mated to young males lived longest followed by females mated to middle aged males, and females mated to old aged males lived shortest. Survival curve analysis showed that female mated with old male survived shorter than female mated with young or middle aged males (Figure 2 and Table 1).

Figure 2 and Table 1 shows that in *D. ananassae* in both monomorphic and polymorphic strains, female mated with old aged male lived significantly shorter period than female mated with young or middle aged males. This suggests significant influence of male age on female longevity, too. It is not known whether accessory gland secretion that is transferred to female during copulation has carried greater harmful effect with increasing male age or the delayed mating of males up to 46

days might have caused carrying greater harmful effect in their ejaculate and form the cause for reduction in female longevity or not. However, these causes have not been tested presently.

Studies in *Drosophila* have also pointed out that during copulation, in addition to sperm, the seminal fluid contains a cocktail of accessory gland proteins that change the post copulation physiological behavior of the female in various ways. Thereby it has been demonstrated that 1) reduce female receptivity to courting males, 2) increase the egg production rate, and 3) decrease female life span. It has been convincingly demonstrated that the reduction in lifespan resulting from exposure to males is a consequence of harmful effects of both male courtship and seminal fluid (Partridge *et al.*, 1987; Chapman *et al.*, 1995; Vahed, 1998; Long and Pischedda, 2005; Nicholas *et al.*, 2008a, b; Bretman *et al*., 2009; Wigby *et al.*, 2009).

Polymorphic strain showed greater fitness than monomorphic strain. This confirms the earlier studies of greater fitness of polymorphic strain over monomorphic strain (Spiess, 1970; Singh, 1985a, b; Singh and Chatterjee, 1988; Singh, 1989; Singh and Som, 2001). Even though the polymorphic strain showed greater fitness over monomorphic strain, the inversion system does not affect the age based fitness.

Acknowledgment: The authors are grateful to the Professor and Chairman, Department of Studies in Zoology, University of Mysore, for providing facilities and also grateful to the Yuvaraja's College, Mysore. M. Prathibha is also grateful to UGC for awarding teacher fellowship to carry out this work.

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Reproductive age and copulation duration in *Drosophila* species with females with low re-mating frequency.

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In this note, we report a by-product information obtained in our research: the reproductive age, overall mating speed, and copulation duration in three *Drosophila* species reported with zero remating frequency (Markow and O'Grady, 2006): *D. acanthoptera* Wheeler 1949, *D. biarmirpes* Malloch 1924, and *D. subobscura* Collin 1936. Nevertheless, McRobert *et al.* (1997) indicated that 26% of the *D. biarmipes* females mate a second time after 8-10 days. On the other hand, Loukas *et al.* (1981) reported that 23% of the females re-mate in their life and Pitnick and Markow (1994) indicated that 46.7% of the *D. acanthoptera* females re-mate 8 days after first mating.

We obtained from the UC San Diego *Drosophila* Stock Center, *D. acanthoptera* 15090-1693.00, *D. biarmirpes* 14023-0361.06, and *D. subobscura* 14011-0131.13. We maintained *D. biarmipes* and *D. subobscura* at 20°C and *D. acanthoptera* at 24°C. We used CO_2 at 5 psi to collect virgin flies within an hour of eclosion, and we placed the flies individually in 2-ml vials until we used them in both tests. We used a modified Bloomington cornmeal recipe to handle our experiment (Castrezana, *personal communication*).

First, we look out the age in which both sexes were sexually mature. We used the following protocol: we aspirated a virgin female and a virgin male inside a vial with food. We discarded the male after 24 hours and we checked for larvae after 72 hours. For the three species, we tested pairs of flies at eight different ages (N > 15 pairs/age). We found that > 93% of the individuals for both sexes are mature at five days-old in *D. biarmipes*, and eight days-old in *D. acanthoptera* and *D. subobscura*. It is known that in some *Drosophila* species the females and the males can mature at markedly different times. Therefore, our second step was to search for possible reproductive age differences between males and females. We repeated the previous protocol but this time one of the partners used was sexually mature (five days-old in *D. biarmipes*, and eight days-old in *D. acanthoptera* and *D. subobscura*). The results are presented in the Table 1. We found a noticeable difference between the time each sex takes to mature in *D. biarmipes* and *D. subobscura*. In addition, we observed that all *D. biarmipes* males who mate successfully in the 0-24 hr period had a black patch in both wings.

Table 1. Reproductive age, by sex, in *Drosophila acanthoptera, D. biarmipes*, and *D. subobscura.* To test *D. biarmipes,* we used five days-old partners and eight days-old for *D. acanthoptera* and *D. subobscura*. Each period of time has N=15.

species	0-24 hrs	24-48 hrs	48-72 hrs	72-96 hrs	96-120 hrs	120-144 hrs	144-168 hrs	168-192 hrs
D. acanthoptera (females)	0.0%	0.0%	0.0%	13..3%	66.7%	86.7%	86.7%	100.0%
D. acanthoptera (males)	0.0%	0.0%	0.0%	0.0%	40.0%	73.3%	93.3%	93.3%
D. biarmipes (females)	40.0%	53.3%	53.3%	73.3%	100.0%			
D. biarmipes (males)	60.0%	66.6%	100.0%					
D. subobscura (females)	0.0%	0.0%	13.3%	13.3%	20.0%	40.0%	60%	93.3%
D. subobscura (males)	0.0%	0.0%	13.3%	60.0%	80.0%	93.3%	93.3%	

On the other hand, we obtained two reproductive behaviors: overall mating speed and copulation duration. Overall mating speed is defined as the sum of the male courtship latency (which is the time when the male went inside the vial until the time it started to court the female) plus the female receptivity (which is the time when the male started to court until the time female accepted to mate). Copulation duration is the time in which the female accepted to mate until the pair finished mating (Castrezana and Markow, 2008).

Table 2. Overall mating speed and copulation duration in *Drosophila acanthoptera, D. biarmipes*, and *D. subobscura* (time in minutes). Pairs were scored at five days-old for *D. biarmipes* and eight days-old for *D. acanthoptera* and *D. subobscura*.

species	Pairs mating within the first three hours encounter	Minimum-maximum overall mating speed time	Overall mating speed average time	Minimum-maximum copulation duration time	Copulation duration average time
D. acanthoptera	25/69	8.8 - 178.8	74.0 ± 11.4	9.5 - 161.3	122.1 ± 5.50
D. biarmipes	54/69	0.3 - 149.91	35.6 ± 5.2	3.2 - 37.8	21.3 ± 0.8
D. subobscura	52/69	3.6 - 179.1	52.5 ± 6.9	4.6 - 28.6	10.7 ± 0.6

For this behavioral section, we aspirated a mature virgin female and a mature virgin male inside a vial with food. Then, we set the vial on its side over a white surface. A well trained observer recorded the time when flies were introduced in the vial, the time when the flies started mating, and the time the flies finished mating. The observer watched the flies for three hours. Pairs of flies that did not mate within the first three hours of encounter were checked 48 hours later. Only two *D. acanthoptera* pairs did not lay eggs 48 hours after initial encounter. We present the overall mating speed and the copulation duration in Table 2.

Our *D. acanthoptera* copulation duration result did not differ from the data presented by Pitnick *et al.* (1991). However, we suspect that both results could be erroneous. Later in our experiment, we noticed that a *D. acanthoptera* male has a long and passive overall mating speed when it is isolated from other males. However, when we observed *D. acanthoptera* males in large population bottles, we noticed that a *D. acanthoptera* courting male is extremely aggressive with other males who tried to court the female. In fact, a male flattering a female pursued other males around the bottle for several seconds. Then, the male returned to continue the courtship. In addition, we noticed that mating pairs of *D. acanthoptera* can come apart easily after some time. We carefully observed a *D. acanthoptera* pair during copulation under 8× Leica microscope. We found that the distiphallus of the aedeagus was retracted after an hour of copulation. Nevertheless, the presisetea continued in contact with the vaginal plates. At this instant, we consider that *D. acanthoptera* may have a type of male guarding behavior. However, this male behavior should be considered "passive", because we could not observe mating pairs disturbed by other males during copulation, an opposite behavior observed in *D. pegasa* Wasserman 1962 and *D. mainlandi* Patterson 1943, where sometimes a second and third male mounted behind the back of the guarding male.

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Population survey of *Drosophila* species in the Philadelphia area, Pennsylvania, U.S.A.

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In 1986, a survey of the *Drosophila* community was conducted in an area just west of Philadelphia, Pennsylvania. In this survey, nine different species were found, with *D. affinis* and *D. melanogaster* being the most prevalent species in the spring and summer, and *D. immigrans* being the most prevalent species in the fall (McRobert and Tompkins, 1986).

Since the 1986 survey, a new invasive species indigenous to Southeast Asia, *D. suzukii*, has entered the U.S. and has been found in the Philadelphia area (Bolda, 2010; Freda, 2013). *D. suzukii* was introduced to Hawaii in the 1980s and spread to California and Florida by 2008 (Carvajal, 2010). The invasion of *D. suzukii* is of concern as this species acts as a true fruit fly, laying eggs in fruit that is still on the vine or tree (Cini, 2012). *D. suzukii* females have large, serrated ovipositors that allow them to cut into soft-skinned fruits to lay their eggs (Walsh, 2011). Thus, oviposition from this species damages fruit and exposes plants to microbial pathogens, ruining crops such as cherries, raspberries, blueberries, blackberries, strawberries, and others (Walsh, 2011).

The purpose of this study was to replicate the 1986 survey and describe the *Drosophila* species in the Philadelphia area in 2013. We hope that this project will serve as a foundation to document the movement of *D. suzukii* into this area and the possible effects of this invasion on the diversity of the *Drosophila* community.

Table 1. *Drosophila* species collected in the Philadelphia area in 2013.

Species	No. of Individuals Collected											
	January	February	March	April	May	June	July	August	September	October	November	December
D. affinis	0	5	115	75	48	87	50	8	22	22	5	2
D. algonquin	0	22	68	41	12	38	8	12	21	18	0	0
D. buskii	0	0	0	3	57	109	51	37	5	9	5	0
D. ducani	0	0	0	0	19	3	109	27	34	106	33	15
D. immigrans	0	0	0	0	0	0	118	118	91	126	73	18
D. melanica	0	0	0	0	0	0	0	0	1	2	2	0
D. melanogaster	0	4	4	7	39	6	75	108	87	24	4	0
D. putrida	0	0	10	0	79	85	74	33	19	19	4	0
D. quinaria	0	0	0	0	0	196	323	0	8	9	3	1
D. robusta	0	0	5	6	82	63	31	12	5	9	3	0
D. simulans	0	0	0	0	11	43	245	86	50	95	6	2
D. suzukii	0	0	0	0	0	0	0	11	427	389	208	74
D. tripunctata	0	0	0	0	20	108	83	41	24	20	2	0
Unknown	0	0	2	1	10	21	22	15	35	25	12	3

From January through December 2013, plastic cups, baited with a banana-yeast mixture and suspended horizontally at eye level from trees, were set in several suburban locations within 5 miles of Philadelphia. Collections of flies were performed every Monday, Wednesday, and Friday, and new bait was added to each trap every Monday. Flies were collected by placing a large funnel over the opening of each cup and allowing the flies to enter a vial. In the lab, the collected flies were

anesthetized using CO_2 and identified using a dichotomous key of North American Drosophilids. Female flies of species requiring male characteristics for identification were held in vials containing Carolina Instant Media + 5% propionic acid + yeast until they produced offspring; then their male offspring could be identified.

During the 2013 survey, we encountered 13 *Drosophila* species, nine of which were found during the 1986 survey, along with four new species: *D. algonquin*, *D. putrida*, and *D. simulans*, and *D. suzukii* (Table 1). *D. suzukii* did not appear in collections until August, possibly a result of die-off during the preceding winter. Perhaps of some concern, when considering total fly counts during the year, the invasive *D. suzukii* ranked as the most common species in the Philadelphia area in 2013 (Figure 1).

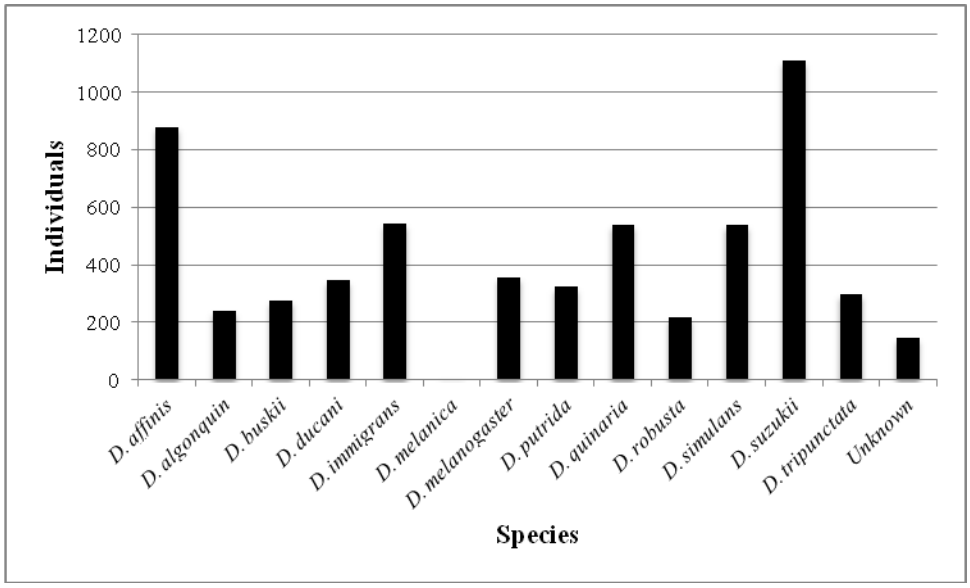

Figure 1. Total individuals/species for 2013.

Acknowledgments: This research was supported by the Saint Joseph's University Summer Scholars Program, a HHMI Undergraduate Education Grant awarded to L.A.T, and a grant from the Saint Joseph's University chapter of Sigma Xi awarded to L.A.T.

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Extension of *Drosophila melanogaster* lifespan by *Decalepis hamiltonii* root extract.

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Introduction

Harman's free radical theory of aging (Harman, 1956) proposes that the free radicals, such as highly reactive derivatives of oxygen, are produced during the normal course of cellular metabolism. The organism fails to counteract all the damage done to macromolecules by free radicals as the balance between oxidants and antioxidants, such as free radical-detoxifying enzymes, acts in favor of oxidants. Over a period of time, these unrepaired damages accumulate due to the altered homeostasis leading to aging and death.

The hypothesis that states free radicals, or more specifically oxygen radicals, cause the biological damage leading to the physiological decline is only now beginning to be adequately appreciated. Although there is no general agreement about the mechanisms underlying aging, the free radical theory of aging has held considerable appeal for many years. Some predictions were made from the free radical theory. According to these, adding antioxidants to the diet that changes the balance between oxidants and antioxidants should increase longevity. Increase in the activity of antioxidant enzymes is also expected to increase longevity. Attempts to relate free radical theory to lifespan in *Drosophila* were initiated by Loeb and Northrup (1917). *Drosophila* is particularly an attractive model to study the aging process as many candidate plant compounds can be easily mixed with food for feeding the larvae or adult flies. The *Drosophila* model could be useful for translational research for discovery of new therapeutic strategies to prevent or ameliorate age-related damage including neurodegenerative disorders (Kim *et al.,* 2011).

Natural compounds with free-radical scavenging activity play a potential role in maintaining human health and preventing diseases. Antioxidant phytochemicals promote human health by exhibiting diversified physiological and pharmacological effects namely, inactivating carcinogens, stimulating the immune system, protecting the heart from free-radical attack, and in preventing the eye lens cataracts (Srivastava *et al.,* 2011). Studies have shown that plant-derived antioxidant nutraceuticals scavenge free radicals and modulate oxidative stress-related degenerative effects (Thatte *et al.,* 2000). There is a great deal of interest in newer natural bioactive molecules with health-promoting potential (Srivastava *et al.,* 2006). Uysal *et al.* (2009) reported that an aqueous extract obtained from the lichen *Usnea* (*Dolichousnea*) *longissima* Ach. (Ascomycota, Parmeliaceae) enhanced lifespan and fecundity of *D. melanogaster* at low doses, but had deleterious effects at higher doses. In addition, the extract obtained from the epiphytic lichen *Lobaria pulmonaria* (L.) Hoffm. (Lobariaceae) caused a dose-dependent increase in the longevity of male and female populations of *D. melanogaster* (Uysal *et al.,* 2010).

Decalepis hamiltonii (family: Asclepediaceae) grows wild in the forests of peninsular India. Its tubers are consumed as pickles and also as juice for its health-promoting properties (Harish *et al.,* 2005; Srivastava *et al.,* 2006). The root extract of *D. hamiltonii* is a cocktail of novel antioxidants that exhibit cytoprotective and chemoprevention potential *in vitro* and *in vivo* (Srivastava *et al.,* 2007, 2011, 2012). *D. hamiltonii* root extract also shows neuroprotective property *in vivo* in *Drosophila* (Jahromi *et al.,* 2013). In view of the health-promoting potential of *D. hamiltonii* root extract, we investigated whether it could have an impact on longevity in *Drosophila*.

Materials and Methods

Drosophila melanogaster (Oregon K strain) obtained from the Drosophila Stock Center, Manasagangotri, Mysore was employed for the study. This stock was maintained in a vivarium at 22 ± 1°C on standard wheat cream agar medium with 12:12 light and dark cycles. Synchronized eggs

were collected (Delcour, 1969) and were raised under uniform conditions of temperature, humidity, food medium, and density. The flies obtained from these cultures were used for the adult lifespan assay. Both methanolic and aqueous extracts of *D. hamiltonii*, which are mixed thoroughly in the cream agar medium, were used for the present study. Three concentrations of aqueous extract (0.1%, 0.5%, and 1%) were prepared and were mixed into the culture medium. The flies obtained from standard experimental cultures served as control. Mixing and feeding of flies with the extract were standardized. This medium was changed every 5-7 days depending upon the condition of the culture media. Five vials for each concentration (20 flies in each vial) were taken. The mortality rate was recorded every day. This procedure was carried out until the death of all flies in all the vials.

Results

Lifespan assay carried out with *D. hamiltonii* extract showed that the methanolic extract does not have much effect in extending lifespan when compared with the aqueous extract in *Drosophila melanogaster*. Therefore, for the current study, only aqueous extract was employed. Our results revealed significant effect of *D. hamiltonii* on lifespan extension in *D. melanogaster* (Figure 1). This result further showed a difference in the male and female lifespan ratio of *D. melanogaster* fed with *D. hamiltonii*. There was a significant survivability of females over males both in flies treated with and without *D. hamiltonii*. There was also significant difference in the lifespan between males and females in both control and *D. hamiltonii*-fed groups. 1% aqueous extract was found to significantly extend the lifespan more than the other two lower concentration extracts. Therefore, 1% aqueous extract was employed for all the experiments that were conducted later.

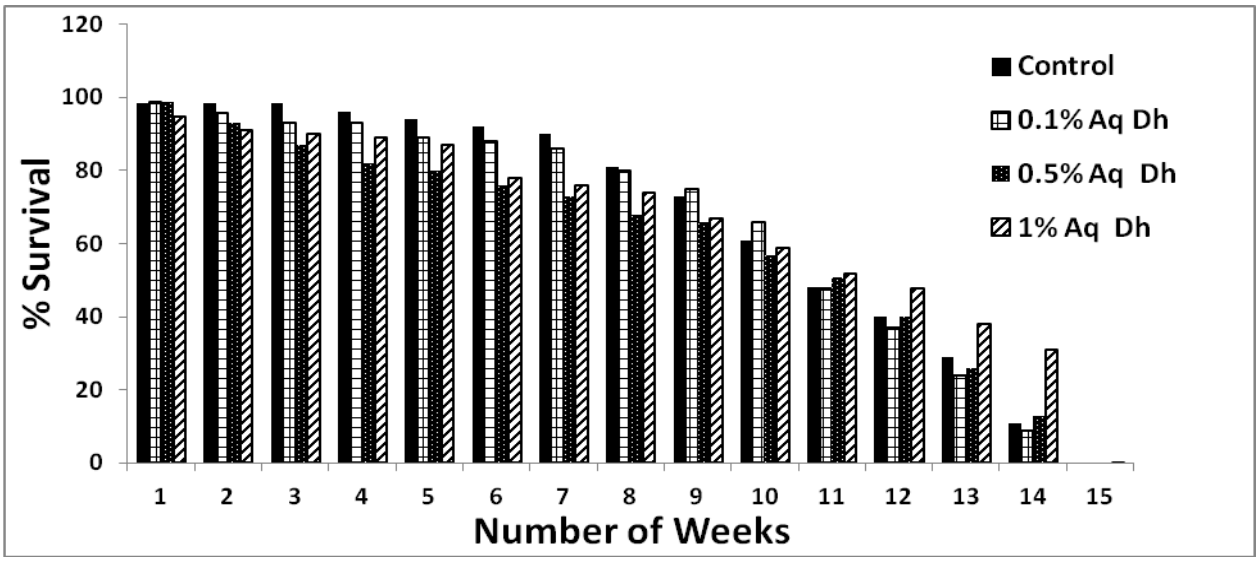

Figure 1. Survival curves of *D. melanogaster* fed with 0.1%, 0.5%, and 1% aqueous (Aq) *D. hamiltonii* (*Dh*) root extract.

Discussion and Conclusion

Oxidative stress occurs when a variety of antioxidant defense mechanisms are overwhelmed by environmental exposure or intrinsic factors, and may lead to cell damage and death. Therefore, major focus of therapeutic research has been to reduce oxidative stress using antioxidant compounds.

Peng *et al.* (2009) have showed that black tea extract, a mixture of epicatechins and theaflavins, when added to the culture medium, prolonged the survival of wild-type *Drosophila* exposed to paraquat or hydrogen peroxide. Similar beneficial effects on survival were observed with combinations of catechin, epicatechin, and glutathione, which have high superoxide-scavenging activity (*cf.*, Kim *et al.*, 1997). It has been earlier shown that the root extracts of *D. hamiltonii* contain at least a dozen distinct antioxidant compounds that scavenge free radicals and chelate metal ions (Harish *et al.*, 2005; Srivastava *et al.*, 2006). In the present study, we have found that the aqueous extract of *D. hamiltonii* significantly extends the lifespan of *D. melanogaster*, which could be attributed to the free radical scavenging and cytoprotective property of the bioactive molecules. Further, we have recently shown that *D. hamiltonii* root extract shows neuroprotective potential in *Drosophila*, which strongly implies that neurodegenerative changes associated with aging are delayed (Haddadi *et al.*, 2013). Therefore, *D. hamiltonii* roots could be useful natural antioxidant-rich supplement that exhibits therapeutic potential in aging individuals.

Acknowledgment: We thank The Chairman of the Department for the facilities. The first author thanks the Department of Science and Technology, Govt. of India, for the financial support under the INSPIRE Program.

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Radiation induced toxicity in *Drosophila melanogaster*.

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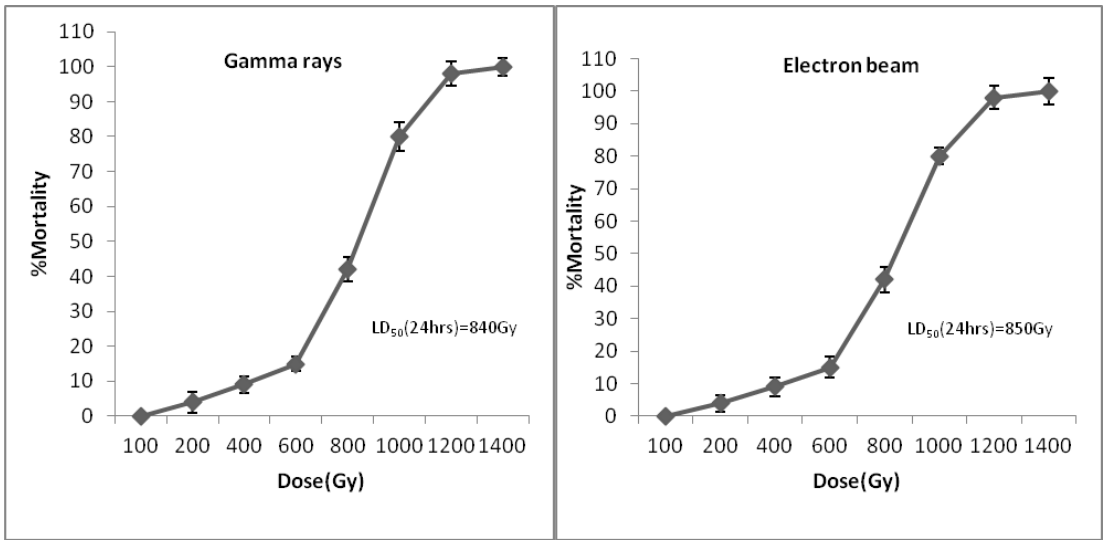
Radiotherapy is an important treatment regimen for cancer that could be used as a single treatment or along with surgery and/or chemotherapy. However, use of ionizing radiation is compromised by the side effects that result from radiation-induced damage to normal tissue. Radiation treatment leads to destruction of proliferating cells in tissues, such as lymphoid organs, bone marrow, intestinal crypts, testes, and ovaries, and long-term fibrotic damage to the soft tissues that limit their function (Hall, 2000). *Drosophila melanogaster* is an excellent model organism for

varied kinds of biological investigations in human disease and toxicological research (Sharma *et al.*, 2011; Mukhopadhyay *et al.*, 2003; Siddique *et al.*, 2008). It is also used by radiation biologists as an *in vivo* model to study radiation induced oxidative stress and radioprotective agents (Bozuck, 1972; Ducoff, 1972). In order to evaluate radioprotective agents, we have investigated the *Drosophila* model for radiation sensitivity and we report herein the radiation toxicity of gamma rays and electron beam in *D. melanogaster*.

D. melanogaster (Oregon K) strain was obtained from the *Drosophila* Stock Center, Department of Studies in Zoology, University of Mysore, Mysore. Experimental stocks of 5 day old flies were built up by the serial transfer method, and these flies were maintained on standard wheat agar medium at 22 ± 1°C and 70–80% relative humidity in a vivarium.

The flies were irradiated with 100 Gy, 200 Gy, 400 Gy, 600 Gy, 800 Gy, 1000 Gy, 1200 Gy, and 1400 Gy of Cobalt-60 Gamma radiation (Gamma chamber 5000) at Centre for Application of Radioisotopes and Radiation Technology (CARRT), (Mangalagangotri, Konaje, Karnataka, India) at a source strength of 14,000 Ci (Curie) that delivers about 9 kGy/hr (kilo Grey per hour), and electron beam current of 50 mA and dose rate of 2 kGy/min was administered to whole body of the *Drosophila* enclosed in the polypropylene tubes of 65 × 25 mm size.

The median lethal dose (LD_{50}), the dose that causes 50% mortality in 24 hrs, was determined. After the exposure of the flies to the radiation they were transferred to new media bottles and the number of dead flies in each dose was counted at 24 hrs and LD50 was calculated by using probit analysis.



A B

Figure 1. Radiation induced mortality in *D. melanogaster* (A) gamma rays (B) electron beam. LD_{50} (24 hrs) is the dose at which half of the population dies at 24 hrs after exposure determined statistically by probit analysis. Values are given as mean ± S.D of mean (Each set contained 25 flies × 6 replicates).

From Figure 1 it is evident that both gamma radiation and electron beam did not cause any mortality at 100 Gy and 200 Gy. At higher doses of 600 Gy to 1000 Gy there was dose dependent mortality in flies exposed to gamma radiation. The pattern of lethality was also similar in flies

exposed to electron beam. LD_{50} (24 hrs) were 840 Gy and 850 Gy for gamma rays and electron beam, respectively. Dose from 100 Gy to 400 Gy was considered as sublethal in both the radiations. From the results, it is clear that *Drosophila* is tolerant to radiation up to 850 Gy. Although there are several studies on gamma radiation induced toxicity in *D. melanogaster*, our study is the first to report the toxicity of electron beam radiation on *D. melanogaster*.

The results of LD_{50} dosage and sublethal dosage of gamma rays and electron beam will be useful in evaluating the natural radioprotective agents in *D. melanogaster*. These studies are currently in progress in our laboratory.

Acknowledgments: We thank The Chairman of the Department for the facilities. The authors thank the Department of Atomic Energy (DAE), Govt. of India, for financial support (2011/34/18/BRNS/0587).

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Drosophilid collection in the Font Groga site, Barcelona (Spain).

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During October 23rd and 24th and November 2012 we collected a sample of drosophilids at Font Groga (Barcelona). This site is located on the foothills of the Tibidabo mountain, which is located on the northwest edge of Barcelona and at approximately 400 m above sea level. The vegetation is typical for the area, and it is mainly composed of a sparse pine forest (*Pinus pinea*) with some oaks (*Quercus ilex*) and Mediterranean brushwood. Flies were netted over 12 baits containing fermenting bananas. Font Groga is a common place for sampling drosophilids (Araúz *et al.*, 2009; Calabria *et al.*, 2012). The distribution of trapped flies, according to species and sex, is presented in Table 1.

Table 1. Number of adult flies collected in Font Groga (Barcelona, Spain) in autumn 2012.

Species	23rd Oct.	24th Oct.	6th Nov.	Total	Percentage
D. subobscura (♂)	3	14	1	18	3.52
D. subobscura (♀)	6	11	0	17	3.33
D. melanogaster (♂)	2	5	0	7	1.37
D. simulans (♂)	75	95	1	171	33.46
D. melalo/simulans (♀)	71	161	4	236	46.18
D. suzukii (♂)	4	7	0	11	2.15
D. suzukii (♀)	3	31	2	36	7.05
D. immigrans (♀)	6	7	0	13	2.54
D. phalerata (♀)	1	0	0	1	0.20
Scaptomiza sp.	1	0	0	1	0.20
Total	172	331	8	511	100

A large proportion of *D. simulans* males was found. The invasive species *D. suzukii* (Calabria *et al.*, 2010; Cini *et al.*, 2012) was detected in a non-negligible quantity. Taking into account the number of males and females, the estimated N_e for *D. suzukii* in the Font

Groga sample was 33.70. A similar value was obtained for *D. subobscura* (34.97). Finally, in the study of species diversity the values obtained for *H'* (Shannon diversity index) and *J* (Shannon uniformity index) were 0.678 and 0.421, respectively. These estimates are very similar to those obtained in September 2009 in Montpellier by Calabria (2012), who reported *H'* = 0.679 and *J* = 0.422, but differ from those reported by the same author in a Font Groga sample of October 2007 (*H'* = 0.904 and *J* = 0.505).

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Evidence of selective mating in *D. malerkotliana*: greater reproductive success of wild flies than Spw mutant.

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Introduction

Mating behavior of *Drosophila* consists of specific actions which are accompanied by orientation movements. Such actions referred to as courtship displays are performed sequentially. Mating occurs only if the female responds by performing acceptance signals. In *Drosophila* mating behavior has been studied using various species, strains and mutants (Merrell, 1949; Reed and Reed, 1950; Rendel, 1951; Bastock, 1956; Petit, 1959; Barker, 1962). These studies have shown the genetic control of this behavior. Though mutants are rare events, they form the source of variations for evolution and through these mutants the functioning of many genes can be understood. Therefore more studies using mutants are warranted.

In *Drosophila* the pattern of mating has been tested using mutants (Merrell, 1949; Crossley and Saul, 1970; Rendel, 1951). Some of these studies showed the occurrence of selective mating while others found a lack of selective mating. The lack of selective mating could be due to changes in their behavior patterns causing them to provide sub-optimal courtship to reduce activity generally (Bastock, 1956; Kyriacou *et al.*, 1978) so that females reject more frequently than wild males. In almost all these studies mutants of *D. melanogaster* have been employed due to availability of mutants in this species. In contrast to this only a few mutants have been identified and described in other species of *Drosophila*, such as *D. hydei, D. virilis, D. subobscura, D. pseudoobscura, D. ananassae, D. bipectinata, D. nasuta*, and *D. malerkotlinana* (Lifechytz, 1974; Strursa, 1983; Mohanty *et al.*, 1988; Lozovskaya and Evengener, 1991; Singh and Sisodia, 1999). Therefore, in the present study Spw mutant of *D. malerkotliana* has been employed to study the role of Spw male and female in pre mating and post copulatory success in *D. malerkotliana*. *D. malerkotliana* is a member of the *bipectinata* species complex of the *ananassae* sub group of the *melanogaster* species group distributed in South East Asia. In the laboratory stock of this species, Spw spontaneous recessive autosomal mutation has been detected (Krishna and Hegde, 1998). Therefore, in the present study wild and mutant spread winged strains of *D. malerkotliana* were used to study the role

of spread winged mutant male and female behavior in pre mating success and post copulatory success in *D. malerkotliana*.

Materials and Methods

Stocks used in the present study were wild and Spw mutant (wings spread at 45°) strains of *D. malerkotliana*. When progeny appeared, flies were distributed to different culture bottles and were maintained under constant temperature (22 ± 1°C). For every generation, flies multiplied in different culture bottles were mixed together and eggs were collected using Delcour's (1969) procedure. Eggs (100) were seeded in fresh quarter pint milk bottles with 25 ml of wheat cream agar medium to avoid larval competition during development. From these culture bottles virgin females and males were isolated within three hours of their eclosion and maintained separately at 22 ± 1°C in fresh food vials containing yeast and aged for 5-6 days.

Virgin females and males (aged 5-6 days) were used to see whether there is any difference in mating success between mutant and wild strains of *D. malerkotliana*. In a multiple choice experiment, 10 females of each of the two strains were introduced into the mating chamber along with 10 males of respective strains and were observed for 60 min. When a pair commenced mating it was aspirated out and the type of individuals mated was recorded. Total 5 trials were run.

In a male choice experiment, a mutant male was introduced together with a mutant and a wild female into the mating chamber. A reciprocal cross was also made with wild male and two females, one mutant and one wild. Similarly, in the female choice experiment, we introduced a wild female together with a wild and a mutant male. Reciprocal crosses were also made here. Total 50 trials were run for each experiment.

Different crosses were made (wild male × wild female; wild male × mutant female, wild female × mutant male; mutant male × mutant female) to study the various components of sexual selection. Twenty five pairwise matings were done for each combination. Courtship latency (time between introduction of male and female together in mating chamber until orientation of male to female), mating latency (time between introduction of male and female together in mating chamber until initiation of copulation of each pair), and copulation duration (time between initiation of copulation to termination of copulation of each pair). Courtship acts such as tapping, scissoring, vibration, licking, circling, ignoring, extruding, and decamping were measured following the procedure of Hegde and Krishna (1997). Soon after mating, mated males were transferred individually into a separate vial to study remating ability of male and mated females. They were then transferred into fresh food vials every 24 h without anesthesia to study fertility. Total number of progeny emerged in such vials were counted over a period of 15 days.

To study remating ability of male, mated males were placed separately in the vials and were provided with virgin females (wild/mutant female depending on crosses). Soon after mating the mated female was aspirated out and replaced with another virgin female, and the same procedure was repeated. The observation was made for 2 h and the number of females mated by a single male was recorded. If there was no mating within 2 h, then the pairs were discarded and mean remating ability of male was calculated.

To study female remating, 25 mated females obtained as above were transferred individually into Elens-Wattiaux mating chamber containing 5-6 days aged male. Each female was allowed to stay with this male for 2 hours. Then the female was aspirated out from the Elens-Wattiaux mating chamber and placed back in its marked food vial. This procedure was repeated every day until the

female mated with a second male. The total number of females remated and the interval in terms of days in each cross was recorded.

To measure longevity, different crosses were made using virgin males and females. The pairs were placed in a fresh food vial containing wheat cream agar medium. Active yeast was also added to vials 48 h prior to use. The vials were renewed daily during measurement of longevity. All cultures were maintained at 22 + 1°C and at 12:12 light/dark cycle.

Results

Mating success of wild and mutant flies of *D. malerkotliana* in multiple choice method showed that in 62 crosses wild males mated with wild females, and in 54 mutant males mated with mutant females as against 44 matings of wild males with mutant females and 32 matings of mutant males with wild females ($F = 78$, df = 3, 188; $P < 0.0001$). This shows homogamic matings were more than heterogamic matings. Even in male choice situations, out of 50 wild males, 37 mated with wild females and the remaining males mated with mutant females ($\chi^2 = 11.52$, df = 1, $P < 0.01$). In reciprocal crosses, out of 50 mutant males, 36 mated with mutant females while remaining 14 mutant males mated with wild females ($\chi^2 = 9.68$, df = 1, $P < 0.001$). In female choice method, wild females mated with wild males. Out of 50, 41 wild females paired with wild males while the remaining 7 females mated with mutant males ($\chi^2 = 20.48$, df = 1, $P < 0.001$). In 50 reciprocal crosses, 44 mutant paired with wild males and remaining 6 mutant females mated with mutant males ($\chi^2 = 28.88$, df = 1, $P < 0.001$). In both the crosses wild males were more successful than mutant males.

Table 1. Mating behaviour and fitness characters of wild and spread winged mutant strains of *D. malerkotliana.*

Parameters	Crosses				
	Wild Male x Wild Female	Wild Male x Mutant Female	Mutant Male x Wild Female	Mutant Male x Mutant Female	F-Value
No. of Pairs	25	25	25	25	
Pairs copulating(%)	92	84	56	60	
Courtship latency	4.15 ± 0.41	5.02 ± 0.45	11.05 ± 0.37	9.00 ± 0.31	99.24**
Mating latency	4.36 ± 0.33	5.08 ± 0.33	11.72 ± 0.27	9.52 ± 0.36	106.02***
Tapping	14.56 ± 0.41	13.00 ± 0.43	9.15 ± 0.43	8.51 ± 0.65	68.00**
Scissoring	18.35 ± 0.56	18.02 ± 0.31	14.31 ± 0.45	12.52 ± 0.61	71.00**
Vibration	10.35 ± 0.35	10.41 ± 0.39	9.12 ± 0.31	8.81 ± 0.26	9.68*
Licking	6.35 ± 0.42	6.02 ± 0.31	5.42 ± 0.16	5.12 ± 0.19	12.15**
Circling	19.38 ± 0.51	18.48 ± 0.26	15.15 ± 0.39	15.85 ± 0.41	56.10**
Ignoring	6.31 ± 0.26	4.49 ± 0.26	7.39 ± 0.36	4.81 ± 0.31	14.12**
Extruding	21.51 ± 0.42	17.21 ± 0.35	25.42 ± 0.51	18.21 ± 0.32	59.37**
Decamping	18.97 ± 0.24	14.31 ± 0.35	21.42 ± 0.62	15.32 ± 0.61	50.37**
Compulation duration	11.52 ± 0.30	9.84 ± 0.32	6.60 ± 0.21	8.28 ± 0.29	51.35**
Fertility	285.00 ± 2.53	243.60 ± 5.64	195.60 ± 3.67	222.24 ± 4.82	71.84**
Male mating ability	4.60 ± 0.23	5.00 ± 0.27	2.56 ± 0.14	3.60 ± 0.28	21.03**
Female remating (%)	63.23	54.51	95.27	61.29	39.21**
Female remating interval (in days)	11.51 ± 0.21	14.32 ± 0.42	4.21 ± 0.162	7.18 ± 0.29	195.21**

(Mean values are reported with standard error)
Df = 3.69; * P<0.05; ** P<0.01; *** P<0.001

Table 1 shows courtship and mating activities of different crosses. Highest mating was noticed in pairs involving wild males and females while lowest mating was observed in crosses involving mutant males and wild females. Courtship and mating latency were shortest in crosses of wild males and females while they were highest in crosses of mutant males and wild females indicating that wild males oriented quickly and mated faster than mutant males. Even in male courtship patterns, such as tapping, scissoring, vibration, and circling, were greater in wild males than mutant males. Rejection responses of females, such as ignorance, extruding, and decamping, were greater in crosses involving mutant males and wild females while these responses were lower in crosses involving wild males and mutant females. Highest copulation duration and fertility were noticed in pairs of wild males and females while lowest copulation duration and fertility were observed in pairs of mutant males and wild females. Therefore, copulation duration and fertility were positively related. Highest male remating ability was noticed in crosses of wild males and mutant females while lowest male remating ability observed in pairs of mutant males and wild females suggesting that wild males inseminated more females than mutant males. All parameters were significantly varied by one-way ANOVA.

Female remating frequency and female remating interval in different crosses showed that the highest percentage of female remating was noticed in crosses involving wild females and mutant males while the lowest percentage of female remating was observed in crosses involving wild males and mutant females. Longevity of wild and mutant flies is provided in Table 1. Wild flies had greater longevity than mutant flies suggesting that wild flies live longer than spread winged mutant.

Discussion

It is known that male activity and female receptivity are the main factors responsible for successful mating in *Drosophila* (Bastock, 1956). It is evident from the present study that in both multiple and male choice situations preferential mating was noticed between wild and mutant flies of *D. malerkotliana* suggesting occurrence of selective matings between spread winged mutant and wild flies *D. malerkotliana*. This agrees with the work of Morpurgo and Nicoletti (1955) and Barker (1962) in *D. melanogaster*. They also found strong selective mating between the white and wild type flies and also between yellow mutant and wild type in *D. melanogaster*. However, in female choice situation wild males were more successful than mutant males suggesting that under competitive condition wild males were more successful in pre-mating than spread winged mutant. This shows that wild males were more active and vigorous than mutant males during pre mating behavior. This agrees with the work of Faugers (1971), who while studying the components of sexual selection in *D. melanogaster* has also suggested that genotype having no influence whatsoever the more active and vigorous males being likely to win their contests. Even in the present study it was noticed that wild males inseminated more females than mutant males (Table 1). This clearly shows greater sexual vigor of wild males than mutant males.

Furthermore, studies have also indicated that variation is more pronounced in males than in females (Spiess, 1970; Parsons, 1973; Gilbert *et al.*, 1983). According to Smith (1956) in males differences in sexual vigor due to genetic difference must exist in natural population enabling selection to operate. In the present study even in no choice situation where there is no competition, wild male crossed with wild female had greater mating percentage, oriented quickly and mated faster than mutant males crossed with wild/mutant females. This is due to greater sexual vigor of wild males than mutant males; therefore, they oriented quickly and mated faster hence their courtship and mating latency were shorter. On the other hand spread winged mutant males have showed sub-optimal behavior to female as seen in their promoting behavior. In *Drosophila* studies have also

shown that successful matings are also dependent on the activities of the courting pairs (Hegde and Krishna, 1997). Further, courtship behavior of one male may also change the behavior of other males (Rendel, 1951). In the present study, we noticed that wild males recognized wild/mutant females more quickly and displayed different courtship acts such as tapping, scissoring, vibration, licking, and circling more frequently than mutant males. Through these courtship acts males not only transmit sexual signals but also stimulate the female (Spieth and Ringo, 1983). On the other hand, wild females showed more rejection responses such as ignorance, decamping, and extruding to mutant males than wild males. Therefore, wild male paired with wild or mutant females had greater mating success than mutant males paired with wild/mutant females. This agrees with the work of Soudergard (Smith, 1956), who found several changes in mating behavior between ebony mutant and wild males of *D. melanogaster*. The ebony males exhibit less licking than wild type and show more frequent breaks in courtship (Crossley and Zuill, 1970). Also the courtship song is changed in ebony males.

According to Elens (1973) the behavior of the males varied according to the genotype of the female they were courting. In the present study in order to test the female behavior we have studied female remating. Female remating frequency and female remating internal varied significantly between wild and mutant males of *D. malerkotliana* (Table 1). Wild/mutant female initially mated with mutant male remated more rapidly and more frequently than it initially mated with wild male. This is because in *Drosophila* females remate only when sperms in the spermatheca are exhausted. According to Gromko and Pyle (1978) the female remating interval seems the result of selection on both sexes. Sexual selection would favor any adaptations that lower female receptivity to remating or that insure most or all of their sperm is used. However, females suffer a decrease in fitness when the amount of sperm stored drops to a low level (Gromko and Pyle, 1978) and selection on females for the maintenance of high reproductive output would favor their remating before the sperm stored from a prior mating is exhausted. In the present study we also found that females (wild/mutant) copulated longer time with wild males than with mutant males. Increased copulation duration increases the number of ejaculations (Hegde, 1979). Hence females initially mated with wild males remated more slowly than it initially mated with mutant males.

Sexual selection is common among animals, because the females reproductive success is limited by the number of eggs she can produce in her lifetime and a male's reproductive success is limited by the number of females he can inseminate. In the present study fertility varied significantly between crosses. Wild male mated with wild female had greater fertility than mutant male mated with wild or mutant female.

According to Krishna and Hegde (1997) higher reproductive success can also be accounted for its longevity. In the present study we found wild flies had higher longevity than mutant flies and varied significantly. Wild male with higher longevity, greater sexual vigor, and fast mating ability could inseminate more females than could mutant males. As a result the total number of progeny they produce is greater. On the other hand, wild female with greater longevity and remating ability could receive more sperms and produce more fertile offspring in their lifespan than mutant females. Thus, in *D. malerkotliana* present observation clearly suggests there is an evidence that selective mating exists between spread winged mutant and wild flies and greater reproductive success of wild flies.

Acknowledgment: The authors are grateful to Chairman, Department of Studies in Zoology, University of Mysore for facilities.

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A novel mechanism underlying axon guidance phenotypes: Indirect disruption of embryonic axon guidance by unspecified cells.

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Abstract

During characterization of the *Netrin* locus, we uncovered a background maternal effect mutation in which a small number of presumptive mesoderm cells fail to express the mesoderm determinant *twist* (*twi*). In contrast to *twi* mutants in which the mesoderm cells adopt alternative cell fates, the affected cells did not appear to differentiate. The cells failed to invaginate with other mesodermal cells and remained at the central nervous system (CNS) midline physically blocking cell migration and axon outgrowth. Due to low penetrance we were unable to map the mutation, but propose that the gene is required for coordination of gene expression throughout the cells of a tissue. We believe that the phenotype represents a novel way for cell fate alterations to disrupt axon guidance, distinct from alterations in neuronal or target cell identity.

Introduction

Like all animals, early development of *Drosophila* embryogenesis is controlled by gene products deposited by the mother in the oocyte (Tadros and Lipshitz, 2009). One of the first zygotic genes to be activated is the *twist* (*twi*) gene, a basic helix-loop-helix (bHLH) transcription factor required for mesodermal cell fate (Thisse *et al*., 1987). In *twi* mutants, the presumptive mesoderm fails to invaginate during gastrulation and adopts alternative cell fates, mainly neurectodermal (Leptin and Grunewald, 1990; Rao *et al*., 1991). The failure of a large number of cells to invaginate leads to an increase in the length of the embryo which, as the embryo is constrained by the egg membranes, leads to twisting of the embryo giving the mutant its name (Simpson, 1983).

The *Drosophila* central nervous system (CNS) is formed by the juxtaposition of neurectodermal tissue from opposite sides of the embryo after the presumptive mesoderm has

invaginated (Leptin, 1999). The site of invagination becomes the CNS midline, an axis of bilateral symmetry. The midline is a source of attractive and repulsive cues that guide axons and cell migration during CNS development (Evans and Bashaw, 2010). Proteins of the Netrin family primarily act as attractive signals. *Drosophila* has two *Netrin* genes, *NetA* and *NetB*, with deletion of both leading to decreased axon crossing of the CNS midline (Mitchell *et al*., 1996; Harris *et al*., 1996; Brankatschk and Dickson, 2006). Here we describe a cell fate alteration that disrupts CNS formation, not by altering neuronal or target cell identity, but producing apparently unspecified cells during gastrulation that persist to sterically hinder cell migration and axon outgrowth.

Materials and Methods

Drosophila Stocks and Genetics

The *KG03586* transposon and transposase stocks were obtained from the Bloomington *Drosophila* stock center. Excision of the *KG03586* insertion was by standard techniques.

Immunohistochemistry

Immunohistochemistry was performed as described in Patel (1994). The anti-Singleminded antibody was obtained from S. Crews. *In situ* hybridizations were performed as described in Kopczynski *et al*. (1996).

Results and Discussion

The *NetA* and *NetB* genes appear to be largely redundant, with functional differences originating primarily in expression patterns (Mrkusich *et al*., 2010; Matthews and Grueber, 2011). Deletion of either gene alone has no discernible effect on CNS formation (Brankatschk and Dickson, 2006), so we were intrigued by subtle alterations to axon and midline cell position present in a stock with a transposon insertion in *NetB* (Figure 1). The *KG03586* transposon is inserted in a 55 kb intron of *NetB*, suggesting it might be altering *NetB* activity in an unusual manner. *NetB* mRNA expression was reduced or absent in embryos mutant for the *KG03586* transposon. An imprecise excision of the transposon restored *NetB* expression, but unexpectedly the CNS phenotype remained (Figure 1D). Analysis of the site of the *KG03586* transposon revealed a small gene, *CG32595*, which we named *hog* (Newquist *et al*., 2013). The CNS phenotype failed to appear when *KG03586* was crossed to a deficiency for the region, or when transgenic RNAi for the *hog* gene was expressed in the female germline (*hog* is maternally deposited in the egg). We conclude that the phenotype is in the background of the *KG03586* transposon. The phenotype occurs at low penetrance and disappeared from our stocks over time, preventing mapping.

Closer examination of the CNS in the *KG03586* background revealed large rounded cells (Figure 1D), which did not stain with neuronal or midline cell markers. These cells appeared to be disrupting midline patterning and axon crossing. The maternal deposition and early disappearance of the *hog* mRNA suggested that the cells might arise as the result of a gastrulation defect. The *twi* gene is normally expressed in future mesoderm cells in a broad ventral band (Figure 2A). We examined *twi* expression in *KG03586* mutants and found that the *twi* mRNA pattern showed single or small groups of cells lacking *twi* expression (Figure 2B). As *twi* is required for mesodermal cells to invaginate, we examined the pattern of Single-minded (Sim) protein staining at the end of gastrulation. Sim marks the CNS midline which is created by invagination of the mesoderm. In *KG03586* mutants, we noticed that the regular array of Sim positive cells was occasionally

interrupted by unstained cells (Figure 2C,D). These cells lie slightly dorsal to the Sim positive cells, suggesting they may have attempted to invaginate. In *twi* mutants, there is a brief but ineffectual attempt at invagination (Leptin and Grunewald, 1990; Ip *et al*., 1994).

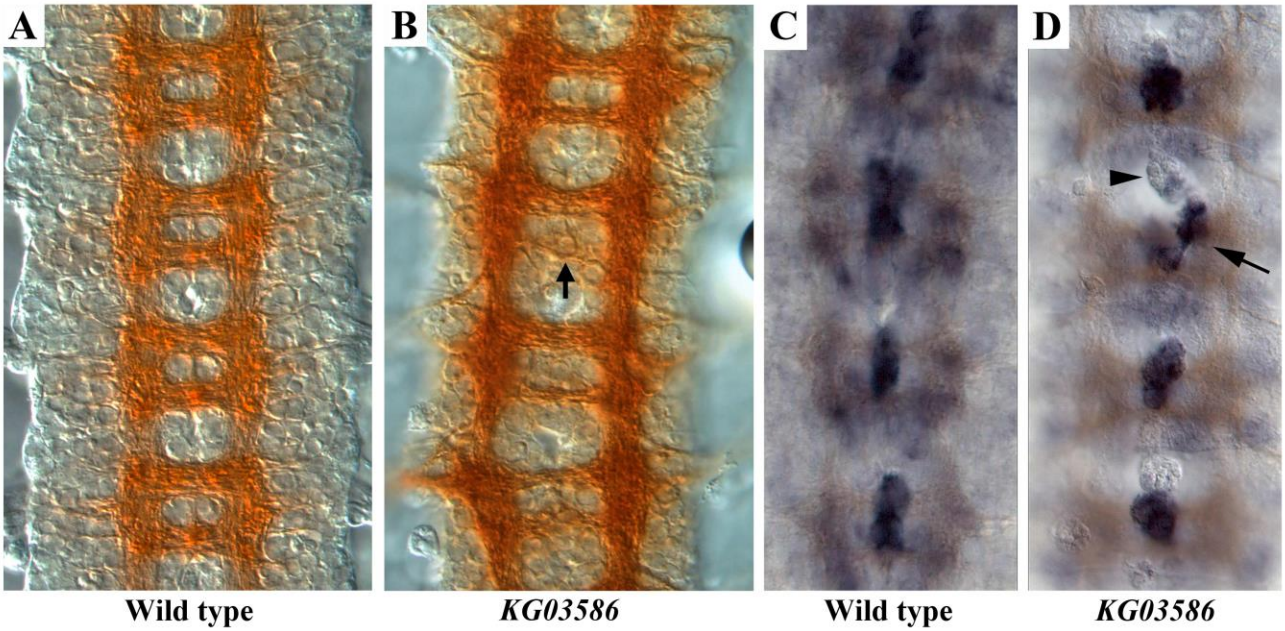

Figure 1. CNS phenotypes of *hog* mutants. Stage 16 (A,B) and stage 15 (C,D) embryos stained with monoclonal antibody BP102, which recognizes all CNS axons (brown), and an *in situ* hybridization probe against NetB (blue; C,D). (A) Wild type. The CNS axon scaffold is visible as a repeated ladder-like pattern. (B) *KG03586* mutant embryo. This is a strong example in which a posterior commissure (arrow) is almost completely missing. (C) Wild type. Strong NetB expression at the CNS midline can be seen in a regular repeated pattern. Additional neuronal expression can be seen lateral to the midline, mainly out of the plane of focus. The CNS axon scaffold is out of focus. (D) *KG03586* mutant embryo. The arrow indicates a cluster of *NetB* expressing midline glia that are mispositioned away from the underlying anterior commissure. Immediately above the midline glia is an abnormal cluster of cells (arrowhead). Similar cells can be seen in the lowest, but not the middle, segment. They are also absent from the wild type embryo in panel C.

We propose that the failure of the unidentified cells to invaginate represents a novel mechanism for indirectly disrupting axon guidance: the creation of physical barriers for migrating cells and growing axons by a failure of cells to migrate to their correct locations. The observed phenotype would probably not be obvious if the unidentified cells did not persist at the CNS midline, a critical organizing center for CNS formation. We speculate that rather than adopting an alternative cell fate as in *twi* mutants, these cells appear to stall in the differentiation process, remaining morphologically distinct and failing to express mesodermal or CNS markers. The rounded morphology is suggestive of an undifferentiated state, but we have been unable to identify a marker for undifferentiated embryonic cells in *Drosophila* to confirm this. The phenotype contrasts with *twi*

mutants in which mutant cells adopt more lateral cell fates. The disruptions to the *twi* mRNA pattern suggest that the gene product is either directly involved in *twi* transcription, or is required for the coordination of transcription throughout the presumptive mesoderm (Lagha *et al*., 2013). It is also possible that the cells may not be able to respond to signals from other mesoderm cells as has been described for the community effect (Gurdon *et al*., 1993). The phenotype is somewhat reminiscent of axon regeneration in the injured spinal cord in which a scar of glial tissue imposes a barrier to axonal regeneration (Rolls *et al*., 2009).

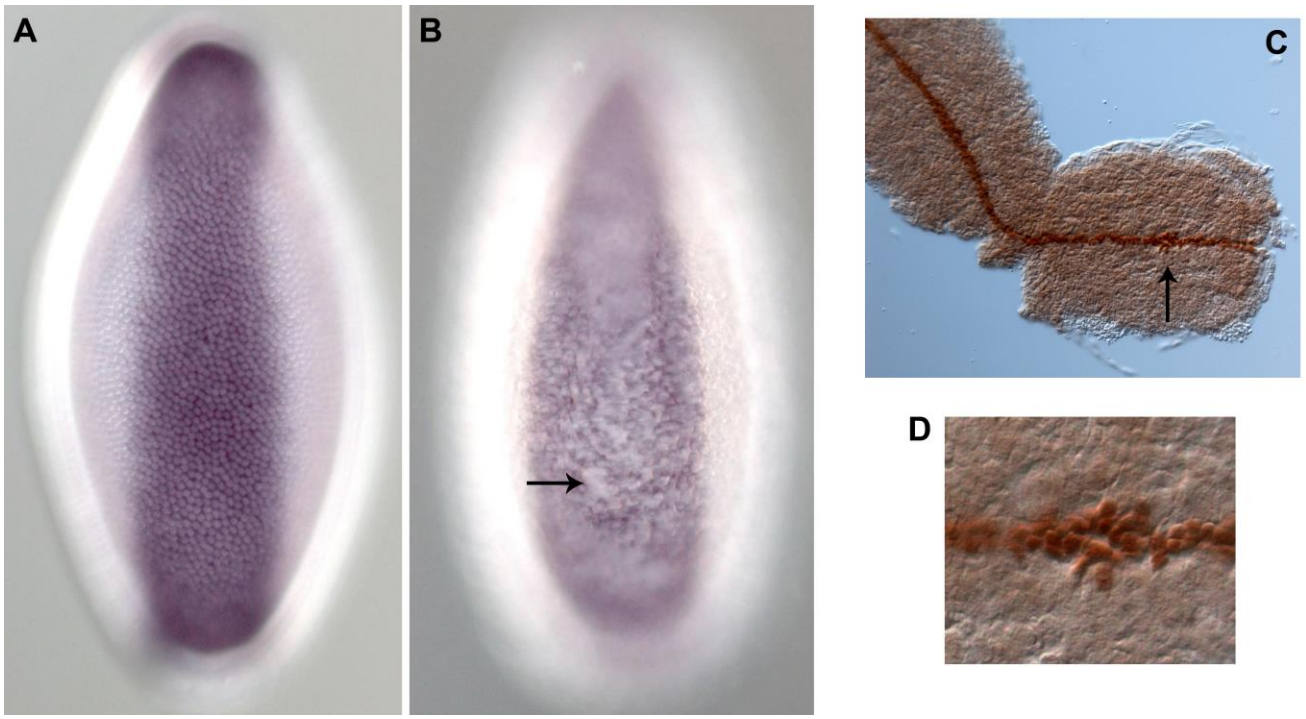

Figure 2. *twi* expression is affected by *hog* mutants. (A,B) Embryos stained with an *in situ* hybridization probe for *twi*. (C,D) Embryo stained with an antibody against Single-minded (Sim). (A) Wild type late stage 5 embryo showing a contiguous sheet of cells expressing *twi*. (B) Early stage 6 *hog* mutant embryo showing gaps in the *twi* mRNA pattern (arrow). Gastrulation has initiated so the sheet of *twi* expressing cells is slightly indented. (C) Stage 9 *hog* embryo showing the future CNS midline as a continuous line of Sim positive cells with a disruption near the posterior end (arrow). (D) Higher magnification view of the embryo in C, showing the disrupted region of Sim staining. A non-staining cell can be partially seen in the center of the disrupted region and other midline cells have been displaced laterally.

Acknowledgments: We thank S. Crews for the anti-Single-minded antibody. We thank G. Andrews and M. Lamanuzzi for advice and technical assistance. Antibodies were obtained from the Developmental Studies Hybridoma Bank (DHSB) developed under the auspices of the NICHD and maintained by the University of Iowa. This project was supported by grants from the National Science Foundation IOS-1052555, National Center for Research Resources (P20RR016464,

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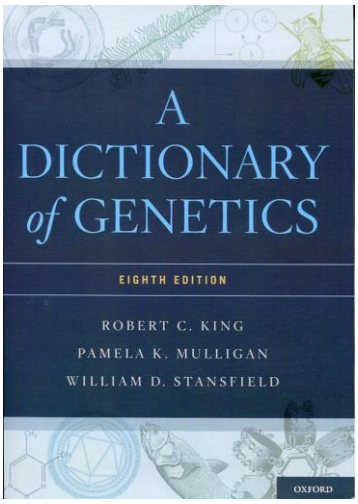
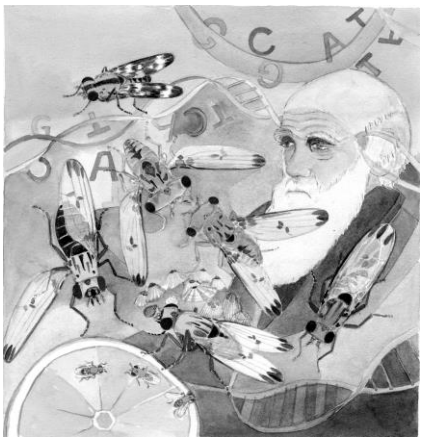
New Book Announcements



A Dictionary of Genetics, 8th edition.

King, Robert C., Pamela K. Mulligan, and William D. Stansfield. 2013. 641 pages. ISBN: 978-0-19-976644-4. Oxford University Press.

This enduring classic resource is much more than just a dictionary for geneticists. It is a ready and reliable resource for information about all topics in our broad field. The definitions are detailed, yet easy to understand. Illustrations are clear and informative. It does not simply define useful terms so we can read the literature in related fields of genetics, it explains important concepts in detail. For that reason, it should be especially useful for graduate students and established professionals in genetics, genomics, molecular biology, and biomedical research.



***Drosophila*, Famous Little Flies**

do Val, Francisca. 2014, 36 pp., illustrated.

This is a new English edition of a short book originally published in Portuguese in 2007 designed to encourage children (and adults) to learn about science. Drawing from the field and laboratory experiences of Dr. do Val and her colleagues, this short book explores the contributions that *Drosophila* research has made to fields like evolutionary biology. Colorful illustrations help make the message accessible to the general reader, including a key target – young students. Dr. do Val is an experienced researcher at the University of Sao Paulo and works in close association with colleagues in the Hawaiian *Drosophila* Project at the University of Hawaii (Pacific Biosciences Research Center), which celebrates **50 years** in 2013. The author can be contacted at: fracdval@usp.br.

Technique Notes

The free-running period of *Drosophila melanogaster* is not affected by the length of the tube in a *Drosophila* Activity Monitor (DAM).

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Drosophila melanogaster has been a widely used model to study the circadian rhythm since the creation of the *period* mutants (Konopka and Benzer, 1971), as there are many similarities in circadian function to mammals at both the molecular and behavioral levels (Helfrich-Foster, 2004). Many different devices were invented and used to observe the behavioral circadian rhythm in fruit flies, but the most commonly used are the *Drosophila* Activity Monitors (DAMs), made by the company Trikinetics Inc. (Waltham, MA) (Klarsfeld *et al.*, 2003). Several articles and chapters describe the procedures for using activity monitors and provide detailed instructions from setting-up the monitors, computers, software, and wiring (Klarsfeld *et al.*, 2003; Rosato and Kyriacou, 2006; Zordan *et al.*, 2007; Chiu *et al.*, 2010; Pfeiffenberger *et al.*, 2010). Basically, the DAMs monitor the activity of individual flies housed in a tube by counting the number of infrared beam crossings within a specific time-frame or bin (which can range from 1 sec to 60 min), and subsequently compiling the activity in a raw data text file. It is then necessary to utilize a separate data-analysis program, such as ClockLab (Actimetrics, Wilmette, IL) or Actiview (Minimitter – Philips Respironics, Bend, OR) in order to extract the information from the raw-data file and to produce an actogram, which is a graphical representation of the individual fly's activity rhythm. The use of fruit flies and DAMs to study the circadian clock is a powerful, yet cost-effective, method to use in both a research and classroom setting (Seggio, 2011).

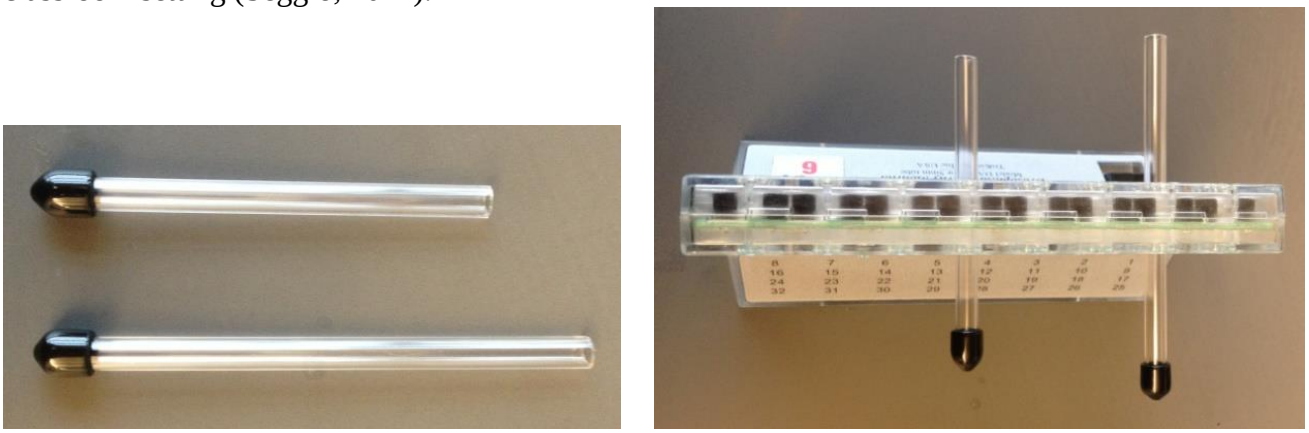

Figure 1. *Left:* A 5 × 65 mm polycarbonate tube with black cap (top) compared with a 5 × 80 mm tube (bottom). *Right:* When placed into a DAM, the 80 mm tube (right) has more room for motion compared to the 65 mm tube (left).

The majority of the projects published using the DAMs have used a 5 × 65 mm tube to house the individual fly, including Seggio *et al.* (2012) and Oh *et al.* (2013), as well as at least thirty other

articles since 2012. Recently, Trikinetics has offered a larger tube size, 5 × 80 mm, for use in the DAMs, but few researchers have used it to date. Lone and Sharma (2012) have recently published using the longer tube, according to a recent google.scholar search for "Trikinetics" and "80 mm"/"80mm" (although Rosato and Kyriacou, 2006, and Zordan *et al.*, 2007, make note of that option in their reviews). This project aims to uncover any differences in circadian activity levels and free-running period between the 65 mm and 80 mm tubes.

Methods

Canton-S (*CS*), *Oregon-R* (*Or-R*), *period Short* (*perS*), *period Long* (*perL*) *Drosophila melanogaster*, reared at 25ºC in a 12:12 Light:Dark (LD) photoperiod were used throughout this study. Flies were raised on instant *Drosophila* medium Formula 4-24, blue, (Carolina Biological Supply Company, Burlington, NC) with 5 mL food and 11 mL of water per vial. Randomly selected one-day-eclosed adults were individually monitored for locomotor activity and circadian free-running period. Single adult flies were placed into individual polycarbonate tubes (either 5 × 65 or 5 × 80 mm tubes) containing approximately 10 mm of 5% (w/v) sucrose and 2% agar (w/v) food with a plastic cap on one end and a cotton plug approximately 10 mm in length on the other end, and placed into activity monitors (DAM2 – Trikinetics Inc., Waltham, MA). The DAMs were placed into an incubator with approximately 200 lux fluorescent light in a 12:12 LD cycle at 25ºC for 3 full days. Following the 3 days in 12:12 LD treatment, measurements continued without interruption for 7-10 additional days in constant darkness (DD) to assay the free-running circadian period of locomotor activity. Activity was monitored using the Trikinetics DAM3 data collection software with activity counts collected in 10-min bins, and assembled into data files for individual flies using the DAMFileScan Software. Mean activity levels and circadian period (an average of the chi-square periodogram and visual inspection) in DD for individual flies were analyzed using ClockLab (Actimetrics, Wilmette, IL). Two-way ANOVAs were used to detect significant differences in mean activity and period between tube-length, fly type, and tube by genotype interaction for the wild-type and *period* mutant flies.

Results

Table 1 shows the mean activity in LD and DD, as well as the free-running period for each of the four genotypes. *CS* and *Or-R* were shown to have significantly different periods ($F_{1,147} = 56.4$, $p < 0.001$), with *CS* having a longer rhythm; however, *Or-R* was found to have higher activity amplitude than *CS* in both LD ($p < 0.001$) and DD ($p < 0.001$). There were no tube-length by genotype interactions for LD, DD, or free-running period (all $p > 0.10$); *i.e.*, Canton-S in 65 mm tubes did not differ from *CS* in 80 mm tubes (the same being observed for *Or-R*). Obviously, *perL* and *perS* have significantly different periods ($F_{1,105} = 4621$, $p < 0.001$). *perS* was found to have much higher activity amplitude in both LD ($p < 0.001$) and DD ($p = 0.001$) than *perL*, a result that was previously found by Ahmad *et al.*, 2013. As before, there was no significant difference found for either tube-length or tube-length by genotype interaction (all $p > 0.10$) for LD and DD activity or free-running period for the *period* mutants.

Based on these results, it appears that there are no differences between the 65 mm and 80 mm tubes in LD or DD activity in terms of beam crosses per 10 min bin or the free-running period among two widely-used wild-type flies and two *period* mutants. While average beam crosses per 10 min bin

were not different among any of the four interactions, it is not known whether the larger tube will alter other activity parameters, such as average bout length, counts per bout, bouts per day, or Light:Dark activity ratio.

Table 1. Average activity (number of beam crossings per 10 min bin ± SEM) in LD and DD, as well as free-running period (hours ±SEM) for the two wild-type flies (CS and Or-R) and the period mutants (perS and perL).

Genotype	Tube Length	N (Sample)	Activity in LD	Activity in DD	Period
Canton-S	65mm	35	5.00 ± .31	5.13 ± .31	24.34 ± .03
(*CS*)	80mm	25	4.28 ± .30	5.14 ± .35	24.33 ± .02
Oregon-R	65mm	45	6.35 ± .37	6.56 ± .31	24.05 ± .04
(*Or-R*)	80mm	46	5.92 ± .44	6.47 ± .48	24.04 ± .04
period Short	65mm	32	6.87 ± .60	7.25 ± .57	19.26 ± .12
(*perS*)	80mm	23	6.79 ± .58	7.65 ± .81	19.29 ± .10
period Long	65mm	28	4.82 ± .37	4.77 ± .42	28.37 ± .11
(*perL*)	80mm	26	5.29 ± .52	4.00 ± .39	28.44 ± .18

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An efficient, practical, and reliable *Drosophila* trap.

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A good *Drosophila* trap should be made of materials that are inexpensive and readily available. Also, the materials should be sturdy enough to be used outdoors. Additionally, a trap should be simple enough that anyone can assemble it quickly. The trap of Medeiros and Klaczko (1999) is well designed, but improvements and simplifications are possible. Using their work as a foundation, an efficient, practical, and reliable trap for live *Drosophila* specimen collection was designed.

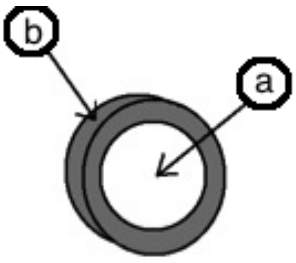

Figure 1. Bottle cap

The first step is to modify a bottle cap for attachment of a plastic culture vial (1¼'' diameter × 4'' high; Carolina Biological Supply item #173120). Using a rotary tool or sharp blade, make a hole approximately ½-inch in diameter in the middle of the bottle cap so flies can travel through it (Figure 1a). Make sure not to damage the threading on the inside of the cap so it can still be tightened onto the bottle. Next, add masking tape to the outside of the bottle cap (Figure 1b). Circle the side of the cap with enough tape so that a culture vial fits snuggly. The tape may need to be cut horizontally so that it does not interfere with the culture vial. Modify multiple caps as a supply for many traps. Alternatively, this trap can be used without a bottle cap by taping the culture vial to the bottle itself. However, the cap makes it much easier to remove and replace the culture vial quickly.

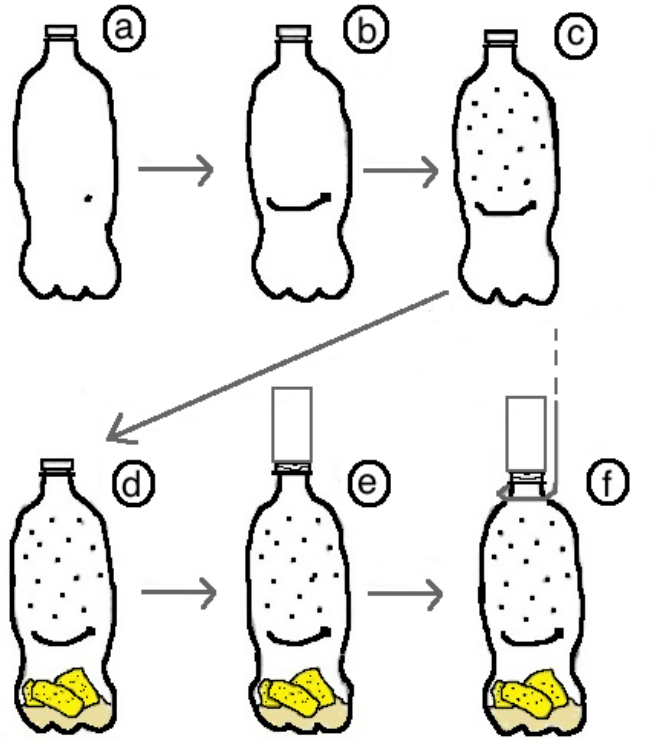

Figure 2. Procedures for making the trap.

The containment portion of the trap is made with one transparent 2-L soda bottle. Alternatively, for a smaller trap, a 20-oz (591-mL) bottle can be used. The first step is to make a small hole near the middle of the bottle (Figure 2a). From this hole, cut a curved slit into the bottle (Figure 2b). The best tool to use for this is a box cutter. Bait will be introduced through this slit. After the trap is hung, this slit can be taped over to ensure larger animals, such as wasps, do not enter the trap. However, this usually is not an issue. Second, using a narrow, sharp tool, puncture small holes (~ 4 – 5 mm in diameter) in the trap at random locations above the curved slit (Figure 2c). Make these holes large enough for fly entry but small enough to limit the entry of larger insects. Make 15 to 30 of these holes for a 2-L trap. Next, add the bait via the curved slit (Figure 2d). Finally, place the culture vial onto the modified cap at the top of the trap (Figure 2e). The trap can be hung from vegetation or hooks using a bent wire coat hanger (Figure 2f).

To collect flies from the trap, have several culture vials and plugs available. Flick the trap by gently tapping it a few times. Flies will migrate upwards into the culture vial. Quickly remove the culture vial and immediately plug it using a foam plug (Carolina Biological Supply item # 173122) while holding the vial upside down. Replace the culture vial with an empty one. Repeat the

collection process until the trap is empty. A filtered aspirator (for example, Bioquip item #1135A) can be used through the slit to selectively remove specimens.

Using a sleeve expedites the collection process. Hollow cardboard mailing tubes (Uline model # S-10723) with plastic end caps (Uline model # S-7020) can be used as a sleeve (Figure 3a). Using a rotary tool, drill, or sharp blade, make a hole into the plastic cap of the tube so that the culture vial can tightly fit through (Figure 3b). Next, cut the tube to roughly the height of the bottle without the shell vial attached (Figure 3c). When collecting, slide the tube over the trap (Figure 3d). Specimens migrate upwards toward light into the culture vial (Figure 3e). Natural light or a lamp may be used.

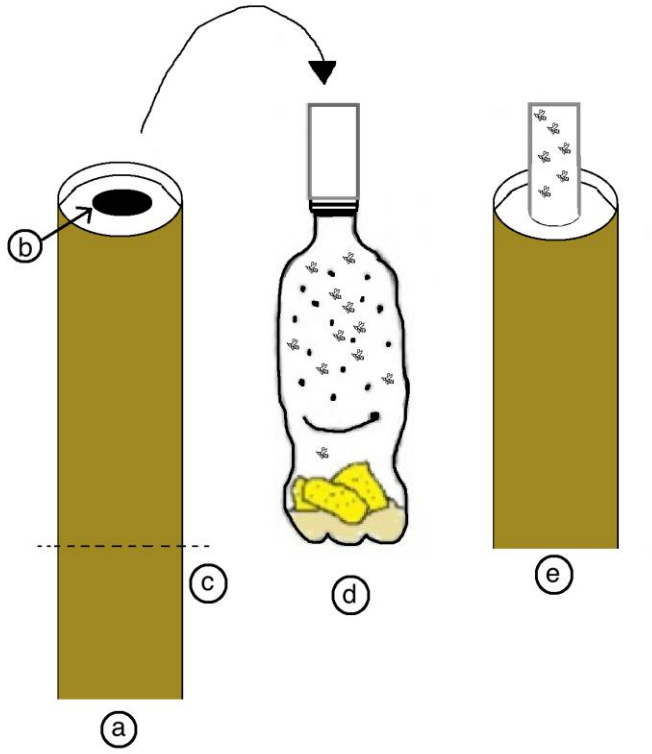

Figure 3. Collecting specimens using a sleeve.

A trap of this design is efficient because it is capable of trapping thousands of specimens 2 to 3 days after deployment, and it can be emptied quickly and easily. This trap has captured flies from many different species, including *D. melanogaster*, *D. simulans*, *D. busckii*, *D. robusta*, *D. affinis*, *D. tripunctata*, *D. immigrans*, *D. suzukii* (Freda and Braverman, 2013), and *Zaprionus indianus*.

References: Freda, P.F., and J.M. Braverman 2013, Entomol. News 123(1): 71-75; Medeiros, H.F., and L.B. Klaczko 1999, Dros. Inf. Serv. 82: 100-102.

The impact of pheromones on sexual behavior in *D. melanogaster*: Recommendations for laboratory protocols.

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Abstract

Pheromones are conspecific chemical signals used throughout the animal kingdom that elicit behavioral responses in other organisms and are essential for intraspecies communication. *D.*

melanogaster utilize chemoreception for food acquisition, aggression, avoidance, courtship behavior, sexual recognition, and mate selection. Specific to sexual behavior, mating processes are mediated by volatile and non-volatile pheromone detection through olfactory and gustatory systems. These chemical signals are present in stock cultures housing *D. melanogaster* and can potentially influence behavior, leading to changes that may confound experimental data collection. Here, we review pheromonal processing and its role in sexual behavior, mating preferences, and courtship behavior; we illuminate specific pheromones and associated receptors and elucidate their involvement in each behavior, providing a thorough analysis of pheromone induced sexual behavior. Lastly, genetic manipulation and pheromonal application can be applied to regulate *D. melanogaster* behavior and enhance genetics research methodologies.
Keywords: Pheromones, *Drosophila*, *D. melanogaster*, fruit flies, laboratory protocols

Pheromones are conspecific chemical signals used throughout the animal kingdom that elicit behavioral responses in another organism. They are involved in behaviors such as trail marking, warning signals, territory marking, mating behavior, and sexual recognition (Wyatt, 2003). *Drosophila melanogaster* is a prominent animal model for genetics research, yet little attention is paid to the role of pheromones in its sexual behavior. *D. melanogaster* responds to many odorants critical for food acquisition, aggression, avoidance, courtship behavior, sexual recognition, and mate selection (Cande, Prud'homme, and Gompel, 2013; Dahanukar and Ray, 2011; Eastwood and Burnet, 1977; Fernandez and Kravitz, 2013; Herrero, 2012). These behaviors are mediated through synergistic cues from the olfactory and gustatory systems (Dickson, 2008; Sengupta, 2013; Smith, 2007; Wang and Anderson, 2013). In this review, we elaborate on how these chemoreception processes affect sexual behavior and their relevance to accurate and reliable experimental designs in the laboratory environment.

First, we discuss their pheromones and receptors that underlie pheromonal communication. Second, we describe courtship behaviors and mating preferences, and how specific pheromones and receptors can induce behavioral changes involved in mating. Next, we describe how current laboratory practices can be confounded with pheromones, and how laboratories could control for chemicals that stimulate chemosensory systems and subsequently affect aggression, courtship, and mating. Finally, we offer recommendations for laboratory protocols targeting the regulation of sexual behavior such as genetic manipulation and pheromonal application.

Pheromonal Detection and Processing

The olfactory and gustatory systems underlie pheromone detection in *D. melanogaster*. The detection of volatile pheromones occurs through the olfactory system, while nonvolatile pheromones are detected by the gustatory system (Dickson, 2008; Montell, 2009). The peripheral olfactory system includes the antennae and maxillary palps, which contain individual sensory neurons, called sensilla (Martin, Boto, Gomez-Diaz, and Alcorta, 2013). Within a sensillum are the cell bodies of the olfactory sensory neurons (OSNs) (See Figure 1). Protruding from each sensillum are smaller sensilla, which contain the dendrites of OSNs expressing multiple receptors. Figure 1B depicts a schematic of the dendritic and synaptic projections within each sensillum (Smith, 2007).

Several external areas of the body of *D. melanogaster*, including the labellum, pharynx, legs, and wing margins, contain gustatory receptor neurons (GRNs) that are expressed in separate sensilla (See Figure 2). Gustatory sensilla are more heavily distributed on the external structures such as the head, legs, and wing margins, and this allows for maximal chemosensory reception (Montell, 2009).

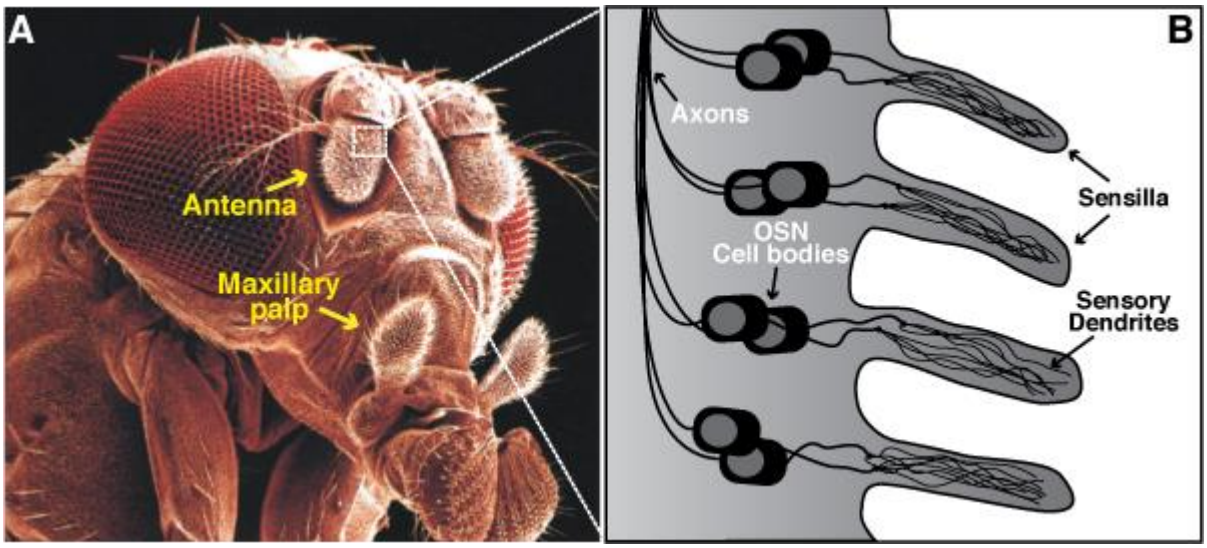

Figure 1. Peripheral olfactory system of *D. melanogaster*. A) Scanning electron image of antenna and maxillary palps. Photograph is courtesy of Juergen Berger, Max-Planck-Institute for Developmental Biology, Tuebingen. B) Schematic of olfactory sensory neurons (OSN) and their sensory dendrites. Schematic is courtesy of Walton Jones, Korea Advanced Institute of Science and Technology, South Korea.

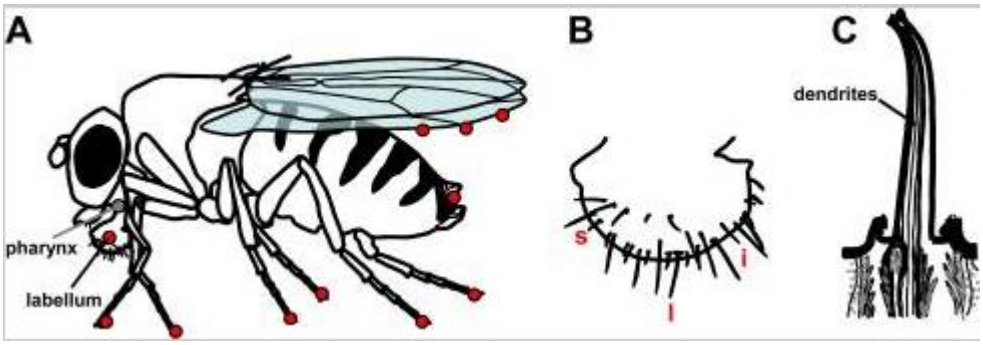

Figure 2. Gustatory system of *D. melanogaster*. A) Full body image of different regions of GRNs. grey dot - pharynx, red dots - (anterior-posterior) labellum, legs, wing margins. B) Labellum shown with protruding s-, l-, i-type sensilla. C) Sensillum with four dendrites extending to the terminal pore. From Montell (2009), reprinted with permission of Elsevier.

Further, this combination of olfactory and gustatory cues allows for a wide range of pheromonal detection.

The information from the peripheral olfactory and gustatory systems is then relayed to the central nervous system where neuroanatomical structures act as the processing centers in pheromonal signaling. Central structures in *D. melanogaster* include the suboesophageal ganglion (SG), antennal

lobes (analogue of the mammalian olfactory bulb), mushroom bodies, and the central complex (Heisenberg, 2003). Each brain region is essential for particular information processing. More specifically, the SG acts as the primary taste center of the central nervous system and receives information from the GRNs (Dickson, 2008). Antennal lobes integrate sensory information from the antennae and serve as downstream targets of OSN projections. The mushroom bodies are involved in odor perception and the memory induction of previous chemical signals, which can influence motor behavior (Crittenden *et al.*, 1998; Dickson, 2008; Heisenberg, 2003), and the central complex (CC) performs a global integration of related information.

Each neuroanatomical region can be broken down into more specialized regions known as glomeruli; OSNs and GRNs that express specific receptors target specific glomeruli (Dickson, 2008; Smith, 2007). Moreover, individual olfactory neurons mediate unique behaviors in response to sex pheromones (Kurtovic, 2007). This specialized chemotopic map aids in spatial and temporal orientation of a pheromone source, a critical mechanism for pheromonal detection and survival (Agarwal and Isacoff, 2011). Other neuronal circuits regulating aversion and attraction are also involved in processing and internalizing pheromonal information (Gao, 2013). This integrative neuronal circuitry thus underlies pheromonal processing and resulting behavior.

Pheromones and Receptors

The pheromones that *D. melanogaster* use to identify conspecifics and potential sexual partners are long-chain cuticular hydrocarbons (CHs) that are produced in the epidermal oenocytes of the abdomen (Krupp and Levine, 2010). Pheromones are composed of species-specific blends of these hydrocarbons and have evolved over many generations (Kent *et al.*, 2007; Billeter *et al.*, 2009). They can allow a male to recognize a conspecific, determine its sex, determine past courtship experience, and finally its receptivity (Billeter *et al.*, 2009; Ejima, 2012; Keleman *et al.*, 2012). The chemical composition of these hydrocarbons may vary in terms of chain length and bond position, including methyls, alkanes, fatty acids, and 5-, 7-, and 9-alkenes (Kent *et al.*, 2007).

Furthermore, the total abundance of each of these blends of compounds, distinguished by chemical composition, may have distinct rhythms of expression and activation over the course of both a 24-hour photoperiod and the individual's circadian free-running activity period (Chatterjee and Hardin, 2010; Kent *et al.*, 2007). The pigment-dispersing factor's (PDF) signaling pathway is a checkpoint in the pheromonal production cycle of the oenocytes, and disruption of this pathway can lead to sexually dimorphic differences in mating behavior (Krupp *et al.*, 2013). There are daily fluctuations of pheromone production in the laboratory setting that must be considered with respect to the time of day that data are recorded and collected.

Several pheromones are important in sexual behavior of *D. melanogaster*. 11-*cis*-vaccenyl acetate (cVA) is a non-volatile CH that is produced in the oenocytes of sexually mature males and is deposited onto females during courtship, and this allows other males to distinguish her as a previously mated individual (Bartelt *et al.*, 1985; Datta *et al.*, 2008; Keleman *et al.*, 2012). Therefore, cVA serves as an inhibitory signal to other males. Ablating the oenocytes in females results in the elimination of this sex-specific inhibitory signal through shorter courtship latency and stronger maintenance of courtship length, and courting by males of other *Drosophila* species. A separate CH alkene, known as 7,11-heptacosadiene (7,11-HD), also functions as a chemosensory signal of heterospecificity in *D. melanogaster* (Billeter, Atallah, Krupp, Millar, and Levine, 2009; Fan *et al.*, 2013). These and additional *D. melanogaster* sex pheromones are listed in Table 1.

Many receptors associated with the sex pheromones are localized to multiple areas in the central and peripheral nervous systems. The SG receives input from the GRNs during courtship,

including a key receptor, Gr68a, and relays these nonvolatile signals to the CC for higher order processing. Or67d and Or65a are two important receptors, among a larger family of OSNs, on the T1 and T3 trichoid sensilla of the antennae, respectively. Axons from the receptors project to the antennal lobe, where primary olfactory information is interpreted as a map of glomerular activation (Agarwal and Isacoff, 2011; Dickson, 2008). DA1 is one glomerulus that receives pheromonal input in the form of coincidence detectors on the antennae, by which the spatiotemporal features of odorants in the local environment are integrated and interpreted (Keene and Waddell, 2007; Perez-Orive *et al.*, 2004).

Table 1. Known pheromones and their associated functions and receptors.

Pheromone	Function	Associated receptor(s)	Reference
11-*cis*-vaccenyl acetate (cVA)	Secreted in male semen; inhibits other males from mating for ~10 days; promotes aggression in pairs of male flies	Or67d, Or65a, DA1	Bartelt et al. (1985); Xu et al. (2005)
z-7 tricosene	Inhibitory signal in males that prevents other males from mating; tastes bitter	Gr32a (?), Gr33a	Lacaille et al. (2007)
7,11 heptacosadiene	Slow down courtship; identifies same species mates; low levels cause hyperstimulated activity	*ppk23*(+) sensory neurons	Billeter et al. (2009); Toda et al. (2012)
7,11 nonacosadiene	Additional female aphrodisiac pheromone		Ferveur & Sureau (1996)
20-hydroxyecdysone	Modulates courtship behavior towards females	DmDopEcR (GPCR)	Abrieux et al. (2013)
ppk25	Stimulatory pheromone	Gr68a	Dahanukar & Ray (2011)

Arborization of the neurons in downstream targets of these glomeruli may be sexually dimorphic. The binding of certain receptors, often by the same pheromone, elicits distinct behavioral responses in males and females (Datta *et al.*, 2008; Dickson, 2008; Kurtovic *et al.*, 2007). For example, there is evidence that the mechanism regulating cVA binding responses is sexually dimorphic, such that it inhibits mating behavior in males and promotes it in females (Kurtovic *et al.*, 2007). This dimorphism in neural circuitry is regulated by the *fruitless* gene, which promotes the development of male-specific interneurons in downstream projections of the glomeruli (Kimura *et al.*, 2005).

DopR1 receptors, expressed in the mushroom bodies, bind signals from dopaminergic neurons that aid in memory formation and learning in the male (Keleman *et al.*, 2012). The mushroom bodies and the CC are two major structures in the *D. melanogaster* brain that regulate different mechanisms for sex-specificity and heterospecificity, respectively (Sakai and Kitamoto, 2006). For example, 7,11-HD functions as both a mating signal in males and as a conspecific identifier (Billeter *et al.*, 2009; Dahanukar and Ray, 2011; Fernandez and Kravitz, 2013).

Courtship Behaviors

Courtship behaviors in *D. melanogaster* are dictated by specific pheromones, their receptors, and subsequent activation through olfactory/pheromonal pathways. The initiation of courtship

involves visual, vibratory, and olfactory signals (Fernandez and Kravitz, 2013; Wicker-Thomas and Hamann, 2008). This allows the individual flies to spatially orient themselves to one another during the mating process and to determine which available conspecifics are optimal mates. To begin, the male will circle around the front of the female. He will then approach her and tap her abdomen with his forelegs, which contain the Gr68a receptors (Dahanukar and Ray, 2011; Fernandez and Kravitz, 2013).

Table 2. Known sex pheromone receptors and corresponding locations.

Receptor	Function	Associated pheromone / ligand	Location	Reference
Or47b	Conveys sensitivity to conspecific odorants	(in accordance with input from the VA1v glomerulus in females)		Vosshall et al. (2000)
Or65a	Required in males for suppressing courtship	cVA	T3 trichoid sensilla on antenna	Ejima et al. (2007); Dahanukar & Ray (2011)
Or67d	Promotes male-male aggression	cVA	T1 trichoid sensilla on antenna	Kurtovic et al. (2007); Dahanukar & Ray (2011)
Or83b	Common subunit in majority of odorant receptors	cVA (in accordance with receptor Or67d)		Wicher et al. (2008)
DmDopEcR	Modulates neuronal signaling in males	20-hydroxyecdysone	Corresponding proteins in mushroom bodies	Abrieux et al. (2013)
Gr32a	Binds male-specific inhibitory signals		Projections to ventrolateral protocerebrum	Miyamoto & Amrein (2008)
Gr33a	Binds male-specific inhibitory signals	(possibly) z-7 tricosene		Moon et al. (2009)
Gr36a	Bitter taste receptor	Inhibitory sex pheromone		Clyne et al. (2000); Weiss et al. (2011)
Gr68a	Induces regular courtship in males and females	ppk25	(In males) on forelegs, in gustatory neurons, auditory neurons in Johnston's organ	Bray & Amrein (2003)

These Gr68a receptors bind nonvolatile pheromones from the female during the mating ritual (Fernandez and Kravitz, 2013) and determine whether the female has any traces of previously deposited sex peptide or cVA, both of which deter the male from further courting. Sex peptide is bound tightly to the sperm of the male (Chapmann *et al.*, 2003; Dickson, 2008). This peptide induces certain female post-mating behaviors, such as the reluctance to mate again (Wigby and Chapmann, 2005). When females mate with mutant males that do not possess the sex peptide, they maintain their desire to continue to mate (Dickson, 2008). However, virgin females are the most likely candidate to accept courtship from males (Odeen and Morray, 2008), and are therefore the most desired targets. Additionally, *ppk25* has been identified as another potential ligand for the male

Gr68a receptor (Dahanukar and Ray, 2011; Fernandez and Kravitz, 2013), functioning as a modulator of the gustatory perception of contact pheromones (Pikielny, 2012).

If the female escapes, the male will pursue her while singing a species-specific song with his wings (Fernandez and Kravitz, 2013). The female may then slow down and open her vaginal plates for the male to commence copulation. If the male is rejected by the female, she will display kicking behavior and will not open her vaginal plates (Fernandez and Kravitz, 2013). The direct contact between the male forelegs and the female abdomen is an important moment when both the olfactory and gustatory systems are incorporated into the mating process.

In the case of successful copulation, cVA is transferred to the female in the male ejaculate and marks her as an already mated female (Dahanukar and Ray, 2011). Males that express Or67d or Or65a receptors receive this pheromonal signal, which acts as an anti-aphrodisiac. In addition to these receptors, LUSH, an odorant binding protein, is necessary to elicit responsiveness to cVA (Dahanukar and Ray, 2011; Fernandez and Kravitz, 2013; Kim *et al.*, 1998; Martin *et al.*, 2013). Additionally, cVA activity is represented in the DA1 glomeruli (Fernandez and Kravitz, 2013). This glomerulus is sexually dimorphic in size and is larger in males, although there is no difference in responsivity to cVA in males or females (Fernandez and Kravitz, 2013).

CH503, another cuticular hydrocarbon, is a pheromone similar to cVA in that it deters males from courting mated females (Dahanukar and Ray, 2011). CH503 has been detected on mated females directly after copulation and lasts 10 days after mating, indicating that it is less volatile than cVA (Dahanukar and Ray, 2011). CH503, along with receptor Or67d, has also been linked to male aggressive behavior (Dahanukar and Ray, 2011). Although CH503 is linked to male-male aggression, we do not yet know the specific quantity or concentration that will induce aggression. If a male *D. melanogaster* lacks the Or67d or Or65a receptors, it will not recognize that the female in question has already mated and is no longer susceptible to its advances. This male-specific olfactory mechanism is used to differentiate between mated and virgin female *D. melanogaster*. Naïve males will still attempt to initiate courtship with previously mated females, albeit with less enthusiasm than with virgin females (Keleman *et al.*, 2012).

An integral part of courtship behavior is a male's ability to decipher rejection from acceptance by females during courtship. The presence of cVA is not the most important factor in mate choice, but rather the previous experience with a particular female and whether the male experienced success or failure at copulating (Fernandez and Kravitz, 2013). Mating success is elevated, through enhancement of sensitivity to cVA, by learning to discriminate between mated females and virgin females.

The most important factor in male *D. melanogaster* learned mating behavior is success or lack of success in mating attempts. Keleman *et al.* (2012) showed that male mating behavior is conditioned by the failure to mate, not simply the rejection. When males were paired with mated females, they learned not to waste their energy or pheromones, while males that were paired with virgins learned to attempt mating with any female, because their advances were always accepted. Thus, males trained by mated females knew not to waste their energy on previously mated females that they encountered subsequently, while males trained with virgins made continuous futile attempts to mate with previously mated females.

Many studies have investigated male-male courtship behavior. Gr33a is a receptor that has been linked to male inhibitory signals with 7-tricosene as a possible ligand (Dahanukar and Ray, 2011; Fernandez and Kravitz, 2013). Additionally, the Gr32a receptor binds male-inhibitory signals, and these signals are then projected to the ventrolateral protocerebrum to induce rejection of male-male courtship (Dahanukar and Ray, 2011; Fernandez and Kravitz, 2013). While the Gr68a receptor helps to regulate normal courtship between males and females, it also works as a receptor for virgin female pheromones and to trigger courtship behavior in males (Dahanukar and Ray, 2011). Given

the range of behavioral responses that are affected as a result of these documented pheromones and their corresponding receptors, changes in commonly used methodologies can be approached.

Laboratory Protocols and Applications

Sexual behavior in male and female *D. melanogaster* may confound an experimental design. *D. melanogaster* exhibit a relatively short time frame from pupal eclosion to sexual maturation (approximately 8 hrs), and experimenters must be consistently vigilant over this time period to limit reproductive contamination and inbreeding. This is accomplished by monitoring the eclosion rate of new offspring in order to maintain generational distinction.

Many protocols require the experimenter to remove any remaining adults one week after making a new cross, given that the female can lay hundreds of eggs during this time. When the offspring begin to eclose from the pupae 2 to 4 days after the adults are removed, the virgin females must be removed from the rest of the offspring (provided that these offspring are to be crossed with genetically different strain). This step is critical in preventing inbreeding with males from the same strain, rather than the target strain. In such a case, it would be impossible to determine whether the offspring of the new cross, which typically arise in large quantities, are from the target cross strain (no contamination), or from the same cross from which the parents were reproduced (contamination). Other experiments require multiple repetitions of this process in a short amount of time. Using pheromones to prolong a period of less contact and attraction between males and females would, therefore, be a practical and non-invasive method of extending the interval of time after sexual maturation occurs when the flies typically begin to interact, in order to prevent contamination in these types of experiments.

Genetic manipulation of pheromone receptors may be a simple way to regulate sexual behavior in *D. melanogaster*. For example, an experimenter may limit sexual behavior by inducing a loss of function mutation to receptors such as Gr68a or *fruitless*. Additionally, one may reduce the frequency of mating rituals by inducing a gain of function mutation to Or65a. These are plausible techniques, but mutant strains could potentially crossbreed with an unwanted strain, in turn affecting sexual behavior and subsequent experiments. However, genetic manipulations may induce permanent changes and inhibit mating behaviors. Pheromonal applications, on the other hand, may provide temporary control over sexual behavior.

Utilizing the intact pheromonal system to regulate sexual behavior can also prove advantageous. CH503 and cVA both deter courtship behavior between males and females. Application of CH503 would deter female-male courtship for 10 days, providing temporary control of *D. melanogaster* sexual behavior (Dahanukar and Ray, 2011; Yew *et al.*, 2009). To induce regular courtship after the application of CH503, the pheromones 7-11HD or *ppk25* could be applied. This is relatively easy and cost effective, and can create a more controlled environment for genetics research. However, limitations of CH503 and cVA application include (1) effects on male-male aggression, (2) necessary reapplication of pheromones, and (3) long term effects of pheromonal application on typical mating behavior.

Conclusion

This review has illustrated the intricacies of the *D. melanogaster* olfactory and gustatory systems and their role in pheromonal reception, examined how pheromones influence sexual behavior, courtship behavior and mate preference, and compiled major known pheromones and

receptors and their specific mechanisms of action. Additionally, genetics research on *D. melanogaster* may benefit through the manipulation of sexual behavior by pheromonal application. This can be used to prolong the interval of time from pupal eclosion to the first mating experience, which is usually relatively short and renders stock cultures easily susceptible to contamination. Extended investigations of application dose levels and interactions between these chemicals would provide a better understanding of how to monitor fecundity levels and keep contamination minimal.

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Utilizing phospho-histone H3 labeling in the *Drosophila* larval central nervous system to generate parametrically testable mitotic index data sets.

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Introduction

In *Drosophila*, the central nervous system (CNS) of the third instar wandering larvae continues to be a highly utilized tissue for cytological studies investigating the mitotic phenotypes of mutations thought to impact cell-cycle progression and/or chromosome dynamics. The larval CNS is unique in that, unlike most other larval organs, it persists into the adult stage (Truman, 1990), and it is comprised of two major cell types all undergoing canonical cell cycles: the neuroblasts and the ganglion mother cells (Hofbauer and Campos-Ortega, 1990). There are many published protocols available in the literature for squashing and labeling larval CNS tissue for the generation of mitotic indices in an effort to characterize mitotic defects (Gatti, 1974; Gatti and Goldberg, 1991; Bently, 2001; Williams, 1992; Bolkan, 2007; Ayeni, 2013). However, in our experience many previously described methods, while useful for analysis of chromosome morphology, did not generate data sets that were amendable to parametric statistical testing such as t-tests and ANOVAs.

In the following report, we describe a variation of our existing protocol (Apger, 2010) for examining the progression of the canonical cell cycle of the larval CNS that utilizes the sensitivity of phospho-histone H3 (PH3) labeling to generate mitotic indices. The added sensitivity of the PH3 labeling method allows for the generation of data that satisfies the assumptions of parametric statistical testing. With these assumptions satisfied, statistical tests, such as t-test and ANOVAs, can now legitimately be used to compare the effects of different mutant alleles on cell-cycle progression.

Materials and Methods

Drosophila Stocks

The w^{1118} line was obtained from the Bloomington Stock Center (Flybase ID: FBst0006326). Flies were maintained at 25°C on *Drosophila* Diet Medium K12 (US Biological Cat #D9600-07B).

Tissue Acquisition

Wandering third instar larvae were collected and placed in a 16 well dissecting dish containing 100 µl of 1× PBS (140 mM NaCl, 2.7 mM KCl, 1.4 mM Na_2HPO_4, 1.8 mM KH_2PO_4).

The CNS was isolated using No.5 tweezers (Electron Microscopy Sciences, Hatfield, PA). All imaginal discs were removed from the CNS tissue following dissection.

Swelling and Fixing Tissue

After dissecting the central nervous system and removing the attached imaginal discs, the tissue was transferred into a new well containing 100 µl of hypotonic solution (0.5% sodium citrate) and allowed to incubate for 10 minutes at room temperature. After 10 minutes, the tissue was moved into another well, containing 100 µl of 4% formaldehyde in dH_2O, for 30 minutes at room temperature.

Squashing and PH3 labeling

Day One: Once fixed, the tissue was transferred to a clean microscope slide, along with 10 µl of fixative. Next, a siliconized coverslip was carefully placed on top of the sample followed by a piece of filter paper and another microscope slide. Using a 4" vise, the sandwiched tissue was squashed for 2 minutes. Following the squash, the slide containing the tissue was lowered into liquid nitrogen for one minute. Once the slide was removed from the liquid nitrogen, the siliconized cover slip was removed with a razor blade. The sample was then incubated in 75 µl of 1× PBS for 6 minutes. After 6 minutes, the 1× PBS solution was removed by blotting around the squashed sample with a Kimwipe®. The sample was then air-dried for 1 minute and placed in 75 µl of 0.5% Triton-X in 1× PBS for 15 minutes at room temperature. Following the 15 minute incubation, excess Triton-X was removed with a Kimwipe®, and the sample was then incubated with 75 µl 3% bovine serum albumin (BSA) in membrane wash buffer (10× PBS, 2% Tween-20) for 30 minutes at room temperature. After 30 minutes, the BSA was removed and the slide was washed with 1× PBS. Using a 20cc syringe equipped with a 22 gauge blunt fill needle filled with Vaseline®, a single line of petroleum jelly was dispensed encircling the squashed sample. Next, 75 µl of primary antibody (rabbit α-phospho-histone H3) diluted 1:750 in 3% BSA was placed on the sample. The slides were then placed into a tupperware container lined with damp paper towels and incubated overnight at 4°C.

Day Two: Following the overnight incubation, the samples were washed by tilting the slide and gently dispensing 1× PBS, three times, making sure to remove the Vaseline® from the slide. The slide was then allowed to air dry for one minute, and subsequently incubated with 100 µl of secondary antibody (anti-rabbit 468) diluted 1:500 in 3% BSA in the dark for 1 hour. After 1 hour, the slides were washed three times with 1× PBS and treated with fresh 3 µg/ml 4',6-diamidino-2-phenylindole (DAPI) solution for 8 minutes. After the DAPI staining the slides were then washed three more times with 1× PBS.

Tissue Mounting

30 µl of Vectashield® Mounting Medium (Cat. No. H-1000, Vector Laboratories, Burlingame, CA) was dispensed on the squashed samples and coverslips (Fisherfinest®, 22 × 50-1, Cat. No. 12-548-5E) were carefully placed on the tissue. With the coverslip on the slide, the corners of the coverslip were gently tapped to ensure the entire area under the coverslip was occupied by Vectashield®. Nail polish was then used to seal the coverslip to the microscope slide.

Imaging the Squashed Sample

Slides were imaged using 63× magnification on the Zeiss LSM700 Confocal equipped with Zeiss's ZEN Black software package. Ten random, highly populated fields of view were imaged

using the 405nm and 615nm wavelength filter for DAPI and PH3 visualization, respectively. Images were saved as .tiff files for analysis using Adobe® Photoshop® elements CS5.1.

Calculating a Mitotic Index

The first step to determining the mitotic index was to count the total number of nuclei in Photoshop® CS5.1 using the count tool. Following this, the numbers of actively dividing mitotic cells (seen by PH3 labeling) were counted. The mitotic index was determined by dividing the number of actively dividing cells by the total number of nuclei. A mitotic index was calculated for each of the ten images. Calculation of the mitotic index for DAPI stained images (without PH3 labeling) was performed as described in Apger (2010).

Statistical evaluation of distributions

Mitotic indices were calculated as described above using Excel®2010, and the square root transformations were conducted in Excel® 2010 by simply taking the square root of each mitotic index value. Mitotic indices were organized by slide ID so that comparisons could be made for both detection methods on a slide by slide basis. Datasets were imported into JMP®10 software package to visualize the distributions using the analyze distribution function. JMP®10 was also used to conduct Levene's test of variance by utilizing the Fit Y by X function using the slide ID as the grouping variable, and selecting "unequal variances". Paired t-tests were also carried out in JMP®10 software using the matched pairs utility. Data sets requiring comparisons were imported into R statistical software to conduct Kolmogorov-Smirnov Goodness of Fit analyses using standard practices.

Results

PH3 labeling provides increased sensitivity and reduces subjectivity from nuclei counts

In order to compare the labeling efficiency and sensitivity of DAPI staining versus PH3 labeling in the central nervous system (CNS) of third instar wandering larvae, ten wild type brains were dissected, squashed, and dual labeled as mentioned in the materials and methods section. From each of those slides, ten random, monolayered, and highly populated fields of view were acquired at 63× using a Zeis LSM 700 Confocal microscope equipped with Zen Black imaging software (representative images shown in Figure 1 A and B). The 100 images were then exported and counted twice using the count feature in Photoshop® CS5.1. One set of counts utilized only the DAPI signal to detect mitotically dividing nuclei and the total number of nuclei per field of view, and the other set used both the DAPI and PH3 signals to record the number of $PH3^+$ nuclei and the total number of nuclei per field of view. A mitotic index was then calculated and recorded for each image, using each labeling method, by dividing the number of mitotically dividing nuclei by the total number of nuclei present in that field of view. The resulting distributions (Figure 1 C and D) show that there is a much higher abundance of zeros when using the DAPI detection method than with the PH3 method. Furthermore, the PH3 labeling method also reduced the individual subjectivity when counting mitotic nuclei such that multiple people could count the same image and reliably obtain the same number of mitotic cells, but this was not the case with the DAPI method of detection, which leaves the counting of some mitotic figures open to individual interpretation.

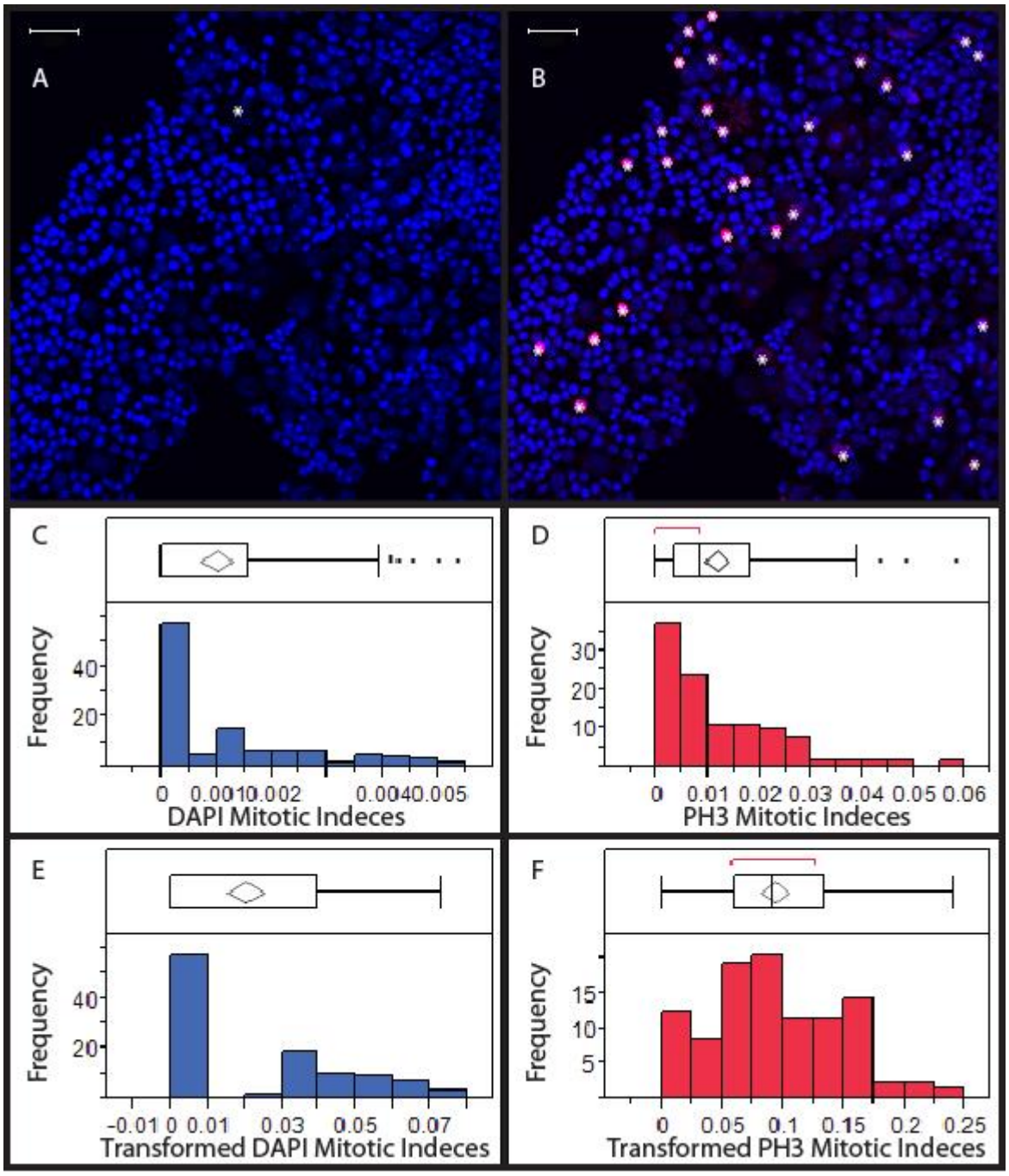

Figure 1. Increased sensitivity of PH3 labeling and its ability to generate "approximately normal" data. A: 63× confocal micrograph of DAPI stained nuclei depicting one unambiguous mitotic nucleus marked with an asterisk. (scale bar = 20 µm); B: 63× confocal micrograph of the same slide shown in A with the PH3 signal represented. The asterisks denote mitotic nuclei that would not have been counted using only DAPI alone. (scale bar = 20 µm); C and D: Visual representations of the distributions of mitotic indices generated using DAPI (C) or PH3 and DAPI combined (D); E and F: Visual representations of the distributions of mitotic Indices using DAPI (E) or PH3 and DAPI (F) once transformed using a square root function.

Statistical evaluation of the DAPI and PH3 mitotic index distributions

To confidently state that the PH3 detection method provides an advantage over the DAPI detection method, the distributions needed to be statistically compared to each other. To begin this comparison, a Kolmogorov-Smirnov Goodness of Fit analysis was conducted to compare the DAPI and PH3 distributions. This test validated that the two distributions were significantly different ($P < 2.2e^{-16}$). Though the PH3 labeling method generates a significantly different distribution, which contains less zeros and reduces ambiguity in counting mitotic figures, observation of the resulting DAPI and PH3 distributions (Figure 1 C and D, respectively) easily show that neither of these distributions appears to be normally distributed. Furthermore, significant results from Levene's test for homogeneity of variance for both DAPI ($P < 0.0001$) and PH3 ($P = 0.0264$) distributions demonstrate that both suffer from heterogeneous variance. Thus, the skewed nature of the distributions and homogenous variance for these data sets would violate two of the three assumptions that must be met to reliably conduct a parametric test.

The DAPI and PH3 distributions were transformed using a basic square root transformation and then reevaluated for the ability to meet the assumptions of normality and homogenous variance. The variances of the transformed distributions were tested again using Levene's test and resulted in a significant result for the transformed DAPI data set ($P = 0.0001$) but not for the transformed PH3 data set ($P = 0.7393$). Therefore the transformed PH3 distribution now meets the assumption of homogeneous variance; furthermore, the square root transformation also makes the PH3 distribution, but not the DAPI distribution, appear much more normally distributed (Figure 1 E and F). However, a significant result from a Kolmogorov-Smirnov Goodness of Fit analysis comparing the transformed PH3 distribution to a generated normal distribution ($P < 2.2e^{-16}$) suggests that the data are "approximately normal" but not completely normally distributed. Finally a paired t-test that was conducted, using slide numbers for grouping, evaluating both transformed distributions and a P-value < 0.0001 indicates that the transformed PH3 data generate a different mean both among and within the groups when compared to the transformed DAPI data set.

Discussion

The use of mitotic indices as means of assessing cell cycle progression, and even as a diagnostic test for the presence of cancerous cells, has been utilized in many model systems from cell culture to canines, displaying the versatility and potential sensitivity of this type of assay (Romansik, 2007; Baak, 2009). In the *Drosophila* community the larval CNS has been used extensively to evaluate mutants for impacts on cell cycle progression and chromatin morphology, and as such there are many different approaches for utilizing these tissues in the literature (Gatti, 1974; Gatti and Goldberg, 1991; Bently, 2001; Williams, 1992; Bolkan, 2007; Ayeni, 2013). The popularity of this specific tissue for this type of analysis can be explained by the ease of dissection of the tissue, and the fact that the CNS is comprised of two major cell types all undergoing canonical cell cycles: the neuroblasts and the ganglion mother cells. The neurobalsts can divide either symmetrically or asymmetrically resulting in two neuroblasts or a neuroblast and a ganglion mother cell, respectively. Ganglion mother cells can then divide only once, producing two daughter cells that differentiate into neurons (Hofbauer, 1990; Truman, 1990). Due to the proliferative nature of these cells, the larval CNS contains proliferation centers that produce characteristic patterns of cell cycle progression in specific regions (Truman 1988; Truman, 1990; Ito, 1991). These stereotypical patterns of cell cycle progression can be visualized using thymidine analogue incorporation assays in the brain (Apger, 2010). For this reason our lab attempts to reduce bias and inflated counts of mitotic nuclei by acquiring 10 random highly populated fields of view from each larval CNS that is analyzed as

opposed to counting single fields of view, acquiring adjacent fields of view, or attempting to count entire brains (Bolkan, 2007; Baak, 2009; Apger, 2010).

As stated previously there are many established protocols in the literature that can be used to visualize mitotic cells in the larval CNS; however, the methods used to calculate mitotic indices vary greatly from one source to another (Gatti, 1974; Gatti and Goldberg, 1991; Bently, 2001; Williams, 1992; Bolkan, 2007; Ayeni, 2013). Furthermore, the statistical methods used to compare these mitotic indices are often not clearly reported, or commonly employ non-parametric testing to elucidate non-quantitative differences between treatments (Gatti, 1974; Gatii and Baker, 1989; Gatti and Goldberg, 1991; Bently, 2001; Williams, 1992; Bolkan, 2007; Ayeni, 2013). Our lab has historically followed the protocol listed in Apger (2010) which is roughly based on the protocols described by Gatti and Goldberg (1991) with some modifications. However, in our experience with this protocol, we commonly acquire data that do not meet the assumptions required for parametric testing (*i.e.*, normality, homogenous variance, and independence of samples/error), thus we must rely upon non-parametric tests for comparisons (Glass, 1972; Zimmerman, 1998). The assumptions that we usually failed to satisfy were those of normality and homogeneous variance, and these issues resulted from an abundance of zeros in our data sets acquired using DAPI staining (see Figure 1 C and E). The approach utilized to solve these issues was to increase our assays sensitivity such that the number of mitotic indices with a value of zero would be reduced, but maintain the same sample size. The added sensitivity of PH3 antibodies to detect mitotically dividing cells throughout M phase is prevalent in the literature and has been conducted in the larval CNS (Hans, 2001; Bolkan, 2007; Pennetier, 2012). Therefore, we decided to utilize the added sensitivity of the PH3 antibody labeling approach with our currently established protocol from Apger (2010).

A comparison of the distributions obtained by the DAPI labeling versus the PH3 labeling strategy (Figure 1 C and D) clearly demonstrated that the added sensitivity of PH3 antibody labeling did reduce the overall number of mitotic indices with a value of zero. However, the data obtained by both methods remained heavily skewed and suffered from heterogenous variance. The reduction in zero values provided by the PH3 labeling approach, but not the DAPI staining approach, allowed for a standard square root transformation to make the PH3 distribution both "approximately normal" and contain homogenous variance. Given that our PH3 distribution now meets the assumptions of homogenous variance and independence of samples/ errors, we can legitimately conduct parametric analyses with "approximately normal" distributions, because the slight violation of normality does not have as much of an impact on parametric tests as violations of the assumptions that our data satisfies (Glass, 1972). Furthermore, reports in the literature state that parametric tests remain more robust than non-parametric tests when using roughly normal data (Zimmerman, 1998).

In summary, we have demonstrated that the use of PH3 antibody to label mitotic cells using the method outlined in Apger (2010) will result in a distribution that is amendable to parametric testing after a standard square root transformation is applied to the data. This will allow for a variety of parametric tests to be conducted on the resulting data in a way that will provide quantitative differences between mutants/treatments.

Acknowledgments: We would like to thank Dr. David Kimmel for statistics advice on this manuscript.

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Protocol: Utilizing phospho-histone H3 labeling in the *Drosophila* larval central nervous system to generate parametrically testable mitotic index data sets

Reagents

1× PBS (140 mM NaCl, 2.7 mM KCl, 1.4 mM Na_2HPO_4, 1.8 mM KH_2PO_4)
0.5% Triton-X in 1× PBS
3% BSA in membrane wash buffer (10× PBS, 2% Tween-20)
0.5% sodium citrate in dH_2O
4% formaldehyde in dH_2O
Sigmacoat® (Cat. No. SL-2, Sigma-Aldrich Co. LLC, St. Louis, MO)
3 μg/ml DAPI (4',6-diamidino-2-phenylindole) in 1× PBS
Primary Antibody (rabbit α-phospho-histone H3)
Secondary Antibody (anti-rabbit 468)

Equipment

Slides (Fisherbrand® 25 × 75 × 1.0 mm, Cat. No. 22-034-486)
Vectashield® Mounting Medium (Cat. No. H-1000, Vector Laboratories, Burlingame, CA)
Coverslip (Fisherfinest®, 22 × 50-1, Cat. No. 12-548-5E)
Liquid nitrogen
No. 5 Tweezers (Electron Microscopy Sciences, Hatfield, PA)
Blotting paper
Vaseline®
4" Vise
Tupperware containers
Paper towels
Kimwipe®
Razorblade

Dissecting and labeling 3rd instar larval CNS

DAY ONE

1. Using a 16 well dissecting dish, dissect the 3rd instar wandering larval CNS in 100 μl of 1× PBS. Make sure to remove the attached eye and wing imaginal discs.

2. Transfer the tissue into a second well filled with 0.5% sodium citrate (hypotonic solution) for 10 minutes.
3. After 10 minutes, move the CNS into another well filled with 4% formaldehyde (fixative) for 30 minutes.
4. Transfer the CNS, along with 10 µl of fixative, onto a clean slide and carefully lower a siliconized coverslip on top of the tissue.
   ***Helpful Hint:** Make sure to mark the location of the tissue on the bottom of the slide after placing the siliconized coverslip.*
5. To create the squashed sample, a vise will be utilized. Create a microscope slide sandwich by placing a sheet of blotting paper and another blank slide on top of the sheet. Place the sandwich in the vise, tighten, and let squash for 2 minutes.
   ***Helpful Hint:** Be careful when handling the microscope slides. Shuffling the slides can result in poor images.*
6. After 2 minutes, freeze the sample to the slide in liquid nitrogen for one minute. Once the slide has been removed from liquid nitrogen, pop off the siliconized coverslip using a razor blade.
7. Next, wash the sample with 75 µl of 1× PBS for 6 minutes.
   ***Helpful Hint:** If performing multiple dissections, you may prolong this wash period.*
8. Remove the PBS solution from the sample by tilting the slide and blotting carefully with a Kimwipe®. Make sure not to disrupt the sample.
9. Treat the dried slide(s) with 75 µl of 0.5% Triton-X solution in 1× PBS for 15 minutes.
10. Remove excess Triton-X solution in PBS as describe above.
11. After removing the solution, incubate the sample in 75 µl 3% BSA for 30 minutes.
12. Following the 30 minute incubation, wash the slide once with 1× PBS.
13. Using a syringe of Vaseline, encircle the sample. This keeps the primary antibody (see next step) on the dissected sample.
14. Place 75 µl of primary antibody, diluted in 3% BSA, onto the sample (if using phospho-H3 perform a 1:750 dilution).
15. Next, place the sample slide(s) in a humid chamber and incubate at 4ºC overnight. Create a humid chamber by lining the interior of a Tupperware container with damp paper towels.

DAY TWO

1. Gently wash the slide(s) three times with 1× PBS.
2. After the third wash, remove excess PBS with a Kimwipe®.
3. Next, incubate the sample in 100 µl secondary antibody, diluted (1:500) in 3% BSA, in the dark for 1 hour.
4. Following this incubation, wash the sample an additional three times with 1× PBS.
5. Again, remove excess PBS with a Kimwipe®.
6. Continuing, place 3 µl of DAPI in 1× PBS [3 µg/ml] onto the sample(s) for 8 minutes.
7. Gently wash the slide(s) three times with 1× PBS and then dry by removing excess PBS with a Kimwipe®.
8. For fluorescence, mount the sample(s) in Vectashield® H-1000 and gently place down a non-siliconized coverslip.
9. Seal the coverslip around the edges with nail polish to prevent Vectashield® from evaporating.

Optimization of 64 microsatellite loci primer pair annealing temperatures of *Drosophila mediopunctata*.

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Drosophila mediopunctata is a model organism that our research group has been studying for almost 30 years (Klaczko, 2006). Laborda *et al.* (2009) developed and characterized 134 microsatellite *loci* for this species. They performed PCR in a gradient temperature (*touchdown PCR*), and, although the temperature range was established, for many *loci* the exact annealing temperature (T_a) was not determined.

Preliminary tests using homokaryotypic strains from the laboratory and samples from natural populations of *D. mediopunctata* revealed unspecific alleles for several *loci*. In an attempt to make our genotyping more reliable, eliminating unspecific alleles from our analysis, we tried to set accurately the annealing temperature (T_a) for several *loci* primer pairs.

Genomic DNA was isolated from four individuals, two from each of a pair of standard homokaryotypic strains (*ITC 229ET* and *ITA 24P*). Polymerase chain reactions (*PCRs*) were performed in 25 µl reactions with: 11.5 µl of Milli-Q water, 1 µl of DMSO, 2.5 µl of buffer 10×, 2.5 µl of dNTP Mix (2 mM), 2 µl of $MgCl_2$ (25 mM), 1 µl of *Taq* DNA polymerase, 1.25 µl of each primer (10 mM), and 2 µl of genomic DNA (approximately 10 ng/µl). Thermocycler parameters were: 95°C for 5 min, 40 cycles of 94°C for 1 min, T_a for 1 min and 15 s, 72°C for 1 min and 30 s, 72°C for 30 min.

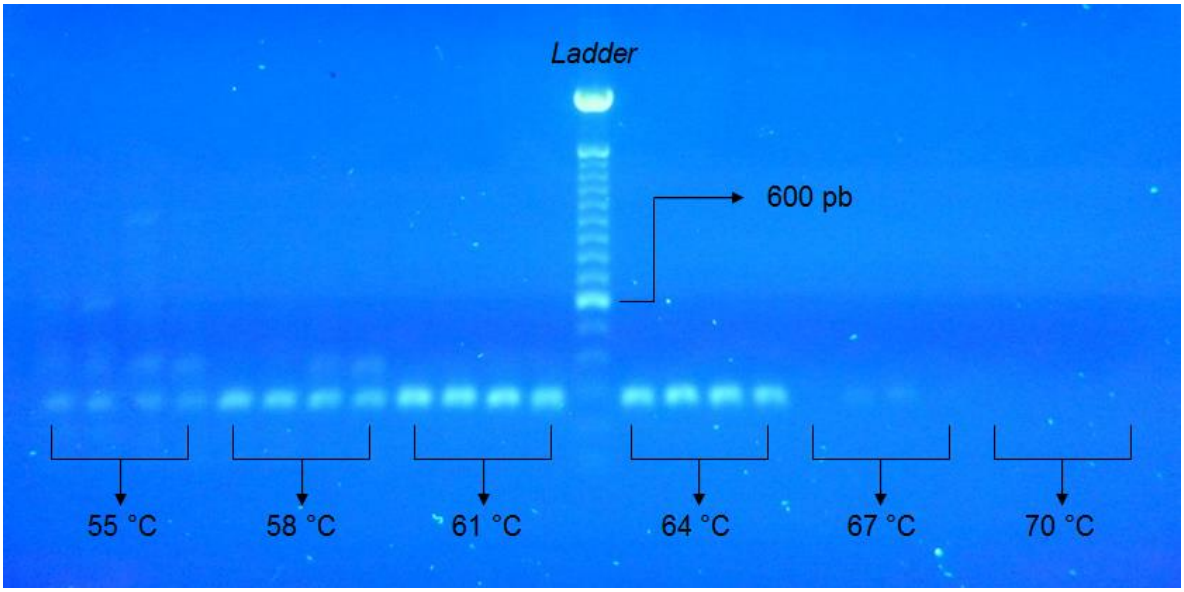

Figure 1. Locus *Dmed*048 amplified products visualized in 1% agarose gel (TBE 1×), stained with *SYBR-safe*. Annealing temperature (T_a) is 64ºC.

We tested the amplification of 64 *loci* in six specific annealing temperatures (T_a = 55ºC; 58ºC; 61ºC; 64ºC; 67ºC; and 70°C). The highest temperature that showed positive amplification with minimum unspecific alleles was assumed to be the optimal annealing temperature for each *locus* primer pair. PCR products were visualized in 1% agarose gel (TBE 1×) stained with *SYBR-safe* (Figure 1). Table 1 shows the specific annealing temperature settled for each locus primer pair.

Table 1. Annealing temperatures (T_a) of 64 primer pair loci of *Drosophila mediopunctata*.

Locus	T_a (ºC)	*Locus*	T_a (ºC)	*Locus*	T_a (ºC)	*Locus*	T_a (ºC)
*Dmed*003	58	*Dmed*040	58	*Dmed*071	61	*Dmed*100	58
*Dmed*006	64	*Dmed*044	61	*Dmed*072	58	*Dmed*102	58
*Dmed*011	58	*Dmed*045	55	*Dmed*074	58	*Dmed*103	64
*Dmed*012	55	*Dmed*046	61	*Dmed*076	61	*Dmed*106	61
*Dmed*014	61	*Dmed*048	64	*Dmed*078	61	*Dmed*107	67
*Dmed*015	61	*Dmed*049	64	*Dmed*080	61	*Dmed*109	61
*Dmed*017	61	*Dmed*051	61	*Dmed*084	58	*Dmed*112	58
*Dmed*018	61	*Dmed*053	58	*Dmed*085	58	*Dmed*113	61
*Dmed*020	61	*Dmed*054	58	*Dmed*086	61	*Dmed*114	55
*Dmed*021	58	*Dmed*058	64	*Dmed*087	61	*Dmed*115	61
*Dmed*025	58	*Dmed*060	61	*Dmed*091	61	*Dmed*117	58
*Dmed*027	61	*Dmed*061	61	*Dmed*094	58	*Dmed*118	61
*Dmed*028	61	*Dmed*064	61	*Dmed*095	61	*Dmed*120	55
*Dmed*030	58	*Dmed*067	64	*Dmed*096	61	*Dmed*123	61
*Dmed*035	61	*Dmed*069	61	*Dmed*097	67	*Dmed*130	61
*Dmed*037	61	*Dmed*070	58	*Dmed*098	67	*Dmed*131	61

Loci names are abbreviated, such as *Dmed*058 for Dmed^{UNICAMP}_ssr058.

Acknowledgments: We thank Iuri M. Ventura, Mitsue T. Brianti, and Maurício P. de Arruda for cogent suggestions; and Claudete do Couto for technical help. Funds were provided by: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq); Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES); Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP); Fundo de Apoio ao Ensino e à Pesquisa (FAEPEX-UNICAMP).

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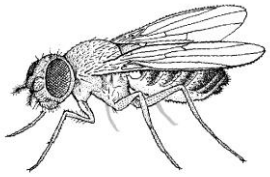

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Mutation Notes

A new mutation in *Drosophila malerkotliana.*

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Drosophila malerkotliana, which was described by Parshad and Paika from Punjab, India, in 1964, belongs to the *bipectinata* species complex of the *ananassae* subgroup of the *melanogaster* species group (Bock and Wheeler, 1972). On the basis of abdominal tip pigmentation in males, *D. malerkotliana* has been classified into two allopatric subspecies, namely, *D. m. malerkotliana*, black abdomen, and *D. m. pallens*, yellow abdomen (Singh and Sisodia, 2008). This species shows incomplete sexual isolation with other members of the *bipectinata* complex (Bock, 1978; Singh, *et al.*, 1981, 1982; Singh and Chatterjee, 1991). Similarly, *D. malerkotliana* shows asymmetrical sexual isolation with *D. parabipectinata* (Banerjee and Singh, 2012). Chromosomal polymorphism has also been studied, and inversions are known to occur in natural populations of *D. malerkotliana* (Bock, 1971; Rahman and Jha, 1973; Tomimura *et al*., 2005). Similarly, persistence of chromosome inversions in laboratory stocks of *D. malerkotliana* was observed by Banerjee and Singh (1995). Behavioral studies have been documented to some extent in this species (Hegde and Krishna, 1997, 1999; Singh and Singh, 2013). Several induced and spontaneous mutations have been reported in this species (Srivastava and Singh, 1995; Siegal *et al*., 2004).

A large number of stocks of *D. malerkotliana* established from flies collected from different geographical localities are being maintained in our laboratory. This note describes an x-ray induced mutation in *D. malerkotliana*. For irradiation experiments the males were taken from a wild type stock collected from Punjab, India, and raised for many generations in the laboratory. The newly hatched males were collected. For irradiation 50 males were irradiated immediately and 50 males were aged for two days and then given radiation. These males were kept in a gelatin capsule and were exposed to X-rays under following conditions:

Target distance – 50 cm
KVP – 120 KVP
Dose rate – 400 r per minute
Total dose given - 1600 r in 4 min.

In each experiment 50 males were irradiated under similar conditions. The newly hatched wild type irradiated males were allowed to grow at least for 2 to 3 days and were then mated for four days with a first set of 40 virgin females (wild type). Similarly, two days old irradiated males were immediately mated with 50 four days old virgin females. After four days these males were separated and mated with another set of 40 wild type virgins. Again after four days these males were separated and mated again with another set of 40 wild type virgin females. After 12-16 days F_1 progeny were collected from all the bottles and observed for any variant. Pair mating was made from these F_1 flies in food vials. F_2 progeny from food vials were carefully examined for any variations from the wild type.

In one of the vials, six males showing brownish eye color were observed. The brownish eye color in these males shows resemblance with *garnet* eye color, a sex linked mutation of *D. ananassae* reported by Hinton (1980). These males were crossed with wild type females and the resulting progeny from this cross were normal. When these flies were pair mated, some of the males obtained from this cross showed *garnet* eye color. These males were pair mated with females from the same cross, which resulted in the production of *garnet* eye color females. A separate homozygous line was established by using females and males showing *garnet* eye color. In order to test the inheritance pattern, virgin *garnet* eye color females were collected from the stock and mated with wild type males. All the F_1 males showed *garnet* eye color demonstrating sex linked inheritance. Figure 1 shows a mutant male with *garnet* (*g*) eye color. Thus it is concluded that *garnet* eye color mutation in *D. malerkotliana* is a sex linked recessive mutation that was induced by X-rays. It is a new mutation being reported for the first time in this species.

Figure 1. Garnet eye color phenotype in *Drosophila malerkotliana.*

Acknowledgments: Thanks are due to the Apex Hospital, Varanasi, and their staff members for providing the X-ray facility. The financial assistance in the form of UGC-BSR Faculty Fellowship Award to B.N.S. and the Meritorious Fellowship to A.S. from the University Grants Commission, New Delhi, is gratefully acknowledged.

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New spontaneous wing mutant *curly* in *Drosophila willistoni* strain GdH4-1.

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Introduction

In the 20th century the family Drosophilidae has become the focus of increased interest in the areas of genetics, development, and evolution, their most known species *Drosophila melanogaster* being used as a model organism in several experimental studies, including of human diseases (Leopold and Perrimon, 2007; Vosshall, 2007). However, the genus *Drosophila* comprises 15 subgenera and about 1,400 species, with high diversity and wide geographic distribution. The subgenus *Sophophora* (Sturtevant, 1939) comprises 332 species divided into eight groups, among which is the *willistoni* group (Bächli, 2008).

In the beginning of the 21st century the complete genome sequencing of 12 *Drosophila* species shows the interest in the genus as eukaryote models (Clark *et al.*, 2007; Schaeffer *et al.*, 2008). Other species can also become important, as their biology and genetics reach an information level to allow its use as experimental models. The *D. willistoni* is regarded as a model organism for evolutionary studies, and aside from *D. melanogaster*, has the largest number of structural genes mapped, which render it the only Neotropical species included amongst the complete genome sequenced 12 *Drosophila* species (Clark *et al.*, 2007; Schaeffer *et al.*, 2008). The strain of *D. willistoni*, selected by the "Drosophila 12 Genomes Consortium" group to be sequenced, was collected on Guadalupe Island and named GdH4-1 (Guadaloupe, France, Tucson Center 14030-0811.33) and does part of the *Drosophila* Species Inventory held at the Laboratório de Experimentação em *Drosophila* (LE*D*-UFPE). The aim of this work is to describe a new mutant phenotype showing rolled up wing tips in the strain GdH4-1 of *D. willistoni*, noticed during its maintenance.

Material and Methods

The *Drosophila willistoni* GdH4-1 strain is cultivated in a rich culture medium containing cereal flours, bananas, yeast, sugar, honey, antifungal methylparaben and phosphoric and propionic acids, in 60 ml glass bottles capped with foam stoppers. For the preparation of the culture medium all nutrients are weighed, then mixed and cooked in a microwave for 15 minutes, stopping every 2 minutes to mix. The room temperature is kept at 22 ± 1°C with constant humidity. The cultivation flasks are maintained by adding water and yeast regularly. During this procedure, in a single culture glass of generation F14, were found 6 specimens with the upward curved wing tips phenotype. These flies were separated from the others by successive changes of glass (without ether anesthesia) and a mass culture was initiated, without counting how many flies were females or males, since certainly the females were already inseminated. This new mutant strain was successively transferred to new vials and selected by the removal of wild-type flies, in order to improve the phenotypic trait (curled wings) until generation F9, when no more wild-type flies hatched.

To determine the inheritance pattern for the new trait, some crosses were performed between the curled wing mutant and the wild-type parental strain GdH4-1 of *Drosophila willistoni*.

Results and Discussion

The first wing mutant phenotype was found in *D. melanogaster* by Morgan (1915) and named *curled* (*cu*) (*appud* Bridges and Morgan, 1923). Since 1915, this same mutant phenotype was described as a series of mutations, sometimes with dominant inheritance pattern and other times as recessive. These mutants includes *Curly* (Ward, 1923), *Upturned* (Ball, 1935), *Curlyoid* (Curry,

1939), *curly* (Goldshmidt, 1944), *Curled 3* (Meyer, 1952), *curlex* (Lindsley and Grell, 1968), and *curled* in X (Krivshenko, 1958). The rolled up wing tips phenotype found spontaneously in the strain GdH4-1 of *D. willistoni* is similar to the *curly* (*Cy*) phenotype described by Ward (1923) in *D. melanogaster*. Classically, the *curly* mutant generally presents curved shape wings with a clearer color membrane, also finer in texture. The degree of the wings curvature varies from a curly bend upward to a rotation of 360°, the more the wing is rolled-over the more wrinkles are present (Ward, 1923). In *D. melanogaster* the *curly* phenotype, which is the better studied of this mutant series, is a dominant trait associated with two large inversions in the second chromosome (Ward, 1923).

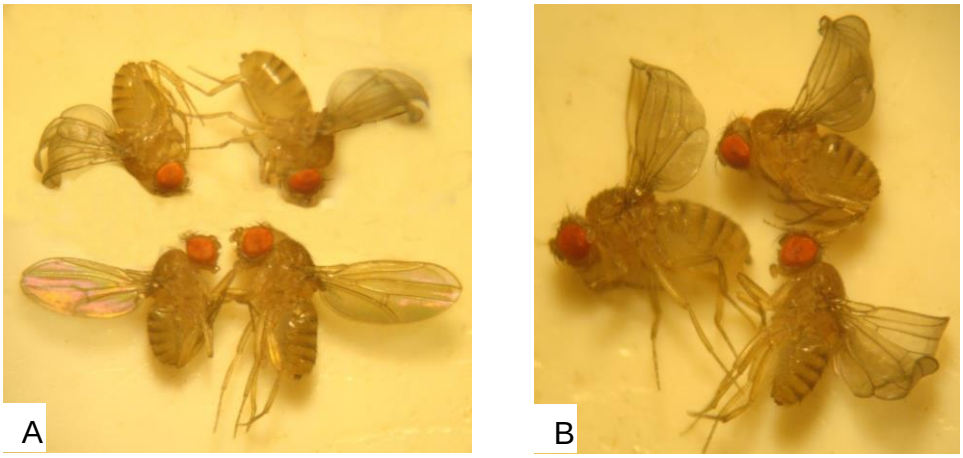

Figure 1: Comparison (A) between individuals of *curly* (*Cy*) mutants (above) and wildtype (below) of *Drosophila willistoni*. Males are positioned to the left, and females at right. The variation in expression of *Cy* phenotype (B), from a subtle to a more severe phenotype.

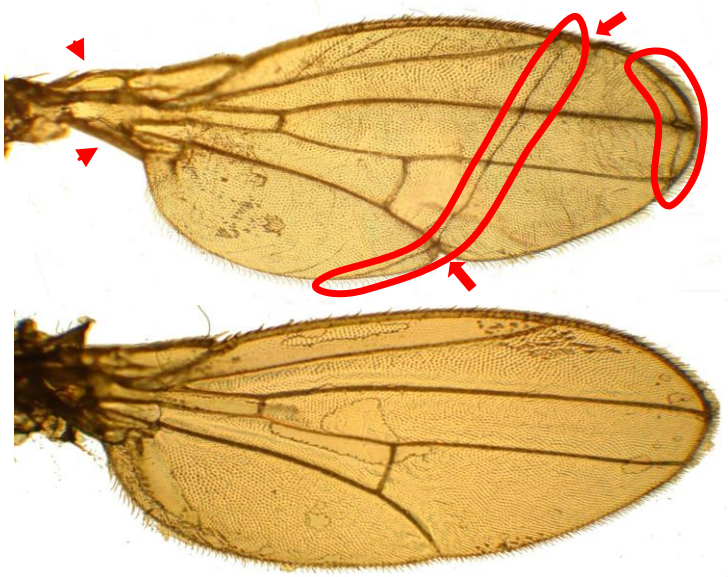

Figure 2: Structure of the wings of *Cy* mutant and wildtype of *Drosophila willistoni*, pointing out the details of the wing of the fly mutant (top), compared to wild (bottom). Modificafion of basal areas (arrowheads), recesses in the perimeter (arrows) and new lines wrinkles (circled) can be seen in the mutant, possibly caused by the curved wing. Both wings are in the same magnification (100×).

The *curly* (*Cy*) mutant phenotype found in *D. willistoni* GdH4-1 is shown in Figure 1, compared to the wildtype wing of the original strain (A), and a series of *curly* mutants (B), varying from a subtle to the most severe phenotype. This series is fully similar to that presented in Pavelka *et al*. (1996) for *D. melanogaster*, so the new mutant of *D. willistoni* is named here as "*curly*". A clearer comparison between mutant and wild type wings is shown in Figure 2. The mutant wings show recesses near vein ends and wrinkles which can be confused with new crossveins. Both recesses in the wing perimeter and the wrinkled lines were probably caused by curvature of the

wings. The effect of full phenotype expression seems also to modify significantly the axillary and jugal areas of the wing base.

The initial impression was that the new *curly* (*Cy*) phenotype of *D. willistoni* extended the life cycle of the strain to approximately 60 days. However, when the experiments to establish the generation time were performed, by generation F11 of the mutant strain, the developmental time was about 21 days, very similar to the GdH4-1 strain.

There was no significant difference between crosses in both directions (*i. e.*, female *Cy* × male *cy* or female *cy* × male *Cy*), which leads to the conclusion that the *curly* mutation of *D. willistoni* is not linked to the X chromosome. The F1 showed a frequency of 96.02% of *Cy* mutants (241:10), very near to 100% expected for a Mendelian dominant allele inheritance. Since the phenotype is highly variable, the individuals marked as wild types could be in fact mutants with the more subtle phenotype, which could made the F1 mutant frequency raise to 100%. This seems to be a situation very similar to that described by Ward (1923), who described that some phenotypically wildtype flies were in fact mutants in a genetic background that promoted the suppression of the *curly* phenotype. The F2 generation has shown the frequency of 2.34:1, which can be fairly accepted as similar to the expected 3:1 Mendelian proportion. The deviation can be due to partial lethality, since in some crosses a very few individuals were recovered in F2. This lethality is presumably dependent on the individual genetic background. The availability of mutants, as the *curly* described here and the series of eye mutants described by Soler and Goñi (2012) and the chromosome gene arrangement of these mutants (Goñi and Valente, 2012), are very important to make *D. willistoni* a candidate to become an experimental model. Its genomic characteristics distinctive from *D. melanogaster* (Clark *et al.*, 2007; Schaeffer *et al.*, 2008) can increase even more such interest.

Acknowledgments: The author thanks the Brazilian agency CNPq, for master fellowship to ESM and Scientific Iniciation to DLO. Thanks are also due to PROACAD/UFPE for financial support to work with flies.

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Erupt-like mutants from a natural population of *Drosophila melanogaster*.

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Mutations affecting head structures (eyes and antenna) were isolated from a natural population of *D. melanogaster* from Nalchik (North Caucasus, Russian Federation). The screen for

visible mutations was performed for several years by establishing isofemale lines. The mutations affecting eye and antenna formation were found in screens of several years in average in one of 100 females tested. All are recessive and demonstrate incomplete penetrance and variable expression. Phenotypically the mutations resemble *er* (*erupt*, 3-70.7) mutant (Lindsley and Grell, 1968; Aubele, 1968). Chromosomal localization of our mutants was not identified, so we denoted them as vm (visible mutation).

Here we report the mutant phenotypes.

Three mutants - $vm^{23\text{-}N2013}$, $vm^{28\text{-}N2012}$, and $vm^{8\text{-}N2011}$ have similar manifestation: eruption of underlying hypodermis in center of one or both eyes. Eruption may be segmented and have hairs. In a few flies legs are deformed.

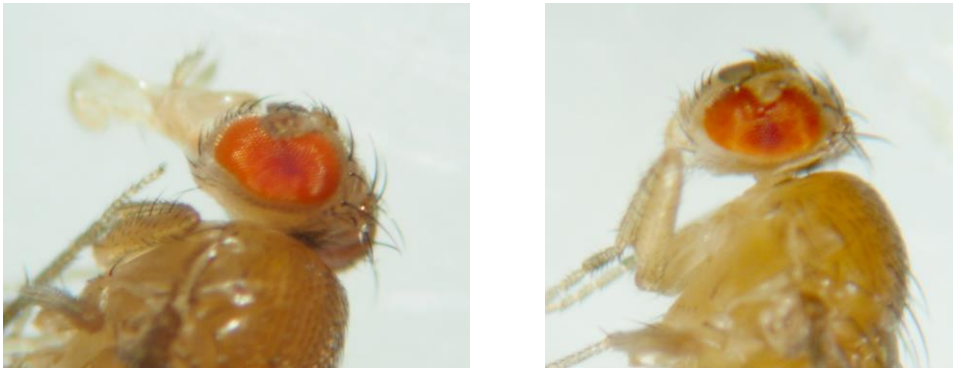

Figure 1. The $vm^{23\text{-}N2013}$ phenotype. A "palp-like" growth protruding from the eye.

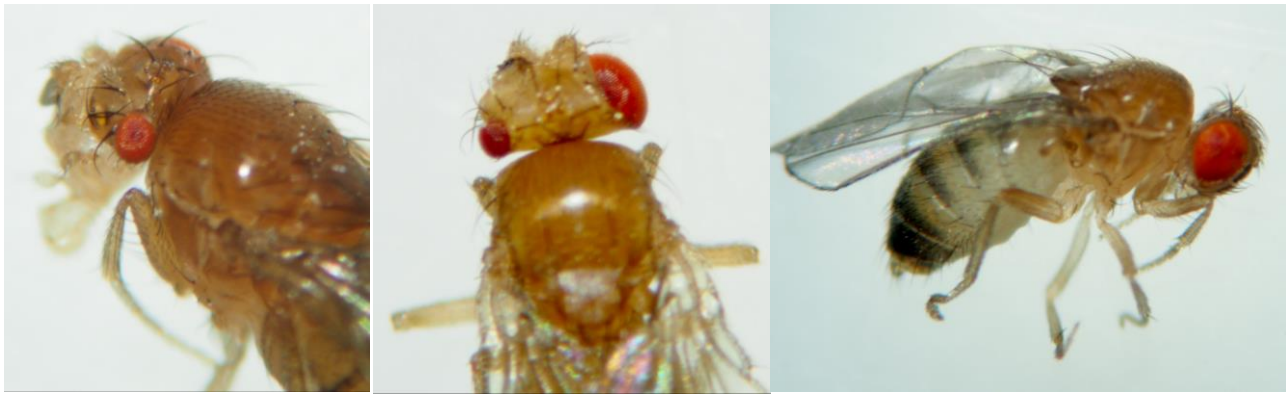

Figure 2. The $vm^{28\text{-}N2012}$ phenotype. The variable expression in eyes and the deformed legs are shown.

The third mutant, $vm^{11\text{-}N2012}$, is different from others. It affects both eyes and antenna. In extreme cases antenna are duplicated and the head expanded. Eyes are malformed. Flies with strong manifestation are sterile, but others have fertility sufficient to maintain a stock. The mutation was mapped to chromosome 2.

Figure 3, see facing page.

References: Lindsley, D.L., and E.H. Grell 1968, Carnegie Inst. Wash. Publ. 627; Aubele, A.M., 1968, Dros. Inf. Serv. 43: 139.

Figure 3. The $vm^{11\text{-}N2012}$ phenotype. In extreme cases the antenna are duplicated on one or both sides.

Guide to Authors

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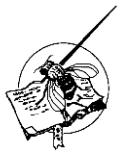
Green, R.L., 1998, Heredity 121: 430-442.

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

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

Teaching Notes



An attempt to select for increased recombination in *Drosophila melanogaster*.

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There are four main reasons why genetic recombination, the exchange of genetic material between homologous chromosomes during meiosis, is so important for the survival and fitness of most organisms (for reviews of this topic see Muller, 1964; Crow and Kimura, 1965; Maynard Smith, 1978; Bell, 1982; Charlesworth, 1989, 1993; Michod and Levin, 1988; Michod, 1995; Barton and Charlesworth, 1998; Otto and Michalakis, 1998; West *et al.*, 1999; Burt, 2000; Otto and Barton, 2001; Rice and Chippindale, 2001; Otto and Lenormand, 2002; Rice, 2002; Gillespie, 2004; Agrawal, 2006; Charlesworth and Charlesworth, 2010; Becks and Agrawal, 2012):

1) Recombination can give rise to new multiple-gene variation that can allow organisms to adapt to changing environmental conditions faster than if there was no recombination (Fisher, 1930; Muller, 1932; Roze, 2012).

2) Recombination allows for the removal of deleterious mutations faster than if there was no recombination. This is because recombination can place different deleterious mutations on the same chromosome, where they can be eliminated together and can generate chromosomes with no, or fewer, deleterious mutations (Kondrashov, 1988, 1993).

3) Recombination is essential for the proper segregation of homologous chromosomes during meiosis, because recombination physically serves to interlock homologous chromosomes and ensure their proper segregation during the first meiotic cell division. Therefore, there is at least one recombination event for each arm of every chromosome during meiosis in humans (Hassold *et al.*, 2004). Failure of recombination can lead to either germ cell death or chromosome aneuploidy (missing or extra chromosomes) (Hawley, 2011).

4) Recombination is needed for repair of double-strand chromosome breakage, which occurs about ten times each day in each cell of higher organisms (Whyman *et al.*, 2004; Lieber, 2010).

Because recombination is essential for the long-term survival of most organisms, it is of interest to determine if selection can act on standing and new genetic variation to modify the frequency of recombination. For example, Chinnici (1971) was able to increase and decrease recombination significantly in *Drosophila melanogaster* for the X chromosome, although others have not been so successful (see discussions in Detlefsen and Roberts, 1921; Parsons, 1958; Acton, 1961; Charlesworth and Charlesworth, 1985; Otto and Barton, 2001). Selection for DDT resistance, geotaxis, and temperature fluctuation has also led to concomitant significant increases in recombination in *D. melanogaster* (Flexon and Rodell, 1982; Zhuchenko *et al.*, 1985; Korol and Lliadi, 1994). Finally, there are known differences in recombination rates in individual humans and *D. melanogaster*, and genes have been identified in both humans and *D. melanogaster* that affect recombination (Ashburner, 1989; Baudat *et al.*, 2010; Kong *et al.*, 2010; Chan *et al.*, 2012).

In this study we have attempted to increase the frequency of recombination for the X-chromosome region between the genetic markers white (white eyes, *w*, map position 1.5) and singed-

3 (singed, short, bristles, *sn*^{3}, map position 21.0), by selecting for increased recombination in each generation for up to five generations.

The following crosses were performed to initially increase the amount of genetic variation in the tested F1 + + / *w sn*^{3} females. The OBL1&2 wild-type stock was initiated by mixing six mated females from a Perrysburg, Ohio, population that were captured by sweeping bananas in 2010.

w sn^3 / *w sn*^{3} females × OBL1&2 males

For each of the five participants in this study, a total of ten or eleven vials of single F1 + + / *w sn*^{3} virgin females were mated with one or two *w sn*^{3} / Y males from the *w sn*^{3} stock. The F2 progeny from these vials were then screened as:

1) Non-recombinants females that were + + / *w sn*^{3} (red eyes and long bristles) or *w sn*^{3} / *w sn*^{3} (white eyes and short bristles); or non-recombinant males that were + + / Y (red eyes and long bristles) or *w sn*^{3} / Y (white eyes and short bristles);

2) Or as recombinants females that were + *sn*^{3} / *w sn*^{3} (red eyes and short bristles) or *w* + / *w sn*^{3} (white eyes and long bristles), or recombinant males that were + *sn*^{3} / Y (red eyes and short bristles) or *w* + / Y (white eyes and long bristles).

From the vial with the highest frequency of recombination in the F2 generation among the ten to eleven tested vials, each of the five participants in this study set up five to ten new vials of F2 single virgin + + / *w sn*^{3} females mated with one or two F2 sibling *w sn*^{3} / Y males. The F3 progeny were then screened as non-recombinants or recombinants. This mating and selection procedure was then continued for four or five generations. Since the first generation was not selected, the selection scheme occurred for four or five generations only. It should be noted that in some generations, fewer than ten vials produced progeny in some experiments.

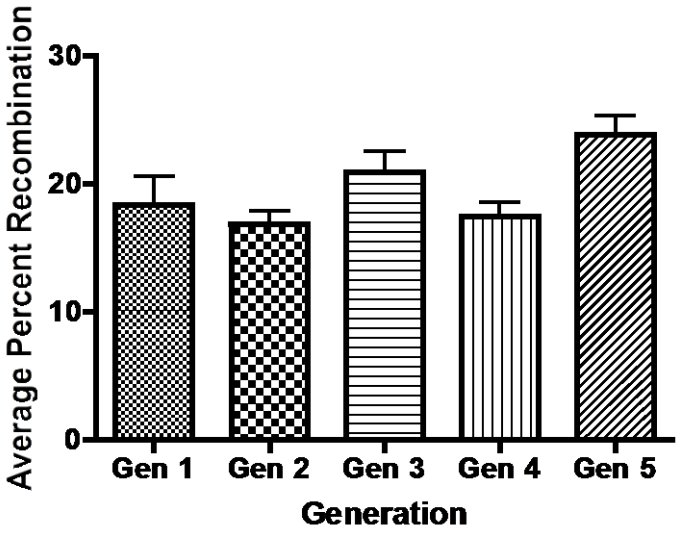

Figure 1. Average percent recombination between the X-linked markers *w* and *sn*^{3} over five generations. There was a significant (P = 0.02) increase in the frequency of recombination over time.

The results of the five selection experiments for increased recombination are shown in Figures 1-5. The beginning frequency (generation one) of recombination for the *w* to *sn*^{3} region was 18% (589/3,270), which was not significantly different (P = 0.12) from the frequency of recombination of 19.5% reported in Lindsley and Zimm (1992). In one of the five selection experiments there was a significant (P = 0.01) increase in the mean frequency of recombination over generations (see Figure 1), and in another the frequency was significantly (P = 0.02) decreased (see Figure 5). The reason for the lack of a significant response of selection for increased recombination in three of the experiments (Figure 2, P = 0.86; Figure 3, P = 0.18; Figure 4, P = 0.17) is not known, but may be because only four or five generations of selection were completed in this study, compared with 33 generations in Chinnici

(1971). The single experiment (Figure 5) that had a significant decrease in the frequency of recombination over time was due to the high frequency of recombination in generation one ($P = 0.67$ for generations two through six).

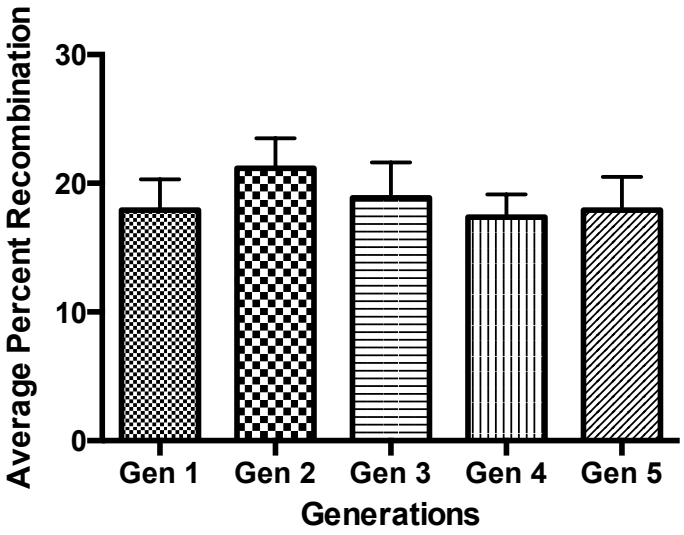

Figure 2. Average percent recombination between the X-linked markers *w* and sn^3 over five generations. Not significant ($P = 0.86$).

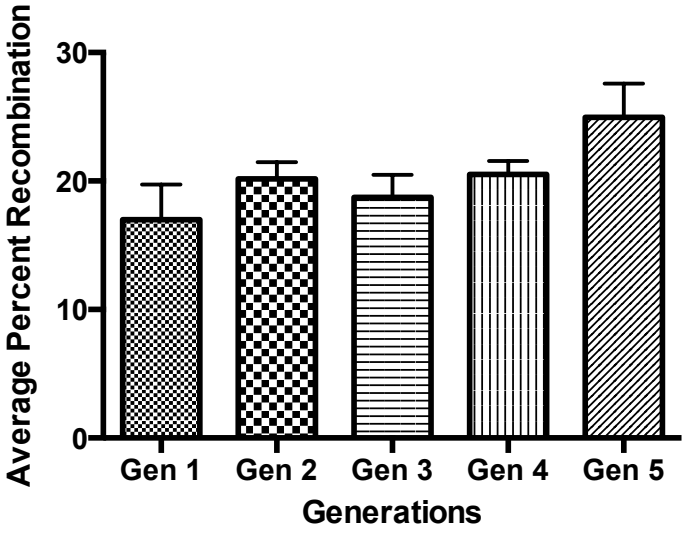

Figure 3. Average percent recombination between the X-linked markers *w* and sn^3 over five generations. Not significant ($P = 0.18$).

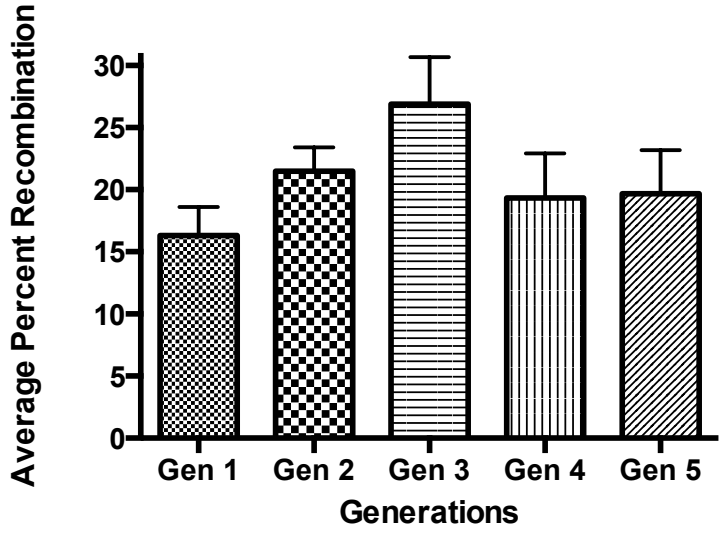

Figure 4. Average percent recombination between the X-linked markers *w* and sn^3 over five generations. Not significant ($P = 0.17$).

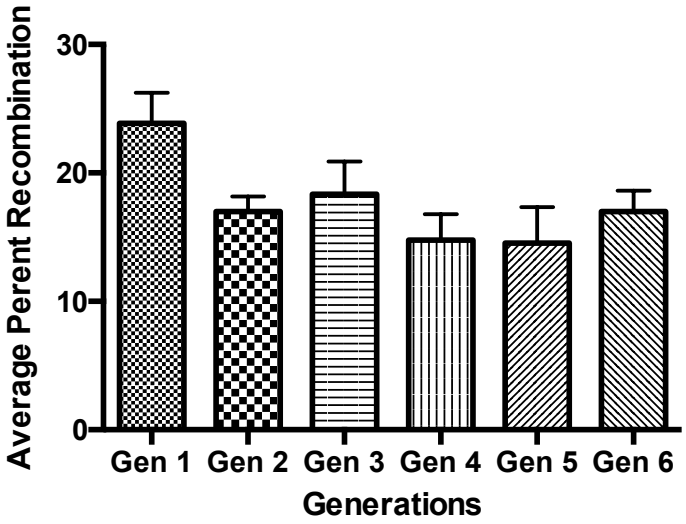

Figure 5. Average percent recombination between the X-linked markers *w* and *sn*^{3} over six generations. There was a significant (P = 0.04) decrease in the frequency of recombination over time.

A class discussion of the results of this teaching exercise could include the following: 1) Under what conditions in nature would one expect the frequency of recombination to increase? This might happen under a changing and stressful environment, such as in the presence of a new parasite or increased temperature (Parsons, 1988). 2) Is the frequency of recombination the same in human females and males? The frequency is almost twice as high in females (Lenormand and Dutheil, 2005). 3) What is the maximum frequency of recombination possible between two genetic markers in an experiment? The answer is fifty percent, because recombination occurs at the four-strand stage of meiosis after DNA synthesis. Even if an exchange occurred in every tetrad, only one-half of the four products of meiosis would be a recombinant. 4) What could be done to increase the ability of observing an increased response of selection on the frequency of recombination? One could discuss using larger numbers of selected lines in each experiment (Chinnici, 1971, used 20 to 40 lines), additional generations of selection, and genetic markers with greater map distances. 5) Why was recombination not measured in males? There is no recombination in *D. melanogaster* males (Morgan, 1912). 6) Are there any organisms that have survived without sex and recombination? This is true for some species of rotifers (Welch and Meselson, 2000).

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2011, Science 331: 870-871; Kondrashov, A.S., 1988, Nature 336: 435-440; Khondrashov, A.S., 1993, J. Heredity 84: 372-387; Kong, A., *et al.*, 2010, Nature 467: 1099-1103; Korol, A.B., and K.G. Lliadi 1994, Heredity 72: 64-68; Lenormand, T., and J. Dutheil 2005, PLOS Biology 3: e63; Lieber, 2010, Annual Review of Biochemistry 79: 181-211; Lindsley, D.L., and G.G. Zimm 1992, *The Genome of* Drosophila melanogaster. Academic Press, New York; Maynard Smith, J., 1978, *The Evolution of Sex*, Cambridge University Press, Cambridge; Michod, R.E., 1995, *Eros and Evolution*. Addison Wesley, New York; Michod, R.E., and B.R. Levin 1988, *The Evolution of Sex: An Examination of Current Ideas*. Sinauer Assoc. Inc., Sunderland, MA; Morgan, T.H., 1912, Science 36: 718-720; Muller, H.J., 1932, Amer. Nat. 66: 118-138; Muller, H.J., 1964, Mutation Research 1: 2-9; Otto, S.P., and Y. Michalakis 1998, Trends Ecology and Evolution 13: 145-151; Otto, S.P., and N.H. Barton 2001, Evolution 55: 1921-1931; Otto, S.P., and T. Lenormand 2002, Nature Reviews Genetics 3: 252-261; Parsons, P.A., 1958, American Naturalist 92: 255-256; Parsons, P.A., 1988, Biological Journal of the Linnean Society 35: 49-68; Rice, W.R., 2002, Nature Reviews Genetics 3: 241-251; Rice, W.R., and A.K. Chippindale 2001, Science 294: 555-559: Roze, D., 2012, PLoS Biology 10: e1001321; Welch, D.M., and M. Meselson 2000, Science 288: 1211-1215; West, S.A., C.M. Lively, and A.F. Read 1999, J. Evolutionary Biololgy 12: 1003-1012; Whyman, C., D. Ristic, and R. Kanaar 2004, DNA Repair 3: 827-833; Zhuchenko, A.A., A.B. Korol, and L.P. Kovtyukh 1985, Genetica 67: 73-78.

Non-crisscross inheritance in crosses between X-linked mutant strains of *Drosophila melanogaster*: treasuring exceptions.

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Introduction

According to Bridges (1916), the exceptional flies that emerged from crosses between an X-linked *vermilion-eyed* female and a *red-eyed* male of *Drosophila melanogaster* (then known by its junior synonym *D. ampelophila*) are those who failed to express the typical crisscross inheritance of daughters with red eyes (as their father) and sons with *vermilion* eyes (as their mother). Instead, the exceptional flies include matroclinous daughters with *vermilion* eyes as their mother and patroclinous sons with red eyes as their father. Trying to save the paradigm of sex chromosomes inheritance, Bridges proposed a then bizarre explanation for the origin of the exceptional flies as being a result of a non-disjunction of the two X chromosomes during anaphases of the first meiotic division. Six years later, Lilian Vaughan Morgan (1922), the wife of Thomas Hunt Morgan, was the first to report the presence of a non-crisscross inheritance of a sex-linked trait in a line where 100% of the offspring were exceptional. Morgan proposed the presence of an attached X chromosome as a possible explanation, which was confirmed through cytological analysis. The attached X chromosomes may originate from a Robertsonian translocation (centric fusion) involving the pericentromeric heterochromatin. As pointed out by Moore (1986) the *Drosophila* group of the Columbia University, under the leadership of T.H. Morgan, learned how to treasure exceptions to get deep understandings of an inexplicable phenomenon.

Whenever a basic genetics experiment is carried out using *Drosophila melanogaster* X-linked mutations, there is a great probability for such exceptional flies (matroclinous females and patroclinous males) to show up in samples from 1,000 to 2,000 indentified flies of the F_1 Generation. However, the X-linked mutant used as a probe to allow the detection of exceptional flies must be present in the parental female, otherwise the crisscross inheritance, typical of sex linkage, will not show up.

The Genetics discipline of the Biological Sciences course at the *Universidade de Sao Paulo* provides freshman students the first opportunity to carry out an experiment on the model organism *Drosophila melanogaster*. The experiment aims primarily to give students tools to discover the inheritance patterns (an important basic genetic concept) of four selected morphological mutations, which are intentionally changed from year to year. The ideal experimental design is that the crosses occur between two double-mutant strains, the male parental line bearing two of the X-linked mutations and the female one autosomal and one X-linked recessive mutation. Once the patterns are determined, students are asked to map the X-linked genes using the male offspring without the need of performing testcrosses. The students dedicate 105 min per week and the whole experiment (three generations) takes only about one and a half months to be completed. It begins with crosses between two homozygous double-mutant lines named unknown Alpha and unknown Beta, which constitutes the parental generation, and follows with the analysis of the next two generations (F_1 and F_2). The mutations present in the Alpha and Beta strains and the offspring (F_1 and F_2 generations) of their crossings are a mystery to be unveiled, *i.e*., a challenge to the students through heuristic learning. Flies must be sexed in the late pupal stage, based on presence/absence of sexual combs, and individualized in vials with usual banana-agar culture medium; males being selected from the Alpha line and females, from the Beta line. Upon emerging a couple of flies must be anesthetized with triethylamine fumes (Fuyama, 1977) by each student to have their mutations identified under stereomicroscope. This substance is recommended, because flies remain anesthetized for at least 45 min and students have enough time to identify them without any stress. We suggest the use of LED illuminator, which does not cause flies to die because of heating during a longer analysis.

Over more than one decade, while carrying out lab experiments with *Drosophila melanogaster* for teaching basic principles of Genetics, we have noticed that most of the times we were more worried about exploring the typical inheritance pattern of different mutations than to take time to explore the by-products of such crosses as those represented by the exceptional flies. Later on, treasuring exceptions that showed up in undergraduate basic Genetics experiments we have realized the extraordinary value of also exploring the by-products that may occur in the experiments. In most cases the exceptional flies detected in the F_1 generation proved, through additional experiments, to be originated from primary nondisjunction of X chromosomes of Beta line females. In this paper we will present a case we considered the most remarkable one.

A special case of non-crisscross inheritance in a basic Genetics experiment

During part of the first semester (from late March through mid May) of 2008, a total of 122 enrolled students were assigned to groups of mostly four students to carry out the same identical experiment, which consisted of five individual crosses (per group of students) between *eosin forked* males and *ebony crossveinless* females. A total sample of 1,483 flies of F_1 generation, taken at random, was identified by all the students regarding their sexes and the presence/absence of one or more out of the four genetic markers. The sample consisted of 656 *crossveinless* males and 827 wild females, which clearly exhibited the crisscross inheritance regarding the *crossveinless* mutation. Additionally 12 exceptional imagines were identified: four *eosin forked* patroclinous males and eight

crossveinless matroclinous females. It is worthwhile to note that two of the males and six of the exceptional females were found in the same vial, *i.e.*, they were descendants of a single female. On the other hand, the remaining two exceptional females were also descendants of another single female and the remaining two exceptional males were descendants of two different females. The four patroclinous males were considered exceptional because they did not receive, as expected, the X chromosome of the mother but received it from the father instead, since it carried the recessive alleles for *eosin* eyes and *forked* bristles. So were the eight matroclinous female daughters, because they did not receive the X chromosome of the father, since it carried the dominant allele for complete wing veins and its influence would have prevailed. The crosses between *crossveinless* males and wild females of the F_1 generation were performed, consisting of 8 individual crosses per group of students. A total sample of 2,760 flies (half of each sex) of the F_2 generation was identified. Males were distributed in 16 phenotypes, while the females in only four.

The exceptional flies, except for one female who did not recover from the anesthetic, were used to carry out additional experiments, aiming to find out an explanation for the amazing condition, *i.e.*, the occurrence of non-crisscross inheritance regarding the X-linked *crossveinless* gene. The four males were individually crossed to virgin females from the enigmatic Beta strain (*crossveinless ebony*), exhibited normal sexual behavior, and copulated from 15 to 21 min. However, two out of the four of them, those descendants from two different females, proved to be sterile, because the females laid large amount of eggs but no larvae emerged.

The two females originated from a single parental female were individually transferred to new vials at least once a week until no sperm remained in the spermathecae and their offspring were analyzed. The results proved that they were the product of a primary nondisjunctional event on the meiosis of their mother, the more frequent numerical chromosomal aberration we have observed in similar experiments. The five females originated from another single parental Beta female were submitted to the same process. Virgin females were obtained by individualizing pupae of the offspring of one of the five females and subsequently crossed to *white-coffee* males, aiming to analyze further the non-crisscross inheritance. Amazingly, for many generations the offspring consisted of *crossveinless* or *crossveinless ebony* females and *white-coffee* males, *i.e.*, a non-crisscross strain was established.

As the *ebony* mutation causes approximately 20% of mortality in mutant flies in relation to wild type (Lindsley and Zimm, 1992), getting rid of it produces a stronger line. Several individual test crossings between *crossveinless* females and *ebony* males and the analysis of offspring allowed us to exclude the heterozygous ones, finally getting a non-crisscross lineage composed only by *crossveinless* females and *white-coffee* males.

Materials and Methods

Two strains of *Drosophila melanogaster*, the classical wild type Samarkand, and one non-crisscross isofemale line (*white-coffee* males and *crossveinless* females), isolated by the senior author and currently maintained in the *Laboratorio de Drosofilideos do Departamento de Genetica e Biologia Evolutiva do Instituto de Biociencias da Universidade de Sao Paulo*, were analyzed.

Aiming to find an explanation for the occurrence of a 100% non-crisscross lineage containing *crossveinless* females and *white-coffee* males, third instar larvae were cytologically analyzed. The larvae were taken at random from among those crawling up the walls of the vials, dissected under a stereomicroscope over a black background, and sexed through the analysis of the paired gonads in the posterior third of the body. Testes are much larger, oval-shaped, and more loosely attached to the opaque fat body than the ovaries (Demerec, 1950).

The pro-metaphase and/or metaphase mitotic chromosomes were prepared according to the technique detailed by Baimai (1977) with some modifications as is follows. The larva was dissected directly in 1% trisodium citrate solution (and not in normal saline solution), and the brain ganglia were transferred to a new drop of the same solution (without pretreatment with colchicine) where it remained for ~ 10 min and then transferred to a drop of fixative (3:1 ethanol-acetic acid solution) for 1 min on the left lateral half of a siliconized microscope slide. The brain ganglia were removed from the fixative solution to a drop of 25 µl of 60% acetic acid placed on the right lateral half the same slide and dissociated with the aid of a pair of minuten pins inserted into a shortened wooden chopstick. The latter drop containing the cell suspension is transferred to a clean microscope slide on a warming plate at about 45ºC with the aid of a 200 µl standard pipette tip in a 5-40 µl micropipette. The cell suspension is heat-dried onto the slide and stained with 10% Giemsa, as detailed by Baimai (1977). At first, the microscope slide was checked from scanning magnification through 40× objective of a light microscope, and then digital photomicrographs of the best plates were taken with a 100× objective of a Photomicroscope.

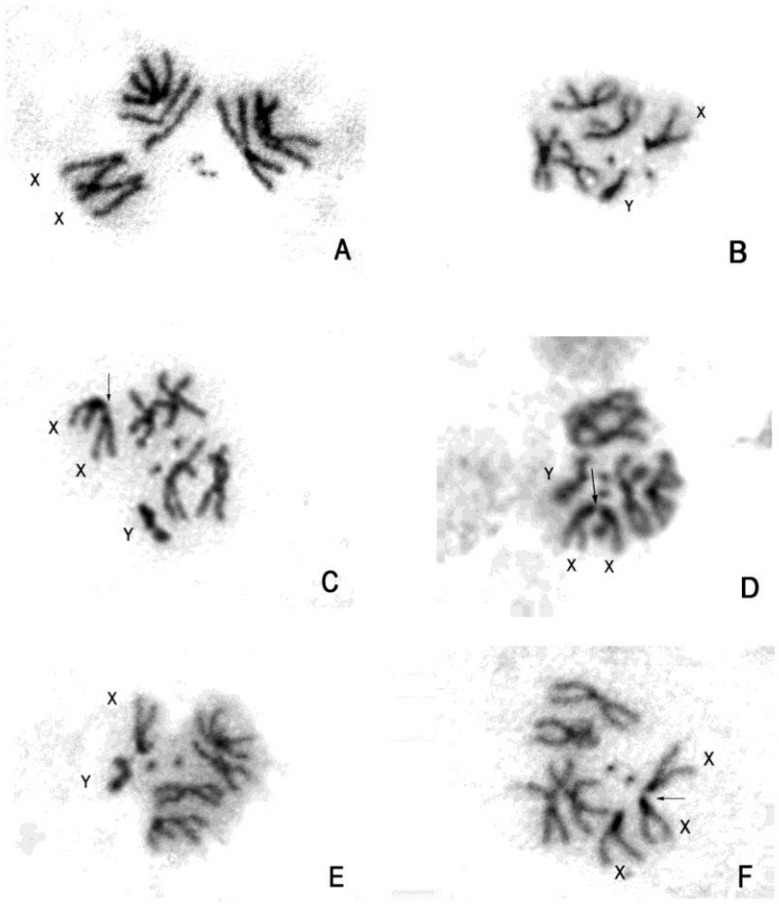

Figure 1. A) pro-metaphase mitotic plate of a wild type female from the Samarkand strain, showing two unattached X chromosomes and three pairs of autosomes (two V-shaped pairs and one dot-shaped pair). B) metaphase mitotic plate of a wild type male from the Samarkand strain, showing one X chromosome and one Y chromosome. C, D) two metaphase mitotic plates of *crossveinless* females XXY, the arrow points to the region of the attachment in the X chromosomes. E) pro-metaphase mitotic plate of a *white-coffee* male, depicting a normal karyotype with one X chromosome and one Y chromosome, like the wild type male. F) pro-metaphase mitotic plate of a wild trisomic female XXX (two attached and one unattached X, forming a trivalent mostly paired through their pericentric heterochromatin); the arrow points to the region of attachment of two out of the three X chromosomes.

Results and Discussion

Mitotic plates of normal female (Figure 1A) and male (Figure 1B), both with a diploid number 2n = 8, were obtained from a classical wild type strain (Samarkand) and photomicrographed for comparison purposes as shown in Figure 1. Analyses of the photomicrographs of the most

frequent type of mitotic plates of the non-crisscross line confirmed the hypothesis of attached X chromosomes in the *crossveinless* female flies, which therefore have a diploid number 2n = 9, consisting of three pairs of autosomes and three sexual chromosomes (XXY); the attached chromosomes X being V-shaped, longer than each of the also V-shaped chromosomes 2 and 3 and usually unpaired to the almost entirely heterochromatic Y (Figures 1E, F). The *white-coffee* males have a normal diploid number 2n= 8 (XY) as depicted in Figure 1C. Rare females also bearing the same diploid number 2n = 9 were also detected; however, they are devoid of a Y chromosome but are trisomic for the X chromosomes instead, two of them being attached (Figure 1D). The karyotypes we have found, except for the triplo-X, are similar to those obtained by Lilian Morgan in 1922.

The technique described by Baimai (1977) results in clear cut mitotic pro-metaphase and/or metaphase plates and, most importantly, it yields sharp differentiation between the euchromatic and heterochromatic regions of the chromosome complement. The photomicrographs show that the attachment of the X chromosomes occurred through their centromeric regions. Figures 1A and 1B depict the chromosomes of Samarkand wild type female and male, respectively. All the analyzed female larvae had a diploid number 2n = 9 and were trisomic for the sexual chromosomes, most of them had two X chromosomes attached to each other in addition to the Y chromosome, as shown in Figures 1C and 1D. The *white-coffee* male has a similar karyotype to the wild type male, showing two pairs of large V-shaped chromosomes, one pair of dot-shaped, and one rod-shaped X chromosome, in addition to one J-shaped Y chromosome, as shown in Figure 1E. Few female individuals, however, had three X chromosomes as shown in Figure 1F that shows the presence of two X chromosomes fused through their centromeric region (largest V-shaped chromosome) and one free, rod-shaped X chromosome.

It should be pointed out that usually attached X strains are not stable, and this instability was first reported by Anderson (1925) soon after Lilian Morgan (1922) had published her findings. He concluded that instability, in his case, whose attachment was induced by X-Ray irradiation, is associated with a detachment rate of X chromosomes, where an attached X adult female is eventually able to produce two types of ova, with one X or one Y chromosome. Anderson (1925) found a proportion of about 1 crisscross inheritance offspring per 1300 non-crisscross descendants in his lineage.

However, in our non-crisscross strain, which probably originated from a spontaneous rearrangement of parental female X chromosomes, we have observed a higher frequency of about 1 crisscross offspring per 300 non-crisscross descendants. We propose that the higher rate we have observed is due to the occurrence of the trisomic females (XXX) that emerge from the puparia as fertile females. Such a hypothesis was suggested because we have found triplo-X third instar larvae as shown in Figure 1C, which will account for the occurrence of fertile wild females, and a higher frequency of regular, *i.e.*, crisscross, offspring in the lineage. The analyses of the different phenotypes that eventually were identified in the lineage are detailed in Table 1. Based on the genetic analysis of the unstable strain along successive generations, we suspect that the three X chromosomes, that paired as an almost perfect trivalent during meiotic metaphase stage, being completely homologues, will have a higher probability of detachment of the attached pair during the subsequent anaphase stage than in a trivalent formed in the XXY females, where pairing occurs more loosely between the two attached X and the slightly, mostly heterochromatic, partially homologous Y chromosome. So, to be maintained, the attached X chromosome strain must be continuously scanned in order to detect and eliminate the wild female flies that promote the instability driving the strain back to the stable XY/XX condition.

Therefore, we believe that the wild and *white-coffee* females, in addition to the *crossveinless* males, were descendants of the fertile trisomic wild type females (XXX). The wild type males were

the result of a recombination event between the *crossveinless* and *white-coffee* loci. However, the wild females, who first appear in any given generation, are most probably the fertile trisomic ones that have escaped dying during the pupal stage, where the lethal effect of the triplo-X condition mainly occurs, as pointed out by Brehme (1937).

Table 1. Phenotypic classes and respective number of individuals sampled from many generations (pooled together) of the non-crisscross strain, composed of *crossveinless* females and *white-coffee* males. A total of 2,103 individuals, 1,000 females and 1,103 males, were sampled. *Crossveinless* females and *white-coffee* males were the most frequent classes, and are the product of a non-crisscross inheritance. The rarer classes found in the offspring were wild females and males, *white-coffee* females and *crossveinless* males.

females			males		
+	*cv*	w^{cf}	+	*cv*	w^{cf}
9	988	3	1	4	1,098

+ = wild type; *cv* = *crossveinless*; w^{cf} = *white-coffee*

Conclusion

The unusual X-linked inheritance pattern exhibit in 100% of the offspring of a lineage of *Drosophila melanogaster*, called non-crisscross inheritance and composed of *crossveinless* females and *white-coffee* males, was a result of the occurrence of a compound (attached) X chromosome. The genetic results are similar to those obtained by Lilian V. Morgan in 1922 and were as well confirmed by the cytological analysis that proved the existence of an attached X in the female matroclinous offspring. We afford the opinion that the instability of the analyzed strain starts whenever one fertile wild type triplo-X female escapes from dying during the critical pupal stage.

Acknowledgments: We are grateful to all 2008 Genetics discipline team at the *Universidade de Sao Paulo*, which included 120 Biological Sciences enrolled students (especially those from the group of the senior author: Enrico de Vincenzo Cacella, Jose Pedro de Queiroz, and Lucas Alvizi Cruz), three additional faculty members (Drs. L. Mori, L.E.S. Netto, and D.S. Sheepmaker), four monitor students (F.B. Bittencourt, C. Garcia, T.A. Hamaji, and K.C. de Oliveira), and two technicians (C.E. Lopes and F. Flauzino), for different reasons, and to Drs. Beatriz Goni and L. Mori for helping with the chromosomes.

References: Anderson, E.G., 1925, Genetics 10: 403–417; Baimai, V., 1977, Genetics 85: 85-93; Brehme, K.S., 1937, Exp. Biol. Med. 37: 578-580; Bridges, C.B., 1916, Genetics 1: 1-52, 107-163; Demerec, M., 1950, *Biology of* Drosophila, John Wiley, New York; Fuyama, Y., 1977, Dros. Inf. Serv. 52: 173; Lindsley, D.L., and G.G. Zimm 1992, *The Genome of* Drosophila melanogaster, Academic Press, San Diego; Moore, J.A., 1986, American Zoologist 26: 583-747; Morgan, L.V., 1922, Biological Bulletin 42: 267-274.

Simple high school laboratory exercise on mate attraction and reproductive isolation in *Drosophila*.

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Species are often defined as groups that fail to successfully interbreed with other groups. Behaviors that keep groups from interbreeding help maintain biodiversity on our planet, and students

can explore such behaviors in K-12 biology classes. The recently retired AP Biology Laboratory 11 Part B exercise on Animal Behavior allowed students to observe the fruit fly courtship sequence. While this activity had value, it came across as somewhat minimal to most students, particularly if they had already worked with flies and had seen courtship occur. Nonetheless, many teachers and college faculty still leverage this activity for demonstrating courtship behavior, and multiple commercial kits are available for it.

Simple additions to this activity make it more engaging and allow students to witness one aspect of the principle of reproductive isolation, for both introductory biology and Advanced Placement classes. AP Biology students are required to be "able to justify the selection of data that address questions related to reproductive isolation and speciation" (College Board Fall 2012 Learning objective 1.23). Many students know that species often produce sterile hybrids after mating with other species, but they often forget that pre-zygotic mechanisms (*e.g.*, disinclination to mate with other species) are also a form of reproductive isolation that maintains the separateness of species. We reinforced this concept by having students observe same-species and different-species pairings themselves directly.

We present both a simple extension that differs trivially in effort from the original AP Biology Laboratory exercise, and an elaborate version that demonstrates the concepts more elegantly but requires additional preparation. These revised exercises keep the students engaged while reinforcing their understanding of behavior and evolution. Each version can be conducted easily within a single class period, though some advance preparation is necessary by the teacher.

Presenting the Concept

We opened by asking the students how one knows whether two groups are separate species. Students often reply that different species "can't reproduce" or that "their hybrids are sterile." Yet, students often fail to appreciate the importance of mate recognition-- animal species (*e.g.*, squirrels, pigeons, mosquitoes, etc.) encounter other animal species all the time without attempting to mate with each other. Even apparently similar species, like dogs and foxes, often exhibit little or no physical attraction to each other. In such a case, would it be relevant if these species can produce hybrid offspring when there's no inclination to try in nature?

Often, two distinct types of pre-zygotic reproductive isolation exist:

– males are disinclined to court females of the other species (species discrimination by males), and

– even if males did court females of the other species, the females would be disinclined to accept their advances (species discrimination by females).

Activity Overview

A culture of male *Drosophila simulans* (available from the *Drosophila* species stock center) is the only material the class will need besides those included in the commercially available AP Biology Laboratory 11 kits (available from Carolina Biological Supply, Ward Scientific, and other vendors). Students work in groups of two to four to observe courtship behavior in within- or between-species pairings. For the basic activity, each group should use four food-containing vials: two vials with each containing an individual *D. melanogaster* female, one vial with an individual *D. melanogaster* male, and one vial with an individual *D. simulans* male. Without anesthesia, the students pair a same-

species male and female in a vial, and the different-species male and female in a separate vial. In the optional elaborate activity, students also pair the two kinds of males with *D. simulans* females.

Students document the behaviors, as described in the standard AP Biology Laboratory 11 exercise. However, they also record differences between the within- and between-species pairings. At the end of the period, the students can consolidate and present their data.

Results and Discussion

We have tried this activity with multiple classes, and we were thrilled (and relieved!) to observe striking differences between within- and between-species pairings within each class period. Courtship was very easy to identify-- the male fly would follow the female very closely from behind, periodically extending one or both wings and vibrating them. After a few minutes of following and wing vibrating, he would attempt to mount the female (whenever she stopped moving).

The first rows of Table 1 illustrate results of the basic activity. Simply, *D. simulans* males are disinclined to court *D. melanogaster* females, demonstrating species discrimination by ***males***. This is due to a cuticular hydrocarbon difference that distinguishes the females of the two species (Jallon, 1984). In contrast, *D. melanogaster* males court and mate with *D. melanogaster* females readily. With the elaborate activity, students see that the reverse is not true-- *D. melanogaster* males will readily court *D. simulans* females, but the females are unreceptive to their advances (running away or not allowing the mount), which demonstrates species discrimination by ***females***. Females rely heavily upon auditory cues produced by the male's courtship song when they decide if a mate is appropriate (Ritchie *et al.*, 1999). The students visibly enjoyed watching the different behaviors, rooting for the poor males who could never mate with their partner and cheering for those males who did mate eventually.

Table 1. Observational data from 21 student groups of within- and between-species pairings.

COURTSHIP	*D. melanogaster* male	*D. simulans* male
D. melanogaster female	100%	19% (all very short)
D. simulans female	67%	100%

MATING	*D. melanogaster* male	*D. simulans* male
D. melanogaster female	76%	0%
D. simulans female	0%	52%

This relatively simple extension on the standard AP Biology exercise increases its pedagogical value, the breadth of concepts presented, and its appeal to students. It can easily be implemented in introductory classes as well, yet it arguably touches upon all four of the "Big Ideas" associated with the AP Biology Curriculum Framework, as well as demonstrating some of the specific concepts. Detailed (and editable) step-by-step instructions are available at http://tinyurl.com/bulmcap , and a YouTube video demonstrating the fly manipulations can be viewed at: http://www.youtube.com/watch?v=8Wc01zIzfEY

References: Jallon, J.M., 1984, Behav. Genet. 14: 441-478; Ritchie, M.G., E.J. Halsey, and J.M. Gleason 1999, Anim. Behav. 58: 649-657.

Experimental verification that crossing-over events within inversion heterozygotes are eliminated in the gametes of *Drosophila melanogaster* females.

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Early in the development of *Drosophila* genetics, heterozygous autosomal "genes", "genetic factors", or "crossover-suppressors" were isolated that eliminated (or almost eliminated) the recovery of recombinant gametes for some genetic markers in germ cells (Muller, 1916; Sturtevant, 1917, 1921; Ward, 1923; Payne, 1924). Sturtevant (1926) hypothesized that these crossover reducers were inversions and that crossing over leading to recombinant gametes did occur in inversion *homozygotes.* Calvin Bridges (1937; quoted in Sturtevant, 1926) confirmed that the crossover reducers were inversions by cytogenetic analysis of salivary-gland polytene chromosomes.

Why are recombination events in inversion heterozygotes eliminated in the gametes of *Drosophila melanogaster*?

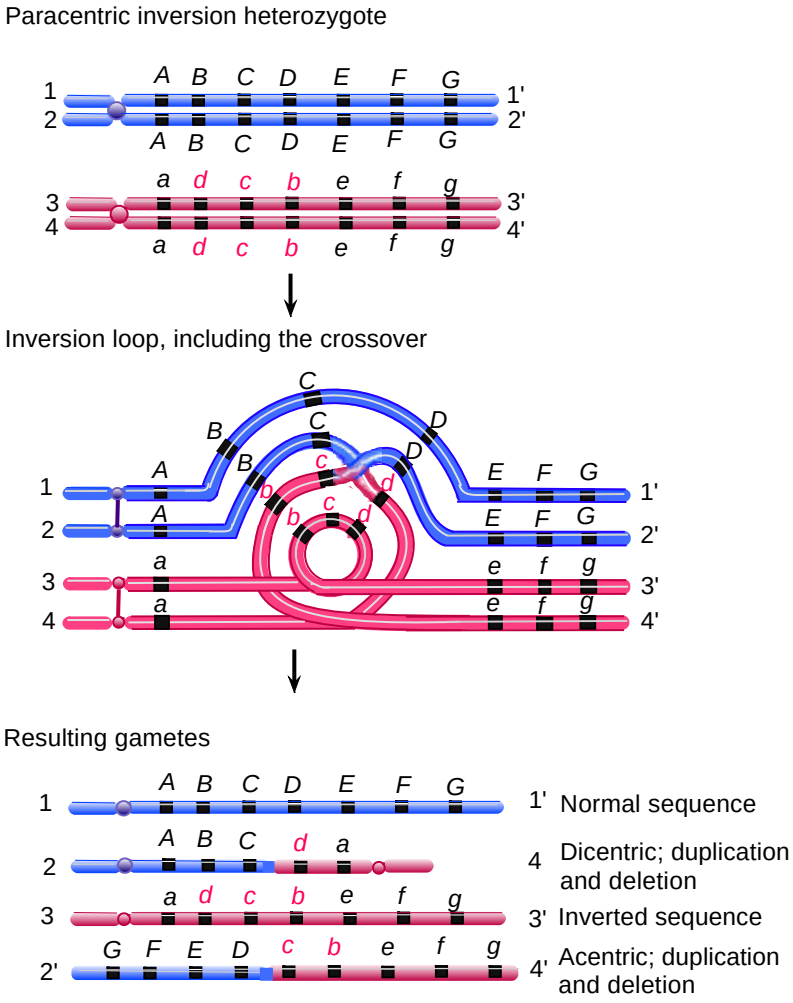


Figure 1. Consequence of crossing-over within a paracentric inversion heterozygote. NCO = non-crossover; SCO = single crossover. (After Klug, William S., Michael R. Cummings, Charlotte A. Spencer, and Michael S. Palladino, 2010, *Essentials of Genetics*, 7th Edition, p. 124. Pearson Education, Inc., Upper Saddle River, NJ).

Recombinant gametes from crossing-over events within inversion heterozygotes have chromosomes with large duplications and deficiencies, plus, in some cases, acentric (no centromere) chromosomes or dicentric (two centromeres) chromosomes (for reviews of this topic see Ashburner, 1989; Kirkpatrick, 2010). These altered chromosomes at meiosis are not included in the egg nucleus of *D. melanogaster*, or early embryos

that contain these chromosome duplications and deficiencies do not survive (Beadle and Sturtevant, 1935; Sturtevant and Beadle, 1936). These chromosome events are shown in Figure 1 for paracentric inversions (centromeres outside the inversions) and in Figure 2 for pericentric inversions (centromeres inside the inversions). In Figure 1, crossing-over events within the paracentric inversion heterozygote give rise to gametes with no or two centromeres and with duplications and deficiencies. In Figure 2, crossover gametes from heterozygous pericentric inversions have the normal number of centromeres, but duplications and deficiencies. The chromosomal changes shown in Figures 1 and 2 have been observed directly by cytology in corn (McClintock, 1933), red trillium (*Trillium erectum*) (Smith, 1935), fungus gnat (*Sciara implatiens*) (Carson, 1946), and *D. melanogaster* (Stone and Thompson, 1935; Hinton and Lucchesi, 1960).

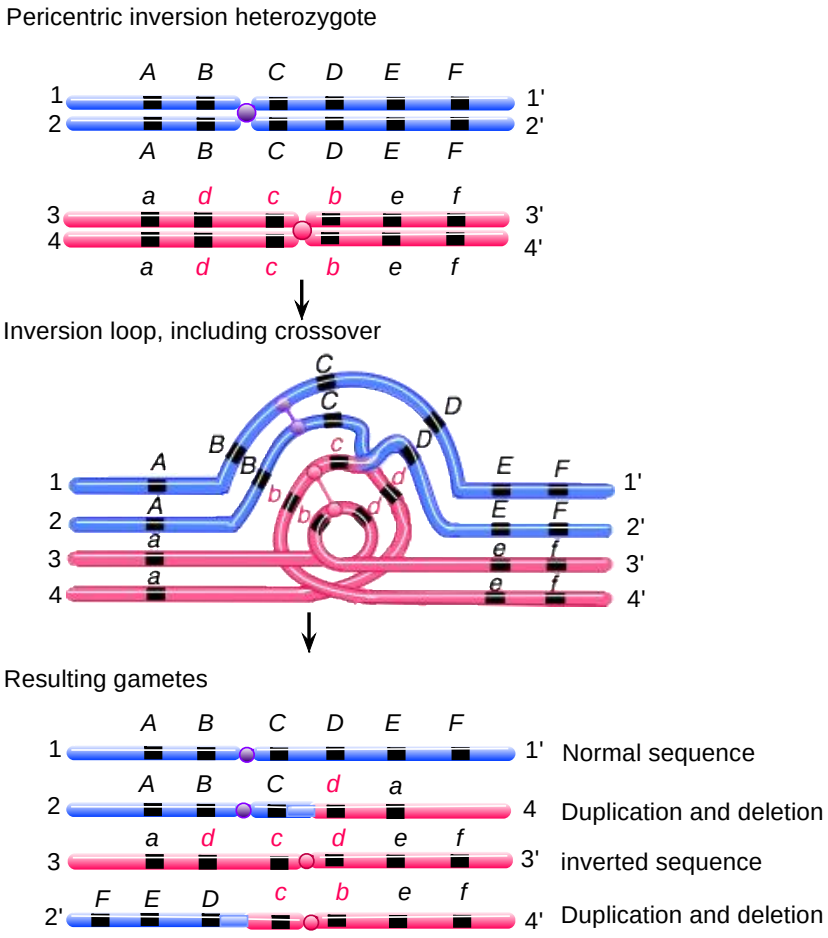


Figure 2. Consequence of crossing-over within a pericentric inversion heterozygote. NCO = non-crossover; SCO = single crossover. (After Klug, *et al.*, 2010, see Figure 1 citation).

In this study we have attempted to confirm genetically the historical observations on the consequence of crossing-over events in inversion heterozygotes by screening for recombination events within an inversion on the right arm of the third chromosome of *D. melanogaster* females. As controls, we also screened for recombination in females homozygous for a structurally normal third chromosome and in males, which are known not to undergo recombination (Morgan, 1912; Sturtevant, 1917; Woodruff and Thompson, 1977).

Screen for possible crossing-over events within In(3R)P / gl^2 e^4 heterozygous females:

To confirm that recombinant gametes are not recovered from crossing-over events within paracentric heterozygotes, we performed the following crosses. In(3R)P is a paracentric inversion with salivary-gland chromosome breakpoints at 89C-D and 96A on the right arm of chromosome three (Lindsley and Zimm, 1992, p. 967). The third-chromosome marker glass-2 (glass eyes, gl^2) is at cytological position 91A1-2 (Lindsley and Zimm, p. 252), and ebony-4 (ebony body color, e^4) is at position 93D2-6 (Lindsley and Zimm, 1992, p.180). Hence gl^2 and e^4 are within the chromosomal

region of the In(3R)P rearrangement (breakpoints 89C-D and 96A). In(3R)Hu, $Antp^{Hu}$ Sp^{spi} / In(3R)P is Indiana University Drosophila Center Stock #3396 and gl^2 e^4 is Indiana University Drosophila Center Stock #507. Sb^{spi} = dominant mutation causing stubble (short) bristles.

In(3R)Hu, $Antp^{Hu}$ Sb^{Spi} / In(3R)P females × gl^2 e^4 / gl^2 e^4 males

In(3R)P / gl^2 e^4 females × gl^2 e^4 / gl^2 e^4 males
(with normal bristles)

Non-recombinants		Recombinants	
gl^2 e^4 / gl^2 e^4 =	glass & ebony	gl^2 + / gl^2 e^4 =	glass
or		or	
+ + / gl^2 e^4 =	wild-type	+ e^4 / gl^2 e^4 =	ebony

From these crosses, we observed one recombinant (+ e^4) and 4,506 non-recombinants, for a map distance of 0.02 (1/4,507 = 0.0002; one map unit is equal to one percent recombination). The one recombinant may have arisen by double recombination or as a gene conversion event (Ashburner, 1989, p. 486). Lindsley and Zimm (1992) give the map distance between *gl* (3-63.1) and *e* (3-70.7) as 7.6 map units. Based on the expected map distance of 7.6, if recombinant gametes from crossing-over in In(3R)P heterozygotes were not eliminated, we should have seen about 343 recombinants and 4,164 non-recombinants instead of one out of 4,507 ($P < 0.0001$). Hence, these results clearly support the early observations that recombinant gametes from crossing-over events within inversion heterozygotes are not included in offspring.

Screen for possible crossing-over events within + + / gl^2 e^4 heterozygous females:

As a control, we also screened for recombination in heterozygous females for gl^2 e^4 and a third chromosome with a normal structure (from the laboratory wild-type stock, Canton-S), by the following crosses.

Canton-S females × gl^2 e^4 / gl^2 e^4 males

+ + / gl^2 e^4 females × gl^2 e^4 / gl^2 e^4 males

Score for non-recombinants and recombinants as shown in the above crosses.

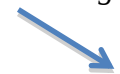
From these crosses, we observed 136 recombinants and 1,840 non-recombinants, for a map distance of 6.9 (136/1,976 = 0.069). In comparison to the expected map distance of 7.6 (150 recombinants and 1,836 non-recombinants), a result of 136 and 1840 for Canton-S / gl^2 e^4 is not

significantly different (P = 0.42) from the expected frequency. Yet, the frequency of recombinants in In(3R)P / gl^2 e^4 females is significantly lower than in + + / gl^2 e^4 females (1/4,508 *vs*. 136/1,976; P < 0.0001). Hence, the lack of recombination in the In(3R)P / gl^2 e^4 females is not due to a crossover suppressor in the gl^2 e^4 third chromosome.

Screen for possible crossing-over events within + + / gl^2 e^4 heterozygous males:

As an additional control we screened for recombination in Canton-S / gl^2 e^4 males that are known not to undergo recombination (Morgan, 1912; Sturtevant, 1917), by use of the following crosses.

Canton-S females × gl^2 e^4 / gl^2 e^4 males



gl^2 e^4 / gl^2 e^4 females × + + / gl^2 e^4 males

Score for non-recombinants and recombinants as shown in the above crosses.

As expected, we observed zero recombinants and 3,742 non-recombinants from the Canton-S / gl^2 e^4 heterozygous males (0/ 3,742 *vs*. 258/3,742, if we recovered the expected 6.9 percent recombination that was observed in females; P < 0.0001).

A summary of the results of this experiment is given in Table 1.

Table 1.

Genotype	Number Recombinants	Number Non-Recombinants	Percent Recombination
In(3R)P / gl^2 e^4 females	1	4,506	0.02[a]
+ + / gl^2 e^4 females	136	1,840	6.9[a,b]
+ + / gl^2 e^4 males	0	3,742	0[b]

[a]P < 0.0001; [b]P < 0.0001

The results from this study clearly support the early observations that gametes from crossing-over events within inversion heterozygotes are not recovered in progeny. Yet, why is this an important observation?

First, recent DNA sequences of homologous chromosomes of individuals have identified many more inversions in each human than previously assumed; for example, 90 inversions in a single diploid genome (Levy *et al*., 2007), some of which are caused by recombination between transposable DNA elements (Hancks and Kazazian, 2012). Hence, inversions and other rearrangements are important in altering the position and expression of genes (position-effect variegation; Spofford, 1976; Dimitri and Pisano, 1989), in some cases causing cancer (Hancks and Kazazian, 2012).

Second, genes within an inversion can evolve together, since they will not be separated by recombination in heterozygotes. This has important evolutionary implications that allow for groups of genes to evolve and interact over time (Hedrick, 2011).

As part of a class discussion, students might be given four examples where there has been

selection for groups of genes within inversions: 1) in *D. melanogaster* for changes in response to global warming (Balanya *et al*., 2006) and body size (Kennington *et al*., 2007); 2) in butterflies for wing color (Joran *et al*., 2011), and 3) in humans for an increase in offspring numbers (Stefansson *et al*., 2005). Yet, the genes have not been identified in the inversions of these four examples. Hence, students might also discuss the two alleles of an odorant-binding protein gene (*Gp-9*) that is located in a large inversion that determines the social structure of fire ants (Krieger and Ross, 2002; Wang *et al*., 2013). For example, the two variants of the *Gp-9* gene determine if ant colonies have one queen or many queens, because of the killing of queens by workers if the queens have the wrong *Gp-9* genotype.

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An attempt to identify new recessive sex-linked visible mutations in *Drosophila melanogaster*.

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The first attached-X chromosome in *Drosophila melanogaster*, where two X chromosomes are attached to a single centromere, was isolated by Lillian V. Morgan in 1921 (Morgan, 1922). This compound stock, which is now called C(1)RM (the two X chromosomes are attached in reverse), and the C(1)DX attached X chromosome (the two X chromosomes are attached in tandem), which was isolated by H. J. Muller in 1943 (Muller, 1943), have been used to isolate new visible mutations on

the X chromosome by the following cross (see Auerbach, 1962, p. 39; Woodruff *et al*., 1979; Norris and Woodruff, 1992; for reviews of this topic). In this cross, *y* is a yellow-body mutation, *f* is a forked (small) bristle mutation, and + is the symbol for wild type (for descriptions of the compound autosome stocks and mutants, see Lindsley and Zimm, 1992).

C(1)DX, *y f* / Y females × single + / Y male

In this experiment, we mated single wild-type males, which were scanned for preexisting visible morphological aberrations, with three C(1)DX, *y f* / Y females, and screened F1 patroclinous males (receiving their X chromosome from their fathers) for new altered phenotypes. In particular, we looked for changes in 20 known X-linked mutations that alter wings (miniature, cut, rudimentary, notch, crossveinless, fused, scalloped, and cut); bristles (forked, bobbed, and singed); eye color (white, vermilion, carnation, garnet, prune, ruby, and carmine); eye shape (lozenge); and body color (yellow) (see Lindsley and Zimm, 1992). Any male progeny with altered phenotypes were mated again to C(1)DX, *y f* / Y females to confirm that the presumptive X-linked mutants bred true (the F2 male progeny have the same new phenotype as the F1 males). Rare dominant autosomal mutations would appear in both male and female F1 and F2 progeny. Any mutants that bred true were mated with wild-type (Canton-S) females to determine if the mutations were recessive (progeny females would be wild type) or dominant (progeny females would be mutant). Rare triplo-X females were also observed at a low frequency in the above cross. The triplo-X females are easy to identify, because they are wild type for *y* and *f*, have small deformed wings, are weak (move slowly), develop slowly (eclose from pupae in older vials), and are sterile (Lindsley and Grell, 1992).

We screened 10,603 F1 males for new recessive visible mutations. A total of 27 presumptive visible mutants were tested to see if they bred true by mating them to C(1)DX, *y f* / Y females. These presumptive mutants included abnormal wings, abdomens, heads, legs, and antennae, or dark eyes. Of these presumptive mutants, eight were sterile and 18 did not breed true. One white-eyed male (from a cross with Canton-S males) did breed true. This white-eyed male was observed with 13 red-eyed males. This gives a rate for X-linked visible mutations of 0.0094 percent (1/10,603, yet, see below). Woodruff, *et al*. (1979) recovered no mutants out of 30,748 males, and Norris and Woodruff (1992) recovered seven mutants out of 23,092 males in similar crosses. We also recovered 149 triplo-X females; 81 of these triplo-X females were mated and all were sterile, as expected.

Additional crosses determined that the new white-eyed mutation was X linked and recessive (see above). To determine if the white-eyed mutation was an allele of the X-linked white locus of *D. melanogaster*, Canton-S females were mated to w^{1118} males, and F1 heterozygous + / w^{1118} females were mated with the new white-eyed males. From these crosses we observed 122 wild-type F2 females and 126 white-eyed F2 females. Hence, the new white-eyed mutation was an allele of the white locus, and we named it w^{bg} (white bowling green).

To rule out the possibility that w^{bg} was not a new mutation of the white locus, but was a contaminant from a laboratory w^{1118} stock, the only white-eye mutant in the laboratory during this experiment, we designed primers that included the first exon of the white locus and ran a PCR analysis of part of the white gene in single males of Canton-S, w^{1118}, which is the result of a 9,000 base-pair deletion that includes the first exon of the white locus (Platts *et al*., 2009), and w^{bg}, which is assumed to have occurred on the Canton-S X chromosome. The PCR primers were: White.Ex1.F (5' - GTC CGC TAT CTC TTT CGC CA - 3') and White.In1.R (5' - ACG CCG CAG ACA ATT TGA TG - 3'). These primers were designed to amplify exon 1 (~385 base pairs) of the white locus. PCR amplifications were conducted in 20 µl volumes containing 4 µl extracted DNA, 2.0 mM $MgCl_2$, 0.5 µM of each primer, 150 µM of each dNTP, 1× Promega GoTaq Buffer, and 1 unit of Promega GoTaq

DNA Polymerase. Samples were amplified using a MJ Research Thermal Cycler with the thermal profile of 35 cycles at 94°C for 30 sec, 61°C for 30 sec, and 72°C for 30 sec. PCR products were visualized under UV light by 1% agarose gel electrophoresis with ethidium bromide staining.

If w^{bg} were a contaminant from the w^{1118} stock, then we would expect to recover no PCR bands from the DNA of w^{1118} and w^{bg} males, but would get a band of 385 base pairs from the Canton-S male. If w^{bg} were a new mutation of the white locus, we would expect to recover no PCR band from the w^{1118} male, but would observe similar sized (385 base pairs) PCR bands from the Canton-S and w^{bg} males. The results of the PCR analyses are shown below (Figure 1). These PCR results do not support the hypothesis that w^{bg} is a new mutation of the white locus. In fact, it seems to be a w^{1118} contamination from our w^{1118} laboratory stock, since a band was not amplified for w^{bg} or w^{1118}. Hence, we isolated no new X-linked visible mutations in this experiment (0/10,603).

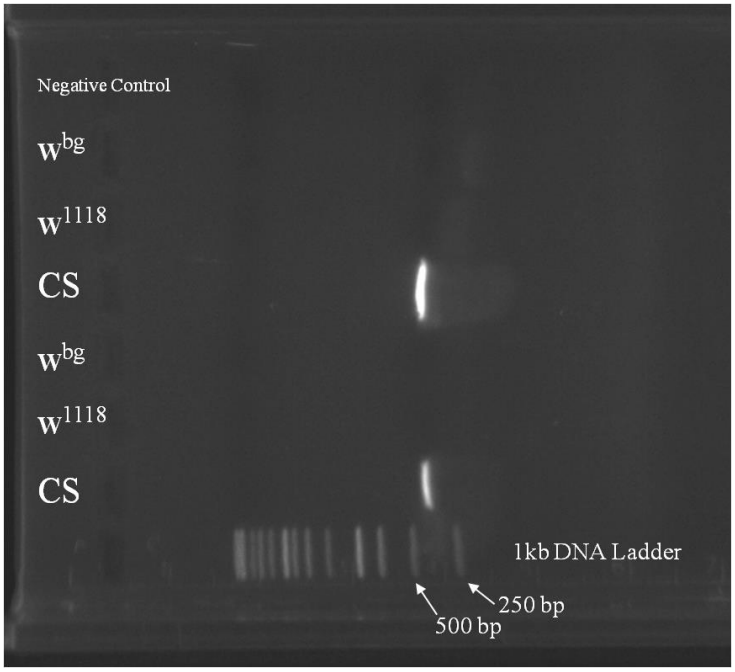

Figure 1.

The lack of recovery of visible mutants among 10,603 males screened in this study was not expected, since we had looked for mutations in at least 20 X-linked genes (see above), giving us over 200,000 gene tests (20 × 10,603) for visible mutations. If the average mutation rate for a gene in *D. melanogaster* is about one in 100,000 (Drake *et al*., 1998), then we should have expected to recover two new mutants.

The deficit of visible mutants in this study, and similar studies (Woodruff *et al*., 1979), is one reason for the new motivation to measure mutation rates as changes in base pairs from genomic DNA sequences. For example, Haag-Liautard *et al*. (2007) have observed a base-pair mutation rate per generation for *D. melanogaster* of 8.4×10^{-9}. Since *D. melanogaster* has about 279,000,000 base pairs in their diploid genome (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/genome/browse/), there would be about two new base-pair mutations per fly each generation (2.79×10^8 times $8.4 \times 10^{-9} = 2.37$). For humans, there are about 75 new base-pair mutations per human each generation (6.47×10^9 bases times 11.6×10^{-9} base-pair mutation rate = 75.05) (Campbell and Eichler, 2013; http://www.ncbi.nlm.nih.gov/genome/browse).

A class discussion of the results of this teaching exercise could include:

1) This experiment and others suggest that new visible mutants of *D. melanogaster* are very rare. Yet, early in T.H. Morgan's laboratory at Columbia University in New York City a number of new sex-linked visible mutants were isolated (Kohler, 1994). How was this possible? Robert Kohler (1994) suggests that there was a burst of mutational activity in the Morgan laboratory due to the movement and insertion of transposable DNA elements (this idea was suggested to Kohler by Edward Lewis, Nobel Prize *Drosophila* geneticist from Cal Tech). One might ask students to go to

FlyBase [http://flybase.bio.indiana.edu] and search for the molecular basis of some of the early sex-linked mutations recovered in Morgan's laboratory. For example, the sex-linked mutants white, forked, vermilion, prune, miniature, and garnet were caused by insertions of transposable DNA elements.

One possible reason for the burst of transposable DNA activity in Morgan's laboratory was that they often screened the progeny of crosses between strains, which is known to activate some transposable DNA elements (Woodruff, Slatko, and Thompson, 1983; Crow and Dove, 1988). In addition, recombination in males was observed in Morgan's laboratory (Muller, 1916; Bridges and Morgan, 1919). These recombination events in males are not the usual exchanges that occur in meiosis of females, but are now known to be caused by transposable DNA elements (Engels, 1989).

2) Students might wonder how one determines the base-pair mutation rate. A review of the techniques is given in Campbell and Eichler (2013, page 576, Box 1). The current method is to sequence the genomes of parents and their offspring and look for differences in base pairs (see Kong *et al*., 2012, where the base-pair mutation rate is estimated as 12×10^{-9} per nucleotide per generation).

3) How does the rate of new visible mutants compare to the frequency of preexisting recessive visible mutations from nature or laboratory stocks? Students might read Woodruff and Onasch (2009) where 10 recessive visible mutants out of 40 tested wild lines of *D. melanogaster* were recovered by inbreeding experiments. This abundance of hidden genetic variation is one reason some consider new mutations to be a weak force in evolution (Hedrick, 2011; Relethford, 2012), although not all agree (see a discussion of the role of new mutations in evolution in Nei, 2013).

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Instant synthetic species: tests of sexual isolation between compound-autosome stocks of *Drosophila melanogaster*.

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In the 1960s and 1970s, compound autosome stocks of *Drosophila melanogaster* were synthesized, making these stocks "instant synthetic species". These stocks are considered new species, because when they mate with *D. melanogaster* that have a normal karyotype, no progeny survive. The progeny of these crosses do not survive, because they have extra and missing autosome arms, and, therefore, triple and single copies of genes on these chromosome arms (see reviws of this topic, including the methods to synthesize the compound autosomes, in Holm, 1976; Ashburner, 1989).

In 1960, Rasmussen reported the synthesis of a C(3L)RM; C(3R)RM stock, which has the two left arms of the third chromosome attached to a centromere and the two right arms attached to a centromere (3L . 3L and 3R . 3R, with "." being the centromere), as compared to the left arms being attached to the right arms (3L . 3R and 3L . 3R) in wild-type *D. melanogaster.* Figure 1 shows what is expected when this compound-autosome stock is mated with a stock with normal third chromosomes (also see Figure 25.2, page 784, Ashburner, 1989); no adult progeny are expected to survive. Since the biological species concept is based on organisms being separate species if they are reproductively isolated (Coyne and Orr, 2004), then the C(3L)RM; C(3R)RM stock is an "instant synthetic species".

Figure 1. Expected results of matings between a C(3L)RM; C(3R)RM stock and a stock with normal third chromosomes (3L . 3R).

	C(3L)RM gamete	C(3R)RM gamete	C(3L)RM and C(3R)RM gamete	No third chromosome gamete
3L . 3R gamete	C(3L)RM / 3L . 3R Three 3L and one 3R Flies do not survive	C(3R)RM / 3L . 3R Three 3R and one 3L Flies do not survive	C(3L)RM; C(3R)RM/ 3L . 3R Three 3L and three 3R Flies do not survive	3L . 3R One 3L and one 3R Flies do not survive

Figure 2. Expected results of crosses between a C(2L)RM, F(2R) stock and a stock with normal second chromosomes.

	C(2L)RM; 2R gamete	2R gamete
2L . 2R gamete	C(2L)RM; 2R / 2L . 2R Three 2L Flies do not survive	2R / 2L . 2R One 2L Flies do not survive

As another example of a compound autosome stock, Grell in 1970 reported the synthesis of a C(2L)RM, F(2R) stock, which has two left arms of the second chromosome attached to a centromere

and two free right arms of the second chromosome, each with a centromere (2L . 2L, 2R., and 2R.). Wild-type *D. melanogaster* have the left arms of the second attached to the right arms (2L . 2R and 2L . 2R) (also see Figure 1, page 66 of Grell, 1970). Figure 2 shows what is expected when this compound autosome stock is mated with a stock with normal second chromosomes (Boulton and Woodruff, 2010). Again, no adult progeny are expected to survive, also making the C(2L)RM, F(2R) stock an "instant synthetic species".

Based on the dates of their synthesis, the C(3L)RM, *ri*; C(3R)RM, e^4 and the C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stocks that are used in this study have been isolated from normal-karyotype *D. melanogaster* for 53 and 43 years, respectively. Since the generation time of *D. melanogaster* laboratory stocks is about three weeks, the C(3L)RM, *ri*; C(3R)RM, e^4 stock has been reproductively isolated from other *D. melangaster* flies with normal chromosomes for about 919 generations, whereas the C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stock has been isolated for about 745 generations. Taken together, the two stocks have evolved separately for 1,664 generations. Has this been enough time for the two stocks to begin to develop reproductive isolation? Do the compound autosome stocks prefer to mate within the same stock more than beween the two stocks?

To begin to answer these questions, we first tested to make sure that the C(3L)RM, *ri*; C(3R)RM, e^4 and C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stocks have rearranged autosomes, and then measured their sexual isolation (mating preference). The mutant genes in these stocks are: *ri* = radius incompletus (short wing vein); e^4 = ebony-4 (dark body color); dp^{ov1} = dumpy-ov1 (wings); *c* = curved (wings); *cn* = cinnabar (eye color); *bw* = brown (eye color); *cn* and *bw* together gives a white-eye color to the C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stock, whereas the C(3L)RM, *ri*; C(3R)RM, e^4 stock has red eyes (Lindsley and Zimm, 1992).

Table 1. Results of crosses between the compound autosome stocks and Canton-S that has normal second and thrid chromsomes.

Females \ Males	Canton-S males
C(3L)RM, *ri*; C(3R)RM, e^4 females	No larva, pupae, or adult progeny were recovered; just unhatched eggs.
C(2L), RM, dp^{ov1}; F(2R), *c cn bw* females	Larvae and pupae were recovered, but no adults. Fitz-Earle and Holm (1978) saw similar results.

Table 2. Expected results from crosses of the two compound autosome stocks.

Females \ Males	2L . 2R; C(3L)RM; C(3R)RM males	C(2L)RM; F(2R); 3L . 3R males
2L . 2R; C(3L)RM; C(3R)RM females	Some adults are expected to survive	Extra or missing chromosome arms. Flies do not survive
C(2L)RM; F(2R); 3L . 3R females	Extra or missing chromosome arms. Flies do not survive	Some adults are expected to survive

I. Genetic tests of the chromosomal structure of the C(3L)RM, *ri*; C(3R)RM, e^4, and C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stocks*:*

Table 1 shows the results from crosses of the two autosomal stocks with the wild-type Canton-S stock, which has normal second and third chromosomes. No adults survived in either cross. These results confirm that the two compound autosome stocks contain rearranged chromosomes.

Next, the two compound autosome stocks were mated with each other. The expected results (no adult progeny are expected to survive) of these crosses are shown in Table 2, and the observed results are shown in Table 3. As predicted, no adult progeny survied, confirming that the two compound autosome stocks have different rearranged chromosomes.

Table 3. Observed results of matings between the two compound autosome stocks.

Females \ Males	C(3L)RM, *ri*; C(3R)RM, e^4 males	C(2L)RM, dp^{ov1}; F(2R), *c cn bw* males
C(3L)RM, *ri*; C(3R)RM, e^4 females	Adults survived	No larvae, pupae or adults were recovered.
C(2L)RM, dp^{ov1}; F(2R), *c cn bw* females	No larvae, pupae, or adults were recovered	Adults survived

II. Sexual isolation (mating preference) experiments:

Using a technique similar to Crossley (1974), we performed all pair-wise crosses between females and males of the two compound autosome stocks, first using one female and two males (female choice) and then one male and two females (male choice). For a review of methods for measuring sexual isolation (mating preference) with *D. melanogaster*, see Spieth and Ringo (1983) and Nanda and Singh (2012).

Unetherized three-day-old virgin females and three-day-old males were placed in empty vials and were observed for matings for up to two hours. When a mating was observed, the identification of the mating male (female choice experiment) or mating female (male choice experiment) was determined. The results of these mating experiments are shown in Tables 4 and 5. In Table 4 (female choice), a single C(3L)RM, *ri*; C(3R)RM, e^4 female or a single C(2L)RM, dp^{ov1}; F(2R), *c cn bw* female was mated with two males, (one C(3L)RM, *ri*; C(3R)RM, e^4 male and one C(2L)RM, dp^{ov1}; F(2R), *c cn bw* male), whereas in Table 5 (male choice) a single C(3L)RM, *ri*; C(3R)RM, e^4 male or a single C(2L)RM, dp^{ov1}; F(2R), *c cn bw* male was mated with two females (one C(2L)RM, dp^{ov1}; F(2R), *c cn bw* female and one C(3L)RM, *ri*; C(3R)RM, e^4 female).

Table 4. Female choice sexual isolation (mating preference) experiment, using one female and two males (one from each compound autosome stock).

Female (one) \ Males (two)	C(3L)RM, *ri*; C(3R)RM, e^4 male	C(2L)RM, dp^{ov1}; F(2R), *c cn bw* male
C(3L)RM, *ri*; C(3R)RM, e^4 female	46[a]	12[a]
	Number matings if random = 29[a]	Number matings if random = 29[a]
C(2L)RM, dp^{ov1}; F(2R), *c cn bw* female	38[b]	40[b]
	Number matings if random = 39[b]	Number matings if random = 39[b]

[a]P = 0.002; [b]P = 1.

As an example of the results in the upper cross of Table 4, 46 of the 58 total matings were between C(3L)RM, *ri*; C(3R)RM, e^4 flies, and 12 were between C(3L)RM, *ri*; C(3R)RM, e^4 females and C(2L)RM, dp^{ov1}; F(2R), *c cn bw* males. The significant P value of 0.002 comes from a Fisher exact test (using the Prism program) of 46 and 12 observed matings compared to the expected 29 and 29, if the matings were at random. See Dodd (1989) for a similar analysis of reproductive isolation tests of *D. pseudoobscura* strains.

In the female choice experiment (Table 4), the C(3L)RM, *ri*; C(3R)RM, e^4 stock preferred to mate with their own karotype ($P = 0.002$), whereas the C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stock mated at random with the C(3L)RM, *ri*; C(3R)RM, e^4 and C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stocks ($P = 1$). In the male choice experiment (Table 5), neither stock showed a mating preference ($P = 0.85$ for the C(3L)RM, *ri*; C(3R)RM, e^4 stock and $P = 0.18$ for the C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stock). Hence, the C(3L)RM, *ri*; C(3R)RM, e^4 stock showed significant sexual isolation (based on the female choice experiment), whereas, the C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stock mated at random.

As an interspecies control, where matings are expected to occur preferentially among species (Sturtevant, 1920; Ashburner, 1989, p. 1186), we also set up female and male choice mating experiments with the two compound autosome stocks mated with a w^{pch} white-eyed mutant stock of *D. simulans*, a sibling species of *D. melanogaster*. The results of these crosses are shown in Tables 6-9.

Table 5. Male choice sexual isolation (mating preference) experiment, using one male and two females (one from each compound autosome stock).

Males (one) / Female (two)	C(3L)RM, *ri*; C(3R)RM, e^4 male	C(2L)RM, dp^{ov1}; F(2R), *c cn bw* male
C(3L)RM, *ri*; C(3R)RM, e^4 female	26[a]	8[b]
	Number matings if random = 28[a]	Number matings if random = 14.5[b]
C(2L)RM, dp^{ov1}; F(2R), *c cn bw* female	30[a]	21[b]
	Number matings if random = 28[a]	Number matings if random = 14.5[b]

[a]P = 0.85; [b]P = 0.18

In the female and male choice experiments, there is a clear preference for matings within species. For example, from Tables 6-9, there are 65 total matings between *D. simulans* and *D. melanogaster*, 172 matings within *D. simulans* (86 × 2, because there are half as many chances for these mating as matings between species), and 224 matings (112 × 2) within *D. melanogaster*, giving a chi-square value of 50.7 ($P < 0.0001$; 65, 172 and 224 *vs* 153.67, 153.67, and 153.67, if matings were at randaom). In addition, there were significant mating preferences within species in Tables 6 and 7.

One possible reason why the C(3L)RM, *ri*; C(3R)RM, e^4 stock has more sexual isolation than the C(3L)RM, *ri*; C(3R)RM, e^4 flies is because the former stock is more active than the C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stock. For example, the C(3L)RM, *ri*; C(3R)RM, e^4 males in Table 4 may have increased activity and, therefore, may simply encounter the C(3L)RM, *ri*; C(3R)RM, e^4 females more often than the C(3L)RM, *ri*; C(3R)RM, e^4 males. Hence, locomotion activity was measured for ten males for each of the two compound autosome stocks by use of the Connolly (1966) circular runway apparatus (Woodruff *et al*., 1999). In this apparatus, three-day-old, unetherized, flies were introduced into a 3 mm diameter channel in a 15.7 cm round runway that is marked with radial lines (see Figure 5, Connolly, 1966). Single, unetherized, males were placed into the runway for 90 seconds to recover

from the transfer process and then the number of radial lines crossed by the fly in one minute was recorded (distance between lines is 2 cm). The locomotion activities of the two stocks were significantly different ($P = 0.04$). Yet, the C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stock had a significantly higher locomotion (136.10 ± 10.68 lines per minute; mean ± SE) than the C(3L)RM, *ri*; C(3R)RM, e^4 stock (99.30 ± 12.79). Hence, the increased sexual isolation of the C(3L)RM, *ri*; C(3R)RM, e^4 stock is not due to increased activity.

Table 6. Female choice sexual isolation (mating preference) experiment, using one female and two males (a *D. simulans* male and a compound autosome male).

Females (one) \ Males (two)	*Drosophila simulans* w^{pch} male	C(2L)RM, dp^{ov1}; F(2R), *c cn bw* male
Drosophila simulans w^{pch} female	44[a] Number matings if random = 28[a]	12[a] Number matings if random = 28[a]
C(2L)RM, dp^{ov1}; F(2R), *c cn bw* female	29[b] Number matings if random = 40[b]	51[b] Number matings if random = 40[b]

[a]P = 0.003; [b]P = 0.11

Table 7. Female choice sexual isolation (mating preference) experiment, using one female and two males (a *D. simulans* male and a compound autosome male).

Females (one) \ Males (two)	*Drosophila simulans* w^{pch} male	C(3L)RM, *ri*; C(3R), e^4 male
Drosophila simulans w^{pch} female	29[a] Number matings if random = 20[a]	11[a] Number matings if random = 20[a]
C(3L)RM, *ri*; C(3R), e^4 female	0[b] Number matings if random = 11.5[b]	23[b] Number matings if random = 11.5[b]

[a]P = 0.07; [b]P = 0.0002

Table 8. Male choice sexual isolation (mating preference) experiment, using one male and two females (a *D. simulans* female and a compound autosome female).

Females (two) \ Males (one)	*Drosophila simulans* w^{pch} male	C(2L)RM, dp^{ov1}; F(2R), *c cn bw* male
Drosophila simulans w^{pch} female	5[a] Number matings if random = 2.5[a]	6[b] Number matings if random = 12[b]
C(2L)RM, dp^{ov1}; F(2R), *c cn bw* female	0[a] Number matings if random = 2.5[a]	18[b] Number matings if random = 12[b]

[a]P = 0.17; [b]P = 0.14

Table 9. Male choice sexual isolation (mating preference) experiment, using one male and two females (a *D. simulans* female and a compound autosome female).

Females (two) \ Males (one)	*Drosophila simulans* w^{pch} male	C(3L)RM, ri; C(3R), e^4 male
Drosophila simulans w^{pch} female	8[a] Number matings if random = 5[a]	5[b] Number matings if random = 12.5[b]
C(3L)RM, *ri*; C(3R), e^4 female	2[a] Number matings if random = 5[a]	20[b] Number matings if random = 12.5[b]

[a]P = 0.35; [b]P = 0.07

In summary, the C(3L)RM, *ri*; C(3R)RM, e^4 stock has more sexual isolation than the C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stock. The C(3L)RM, *ri*; C(3R)RM, e^4 flies prefer to mate within the stock more than the C(2L)RM, dp^{ov1}; F(2R), *c cn bw* stock. Furthermore, the mating preference of the C(3L)RM, *ri*; C(3R)RM, e^4 stock is not due to an increase in locomotion. Yet, it is impossible to know if the observed isolation differences have occurred during the 1,664 generations of separation of the two stocks, or were present in the base stocks before they were synthesized into compound autosome stocks. Although most natural population lines of *D. melanogaster* mate at random (Henderson and Lambert, 1982), significant differences in sexual isolation have been observed for laboratory and wild stocks of *D. melanogaster* (for example, Sturtevant, 1915; Wu *et al*., 1995; Haerty *et al*., 2002; Takahashi and Ting, 2004; Alipaz *et al*., 2005).

A class discussion of the results of this teaching exercise could include:

1) For students to see the extent of research on sexual isolation in *Drosophila*, they could go to FlyBase [http://flybase.bio.indiana.edu] and search under references for the key words, sexual isolation, mating preference, and reproductive isolation; they will recover 922 articles with these words in titles or in abstracts from 1938 to 2013.

2) How would larvae and pupae survive from crosses of C(2L), RM, dp^{ov1}; F(2R), *c cn bw* females with Canton-S males, even though the larvae and pupae have extra and missing chromosome arms (see Table 1 and Fitz-Earle and Holm, 1978)? It is known that aneuploidy (missing or extra chromosomes) in humans leads to early embryo death, unless the aneuploidy involves the sex chromosomes, or chromosomes 13, 18, or 21 (Strachan and Read, 2004). Students might consider that triploid *D. melanogaster* with three of each chromosome, survive (Bridges, 1922, Figure 1); there are no triploid humans.

3) Students might be asked to go to FlyBase [http://flybase.bio.indiana.edu] and see if they can find other examples of compound autosomes. For example, there are C(2)EN, C(3)EN, and C(2;3)EN stocks (EN = entire) (Ashburner, 1989). The C(2:3)EN stock has the 2L, 2R, 3L, and 3R chromosome arms attached to one centromere. Novitski *et al*. (1981) reviews the synthesis of the C(2)EN, C(3)EN, and C(2;3)EN chromosomes.

4) One could also discuss other uses of the compound autosome stocks. For example, they have been used to measure fitness of *D. melanogaster* stocks (for reviews of this topic, see Jungen and Hartl, 1979; Sved, 1989). Suppose the intended goal is to measure the fitness of two wild-type

stocks (A and B) that have normal chromosomes. You could not mix the two stocks together and compare offspring production, because the two stocks have the same, wild type, phenotype. Yet, if you place stock A in a bottle with a compound autosome stock that is marked with a visible mutation, you could measure the proportion of A offspring and compound autosome offspring. This progeny ratio could then be compared to the results of B flies and the compound autosome. This would give the fitness of A and B stocks compared to the compound autosome stock and, therefore, the fitness of stocks A and B compared with each other. The compound-autosome technique is a one-generation test of overall fitness, including mating ability, fecundity, fertility, and viability, and has been used to measure the fitness of stocks in the presence and absence of active transposable DNA elements (Belyaeva *et al*., 1982; Woodruff *et al*., 1999) and the fitness of stocks that are resistant or sensitive to insecticides (Minkoff and Wilson, 1992).

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Drosophila smoking: using flies in a smoke-free class.

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Overview

Smoking habit is responsible for 71% of cases of lung cancers, 42% of deaths for chronic respiratory disease, and 10% of cardiovascular diseases (Öberg *et al*., 2010). According to the World

Health Organization (WHO), tobacco caused 100 million deaths in the twentieth century (WHO 2011).

Adult *Drosophila melanogaster*, like many vertebrates, when exposed to cigarette smoke or nicotine are initially stimulated, but higher concentrations are lethal to flies (Wolf and Heberlein, 2003). There is wide genetic variation for resistance to nicotine in natural populations and laboratory stocks (Passador-Gurgel *et al*., 2007). These characteristics make *Drosophila* an excellent model to class activities, allowing the students to observe directly the harmful action of smoking. We hope that this experience would collaborate to reduce the smoking habit. Also, the activity allows students to conduct experimental research using hypothesis tests and statistical analysis. It has been suggested that it is crucial for scientific literacy that the students can experience the nature of science -NOS (Miller *et al*., 2010). The activity here described offers a possibility to understand better how scientific knowledge is produced.

The experiment consists in comparing the recovery time and mortality rates for different *Drosophila* strains exposed to CO_2, cigarette smoke, and smoke provided by burning of different vegetables, such as grass, tea, or some seasonings. The flies are exposed to a saturated atmosphere with CO_2 or smoke. For the recovery time is registered the time at which the first and last fly begins to move. The flies that do not recover are registered as dead for quantification of mortality rate.

The experiment proposed can be useful in many school levels, from fundamental school to under-graduation. Of course, the amplitude and profundity should be modulated by the objectives of the course, the syllabus, age of students, and proposal of the teachers. In the fundamental school can be a concrete and immediate demonstration of the damage caused by cigarette smoke, associated with learning of healthy habits and combat to smoking. At higher levels of schooling others aspects can be added. For example, different experimental designs can be proposed, as the control of variables as exposition time, different smoke, genotype of the flies, and so forth. Also, the sample size (flies for experiment) and the number of experimental replicas can be included as themes for learning.

Constructing Some Apparatus for the Experiment

To expose the flies to cigarette smoke, the teacher could simply aspire the smoke and, using a tube, to expire the fume to the bottle in which there are the flies. However, as we want to discourage the smoking habit, this procedure is strongly not recommended. To expose flies to CO_2 and the fume, we have made the apparatus shown in Figure 1. For CO_2 we have used a small PET bottle and, in the cap, is done a hole and fixed a plastic tube using epoxy putty. Water is placed in the PET bottle and the CO_2 is produced by placing an effervescent antacid tablet in it. The CO_2 is transferred to the bottle with the flies by the plastic tube (Figure 1-A). Also, the gas from a soda can be used, putting soda in the PET bottle and shaking.

The smoke is produced using another apparatus. A wash bottle is attached to a plastic tube, and this one is linked to the cigarette into a glass vial. From the glass vial is another plastic tube leading the smoke to the bottle with the flies (Figure 1-B).

To avoid secondhand smoke it is highly recommended to use fume hoods or, alternatively, this part of the activity could be done in an open environment. The reasons of such care should be discussed with the students.

Observed Results

Figure 2 shows an example of results that can be obtained in a classroom activity. The objective was to compare the recovery time after 30 minutes of exposition to CO_2 or to cigarette smoke. Four strains of flies were used in this activity: *D. melanogaster* wild type (Dm wt), *D.*

melanogaster sepia mutant (Dm se), *D. simulans* wild type (Ds wt), and *D. simulans* yellow mutant (Ds ym).

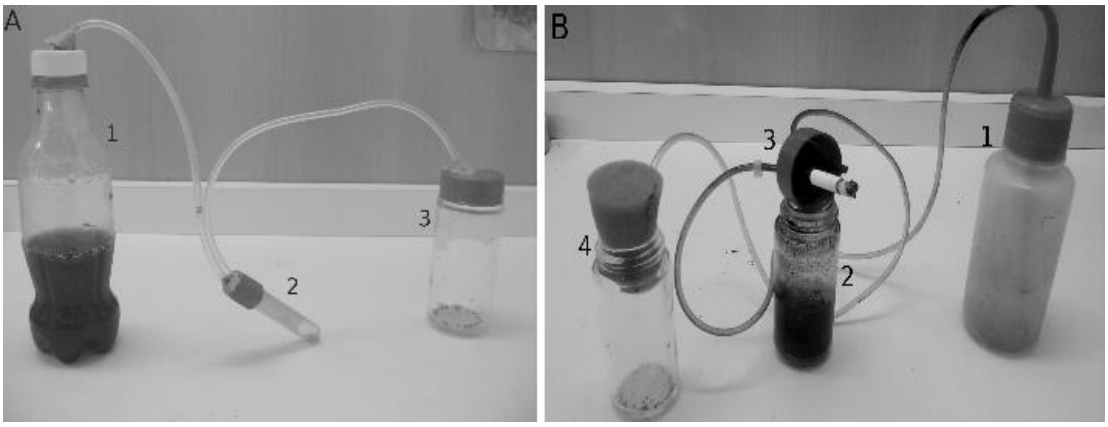

Figure 1. A) Apparatus for CO_2 exposure of flies. A PET bottle (1) with water and an effervescent antacid tablet is connected by a tube to a vial with the flies (2). We include a reservoir between the bottle that is source of CO2 and the vial with flies, as a security system, to avoid liquids reaching the flies (3). B) Apparatus for exposing the flies to smoke. A wash bottle (1) is connected by a plastic tube to a glass vial (2). In a CAP of this vial is attached a 1 ml micropipet tip to affix the cigarettes (3). From this vial one plastic tube is connected to another bottle with the flies (4).

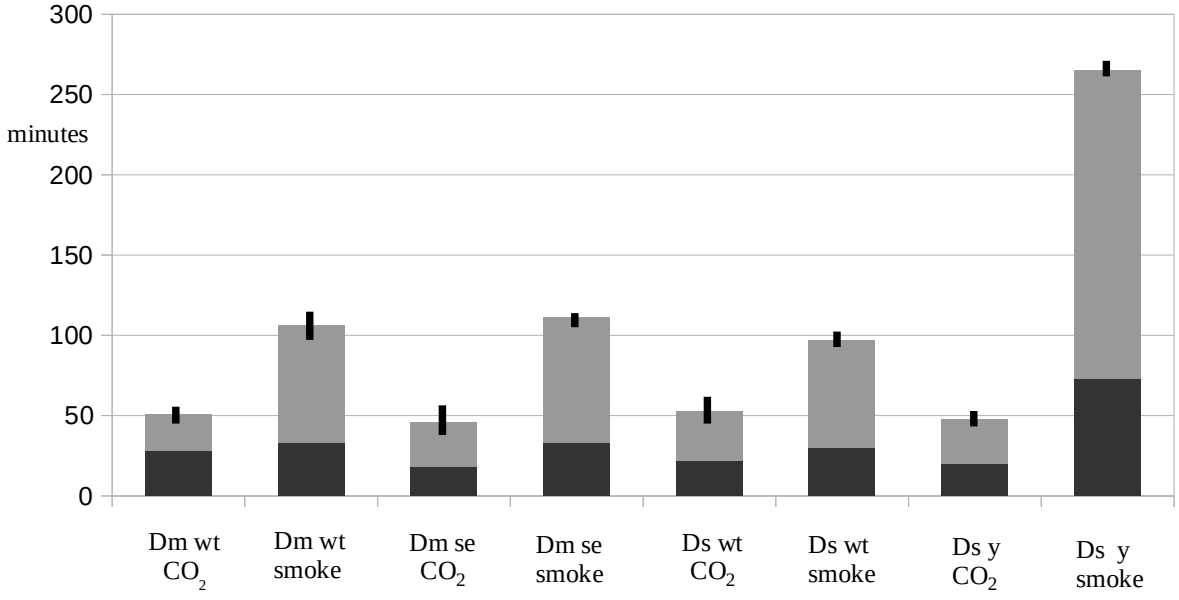

Figure 2. Time, in minutes, in that the first fly becomes active (dark gray), and that all the flies become actives (light gray) after having been exposed for 30 minutes to an atmosphere saturated with CO_2 or cigarette smoke. Dm wt = wild type strain of *D. melanogaster*; Dm se = sepia strain of *D. melanogaster*; Ds wt = wild type strain of *D. simulans*; Ds y = yellow strain of *D. simulans*. Black lines in the bars represent the standard deviation.

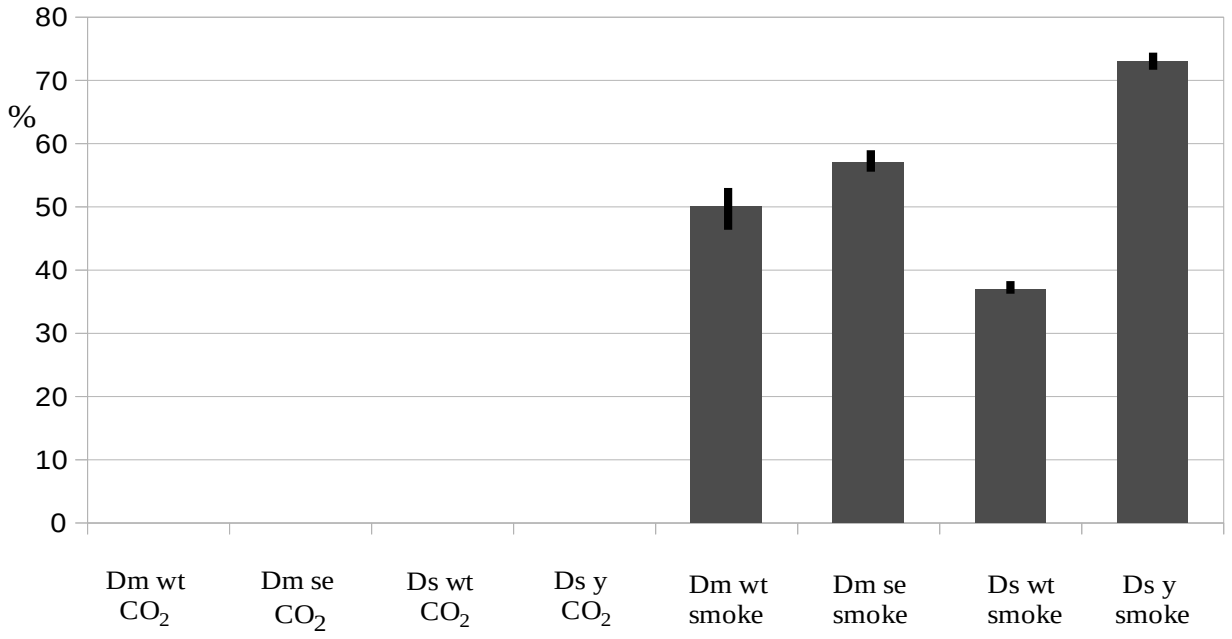

Figure 3. Mortality rate (in percentage) for flies exposed to CO_2 and to cigarette smoke. Dm wt = *D. melanogaster* wild type; Ds se = *D. melanogaster* sepia; DS wt = *D. simulans* wild type; Ds y = *D. simulans* yellow. Black lines in the bars represent the standard deviation.

The recovery time reported is the average from three independents replicas, each one using a sample of approximately 30 flies. The exposure to cigarette smoke was standardized; in each trial the flies were exposed to the burning of two centimeters of cigarette, during 30 minutes. The recovery time and mortality were registered. The recovery time was around fifty minutes for CO_2 for all strains, and for flies exposed to cigarette smoke, this time was twice. The exception was the *D. simulans* yellow strains that spent about 270 minutes to recover from the exposition to fume.

Analysis of variance (ANOVA) showed that the treatments using CO_2 and cigarette smoke differ significantly in the samples. The "t" test showed us that *D. simulans* yellow is more susceptible to cigarette smoke than other strains, and it is a good example of genetic diversity in response to environmental factors.

A more remarkable result was the high rate of mortality observed in the flies exposed to cigarette smoke as compared the absence of mortality in flies exposed to the CO_2 (Figure 3). Differences among the strains can be observed, suggesting genetic variation to the resistance or tolerance to the cigarette smoke.

Once well settled on the protocols and the procedures to expose the flies to cigarette smoke, new treatments were applied to the four *Drosophila strains*. We have done some "cigarettes" using grass (*Paspalum notatum)* or teas, as for example leaves of herb mate (*Ilex paraguariensis*). However, when the flies were exposed to smoke of these "cigarettes" the results were similar to those observed using CO_2 , showing that it is the chemical compounds present in the tobacco that are responsible for the mortality of flies and not from any fume.

Experiments using shorter exposition time, as 10 or 15 minutes, produce similar results and the class duration will be, also, shorter.

Didactic Possibilities

Different types of experiments in which *Drosophila* are exposed to CO_2, cigarette smoke, or smoke from other plants can be configured. Demonstrative activities allow the observation of the damage caused by smoking. While CO_2 and fume produced by the burning of most plants only produce anesthesia in the flies, cigarette smoke produces a high mortality. These observations can be associated to discussion and advertisements about smoke-free. Higher skills like setup of experiments, control of variables, statistical analysis, and so forth can be developed if the activities were done as a project. For example, analyses of genetic variability for nicotine susceptibility allow discussion of themes like pharmacogenetics. The literature reviews on the phenomena observed can lead to a deeper understanding in physiology, biochemistry, genetics, and other disciplines.

Acknowledgments: We thank PIBID/CAPES and CNPq for fellowships.

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54th Annual *Drosophila* Research Conference

The 54th Annual *Drosophila* Research Conference was held on 3-7 April 2013 in Washington, D.C. The 2013 Organizing Committee was Richard Mann (Columbia University, New York, NY), Hannele Ruohola-Baker (University of Washington, Seattle, WA), Kristin Scott (University of California, Berkeley, CA), and David Stern (Janelia Farm Research Campus, Ashburn, VA). The conference was sponsored by The *Drosophila* Board in association with the Genetics Society of America, 9650 Rockville Pike, Bethesda, MD 20814-3998. The Program and Abstracts Volume lists two Plenary sessions, 156 platform session talks, 807 posters, and 12 workshops.

Historical Address Speaker

Jules A. Hoffmann (IBMC, University of Strasbourg, Strasbourg, France). Innate immunity: From flies to humans.

Plenary Lectures

Marc R. Freeman (University of Massachusetts Medical School/HHMI, Worcester, MA). Molecular mechanisms of axon degeneration.

Tom Clandinin (Stanford University, CA). Genetic approaches to dissecting neural computation in the visual system.

Chris Jiggins (University of Cambridge, Cambridge, UK). The genomics of speciation and pattern evolution in (butter)flies.

Naama Barkai (Weizman Institute, Rehovot, Israel). Creating gradients by morphogen shuttling.

Leanne Jones (Salk Institute, La Jolla, CA). Maintenance of niche function and tissue homeostasis during ageing.

Jürg Müller (Max-Planck Institute of Biochemistry, Martinsried, Bavaria, Germany). Histone genetics in *Drosophila*.

Terry Orr-Weaver (Whitehead Institute, MIT, Cambridge, MA). Diamonds in the rough: Finding paradigms in *Drosophila* developmental strategies.

Scott Barolo (University of Michigan Medical School, Ann Arbor, MI). Information, enhancers, and cell signaling: A view from the binding site.

Greg Hannon (HHMI, Cold Spring Harbor Lab, Cold Spring Harbor, NY). The piRNA pathway: A small RNA-based innate immune system.

Ilaria Rebay (University of Chicago, IL). Transcription factor network dynamics in development.

Martin W. Hetzer (Salk Institute, La Jolla, CA). The role of nuclear pore proteins in developmental gene regulation.

Andrea H. Brand (The Gurdon Institute, University of Cambridge, Cambridge, UK). Stem cells to synapses: Regulation of self-renewal and differentiation in the nervous system.

Nancy M. Bonini (University of Pennsylvania/HHMI, Philadelphia, PA). Neurodegeneration and aging: Insight from *Drosophila*.

Workshops

Ecdysone Workshop

Organizers: Robert Ward (University of Kansas) and Ginger Carney (Texas A&M University)

Federal Funding Opportunities and the Proposal Review Process

Organizers: Joyce Fernandes (National Science Foundation) and Leslie Pick (National Science Foundation)

Undergraduate Plenary Session and Workshop

Organizers: Karen Hales (Davidson College, NC) and Beth Ruedi (Genetics Society of America)

The Future of *Drosophila* Genomics

Organizers: Susan Celniker (Lawrence Berkeley National Laboratory) and Gary Karpen (Lawrence Berkeley National Laboratory)

Apoptosis, Autophagy, and Other Cell Death Mechanisms

Organizers: Andreas Bergmann (University of Massachusetts Medical School) and Michael Brodsky (University of Massachusetts Medical School)

Everything You Ever Wanted to Know About Sex

Organizers: Michelle Arbeitman (Florida State University) and Mark Siegal (New York University)

Effectively Integrating Undergraduates into a Research Program

Organizers: Joyce Fernandes (Miami University, OH) and Larry Wimmers (Biology Division of the Council on Undergraduate Research)

Myofibril Assembly

Organizer: Frieder Schoeck (McGill University)

The North American *Drosophila* Board

The Board's duties include: overseeing community resource centers and addressing other research and resource issues that affect the entire *Drosophila* research community. The Board also administers the finances for the annual North America *Drosophila* Research Conference and its associated awards, and it chooses the organizers and the site of the annual meeting. The Board consists of nine regional representatives and four international representatives, who serve 3-year terms. The three elected officers are President, President-Elect, and Treasurer. In addition, the Board has *ex officio* members who represent *Drosophila* community resources or centers. For more information about the Board and the summaries of the annual Board meetings, see: Fly Board under the News menu at the FlyBase web site: flybase.bio.indiana.edu.

Drosophila Board Membership as of 54th Annual *Drosophila* Research Conference April 2013

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