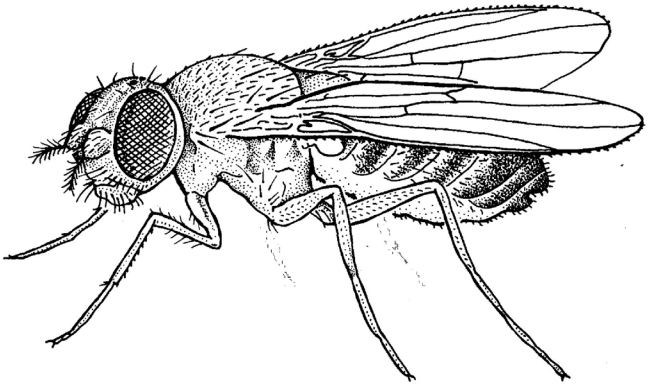
Drosophila Information Service



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Preface

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

The production of this volume of DIS could not have been completed without the generous efforts of many people. Except for the special issues that contained mutant and stock information now provided in detail by FlyBase and similar material in the annual volumes, all issues are now freely-accessible from our web site: www.ou.edu/journals/dis. For early issues that only exist as aging typed or mimeographed copies, some notes and announcements have not yet been fully brought on line. But we intend to fill in those gaps for historical purposes in the future. If you would pdf copies of material missing from the on-line issues, please contact Jim Thompson.

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

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Lithium and caffeine interact to alter circadian activity rhythms in *Drosophila melanogaster.*

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Bipolar disorder (BPD) is a psychiatric disorder associated with disrupted rest/activity cycles and cyclical expression of symptoms, hence circadian clock function has been investigated as a mechanism underlying bipolar cycles of mania and depression (Logan and McClung, 2016). Lithium is a focal point of research on BPD since it is a widely used treatment (Sawai *et al*., 2019). Drugs that interact with lithium, therefore, are of interest because they may alter lithium's therapeutic effects, and BPD patients have high rates of substance use disorder comorbidity (Nascimento *et al*., 2015). An increase in manic episodes and suicide risk has been associated with coffee consumption in bipolar patients on lithium (Baethge *et al.*, 2009), though caffeine is generally associated with positive effects on health (Poole *et al*., 2017). While studies of lithium's effects on circadian rhythmicity have been conducted using mice as a model (*e.g.,* Li *et al.*, 2012; Nascimento *et al.*, 2015; Logan and McClung, 2016), few studies have used *Drosophila* (Dokucu *et al.*, 2005; Padiath *et al*., 2009). *Drosophila melanogaster* has great potential to be used as a model system for studying mechanisms mediating drug interactions because of its short life cycle, high efficiency and convenience in *vivo*, low cost, and extensive homology with the human genome (Mackay and Anholt, 2006). Padiath *et al*. (2004) and Dokucu *et al.* (2005) showed that lithium lengthens the free-running circadian period in male *Drosophila*, and caffeine has been shown to lengthen circadian clock period in *Drosophila* (Wu *et al*., 2009) and in mice (Oike *et al*., 2011). We present evidence that lithium and caffeine interact to alter the distribution of circadian locomotor activity between the light and dark phases of a 12:12 LD photoperiod in both male and female fruit flies.

Male and female *Drosophila melanogaster* from wild type strain *Oregon-R* were obtained from Carolina Biological Supply (Burlington, North Carolina, USA) and maintained at Skidmore College for 37 years. Four males and four females were placed in each of 20 plastic vials (65 mm long and 23.5 mm diameter) with Carolina Instant *Drosophila* Medium (Carolina Biological Supply) and incubated at 25°C in a 12:12 LD photoperiod. The parents were cleared on day seven and offspring, aged 1-4 days old, were collected at day 14. Individual adult male and female flies (N = 192) were placed in plastic tubes (65 mm long and 5 mm in diameter from Trikinetics, Waltham MA, USA) and received control medium, lithium chloride (20 mM), caffeine (0.01%), or a combination of both drugs in agar food (2% agarose and 5% sucrose). Experimental tubes had agar food sealed with a plastic cap at one end and a cotton plug at the other end and were inserted into *Drosophila* Activity Monitors (DAM; Trikinetics). Daily locomotor activities were assayed for two days in a 12:12 LD photoperiod and for nine days in constant dark at 25°C, using DAMs, as instructed by Seggio (2011). Each tube was bisected by an infrared photobeam, and activity was recorded as the number of infrared beam crossings in a 10-min interval. Flies were included in the analysis only if they lived for at least eight days, in order to optimize both sample size and the number of cycles in DD (constant dark) to estimate the free-running circadian period (tauDD) as precisely as possible. Activity data were analyzed with the program Rhythm Watch (Minimitter, Bend, OR USA) to estimate average light activity in the two days in LD (XL), average dark activity in LD (XD), average LD activity (XLD), and average constant dark activity (XDD). The distribution of day and night activity was estimated by calculating the ratio of light activity to total LD activity (XL/LD = LDratio). The free-running circadian period (tauDD) was estimated using the program Actogram J (Schmid *et al*., 2011) by averaging the results of the Sokolove-Bushell's chi-square periodogram and the Lombard-Scargle periodogram, and only including the data if both periodograms were statistically significant ($p < 0.05$). All variables were analyzed using the ANOVA procedure in SAS (SAS

Institute, Carey, NC USA) to assess differences among treatments and interaction between lithium and caffeine for each variable.

Table 1. Means ± SEM of treatment and sex differences for measurements of circadian locomotor activity rhythms.

	Sex	Control	LiCl	Caffeine	LiCl*Caffeine
XLD	F	7.29 ± 0.45	11.42 ± 1.01	8.80 ± 0.73	10.28 ± 0.96
	M	6.05 ± 0.56	10.23 ± 1.06	5.86 ± 0.43	11.28 ± 0.91
XL	F	8.54 ± 0.60	11.82 ± 1.09	8.65 ± 0.75	9.78 ± 0.84
	M	5.52 ± 0.72	7.98 ± 0.91	4.16 ± 0.54	8.68 ± 0.86
XD	F	6.04 ± 0.51	11.02 ± 1.03	8.94 ± 1.04	10.80 ± 1.16
	M	6.59 ± 0.54	12.47 ± 1.34	7.56 ± 0.57	13.88 ± 1.07
LDratio	F	1.17 ± 0.04	1.03 ± 0.03	1.00 ± 0.05	0.95 ± 0.03
	M	0.89 ± 0.06	0.78 ± 0.04	0.69 ± 0.06	0.75 ± 0.03
XDD	F	8.40 ± 0.53	11.07 ± 0.99	9.58 ± 0.62	10.79 ± 0.86
	M	7.42 ± 0.75	11.24 ± 1.24	6.92 ± 0.69	9.54 ± 1.04
tauDD	F	24.67 ± 0.07	25.04 ± 0.28	24.70 ± 0.13	25.07 ± 0.07
	M	24.09 ± 0.09	24.51 ± 0.19	24.25 ± 0.07	24.38 ± 0.17

Table 2. ANOVA treatment effects and interactions: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

	Sex	LiCl	Caffeine	Sex* LiCl	Sex* Caffeine	LiCl* Caffeine	Sex* LiCl* Caffeine
XLD	*	***	ns	ns	ns	ns	ns
XL	***	***	ns	ns	ns	ns	ns
XD	ns	***	ns	ns	ns	ns	ns
LD ratio	***	ns	**	ns	ns	*	ns
XDD	ns	***	ns	ns	ns	ns	ns
tauDD	***	**	ns	ns	ns	ns	ns

The lithium only, caffeine only, and combined lithium plus caffeine treatments all had significant effects on at least one variable (Table 1 and 2, Figure 1 and 2). Lithium alone increased activity levels for both male and female flies during both daytime and nighttime in 12:12 LD, and in constant dark. The LDratio was not altered by lithium since lithium's effects on activity were similar regardless of time of day. The effect of caffeine on activity showed the opposite pattern from lithium. There was no significant effect of caffeine on any measure of activity, but the ratio of activity between light and dark was significantly altered since caffeine increased the proportion of activity expressed in the dark phase of the 12:12 LD photoperiod. This effect of caffeine was amplified by lithium resulting in a subtle, but significant interaction between lithium and caffeine for the LDratio. Our results for the circadian period-lengthening effects of lithium were consistent with those reported by Padiath *et al*. (2004) and Dokucu *et al*. (2005) and demonstrate, for the first time, that lithium can lengthen circadian period in female *Drosophila melanogaster*. We did not observe a lengthened circadian period in response to caffeine, possibly because of the low dosage of caffeine treatment used in our experiment compared to Dokucu *et al*. (2009), or a strain-dependent circadian response to lithium since we used *Oregon-R* wild type flies, and Dokucu *et al*. (2009) used the caffeine-sensitive RC1 strain.

Females were significantly more active than males in the daytime, and displayed a longer tauDD, both of which are typical of *D. melanogaster* circadian activity rhythms (Helfrich-Förster, 2000).

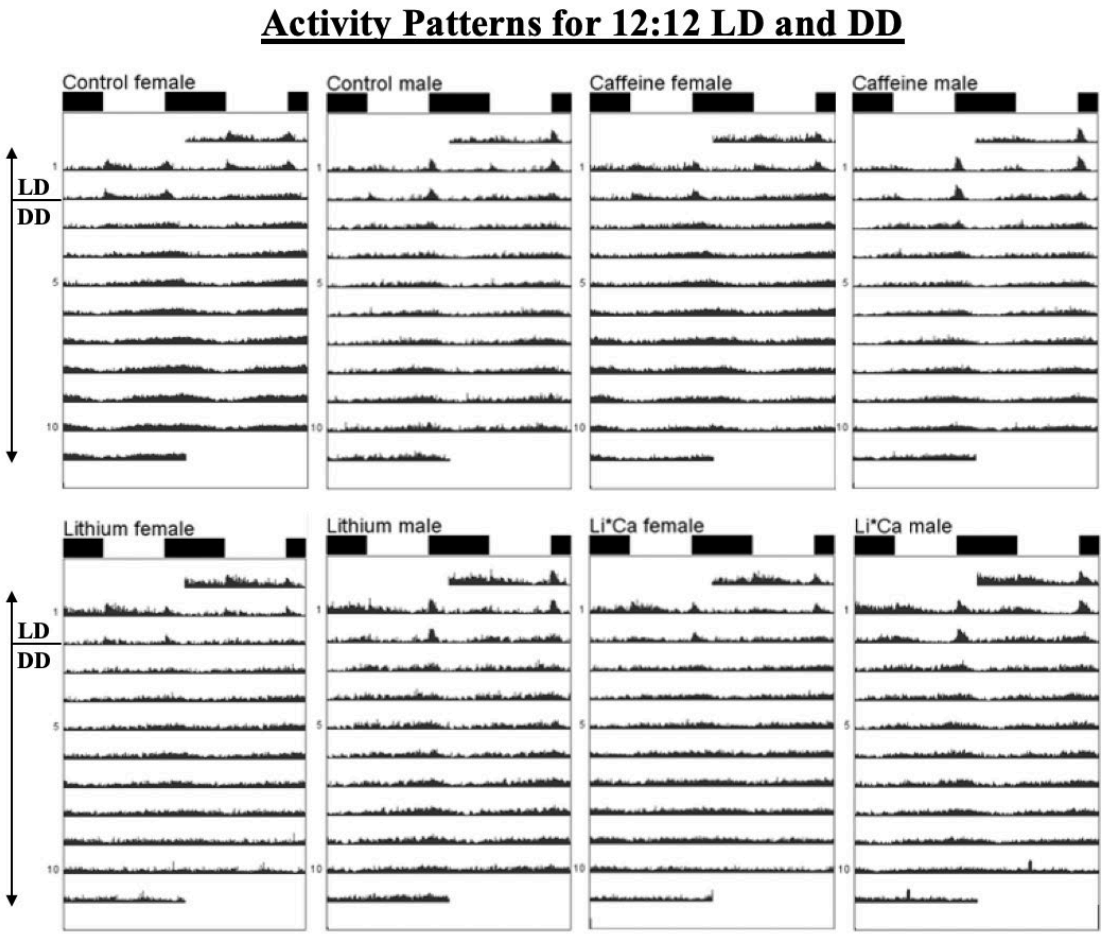

Figure 1. Compositive actograms depicting the circadian locomotor activity patterns for 12:12 LD and DD photoperiods. Dark and light bars represent night and day, respectively, in the 12:12 LD photoperiod.

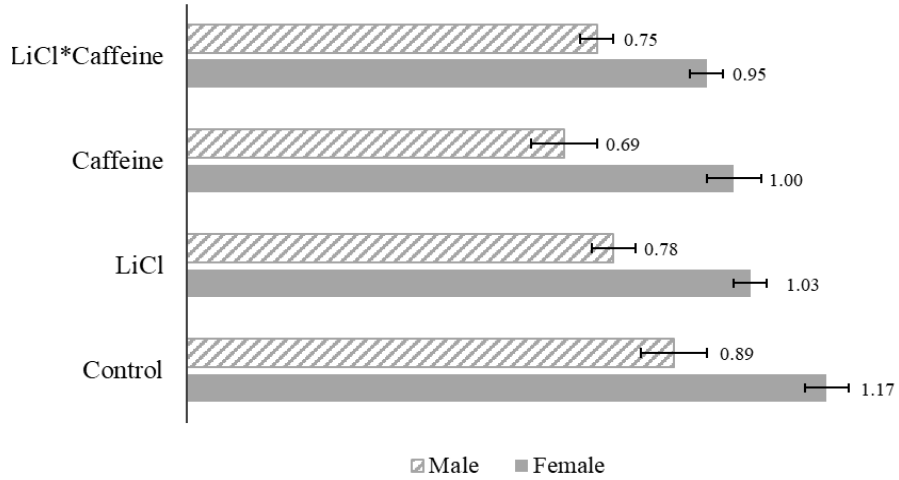

Figure 2. Light/Dark activity distribution (LDratio) of different treatments and sexes in two days of LD.

There were no interactions between sex differences and either drug treatment. Our results suggest that it may be useful to further investigate drug interactions with LiCl affecting circadian rhythms in *Drosophila*, particularly with respect to mechanisms mediating circadian regulation of activity that are peripheral to the circadian clock mechanism (Liang *et al*., 2019).

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Induced somatic mutation during chronic exposure of chlorfenson on *Drosophila melanogaster* OregonR (wild-type).

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Introduction

Rapidly growing world population is one of the major problems of today. Depending on the increasing population, new settlements are established. Therefore, agricultural lands are rapidly decreasing. Due to the decrease in the amount of agricultural products in many regions of the world hunger threatens people. In this case, it is aimed to get more productivity from farmers. For these reasons, insecticides are used extensively in stages of the production and storage of the product. Insecticides are toxic chemicals that are deliberately introduced into the environment for the sole purpose of killing insects. However, when insecticides are used against certain pests, they do not only kill the target species; they also affect many harmless creatures (Sayılı and Akman, 1994; Özyurt *et al*., 2018). Even sublethal doses of some pesticides could be mutagens (Abd-Alla *et al.,* 2003) and lead to diseases such as cancer has been reported (Atamanalp and Cengiz, 2002). Chlorfenson is an obsolete insecticide. The chlorfenson (CHF) was extensively used as insecticide and acaricide in agriculture (Lewis, 1993), for pest control in forestry, and vector control in hygiene against diseases like malaria and typhus. CHF, characterized by its long residual ovicidal activity, is used against mites on citrus, fruit, vegetables, and ornamentals (Gerhartz, 1985). CHF is highly acutely toxic, cholinesterase inhibitor and known/probable carcinogen. According to the European Union, this substance is very toxic to aquatic life with long lasting effects, is harmful if swallowed, and causes skin irritation. It has been banned in many countries of the world because of the increase in the environmental residues of organochlorine insecticides and their harmful effects on human and animal health. Therefore, the study with this insecticide is almost absent.

In this study, we used *in vivo* somatic mutation and recombination test (SMART) in *Drosophila melanogaster* to evaluate the mutagenic and recombinogenic effects of CHF as an organochlorine insecticide.

Materials and Methods

Chemicals

The chlorfenson (other name of CHF: 4-chlorophenyl 4-chlorobenzene-1-sulfonate 99.0% purity, CAS No.0-33-1), dimethyl sulfoxide (99.5% purity CAS No. 67-68-5), ethyl methane-sulfonate (100% purity; Cas no. 62-50-0), which were used as positive control, were obtained from the Sigma-Aldrich Company (St Louis, Missouri, USA), while *Drosophila* Instant Medium was obtained from the Carolina Biological Supply Company (2700 York Road, Burlington, USA). The molecular formula of CHF is $C_{12}H_8C_{l2}O_3S$ and its structural formulae is shown in Figure 1.

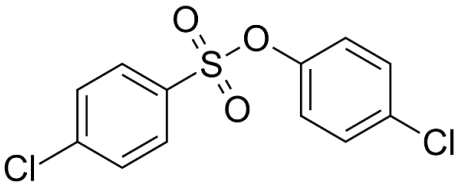

Figure 1. Structural formula of CHF.

Drosophila stock

To investigate the possible genotoxic effects of CHF, two *Drosophila* strains were used: the multiple wing hairs strain (*mwh/mwh*) and the *flr*^{3}strain (*flr*^{3}/*TM3*,*Bd*^{S}). The *mwh* and *flare-3* genes are located on the left arm of the third chromosome at map positions 0.3 and 38.8, respectively. In the *mwh* phenotype, the wing cells contain three or more hairs instead of one hair per cell as in wild type; the *flr*^{3} phenotype exhibits quite a variable expression, ranging from pointed, shortened and thickened hairs to amorphic, sometimes balloon-like extrusions of melanotic chitinous material. More detailed information on the genetic symbols and markers can be found in Lindsley and Zimm (1992).

Somatic mutation and recombination test (SMART)

SMART is based on the loss of heterozygosity, which may occur through various mechanisms, such as mitotic recombination, mutation, deletion, half-translocation, chromosome loss, and non-disjunction. Genetic changes occurring in somatic cells of the wing's imaginal discs cause the formation of mutant clones on the wing blade. The principles and basic procedures for the *Drosophila* wing spot test were described by Graf *et al.* (1984). In order to generate transheterozygous larvae, *flr*^{3} virgin females were crossed with *mwh* males.

P: ♀ *flr*^{3}/*TM3*,*Bd*^{S} × ♂ *mwh/mwh*

Eggs were collected optimally at 8 hour periods and the third instar transheterozygous larvae (72 ± 4 hours old) with two recessive markers, multiple wing hairs (*mwh*) and flare (*flr*^{3}), were treated with CHF. The larvae were fed this medium with different concentrations (2.5, 5, 7.5, and 10 ppm) of the CHF. Outside of experimental groups, control groups were prepared with distilled water and 1% DMSO as negative control and 1 ppm EMS as positive control. All the experiments were conducted at a temperature of 25 ± 1ºC (rearing temperature) and at a relative humidity of 60-70%. The hatching adult transheterozygous flies were collected in the treatment vials and stored in 70% ethanol in +4ºC at the refrigerator. The wings were removed and mounted in Faure's solution on microscope slides and inspected under 400× magnification for the presence of spots.

Statistical Analysis

The mutant clones were classified into three types: (1) small single spots (one or two *mwh* cells in size), (2) large single spots (three or more of *mwh* or *flr*^{3} cells), and (3) twin spots (consisting of adjacent *mwh* and *flr*^{3} cells). Each type was evaluated separately. The frequency of each type of mutant clone, the total spots, and the total *mwh* spots per fly for each treatment were compared with the frequency of the controls

(DMSO). The multiple-decision procedure of Frei and Würgler (1988) was used to determine whether the result was positive, negative, or inconclusive.

Results and Discussion

The findings obtained from distilled water, DMSO, EMS, and CHF application groups for the normal wings (*mwh/flr*^{3}) and serrate wings (*mwh/TM3*) phenotypes are given in Table 1. As shown in Table 1, there were no significant differences between the values, which were obtained with distilled water and 1 ppm DMSO applications for both normal and serrate wing phenotype. When the all CHF application groups (2.5, 5.0, 7.5 ppm) were compared with the DMSO application group, inconclusive (i) results were observed for all spots, despite the increased mutation frequency in two phenotypes (Figure 2).

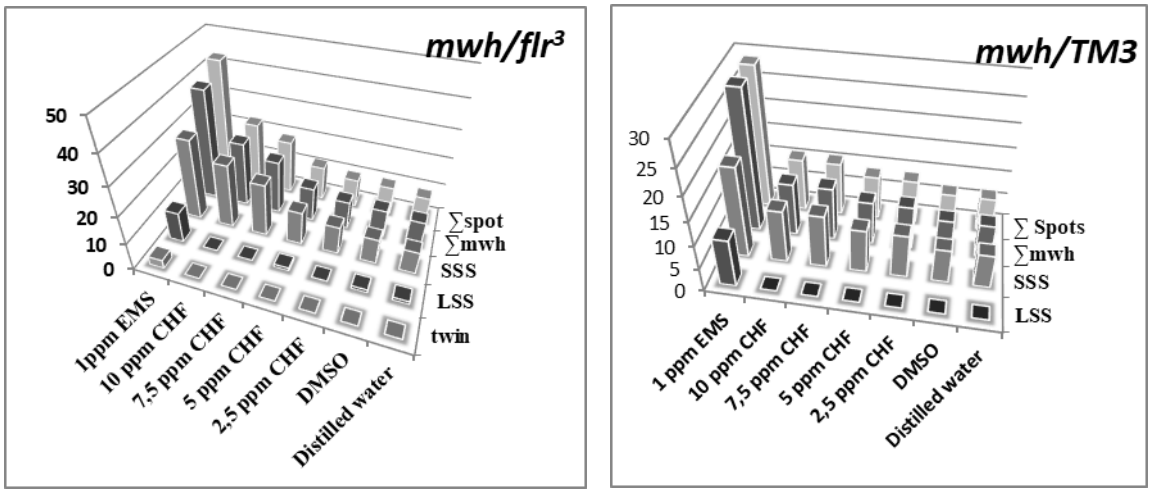

Figure 2. Comparison of the mutagenic effect of CHF.

Table 1. Statistical significance of wing spot test induction after exposure of CHF.

Compound Concentration (ppm)	N	Small single spots (1-2 cells) (m = 2)			Large single spots (> 2 cells) (m = 5)			Twin spots (m = 5)			Total *mwh* spots (m = 2)			Total spots (m = 2)			Clone induction frequency
		No	Fr.	D	No	Fr.	D	No	Fr.	D	No	Fr.	D	No	Fr.	D	CIF
Normal wings (*mwh/flr*^{3})																	
Distilled water	80	8	(0.10)		1	(0.01)		0	(0.00)		9	(0.11)		9	(0.11)		0.46
DMSO	80	9	(0.11)	i	1	(0.01)	i	0	(0.00)	i	10	(0.13)	i	10	(0.13)	i	0.51
EMS	80	29	(0.36)	+	11	(0.14)	+	3	(0.04)	i	39	(0.49)	+	43	(0.54)	+	2.00
2.5 CHF	80	10	(0.13)	i	0	(0.00)	-	0	(0.00)	-	10	(0.13)	i	10	(0.13)	i	0.51
5 CHF	80	11	(0.14)	i	1	(0.01)	i	0	(0.00)	-	12	(0.15)	i	12	(0.15)	i	0.61
7.5 CHF	80	19	(0.24)	i	0	(0.00)	-	0	(0.00)	-	19	(0.24)	i	19	(0.24)	i	0.97
10 CHF	80	23	(0.29)	+	0	(0.00)	-	0	(0.00)	-	23	(0.29)	+	23	(0.29)	+	1.17
Serrated wings (*mwh*/TM3)																	
Distilled water	80	7	(0.09)		0	(0.00)					7	(0.09)		7	(0.09)		0.35
DMSO	80	7	(0.09)	i	0	(0.00)	i				7	(0.09)	i	7	(0.09)	i	0.35
EMS	80	19	(0.24)	-	10	(0.13)	+				29	(0.36)	+	29	(0.36)	+	1.49
2.5 CHF	80	9	(0.11)	i	0	(0.00)	-	Balancer chromosome TM3 does not carry the *flr*^{3} mutation			9	(0.11)	i	9	(0.11)	i	0.46
5 CHF	80	9	(0.11)	i	0	(0.00)	-				9	(0.11)	i	9	(0.11)	i	0.46
7.5 CHF	80	11	(0.14)	i	0	(0.00)	-				11	(0.14)	i	11	(0.14)	i	0.56
10 CHF	80	11	(0.14)	i	0	(0.00)	-				11	(0.14)	i	11	(0.14)	i	0.56

N: number of wings; DMSO: dimethyl sulfoxide; EMS: ethyl methanesulfonate; CHF: Chlorfenson; No: number of clones; Fr: frequency; D: Statistical diagnosis according to Frei and Würgler (1988); +: positive; -: negative; i : inconclusive; m: multiplication factor; Probability levels = 0.05.

In addition, a positive result (+) was determined for the 10 ppm CHF application group in normal wing phenotypes ($P < 0.05$). While the CIF value for *mwh/flr*^{3} genotype in the application of 10 ppm CHF was 1.17, this value for the DMSO application group was calculated as 0.51. This study concluded that the mutation increases by CHF in *D. melanogaster* have been considered to be an indicator of the damage at genetic materials. Conversely, no significant differences were observed for all spots in the *mwh/TM3* genotype of CHF application group (Table 1).

Physical factors such as solar radiation, X-rays, and numerous chemicals such as insecticides can damage cellular DNA. Pesticides form an important group of man-made noxious chemicals. Their potential synergistic or antagonistic side effects in humans have not yet been extensively investigated (Demsia, 2007). Today, however, research on this subject is ongoing. All alive outside of the target organism are exposed to the insecticide by way of air, water, and food (Özyurt *et al*., 2018). Almost all insecticides are not specific only to kill the target organisms; also vertebrates and invertebrates are affected.

But, we did not find any studies with CHF in the literature. Therefore, our results are compared with similar insecticides. A kind of herbicides that alachlor is an organochlorine such as CHF, in *Drosophila melanogaster*, it induced somatic mutation and recombination in the wing cells (Torres, 1992). Similarly, it was observed that organochlorine endosulfan increased sperm abnormalities due to increased dose (Pandey, 1990). Furthermore, significant increase in the incidence of micronuclei in chicken bone marrow cells was induced by all three doses (100, 75, and 50 mg/kg) organochlorine insecticide lindane (Bhunya, 1992). Just like CHF, it caused genotoxicity in profenophos and dimethoate insecticides (Kasımoğlu and Uysal, 2014; Kızılet *et al*., 2019). Moreover, Ennaceur *et al.* (2008) says "genotoxic risk is associated with the exposure to organochlorine dichlorobiphenyl trichloroetane at concentrations 80 mM and above". In another study, CHF was given to rats at dose levels of 300, 1000, and 3000 ppm. In the 1000 and 3000 ppm dosage levels liver weight was increased and slight degeneration was observed. The 200 ppm dose level caused an increase in liver and thyroid weight and even at the 50 ppm dose level histological changes were observed in these organs (Verschuuren, 1973). According to Engelina *et al.* (1974), the mutation induction capacity of CHF is of the same order of magnitude as that of DDT.

In this study, we found that the 10 ppm concentration of CHF demonstrated a positive value. This suggests that somatic mutations are stimulated. The somatic mutation inductions depending on increasing dose of CHF in *D. melanogaster* have been considered to be an indicator of the damage in genetic materials. Our results are in line with other studies conducted in a similar way.

The insecticides such as CHF, etc., exert their toxic effects by two ways. They prevent membrane transport of sodium, potassium or chloride ions in neurons (such as organochlorine (CHF) or pyretroid insecticides) and inhibit specific enzymes or affect chemical neurotransmitters in neuronal endings (such as organic phosphates, carbamates, and neonicotinoids) (Vural, 2005). Thus, activation of proteolytic enzymes such as nitric oxide synthase, which causes oxidative stress, leads to increased free radicals (Kaymak *et al*., 2014). Free radicals formed as a result of oxidative stress lead to lipid peroxidation, leading to impaired permeability of the cell membrane. This chain of reactions results in unsaturated fatty acids in the organelles of the cell, as well as proteins, carbohydrates, and nucleic acids that are involved in the formation of various enzymes (Ikeda and Long, 1990; Schmidley, 1990; Hermes-Lima and Zenteno-Savín, 2002).

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Protective role of roseship fruit extracts against longevity toxicity of the insecticides 'Profenofos' on *Drosophila melanogaster.*

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Abstract

In this study, the possible toxic effects of profenofos (PRO), which are organophosphate insecticides, have been investigated on the longevity of female and male individuals belonging to Oregon R strain of *Drosophila melanogaster*. Also the healing properties of the water (RC_{wtr}) and ethanol (RC_{eth}) extracts of rosehips plants (*Rosa canina*) have been determined against toxic effects of PRO. For this purpose, four different application doses (0.025, 0.05, 0.075, and 0.1 ppm) of PRO and 1 ppm concentration of rosehip extracts were chosen by means of preliminary analysis. In the control group, the maximum lifespan was determined to be 78 days for ♀♀, 76 for ♂♂. The maximum life span for the lowest (0.025 ppm) and highest (0.1 ppm) application groups among the adult populations of *D. melanogaster* exposed to PRO were observed to be 52-24 days for ♀♀ and 67-45 days for ♂♂. These values indicate a negative correlation (R= - 0,718 for ♀♀ and R= - 444 for ♂♂) between the maximum lifespan of the application groups and changing PRO concentrations. It has been concluded that PRO has the toxic effect to decrease maximum and average lifespan in the both female and male individuals of *D. melanogaster* in our experiments. It was determined that the observed toxic effects as a result of PRO application decreased with RC_{wtr} and RC_{eth}. It was found that the differences between the 0.1 ppm PRO and application groups (0.1 ppm PRO + RC_{wtr} and RC_{eth}) were statistically important ($p < 0.05$). Keywords: Profenofos, *Rosa canina*, *Drosophila melanogaster*, Longevity, Toxicity

Introduction

Pesticides lead to various problems despite the benefits such as increasing agricultural production, the protection of food and public health. It was expressed that pesticides affect other non-target organisms and lead to poisoning in humans (Cakir and Sarikaya, 2005). The World Health Organization (WHO) has stated that the annual three million cases of pesticide poisoning in the world were seen and these poisonings resulted in the death of 220,000 (Darko and Akoto, 2008). Profenofos which is one of the broad-spectrum organophosphate insecticides has been used to control insects and mites in cotton, fruit trees, and vegetables (Habiba *et al.,* 1992; He *et al.,* 2010). It was reported that profenofos indicated neurotoxic effects (Rao *et al.,* 2003), inhibited the acetyl cholinesterase enzyme, and induced oxidative stress (Kavitha and Rao, 2009). Profenofos has high toxicity on zooplankton and insects, medium on fish, and low on mammals (Ismail *et al.,* 2009). It is located in moderately toxic substances according to the World Health Organization (WHO).

Various plants are used against different effects of chemicals at present. *Rosa canina* L. (Rosaceae) is one of the plants that are used for alternative treatment purposes. According to various researchers (Ozcan, 2000), the rosehip plant (*R. canina*) has antioxidant properties. Rosehip fruits are known to be rich in terms of

vitamin C and phenolic compounds (Montazeri *et al.,* 2011), which have antioxidant properties. It was reported that the rosehip reduces the DNA damage caused by oxidative stress (Kılıçgün and Dehen, 2009).

In this study, the effects of profenofos were investigated on longevity of the female and male individuals belong to Oregon strain of *D. melanogaster*. In addition, the curative properties of the water and ethanol extracts of rosehip plants were determined on the possible toxic effects of profenofos.

Materials and Methods

Chemicals

The profenofos (98.2% purity, CAS No. 41198-08-7), ethanol (99.8% purity, CAS No. 64-17-5), dimethyl sulfoxide (99.5% purity, CAS No. 67-68-5), and propionic acid (99.0% purity, CAS No. 79-09-4) were supplied by Sigma-Aldrich Company (St Louis, Missouri, USA), while *Drosophila* instant medium was obtained from the Carolina Biological Supply Company (2700 York Road, Burlington, USA).

Experimental animals

The flies used in the experiments were Oregon R wild type (w.t.) strain of *D. melanogaster* Meigen (1830) (Diptera; Drosophilidae). This stock has been maintained for many years in the laboratory at the Department of Biology, Ataturk University, Erzurum and was, therefore, highly inbred with little genetic variation (Uysal *et al.,* 2006).

Plant extraction

Berries of the rosehip plants were collected from natural areas at an altitude of 2000-2200 m in the highlands of Erzurum, Turkey, in September 2016, during the maturation period. The plant was identified by Meryem Şengul Koseoglu (Atatürk University, Turkey). Voucher specimens are deposited in the Herbarium of Atatürk University's Faculty of Science (Erzurum, Turkey). Rosehip berries were dried in indirect light and a clean environment. Then, both the berries and seeds in the fruit were milled with the help of a blender. Ethanol extract of rosehips was prepared according to Cakir *et al.* (2005). To prepare the ethanol extract, the milled rosehip samples were kept in ethanol (95%) for 4 days. Plant-ethanol mixture was filtered at the end of each day and ethanol was removed from the released solution. The remaining extract was used as the ethanol extract (RC_{eth}). The water extract of rosehips was prepared on the basis of the method applied by Halici *et al.* (2005). According to this, 100 g of the milled rosehips was put in 200 ml of distilled water. Rosehip-water mixture was afflicted in the water-bath which was adjusted 50°C for 2 h and subsequently filtered. After the released solution was passed the lyophilizer, the water extract of rosehip plant (RC_{wtr}) was obtained.

The application of insecticides and water and ethanol extracts of plants to adult individuals

In this study, the effects of insecticides and water and ethanol extracts of *R. canina* on longevity were studied separately in female and male populations of *D. melanogaster*. Experimental conditions were strictly controlled and experiments were repeated three times for each group. On average, 100 individuals for each group were collected from among the same aged female and male flies which were not mated and obtained from pupa. The gathered individuals were put into the empty culture vials and they were starved for 2 hours before the insecticides and insecticides+plant extract applications. For the insecticide applications, PRO (98.2% purity) was separately dissolved in 1% dimethyl sulfoxide (DMSO). Then, two layers of blotting papers were placed into each culture vial; PRO in four different concentrations (0.025, 0.05, 0.075, and 0.1 ppm) were absorbed into these papers. Less than 0.025 ppm and greater than 0.1 ppm doses were not used, because no exchange was observed for less than 0.025 ppm application groups when compared to the control in terms of longevity.

In applications higher than 0.1 ppm mass deaths occurred in mature individuals and they could not be perpetuated. Furthermore, different sets of experiments were prepared as control, DMSO control, and separately four different doses PRO (0.025, 0.05, 0.075, and 0.1 ppm), 0.1 ppm PRO + 1 ppm water extracts, and 0.1 ppm PRO + 1 ppm ethanol extract. For each group's ♀♀ and ♂♂ individuals, separated by four groups to simplify censuses and 25 individuals were placed into culture flasks. All the vials were kept in appropriate thermal cabins. During the experiments, food was replaced with fresh food twice a week. The

number of individuals was counted both at the beginning and at the end of each application day, and the dead individuals were recorded and then removed from the culture vials. The applications were carried out until the last individual died.

Statistical analyses

The obtained data were analysed with SPSS (version 13.0). One-way analysis of variance and Duncan's multiple range tests were used for the mean longevity values of the control and applications groups. The differences between groups were considered significant at $p < 0.05$.

Results

In our study, the effect of PRO insecticide was firstly investigated. Then, RC_{wtr} and RC_{eth} together with the highest concentration of PRO were applied. Maximum and average longevity values of the female and male population of *D. melanogaster* were determined for both DMSO negative control and the treatment groups.

Effects of PRO on the longevity

Maximum longevity values have been observed as 78 and 74 days for ♀♀, 76 and 74 days for ♂♂ in control, and DMSO control groups, respectively. The differences between the two control groups were not significant statistically ($P > 0.05$). Four different concentrations (0.025, 0.05, 0.075, and 0.1 ppm) were prepared for PRO treatment groups. The maximum longevity values decreased depending on the dose increases (Figures 1 and 2). Namely; the maximum longevity declined from 52 days (0.025 ppm) to 24 days (0.1 ppm) in the female populations which RRO was applied. These values for the male populations were defined as 67 days (0.025 ppm) and 45 days (0.1 ppm) (Table 1). Differences between the control and experimental groups were statistically significant ($p < 0.05$). These values indicate a negative correlation (R = - 0,718 for ♀♀ and R= - 444 for ♂♂) between the maximum longevity of the application groups and changing PRO concentrations.

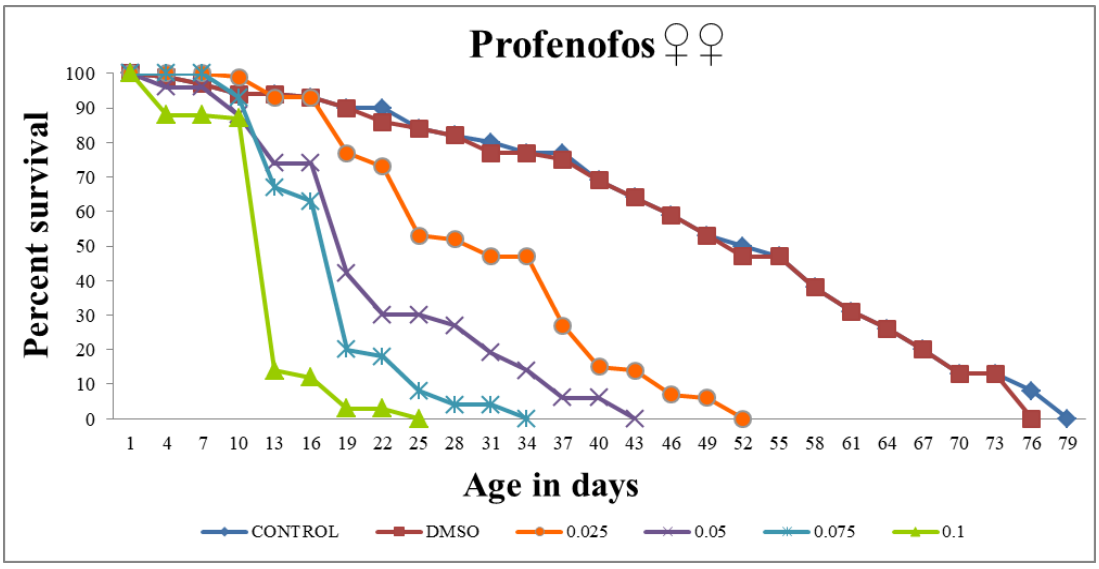

Figure 1. The survival lines of Oregon R wild type of *Drosophila melanogaster* female exposed to profenofos.

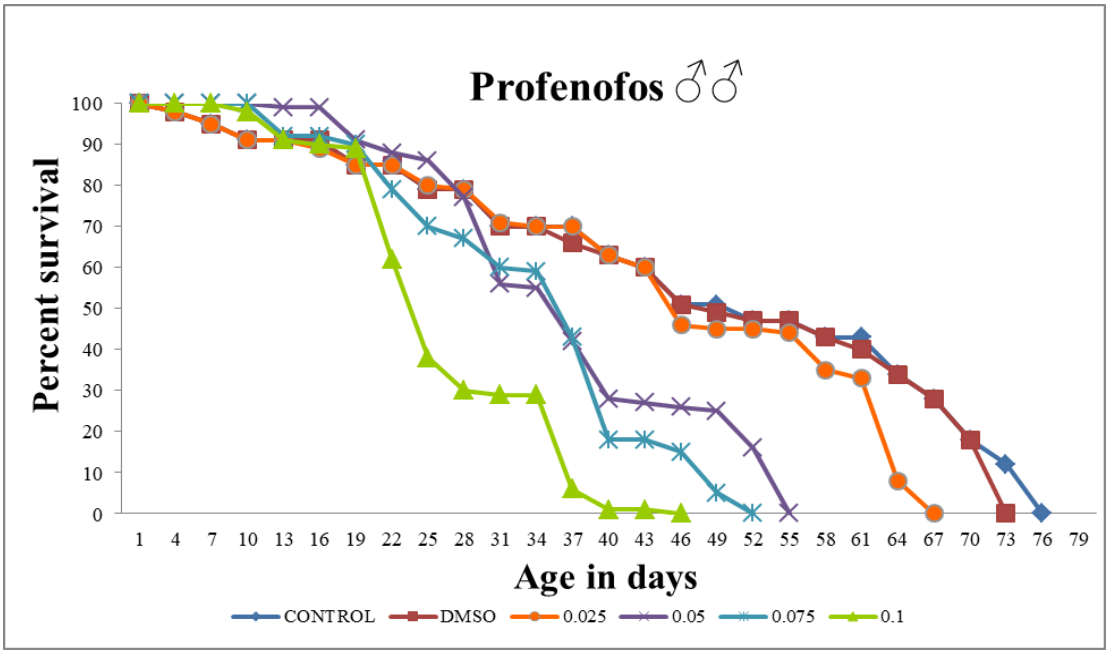

Figure 2. The survival lines of Oregon R wild type of *Drosophila melanogaster* male exposed to profenofos.

Table 1. The comparison and statistical analysis between maximum and average life spans of male and female populations of *D. melanogaster* in which profenofos and *R. canina* extract.

Profenofos (ppm)								
	Female Population				Male Population			
Experiment Groups (ppm)	N	Max. Lifespan	Mean Lifespan± SE	*p*	N	Max. Lifespan	Mean Lifespan± SE	*p*
Control (1)	100	78	49.15 ± 1.99	1-3*	100	76	48.48 ± 2.23	
DMSO (2)	100	74	48.83 ± 1.94	1-4* 1-5*	100	74	48.30 ± 2.21	1-4* 1-5*
0.025 (3)	100	52	29.03 ± 1.38	1-6*	100	67	45.12 ± 1.43	1-6*
0.05 (4)	100	41	21.23 ± 1.41	2-3* 2-4*	100	55	35.57 ± 1.46	2-4*
0.075 (5)	100	33	17.21 ± 1.29	2-5* 2-6*	100	52	33.18 ± 1.62	2-5* 2-6*
0.1 (6)	100	24	12.00 ± 1.09		100	45	25.50 ± 1.65	
0.1 ppm PRO + 1 ppm Rosehip Extracts								
PRO+RC_{wtr} (7)	100	40	18.31 ± 1.42	6-7*	100	53	34.89 ± 1.26	6-7*
PRO+RC_{eth} (8)	100	40	18.30 ± 1.28	6-8*	100	50	30.74 ± 1.19	6-8*

N: Total number of individuals, Max.: Maximum, SE: Standard Error, p: Probability levels between groups, *: The mean difference is significant at the 0.05 level.

The average longevity values in control and DMSO control groups was, respectively, found as 49.15 ± 1.99 and 48.83 ± 1.94 for the female population; 48.48 ± 2.23 and 48.30 ± 2.21 for the male population (Table 1). Differences between control and DMSO control groups for the female and male population were

statistically significant ($P > 0.05$). The average longevity dropped from 29.03 ± 1.38 days (0.025 ppm) to 12.00 ± 1.09 days (0.1 ppm) for the female population; from 45.12 ± 1.43 days (0.025 ppm) to 25.50 ± 1.65 days (0.1 ppm) for the male population in PRO treatment groups (Table 1). The differences between DMSO negative control and the application groups were statistically significant ($p < 0.05$).

Effects of the Rosehip extracts on the longevity

The most toxic effects of PRO insecticide were observed in 0.1 ppm application groups (Table 1). Another research group was formed to prevent the decrease of the longevity. For this purpose, the water and ethanol extracts of *Rosa canina* were added to the highest PRO application group (0.1 ppm). All plant extracts increased the maximum and average lifespan in both female and male populations according to the highest PRO application group (Table 1 and Figures 3-4).

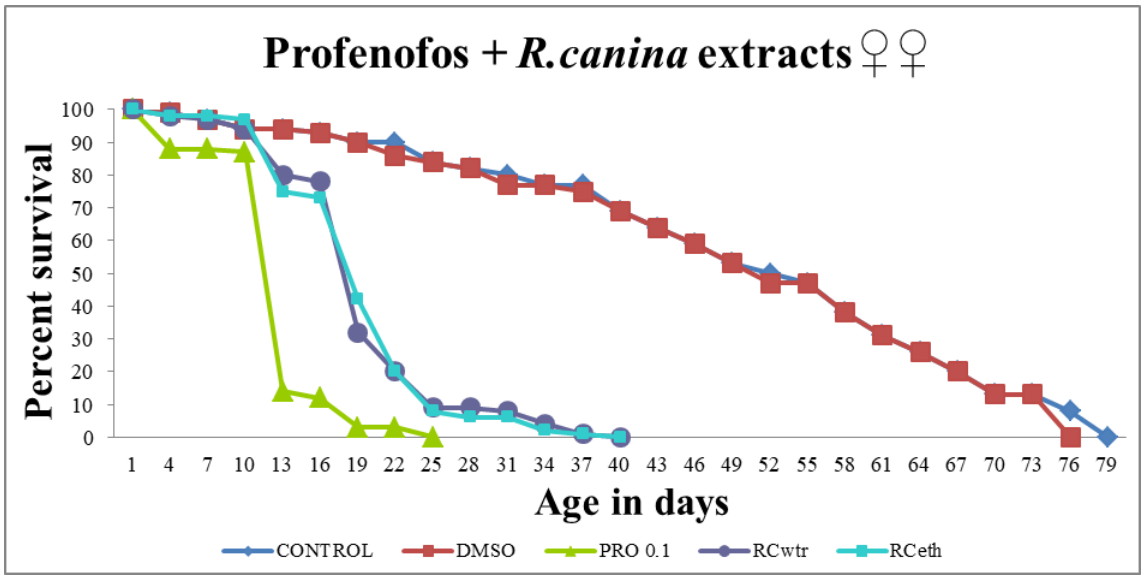

Figure 3. The survival lines of Oregon R wild type of *Drosophila melanogaster* female exposed to profenofos and plant extracts.

The maximum longevity values in RC_{wtr} and RC_{eth} application groups were found as 40 and 40 days for ♀♀; were 53 and 50 days for ♂♂ (Table 1). The observed increases in longevity in both sexes are statistically significant ($p < 0.05$). The average longevity values in 0.1 ppm PRO application groups are 12.00 ± 1.09 days for ♀♀; 25.50 ± 1.65 days for ♂♂. These values in the application groups of 0.1 ppm PRO + 1 ppm *R. canina* extracts (RC_{wtr} and RC_{eth}) were, respectively, determined as 18.31 ± 1.42 and 18.30 ± 1.28 for ♀♀; 34.89 ± 1.26 and 30.74 ± 1.19 days for ♂♂ (Table 1). As a result of application of plant extracts, the observed increases in average longevity were found to be significant ($p < 0.05$).

Discussion

As a result of our study, profenofos has been determined to show toxicity. Several studies in which profenofos has shown toxic effects are available. For example; it was found that profenofos caused apoptosis and necrosis in human lymphocyte cells (Das *et al.,* 2006). Profenofos had a highly lethal effect on tadpoles of frogs (Li *et al.,* 2010), caused histopathological changes in mice testes (Moustafa *et al.,* 2007), led to

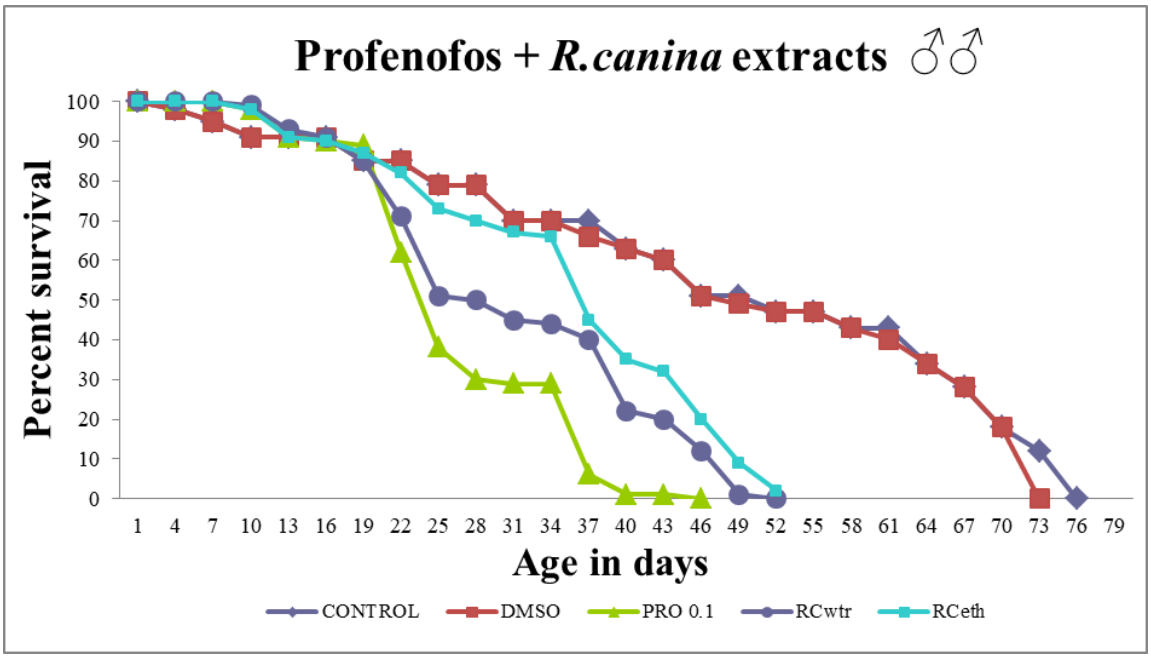

Figure 4. The survival lines of Oregon R wild type of *Drosophila melanogaster* male exposed to profenofos and plant extracts.

pathological changes in liver, kidney, spleen, and testicle tissues of white albino rat (Hammam and Abd el Mottaleb, 2007). It was observed that serum acetyl cholinesterase activity was inhibited and the amount of serum testosterone significantly reduced in rats which fed with profenofos for 65 days (El-Hoda and Zidan, 2009). Mansour *et al.* (2009) concluded that profenofos caused increases in the catalase activity and lipid peroxidation in the male rats, disrupted some biochemical parameters and led to changes in antioxidant enzyme. Profenofos affected the activity of antioxidant enzymes in black Mozambique fish (*Oreochromis mossambicus*) (Kavitha and Rao, 2009). It was observed that profenofos inhibited 40% the acetyl cholinesterase enzyme in Australian freshwater shrimp (*Paratya australiensis*) (Abdullah *et al.,* 1994) and caused balance, movement, and behavioral disorders in carp (*Cyprinus carpio*) (Ismail *et al.,* 2009).

The water and ethanol extracts of *R. canina* were used to remove toxic effects of profenofos. Increases in both the maximum and average longevity values of rosehip extracts were found to be statistically significant ($p < 0.05$). Therefore, it was observed to have curative properties of rosehip extract against the toxic effects of profenofos. These effects are thought to result from the antioxidant properties of *R. canina*. The curative effects of rosehip have been shown with various studies. According to a lot of researchers (Ozcan, 2000; Serteser *et al.,* 2008), the rosehip plant (*R. canina*) has antioxidant properties. Tumbas *et al.* (2012) has determined that there is a positive correlation between antioxidant capacity with vitamin C and phenolic compounds in the rosehip tea**.** In another study, rosehip extract has inhibited 83.7% of lipid peroxidation *in vitro* (Gao *et al.,* 2000). The rosehip extracts reduced the formation of free radicals in the polynuclear neutrophils cell with a study conducted by Daels-Rakotorison *et al.* (2002).

Kızılet *et al.* (2013), reported that ethanol extract of rosehip reduced genotoxic effects of EMS in *Drosophila melanogaster*. Ascorbic acid which is abundantly found in *R. canina* has decreased the genotoxic effects of mutagen compounds such as EMS (ethyl methanesulfonate) (Kaya, 2003). Protocatechuic acid which is found in *R. canina* (Khadem and Marles, 2010) has antigenotoxic effects (Anter *et al.,* 2011). Methyl gallate which is another compound in *R. canina* (Hvattum, 2002) has potently inhibited the formation of micronucleated reticulocytes in the mice which have been applied $KBrOP_3$ *in vivo* (Lee *et al.,* 2005). Because of these healing effects, the rosehip has been used in the treatment of various diseases such as cold, grippe (Tumbas e*t al.,* 2012), rheumatism (Willich *et al.,* 2010), and diabetes (Orhan *et al.,* 2009). There are made variety researches with different plants. Extracts of *Salvia lavandulifolia* Vahl. (Lamiaceae) gave successful results with antioxidant properties in the treatment of Alzheimer's disease (Perry *et al.,* 2003). Extracts of

Urtica dioica L. (Urticaceae) have been shown to protect the body from the harmful effects by acting against free radicals (Kan *et al.,* 2009). *Nigella sativa* L. (Ranunculaceae) plant has antioxidant (Worthen *et al*., 1998) and anti-tumor (Burits and Bucar, 2000) properties. In a study conducted by Uysal *et al.* (2015), the methanol extract of *Echium amoenum* (Fisch. and Mey) (Boraginaceae) decreased the genotoxic effects of EMS.

Consequently, profenofos insecticide has shown toxic effects on both female and male of *D. melanogaster*. The extracts of rosehip plant have reduced the toxic effects of profenofos. In a result of this study, it is thought that *Rosa canina* has healing effects and could be used in alternative medicine.

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Autumnal fauna of drosophilids at Font Groga site (Barcelona, Spain).

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On 16th October 2018 we trapped flies at the Font Groga site (Tibidabo mountain), on the city limits of Barcelona (Araúz *et al.,* 2009). The vegetation is typically Mediterranean, dominated by pines (*Pinus pinea*)

and ilexes (*Quercus ilex*). The flora of the undergrowth is composed, among other plants, by *Arbutus*, *Ruscus*, *Erica*, *Hedera*, *Rubus*, and *Smilax*. Ten fermented banana baits were left along a trail separated each other approximately by 10 meters. Flies were netted from 4 to 6:30 p.m., in intervals of 15-30 minutes. The climatic variables, recorded from the Observatori Fabra, which is located only 2 km from the trapping place, were: Maximum temperature: 22.4ºC, Minimum temperature: 14.8ºC. That afternoon there was a fine intermittent drizzle. Flies were classified next day in the laboratory and results are presented in Table 1.

Table 1. Classification of the flies according to species and sex (Font Groga site, Barcelona).

Species	Number	Percentage
D. subobscura (♂)	192	23.67
D. subobscura (♀)	229	28.24
D. simulans (♂)	130	16.03
D. melanogaster (♂)	4	0.49
D. melano/simulans (♀)	207	25.52
D. suzukii (♂)	4	0.49
D. suzukii (♀)	13	1.60
D. immigrans (♀)	2	0.25
D. phalerata (♂)	11	1.36
D. phalerata (♀)	16	1.97
D. hydei (♂)	1	0.12
D. hydei (♀)	1	0.12
D. kuntzei (♂)	1	0.12
Total	811	100

As expected, the most abundant species was *D. subobscura*, because it presents a peak of expansion in autumn (Krimbas, 1993; Argemí *et al.,* 1999). *D. simulans* was at a fairly high frequency, due to its autumnal expansion period in Mediterranean populations (Argemí *et al.,* 1999, 2003). The invasive species *D. suzukii* was also found, and it seems that it has established a rather stable way in this place, because it has been reported in different collections (Canals *et al.,* 2013; Pineda *et al.,* 2014; Esteve and Mestres, 2015; Rosselló *et al.,* 2016; Madrenas *et al.,* 2017; Lagares and Mestres, 2018). The remaining species are also regularly observed at Font Groga site. With this composition of drosophilids, the values of *H'* (Shannon diversity index) and *J* (Shannon uniformity index) were 0.929 and 0.477, respectively. They are similar to those estimated in 2013 (Pineda *et al.,* 2014) and 2014 (Esteve and Mestres, 2015).

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Egg density effect on individual reaction norm of genotype in *Drosophila pseudoobscura* population from Strawberry Canyon, California.[1]

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[1]Dedicated to the memory of Drs. Stephen Jay Gould (Harvard University) and Joseph Fail, Jr. (JCSU).

Numerous genetic studies on the effect of different external environmental conditions for the development of a phenotype from a genotype *(reaction norm)* in *Drosophila* are a well-known phenomenon. Analogous genetic studies are also accessible at the molecular level. Both types of studies, however, are pertinent to the gene expression and have led to understanding the regulation of development for a phenotypic trait. The present study is designed to *compare* the *same genotype* at two egg densities under three different external environments. This was done for parental strains and their hybrids (Gupta, 1978).

Experimental Procedure

For experimental purposes, 10 laboratory strains iso-chromosomal for second chromosome of *D. pseudoobscura* from Strawberry Canyon, California, 200 ft. above sea level, were used. Lines were derived by the standard dominant marker-inversion technique (Dobzhansky and Spassky, 1944). Heterozygotes between lines were created by mating pairs of iso-chromosomal strains at random (1 × 2, 3 × 4, 5 × 6, …9 × 10) so as to reconstitute the variety of genotypes present in nature. These strains were maintained in half pint milk bottles on Carpenters Medium at 24ºC before they were used for the experimental work. The fertile eggs collected varied from 6 to 14 h of age. For each parental homozygote and their heterozygous offspring, the following was done. Eggs were collected and cultured at two egg densities and at three temperatures (14º, 21º, and 26ºC). It is important to note that with present knowledge of the ecology of *D. pseudoobscura*, it is impossible to predict whether the densities chosen lie in the normal stress range for the species in nature (Gupta and Lewontin, 1982). Viability was measured as the percent of adults hatching.

Results and Discussion

Table 1 shows that the mean viability for genotype 1 at 14ºC, 21ºC, and 26ºC is lower at 140 egg density compared to 40 egg density. It is to be noted that at 21ºC is 20.00012% at 140 egg density compared to 41.78597% at 40 egg density. This provides the evidence that the norm of genotype 1 is different at 140 egg density than at 40 egg density. At 140 egg densities the genotype 2 shows higher mean viability at 14ºC, but lower at 21ºC and 26ºC compared to 40 egg density. This indicates that the norm of this genotype differs from one egg density to another. The genotype 3 shows lower mean viability at 14ºC and 21ºC using 140 egg density when compared with 40 egg density, while the same genotype shows higher viability at 140 egg density compared to 40 egg density. This indicates that the norm of the same genotype differs at two different egg densities depending upon the temperature (external environmental effect). A comparison of genotype 4 at two egg densities demonstrates lower viability at 140 egg density than at 40 egg density at each of the three temperatures, showing the effect of population density. Genotype 5 using 140 eggs shows lower mean viability at 14ºC and at 26ºC compared to 40 egg density. It is interesting to note that the same genotype has a different mean viability of 58.21464% at 140 egg density while at 40 egg density it is 7.50005% (a ratio of 7.76190 to 1.0000). This is clearly an evidence of population density effect of the reaction norm of the same genotype. Genotypes 6, 7, 8, 9, and 10 show lower mean viability at 14ºC, 21ºC, and 26ºC at 140 egg density

Table 1. Mean viability (%) of ten genotypes of *D. pseudoobscura* at two egg densities and at three temperatures.

	Mean viability (%) at 140 Egg Density			Mean viability (%) at 40 Egg Density		
Genotype	14^0C	21^0C	26^0C	14^0C	21^0C	26^0C
1	43.57169	20.00012	17.42032	57.50035	41.78597	24.28596
2	46.42885	31.42876	10.00742	44.28596	53.21461	11.42864
3	30.71447	14.08451	29.28571	36.42879	25.00015	25.71444
4	57.1432	30.35733	18.57154	58.57178	53.21461	38.28592
5	36.07165	58.21464	19.28583	56.07177	7.50005	41.07168
6	32.5002	15.71438	10.71435	43.92884	23.57157	18.57154
7	43.21455	19.28583	41.42882	51.07175	28.21446	55.00033
8	56.35748	45.00027	55.71462	74.64331	45.71456	61.42894
9	45.71456	3.57145	19.28583	54.64319	68.57184	27.14302
10	50.35745	34.28592	35.00021	57.50035	41.42882	54.28604

when compared to 40 egg density. It is to be noted that the genotype 9 here has a very low mean viability (3.57145%) at 140 egg density when compared with the mean viability at 40 egg density (68.57184%) at 21ºC, with a ratio of 1.00000: 19.20000 providing the effect of population density on the individual reaction norm of a genotype.

Comparison of the reaction norm of heterozygote (F_1) at 40 eggs and 140 egg densities:

Table 2. Mean viability (%) of heterozygotes (F_1) at 140 egg density and 40 egg density of *D. pseudoobscura*, Strawberry Canyon, California (From Gupta, 1978). 1´, 2´, 3´, 4´, and 5´ are reciprocal crosses to represent heterozygotes. For example: Genotype 1 female X genotype 2 male; and is represented as genotype 1´.

	Mean viability (%) at 140 Egg Density			Mean viability (%) at 40 Egg Density		
Genotype	14^0C	21^0C	26^0C	14^0C	21^0C	26^0C
1	59.60499	45.78926	30.78933	26.44725	39.86824	32.36827
1´	21.31569	41.44718	29.21039	50.52608	38.68403	35.52615
2	63.15769	14.21046	14.21046	62.36813	28.42092	24.47357
2´	48.15767	42.63138	14.21046	61.18393	50.52608	22.89463
3	35.13142	30.78933	19.73675	52.10502	36.31562	34.73668
3´	43.81559	24.47357	20.13149	56.05237	26.05251	31.18407
4	54.0787	37.89456	36.31562	41.84191	48.94714	48.15767
4´	19.73675	21.21039	30.3946	75.78912	29.21039	36.31562
5	39.07877	44.21032	18.15781	69.8681	48.15767	39.4735
5´	56.05237	42.63138	20.52622	63.94707	51.31555	33.15774

Table 2 lists the mean viability (%) for heterozygotes (F_1) at 14ºC, 21ºC, and 26ºC for 140 egg density and 40 egg density. Heterozygote 1 shows higher viability at 14ºC and 21ºC at 140 egg density compared to 40 egg density, while the same heterozygote shows a lower viability at 140 egg density at 26ºC than at 40 egg density, while its reciprocal heterozygote shows at 14ºC and 21ºC for 140 egg density but higher at 21ºC compared to 40 egg density. Heterozygote 2 has higher viability for 14ºC at 140 egg density than at 40 egg density. The same heterozygote has lower viability at 140 egg density than at 40 egg density for 21ºC and 26ºC. However, its reciprocal cross has lower viability at 21ºC and 26ºC using 140 egg density compared to 40 egg density. Heterozygotes 3 and 3´ each have lower viability at all the three temperatures using 140 egg density than at 40 egg density. Heterozygote 4 has higher viability at 14ºC using 140 egg density compared to 40 egg density. However the same heterozygote has lower viability at 21ºC and 26ºC at 140 egg density compared to using 40 egg density. It clearly indicates the norm for this heterozygote depends upon the population density. The heterozygote 5 and the reciprocal heterozygote 5´ each has lower viability at each of the three temperatures (14ºC, 21ºC, and 26ºC). However, a difference in the mean viability at each of these three temperatures is observed between the heterozygote 5 and its reciprocal heterozygote 5´ providing the evidence of the effect of population density (egg density) on viability, leading to the evidence that the reaction norm of heterozygote changes depending upon the egg density.

Conclusion

It is concluded that there is an egg density effect on viability.

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The use of the trophic resource by exotic and native species Drosophilidae: fruit colonization on the plant.

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Abstract

In this study is reported a collection, identification, and relative frequency of exotic and native species in different fruits on the plant, and investigation of them as potential breeding sites. Key words: Coexistence, competition, decaying stage.

Introduction

The availability of resources necessary paralleled by seasonal variations are extremely important for the growth and reproduction of individuals, thus limiting population growth (Giller, 1984; Mata *et al*., 2015). Thus, knowing how the availability of resources occurs and, therefore, how they affect populations, is critical to understanding how interactions between species work (Valadão *et al*., 2010; Mata *et al*., 2015; Marco Silva Gottschalk, unpublished data).

Species can share trophic resources and should be susceptible to competitive exception, depending on their success in exploiting it (Mata *et al*., 2015; Valadão *et al*., 2010). Drosophilidae feed on microorganisms present in biological materials in decomposition, such as fallen fruit, flowers, fungi, and others, and their abundance shows a marked seasonal variation (Dobzhansky and Pavan, 1950; Mata *et al*., 2015; Valadão *et al*., 2010). Most species of Drosophilidae oviposit in decayed fruit, and a way to avoid competition is to use the resource at different stages of decomposition (Kondo and Kimura, 2008; Mata *et al*., 2015; Marco Silva Gottschalk, unpublished data).

There are many studies with family emergence on fruits, however, except for *Zaprionus indianus* Gupta 1970 in *Ficus carica* L., in natural environments there are no records of these organisms using fruits still in the plant, because they do not have the capacity to pierce the fruits, and therefore they are not attracted to them (Leão and Tidon, 2004; Pasini and Link, 2012; Roque *et al*., 2009; Tidon *et al*., 2003; Vilela and Mori, 2014).

Brazil has a large fauna of drosophilids and a wide variety of ecological niches; however, knowledge is still incipient on the places used by these flies for egg laying (Mata *et al*., 2015; Tidon, 2006). This study describes the guild of drosophilids associated to different fruits on the plants. Thus, another collection of drosophilids was obtained in fruits, with the objective of investigating if their maturations still in the plant can be used as trophic resource for larval Drosophilidae.

Materials and Methods

The fruits were collected in Horto Botânico Irmão Teodoro Luís a Restinga forest located in the municipality of Capão do Leão (31°47'48"S, 52°15'45"W) in the state of Rio Grande do Sul, RS, Brazil. We sampled fruits on the plant in *Psidium guajava* L. (N = 27), *P. cattleyanum* S. (N = 30), *Butia capitata* (Mart.) Becc. (N = 109), *B. leiospatha* (Mart. ex Drude) Becc. (N = 63), *Eugenia uniflora* L. (N = 16), *Strychnos* sp. (N = 155), and *Ficus insipida* Willd. (N = 75).

The samples occurred in March, April, and May of 2012, January, February, March and June of 2013, and March and April of 2015. Posteriorly, the fruits were accommodated individually in plastic containers

with a display containing sand sterilized and maintained for two weeks (2012 and 2013) and four weeks (2015) in a heated chamber (25 ± 3ºC temperature, 70 ± 10% relative humidity, and 12 h photophase).

The emerged specimens were preserved in 70% ethanol and identified based on their external morphology according to the current literature. Male terminalia of sibling species were dissected according Bächli *et al*. (2004) for species-level identification. Females of sibling species were identified by external morphology and, when possible, the species level was determined according to the quantities of males in each trap for analysis purposes.

Results and Discussion

A total of 1,312 individuals emerged from the 498 fruits, belonging to seven species and one subgroup. On the fruits collected on the plant, 32.15% were colonized. In Table 1, the abundance of the *taxa* sampled in fruits with emergence register is presented. The most abundant species are the exotics *Drosophila suzukii* Matsumura 1931, *D. simulans* Sturtevant 1919, and *Zaprionus indianus* Gupta 1970, but only the last two are present in all species of fruits from where the emergence was registered (Figure 1).

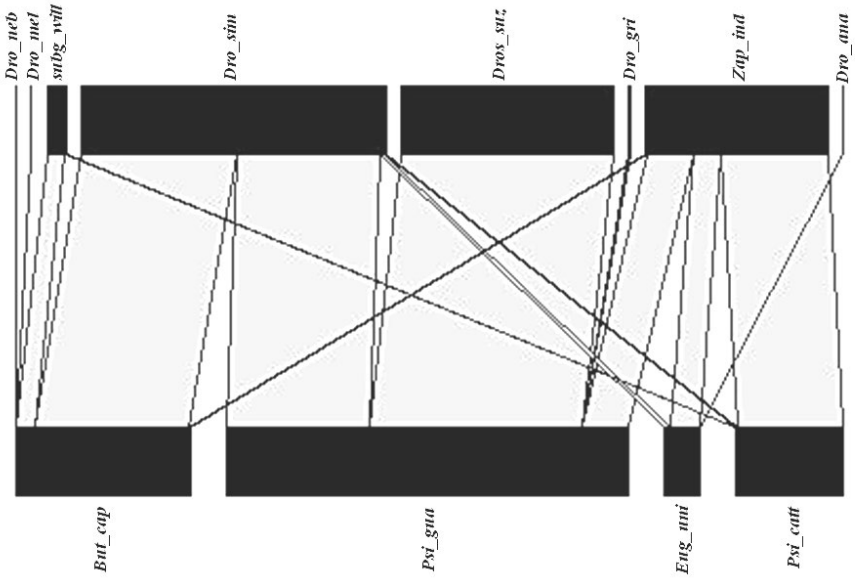

Figure 1. Illustration of the network of mutualistic interactions Drosophilidae/fruits plants, sampled in the Horto Botânico Irmão Teodoro Luís, Rio Grande do Sul, RS, Brazil. Lines/bars in grays represent the interactions and their width represents the frequency of the interaction.

The fruits of *Butia capitata* also were resources for the native species of the *Drosophila nebulosa* Sturtevant 1916 and subgroup *D. willistoni*, but these are present in low abundance. Given the small richness presented, the colonization of the fruits in the foot can be a way to avoid interspecific competition.

Of the 498 fruits, only 27 were colonized by more than one species, but the rate of fruit colonization reflected by the relative frequency is high only for fruits such as *Eugenia uniflora*, *Psidium guajava*, and *P. cattleyanum*. The exotic species present high capacity of use of resources in different stages than the native subgroup *D. willistoni*.

Drosophila suzukii reached 64% of the guild associated to guava; however, it was dominant in these fruits from sampling starting 2015. Its high incidence of fruits on the plant was probably because it is the only Drosophilidae in Brazil that can pierce the fruit (Bueno *et al*., 2018; Deprá *et al*., 2014). This result reinforces the competence of *D. suzukii* to pierce intact fruit during egg-laying, to occupy different environments and help track the occupation and dispersal of this fly.

Drosophila and *Zaprionus* genera are often also collected associated to fruits in other studies, but are not mentioned in fruits still in the plant (Carson, 1971; Silva *et al*., 2005; Garcia *et al*., 2008; Mata *et al*., 2015; Marco Silva Gottschalk, unpublished data), demonstrating its versatility in resources. The species that use fruits on the plant must have competitive advantage, because the colonization happens earlier than in other species (Sevenster, 1996; Silva *et al*., 2005; Mata *et al*., 2015). Further, the colonization of the fruits for these

species should change the resource physically and chemically (Sevenster, 1996; Sevenster and Alphen, 1996).

The present study suggests the use of the fruits in the foot by Drosophilidae, although detailed analyses are necessary for the association of the colonization in the fruits on the plants with maturation of the fruits or with the presence of organisms that promote the opening of the fruit, such as those of the *D. suzukii*, family Tephritidae or others organisms, for example.

Table 1. Register occurrence of plants host, absolute abundance of Drosophilidae species and relative frequency (%) of emergence on the fruits sampled.

Family	Species fruits	Distribution	Species	Number	Percentage
Myrtaceae	*Eugenia uniflora* N = 16	BA, MS, ES, MG, RJ, SP, PR, RS, SC	*Drosophila simulans*	10	18.7
			Zaprionus indianus	53	75.0
	Psidium guajava N = 50	AC, AM, AL, BA, CE, MA, PE, PI, SE, MS, MT, ES, MG, RJ, SP, PR, RS, SC	*Drosophila suzukii*	393	64.0
			D. simulans	265	35.1
			D. griseolineata	1	2.0
			D. ananassae	2	4.0
			Z. indianus	86	7.4
	P. cattleyanum N = 30	BA, MS, MG, RJ, SP, PR, RS, SC	*Drosophila simulans*	2	6.6
			subgrupo *D. willistoni*	1	3.3
			Z. indianus	196	56.7
Arecaceae	*Butia capitata* N = 109	SP, PR, RS, SC	*Drosophila simulans*	287	18.3
			D. nebulosa	1	0.9
			D. melanogaster	1	0.9
			sgr *D. willistoni*	34	7.3
			Z. indianus	1	0.9
			Total	1.312	

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Circadian activity rhythms in mutant strains for alcohol dehydrogenase Fast, Slow, and Null alleles.

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Here we present profiles of circadian locomotor activity for mutant strains of *Drosophila melanogaster* carrying Fast, Slow or null alleles at the alcohol dehydrogenase (*Adh*) locus. Seggio *et al*. (2012) and Ahmad *et al*. (2013) have shown that exposure to ethanol (etoh) alters circadian locomotor activity in *D. melanogaster*. Etoh treatment altered both the circadian free-running period under constant laboratory conditions and transcription from circadian clock genes, demonstrating an effect on the circadian pacemaker driving the rhythms in activity. Liao *et al*. (2016) also demonstrated that mutations in circadian clock genes alter sedation response and Adh activity after exposure to alcohol. Additionally, Pohl *et al*. (2013) showed that having a functional circadian clock is necessary for exhibiting ethanol tolerance as both arrhythmic mutant strains and flies kept in constant light have no ethanol tolerance. We examined mutant strains for the *Adh* locus to determine whether disrupting alcohol metabolism genetically would also alter circadian activity rhythms, even in the absence of exposure to etoh.

Mutant stocks were obtained from the Bloomington Stock Center (Bloomington IN, USA) and were assayed over two experiments. Genotypes and stock numbers in Experiment One were null mutants Adh[fn23]pr[1]cn[1] (#1152) and Adh[n1] (#3976), Slow allele w[1118];Adh[S] (#4062), Fast allele Adh[F] (#6040) and controls were w[1118] (#5905) and Oregon-R (originally obtained from Carolina Biological Supply, Burlington, North Carolina, USA and maintained at Skidmore College for the past 36 years). Experiment Two strains were Oregon-R controls (Skidmore College), null allele Adh[n5]pr[1] (#3977), Fast allele Adh[F'] (#3975) and Slow allele b[1]Adh[S]pr[1]cn[1] (#6042). Flies were raised in plastic vials 65 mm long and 23.5 mm diameter on Carolina Instant *Drosophila* Medium (Carolina Biological Supply) at 25°C under a 12:12 LD and tested under the same temperature and photoperiod, using methods described by Seggio *et al*. (2011). Individual adult males, aged 1-5 days old, were placed in plastic tubes 65 mm long and 5 mm in diameter, with agar (2% agar and 5% sucrose) food sealed with a plastic cap on one end and a cotton plug at the other end. The tubes were inserted into *Drosophila* Activity Monitors (DAMS: Trikinetics, Waltham MA, USA) to record locomotor activity by counting breaks in a photobeam, bisecting the tube, for consecutive 10-minute intervals. Activity was recorded for two days in 12:12 LD followed, without interruption, by nine days in constant dark. Only flies surviving for eight days in Experiment One, and nine days in Experiment Two were analyzed in order to optimize sample sizes with as many cycles of DD data as possible for estimates of tauDD, while still standardizing the data for age and environmental conditions. Fewer cycles in DD reduce the precision of tauDD estimates, but more cycles reduce the sample size because survival rates decrease. Average activity counts per 10-minutes were analyzed for two days in LD (XLD), the 12-hours of light in LD (XL), the 12-hours of dark in LD (XD), constant dark (XDD), and the ratio of daytime activity to total activity as a measure of the distribution of activity between day and night (LDRATIO). The average number of days each fly survived during the activity monitoring was recorded (DAYS), and only flies surviving for six days in Experiment One, and seven days in Experiment Two were used for data analysis. The free-running circadian period in constant dark (tauDD) was estimated using both the Sokolove-Bushnell and Lombard-Scargle chi-square periodograms in Actogram-J (Schmid *et al*., 2011). Only flies with significant circadian periods (alpha level 0.05) for both measures were included in the tauDD estimates, and the others were labelled as arrhythmic, resulting in a "percent rhythmicity" score (PR) for each strain. Contingency chi-square was used to compare PR among strains. The w1118, OR-R, Adh[fn23]pr[1]cn[1][fn23], Adh[n1], w1118; Adh[S] and Adh[F] strains were assayed in Experiment One, and the OR-R, Adh[n5]pr[1], Adh[F'] and [b1]Adh[S]pr[1]cn[1] strains were assayed in Experiment Two. Initial sample sizes were 30 per strain in Experiment One, and 32 per strain in Experiment Two. Analysis of variance (SAS, Carey, North Caroline USA) was used to assess strain differences.

Table 1. Sample size, mean ± SEM, and p-values for ANOVA main effect of strain for measures of circadian locomotor activity.

Experiment 1									
	n	XLD	XL	XD	XDD	LDRATIO	tauDD	Days	PR
Strain Effect		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.009	0.001
OR-R	27	5.6 ± .4	3.3 ± .4	7.8 ± .8	7.6 ± .5	0.59 ± .1	24.43 ± .2	9.4 ± .4	0.56
W1118	59	8.3 ± .6	7.2 ± .5	9.4 ± .8	9.2 ± .7	0.89 ± .1	24.04 ± .1	10.3 ± .5	0.93
Nullfn23	24	2.6 ± .3	2.4 ± .2	2.7 ± .4	3.9 ± .5	0.94 ± .1	24.38 ± .3	8.5 ± .7	0.67
Nulln1	28	9.4 ± .2	6.3 ± .9	11.1 ± 2	11.3 ± .8	0.83 ± .1	23.82 ± .2	9.3 ± .3	0.93
Fast	20	12 ± 1	14 ± 2	11 ± 2	9.7 ± .1	1.11 ± .1	24.65 ± .1	8.1 ± .7	0.75
Slow	30	7.7 ± .4	8.1 ± .5	7.4 ± .6	6.1 ± .4	1.06 ± .1	23.87 ± .1	10.3 ± .2	0.90
Experiment 2									
	n	XLD	XL	XD	XDD	LDRATIO	tauDD	Days	PR
Strain Effect		0.0007	0.0001	0.0001	0.0001	0.0001	0.0001	0.01	0.09
OR-R	23	6.0 ± .5	5.7± .6	6.3 ± .5	8.8 ± .6	0.93 ± .1	23.96 ± .1	6.3 ± .7	0.74
Null	31	6.0 ± .4	7.6 ± .5	4.6 ± .5	6.9 ± .6	1.30 ± .1	23.96 ± .1	8.6 ± .3	0.74
Fast	29	4.8 ± .5	5.0 ± .6	4.7 ± .6	6.8 ± .6	1.08 ± .1	23.69 ± .1	8.3 ± .4	0.97
Slow	28	7.4 ± .7	2.3 ± .3	12.4 ± 1.2	11.9 ± 1	0.34 ± .1	24.53 ± .1	7.7 ± .5	0.81

There were significant differences among strains for all variables assayed, except for PR in Experiment Two, but no discernable pattern of differences, for any variable, was associated with wild-type *vs* mutant behavior, or Fast *vs* Slow *vs* null mutations (Table 1). Our results suggest that etoh affects circadian rhythms and clock function by acting directly on circadian clock mechanisms rather than by indirect action mediated through metabolic pathways involving *Adh*. The absence of any pattern of differences in circadian measures associated with null, Fast or Slow mutant strains suggests that the variation observed among these *Adh* mutant stocks is likely a function of different genetic backgrounds among independently derived strains, although isolation of *Adh* mutants against common genetic backgrounds is necessary to definitively separate effects of mutations from genetic background, and to examine epistatic interactions between Adh mutations and genetic background differences.

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Experimental revelation on the Total Phenotypic Variance Equation based on the Reaction Norm Curve using *Drosophila pseudoobscura*.

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Scientific research studies (experimental, mathematical, and statistical models) have proven that *Drosophila* is a very influential tool in studying the genetics and the environmental experiments leading to evolution.

Environment is the surroundings or conditions in which a person, animal, or plant lives or operates. A distribution of environments is reflected biologically as a distribution of phenotypes. The transformation of environmental distribution into phenotypic distribution is determined by the Reaction Norm (norm of reaction).

A reaction norm (norm of reaction) in genetics and ecology refers to the pattern of phenotypic expression of a single genotype across a range of environments. One evolutionary usage of reaction norms is in describing how different species (especially related species) respond to the varying environments, whereas the Range of reaction (reaction range) is a concept in psychology, genetics, and related fields that the expressed trait (phenotype) of an organism depends both on genetic characteristics (genotype) and the environment.

Norm of Reaction Curve is a curve that relates, for a given genotype, the contribution of environmental variation to observed phenotypic variation. Such a *curve* can be thought of as a genetic mirror that reflects the environmental into phenotypic space.

An experiment was conducted to study the reaction norms on viability at three temperatures using *D. pseudoobscura* from Santa Cruz, California (Gupta, 1978). Figure 1 shows the norm of reaction on viability for eight genotypes at three different temperatures. Each genotype interacts differently at three different environmental conditions (three different temperatures) and thereby produces the different phenotypes. However, it is interesting to note that the genotype 5 produced 'V' shaped, while genotype 3 produced an inverted '^' shaped curve. These two genotypes cross each other between 14 and 21 degrees centigrade; 21

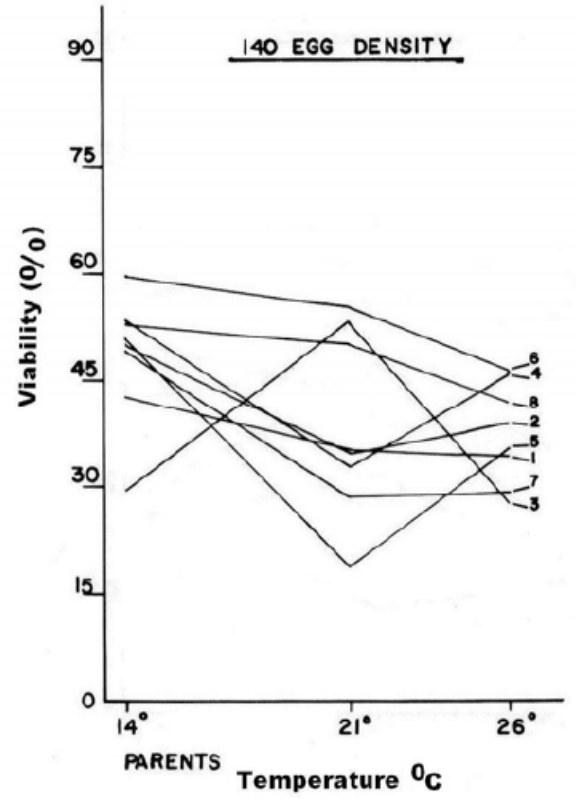

Figure 1. Viability of eight genotypes of *D. pseudoobscura* (numbered 1 to 8), Santa Cruz, California. Each raised at three temperatures (14ºC, 21ºC, and 26ºC). Each genotype interacts differently with the temperature and provides evidence for the gene × environment interaction effect (Gupta, 1978 and 2009).

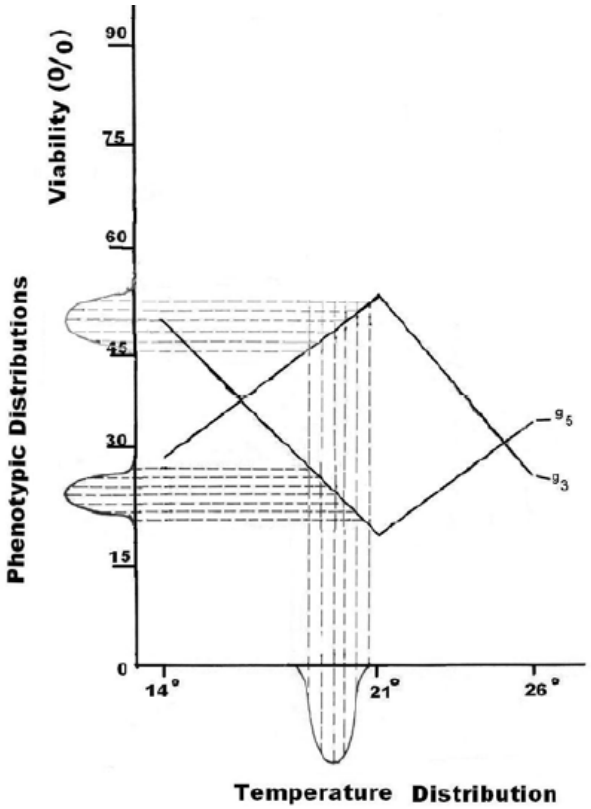

Figure 1a. Phenotypic Distributions (Two different phenotypic curves): The viability data, illustrating the *two* phenotypic distributions, for two genotypes, g3 and g5, taken from Figure 1 at the temperature range of 18.5ºC and 20.6ºC provides the evidence of mostly genetic effect.

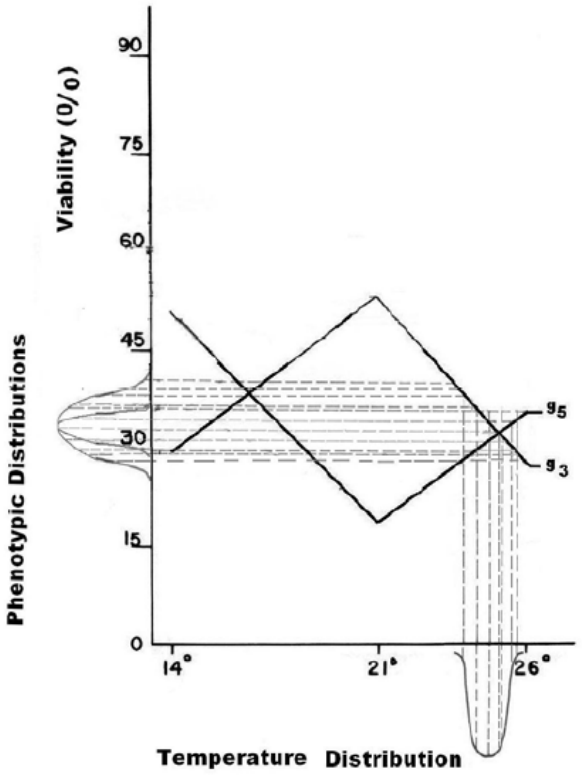

Figure 1b. Phenotypic Distribution curve: The viability data, demonstrating the only *one* phenotypic distribution, for two genotypes, g3 and g5, taken from Figure 1 when the temperature range varies from 23.6ºC and 25.6ºC provides the evidence of mostly non-genetic or environmental stimuli expression effect.

and 26 degrees centigrade. It provides the experimental evidence of no effect of the environment on the development of a phenotype from a given genotype. Based on the reaction norm of these two different genotypes selected, Figure 1a shows two phenotypic distributions (curves) demonstrating mainly the genetic effect, whereas Figure 1b shows mainly one phenotypic curve demonstrating mostly the non-genetic (environmental) effect.

Thus, the graphical evidence (Figure 1a and Figure 1b) from the experimental data on the norms of reaction curve(s) leads to the value-added formula of Total Phenotypic Variance Equation.

Total Phenotypic Variance Equation

In quantitative genetics,

$$\sigma^2_{TP} = \sigma^2_G + \sigma^2_E + (\sigma^2_G \times \sigma^2_E) \quad \text{……..(1)}$$

σ^2_{TP} = Total phenotypic variance; σ^2_G = Genetic variance
σ^2_E = Environmental variance
$(\sigma^2_G \times \sigma^2_E) = \sigma^2_{GE}$ = Genetic × Environmental Interaction Variance

The experimental data presently on the *norm of reaction* evidenced that the genetic variance is impacted by the environmental variance. Similarly, the environmental variance is impacted by the genetic variance. Based on the experimental evidence presented here, the equation (1) for Total Phenotypic Variance amends to:

$$\sigma^2_{TP} = (\sigma^2_{gi} + \sigma^2_{ei}) + (\sigma^2_{ei} + \sigma^2_{gi}) + (\sigma^2_g \times \sigma^2_e) \text{……(2)}$$

Where:

σ^2_{TP} = Total phenotypic variance; σ^2_{gi} = Genetic variance impact;
σ^2_{ei} = Environmental variance impact;
σ^2_g = genetic variance; and σ^2_e = environmental variance
$(\sigma^2_g \times \sigma^2_e) = \sigma^2_{ge}$ = Genetic × Environmental Interaction Variance

The equation #2, consequently, reveals that it takes into account that the genetic variance is impacted by the environmental variance. Similarly, the environmental variance is impacted by the genetic variance (however, in equation # 1 the genetic variance is not impacted by the environmental variance; and also the

environmental variance is not impacted by the genetic variance), thus providing the experimental evidence for the newly developed formula at this juncture for calculating the Total Phenotypic Variance.

References: Gupta, A.P., 2009, Dros. Inf. Serv. 92: 32-37; Gupta, A.P., 1978, Norms of Reaction of genotypes in *Drosophila pseudoobscura*, Ph. D. Thesis, Harvard University, Cambridge, MA.

Relative frequencies of deleterious genes in natural populations of *Drosophila melanogaster* originating from the nucleoelectric plant of Laguna Verde, Veracruz.

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Abstract

In order to obtain information about possible changes and/or damages that could be caused by the operation of the nuclear reactors of the Laguna Verde Nuclear Power Plant, over populations of *Drosophila melanogaster* that live in the area, a series of semi-annual collections (summer and winter) during the years 1991-1992 and 1996-1998 were done and later subjected to a battery of tests. Flies of this species after being captured were carried to the ININ Biology laboratory where they were subjected individually to a series of crosses that allowed us in the third generation to detect the presence of deleterious genes that, depending on their viability, were classified as normal, lethal, or semi-lethal according to Wallace's methodology. In this way a total of 933 second chromosomes were analyzed and from them the relative frequencies for each of the categories calculated, as well for each sampling season. This information is shown in Tables 1 and 2. Results from the statistical tests indicate that there are no significant differences between the populations and that the differences, if they existed, are due only to environmental changes, which normally occur in all populations. Due to this we can point out that the presence of the reactors does not seem to negatively influence the behavior of the populations that live in the area.

Introduction

The current energy needs require the use of new technologies, among which the generation of electricity through the construction and operation of nuclear power plants partially cover these requirements. The operation of these plants is coupled with the concern of governments to inform the community of the advantages and disadvantages that this type of facility may cause. Mexico has two nuclear power plants located in Laguna Verde, Veracruz. Their construction began in 1986 and one started to operate in 1989 and the second in 1994. Consenting to the responsibility of evaluating the possible biological effects that the operation of a nuclear power plant represents, the Mexican authorities, in this case the Instituto Nacional de Investigaciones Nucleares (ININ), approved a long-term project to study the possible consequences of ionizing radiation in populations of organisms that inhabit the area of influence of the two reactors. The organisms selected for the study are two dipterous species widely used in biological research: *Drosophila melanogaster* and its sister species *D. simulans*. The use of these species makes it possible to compare the responses of similar genomes, which will serve as an indicator of possible changes due to exposure to the same factors, as well as an extensive knowledge of their biology.

The study would include the answer to the following biological parameters: relative frequencies of the species, resistance to desiccation, displacement capacity, radio resistance, and egg-adult viability and others that could be incorporated during the development of the project. It should be noted that these parameters by themselves represent the natural response of organisms to natural adversities.

Preliminary studies of these biological parameters, during the construction phase, were presented by Levine *et al*. (1989), de la Rosa *et al.* (1989), Rockwell *et al*. (1991), and Olvera *et al*. (1993), information that serves as a reference for later studies. Recently, and considering the information regarding the operational phase of the two reactors, there are already responses of both species to the different parameters, thus Pimentel *et al*. (2003) report on radio resistance, Pimentel *et al*. (2004) with reference to adult egg viability and Pimentel *et al*. (2007) with respect to resistance to desiccation. These three studies are comparative between the two species involved and considering the response over a period of ten years and in none of them was found evidence of negative impact.

Now our attention is focused on the genetic load that will give us an idea of its variability because it determines the frequency of recessive lethal genes. With respect to this parameter there is a lot of information and here we present some reports considered as pioneers in their region and therefore of greater relevance.

Thus, the genetic variability in natural populations of *Drosophila melanogaster* has been widely analyzed, highlighting those studied by Ives (1945), in populations of USA; Dubinin (1946) in Russia; Goldschmidt *et al.* (1955) and Dawood (1961) in the Mediterranean; Paik (1960) in Korea, and Minamori and Saito (1964) in Japan and in Mexico among others by Salceda a and b (1977), Espinoza-Velázquez and Salceda (1977), and Maganhotto *et al.* (1979). In all of them, reference is also made to the average viability of the carriers of different categories of deleterious genes. This parameter was selected because, among other aspects, it gives us information regarding the hidden variability present in the populations that are studied. And although this way of measuring the variability has been surpassed by molecular techniques based on DNA and that are more precise, however, the advantage of using the genetic load is that it allows us to have additional information regarding the genetic health of the population by quantifying the presence of deleterious genes present in the populations and also to obtain values of viability and average fecundity in the populations. Pioneering studies of this type are those of Wallace (1956).

It is known that a good number of mutations are lethal or semilethal when presented in homozygous condition, but the effects of those lethals in heterozygous flies are scarcely known and conflicting views about these effects have been expressed by various authors. Thus, Berg (1945) demonstrated that the lethal ones found in natural populations and Stern and Novitski (1948) using experimental populations showed that the lethal genes are deleterious in heterozygous condition, both authors employing *D. melanogaster*.

On the other hand, Wright *et al*. (1942) using *D. pseudoobscura*, found that autosomal lethals are less frequent in natural populations than they would be if they were completely recessive. For its part Cordeiro (1952) showed that on average the viability of lethal heterozygotes is significantly lower than in individuals free of lethal, and both types of chromosomes, carriers and non-carriers of lethals, vary significantly with respect to the effects of viability when combined with other chromosomes.

Also it will be possible to propose this biological system as a test to monitor in similar facilities, the possible existence of leaks or to detect damages caused by extremely low doses due to gaseous emissions, if they occur.

Material and Methods

The collection of the biological material was done in two sites of Laguna Verde in which the first electric-power plant of the country was established, which is located 72 km north of the city of Veracruz, and its coordinates are: 96º 24'30" longitude W and 19º 43 '24" of latitude N. These sites were established by Levine *et al*. (1989), designated Site I the one located in the residential area at 1350 m WNW of the reactor and corresponds to the control population, while Site II is located 350 m from the exit of the reactor cooling water in a SSW direction of the first site and corresponds to our experimental population. Subsequently, a third site was included outside the facilities approximately 5 km north of them.

The plant started its operation in February of 1989 and although collections of *Drosophila* were done in the stage prior to its operation, there is no information regarding the frequency of lethal genes in these populations. The collections of biological material analyzed here were done in three successive seasonal periods: July 1991, January 1992, and July 1992, and correspond to the first years of operation of the plant. The collections were suspended for four years and continued on the following dates, where information on Site III is already included: summers and winters of 1996, 1997, and 1998, the last date on which samples were

analyzed. Even though we have looked for more collections, the Authorities of the Plant did not allow the entrance for security regulations.

The flies were captured by means of traps containing fruit in fermentation as an attractant and with the use of an entomological network. Once captured and selected, they were transported to the ININ laboratory where the analyzes were carried out. For this, the flies were subjected to a series of experimental crosses according to the Cy L / Pm technique; H / Sb described by Wallace (1956) and widely used in similar studies that schematically is as follows:

P1 male + / + nature × female Cy L / Pm; H / Sb
This initial cross serves to isolate or extract a second chromosome from the male of nature
F1 male + / Cy L × female Cy L / Pm; H / Sb
This cross acts as a multiplying agent of the isolated chromosome, since all the descendants will be identical
F2 males + / Cy L × females + / Cy L
these crosses will originate the test generation
F3 Cy L / Cy L: Cy L / +: + / +
1 (dies): 2 (heterozygote): 1 (wild),

where the Cy L / Cy L individuals die because the markers in turn carry in association a recessive lethal gene; CyL / + individuals are heterozygous for the marker chromosome and the one from a male of nature. As noted in the diagram, in the third generation two types of individuals are obtained: the heterozygotes carrying a chromosome with markers and the other a replica of one of the second chromosomes of the male originating from nature and whose phenotype is Curly wings. The other type of individual that appears in this third generation corresponds to homozygous individuals for that second chromosome of the captured male and wild phenotype; the proportion of these two genotypes is 2: 1 or in percentage 66.67 against 33.33. The absence of wild individuals is indicative of the presence of a lethal gene and deviations to the 2: 1 ratio indicate differential viability. When the fraction corresponding to wild types is greater than zero but less than ten percent of the total number of flies counted in the culture, they are individuals carrying semilethal genes. This characterization helps us to determine which category corresponds to each chromosome so extracted from the population and can, therefore, determine the relative frequencies of these genes in each sample.

With this test we could determine the frequency of recessive genes, lethal, semilethal and normal for each population and with this information suggest the existence or not of genetic damage possibly caused in a natural way and in case of significant differences with the control population suggest the possible effect caused by other agents present in the area, which according to our hypothesis could be due to emanations from the reactors. The absence of changes in the relative frequencies of these genes indicate that there is no damage caused by the presence and operation of the reactors.

The determinations of the type of gene extracted in this way were made by quantifying the number of heterozygous descendants carrying the wild type and the markers (+ / Cy L) *vs* wild (+ / +) that in a 2: 1 ratio indicate normality and deviations of the same degree of viability being the proportion 100% + / Cy L indicative of lethality.

The comparisons that took place were of two types: comparison of the frequencies of deleterious genes with regard to the different seasons of the year, that is, winter *versus* summer, since the collections were in January and July and those between the sites. The different comparisons and their differences were analyzed by applying the χ^2 test, which are observed in the respective tables.

All the cultures were made in ¼ liter jars containing fresh food consisting of a mixture of agar-agar, corn flour, sucrose, dextrose, and brewer's yeast to which tegosept and propionic acid were added as a fungicide and preservative; the cultures were maintained at a temperature of 25 $\pm$ 1ºC and a relative humidity of 65%.

Results

Once all the counts have concluded, data were collected, tables prepared and the results analyzed, they are shown in Tables 1, 2, and 3.

Table 1. Percentage frequency of normal, lethal and semi-lethal genes in natural populations of *Drosophila melanogaster* originating from Laguna Verde, Ver.

Population	Normal	Lethal	Semi-lethal	n
SI, July, 1991	100	---	---	11
SI, January, 1992	62.2	13.5	24.3	37
SI, July, 1992	65.4	7.7	26.9	26
SI, July, 1996	94.4	5.6	---	18
SI, January,1997	92.9	7.1	---	14
SI, July, 1997	95.6	---	4.4	45
SI, January,1998	86.1	8.3	5.6	36
SI, July, 1998	95.3	1.9	2.8	108
SII, July, 1991	80.0	5.7	14.3	35
SII, January,1992	89.1	3.9	7.0	129
SII, July,1992	84.6	7.7	7.7	13
SII, July, 1996	96.3	3.7	---	27
SII, January,1997	100	---	---	12
SII, July, 1997	96.9	3.1	---	65
SII, January,1998	87.0	13.0	--	23
SII, July, 1998	90.3	7.8	1.9	103
SIII, July, 1996	100	---	---	2
SIII, January, 1997	100	---	---	25
SIII, July,1997	92.3	---	7.7	13
SIII, January, 1998	90.5	7.1	2.4	42
SIII, July, 1998	91.3	3.4	5.4	149
Average	90.01	6.3	9.2	933
SG	90.01	4.44	5.92	148

SI= Site I; SII= Site II; SIII= Site III; n= population size; SG= Salceda and Gallo, (2002).

Table 2. Seasonal differences of the relative frequencies of normal, lethal and semi-lethal genes in natural populations of *Drosophila melanogaster* originating from Laguna Verde, Ver.

	Normals	Lethals	Semi-lethals	n
SI Winters	77	10.3*	12.6	87
SI Summers	91.8	2.4	5.8	208
SII Winters	89.6	4.9	5.5	164
SII Summers	90.9	5.8	3.3	243
SIII Winters	94.0	4.5	1.5**	67
SIII Summers	91.5	3.0	5.5	164

* Significant $p \leq 0.05$; SI = Site I; SII = Site II; SIII= Site III.

Table 3. Inter-population differences for the relative frequencies of normal, lethal and semi-lethal genes in three natural populations of *Drosophila melanogaster* originating in Laguna Verde, Ver.

	Normals	Lethals	Semi-lethals
SI January	77	10.3	12.6*
SII January	89.6	4.9	5.5
SI July	91.8	2.4*	5.8
SII July	90.9	5.8	3.3
SI January	77	10.3	12.6**
SIII January	94	4.5	1.3
SI July	91.8	2.4	5.8
SIII July	91.5	3	5.5
SII January	89.6	4.9	5.5
SIII January	94	4.5	1.5
SII July	90.9	5.8	3.3
SIII July	91.5	3	5.5

Significant * $p \leq 0.05$; ** $p \leq 0.01$ SI = Site I; SII = Site II; SIII= Site III

As a result of the study, a total of 933 chromosomes were extracted or isolated from the same number of males captured in the sampling sites and seasons; distributed in the three categories of genes as shown in Table 1, in which in order to have reference data from Salceda and Gallo (2002) from a locality in the vicinity of the City of Veracruz are included as reference.

The most important aspect of the study and from which the subsequent analyzes are derived is to determine the amount of the genetic load, that is, the relative frequencies of normal, lethal and semi-lethal genes in each of the populations. This information is shown in Table 1. On the other hand, in Tables 2 and 3 we present the result of the application of the statistical test χ^2, in which to increase the sample size that allows us to make comparisons, the data of all the collections were added to obtain the corresponding value per site and per season of the year, which were compared among themselves to be subjected to the afore mentioned test. The next step was first to compare the observations between seasons, that is, summers against winters for each site as shown in Table 2; the values between site and site were then compared for each season, as shown in Table 3.

Discussion

From the enormous amount of existing data in the bibliography regarding the amount of the genetic load or accumulation of lethal genes in natural populations of *D. melanogaster*, it is inferred that this value fluctuates between 5 and 20 percent for the frequency of this type of genes, depending on these values of both the time of year in which the sample was taken and the geographical position of the sample. Regarding the frequency of semi-lethals there is little information and it is only indicative of the genetic health of the population since it does not have more information of an adaptive nature.

The effects caused by irradiation, however, vary according to the type and dose of radiation and in this respect little or nothing is known regarding the effect of emanations of nuclear plants.

The information we detected as shown in Table 1, indicates that our populations do not differ from those studied by other authors in studies of natural populations because these are the ones that serve as the basis for any comparison. As the values detected by us are similar to those of other authors we consider that these populations are within the range of normality for the parameter analyzed.

Our results clearly show that the frequencies of lethal genes fall within the ranges of normality, in addition, since there are no differences between the values obtained for each population, it is suggested that the absence of damage caused by possible leaks or gaseous emanations proper to the functioning of the reactor do not represent any alteration of the values.

When no significant differences were found or were very small between the two populations, it was decided to do another type of analysis, consisting of comparing the behavior of the populations in relation to the parameter under study but now taking into consideration the temporal differences, that is, seasonal changes.

As the data were collected during several successive seasons two summers, July 1991 and 1992 and a winter January 1992 in a first stage, as well as the summers of 1996, 1997, and 1998 and the winters of 1997 and 1998, it is a possibility to detect changes due to the change of seasons. Since the summer samples were small in size and there were no differences in the relative frequency of lethal genes in both populations, we opted to add the data of both years for this season and try to see differences, as shown in Table 2.

The result of these observations and when doing the statistical tests was the response that the differences between the summer frequencies with respect to the winter ones are so small and probably due to the sample size. These small differences can, therefore, be due exclusively to seasonal changes fundamentally due to the different temperatures and humidity that prevail in the area.

It is known that in most natural populations in which this type of analysis has been carried out, the behavior is similar and the changes detected are only a reflection of the changes caused by the alternation of the seasons, in our case dry and cold against wet hot.

Having not found differences between the seasons, we will now deal with those present in the different sites. By our design and following that established by Levine *et al*. (1989), in addition to Site III since no differences were found during the first stage between Sites I and II, we consider it pertinent to include a third sampling site. Site I represents our witness against which to compare the values of Site II or experimental population, the values of these comparisons are seen in the first four rows of Table 3. In the table it is noted that the only two differences found correspond to the semi-lethal frequencies between Site I and Site II in the winter; for lethal in July between the same sites for summer and for semilethals in winter between Site I and Site III.

The difference in the summers between sites I and II may be due to the fact that Site I is located in an urban area and since it frequently suffers bottlenecks in terms of abundance of individuals, our samples probably suffered alterations in which individuals that started the repopulation were those free of a lethal, and probably the same happens in the case of the semilethals in the winter comparisons between Sites I and II and between Sites II and III.

Therefore, we can conclude that the differences between the three populations are not significant and this suggests the absence of the possible induction of damage due to the possible effect of gas emanations from the reactor and that the differences found are due to seasonal changes normally occurring in natural populations.

Our information regarding the frequencies of deleterious genes present in natural populations of *D. melanogaster*, with respect to the possible impact due to the presence and operation of the reactors in the Central Nucleoeléctrica de Laguna Verde, Ver., coincides with the studies reported by Pimentel *et al.* (2003) about radio resistance, Pimentel *et al*. (2004) with reference to adult egg viability, and Pimentel *et al*. (2007) with respect to drying resistance. These three studies conducted in the same populations presented here indicate that there is no impact whatsoever on the operation of the reactors, and we agree with them in their statement that the small differences are only responses to environmental changes.

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Description of *a Drosophila* species of subgenus *Pholadoris,* and replacement of phylogenetic rank to genus *Scaptodrosophila* (*Drosophilidae-Diptera*).

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Abstract

The present study aims to describe a species belonging to subgenus *Pholadoris* of genus *Drosophila* collected from Lahore, Pakistan. Diagnostic features like presence of prescutelar bristles and wide sub median spines in novasternum of male phallic organ, the reported species was placed in subgenus *Pholadoris* of genus *Drosophila*. Wing indices and anatomy of male genital organs revealed similarities with *Drosophila setaria* of *levis* group of Pholadoris, which have been previously reported from India. According to new hierarchies based upon improved tools in taxonomy and molecular phylogeny, the members of subgenus *Pholadoris* have replaced in a separate genus *Scaptodrosophila* of family *Drosophilidae* of *dipteran* flies. The characteristics like the presence of three katepisternal setae and irregularly arranged acrostichal setulae found in the described species warrants its inclusion in *Scaptodrosophila.* Features of Periphallic organ such as cerci without thick layer of bristles found to be similar to the rest of described members belonging to genus *Scaptodrosophila.* Further investigations such as barcode analysis will be helpful in identification of observed *Scaptodrosophila* up to species level. Keywords: *Drosophila*, *Pholadoris*, *Scaptodrosophila*, Lahore, Pakistan.

Introduction

Drosophilidae is a widely distributed family of dipteran flies whose phylogeny is being well studied (Sturtevant, 1942). More than four thousand species placed in seventy-five different genera of this family have been recently classified by using modern molecular approach (Yassin, 2013). Initially it was Throckmorton (1975) who proposed various hypotheses for different radiations and clades of *Drosophilidae* family, based upon comparative morphology and reproductive isolation. At the end of the 20th century classification on molecular phylogeny was presented to propose relationships at genus level among the family *Drosophilidae* (DeSalle, 1992; Pelandakis and Solignac, 1993; Russo *et al*., 1995; Remsen and DeSalle, 1998; Kwiatowski and Ayala, 1999; Tatarenkov *et al*., 2001; Remsen and O'Grady, 2002). Thus, beside slight differences identified by these researchers, most of the key findings about phylogenetic relationships were consistent. Following the up-to-date approach of modern systematics of *Drosophilidae* described by Bachli (2000) it has been realized that several genera of this family do not exhibit monophyletic origin. The radiation at the base of this family is consistently occupied by subfamily *Steganinae*, while *Scaptodrosophila* is described as derivative of basal radiation *Steganinae* according to phylogenetic hypothesis of *Drosophilidae* based on Grimaldi (1990). Phylogenetic analysis founded by Kwiatowski and Ayala (1999) and Tatarenkov *et al*. (2001) also proposed basal position of *Scaptodrosophila*. Thus, according to Sturtevant (1942) genus *Drosophila* was divided into six subgenera named as *Hirtodrosophila*, *Pholadoris*, *Dorsilopha*, *Phloridosa*, *Sophophora*, and *Drosophila*. *Scaptodrosophila* originated in Asia, and the highest fauna of *Scaptodrosophila* occur in south-east Asia, Australia, and Africa, with a very limited number of species in Europe and South and North America (Liu *et al*., 2017). Twenty-six species belonging to genus *Scaptodrosophila* have been described from India (Sundaran and Gupta, 1991).

Methodology

Drosophila flies were trapped by traditional method of baits hung on the branches of fruit trees. Mature female flies were used to raise isofemale pure cultures by using porridge, yeast, agar, and sugar media according to ingredients used by Durr-e-Samin *et al*. (2014), and male flies were preserved in 3:1 mixture of acetic acid and ethanol. Male flies from cultures were used to describe anatomical features of species under observation by making micro-dissections under dissecting microscope (Leitz, Wetzler, Germany). Flies were soaked in 10% KOH (potassium hydroxide solution) twenty-four hours before dissections.

Results

Morphological and anatomical studies

Arista with 4 branches above and 2 below in addition to the terminal fork (Figure 1). Palpus with two prominent and a few small bristles. Second orbital being half the size of the other two orbitals. Thirteen rows of acrostichal hairs were found to be irregularly arranged on the thorax of all observed male flies. Prescutellar bristles are present originating slightly above the mesonotum. Ventral side of episternum called katepisternum possess three pairs of almost equal katepisternal setae. Prescutellars present. Both anterior and posterior scutellars convergent (Figure 2). Abdominal tergites are yellowish with dark brown uninterrupted bands. Preapicals prominent on all tibiae. There is no sex-comb on forelegs of males. Various wing indices in the males are approximately as follows: Costal index: 1.77; 4V index: 3.00; 4C index: 1.55; 5X index: 2.83.

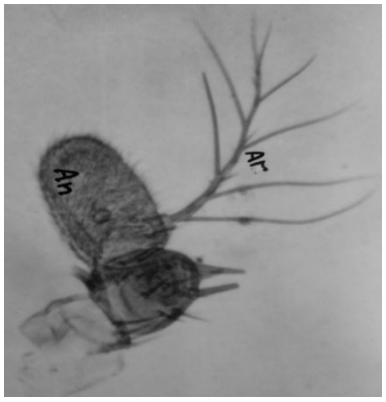

Figure 1 (left). An: antennae; Ar: arista.

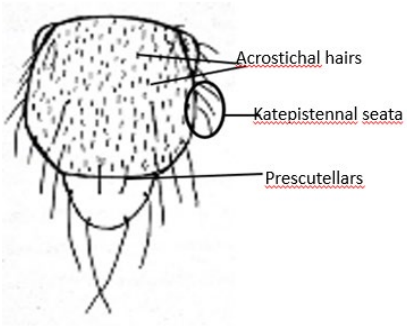

Figure 2 (right). Drawing of chaetotaxic features of thorax.

Periphallic organs: Genital arch pubescent narrow above lightly bristled and broader below upper posterior margin with 4-6 bristles, toe rounded broad with 8-9 bristles of almost equal size. Cerci boat-shaped pubescent with about 28 bristles and not contiguous to genital arch, clasper single primary teeth 11-12 arranged along the concave margin of the clasper marginal bristles 3. Heel prominent nearly at the level of toe and not extending beyond the primary teeth row (Figure 3).

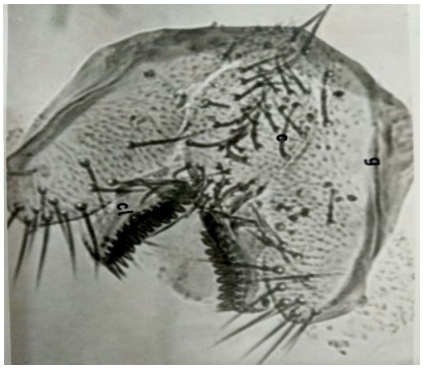

Figure 3. Periphallic organ. c: cerci; cl: clasper; g: genital arch.

Phallic organ: Aedeagus bifid, apically narrowing and curved. Anterior paramere broad and fused to form V-shaped structure. Posterior paramere longer than aedeagus being apically narrowing with 3-4 sensilla arranged in a row. Novasternum broad posteriorly concave pubescent with a pair of long stout sub median spines. Ventral fragma quadrangular. Basal apodeme longer than aedeagus and extends the ventral fragma (Figure 4).

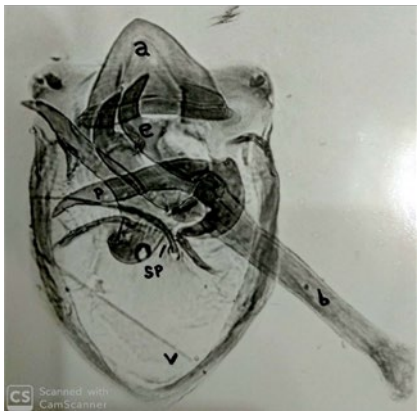

Figure 4. Phallic organ. a: anterior paramere; b: basal apodeme; e: aedeagus; p: posterior paramere; sp: sub median spine; v: ventral fragma.

Discussion

Bock and Parsons (1978) considered *Scaptodrosophila* genus as one of subgenus of *Drosophila* and considered it synonymous to *Pholadoris* (Sturtevant, 1939). Throckmorton (1975) suggested that some sub genera *Hirtodrosophila* and *Scaptodrosophila* of genus *Drosophila* would place as separate genera of the family. Afterwards, Grimaldi (1990), by adopting cladistic classification, placed these groups in the rank of genera and verified that these groups occupy an independent position from *drosophilidae*. This reranking was based upon analysis of more than 200 morphological features. *Scaptodrosophila* comprised of endemic origin with large fascinating assembly with around 200 species found in the continent of Australia (Bock and Parsons, 1975). Since little work regarding phylogenetic position has been done on the genus *Scaptodrosophila* thus yet it is described as monophyletic radiation instead of existing in a large group (Bock and Parsons, 1978).

According to Okada (1955) the characters such as the presence of prescutellar bristles and exceedingly long and stout submedian spines of male novasternum warrant the inclusion of the species in question in the subgroup *Pholadoris*. The characters such as the convergent anterior scutellars, acrotichal hairs in more than ten irregularly arranged rows, genital arch lightly bristled below, cerci without dense bristles especially at lower tip, clasper without numerous fine hairs, and heel not extending beyond primary teeth row qualify this species for inclusion in the *levis* species group (Parshad and Singh, 1971).

The *levis* species group of the subgroup *pholadoris* includes two closely related species namely *D. levis* and *D. setaria*. Both species have common features such as equal number of branches of arista, coastal index, 4C and 5X indices have about the same value and the number of primary teeth of clasper are equal. However, these two species differ from each other with respect to 4V-index and the shape of the heel and toe. The comparison species under observation with these two species revealed its resemblance with *D. setaria* (Parshad and Singh, 1971) in having rounded heel and toe and the 4V-index equal to 3.00. Moreover, in contrast with *D. levis* the genital arch of the species in question is pubescent. Further investigations of reproductive isolation and molecular barcode analysis are required for confirmation of this pholadorian at species level.

Phylogenetic position according to new hierarchy in genus Scaptodrosophila *of family Drosophilidae*

One of the main features for identification of genus *Scaptodrosophila* is presence of Katepisternal median seta on mesonotum which is not considerably shorter than anterior setae and existence of prescutellar elongated setae on scutellum (Bachli, 2005). Most of the species belonging to *Scaptodrosophila* genus like members of subfamily *Steganinae* possess prescutellar acrostichal setulae as the characteristic of basal position of members of *Drosophilidae* (Bachli, 2016). Numerous molecular findings done to date also support the

basal location of genus *Scaptodrosophila* in the family *Drosophilidae* (DeSalle and Grimaldi, 1991, 1992; DeSalle, 1992). Thus, the phylogenetic ranking as *Scaptodrosophila* is correct for described species.

Bachli (2016) reported worldwide distribution of 280 species belongs to genus *Scaptodrosophila* distributed in Nearctic, Neotropic, Oriental, Afrotropic, Ausralian, and Asian countries. This genus is divided into several sub-genera, the following of which have been described from India: *Paradrosophila* Duda, 1923; *Pholadoris* Sturtevant, 1942; *Pugiodrosophila* Duda, 1924; *Supristyloptera* Duda, 1923, and *Tanygastrella* Duda, 1924 (Sundaran and Gupta, 1991). However, the species belonging to *Scaptodrosophila* genus have been described the first time from Pakistan. None of the species belonging to this genus have ever been reported from Pakistan.

Acknowledgment: Anatomical section of this paper is part of my M.Phil. dissertation. I would like to thank my supervisor Dr. Mahmood Ahmad and lab fellows at Quaid-i-Azam University, Islamabad, Pakistan.

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Drosophila - A highly potent research tool, neglected in Pakistan as compared to other Asian countries.

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Summary

Pakistan is rich in biodiversity because of suitable climatic conditions and varied kinds of topography. Despite of this fact, only twenty species belonging to genus *Drosophila* have yet been reported. This number is less than ten percent of species explored in some neighboring Asian countries. Thus, we are neglecting an organism of utmost importance. As a result we have less potential in our research designs. More than a hundred years of experience of use of *Drosophila* along with lots of scientific development and remarkable laboratory instruments have given a way for marvelous progress. Thus, using *Drosophila* as a model organism leads to developing the modern concept at gene expression level, underlying the various serious groups of ailments related to neurology and cancer. Through the current article, the author wants to draw the attention of Pakistani biologists towards the use of members of the genus *Drosophila* in their laboratories. With this point of view a *Drosophila* laboratory has been established at Abdus Salam School of Science, Nusrat Jahan College, Rabwah and different species have been maintained to be used for research in various disciplines of biology especially in the field of oncology.

Introduction

The genus *Drosophila* of family *Drosophilidae* occupies a very important position among organisms used in different areas of biological sciences. This is one of the favorite organisms used for genetic studies because of its small size, short life cycle (8-14 days at 25°C), high reproductive rate (as an adult female laid 400-500 eggs in 10 days), and ease to culture, handle and study in laboratory. A wide range of species is present all over the world with lots of naturally occurring and artificially induced variants available to study inheritance.

T.H Morgan and his students C. Bridges, H.J. Muller, and A.H. Sturtevant pioneered the field of *Drosophila* genetics during first half of the twentieth century. During the first fifty years (1910 to 1960) the research revolved around concepts of genetics and deep understanding of principles of inheritance (Sturtevant, 1966). Meanwhile, investigators apprehend that this remarkable fly could be used for a broad range of research other than genetics. As a result a diverse range of biological phenomena were explored during the period of 1960 to 2010 (Bellen *et al*., 2010). Important discoveries were made by exploring several genes, physical mapping and use of tools like balancer and polytene chromosome in *Drosophila* (Muller, 1918; Bridges, 1935). Information about complete sequence of the euchromatic region of *Drosophila melanogaster* was published in the journal "Science" in year 2000, and now genome sequencing of 12 species has been completed in Lawrence Berkeley National laboratory in 2011.

In a report published to explore global trends regarding use of model organisms in biological research, Michan *et al*. (2010) found that *Drosophila* is next to *E. coli* on which most of the research articles have been published. Another comparison presented by the same authors revealed the temporal trend of *Drosophila* research for additional four subjects (beside genetics) *viz* ecology, zoology, evolutionary biology, and toxicology. According to this comparison *Drosophila* is almost equally extensively used in all these fields since 1975 to 2005 and definitely to date.

Drosophila all around the world

Drosophilists all around the world have explored and are still exploring *Drosophila* fauna and using it in laboratories as model organism for investigations. So far 1579 species have been described and reported (Brake and Bächli, 2008). It is expected to have thousands more unexplored species (Patterson and Colin, 1999). Powell (1997) in one of his books stated that great diversity and variety, which is present in genus *Drosophila* with thousands of species in a single genus, is seldom found in any other group.

Many species of *Drosophila* have been explored in Asia. A lot of work is going on in Japan, China, and India. The countries such as Taiwan, Nepal, Malaysia, Korea, and Fiji have also reported a few native species. Not a single species has been explored as endemic to Pakistan.

Among the Asian countries Indian Drosophilists are busy in the field of taxonomy and systematics of genus *Drosophila* since 1920. As a result 275 species have been reported from India; 65 of which were described as native species (Fartyal and Singh, 2001). Kikkawa and Peng (1938) were the first to survey Japan and adjacent countries. Biologists of China, too, have used *Drosophila* to make several achievements on perception of the basic questions in a wide range of areas of biology. In more recent years they began to use this organism for the research in the areas of neurobiology and oncology (Cheng *et al*., 2018). Several species have been reported to investigate recent trends in genetics like epigenetic mechanism (Liu and Li, 2012).

The survey of the Indian sub-continent has revealed that most of the species belong to *melanogaster* and *immigrans* species groups of sub genera *Sophophora* and *Drosophila*, respectively, which supports the view of Bock and Wheeler (1972) that South-East Asia is a fertile region for rapid diversification and speciation of the members of *melanogaster* and *immigrans* species of genus *Drosophila* (Sharma, 1988).

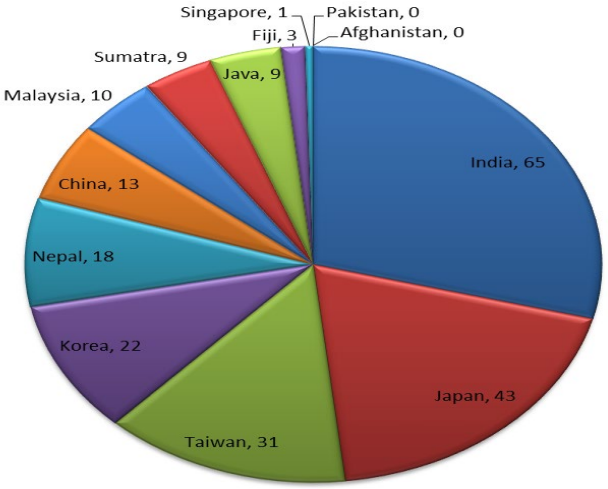

Figure 1. Comparison between different countries in respect of native species of *Drosophila*.

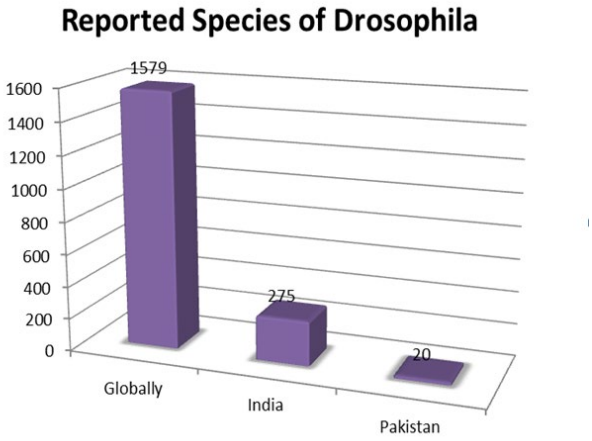

Figure 2. Reported species of *Drosophila* by India, Pakistan, and other countries of the world.

Drosophila **in Pakistan**

Pakistan has a luxuriant flora along with the suitable climatic conditions so it is expected that this region is also rich in *Drosophila* fauna. Unfortunately fauna of this remarkable research organism has so far remained virtually unexplored. Very few attempts have been made in this regard. Din and Mazhar conducted a survey of Islamabad and identified 10 species, *viz*. *D. melanogaster, D. immigrans, D. takahashii, D. suzukii, D. nepalensis, D. hydei, D. jambulina, D. malerkotliana, D. leontia, and D. bifasciata* (Din *et al*., 2005). Another attempt was made by Shahjehan *et al.* (2004) who reported 9 species from NWFP (KPK). This survey was carried out in 15 cities of KPK reporting nine species named *D. szentivanii, D. immigrans, D. rufa, D. takahashii, D. sahyadrii, D. prolongata, D. prostipennis, D. melanogaster,* and *D. trapezifrons.* The following ten species were reported from Lahore: *D. busckii, D. setaria, D. immigrans, D. melanogaster, D. takahashii, D. nepalensis, D. malerkotliana, D. ananassae, D. jambulina,* and *D. brevis* (Tahir, 2014). Some very short term surveys were done to explore *Drosophila* fauna of Murree, Barian, Nathia Gali, and Karachi, reporting *D. melanogaster, D. immigrans, D. takahashii, D. ananassae,* and *D. jambulina* from theses virtually unexplored areas (Tahir *et al.,* 2016). That makes a total twenty species none of which was endemic.

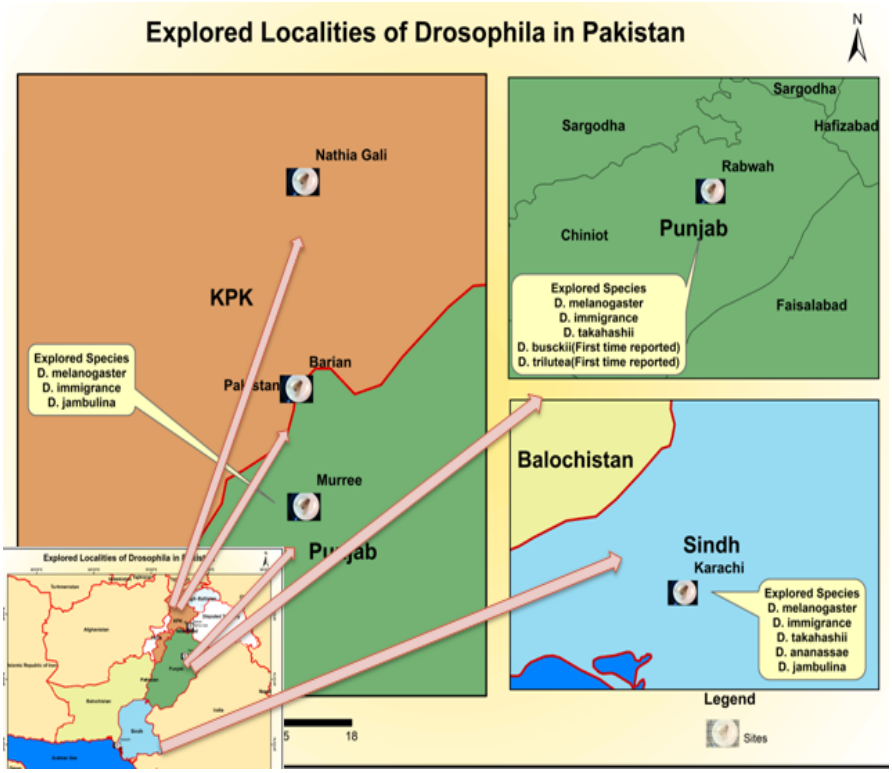

Figure 3. Different localities in Pakistan, which have been partially explored for *Drosophila* fauna.

Proposed plan for exploring fauna of genus *Drosophila* in Pakistan

Zonal distribution:

To explore *Drosophila* fauna of Pakistan, there is need to work systematically to cover all varied kinds of regions. Regarding topology and climatic conditions, zonal distribution would be made as follow:

1) Coastal Areas: Karachi division and Makran division
2) Plain Areas: Sindh, South and Central Punjab
3) Pothohar: Chakwal, Jehlam, Rawalpindi
4) Northern Areas: KPK, GilgitBaltistan and Kashmir
5) Deserts: Thar and Cholistan

Randomly collected flies would be propagated in laboratory to maintain live cultures to be used in different projects of biological sciences especially for research in oncology. It is expected that less than five years of extensive surveys of all zones would be enough to explore *Drosophila* fauna of Pakistan. Species captured from wild or propagated in laboratory would be identified either by traditional morphological and cytological parameters or by using modern phylogenic systematic of Barcode analysis. Author expects diversity of at least hundred species in this area.

Initiative to development of Drosophila *laboratory:*

A *Drosophila* laboratory has been developed by faculty belonging to Abdus Salam research forum, at the Department of Life Sciences, Nusrat Jahan College, Rabwah, Pakistan. Local species propagated successfully are raised from single female cultures. Figure 4 shows the different species of *Drosophila* which have been propagated.

Figure 4. Species of *Drosophila* collected from different localities of Pakistan.

Concluding Remarks

All around the world *Drosophila* is used for genetic research as it is the best eukaryotic model organism. In scientifically developed countries, *Drosophila* is used to teach genetics to undergraduate students. There are lots of workshops, conferences, stock centers, and fly labs to promote the research program on *Drosophila* in different fields of biology. The genomes of 12 *Drosophila* species have been published. This initiative will invigorate *Drosophila* research for the next 100 years. In Pakistan *Drosophila* is not even used at the post-graduate level (in most universities) to teach students genetics or for research. So by carrying out this proposed kind of study we can achieve several targets like:

i) Promote use of *Drosophila* to teach genetics to students and use it in their research projects.
ii) To develop stock centers by maintaining live cultures and stocks in laboratory.
iii) To use different species for carrying out research in ecology, population genetics, evolutionary biology, and many more.
iv) To make use of different species in advanced research in genetics like genomics, proteomics, gene silencing, and RNA interference, epigenetics.
v) To develop methods of barcode analysis for determination of phylogenetic relationships.
vi) To use as model organism for research in neurobiology and oncology.

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Drosophila as a model in major neurological disorders and cancer.

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Summary

Drosophila has been a powerful model organism that has played a vital role in discovering essential conserved neurological and developmental pathways. About 75 percent genes of the human genetic diseases have homologues in the fruit fly. The conserved nature of most of the signaling pathways among human and *Drosophila* makes it easy to genetically manipulate the organism. By using a range of genetic tools to analyze and express these proteins, wide-ranging genetic screens can be performed in *Drosophila* to determine other components of the genetic pathways in humans. This review is aimed at highlighting the usefulness of *Drosophila* as a tool for identifying complex neurological disease pathways for potential therapeutic discoveries and to discuss its importance in studying the events that lead to the development of tumorigenesis.

Role of *Drosophila* as model in neurodegenerative diseases

Neurodegenerative diseases in humans are characterized by a gradual loss of neurons leading to physical and behavioral defects that might eventually cause death of the affected individual (Beal *et al.,* 2005). Many of the neurodegenerative diseases that are related to old age have been found associated with toxic protein aggregates in the intracellular regions (Taylor *et al*., 2002). The presence of mutations in familial cases of these diseases has led to the development of *in vivo*/*vitro* models in order to evaluate the abnormalities at cellular and molecular levels which involve products of a mutant gene. Investigators are of the view that some proteins tend to acquire pathological conformations by misfolding into pathologic aggregates as compared to other proteins. The onset of neurodegenerative disease is believed to be linked to the fact that the amount of neurotoxic proteins inside the cell exceeds the capacity of the cell to dispose of them or when such proteins overcome the cell's quality control system. This concept has developed the possibility of planning novel treatment options by keeping a focus on the cell's protein disposal mechanism (Pandey *et al*., 2011).

The use of humans in biomedical experiments has both ethical and practical limitations. Model organisms with sequences closely related to humans are widely used in biological experiments. They range from unicellular organisms such as *E. coli* to mammals such as mice. *Drosophila* is one of the most widely used model organisms in studies related to genetics, development of embryo, behavior, learning, and the aging process (Jennings, 2011). The full genome sequences of *Drosophila melanogaster* and humans have shown to have an approximate 75% similarity between them, thus validating its position as a model organism (Reiter, 2001).

Several features make *Drosophila* a suitable model organism including a short life cycle of about 40-120 days, cheap and easy to retain cultures in the laboratory, and a large number of genetically identical progeny (Piper *et al.*, 2005; Pletcher *et al.*, 2005). Moreover, it shows complexity in behavior, possessing memory and learning abilities (McGuire *et al.*, 2005; Margulies *et al.*, 2005) controlled by a complex brain and the nervous system (Nichols, 2006).

Trinucleotide repeat expansion diseases

Trinucleotide repeat expansion diseases (TREDs) are related to more than 16 disorders of neurological nature caused by abnormal triplet repetitions in either coding/non-coding regions of genetic loci linked to disease. The result is a dysfunction of the resultant protein that eventually leads to neurodegeneration and death of the patient. The most common cause of TREDs is the abnormal extension of CAG repeats that code for glutamine (polyglutamine, PolyQ). Other than codon repetition, TREDs vary in the length of codon repeats regarding neuropathology and clinical symptoms in different diseases. All the genetic loci that are affected by the trinucleotide repeat expansion have been identified except the androgen receptor. Most significant worth remembering fact is that a fly homologue for each of the genetic loci exists (Hirth, 2010).

Fragile X syndrome, Friedreich's ataxia (FXTAS), Huntington's disease-like 2 (HDL2), spinocerebellar ataxia type 8 (SCA8), and Myotonic dystrophy type 1 (DM1) are all examples of TREDs that exhibit repeat expansions in non-coding sections of RNAs (Ranum and Cooper, 2006; Orr and Zoghibi, 2007; Li *et al.,* 2010).

Drosophila has proved to be an excellent and efficient system that presents detailed insight into neurological disorders related to human situation (Zoghbi and Botas., 2002; Cauchi and Heuvel, 2006). Currently *Drosophila* models have been used to study interactions of pleiotropic Ataxin 2 and role of CAGRNA in disorders related to polyglutamine, antisense transcription in TREDs as well as contribution of RNA foci in diseases related to CUG expansion. *Drosophila* has been successfully used as model to study RNA-induced toxicity mechanisms related to CAG triplet repeat disorder (Li *et al*., 2008). *Drosophila* is also being used as a model in studies related to repeat stability (Yu and Bonini, 2011).

Myotonic dystrophy type 1 (DM1) is an example of a TRED that is characterized by modification of the regional chromatin caused by sense and antisense transcripts (Cho *et al*., 2005). The cause of mutations in DM1 is repeat expansions of CUG. The repeat RNAs formed by the expanded CUG, misguide and misregulate the proteins such as MBNL1 and CUG-BP1 that bind to RNA and are involved in alternative splicing (Savkur *et al*., 2001; Mankodi *et al*., 2002).

Transgenic *Drosophila* models were prepared to study toxicity caused by CUG RNA in DM1. These transgenic flies exhibited a major similarity to the human diseases caused by CUG expansion with 70% of genes showing anti-sense transcription. These flies were then used to identify neurotoxicity of co-expressed CTG and CAG transcripts. The results revealed an increased toxicity when CTG and CAG were co-expressed. Useful conclusions from the transgenic *Drosophila* model were drawn that suggested that expanded transcripts interact *in vivo* and are believed to generate small RNAs such as the siRNA that significantly promotes pathology in the neurological disorders (Yu *et al.,* 2011). Jung and Bonini (2007) used *Drosophila* as a model to study spinocerebellar ataxia type 3 caused by CAG repeat instability in humans. The results showed similar key features of CAG repeat instability in *Drosophila*.

The genomic context of the repeat instabilities caused by expanded CAG and CTG repeats in trinucleotide disorders has been poorly understood. The use of the transgenic *Drosophila* model in attempting to study the molecular basis of a similar repeat instability in spinocerebellar ataxia type 7 (SCA7) proved it to be an unsuitable system for doing so, since it displayed preserved stability of CAG repeats (Jackson *et al.,* 2005).

Expanding role of *Drosophila* in cancer research

Long before *Drosophila* became the most prevalent model organism for research, Bridges and Stark, in 1916, found tumor-like melanotic granules in the *Drosophila* larvae which presented the first evidence of tumor development in flies (Stark, 1918). Research studies on flies have revealed that they possess a large number of genes that are homologs of tumor suppressors and oncogenes in humans (Miklos and Rubin, 1996). Studies on these cancer gene homologs have added a lot to the understanding of genetic pathways, developmental functions, and molecular activity in these genes. Currently, investigations are being conducted on around 76 mammalian cancer gene homologs in flies. A list of a few has been given in Table 1.

Table 1. Cancer genes with homologues in *Drosophila.*

Myeloid Leukemia (ABL1)	E-Cadherin (CDH1)
Acute Myeloid Leukemia (DEK) Chronic	Nijmegen Breakage (NBS1)
Adenomatous Polyposis Coli (APC)	Tumor suppressors (P53, RB1, PTEN, P21)
B-cell Lymphoma 2 (BCL2)	Ewing Sarcoma (FL1-1)
Bloom (BLM)	Colon Cancer (MSH-2, -3, -6, MLH1, PMS2)
Burkitt's Lymphoma (MYC)	Lymphoma (MCF2)
Chk2 Protein Kinase (CHK2)	Neurofibromatosis (NF1, NF2)
Chronic Myeloid Leukemia (BCR)	Multiple Exostosis (EXT1, EXT2)
Cyclin D1 (CCND1)	Multiple Endocrine Neoplasia (MEN1, RET)
Cyclin-dependent Kinase 4 (CDK4)	Pancreatic Cancer (DPC4/MADH4)
Epidermal Growth Factor Receptor (EGFR)	Tuberous Sclerosis (TSC-1, -2)
Oncogenes (RAS, REL, AKT1, ERBB2)	Von Hippel Lindau (VHL)
Peutz-Jeghers (STK11)	Xerod. Pigmentosum (XPA, ERCC3, XPD, XPF, XPG)

Signal transduction pathways are conserved from flies to humans

A large number of extensively researched signal pathways have been found to be conserved in both humans and flies. Studies on *Drosophila* genetics have added a great deal to the understanding of these signal pathways. An example is the Ras proto-oncogene pathway which was first discovered by studying the development of photoreceptor cells in the eyes of *Drosophila* (Wassarman *et al*., 1995), and by reviewing the development of vulva in *Caenorhabditis elegans* (Sternberg and Han, 1998).

Due to the conserved nature of these pathways, studies on fly genes and their genetic pathways are adding to the current knowledge of human cancer research. An example is the discovery of tumor suppressor gene, *Patched,* which exhibits a mutation in the nevoid basal cell carcinoma syndrome. The discovery of *Patched* gene has led to an increased research interest in the *patched*/*hedgehog* pathway in *Drosophila* for possible evidence of other pathway constituents that act as oncogenes or tumor suppressor elements in humans. Three additional components of the *patched*/*hedgehog* pathway have been found to play a role in tumor formation in mammals (Hahn *et al*., 1996; Xie *et al*., 1998; Oro *et al*., 1997; Dahmane *et al*., 1997).

While numerous tumorigenesis-related signal transduction pathways from *C. elegans* to humans are conserved, there might be differences in the biological functions of these pathways. As an example, the *Ras* pathway in both humans and *Drosophila* is involved in determination of cell fate as well as cell proliferation while in *C. elegans,* the *Ras* pathway determines only the cell fate (Yochem *et al*., 1997; Karim *et al*., 1998). Furthermore, the determination of cell fate in *C. elegans* is more related to lineage which suggests that some pathways related to human tissue patterning will either be missing or functionally altered in *C. elegans.* Moreover, genome sequencing of *C. elegans* has revealed very dissimilar core components compared to the signaling pathway in *hedgehog* (Ruvkun *et al.,* 1998). The above facts highlight the need of *Drosophila* as a model organism in investigating the biology of human tumors.

Use of *Drosophila* to study tumorigenesis

Current advancements in the experimental practices offer valuable advantages in the study of developmental aspects of cancer-causing genes in flies including ectopic gene expression. This is particularly helpful for reviewing the tumor etiology since oncogenes can either be over-expressed such as *cyclin D* or activated aberrantly as in *Ras.*

Drosophila researchers can analyze the process of gene over-expression in flies without having to kill the animal by utilizing specific promoters for ectopic gene expression. The fly biology is unique in the sense

that it provides a range of well-defined promoters, including universal promoters as actin/heat shock promoters or tissue-specific promoters such as eye-specific or neuronal specific promoters (Potter *et al*., 2000).

It is also possible to temporarily control the gene expression through the induction of heat-shock promoter for a defined time of interest. Similarly, *in vitro* gene modification is also possible for the analysis of oncogenic mutations including point mutations and deletions with subsequent assays *in vivo* to detect any oncogenic activity; this approach has helped in the confirmation of mutant *Ret* gene which is hyper-activated in the multiple endocrine neoplasia 2B (MEN2B) tumors (Read *et al*., 1998).

The biology of *Drosophila* provides a valid model for cancer research

Drosophila offers researchers an exceptional opportunity to analyze processes that lead to the development of cancer within imaginal discs that are very similar to cancer susceptible mammalian cells. Imaginal discs consist of sacs of specific epithelial cells that form a majority of the adult fly structures. These discs, which are initially single-layered, proliferate throughout the larval stages to give rise to mature discs having distinct morphologies differentiating them into adults (Bryant *et al*., 1990).

Specialized diploid epithelial cells undergo multiplication and differentiation having cell cycle comparable to mammalian cells comprising G1, G2, S, and M phases (Orr-Weaver *et al*., 1994; Edgar *et al*., 1999). The resemblance between the mammalian and fly cell cycles is not only limited to the over-all organizational level but the molecular level is also conserved.

The resemblance between human and *Drosophila* regulatory pathways and cell-cycle machinery advocates for the value of *Drosophila* as a model organism for studying proliferation during the course of tumorigenesis. The process of determination of cell fate in mammals, which plays a role in tumorigenesis, can also be investigated in fly imaginal discs (Basler *et al*., 1991; Rooke *et al.,* 1998). It is now evident that many of the molecular pathways are conserved among flies and mammals.

Conclusion

The present day efforts of the *Drosophila* researcher's community in refining and developing better genetic techniques will further substantiate the role of *Drosophila* as an ideal organism for research in the fields of neurobiology and oncology. Keeping in view its versatility in providing solutions to many queries and its direct relation to mammalian tumorigenesis, *Drosophila* has a lot to offer in the upcoming research about cancer treatment and neuropathies.

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First report of *Chymomyza vaidyai* Okada (Diptera: Drosophilidae) from South India.

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Abstract

This study reveals the first report of *Chymomyza vaidyai* Okada from Dharwad, Karnataka state, South India. The species was found at vegetable/fruit markets of Dharwad city. A few important morphological characters of the species are discussed.

Introduction

Taxonomy and faunal study of the family Drosophilidae is extensively known all over the world, as there are around 4541 species (Bachli, 2019). In India Drosophilid fauna is around 319 and much of the study is concentrated on the genus *Drosophila* which encompasses around 149 species (Kandapal and Singh, 2010). Particularly in South India, there are only 50 species of *Drosophila* (Hegde *et al*., 2001). Later three more rare species were reported (Srinath and Shivanna, 2014, 2017), whereas a detailed faunal survey of Drosophilidae has not been done from all the places of Karnataka. Present survey reports *Chymomyza vaidyai* Okada for the first time from South India.

Materials and Methods

Flies were collected from different spots of Dharwad market using net sweeping method. Later the flies were separated according to their sexes. Females were cultured individually in vials containing wheat cream agar medium, prepared according to the procedure described by Shivanna *et al.* (1996) and males were directly used for identification of species.

Results

The morphological characters are as mentioned below.

Chymomyza vaidyai Okada 1976 (Figure A)

Body length: Male – 2.54 mm

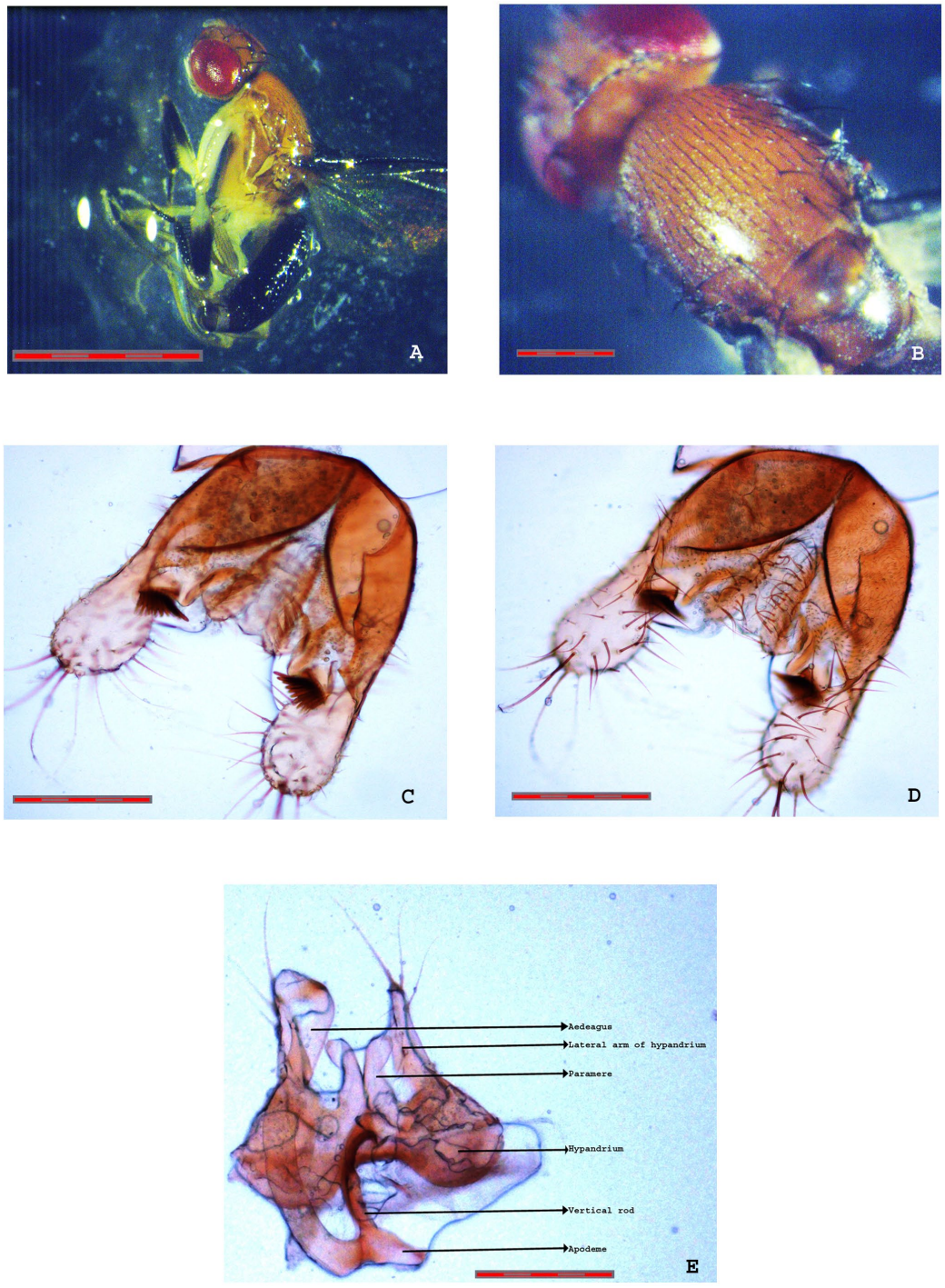

Figure 1. *Chymomyza vaidyai*. A, lateral view of male; B, dorsal view of thorax; C and D, periphallic organ; E, phallic organ. Scale: Figure A – 1 mm; B – 0.4 mm; C, D, and E – 0.2 mm.

Head: Arista plumose with 9 branches – 3 above, 2 below and 4 inner branches except terminal fork. Eye color red; orbital bristles – 1st and 3rd reclinate, 2nd proclinate and very close to 1st. Ocellar triangle slightly darker, postocellar setae present and smaller than ocellar setae, there are extra pair of bristles present in between the ocellar triangle just before ocellar setae. Inner and outer vertical bristles are present, frons yellow;

gena not prominent. Carina broad and bulged posteriorly, depressed centrally. Vibrissae and sub-vibrissae are similar in size.

Thorax: Yellow in color. Presence of 6 irregular rows of acrostichal hairs in between two pairs of dorsocentral bristles (Figure B). Scutellum is yellowish in appearance; basal scutellar bristle present and are convergent. Supra-alar and post-alar bristles are present. Sternum has two bristles, posterior is larger than anterior. Sterno –index is 0.63.

Legs: Femur partially dark. Tibia is dark. Dorsal segments are white and portion before femur is also white. Femur has few prominent bristles on forelegs.

Wing: Presence of white patch apically; costa and C-cell black.

Wing indices calculated according to Okada (1956)

C index	4V index	4C index	5X index
1.5	1.7	1.1	3.4

Abdomen: Tergites dark; sternite white.

Periphallic organ: Genital arch is brown and narrower in the middle, lower portion bulbous with about 28 bristles, upper portion pubescent elliptical. Primary clasper with 9 to 10 teeth; secondary clasper is absent. Anal plate is oval and reduced because of elliptical nature of genital arch on the upper side, pubescent and separate (Figure C and D).

Phallic organ: Aedeagus is curved gently and truncated apically. Apodeme is short; aedeagal structure is connected to hypandrium by vertical rod. Hypandrium is broad basally on either side and curved in the median region. Parameres are almost the size of lateral arms of hypandrium (Figure E).

Place of Collection: Dharwad vegetable and fruit market; 15°.60 N and 75°.01 E.

Specimens examined: 2 ♂, INDIA: Karnataka, Dharwad. 2♂ preserved, Coll: Anurag Samaje

Distribution: Pune (Vaidya and Godbole, 1973; Okada, 1976), Dharwad, South India (new report).

Discussion

About 30 species of *Chymomyza* have been described from various parts of the world (Okada, 1976). The species reported in the present study, *C. vaidyai*, was first described by Vaidya and Godbole (1973) from University of Poona as *C. pararufithorax* sp. nov. and later redescribed by Okada (1976). However, original description by Vaidya and Godbole (1973) indicates that the described species is not of *Chymomyza* but of *Lissocephala* (personal communication with Prof. M.J. Toda). Hence in the present study the name of the species is retained as *Chymomyza vaidyai* Okada, 1976. From the description of Vaidya and Godbole (1973) and Okada (1976) the fly consists of 8 irregular rows of acrostichal hairs whereas the present species consists of 6 irregular rows (Figure B). 1st abdominal tergite is yellow as mentioned by Vaidya and Godbole (1973); in the present species all the abdominal tergites are dark. Scutellum is darker as mentioned by Okada (1976), whereas in the present species it is dark in the median. Sterno index is 1.1 as mentioned by Vaidya and Godbole (1973) and 0.8 according to Okada (1976), whereas in present species it is 0.63. The present species also has a pair of minute bristles present in between the ocellar triangle which is absent in specimens described by above authors. Except these all the other characters described are similar.

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Microsatellite variants in two closely related species of *Drosophila*: *D. bipectinata* and *D. malerkotliana.*

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Studies conducted on *Drosophila bipectinata* species complex have now well established that the four species coming under this complex, *D. bipecinata, D. malerkotliana, D. parabipectinata*, and *D. pseudoananassae*, are closely related and share a number of genetical characteristics (Bock, 1971; Bock and Wheeler, 1972; Matsuda *et al.*, 2005; Tomimura *et al.*, 2005; Mishra and Singh, 2006; Banerjee and Singh, 2012, 2016, 2017; Singh, 2019). Two species of this complex, *D. bipecinata* and *D. malerkotliana*, have been utilized comparatively more than their other two allies for their genetical, behavioral, and evolutionary analysis only because they have a wide distribution in the Indian subcontinent (Kopp and Barmina, 2005; Singh, 2017; Singh, 2019). These two species have been studied for their chromosomal polymorphism to a considerable extent to see the level of genetic differentiation in their natural populations (Gupta and Panigrahy, 1990, Das and Singh, 1992; Banerjee and Singh, 1996; Singh and Singh, 2015, 2016, 2018, 2020). However, polymorphism at the level of nucleotides has not been undertaken in these two species. Microsatellites are tandem repeated sequences of DNA having 1-9 bp repeats represented about 5-60 times (Litt and Luty, 1989). These repeats are also referred to as Simple Sequence repeats (SSRs) or Short Tandem Repeats (STRs). Microsatellite analysis has now become one of the most popular and effective genetic markers in the field of evolutionary and population genetics to measure genetic variation between the populations of a species. Microsatellite markers have high levels of polymorphism and presumptive selective neutrality makes them the best tool for investigation of population structure, migratory trends, temporal and spatial population dynamics, and evolutionary patterns. Bettina and Christian (2004) studied population genetic aspect at the molecular level in four species of *D. melanogaster* species complex and were able to amplify forty-seven microsatellite loci. They observed that its two cosmopolitan species, *D. melanogaster* and *D. simulans*, were the most variable followed by *D. mauritiana* and *D. sechellia*. Schug *et al.* (2004) isolated and characterized eighty five dinucleotide repeat microsatellite sequences and developed assay conditions for genotyping using PCR in *D. ananassae*. They have also mentioned cross - species PCR amplification of microsatellite repeats in a number of *Drosophila* species. In this short communication, we are reporting the microsatellite variants observed in the natural populations of *D. bipecinata* and *D. malerkotliana* by employing four different microsatellite primers.

DNA was extracted from a single fly, randomly selected from the population of a species. The main chemicals and the major steps employed to do this were as follows:

Required Chemicals:

Squishing buffer: **1.** 10 mM Tris-HCl (pH 8.2); **2.** 1 mM EDTA; **3.** 25 mM NaCl; and **4.** Proteinase K: 20 mg/ml stock

Procedure

1. Prepared squishing buffer (SB), 10 µl/ml Proteinase K was added to the SB.
2. Added 5 µl of SB into the PCR tube. Placed a single fly into the tube, mashed it (homogenate) with a 200 µl pipette tip.
3. Added 45 µl of SB in the PCR tube.
4. Incubated it at 30-37°C for 1 to 2 hours.
5. Inactivated the Proteinase K by heating the content at 95°C for 3-5 minutes.
6. Centrifuged the content for 10 minutes at 12000 rpm.
7. Transferred the supernatant (genomic DNA) into a fresh PCR tube, stored extracted DNA at -20°C.

This preparation can be stored for longer duration if kept at -20°C. Usually 4 µl of DNA preparation is used in a 20 µl reaction volume (10 µl master mix + 2 µl primer + 4 µl MQ water + 4 µl DNA). PCR product generally starts appearing after 24-25 cycles, but 30-35 cycles give maximum yield. In the present study, PCR amplification was done for 35 cycles. Annealing temperature was standardized for individual primers and the most appropriate temperature that yielded satisfactorily for specific primer is depicted in Figure 1. The nucleotide sequences (forward and reverse) of four different primers used in the present study to get microsatellite variants of both species is given in Table 1. Two percent agarose gel was used to differentiate microsatellite variants.

Table 1. Showing the nucleotide sequences (forward and reverse) and number of loci of four different primers used to get microsatellite variants of both species.

S.N.	Primer (5′------- 3′)	No. of loci
1	DAN45: F- AGCGGGCCAGTGACAAAA R- TAGCAAACAAAACGCTCACC	1 (both species)
2	DAN73: F- TGACACATACCAATCTATTCACACC R- TATTGGCAGCACTGTGGAAA	1 (both species)
3	DAN76: F- CGTGCGTATGTGAGTGTGTG R- CCCTTTATTCGCCATCATGT	2 (both species)
4	DAN79: F- CTAAACAGCGTCGGTCCTCT R- TCCAAAAGTATCTGTGGCTGTG	1 (*D. bipectinata*) and 2 (*D. malerkotliana*)

We followed the information provided in the research publication of Schug *et al*. (2004) pertaining to isolation and characterization of dinucleotide repeat microsatellites in *Drosophila ananassae*. The four primers chosen in this study have also been mentioned in this paper and have been shown to be effective for amplifying microsatellite segments in the four species that belong to *D. bipectinata* species complex. The preliminary studies indicate the expression of five gene loci in *D. bipectinata* and six loci in *D. malerkotliana*. Each locus of these loci of both species is polymorphic in nature because more than single variant has been

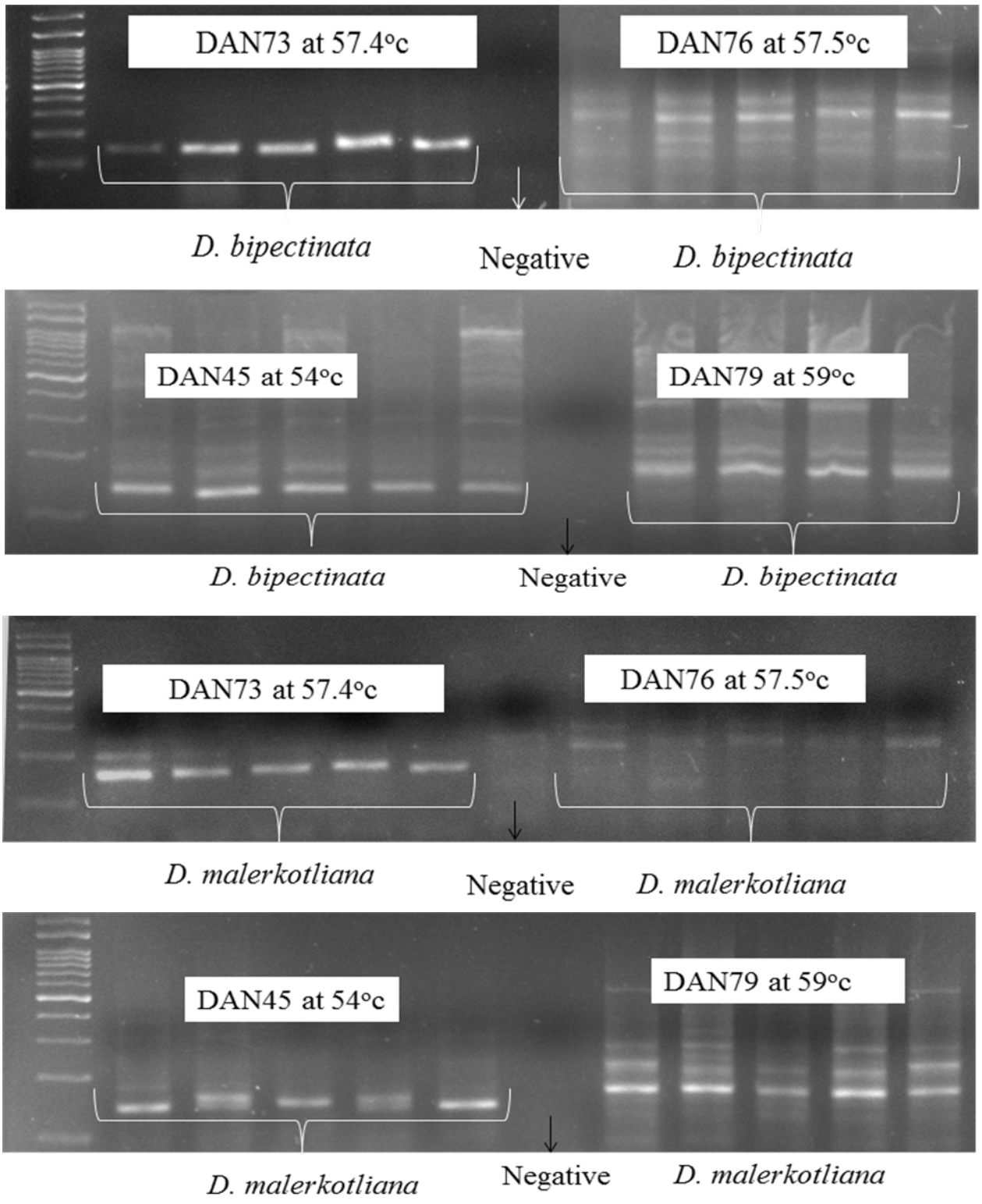

Figure 1. Agarose gel showing primers, their annealing temperatures and microsatellite variants in *D. bipecinata* and *D. malerkotliana.*

observed for them. Inspection of these microsatellite bands also indicates considerable level of heterozygosity. Further, comparison of the microsatellite bands of these two species obtained by using a specific primer do

indicate similarity between them that in itself is an indication of phylogenetic closeness between *D. bipectinata* and *D. malerkotliana*.

Acknowledgments: We are grateful to our laboratory research team for their help and discussion regarding the results of this study. Funding for this research has been done by University Grants Commission (UGC) and Indian Council of Medical Research (ICMR), New Delhi, in the form of research fellowship to GS.

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Diversity of Brazilian savanna drosophilids in two forest fragments with contrasting degrees of conservation.

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Introduction

Diversity distribution patterns vary across time and space because of environmental heterogeneity. The Brazilian savanna, also known as Cerrado biome, holds a complex of very rich vegetal formations exposed to intense seasonality, and to high rates of anthropogenic changes (Klink and Machado, 2005). Thus, this biome is an excellent system to evaluate the effects of environmental heterogeneity on diversity of resident species.

Although gallery forests occur naturally in the Brazilian savanna as patches, the high rates of destruction of theses environments have contributed to the loss and further isolations of the remaining areas. These forests occupy only 5% of this biome, but they harbor the majority of its animal and plant species (Mittermeier *et al*., 2005). Therefore, this high diversity is associated with current severe threats (fragmentation, deforestation, and fire), generating the need for local studies focused on biodiversity monitoring and conservation planning of the gallery forests.

In the Brazilian savanna, drosophilid populations fluctuate over time and space (Tidon, 2006). Neotropical drosophilid populations increase in frequency during rainy seasons; exotic drosophilids, in turn, dominate stressful weather periods. On the effects of spatial heterogeneity on such organisms, studies conducted in protected areas suggest that neotropical drosophilids are more frequent in highly heterogeneous ecosystems, for example, in gallery forests; exotic drosophilids have been abundant in less complex or relatively degraded plant formations (Tidon, 2006; Mata *et al*., 2008, 2010). Thus, the drosophilids of the Brazilian savanna represent a suitable model for investigating the impact of environmental heterogeneity on biodiversity. As studies investigating variation in drosophilid assemblages across scales of different levels of spatial heterogeneity while considering the same habitat type is still lacking in the Brazilian savanna, the results of this study can improve the understanding of the effects of spatial heterogeneity on such animals.

In this study, we collected Brazilian savanna drosophilids in two forest fragments with contrasting degrees of conservation to understand how such spatial variations affect the diversity of local drosophilids. Specifically, we identified (1) the local drosophilid fauna and (2) differences in the diversity of drosophilids associated with these forests to answer the following questions: i) Are the patterns of richness and abundance of drosophilids similar between the researched forests? and ii) Does the composition of drosophilid species differ between such forests?

Material and Methods

We conducted this study in Paracatu, a town located in the northwestern part of Minas Gerais State, 230 km from Brasília, the capital of Brazil. According to Köppen's climatic classification, Paracatu's region belongs to the type Aw—humid tropical savanna climate, with dry winter and rainy summer. In this town, we selected two contrasting forest fragments. Here, we treated the fragment located in downtown Paracatu as a quite degraded forest (QDF), because it is surrounded by urban areas and has grasses, clearings, and a river bed in an advanced silting process in its interior. The second forest fragment, located approximately 5 km from downtown, was considered by us as a relatively preserved forest (RPF), because it is surrounded by a preserved savanna matrix, and presents a diverse vegetation of shrubs and trees bordering a riverbed (Figure 1, drawn in QGIS 3.0-QGIS Development Team, 2019).

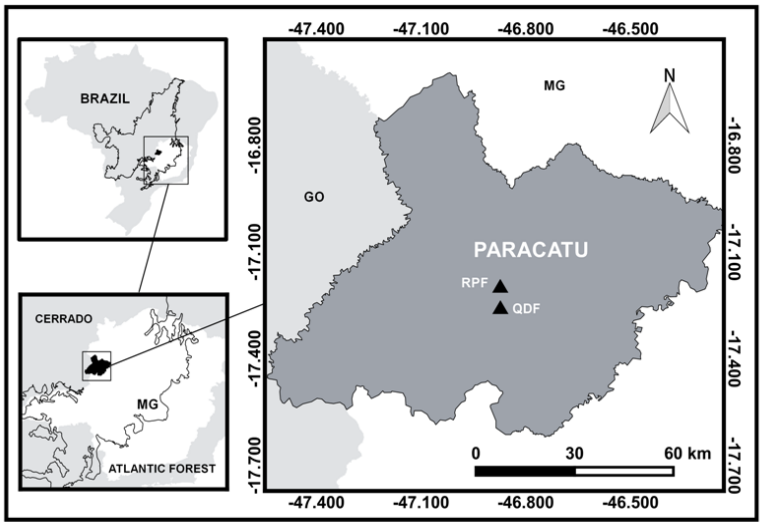

Figure 1. Location of the studied area. **MG**: Minas Gerais states; **GO**: Goias states; **QDF**: quite degraded forest; **RPF**: relatively preserved forest.

We performed four collections of adult drosophilids from October to December 2014. In each collection, we evaluated the interior of each forest fragment through five retention traps (Roque *et al.,* 2011) separated by at least 30 m. The traps were baited with fermented bananas and left inside the forests for three consecutive days.

Captured flies were specifically identified and separated as exotic (EXO) or neotropical (NEO) species. We deposited voucher specimens preserved in ethanol 70% at the Collection of the *Laboratório de Biologia Evolutiva* of the *Universidade de Brasília* for comparisons.

To evaluate the patterns of richness and abundance of drosophilids between the researched forests, we constructed a frequency table of the species collected in each forest and calculated the heterogeneity (Shannon Index - H') and the evenness (J) of these assemblages. To evaluate the composition of drosophilid species, we first characterized the proportions of EXO and NEO between forests by graphics of abundance and then evaluated differences in these proportions using the Chi-squared test (Contingency Table). All analyses were conducted in Past 2.16 (Hammer *et al*., 2001).

Table 1. Brazilian savanna drosophilids associated with two forest fragments with contrasting degrees of conservation. Data collected between October and December 2014 in Paracatu, MG, Brazil. QDF: quite degraded forest; RPF: relatively preserved.

	Collections										
	25/Oct		22/Nov		9/Dec		18/Dec		Total		
Drosophilids	QDF	RPF	QDF	RPF	QDF	RPF	QDF	RPF	QDF	RPF	Total
*Drosophila simulans**	X	—	X	—	X	X	X	X	1,705	25	1,730
*Zaprionus indianus**	X	—	X	—	X	X	X	X	1,009	216	1,225
D. nebulosa	X	—	X	—	X	X	X	X	333	157	490
*D. malerkotliana**	X	—	X	—	X	X	X	X	307	88	395
D. willistoni	—	—	X	—	X	X	X	X	14	30	44
D. sturtevanti	—	—	—	—	—	X	X	X	3	41	44
D. cardini	X	—	X	—	—	X	X	X	24	10	34
*D. ananassae**	X	—	—	—	—	—	—	—	24	—	24
D. saltans	—	—	—	—	—	—	X	X	2	5	7
D. mercatorum	X	—	—	—	—	X	X	X	4	2	6
*D. immigrans**	—	—	—	—	—	X	X	—	1	4	5
D. fumipennis	—	—	—	—	—	X	—	X	—	3	3
D. polymorpha	—	—	—	—	—	—	X	X	1	2	3
D. mediostriata	—	—	—	—	—	—	X	—	2	—	2
Rhinoleucophenga punctulata	—	—	—	—	—	X	—	—	—	2	2
D. ararama	—	—	—	—	—	—	X	—	1	—	1
D. cardinoides	—	—	—	—	—	—	X	—	1	—	1
D. hydei	X	—	—	—	—	—	—	—	1	—	1
D. neocordata	—	—	—	—	—	—	—	X	—	1	1
D. ornatifrons	—	—	—	—	—	—	X	—	1	—	1
*Scaptodrosophila latifasciaeformis**	—	—	—	—	—	—	—	X	—	1	1
Total	1,702	—	147	—	237	252	1,347	335	3,433	587	4,020
Shannon index (H')	—	—	—	—	—	—	—	—	1.278	1.728	1.439
Evenness (J)	—	—	—	—	—	—	—	—	0.211	0.375	0.2

**exotic species*

Results

Overall, we caught 4,020 drosophilids representing 21 species of the genera *Drosophila*, *Rhinoleucophenga*, *Scaptodrosophila*, and *Zaprionus*. All collections performed in QDF produced results (3,433 drosophilids belonging to 17 species), while in RPF only two did (587 drosophilids belonging to 15 species). In both forests, *Drosophila* was the genus with the highest number of species (QDF = 16; RPF = 12). *Drosophila simulans* was the most abundant species in QDF (N = 1,705), while in RPF, this was *Zaprionus indianus* (N = 216) (Table 1). Moreover, RPF recorded higher heterogeneity (H' = 1.73) probably due to a more homogeneous distribution of species abundances (higher evenness). Therefore, the drosophilid diversity varied between forests.

Although the total species considered NEO was always higher than EXO in both forests, the total abundance, in turn, changed. EXO species accounted for approximately 90% of the total abundance in QDF

and approximately 60% in RPF. Because of this, the species composition also varied between our focal forests (Chi-squared = 379.36; df=1; $p = 0.0001$) (Figure 2).

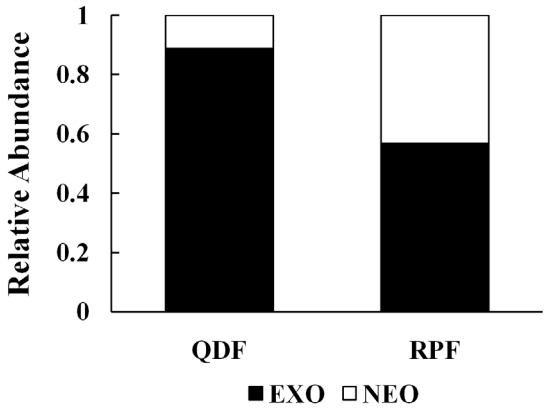

Figure 2. Relative abundance of exotic (EXO) and neotropical (NEO) drosophilids collected in two forest fragments with contrasting degrees of conservation: a quite degraded forest (QDF) and a relatively preserved forest (RPF). Data collected in Paracatu, MGs, Brazil, from October to November 2014.

Discussion

The drosophilids of Paracatu were initially inventoried by a research team of the *Laboratório de Biologia Evolutiva* of the *Universidade de Brasília* (Chaves and Tidon, 2008). Such survey registered *Drosophila busckii*, *D. cardini*, *D. fuscolineata*, *D. hydei*, *D. immigrans*, *D. maculifrons*, *D. mediostriata*, *D. mercaturum*, *D. meridionalis*, *D. nebulosa*, *D. nigricruria*, *D. polymorpha*, *D. prosaltans*, *D. simulans*, *D. sturtevanti*, *Scaptodrosophila latifasciaeformis*, and *Zaprionus indianus*. This study collected eleven of the species previously recorded and added the following drosophilids to the list: *D. ananassae*, *D. ararama*, *D. cardinoides*, *D. fumipennis*, *D. malerkotliana*, *D. neocordata*, *D. ornatifrons*, *D. saltans*, *D. willistoni*, and *Rhinoleucophenga punctulata*. Thus, the current drosophilid fauna of Paracatu consists of 27 species.

The richness and abundance patterns of the drosophilids varied between the researched forests. While the total species richness was similar in both forests, the abundance of individuals was extremely higher in QDF. As many ecosystem disturbance are prevalent in QDF, our results support the idea that environmental degradation interferes with the diversity of living beings in space and that drosophilids are excellent models for assessing the impacts of anthropic transformations. In this scenario, populations become more susceptible to extinction (Kageyama *et al.,* 1998), contributing to the reduction of the environmental heterogeneity of the Brazilian savanna.

The drosophilid species composition also differed between the studied forests. Since EXO were numerically higher in QDF, where they accounted for approximately 90% of the total abundance, our data support the idea that exotic species are more common in disturbed environments (Ferreira and Tidon, 2005; Mata *et al*., 2008). Such dominance occurred because of the high population peaks of *Drosophila simulans* (N = 1,705) in this forest. Studies conducted in contrasting neotropical ecosystems also observed a positive association between the abundance of exotic species and the degree of ecosystem disturbance (Tidon, 2006; Mata *et al*., 2008; Garcia *et al*., 2012; Poppe *et al*., 2014). Thus, forest encompassing the urban matrix may have lost most of its environmental quality.

Conclusions

This study was conducted in gallery forests of the Brazilian savanna non-protected area and in the transition between the seasons of this biome. In this context, it revealed a significant reduction in abundance of native species in QDF. In view of these facts, we reinforce the importance of preserved environments to maintain the biodiversity of neotropical drosophilids. Thus, we suggest that altered forests be targets of conservational programs and that management procedures be developed to avoid additional reductions in their size in order to restore their role of maintaining the neotropical biodiversity. To conclude, we recommend the

protection of biological resources in still-unprotected forest environments, whose loss or alteration may contribute to a reduction of the heterogeneity and biodiversity of the neotropics.

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Reversion of the negative effects of social isolation on morphological, physiological, and conductual development in *Drosophila melanogaster* larvae.

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Introduction

Our team reported that third-stage larvae of the *D. melanogaster* species bred in social isolation were lighter, smaller, and had a very different locomotive behavior to group-raised larvae, genetically very similar (Del Pino, 2018). Once the adverse effects of social isolation have been verified, we wonder if it was possible to reverse the negative effects of social isolation on larval development, modifying the conditions of the breeding substrate, adding stimuli from the presence of congeners.

Material and Methods

We breed in 4 cm diameter Petri dishes, isolated larvae (n = 30). Then, we subjected these treatments to four parenting environments: (i) Burdick's compact culture medium, (ii) Burdick's raked medium, (iii) plastic rods buried in the culture médium, and (iv) medium with chemical signals of congeners. Our goal was to understand the effect of decompressing the culture medium, body contacts and the presence of chemical signals generated by congeners on the larval development of *D. melanogaster*. At 96 hours of development we proceeded to weigh (g), measure the length (mm), and record the larval locomotive activity.

Results

Our results (Figure 1) show that socially isolated larvae in a raked environment, in the presence of inert objects and congener chemicals, increase significantly their weight and larval size with respect to larvae raised in a compact environment, without odor or tactile stimuli (Figures 1 A and B) (ANOVA, $F_{1,156} = 129$; $P < 0.0001$). These results are in accordance with what Dabrowsky reported in the sense that *Drosophila*

larvae need other larvae to excavate the breeding substrate and thus obtain more food (Dombrovski, *et al*., 2017).

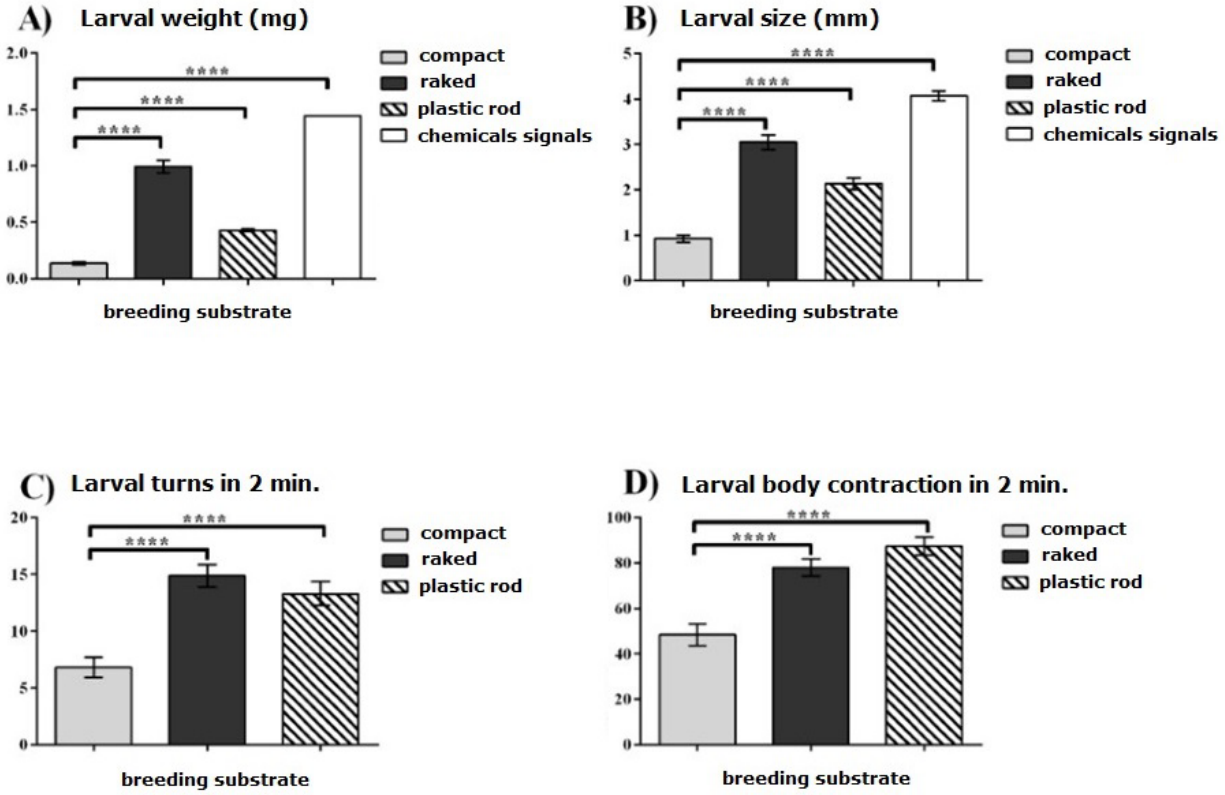

Figure 1.

On the other hand, isolated larvae in raked medium and plastic rod medium have a locomotive behavior far superior to those obtained in the absence of those stimuli (Figures 1 C and D) (ANOVA, $F_{1,156} = 262$; $P < 0.0001$).

These findings show the importance of social interactions as sources of sensory stimulation necessary for adequate larval development in *D. melanogaster* and for living beings in general. In this research we show that it is possible to reverse the negative effects of social isolation, sensory stimulation of isolated individual.

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Taxon F of the *Drosophila nasuta* subgroup and its mating behavior.

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The *Drosophila nasuta* subgroup has been the focus of hundreds of research publications, including many degree theses. The reasons for this include its recent origins, its high number of species and subspecies showing a great deal of inter-fertility, its complex pattern of sympatry and allopatry, its diverse visual mating behaviors, and its complex genetics. The last point includes inter-chromosomal fusion and very high

frequencies of floating inversions in some taxa, but not others, and the sharing of inversions between taxa that are not immediate relatives. In addition, its heterochromatin rapidly remodels, and all this within a clade that is only a couple of million years old.

Major collecting of this subgroup along with studies were undertaken by Wilson and colleagues (Wilson *et al.,* 1969) and Kitagawa and colleagues (1982). On the first set of cultured stocks, Spieth (1969) described the behaviors he saw. Spieth is well-known for his studies of mating behavior in *Drosophila* and was a student of the widely known sexologist Alfred Kinsey. From 1971 to 1982 Kitagawa and colleagues collected what appeared to be a number of new species or subspecies, which they called Taxon F to J. Taxon G from New Guinea was described as *D. niveifrons* by Okada and Carson (1982). Since then, specimens that appear to be very similar have been collected in adjacent areas, such as North Eastern Australia (McEvey and Bock, 1982). In correspondence with Dr. H-Y. Chang, it was reported Taxon H may have been a stock of *D. pulaua* from New Guinea, which is well beyond its otherwise recorded range.

Taxa I (from New Guinea) and J (New Caledonia) appear to be close relatives of *D. pallidifrons* from Ponape Island. However, their inter-fertility with each other and *pallidifrons* is markedly lower than between stocks of *pallidifrons*, or indeed, nearly all other crosses within taxa in the subgroup (Asada and Kitagawa, unpublished in Kitagawa, 1991) including some crosses between recognized taxa. In addition, Waddell (1990) notes differences in behavior that are larger than those typically seen within stocks of the same species in this subgroup, although only one stock of I and J was available. Finally, Taxon F from Borneo appears to be a quite distinct lineage (Kitagawa *et al.,* 1982; Kitagawa, 1991). It has no pollinosity on the frons, and thus is morphologically like *D. pallidifrons*, but in terms of many other characteristics, it appears markedly closer to *D. kohkoa* (Kitagawa *et al.,* 1982; Waddell, 1990, Kitagawa, 1991).

Professor Kitagawa died following his retirement in the 1990's without completing his studies of these taxa. He kindly shared stocks in the 1980's and this is a description of the behavior of Taxon F. This is important, as it is the best and perhaps the only sure way to identify the species.

The behavior of Taxon F

The majority of this report is based on Waddell (1990), itself based on 3 years of observations including stocks of all known taxa within the subgroup. The thesis meets all requirements including an expert examiner external to Auckland. Descriptions of visible behavior are more detailed than those in Spieth (1969) and other publications of *nasuta* subgroup behavior. All stocks received and examined conformed to morphological and behavioral expectations; there was no evidence of any stock mislabelling or mixing at any time. During this study, all stocks were fully and exclusively maintained by the author, including media preparation, and flies were in excellent health for these observations.

The description of the behavior of this species is based on two iso-female stocks labelled B-208 and B-223, wild caught iso-female lines collected 6/6/1971 at Kuching, Sarawak, Malaysia on the island of Borneo. Behavior was observed in a variety of settings such as single pairs in a vial. However, the preferred way to obtain large numbers of detailed observations were 10 to 15 pairs of virgin flies aged 5 to 7 days in unscratched 25 × 100 mm glass vials, with a thin film of media run down the side. Observations were made in the first three hours of the day (during which most mating behavior occurred), and then throughout the day as activity was noticed. These were backed up with a wide range of observations of flies housed in the culture/breeding bottles. These were new highly transparent polyethylene bottles, with a 100 mm wide base, about 120 mm high and a 30 mm throat. Extensive use of a binocular microscope at 10× power greatly facilitated observation of fine details.

One of the reasons these observations were so prolonged and detailed was that, concurrently, the behavior was being broken down into components for scoring in a cladistic data matrix. Thus, if a new character or variation of a character was observed (or a recoding of characters was considered), it would be rechecked in detail in stocks of all species to achieve operational homology, preferably with side-by-side direct comparisons.

Taxon F: The most common wing display

The male would tap the female then promptly orient himself to a position at the perpendicular rear of the female. The male would initiate his main wing display (display 1) by extending the wing nearest the female's head out 70 to 90 degrees simultaneously vibrating it with a large vertical amplitude of +/-17.5 to 30 degrees. Each burst of vibration lasted ~0.4 to 0.8 seconds. There followed a short pause of similar duration followed by another burst of vibration. Anything from 2 to 20 bursts of vibration would constitute an uninterrupted sequence. While the male was vibrating his wing he would (proportion of displays showing this ~0.3, frequency within this display type 40%) vibrate (rapidly tap) one of his forelegs against the female's abdomen.

Following a sequence of wing vibration the male would pause, at this same position for 2 seconds or more before initiating another bout of wing vibration. Alternatively he would break off contact with this female, usually immediately inspecting another nearby female. Only rarely would he crab-walk to a mirror image position on the opposite side of the female. When he did so, he invariably walked about the rear of the female (never in front of her) at a moderate to slow pace while simultaneously rhythmically bobbing his body up and down in large amplitude. During the pause before and after the completion of these short walks, and occasionally while pausing between sequences of wing displays, the male would bob his body up and down with moderate amplitude.

Other types of wing display

In addition to the above display, two other displays (or major variations of the above) were shown by males of this taxon. The first of these (display 2) usually occurred after a considerable amount of courtship interaction, often later in the day, usually beyond 2 hours since dawn. At these times males would (Prop. 0.25, freq. from once per courtship up to 60% of all displays) show a much slowed example of wing vibration. The position that this behavior occurred at was variable, occurring at the perpendicular, diagonal or direct rear (a very similar form of wing movement was also seen in male *D. sulfurigaster albostrigata* and *D. s. neonasuta*). The male would extend one wing out (not always that closest to the female's head) 70 to 90 degrees, rotating it anti-clockwise 60 to 80 degrees. Maintaining the wing in this position the male would then vibrate it slowly up and down +/- 7.5 to 15 degrees. A considerable amount of the wing movement occurred in the horizontal plane due to the angle of wing rotation. The male would either leave the wing fully extended, or, at least, only partially retract it, before a subsequent identical bout of this wing display. More rarely the wing was extended but not vibrated, or else was moved in short jerky movements akin to the way *D. pulaua* males vibrate their wings. These last actions in Taxon F males typically occurred at a lower frequency of wing movement at approximately 1 beat per second. The occurrence of this behavior did not just start late, it also became more frequent as the day progressed. Mating behavior in this taxa was common for at least the first 4 hours, which is markedly longer than typical of *nasuta, albomicans, kepulauana* or the *sulfurigaster* clade.

The third type of wing display (3) which occurred at any time of the day was wing flicking. It occurred in a proportion of greater than 75% of all mating displays in this taxa. Wing flicking occurred at the perpendicular and direct rear positions and would at times occur alternately with wing vibration (of display type 1 mostly). The male repeatedly flicked the wing closest to the female's head quickly out 30 to 50 degrees, forming a sequence of 4 to 15 such actions in succession. The wing was either not vibrated or vibrated only in small amplitude. This wing-flicking action was very similar to wing flicking by *D. kohkoa* males. In Taxon F males, wing flicking was not observed to culminate in large amplitude wing vibration (*c.f.*, *D. kohkoa*). When a bout of wing flicking was completed, there was always a distinct pause before any further action (typically a bout of wing vibration or more wing flicking).

It was not uncommon to see a male, oriented to the perpendicular or direct rear of a female, showing the 2nd or 3rd types of main wing display while another male was oriented at the opposite perpendicular rear position performing the first type of wing display, to the same female. Wing display types (2) and (3) **were not** observed to directly elicit the female acceptance response. Wing flicking (3) was, however, often a part the mating behavior of males observed to copulate.

Wing displays intermediate between types (1) and (2) were not apparent during these observations. This adds to the evidence that these are truly different wing displays producing distinct mating sounds (rather than 2 simply being more lethargic examples of 1).

Males of this taxon rarely and briefly showed a behavior that appeared like the wing fluttering that *D. pallidifrons* males displayed. Due to its intermittent occurrence it was not possible to look at this behavior and side-by-side directly compare it with that in *D. pallidifrons*. Very little male to male interaction or conflict was observed when two or more males of this taxa were courting one female and indeed generally. This is perhaps due to the sedentary nature of the displays in this species.

Males of both Taxon F stocks showed very similar behavior in Waddell (1990). Interestingly, it now seems a stock of Taxon F may have changed. The stock labeled B-208 still looks fairly close to this description, but what seems to be a mislabeled stock of Taxon F (labeled *pallidifrons*, PNI-75) was observed showing aberrant behavior (and none of the defining features of *pallidifrons* which has a very different display performed in front of the female).

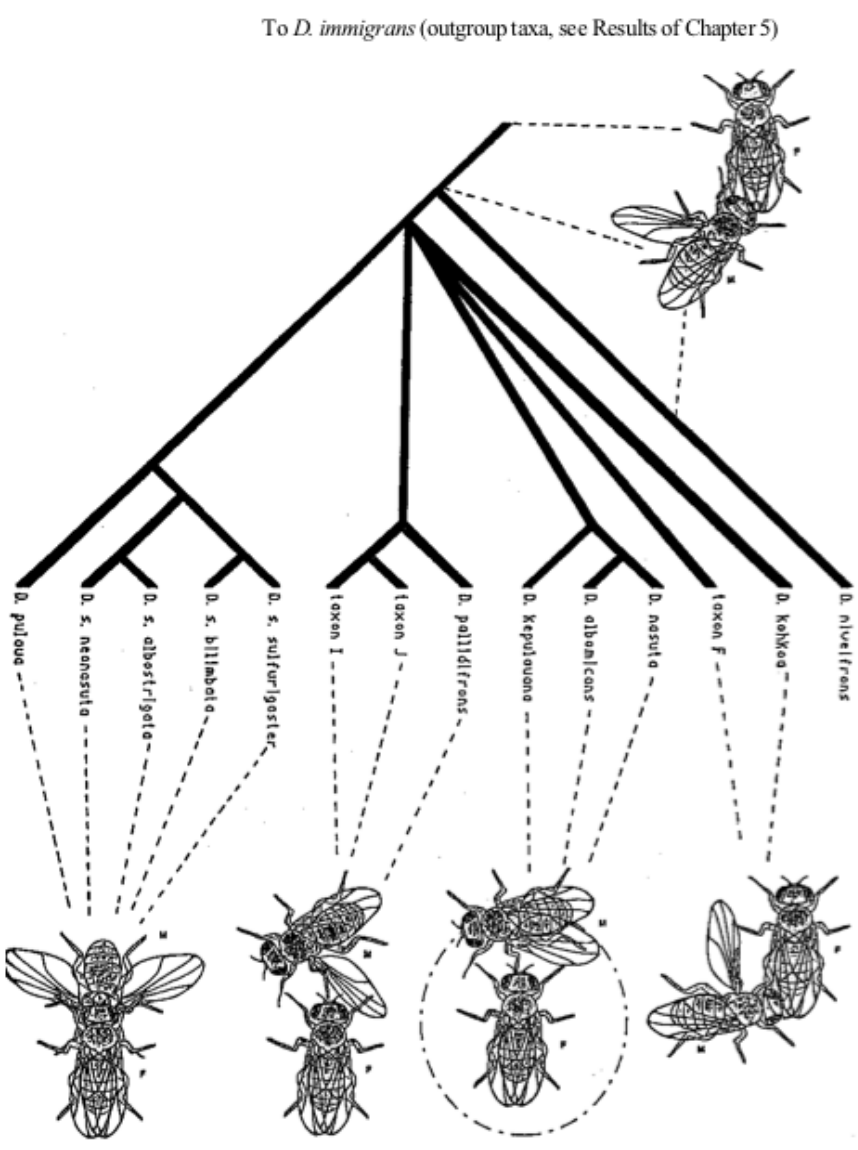

Figure 1. Reproduced from Waddell (1990) Figure 6.1: "General patterns of mating behavior for individuals of *nasuta* taxa (represented by males performing their main wing display) superimposed on the consensus tree produced in Chapter 5. Details of the mating behavior of *D. immigrans* come from Spieth (1952)." This tree agrees with the whole genome tree in Waddell *et al.* (this issue), except that *pulaua* should be sister to *sulfuri-gaster* and *bilimbata* alone, as it was in some of the most parsimonious behavioral trees. While the genome tree is an average, within Waddell (1990) there is good evidence of alternative arrangements consistent with introgression, and within the genomic data, there are signals of some of these alternative pathways of gene sharing (Waddell, Wang, and Jones, this issue and unpublished).

Phylogeny and behavior

In general form, or in specific details, Taxon F's behavior seems closest to that of *D. kohkoa*, with which it is both interfertile and will mate at average rates of about 50% when housed in confinement for some time. Only one other taxon within the *nasuta* subgroup has a similar overall behavior, also dominated by wing displays at the rear of the

female, and that is *D. niveifrons*. When behavior was broken down into discrete components, this same pattern emerged in the most parsimonious trees of the preferred encoding of the observations, that is *kohkoa* is the closest relative to Taxon F, and *niveifrons* was the next closest. *D. immigrans* served as an outgroup, its behavior shared features seen only in *niveifrons* in this subgroup (Waddell 1990). Overall, in Waddell (1990) it was assessed that *D. niveifrons* was the most likely sister to all other *nasuta* subgroup taxa. This has now been corroborated by whole genome sequencing and analysis (Waddell *et al.,* this issue). In alternative codings of the behavioral data, if the root put on the edge to *niveifrons* alone, then Taxon F appears sister to all other *nasuta* taxa, and then *kohkoa* branches off next deepest. Interestingly, in some encodings and analyses of the genomic data, this pattern is seen (Waddell *et al*., uupublished). Either way, a pattern of behaviors with the main wing display performed nearly exclusively at the rear of the female would seem to be the most likely ancestral form for the whole subgroup.

In terms of specific parts of the display, display type 2 is strikingly similar to the second main type of wing display in *albostrigata* and *neonasuta*, but not their closest relatives, *sulfurigaster*, *bilimbata*, or *pulaua*. That suggests the possibility of introgression between these lineages (Waddell 1990). Further, the wing fluttering behavior shared between Taxon F and the *pallidifrons* clade raises the possibility of shared derived features there also. It is doubtful that on average, across the genome, that *pallidifrons* is closer to Taxon F than *kohkoa* given the trees of Waddell *et al.* (this issue), but this snippet of behavior might be a shared derived feature given the propensity of the group for introgression. It is speculated that introgression may be responsible for lineages such as *albostrigata, albomicans, kohkoa*, and *kepulauana* sharing floating chromosome inversions (Waddell 1990), not just that these are unfixed ancestral polymorphisms (Wilson *et al.,* 1969; Lambert, 1978).

While I am unaware of any other written reports of the mating behavior of Taxon F, there is a study by Shao *et al.* (1997) that claims to have studied its acoustics, and they report that it produces no sound, which is surprising. The Taxon F stock they report on is U-88 from Miri, Sarawak, Malaysia; a location and stock I have not examined, yet it is geographically very close to Kuching, the source of the stocks described here. However, stock B-208 definitely does produce a range of courtship sounds (Kim and Waddell, unpublished).

Differences with *D. kohkoa*

While there are differences in how far out the wing is extended, the order of flicking actions versus wing vibration, and so on, one clear difference of these two species is that Taxon F very rarely crab walks about the female, and never in front of her. This “always facing the female crab-walking” is a common behavior of *kohkoa,* while *niveifrons* would also crab-walk in front of the female. While it is tempting to associate the lack of pollinosity on the forehead of Taxon F directly with this behavior, the phylogenetic correlation of aspects of behavior and morphology is weak. The other taxa in the group that do not have frontal pollinosity, the *pallidifrons* clade, do crab walk in front of the females eyes, and also stop right in front facing her. In contrast, *albomicans* and *nasuta* have full frontal pollinosity but rush about the female tangentially, never crab walking. Sometimes a male might stop his run facing the female, but would quickly reorient himself usually perpendicular to her rear. Some *nasuta* females responded to the males by allowing them to mate without their having stopped facing them, and thus not seeming to offer any clear glimpse of their pollinosity. In the case of Taxon F, both crab-walking in front of the female and pollinosity have been lost, but whether one triggered the other or if both were made redundant by a third factor in the display, such as an overwhelming role for “song”, is unclear. So too is why *nasuta* and *albomicans* lack a crab walk when they have full frontal pollinosity which is not fully displayed. Whether the brownish dorsal stripe along the pleura of the thorax picks up a consistent signaling role is also unclear, not least since not all taxa with it display it prominently to the female.

Conclusions

Taxon F is clearly a distinct lineage studied intensely by Professor Osamu Kitagawa. In terms of morphology it is very close to *D. pallidifrons* (described in Wheeler *et al.,* 1969). It seems the best way to reliably phenotypically separate it from other closely related taxa is on the basis of its mating behavior.

Interestingly, there have also been a half dozen or so reports of *nasuta* subgroup members without pollinosity in places such as Taiwan and the Philippines. In at least one instance a stock was brought into culture and studied (van der Linde, 2007). These flies readily interbreed with *sulfurigaster* stocks, suggesting it was in the *sulfurigaster* clade. It varied in factors such as starvation resistance and habitat distribution from *D. s. albostrigata* collected nearby, and it was suggested this may be due to changes in a single gene. Loss of pollinosity is probably much easier than the original changes required to first gain it, and probably also much more probable than reactivating its development once lost. Clearly, there is much to understand about the total diversity of lineages within this subgroup that takes on many aspects of being a species complex with potential pathways of gene flow between most members.

Acknowledgments: Sincere thanks to Prof. Osamu Kitagawa providing a wide range of stocks and his extensive efforts into understanding this exceptional subgroup of *Drosophila*. To Mayoshi Watada (Ehime), the UCSD stock center and the Corbin Jones Lab for materials to recheck old stocks. This work was partly supported by NIH grant 5R01LM008626 to PJW.

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Stock mislabelling and its impact on phylogenomic analysis in the *Drosophila nasuta* subgroup/species complex.

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Abstract

In the course of our 28 *de novo* genome study of the *nasuta*-subgroup of the *immigrans* species group, we have come to suspect that several stocks acquired from the major stock centers may have either been mislabeled or mixed up in other ways. This is a short note to indicate which stocks these are, how they were diagnosed, and how an alignment and assembly free (AFAF) phylogenomic analysis sheds further light on what probably occurred.

Introduction

While flies of this subgroup are quite distinct from their closest relatives such as the *immigrans* subgroup, *hypocausta* subgroup, and *neohypocausta* subgroup, identifying the specific species in the *nasuta*-subgroup faces several challenges, such as cryptic species. However, on the frons (the area between the eye orbits), there are three main patterns of pollinosity or shiny silvery reflective surface, like that on the sides of some of the *Zaprionus* species. This distinctive characteristic allows clumping into three groups of species, of which at least one (the *sulfurigaster* clade, Wilson *et al.,* 1969; Kitagawa *et al.,* 1982) appears to be monophyletic. However, perhaps the best way to categorize to species or subspecies level is by their sexual display behavior. The behavior of many species was described by Spieth (1969). A more extensive comparative analysis of all recognized lineages was made in Waddell (1990), including stocks of Taxon F, I, and J, three lineages not yet formally described, but collected by Osamu Kitagawa and colleagues (Kitagawa *et al.,* 1982; Kitagawa, 1991).

There were two major efforts bringing stocks of these species into long-term and accessible culture. The first were those of Wilson and colleagues in the late 60's (Wilson *et al.,* 1969). Many of these stocks have been available via the US Stock Centers. The other involved collecting in 1971, 1979 and 1981 via Kitagawa and colleagues (Kitagawa *et al.,* 1982). Some of these stocks persist in the Ehime collection and in individual laboratory collections such as Tokyo Metropolitan University. Altogether, in the 1980's, hundreds of stocks of diverse provenance were in culture, covering all known taxa resulting in dozens if not hundreds of research papers.

Active curation of these stocks—as often happens for less studied species—waxed and waned over the subsequent decades. Currently this species group is receiving renewed attention and ideally new isolates will be collected from the field. However, as much of the current work hinges on the extant stocks we believe that it is imperative that the current state of these stocks be assessed and curated.

Incongruent phenotypes among extant stocks

As part of a follow up to Waddell (1990), we received and cultured 23 stocks (all nominally of the *nasuta*-subgroup except for 5 outgroup stocks) from the University of California San Diego Stock Center in 2013 and 2014. Over the course of receiving and establishing these stocks, we noted several concerns. For example, a stock labeled *D. kohkoa* (15112-1771.04, Rizal, Luzon, Philippines, 1972) did not fit either the morphological or behavioral repertoire expected. Its behavior conformed to that of *D. sulfurigaster albostrigata* (Spieth, 1969; Waddell, 1990), which is practically inseparable from that of *D. s. neonasuta* (Waddell, 1990). Like Suzuki and Kitagawa (1990), we view *neonasuta* as a sub-population of *albostrigata*, and hence also a junior synonym. Further, rather than pollinosity across the whole frons, males of1771.04 have wide stripes of pollinosity on the frons along the orbits, exactly the morphology expected of *D. s. albostrigata.*

Another UCSD stock that was anomalous was a stock labeled *D. s. albostrigata* 15112-1811.06 from Luzon, Philippines. Unlike *nasuta* subgroup flies, which are mid-sized *Drosophila* with a honey brown color predominating, these flies were much larger with darker blackish grey tones. The behavior conformed to that of *D. siamana* of the *hypocausta* subgroup (Asada *et al.,* 1992).

Via the Ehime stock collection and Dr. Mayoshi Watada, five stocks were obtained of which three appeared anomalous. The first was labeled *D. niveifrons* from Lae in Papua New Guinea (O-30, collected 1979). This showed sexual dances specific to the *D. s. sulfurigaster* and *D. s. bilimbata* lineages, while both effectively show the same behavior (Waddell, 1990). While *bilimbata* is described as a sub-species, it might well be a human spread population, or series of populations, of *D. s. sulfurigaster*. The case for its synonymy, however, is not as clear cut as with *D. s. neonasuta*. The banding patterns on the frons of "O-30" was of the *D. sulfurigaster* type; quite unlike that of *D. niveifrons* which has silvery pollinosity across the frons. Justt avoid confusion, the stock of O-30 studied in Waddell (1990) was indeed *D. niveifrons*.

The stock labeled *D. pallidifrons* (PNI-75, Ponape, 1979) from Ehime did not show any pollinosity on the frons as was expected. However it did not show any of the distinct sexual behaviors of the *pallidifrons* taxa (*pallidifrons*, Taxon I and Taxon J) either. Its behavior was erratic, and infrequent, but what was

observed was consistent with Taxon F, the only other *nasuta*-subgroup lineage in culture at Ehime meeting the general morphological description. PNI-75, when supplied by Osamu Kitagawa, is listed in Waddell (1990) as being collected June 27, 1981, and Suzuki *et al*. (1990) indicate that all stocks of *pallidifrons* they analyzed were from 1981. Taxon F (labeled B-208) looked and behaved as expected (Waddell, 1990). This stock was communicated to me in 1989 (along with another stock called B-223) and both had collection details of Kuching, Sarawak (Borneo), Malaysia 6/6/1971.

Finally, it seems there was only one stock of Taxon J in captivity by the late 1980's and it was labeled Nou-98, collected August 4, 1981 in Noumea, New Caledonia. Its behavior is described in Waddell (1990) and is close to that of *D. pallidifrons*. The stock labeled Taxon J Nou-98 that arrived via the Ehime center, had pollinosity across the whole frons and showed the typical sexual behavior of *D. albomicans* and *D. nasuta*. The behavior of these last two species is not visibly different, nor do they show any evidence of assortative mating (*e.g*., Kim *et al.,* 2013). The main distinguishing innate feature is that *albomicans* has fused the sex chromosomes to the large autosomal chromosome three. In addition, these species are allopatric except for a hybrid zone in the region of North East India.

Estimates of the species tree using whole genome sequencing sheds further light

We are also using data on these taxa to develop robust and improved methods of assembly free and alignment free (AFAF) methods of phylogenetic analysis. These methods can help deconvolute problems of stock identification among cryptic species. The raw reads for these genomes are particularly well-suited to exploring AFAF analysis as they were all sequenced with the exact same chemistry, the same technology, on the same machines with mixed libraries and all have moderate to deep coverage. AFAF analyses have a number of advantages over assembly and alignment, including circumventing a range of ascertainment and other biases that can be difficult and/or time consuming to uncover.

We calculated a Neighbor-Joining tree using PAUP* (Swofford, 2000) based on Poisson corrected Jaccard index-based distances. These distances were based on a random sample of 100,000 21mers found in the raw reads using MASH (Ondov *et al.,* 2016). A minimum kmer frequency filter of 5 was used to screen out sequencing errors and low level contamination. Kmers of length 21 have a very low probability of occurring by chance (on average, well less than one in a million in these genomes).

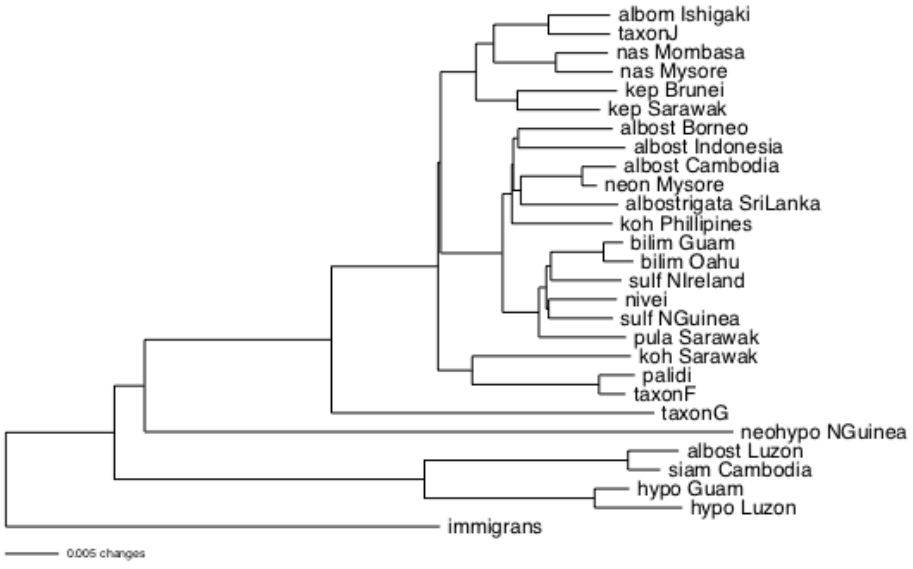

Figure 1. A Neighbor-Joining tree based on kmer distances.

Phylogeny corroboration with another approach

We validate this genome-wide species tree estimate with a novel character-based analysis we have developed. First, randomly choose a 21mer from all those present in all sequences of all stocks. Its presence (character C) or absence (A) is assessed across all stocks. To be kept that kmer must have a minimum frequency of 5 in at least one stock and maximum frequency of no more than twice the average coverage of that stock, across all stocks. Else, resample another kmer randomly. This was repeated until 2 million such kmers were kept. This matrix was

then analyzed in PAUP* using SVD quartets (Chifman and Kubatko, 2014). David Swofford has kindly extended the implementation of SVD quartets in PAUP* to deal with purine/pyrimidine DNA sequences, but here it works equally well for kmer presence/absence encoded in this way. The resulting tree with the results of 100 bootstrap replicates is shown in Figure 2.

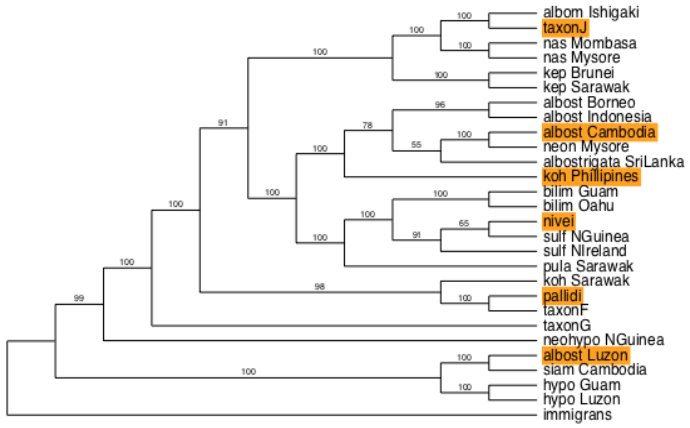

Figure 2. The SVD quartets-based tree from PAUP* based on 2 million randomly sampled 21-mers from across the genomes of these stocks. Support values are based on 100 bootstrap resamplings of the data matrix. Highlighted are the stocks we diagnose as mislabeled; in the additional case of *albostrigata* Cambodia, we suspect stock introgression.

This tree is mostly consistent with expectations based on the extensive phylogenetic analyses of Waddell (1990) analyzing a wide range of data and trees (see also the analyses of Kitagawa 1991). Note, later analyses, such as Yu *et al.* (1999) and Bachtrog (2006) appear compromised by mislabeled stocks and/or apparent mtDNA interspecies transfers (Waddell *et al.,* unpublished). The clade of *pulaua* + *sulfurigaster* + *bilimbata* seen in Figures 1 and 2 is consistent with some of the most parsimonious trees based on scoring behavioral attributes (Waddell, 1990). However, the very close association of *pallidifrons* with Taxon F is not consistent. Our best estimate based on the phylogenetic results in Waddell (1990) and Kitagawa (1990) is that, on average, the *pallidifrons* group branches deeper than the *kohkoa* + Taxon F association, but not as deep as Taxon G (a true *D. niveifrons* stock known by its old name). "On average" is an appropriate qualifier, as this subgroup/species complex is primed for between species introgressions (Waddell, 1990).

These trees confirm that morphological and behavioral clues reliably predict the true identity of these stocks. For *kohkoa* Philippines, the stock is clearly of the *albostrigata* lineage. Further, its location on our trees, which have multiple stocks from across the range of *albostrigata*, is biogeographically consistent with a stock of *albostrigata* from the Philippines (see also Suzuki *et al*., 1990). Further, as behavior and body form suggest, *albostrigata* Luzon is not of the *nasuta* subgroup and appears *prima facie* to be a stock of *siamana.* Whether it is consistent with a stock of *siamana* from the Philippines awaits a biogeographic analysis of *siamana* stocks.

Referring to our species trees and the case of the *D. pallidifrons* stock PNI-75 (not PN175 as some have written), the evidence, particularly the low level of genetic divergence, supports the hypothesis that this is a wrongly labeled stock of Taxon F. In the case of the Taxon J stock, it indeed appears to be a stock of *D. albomicans*. That the mtDNA of this stock reported in Yu *et al.* (1999) clusters with *D. albomicans* sequences suggests that the confusion of this stock may be quite early, but not as early as 1989 when this stock was observed to show a distinct behavior and morphology quite like true *D. pallidifrons* (Waddell, 1990). In the case of the stock labeled *D. niveifrons*, this indeed appears to be a stock of *D. s. sulfurigaster* or *D. s. bilimbata.* That this stock often clusters sister to a *D. s. sulfurigaster* stock from Wau in Papua New Guinea, suggests that the recorded collection locality of this mislabeled stock might indeed be correctly retained, that is, Lae, Papua New Guinea.

Concerns over the *D. albostrigata* Cambodia stock (15112-1811.04, Siam Reap, Cambodia, 1968) show up in Figures 1 and 2. Based on biogeography and the pattern of shared chromosomal inversions (Suzuki *et al*., 1990), *neonasuta* should be just another stock of *albostrigata* from southern India. That being the case, it is expected to be most closely related to Sri Lankan *albostrigata*. This is not the case and the Cambodian stock is of surprisingly low genetic differentiation from *neonasuta* Mysore; an anomaly worth investigating.

Diagnosing the anomalous *albostrigata*_Cambodia

In a situation like this, a look at the NeighborNet (Bryant and Moulton, 2004) representation can be very useful. NeighborNet measures discrepancies of the evolutionary distances between taxa to detect non-tree signals, such as those due to introgression or lineage mixing. It then aims to show these discrepancies in the form of a planner graph (a diagram, that can be written on a page without need of 3 or more dimensions to represent all predicted distances). For an explanation of how NeighborNet results relate to other tests of possible introgression, such as ABBA-BABA tests, see Waddell (2018). That article also describes how the genomic sequence of a single non-admixed individual can well represent its ancestral population in this type of analysis, due to its genome being many effectively unlinked genetic loci from just that population.

The NeighborNet of just the *sulfurigaster* group stocks seen in Figure 3 seems a very good representation of the data, showing over 99.975% of the variance in the data. It is clear that the largest non-tree splits involve *albostrigata* Cambodia showing genetic material similar to both *neonasuta* and *albostrigata* Indonesia. Consistent with this view, when this stock is removed from the analysis, the NeighborNet becomes much more tree-like (Figure 3). Now, the *neonasuta* stock segregates much more strongly with *albostrigata* SriLanka. This does not prove that there was stock contamination, for example, but is highly suggestive that either this occurred or the populations of *albostrigata* in Cambodia and/or southern India have a complex evolutionary history somewhat in contradiction to the biogeographic expectations and the structure seen with other sampled populations of *albostrigata*. Note, there are other non-tree signals also showing up, with the most prominent and biogeographically understandable being *pulaua* from Borneo apparently sharing more alleles with some *albostrigata* stocks than with others. These species are sympatric, interfertile and will mate with each other in confinement (Kitagawa *et al.,* 1982), introducing the possibility of interspecies introgression in the wild.

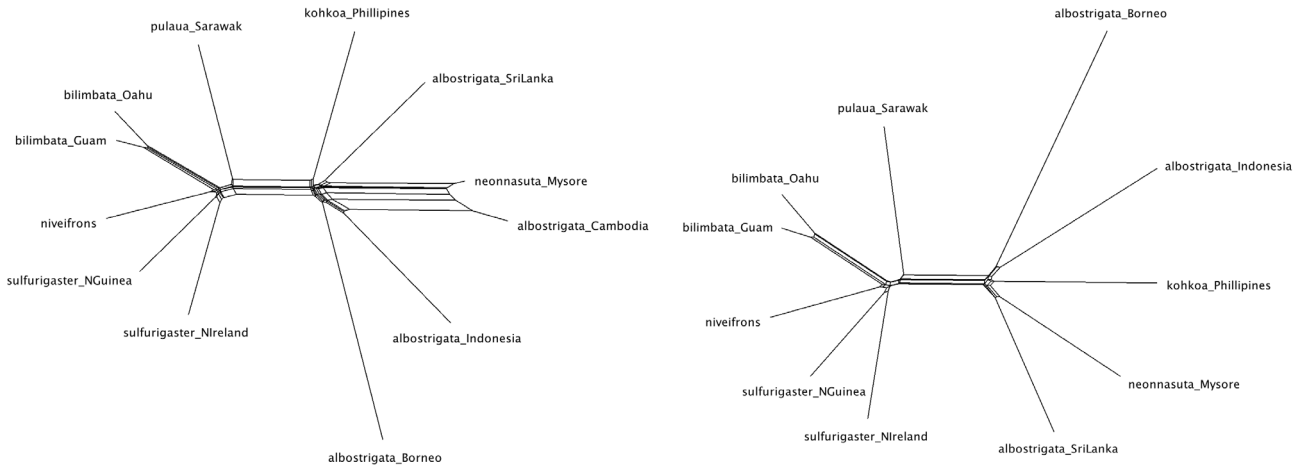

Figure 3. (Left) The NeighborNet, via SplitsTree4 (Huson and Bryant, 2016), of *sulfurigaster* group stocks based on whole genome 21mer Poisson corrected distances passing a minimum 5 and maximum 100 filter. The fraction of the total variance of the data explained by the graph is 0.99975. (Right) With *albostrigata* Cambodia removed. The fraction of the total variance of the data explained is 0.99973.

Conclusions

The diverse natural history, unique phenotypes, sexual dynamism, and volatile chromosomal biology of the *nasuta* group all bring interest to the group making it useful to have cultured stocks of this group available for study. At the same time, long term culture runs the risk of mislabelling of stocks and/or

introgressing stocks. In this sample of 28 stocks, detected rates of such problems are as high as 6 out of 28 or ~21%. These issues were reported to stock centers at the time, but due to limited resources and declining Federal support little could be done. Since then both stock centers are now under new management, and in new locations. The US Drosophila Species Stock Center has discontinued nearly all their *nasuta*-subgroup stocks, while many of the original Ehime stocks continue at the Kyorin-fly stock center in Japan.

We suspect that the geographic locations of stocks that are often used as a second part of the stock description has been both a hindrance and a help. The first because, if it is the only recognized part of a label, it could lead to the assumption it is another species sharing at least part of the locality name, something that may have happened with some of these mislabelled stocks. A help, because in many cases, along with reliable genetic data, it may lead to a dominant probable hypothesis of what the stock really is.

Finally, we call on the larger *Drosophila* community to make a push for continued robust support of our stock centers, to reassess and curate extant stocks, and to invest in genetic barcoding of stocks when they are accepted into long-term culture. Mislabelling and related issues such as these can have lasting impact. For instance, these mixed up stocks may have compromised the results of a number of papers. For example, in the paper of Yu *et al.* (1999) the Taxon J stock shows an *albomicans* type mtDNA sequence further confusing an already confused mtDNA tree (due to multiple real introgressions, Waddell *et al.,* unpublished). The stock marked *D. kohkoa* Rizal, Philippines that we have diagnosed as a mislabeled *albostrigata* stock was also used in a variety of papers.

When authors lodge their sequences in GenBank, this allows these possibilities, along with other conclusions of their papers , to be tested. In the case of Yu *et al.* (1999) this was done, and we observe that the mtDNA sequence of their Taxon I and J is identical to our Taxon J, while their *pallidifrons* shows two transversion differences only, suggestive of sequencing error. These sequences cluster tightly on phylogenetic trees to the exclusion of all other sequences, suggesting all three are mislabelled *albomicans,* possibly the very same stock. *That said, both the high rates of stock mislabelling and the failure to spot such errors in peer reviewed publications reinforces the need for good biological practices of species identification before reporting DNA sequences or analyses*. Further, flagging anomalous stocks such a *D. s. albostrigata* Cambodia for further investigation requires the aforementioned background of properly identified stocks along with diverse genetic data and an analysis sensitive enough to detect possible genetic mixing. Both types of diagnosis are essential if either phylogenetic or genetic conclusions from long cultured stock studies are to be trusted.

Acknowledgments: Sincere thanks to Prof. Osamu Kitagawa for the wide range of stocks he made available to the community and his extensive efforts into understanding this exceptional subgroup of *Drosophila*. To Mayoshi Watada (Ehime) and the UCSD Stock Center for sending stocks most recently. Special thanks to David Swofford for all the unsung work in PAUP*. This work was partly supported by NIH grant 5R01LM008626 to PJW.

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Altered expression of *Rbf* in the *Drosophila* eye alleviates the characteristic *foxo* phenotype.

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Introduction

The transcription factor foxo influences multiple cellular processes such as insulin signalling, stress response, energy metabolism, autophagy, cellular differentiation, and cell death (Greer and Brunet, 2005). When the insulin receptor signalling pathway is activated, it is responsible for the exclusion of foxo from nucleus in the cytoplasm. The activity of foxo provides stress resistance, during different cellular stresses, and longevity by transcription of responsible genes (Kramer *et al*., 2003). However, the overexpression of *foxo* is toxic and can lead to apoptosis in unstressed environment. The directed expression of *foxo* in developing eye of *Drosophila* gives a characteristic phenotype of reduced ommatidia number and little to no interommatidial bristles (Todd and Staveley, 2013). The protective role of *foxo* in stress conditions is reversed in normal situations.

Altered insulin receptor signalling can play a key role in development and disease. The role of *foxo* in the insulin receptor signalling pathway has been established by genetic and biochemical evidence. Recently, Rbf has been reported to target genes of multiple pathways including signalling pathways related to insulin (Acharya *et al*., 2012). The transcription factor Rbf, the *Drosophila* version of the human pRb protein responsible for G1/S transition, plays role in regulation of various other processes such as DNA replication, DNA repair, cell cycle, transcription, oncogenesis, tumor suppression, and apoptosis (Chakraborty *et al*., 2007; Classon and Harlow, 2002). Rbf1 is component of the DREAM complex, a multi protein complex, that can act as transcription activator or repressor depending upon the cellular environment (Ariss *et al.,* 2018). We coupled the directed expression of *foxo* with altered *Rbf* expression to determine possible influence of *Rbf* upon the characteristic *foxo* phenotype in the eye.

Material and Methods

Drosophila *stocks and culture media*

The *GMR-gal4*^{12}; *UAS-lacZ*$^{4\text{-}1\text{-}2}$; the *UAS-Rbf (w[*]; P{w[+mC]=UAS-Rbf.D}III);* the *UAS-Rbf RNAi1 (y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS03004}attP2/TM3, Sb[1])*; the *UAS-Rbf RNAi2 (y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.GL01293}attP40)* stocks were obtained from Bloomington Drosophila Stock Center at Indiana University, Bloomington, Indiana, USA. The *UAS-foxo* line is described (Kramer *et al*., 2003) and the *GMR-gal4 UAS-foxo* line was produced through standard methods

(M'Angale and Staveley, 2016). All flies were maintained on standard cornmeal/molasses/yeast/agar media treated with propionic acid and methylparaben to resist fungal growth. Stocks were maintained at room temperature (22 ± 3°C), whereas crosses and experiments were kept at 25°C.

Biometric analysis of the Drosophila melanogaster *eye*

Female virgins of the *GMR-gal4* genotype were collected every 8 to 12 hours for several days. The confirmed virgins were then crossed with the males of *UAS-lacZ*, *UAS-Rbf*, *UAS-Rbf-RNAi1* and *UAS-Rbf-RNAi2* genotypes. Critical class male progeny was collected for each genotype. The collected flies were kept as cohorts of 10 flies or less upon fresh media and allowed to age for 3 to 4 days. The flies were prepared for scanning electron microscopy following the standard protocol (M'Angale and Staveley, 2017). Ommatidia and interommatidial bristle counts were performed on 10 or more flies of each genotype using National Institute of Health (NIH) ImageJ software. The disrupted eye area was calculated by measuring the area of eye without ommatidium; done on 10 eyes of each genotype. The Biometric analysis was performed using GraphPad Prism version 8 statistical software. Significance was determined at 95% confidence level ($P \leq 0.05$).

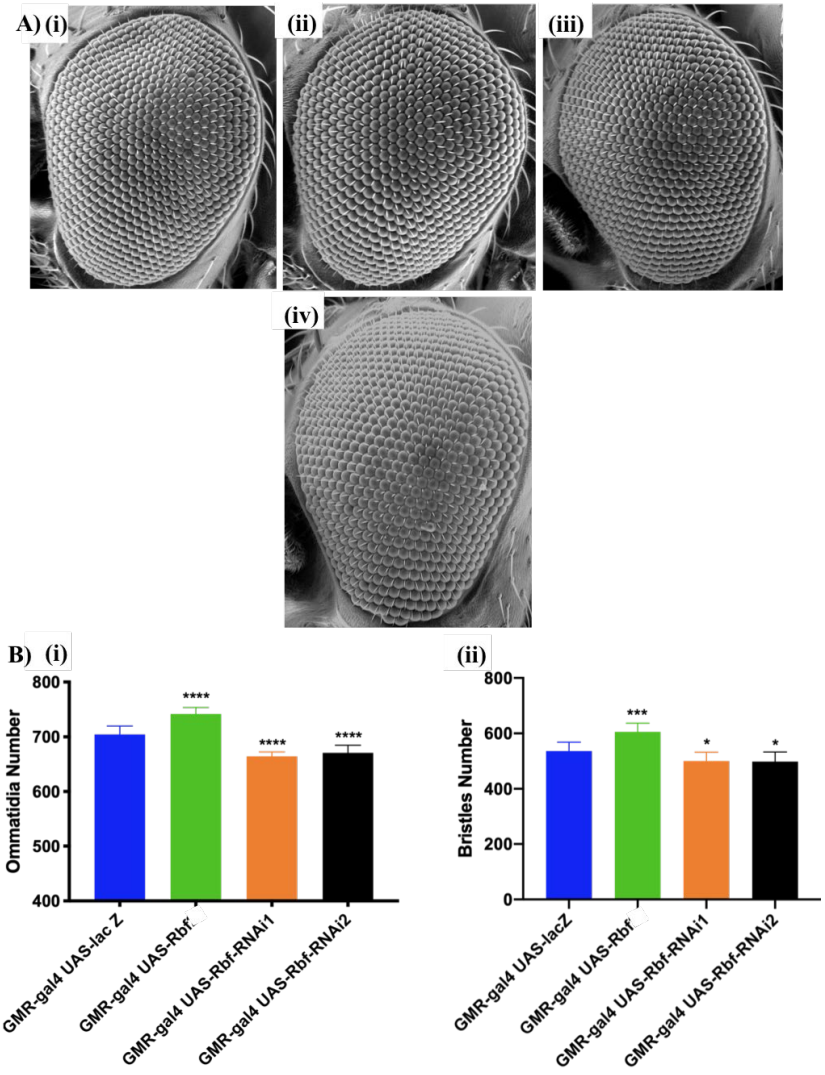

Figure 1. The phenotypic effects of the directed ex-pression and inhibition of *Rbf* in the developing eye. A) The scanning electron micrograph of (i) *GMR-gal4 UAS-lacZ;* (ii) *GMR-gal4 UAS-Rbf;* (iii) *GMR-gal4 UAS-Rbf-RNAi1;* (iv) *GMR-gal4 UAS-Rbf-RNAi2*. B) The biometric analysis of (i) Ommatidia Number; (ii) Interommatidial Bristle Number. The ommati-dia and interommatidial bristle number is decreased signif-icantly upon directed inhibition through *Rbf RNAi's*. The directed expression through *Rbf* overexpression line increased ommatidial and interomma-tidial bristle count.

Results and Discussion

The directed expres-sion of *Rbf* in the developing eyes of *Drosophila* result in altered phenotype. The di-rected expression of *UAS-Rbf* resulted in increased omma-tidia and interommatidial bristle count. The directed expression of *UAS-Rbf-RNAi* lines led to a decrease in ommatidia and inter-ommatidial bristle count (Figure 1B). The decrease in the ommatidia and bristle count is due to the crucial role of *Rbf* during development (Tanaka-Matakatsu *et al*., 2009). Not only *Rbf* inhibition but differential expression of *Rbf* can affect cellular processes differently, hence result in different phenotypes.

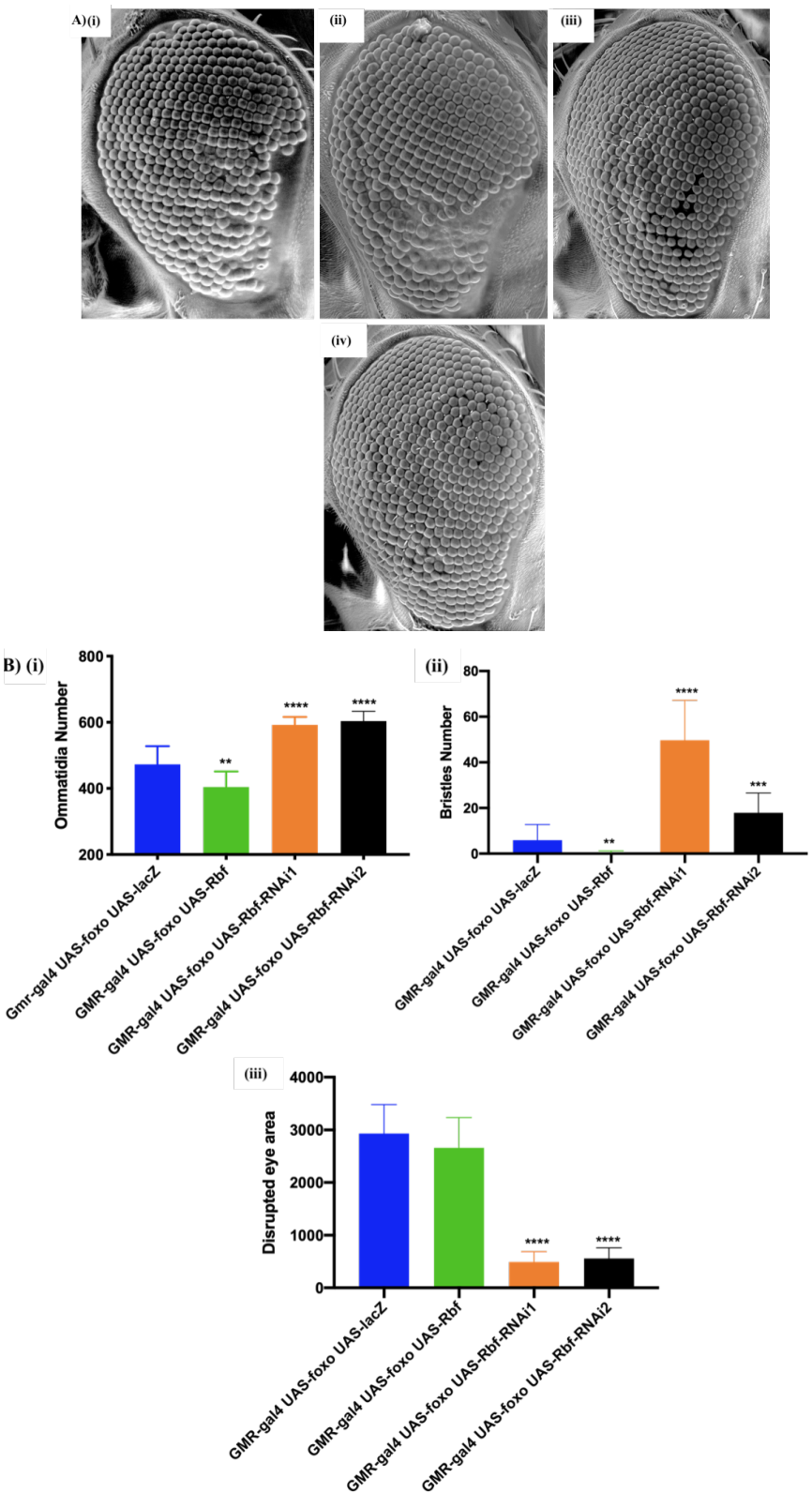

Figure 2. The phenotypic effects of the directed expression and inhibition of *Rbf* along with *foxo* in the developing eye. A) The scanning electron micrograph of (i) *GMR-gal4 UAS-foxo UAS-lacZ;* (ii) *GMR-gal4 UAS-foxo UAS-Rbf;* (iii) *GMR-gal4 UAS-foxo UAS-Rbf-RNAi1;* (iv) *GMR-gal4 UAS-foxo UAS-Rbf-RNAi2*. B) The biometric analysis of (i) Ommatidia Number; (ii) Interommatidial Bristle Number; (iii) Eye epithelial without ommatidia or disrupted eye area. The ommatidia and interommatidial bristle number is increased significantly when inhibition through *Rbf RNAi's* is coupled with *GMR-gal4 UAS-foxo*. The disrupted eye area is decreased when directed expression of *foxo* is combined with directed inhibition of *Rbf-RNAi's*. The directed expression through *UAS-Rbf* result in decreased ommatidial and interommatidial bristle count, the disrupted eye area has increased significantly.

The directed expression of *foxo* in the developing fly eye gives a characteristic phenotype of decreased ommatidia and interommatidial bristle counts (Kramer *et al*., 2003). The *foxo* phenotype can be alleviated by the directed expression of *PI3K*, *Akt*, or other upstream regulators. As *foxo* plays an important role in the regulation of the insulin receptor signalling, it is essential in the maintenance of homeostasis. The overall regulation of many aspects of normal cellular processes is dependent on foxo, Rbf and other transcription factors (Wei *et al*., 2016). Here we demonstrate that the directed expression of *Rbf* along with *foxo* enhances the phenotype (Figure 2A (ii)). In contrast, the directed expression of *foxo* along with the inhibition of *Rbf*,

through *UAS-Rbf-RNAi's*, suppresses this characteristic phenotype. Future studies are planned to investigate the importance of expression level of *Rbf* and its direct and indirect relationships with the activity of *foxo* and the transcription factor that it encodes.

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Skeleton photoperiods shed light on sex-differences in *Drosophila* circadian activity patterns in wild-type and *radish* mutant strains.

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Abstract

We compared circadian locomotor activity rhythms in four independently derived *Drosophila melanogaster* strains mutated at the *radish* locus to wild-type Canton-S controls in full 12:12 LD photoperiods (12 hours of light and 12 hours of dark), and in "skeleton" photoperiods with 15-minute light pulses 12-hours apart and no light in-between (*e.g*., 30 minutes of light and 23.5 hours of darkness per cycle). All strains showed elevated daytime activity levels in the skeleton photoperiod compared to the standard LD cycle. All four *radish* strains displayed lower nighttime mean activity levels in females and a higher proportion of total activity in the daytime under the skeleton photoperiod in both sexes, suggesting that further investigation of the role of the radish gene in regulating the distribution of circadian activity between night and day may be productive. Wild-type flies and all four *radish* strains showed a strong sex difference in the distribution of activity between day and night with females displaying more activity in the daytime in the full 12:12 LD photoperiod. This sex difference in diurnal behavior was eliminated under the skeleton photoperiod, demonstrating that the sex-difference in diurnal behavior observed in the full photoperiod was caused by greater inhibition of activity by light during the daytime in males compared to females. Our results suggest that masking by light is a major contributor to this commonly observed sex-difference in laboratory *Drosophila* circadian locomotor activity rhythms.

Introduction

Light plays a dual role in the regulation of circadian rhythms. The daily photoperiod entrains endogenous circadian clocks to synchronize them with the external light-dark cycle (Pittendrigh, 1964), and the direct effects of light may have non-circadian "masking" effects on behavior that shape the overt expression of entrained circadian rhythms (Pingkalai *et al*., 2019). Under standard laboratory photoperiod and temperature conditions (12:12 light-dark (LD) cycle at 25°C) wild-type *D. melanogaster* typically display morning (M) and evening (E) peaks of locomotor activity, plus transient spikes in activity in response to the abrupt transitions between light and dark (Helfrisch-Förster, 2000). Continuous exposure to bright light during the day suppresses activity levels of wild-type *D. melanogaster*, usually referred to as a "masking" effect of light (Mrosovsky, 1999). Masking of locomotor activity by light in *D. melanogaster* is evident from increases in activity observed when light during the day is removed by using a "skeleton" photoperiod (Pittendrigh,

1964) with brief (*e.g*., 15-minute) pulses of light 12 hours apart that simulate "dawn" and "dusk" and entrain circadian rhythms to the photoperiod (Sheppard *et al*., 2015). While the endogenous circadian clock mechanism generating circadian locomotor activity rhythms in *D. melanogaster* is well-described (Hardin, 2011), mechanisms mediating masking effects of light are not well understood (Pingkalai *et al*., 2019). Skeleton photoperiods, therefore, are a useful tool for characterizing the effect of light induced modulation of locomotor activity by comparing activity rhythms between skeleton and full photoperiod treatments.

Drosophila melanogaster radish mutants were originally identified from deficiencies in consolidated memory (Folkers *et al*., 1993). The *radish* gene is highly expressed within the mushroom bodies (MB) which are implicated in olfactory memory (Folkers *et al*., 2006). Additionally, MBs have been implicated in the shared underlying circuitry between visual and olfactory memories in *Drosophila* (Vogt *et al*., 2014). We explored *radish* locomotor activity further because *radish* mutants show hyperactivity and reduced attention span in response to visual stimuli when compared to wild-type controls (van Swinderen *et al*., 2010), and these differences are significantly reduced by methlyphenidate treatment, suggesting that *radish* mutants may serve as a model for attention-deficit-like behaviors. Although Folkers *et al*. (1993) showed that *radish* flies display wild-type activity levels measured by the "fast phototactic response" assay, the constitutive activity level of *radish* mutant flies over complete 24-hour cycles of circadian activity have not previously been described. We examined daily circadian locomotor activity rhythms of *radish* flies in standard and skeleton laboratory photoperiods to determine whether they display constitutive hyperactivity, differences in circadian timing of activity, or masking of activity by light compared to wild-type flies.

We compared four independently derived *radish* mutant strains of *Drosophila melanogaster* to Canton-S wild type flies for circadian locomotor activity. We show that the four radish mutant strains are significantly more diurnal under a skeleton photoperiod than the Canton-S wild-type flies, and that daytime light causes significant masking in all four *radish* strains, suggesting that the *radish* gene plays a role in elevating daytime activity in the absence of light. Finally, we show that the commonly observed sex difference in diurnal circadian locomotor patterns in *Drosophila* is eliminated, in these strains, by the skeleton photoperiod.

Materials and Methods

Experimental Design

Experiment one compared three *radish* mutant strains derived by transposon insertions to the wild-type Canton-S strain. Experiment two compared a strain carrying the $radish^{1}$ mutation to a second sample of Canton-S wild-type flies. All other methods were the same for both experiments.

Fly strains

Wild-type Canton-S *D. melanogaster* (from an established stock at Skidmore College) and four independently derived strains of *radish* mutants, $rad^{MB00656}$, $rad^{MI10840}$, $rad^{MI12368}$ and $radish^{1}$ obtained from the Bloomington Indiana Stock Center (stock numbers 22862, 56255, 58514 and 79201, respectively) were used in these experiments.

Three *radish* mutant alleles were generated by the *Drosophila* gene disruption project from *Minos* transposon insertions (Bellen *et al*., 2011) and the original $radish^{1}$ mutation was derived by ethyl methanesulfonate mutagenesis (Folkers *et al*., 1993) from a Canton-S strain.

Stock rearing and maintenance procedure

Flies were maintained on Carolina Instant *Drosophila* Medium in 23.5 mm (outer diameter) by 75 ml (length) polystyrene vials and were raised and tested in a 12:12 h LD cycle (lights on 0800 h) in a 25°C incubator. Stocks were moved to a new medium (Carolina Biological Instant *Drosophila* Medium) every three weeks. Flies were five days old at the start of the experiment.

Activity monitoring

Flies were anesthetized using *Flynap* (Carolina Biological Supply). Individual flies were placed in clear plastic tubes (65-mm length, 5-mm diameter). One end of the vial was plugged with cotton and the other

end contained agar food (2 g agarose, 5 g sucrose/100 mL dH_20) sealed with a plastic cap. Locomotor activity was recorded by placing vials in *Drosophila* Activity Monitors (DAM, Version 3.0) (TriKinetics, Waltham, MA, USA) as described by Seggio (2011), which recorded activity through photobeams bisecting each tube. Locomotion was recorded by the number of beam interruptions per ten-minute interval. Sixteen flies of each sex were assayed from Canton-S and each of the three *radish* mutant strains, and 32 flies of each sex and strain were assayed in experiment two comparing Canton-S flies to *radish*^{1} mutants.

Photoperiod treatment

DAMs were placed in light-proof incubators and data were collected over seven consecutive days of treatment. Three separate conditions were used: two days of 12:12 light-dark photoperiod (fluorescent lighting, approximately 600 lux) with lights on a 0800 h and off at 2000 h, followed by one day of a skeleton photoperiod with light exposures of two 15-minute intervals per 24 hour cycle at 0800-0815 h and 1945-2000 h followed by four days of constant darkness to show continuity of the activity patterns between the entrained and free-running rhythms and to demonstrate circadian clock regulation of the underlying daily locomotor activity rhythm. The four days of DD was considered too short to reliably estimate free-running circadian periods.

Statistical Analysis

Flies with no beam crosses for any 24-h period were excluded from analysis. Final sample sizes ranged from 11-16 per group for experiment one, and 30-32 per group for experiment two. ANOVA was used to analyze main effects and interactions of genotype and sex, and the Tukey *post hoc* test was used to compare pairwise strain differences for variables showing a significant strain effect (SAS, Carey, North Carolina, USA). Composite actograms were constructed using Actogram J (Schmid *et al*., 2011).

Variables

Variables assayed were: average overall activity during the standard (XLD) and skeleton (SKXLD) photoperiod, average "daytime" activity during the standard (XL) and skeleton (SKXL) photoperiod, average "night-time" activity in the standard (XD) and skeleton (SKXD) photoperiods, and the ratio of daytime to total activity during the standard (LDRATIO) and skeleton photoperiod (SKLDRATIO). Masking effects of light in 12:12 LD (MASKING) were measured by subtracting XL from SKXL.

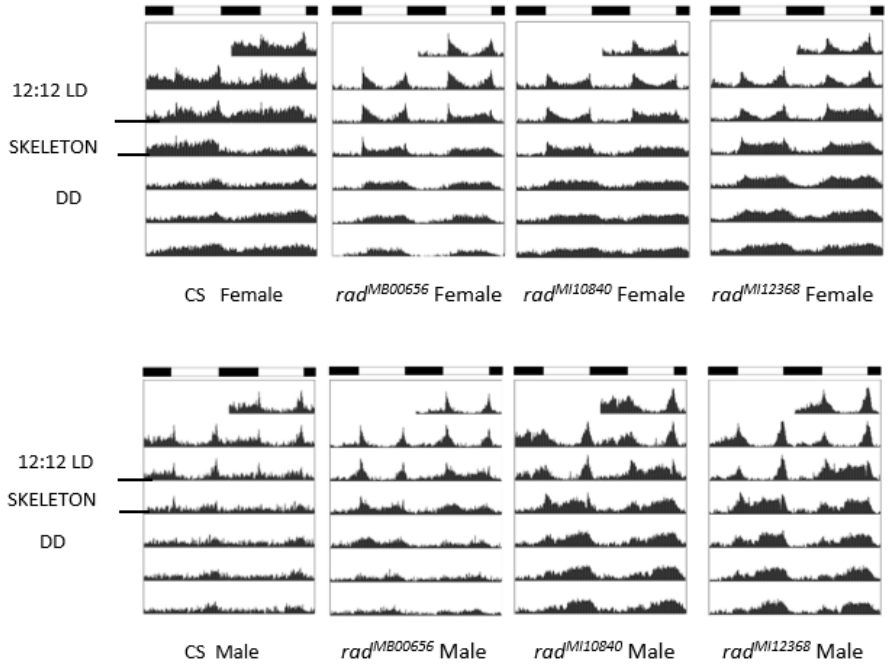
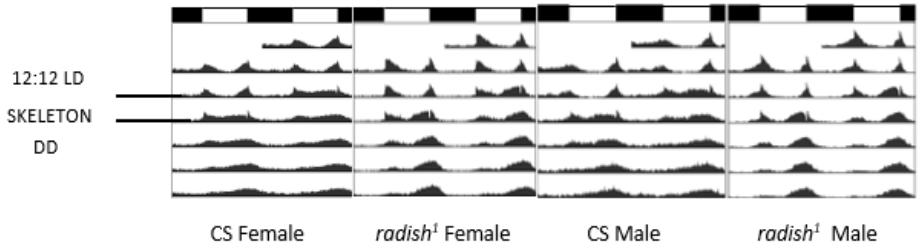


Figure 1. Double-plotted composite actograms (x-axis: 48-h and y-axis: average locomotor activity per 10-minute interval) for female (Top) and male (Middle) Canton-S, *rad*^{MB00656}, *rad*^{MI10840}, and *rad*^{MI12368} strains, and female and male Canton-S and *radish*^{1} strains (Bottom). Actograms show, consecutively, two days of standard photoperiod (12:12 LD activity with lights on at 0800 h and off at 2000 h, one day of a skeleton photoperiod (light exposures of two 15-minute intervals per 24 hour cycle at 0800 and 1945 h) and three days in constant dark.



(Figure 1, continued)

Results: Experiment One

Standard Photoperiod

There were no significant differences among the *radish* and wild-type strains for XLD or XL, but XD varied significantly among strains (Table 1; Figure 1) with all the *radish* strains showing a lower XD in females compared to CS flies. Overall, females were less active in XD than males, with a significant strain by sex interaction. Relatively small sex differences in the $rad^{MB00656}$ and CS strains and much larger sex differences in the rad^{MI1084} and $rad^{MI12368}$ strains were observed. LDRATIO was significantly different among strains (Table 1). Post-hoc tests showed that LDRATIO in $rad^{MB00656}$ was significantly higher than Canton-S and the other two *radish* strains ($p < 0.05$). There was a sex difference for LDRATIO ($p < 0.001$, Table 1) with all strains displaying higher ratios in females, but no sex by strain interaction.

Table 1. Mean (± SEM), sample size and ANOVA p-values for circadian locomotor activity measures, for Canton-S wild-type vs. *radish* mutant strains in the standard 12:12 LD photoperiod.

	XLD	XL	XD	LDRATIO
ANOVA				
Strain	p > 0.07	p > 0.61	p < 0.003	p < 0.004
Sex	p > 0.39	p > 0.09	p < 0.0001	p < 0.0001
Strain by Sex	p > 0.1213	P > 0.36	p < 0.02	P > 0.22
Canton-S				
Male	11.6 ± 1.3 (11)	09.0 ± 1.0 (11)	14.2 ± 1.9 (11)	0.80 ± 0.06 (11)
Female	13.7 ± 3.4 (12)	16.1 ± 4.4 (12)	11.7 ± 3.1 (12)	1.21 ± 0.09 (12)
$rad^{MB00656}$				
Male	06.9 ± 1.2 (15)	07.2 ± 1.1 (15)	06.7 ± 1.4 (15)	1.08 ± 0.07 (15)
Female	09.8 ± 1.0 (14)	13.7 ± 1.6 (14)	06.0 ± 0.7 (14)	1.38 ± 0.05 (14)
$rad^{MI10840}$				
Male	14.3 ± 1.6 (16)	10.5 ± 1.7 (16)	18.1 ± 2.1 (16)	0.74 ± 0.05 (16)
Female	10.3 ± 1.3 (16)	14.2 ± 2.1 (16)	06.5 ± 0.7 (16)	1.30 ± 0.06 (16)
$rad^{MI12368}$				
Male	15.3 ± 3.7 (14)	15.4 ± 6.4 (14)	15.3 ± 3.1 (14)	0.86 ± 0.10 (14)
Female	10.7 ± 0.9 (16)	13.1 ± 1.3 (16)	08.3 ± 0.8 (16)	1.22 ± 0.04 (16)

Skeleton Photoperiod

Significant strain effects were observed for SKXLD and SKXL, but not for SKXD (Table 2; Figure 1), with no sex differences, or sex by strain interactions. SKXLD for $rad^{MI12368\ was}$ significantly greater than Canton-S and $rad^{MB00656}$, and SKXL was significantly greater in both $rad^{MI10840}$, and $rad^{MI12368}$ compared to Canton-S and $rad^{MB00656}$. SKLDRATIO was significantly higher in all three *radish* strains compared to CS wild-type flies with no sex difference or strain by sex interaction, and no significant differences among radish strains (Table 1). Overall, the skeleton photoperiod revealed significant strain differences in daytime activity

and SKLDRATIO that were not evident in the standard photoperiod and eliminated the sex-differences in LDRATIO observed in the standard photoperiod.

Masking

MASKING was significantly different among strains, and significantly greater in males, with no strain by sex interaction (Table 2; Figure 1). The *rad*^{MI10840} and *rad*^{MI12368} strains showed significantly more masking than Canton-S wild-type flies. Each of the *radish* strains increased their daytime activity significantly in the skeleton photoperiod compared to the standard photoperiod (paired t-test, $p < 0.01$), but the increase in Canton-S was not significantly different from zero.

Table 2. Mean (± SEM), sample size and ANOVA p-values for circadian locomotor activity measures, for Canton-S wild-type vs. *radish* mutant strains in the 12:12 LD skeleton photoperiod.

	SKXLD	SKXL	SKXD	SKLDRATIO	MASKING
ANOVA					
Strain	p < 0.001	p < 0.0001	p > 0.26	p < 0.0001	p < 0.005
Sex	p > 0.11	p > 0.16	p > 0.16	p > 0.46	p < 0.005
Strain by sex	p > 0.20	p > 0.09	p > 0.48	p > 0.66	p > 0.55
Canton-S					
Male	10.3 ± 1.2 (11)	11.5 ± 1.3 (11)	09.1 ± 1.3 (11)	1.13 ± 0.03 (11)	2.4 ± 0.7 (11)
Female	11.9 ± 3.6 (12)	14.4 ± 3.5 (12)	10.2 ± 4.1 (11)	1.21 ± 0.10 (12)	-1.7 ± 2.9 (11)
rad^{MB00656}					
Male	10.9 ± 1.5 (15)	14.4 ± 1.9 (15)	07.3 ± 1.3 (15)	1.32 ± 0.04 (15)	7.2 ± 1.7 (15)
Female	10.9 ± 1.2 (14)	15.5 ± 2.0 (14)	06.3 ± 0.7 (14)	1.38 ± 0.08 (14)	1.8 ± 1.3 (14)
rad^{MI10840}					
Male	17.1 ± 1.5 (16)	23.3 ± 1.9 (16)	10.9 ± 1.7 (16)	1.39 ± .07 (16)	12.8 ± 1.5 (16)
Female	12.0 ± 1.2 (16)	17.3 ± 1.7 (16)	06.8 ± 0.8 (16)	1.43 ± 0.03 (16)	03.1 ± 1.4 (16)
rad^{MI12368}					
Male	18.6 ± 1.8 (14)	26.8 ± 2.3 (14)	10.3 ± 1.4 (14)	1.47 ± 0.03 (14)	11.4 ± 6.4 (14)
Female	15.5 ± 1.1 (16)	22.03 ± 1.6 (16)	09.0 ± 0.8 (16)	1.41 ± 0.04 (16)	09.0 ± 1.5 (16)

Table 3. Mean (± SEM), sample size and ANOVA p-values for circadian activity measures, for Canton-S wild-type vs. *radish*^{1} mutants in the standard 12:12 LD photoperiod and the 12:12 skeleton photoperiod. One asterisk signifies $p < 0.05$, two signifies $p < 0.01$ and three signifies $p < 0.001$ and "ns" indicates $p > 0.05$.

	Canton-S		*radish*^{1}		ANOVA		
	Male	Female	Male	Female	p-value		
					A	B	A*B
XLD	9.7 ± 1.0 (30)	10.1 ± 1.0 (31)	8.3 ± 0.6 (31)	8.3 ± 0.5 (32)	ns	ns	ns
XL	8.0 ± 0.8 (30)	11.2 ± 1.1 (31)	9.1 ± 0.6 (31)	11.0 ± 0.7 (31)	ns	**	ns
XD	11.4 ± 1.5 (30)	9.0 ± 1.3 (31)	7.5 ± 0.8 (31)	5.6 ± 0.4 (32)	***	ns	ns
LDRATIO	0.9 ± 0.08 (30)	1.2 ± 0.06 (31)	1.2 ± 0.05 (31)	1.3 ± 0.03 (32)	***	***	ns
SKXLD	14.3 ± 1.3 (30)	12.6 ± 1.0 (31)	9.1 ± 0.7 (31)	10.7 ± 0.7 (32)	***	ns	ns
SKXL	18.9 ± 1.5 (30)	16.9 ± 1.3 (31)	13.6 ± 0.9 (31)	16.3 ± 1.2 (32)	*	ns	ns
SKXD	9.9 ± 1.2 (30)	8.6 ± 1.0 (31)	4.5 ± 0.6 (31)	5.2 ± 0.4 (32)	***	ns	ns
SKLDRATIO	1.4 ± 0.04 (30)	1.4 ± 0.03 (31)	1.5 ± 0.05 (31)	1.5 ± 0.03 (32)	***	ns	ns
MASKING	10.9 ± 1.2 (30)	5.6 ± 1.3 (31)	4.5 ± 0.7 (31)	5.3 ± 0.8 (32)	**	*	**

Experiment Two

Differences between *radish*^{1} and Canton-S wild-type flies were similar to experiment one (Table 3; Figure 1). *Radish*^{1} females were less active in the dark in the standard and skeleton photoperiods, and *radish*^{1} flies displayed a greater proportion of activity in the daytime in both photoperiods. A significant sex-difference in LDRATIO was eliminated by the skeleton photoperiod, as in experiment one.

Discussion

The circadian locomotor activity rhythm of *Drosophila radish* mutants differed from wild-type flies in two ways: female *radish* flies were less active than control females during the night regardless of the photoperiod, and *radish* flies were more diurnal in the skeleton photoperiod than controls for both sexes. These observations suggest that the *radish* gene may play a role in regulating circadian locomotor activity in *Drosophila* in at least two ways- first, during the night, in a manner that is independent of direct exposure to light, and second, during the daytime as a result of an enhanced sensitivity to the masking effects of light on daytime activity. Since masking effects of light are important for shaping overt circadian activity rhythms in *Drosophila* through mechanisms that are not well studied (Pingkalai et al., 2019) it would be useful to explore the potential role of *radish* in mediating masking effects of light further. Our results are only suggestive since we used a single wild-type strain and did not isolate the *radish* mutations against a homogeneous genetic background, but the consistency of these results across four independently derived *radish* mutations and two separate experiments is intriguing. Since mushroom bodies in the *Drosophila* brain have high levels of *radish* expression (Folkers *et al*., 2006) and are known to regulate sleep and locomotor activity in *Drosophila* (Goodwin *et al*., 2018; Helfrich-Förster, 2002), they are a potential neural substrate for further study of the effects of light on activity.

A second observation in the current study is that a stronger diurnal pattern of locomotor activity in females compared to males, commonly observed in *Drosophila* (Helfrich-Förster, 2000; Ferguson *et al*., 2015) was observed in all strains in the standard photoperiod but was effectively eliminated by the skeleton photoperiod. This observation suggests that differential sensitivity to the masking effects of light play a major role in generating sex-specific patterns of circadian locomotor activity, and that the simple method of subjecting flies to a single day of entrainment under a skeleton photoperiod can separate masking effects of light from other mechanisms regulating circadian locomotor activity, such as endogenous circadian clock regulation, coupling pathways and peripheral oscillators.

The underlying molecular mechanisms driving behavioral response to light, including masking effects of light on circadian rhythms, are poorly understood (Pingkalai *et al*., 2019). Overall, our results suggest additional functions for the *radish* gene beyond learning and memory (Folkers *et al*., 1993; van Swinderen and Brembs, 2010) that may be useful for further research on mechanisms mediating interactions between the role of light in entrainment of circadian pacemakers and the role of light in shaping overt daily rhythms through masking effects.

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Constraints in pupation site selection: insight from *Drosophila.*

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Abstract

Every organism is fit to live in its natural habitat, thus prefer a unique ecosystem. However, during lifetime, organisms have to deal with various environmental challenges and try to cope with them through adaption. Adaptation is an evolutionary process wherein organisms use different ways to adapt themselves to different environmental stress conditions and as a result of which, diversity in traits appears in natural populations and the traits under positive selection became prevalent through time and space. Traits with more genetic variation have higher genotype-environment interactions and, thus, possess a higher rate of phenotypic plasticity and flexibility in nature. Adaptation with changing ecology under evolutionary constraint(s) is regulated by two mechanisms, namely, selection and plasticity, which together lead to speciation. Intra- and interspecies trait variations observed in natural populations provide clues to the cause and mechanism of adaptation and the evolutionary forces acting in any particular environment. *Drosophila* is an established model to understand various ecological constraints affecting trait evolution. In the present review, we focused on the factors affecting the pupation site selection in *Drosophila*. Pupa is one of the stationary phases in the insect life-cycle. The significance of studying factors involving the larval - decision making in pupation site selection is important both to save the economically important forest insects from predators and also to reduce the disease causing insect population to a threshold level. Keywords: Adaptation, Ecological constraints, Pupation site selection, *Drosophila*

Introduction

Individuals compete for better survival against various environmental challenges during their lifetime and evolve themselves by adapting to those challenges (Jimenez, 2014). Adaptation is termed as conscious and intentional change through which an organism passes in order to respond towards various stimuli and stress conditions (Nelson *et al.*, 2007). Adapting ability of an individual under different environmental challenging conditions is termed as property of life (Sisodia and Singh, 2012). Adaptation may occur at morphological, cellular, physiological, biochemical, and anatomical levels (Abdul *et al.*, 2015). Environmental challenges comprise both abiotic (thermal, nutritional, starvation, desiccation and humidity-related stress, and pH tolerance) and biotic stress (presence of parasites, predators, pathogens and other competitors in nature) conditions (Rajpurohit *et al.*, 2008; Gassmann *et al.*, 2016; Kwenti 2017). Eco-evolutionary studies across species enable a better understanding towards genetic and molecular basis of trait variations (Ouborg and Vriezen, 2007). The phenotypic studies conducted in laboratory (under controlled parameters) and field (under natural conditions) provide further information in establishing the genetic and ecological basis of trait variations.

Selection of particular phenotypes through eco-evolutionary phenomenon comprises of mutations and natural selection which results in modification of certain trait variations among species (Ungerer, 2008). These modified trait variations at genetic and phenotypic level bring changes in gene arrangement, expression and at genome organisational level and improve organism's survival-reproduction rate and become prominent in generations over time (Philip, 2015; Vitti, 2013). Phenotypic plasticity is the ability of particular genotype to express different phenotypic expressions in response towards surrounding ecological conditions (Samuel and Hilary, 1999). Traits with more genetic variations have higher genotype-environment interactions and, thus, possess a higher rate of phenotypic plasticity and flexibility in nature (Trevor, 2003). Adaptation with changing ecology under evolutionary constraint is regulated by two mechanisms, namely, selection and

plasticity, which together lead to speciation (Grenier, 2016; Noor and Feder, 2006; Rebecca and Patrick, 2012). Traits vary with differences in their ecological niches in order to survive and maintain themselves within their ecological communities under diversified evolutionary patterns and give rise to inter- and intra-species competition (Rocio *et al.*, 2017). Trait variations can also be understood in context with sibling species as traits, and genes within these closely associated species reproductively evolve and differentiate under ecological constraints due to natural selective pressure for existence (Raul and Jose, 1998).

Drosophila consists of four stages in its life cycle, namely egg, larvae, pupa, and adult. Larvae is most crucial stage which signifies the individual fitness level and pupation site selection (Casares and Carracedo, 1987; Pant and Shivanna, 2012).

Variations in pupation site preferences and larval behaviors under different ecological conditions among *Drosophila* species provide the knowledge to predict the rate of evolution and mechanism of adaptation of traits in nature (Sunitha *et al.*, 2015). Pupation is a well-studied mechanism. It is larvae dependent and is regulated by multiple abiotic factors (fluctuations in relative moisture, change in pH level, temperature, humidity and light) and biotic factors (presence of predators, parasites, difference in sex of larvae, density of larvae, and other intra- and inter- specific competitors in nature) (Seema and Girish, 2019; Simon and Paul, 1998; Philip and Marla, 1994). Nowadays, use of insecticide and pesticides primarily target at early stages-larvae and pupae (juvenile stages) as adult insects/pests develop more quickly resistance towards them (Hudson *et al.*, 2014). Thus, study of pupation site selection has significance in medical entomology. Also the information will be helpful to save economically important insects from their predators as mostly they are attacked at larval and pupal stages.

Various constraints affecting pupation

Diet

It possesses direct impact on structural and functional development, reproduction, morphology, physiology, and behavior, such as alternations in timing (pupa to adult emergence period), morphology (body weight, length, and height of larvae and pupae), and behavior of development stage in *Drosophila* species. Development time required by larvae to reach the stage of pupation is regulated by dietary intake (Ormeroda *et al.*, 2017). Studies reveal that pupation rate also depends upon larval feeding patterns. It is found that larvae with higher feeding patterns shows faster rate of pupation. Protein intake in diet directly co-relates with multiple traits among *Drosophila* species: pupation time, pupation height, development, and pigmentation (Krittika, 2019). It is reported that flies show different response towards protein restricted diets and can sustain with minimum protein concentrations by increasing their pupation period (Krittika, 2019). Studies also reveal that different components in diet have different impacts on pupation site selection. For example, flies with soy hydrolysate diet comparatively pupates highest whereas, flies failed completely to pupate under corn hydrolysate diet (Chan *et al.*, 1990). Literature also reveal that larvae exposed to particular diet in one generation shows prolonged effects to many coming generations (Matzkin *et al.*, 2013). It is found that progeny of flies that were exposed to high protein with respect to sugar in diet have higher reproduction and development rate relative to the progeny of flies that were exposed to low protein with respect to sugar in diet. Earlier studies also report that offspring of flies with high sugar intake in diet have more weight and much longer pupation period (Matzkin *et al.*, 2013). It is also reported that among *Drosophila,* increased amylase content in diet increases the glucose content, delays their pupation rate, increases their body weight, and also decreases their mortality (Sakaguchi and Suzuki, 2013).

Temperature

Studies reveal that there is a strong relation between temperature and growth and development period in insects. The temperature range between 15 to 25°C is found to be optimum range for larva to pupate and for pupa to emerge into an adult (Loeb and Northrop, 1917). However, it is reported in earlier studies that increase in temperature shows faster growth and development which comparatively declines with low temperature (Chen *et al.*, 2013). At temperature 6°C - 7°C, although larvae survive, proper pupation does not occur. With little increase in temperature like 9°C - 10°C, larvae continue to develop to pupate but pupa does

not hatch and emerge into an adult. However, it is found that single increment in temperature range (11 to 12 degrees) raises the larval capability to pupate and also its pupation mortality (Kostal *et al.*, 2016).

It is also reported from earlier studies that temperature has an effect on pupation height, such as at extreme high temperature (> 35°C), all pupae possess no movement or merely minimum height. Similarly, at extreme low temperatures maximum height is observed in some species belonging to *melanogaster, willistoni,* and *repleta,* whereas other species belonging to same group did not show any significant upward movement. It can be concluded that temperature effect varies from species to species depending upon their genetic constituents and ecological parameters (Schnebel and Grossfield, 1992). Study conducted between strains of *D. ananassae*, *D. bipectinata*, *D. biarmipes*, and *D. malerkotliana* concludes that at 20 °C their larvae pupate closer to food surface, at 24°C significantly above the food surface, and at 30°C mostly on the medium (Singh and Pandey, 1993).

Therefore, it is seen through literature that flies captured from warm localities have different pupation patterns and preferences as compared to flies belonging from colder localities. However, from experiments it is observed that pupation is a more heat resistant stage in *Drosophila* life cycle (Dillon, 2009).

Photoperiod

Limited studies were conducted to see the effect of light influencing the life event in *Drosophila* species, such as total darkness and total lightness disrupts the natural circadian patterns among *Drosophila* species (Fisher *et al.*, 2015). It is reported that larvae departure from food to pupate in order to emerge into an adult is regulated by photoperiod mechanism; however, different species have different responses towards light and dark regimes relative to pupation site. For example, larval wandering to pupate does not effectively occur under constant light in *D. melanogaster* (Roberts, Henrich *et al.*, 1987) and possess maximum height under darkness as compared to its sister species *D. simulans* (Casares, 1997).

Substrates (pH, moisture, humidity, texture)

Literature shows that although there is no such relation evidenced among pH and pupation in nature, but under lab conditions a strong relation exist between pH and pupation. Under controlled lab conditions it is found that higher the acidic pH of media, more closely the larvae pupates to the media (Hodge and Caslaw, 1998). Studies also reveal that pupation site preferences are dependent on moisture and texture content of food source and also on the humidity in environment. Water content and moisture of food source plays a vital role in survival of pupa. It is found that when moisture content reaches to saturation point, pupal rate of survival declines due to dropped level of oxygen content. At low moisture, pupae show better survival rate (Al-Saffar, 1995). Relative humidity and survivability of pupae are oppositely regulated by nature. Moisture and humidity go hand in hand with fluctuations in temperature range and affects the pupation height (Dillon *et al.*, 2009).

Parasites

Parasites are most prominent natural enemies among *Drosophila* in nature that attack the species more easily at juvenile stages (larvae and pupa). Among, *D. melanogaster* most common parasitoids are Asobara and Leptopilina (Fellowes and Godfray, 2000). Studies reveal that larvae exposed with bacteria and microalgae in nature show delay in pupation emergence period and development (Sakaguchi and Suzuki, 2013). It is also reported that adult males emerge early from pupa and survive better with respect to females under parasitic influence. Among other bacteria, *E. coli* and *Asaia* species result in shorter development time of pupa and at the same time possess superior survival span (Sakaguchi and Suzuki, 2013). *Trichopria drosophilae* infects *Drosophila* at pupa stage, whereas *Ganaspis* and *Leptopilina* infects *Drosophila* at larval stage. If infection succeeds it signifies the reduction in pupation development time duration of host but host retains the capability to defend and continues to develop further into an adult (Small, 2012). Another study reveals that presence of trypanosomatids while culturing the flies leads to emergence of adult flies with reduced fecundity capability and at the same time took longer for pupation to occur (Hamilton, 2015). Some parasites on successful transmission eventually require the death of the host. Study between *D. melanogaster* and *L. Boulardi (*host-parasite) illustrates that both of them possess significant impacts on each other's genotype and ultimately makes conditions extremely difficult for both of them to survive (Leitao,

2019). If *Drosophila* gets infected at larval stage, it is seen often the pupa dies. Thus, parasitoids are reported to have most harsh effect on host which directly aims to cause death of host (Leitao, 2019).

Niche effect

Nature favors species to be in their own microenvironment (niche) in order to reduce the overlapping of resources availability in ecosystems. Community richness of species is influenced with size of niche (Valladares, 2015). However, within the niche, there is always a competition among individuals belonging to same species (intra- species) or different group of species (inter-species). This competition has effects on growth, fitness, and survival depending upon ecological parameters. Natural selection under varying ecological conditions regulates the phenomenon of trait divergence among species belonging to their respective niches and helps in understanding the relationship with each other (Collins, 1986). Similar observation has been evidenced from pupation site preference studies across *Drosophila* species sharing similar niche and it is observed that different species pupate at different heights to minimize the competition in the niche.

Pupation site preference across *Drosophila* species

Most of the studies on pupation site selection have been conducted in *D. melanogaster* and have found that pupation height is regulated by larval density in culture, variability in photoperiod patterns, and humidity (Paranjpe *et al.*, 2004; Shenoi *et al.*, 2016; Casares, 1987). It is observed that maximum pupation height attained at constant darkness, minimum under constant light - dark fluctuations, and intermediate at constant light (Paranjpe *et al.,* 2004). Another study reveals that humidity has an impact on both pupation height and larvae to pupa development time. It is found that in *D. melanogaster*, pupation height negatively correlates with larvae development time due to increased humidity (Casares, 1987).

Pupation site preference experiments were also conducted in sibling species *D. melanogaster* and *D. simulans* with respect to sex, location, photoperiod, humidity, and gravity. It is found that pupation pattern differs with sex in terms of time and height. For example, females take longer development time as compared to males which signifies that male pupates much more rapidly than females. Research findings also say that males on average pupate higher (travel up to top of the vial) in comparison to females (Casares *et al.*, 1987). Further, it was reported that pupation site varies among *D. melanogaster* and *D. simulans,* as *D. melanogaster* prefers to pupate on surface of vials and *D. simulans* prefers to pupate at media whereas, the hybrid (male *D. melanogaster* and female *D. simulans*) prefers to pupate at media (Pant and Shivanna, 2012). Other study also states that *D. melanogaster* chooses to pupate under dark conditions in comparison to *D. simulans* (Manning and Markow, 1981). Also both these sibling species show variation towards gravity as *D. melanogaster* pupates higher towards geonegative in context with *D. simulans.* Among both species larvae with high humidity sensitivity pupate higher and possess faster locomotion (Casares, 1997). The literature also reveals that single gene of X chromosome, known as "tilB" gene, possesses altered levels of expression among *D. melanogaster* and *D. simulans* and is responsible for differences in their pupation site preference (Pischedda, 2019).

Various studies were also conducted in other species of *Drosophila,* such as in *D. jambulina*, show change in temperature range affects the pupation site choice. At high temperature it pupates at food surface and at low temperature it pupates at much higher surface of vials (up to the height of cotton plug) (Ramniwas, 2019). Also it is reported that pupation height also affects the body weight in *D. jambulina.*

In *D. ananassae*, studies reveal that pupation height is under control of multiple genes along with additive ecological factors in nature. Clear divergence is observed even among its different lines; some lines show very high pupation site whereas some shows very low. Further, hybrids generated from crosses between a high pupation line and low preference line shows intermediate pupation site (Singh and Pandey, 1993). Another comparative study conducted between *D. ananassae*, *D. bipectinata,* and *D. malerkotliana* for pupation site preference clearly shows that *D. ananassae* pupates at much higher height, whereas *D. bipectinata* pupates very close to media in respect to *D. ananassae* and *D. malerkotliana* (Singh and Pandey, 1993).

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Teaching Notes

Interaction of inbreeding and temperature on viability of *Drosophila melanogaster*: A test of the impact of inbreeding in future global warming conditions.

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The detrimental influence of physical and chemical stressors on viability, and other measures of fitness, is known to be increased by inbreeding (Hoffman and Parsons, 1991; Armbruster and Reed, 2005; Frankham, Ballou, and Briscoe, 2010; Bijlsma and Loeschcke, 2011; Woodruff, Marvin, and Wagner, 2011). For example, inbred lines of *D. melanogaster* in high temperature environments have reduced viability (number of progeny) compared to non-inbred lines (Miller, 1994: Bijlsma *et al*., 2000). This could mean that long-term adaptation of organisms to rising global temperatures may be more difficult in small inbred populations or species (Schiegg *et al*., 2002; Allendorf and Luikart, 2007).

To give students hands-on experience with the severity of inbreeding in a high-stress environment, we have tested the interaction of inbreeding and high temperature on viability in non-inbred and inbred lines of *D. melanogaster* derived from the same base stock. We hypothesized and observed that inbreeding reduces the number of progeny produced in a high temperature stressful environment, while progeny counts remained unchanged in a non-stressful temperature environment. We also observed that a single generation of interline crosses is sufficient to reduce the influence of inbreeding depression at high temperature.

The base stock used in this experiment (called the Mixed line) was derived from mixing two natural population lines captured in Perrysburg, Ohio in different years (2010 and 2013) and maintained in large numbers in bottles. Hence, this Mixed line should be heterozygous for most mutant genes. To derive an inbred line, single sibling females and males from the Mixed line were mated in vials for three generations. The viability of this inbred line at generations two and three was then tested against the viability of the non-inbred Mixed line at room temperature (23ºC) and high temperature (32ºC). Progeny were counted from the time the parents were initially mated at room temperature for 21 days and at 32ºC for 17 days.

To better understand how quickly inbreeding depression can be mitigated by introgression of another population, we also tested the influence of 32ºC on the viability of the progeny of interline crosses using the third-generation inbred lines. The interline crosses were set up by mating two virgin females from one inbred line and two males from another inbred line. The progeny of these interline crosses should have a decreased proportion of homozygous deleterious genes compared to the original inbred lines as well as a reduction in inbreeding depression, leading to an overall increase in progeny numbers (see a discussion of this topic in Lynch and Walsh, 1998; Hedrick, 2011; Kondrashov, 2017).

Inbreeding and Room Temperature (23ºC)

As shown in Figure 1, there were no significant differences in progeny numbers at room temperature between the Mixed population, 2nd generation inbred line, and 3rd generation inbred line ($P = 0.43$ by a one-way ANOVA). The mean number of progeny (± SEM) for 10 vials of the non-inbred Mixed line was 75.00 ± 4.93, the second-generation inbred line for 10 vials was 80.20 ± 3.24, and the third-generation inbred line for 10 vials was 70.70 ± 6.55. Hence, up to three generations of brother-sister inbreeding did not significantly influence viability at a non-stressful room temperature.

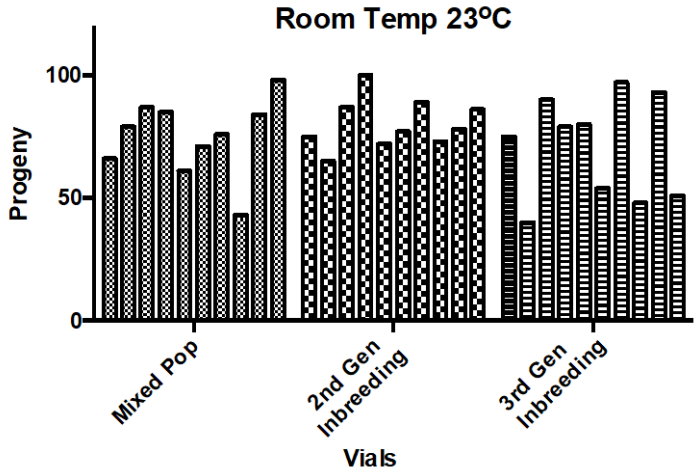

Figure 1. Non-significant interaction of inbreeding and room temperature (23°C) on viability (P = 0.43 by a one-way ANOVA).

Inbreeding and 32ºC:

The influence of two and three generations of sibling inbreeding, and interline crosses, on viability in a stressful 32ºC environment is shown in Figure 2. There was a significant reduction in the number of progeny in the two-generation inbred line *vs*. the non-inbred Mixed line (P = 0.0002), for the three-generation inbred line *vs*. the non-inbred Mixed line (P < 0.0001), and for the two-generation and three-generation inbred lines (P = 0.007). In addition, there was a significant increase in the number of progeny seen in the interline crosses *vs*. the three-generation inbred line (P = 0.002), but not *vs*. the two-generation inbred line (P = 0.88). This shows that only one generation of crosses between the third-generation inbred lines significantly reduced the effect of three generations of inbreeding. The mean number of progeny (± SEM) for 14 vials of the non-inbred Mixed line was 70.79 ± 3.58, for 14 vials of the second-generation inbred line was 42.29 ± 4.57, for 10 vials of the three-generation inbred was 19.30 ± 5.98, and for the 10 vials of the interline crosses was 47.30 ± 4.90. Hence, inbreeding significantly reduced viability at 32ºC and the inbreeding effect was reduced by one generation of interline crosses.

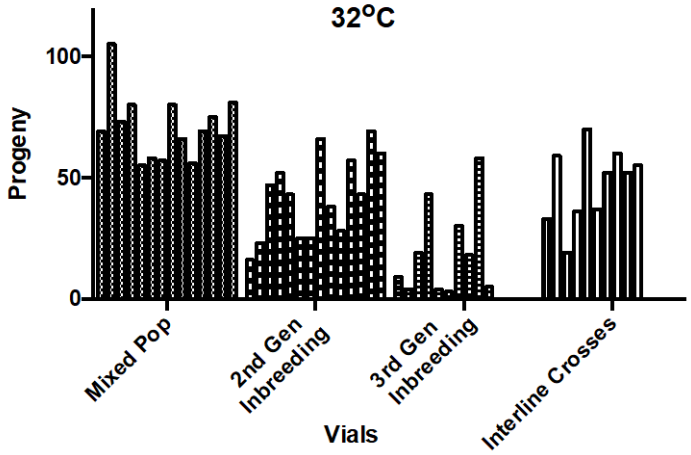

Figure 2. Significant interaction of inbreeding and temperature of 32°C on viability. Mixed Pop *vs*. 2nd Gen Inbreeding, P < 0.0002; Mixed Pop *vs*. 3rd Gen Inbreeding, P < 0.0001; 2nd Gen *vs*. 3rd Gen, P = 0.007; 3rd Gen *vs*. Interline Crosses, P = 0.002; and 2nd Gen *vs*. Interline Crosses, P = 0.88.

Some plants and animals are already adapting to global warming conditions, whereas others are not (see discussions of this topic in Davis *et al.,* 2005; Parmesan, 2006). For example, red-cockaded woodpeckers in the USA lay their eggs earlier in response to changing climate, while inbred woodpecker populations are failing to adapt to increasing temperatures (Schiegg *et al.,* 2002).

In support of the observed interactions of temperature and time of woodpecker egg laying, the results of our study show that inbred populations of the model organism *D. melanogaster* are affected more by stressful high temperatures than are non-inbred populations. This suggests that threatened populations or species at low numbers, where inbreeding depression is higher, may have a more difficult time adapting to increasing temperatures. This is mainly because of the homozygosis of recessive deleterious alleles and the loss of rare alleles by inbreeding and genetic drift, causing a loss of fitness in these small populations. These

rare alleles may be needed for future adaptations to arise in response to rapidly changing environments, such as new parasites and increased temperatures (Frankham, Ballou, and Briscoe, 2010; Hedrick, 2011). Hence, it would be of interest to test other environmental stressors, such as desiccation, starvation, and interspecies crowding, on the viability of inbred and non-inbred lines of *D. melanogaster*, to see if these interactions between stressors and inbreeding hold true.

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The effect of interspecific crowding of *Drosophila simulans* on the viability of *Drosophila melanogaster*.

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Crowding can affect life-history traits and fitness (see reviews of this topic in Hoffman and Parsons, 1991; Klepsatel *et al*., 2018). For example, adult and larval crowding in *Drosophila* has been observed to influence the rate of mutations (Kondrashov and Houle, 1994), lifespan (Klepsatel *et al*., 2018), quantitative variation of morphological traits (Imasheva and Bubliy, 2003), responses to selection (Birch, 1955), body size (Miller and Thomas, 1958), thermal stress resistance (Sorensen and Loeschcke, 2001), and fitness (Bijlsma *et al.,* 1999). Yet, most crowding studies were performed within a single *Drosophila* species and not between closely related *Drosophila* species.

Interspecific crosses were performed by Joly and Lachaise (1988), who observed that hybrid offspring viability of crosses between *Drosophila melanogaster* and *Drosophila simulans* was reduced under male crowing, and by Morgan and Woodruff (2006), who observed that crowding of *D. melanogaster* by *D. simulans* increased mutation rates in *D. melanogaster*.

In this study we hypothesized that crowding of *D. melanogaster* by its sibling species *D. simulans* would decrease the viability (fitness as measured by progeny numbers) of *D. melanogaster*. As predicted, crowding with a mutation-marked *D. simulans* line did reduce the viability of wild-type *D. melanogaster*.

To measure the influence of interspecific crowding on viability, we set up 29 vials of four females and four males of *D. melanogaster* (as uncrowded control vials) and 29 vials of four females and four males of *D. melanogaster*, plus four females and four males of *D. simulans* (crowded vials). The vials were maintained at room temperature (21 to 23°C) on standard cornmeal agar medium, parents were discarded after seven days, and progeny were counted each day for a total of 21 days from the day the crosses were initiated. The *D. melanogaster* line was a wild-type (red eyes) line collected from Perrysburg, Ohio, whereas the *D. simulans* line was marked with the X-linked w^{pch} eye mutation, which produces a peach eye color. If *D. melanogaster* flies mate with *D. simulans* flies, the progeny will be dead or sterile (Ashburner, 1989).

The results of these crosses are shown in Figure 1. There was a significant reduction in progeny numbers from the crowded crosses compared to the uncrowded crosses (P = 0.03; Mann-Whitney one-tail test;

Whitlock and Schluter, 2009). The mean number of progeny (± SD) of the uncrowded crosses was 72.76 ± 30.74, whereas the mean number of progeny of the crowded crosses was 60.72 ± 24.63.

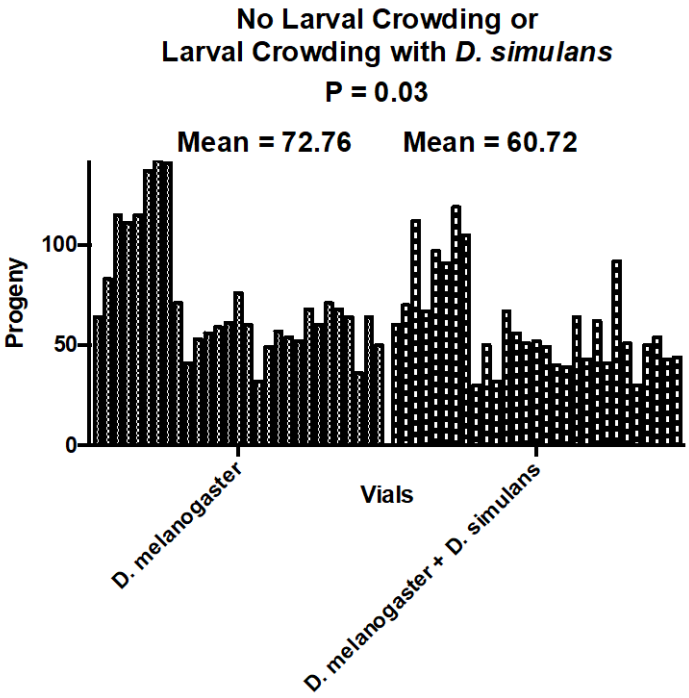

Figure 1. Influence of crowding on viability of *Drosophila melanogaster*.

In this study, the significant crowding effect could occur between adults of *D. melanogaster* and *D. simulans* searching for mates or sites to lay eggs. Crowding could also be between larvae searching for food or pupation sites. For example, crowding does affect the height of pupation in laboratory cultures of *D. melanogaster* (Ringo and Wood, 1983). Hence, it would be of interest to measure the influence of interspecific crowding on pupation height, and other life-history traits, in laboratory conditions.

The results of this study show that crowding can influence viability. From these results, one could also hypothesize that increasing the number of *D. simulans* flies in vials with the same number of *D. melanogaster* would give a concomitant greater decrease in the number of *D. melanogaster* progeny. To test this hypothesis, one could place six and then eight *D. simulans* females and males in vials with four *D. melanogaster* females and four males, expecting a relationship to occur between increased crowding and a decrease in the number of *D. melanogaster* progeny.

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Influence of stress on the somatic movement of the *mariner* DNA element in *Drosophila simulans*: I. Temperature.

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The *mariner* DNA element has been observed to move in the genome of somatic cells resulting in changes in gene expression, including mosaic spots in the eye of the *mariner*-inserted w^{pch} (white peach) mutation of *Drosophila simulans* (see reviews of this topic in Hartl, 1989; Chakrani *et al*., 1993; Nikitin and Woodruff, 1995; Robertson, 1995; Russell and Woodruff, 1999; Woodruff and Thompson, 2001). It has also been observed that high temperature stresses can increase the rate of movement of *mariner* DNA elements (Chakrani *et al*., 1993; Capy *et al*., 2000). In addition, other stressors, such as irradiation and chemicals, can increase the movement rate of DNA elements, although the rate of somatic movement of the *mariner* DNA element has not been tested against these stressors (McClintock, 1984; Eeken and Sobels, 1986; Groriev *et al*., 1990; Arnault and Dufournel, 1994; Giraud and Capy, 1996; Hoffman and Parsons, 1992; Vasilyeva *et al*., 1999; Belyayen, 2014; Piacentini *et al*., 2014; Fitzerald *et al*., 2017). If mariner does respond to these stressors, the identification of an increase in mosaic eyes in treated w^{pch} flies might be used to identify mutational stressors in the environment.

In this report, therefore, we begin a series of studies on the influence of stressors on the rate of somatic movement of the *mariner* DNA element in *D. simulans*, beginning with increased temperature. In additional studies, we will test other stressors for their ability to alter the rate of movement of the *mariner* DNA element.

To measure the influence of temperature on the rate of excision of *mariner* DNA elements from the X-linked w^{pch} mutation of *D. simulans*, we performed the following cross. The OK1 and OK2 lines were isolated from two *D. simulans* isofemales collected at the University of Oklahoma Biological Station in May of 2019, whereas the Per line was isolated from a single *D. simulans* isofemale collected at Perrysburg, Ohio in May of 2019. The w^{pch} mutation is caused by a 1,300 base-pair insertion of the *mariner* element, which can be excised out of the white locus by *mariner* transposase (see references and photographs of mosaics in Hartl, 1989).

P w^{pch} / w^{pch} virgin females × either OK1, OK2, or Per wild-type (+ / Y)males

The eyes of F1 w^{pch} males from each of the three P crosses were screened for red mosaic spots in a white-peach background, which are caused by the excision of the *mariner* DNA element from the w^{pch} mutation. The w^{pch} stock was stable; no mosaic spots were observed at room temperature among 449 flies (216 w^{pch}/w^{pch} females and 233 w^{pch}/Y males), and no mosaic spots were observed at 28°C among 311 flies (195 w^{pch}/w^{pch} females and 216 w^{pch}/Y males).

There was a significant increase in somatic spots at 28°C as compared to room temperature (22°C) in at least one eye of the F1 w^{pch} males with all three genetic backgrounds (OK1, $P < 0.0001$, see Figure 1; OK2, $P < 0.0001$, see Figure 2; and Per, $P = 0.04$, see Figure 3). These results confirm previous reports that *mariner* in the w^{pch} mutation excises at a significantly higher rate at higher temperatures (Chakrani *et al*., 1993; Capy *et al*., 2000).

The results of this study show that the transposase enzyme of the *mariner* transposable DNA elements is located in the autosomes of wild-type *D. simulans*, leading to excision events and the formation of red mosaic eye spots in a w^{pch} background. In 2001, it was reported that active *mariner* transposase was present in natural populations from the same locations as this study (University of Oklahoma Biological Station, Willis, Oklahoma and Perrysburg, Ohio). Active *mariner* transposase has also been identified in *D. simulans* populations from around the world (see references in Capy *et al.,* 1990; Russell and Woodruff, 1999). It is not

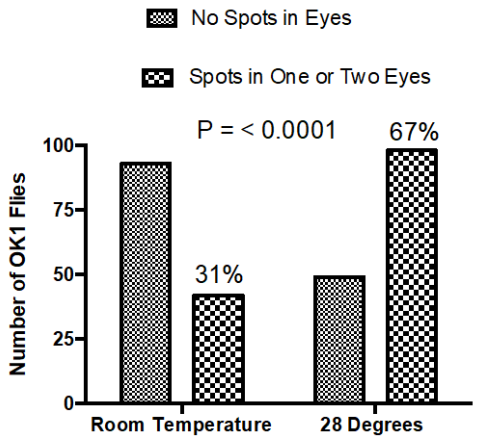

Figure 1. Significant (P = < 0.0001) increase in mosaic eyes in w^{pch} mutations of *D. simulans* at increased temperature due to movement of the *mariner* DNA element in a wild-type OK1 genetic background. There were 135 flies scored at room temperature and 147 at 28 degrees.

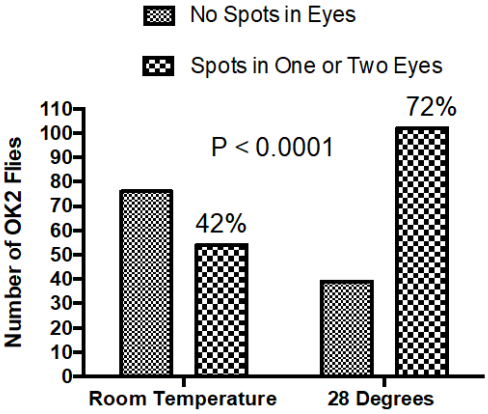

Figure 2. Significant (P = < 0.0001) increase in mosaic eyes in w^{pch} mutations of *D. simulans* at increased temperature due to movement of the *mariner* DNA element in a wild-type OK2 genetic background. There were 130 flies scored at room temperature and 141 at 28 degrees.

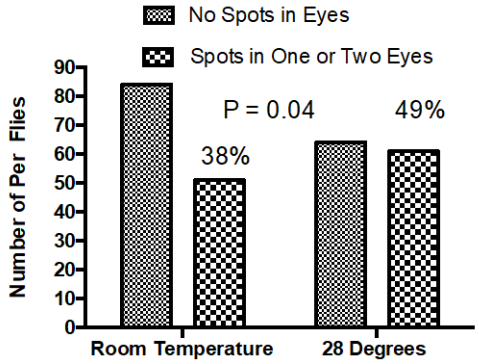

Figure 3. Significant (P = 0.04) increase in mosaic eyes in w^{pch} mutations of *D. simulans* at increased temperature due to movement of the *mariner* DNA element in a wild-type Per genetic background. There were 135 flies scored at room temperature and 125 at 28 degrees.

clear why a transposase, which can lead to deleterious excision and insertion events in nature, would be maintained in nature over time. Maybe the transposase is not active in nature, or as reported in this study, stress can lead to the excision of *mariner* elements, giving rise to new genetic variation that may be needed during changes in the environment, such as global warming. It has even been proposed that variation in maternal care can mediate the mobilization of transposable DNA elements (Bedrosian *et al.,* 2018), and that the movement of DNA elements could be one mechanism for speciation (Ginzburg *et al.,* 1984).

Since the results of this study confirm that stress (increased temperature) can cause an increase in the rate of movement of the *mariner* DNA element in *D. simulans*, we next intend to test other stressors, such as an increased salt and copper sulfate concentration in the diet, overcrowding with other *Drosophila species*, desiccation and starvation, on their ability to increase the rate of excision of *mariner* elements in the w^{pch} mutation of *D. simulans*. The results of these tests of additional stressors on the excision rate of *mariner* will tell us if the mariner/w^{pch} system can be used to identify other stressors in the environment.

References: Arault, C., and I. Dufournel 1994, Genetica 93: 149-160; Bedrosian, *et al.*, 2018, Science 359: 1395-1399; Belyayev, A., 2014, J. Evol. Biol. 27: 2573-2584; Capy, P., *et al.,* 1990, Proc. R. Soc. Lond. B 242: 57-60; Capy, P., *et al.,* 2000, Heredity 85: 101-106; Capy, P., and P. Gibert 2004, Genetica 120: 5-16; Chakrani, F., P. Capy, and J.R. David 1993, Genet. Sel. Evol. 25: 121-132; Eeken, J.C.J., and F.H. Sobels 1986, Mutation Research 175: 61-65; Fitzgerald, D.M., *et al*., 2017, Annu. Rev. Cancer Biol. 1: 119-140; Georgiev, P.G., *et al*., 1990, Mol. Gen. Genet. 220: 229-233; Ginzburg, L.R., P.M. Bingham, and S. Yoo 1984, Genetics 107: 331-341; Giraud, T., and P. Capy 1996, Proc. R. Soc. Lond. B 263: 1481-1486; Hartl, D.L., 1989, Transposable element *mariner* in *Drosophila* species. *In*: *Mobile DNA* (Berg, D.E., and M.M. Howe, eds.). pp. 531-536. American Society for Microbiology, Washington, D.C.; Hoffman, A.A., and P.A. Parsons 1991, *Evolutionary Genetics and Environmental Stress*. Oxford University Press, New York; Nikitin, A.G., and R.C. Woodruff 1995, Mutation Research 338: 43-49; Piacentini, L., *et al.,* 2014, Chromosoma 123: 345-354; Robertson, H.M., 1995, J. Insect Physiol. 41: 99-105; Russell, A.L., and R.C. Woodruff 1999, Genetica 105: 149-164; Woodruff, R.C., and J.N. Thompson, jr. 2001, Dros. Inf. Serv. 84: 2134-215; Vasilyena, L.A., *et al.*, 1999, Genet. Res., Camb. 74: 111-119.

Play-doh activity to enhance student understanding of restriction enzymes and plasmid cloning.

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Introduction

Undergraduate laboratory courses in biology, biochemistry, and molecular biology often involve experiments in which students manipulate DNA with restriction enzymes and clone a DNA fragment into a plasmid vector (Johanson and Watt, 2015). While there are many excellent textbook figures and online animations that visually show how restriction enzymes function and how they can be used, learning may be enhanced by hands-on activities. I have used textbook figures and online animations in the past as part of a unit on restriction enzymes and gene cloning in a genetics lab course that I teach. However, even after students use these resources and carry out an experiment to clone a DNA fragment into a plasmid vector, some students have difficulty conceptualizing how restriction enzymes work. These students struggle to accurately complete a lab worksheet (see below) in which they need to draw DNA molecules that have been cut with restriction enzymes and illustrate the process of cloning a DNA fragment into a plasmid vector.

Because models have been shown to be useful tools to allow learners to visualize complex ideas or processes (Gilbert, 2005), I developed a play-doh modeling exercise that is relatively easy and inexpensive. Since I began using this exercise, most students have been able to successfully complete the lab worksheet, suggesting that the play-doh exercise enhances student learning and conceptual understanding. The lab worksheet questions are written below, followed by a step-wise description of the play-doh exercise.

Lab Worksheet Questions

Worksheet question 1. Figure 1 shows a segment of the fruit fly genome that contains *geneX*, which is flanked by an *EcoRI* site on one side and by a *HindIII* site on the other side. The points at which *EcoRI* and *HindIII* cut each strand of DNA are indicated with arrows. To avoid writing out a long DNA sequence, the notation $(N)_{600}$ has been used to indicate that *gene X* contains 600 nucleotides that are not spelled out in the image. After the fruit fly DNA is digested with *EcoRI* and *HindIII*, there will be three fragments of DNA that contain or flank *geneX*. Draw all three fragments. Also label each strand of each fragment with 5' and 3' marks.

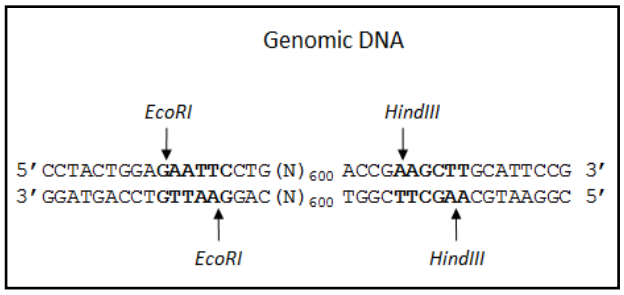
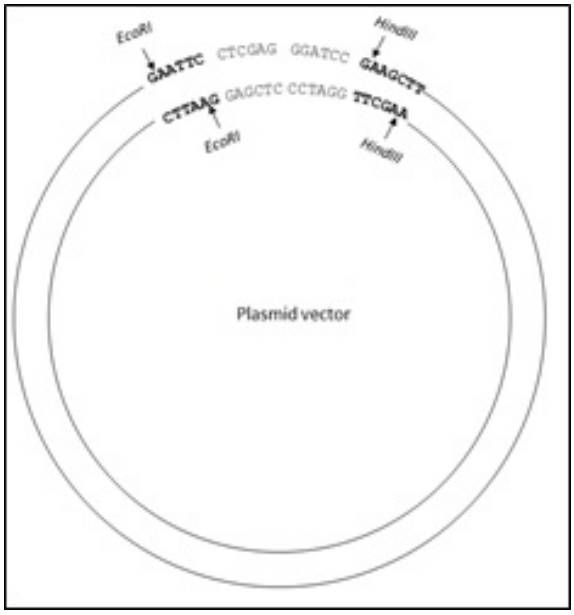


Figure 1. Genomic DNA in the region of *geneX*, flanked by *EcoRI* and *HindIII* restriction sites.

Worksheet question 2. Figure 2 shows a simplified plasmid vector, with only part of the polylinker sequence shown. Draw the plasmid molecule that would result if *EcoRI* and *HindIII* were both used to digest the plasmid vector. Label the cut ends of each strand of DNA with 5' and 3' marks.

Figure 2. Shown is a simplified plasmid vector. The arrows where *EcoRI* and *HindIII* enzymes cut the DNA on each strand.



Worksheet question 3. Draw the recombinant plasmid that would result if the sticky ends of *geneX* that had been doubly digested with *EcoRI* and *HindIII* were to hydrogen bond with the sticky ends of the plasmid that had been doubly digested with *EcoRI* and *HindIII,* and if DNA ligase was added to catalyze the formation of phosphodiester bonds.

Play-doh Activity to Model Using Restriction Enzymes in a Plasmid Cloning Experiment

In this activity, students use paper templates and play-doh to model what occurs when two restriction enzymes, *XbaI* and *BamHI*, are used in a plasmid cloning experiment. Each student needs two different colors of play-doh, a pair of scissors, and the paper templates of plasmid DNA and genomic DNA that are shown in Figure 3. These templates can be enlarged with a copy machine.

The steps that I ask students to follow are listed below. So that students can see what happens in each step, I use an overhead projector to demonstrate each step before students do that step.

Use the plasmid DNA template (enlarged from Figure 3) for steps 1-5.

1. Draw lines on the plasmid DNA template (Figure 4A) to represent "cuts" to the phosphodiester bonds between nucleotides within the restriction enzyme recognition site for *XbaI*. Repeat this for the recognition site for *BamHI*.

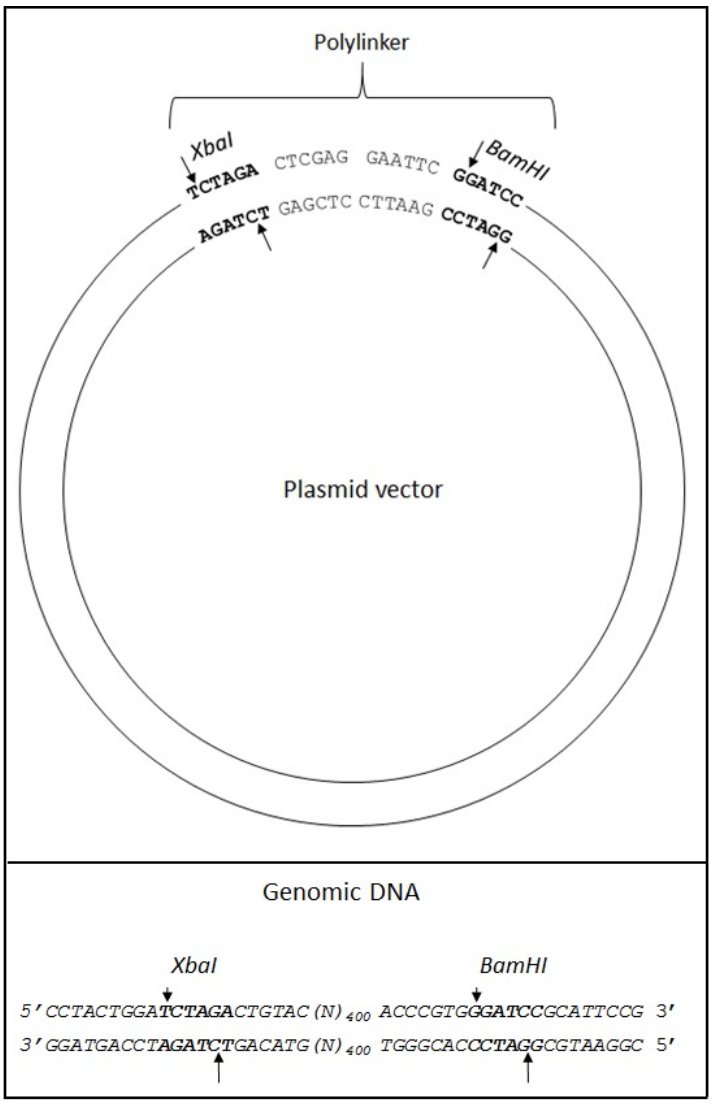


Figure 3. Paper templates to be copied and given to students. Only a short section of polylinker is drawn out for the plasmid DNA in the top portion of the figure. A hypothetical gene, "gene X" is contained in the genomic DNA and is flanked by *XbaI* and *BamHI* restriction sites.

2. Draw two lines to "link" the lines that were drawn in step one (Figure 4B). The area that becomes boxed in will be the section of DNA that will be removed from the plasmid vector after it is cut with the two restriction enzymes. Also, draw a series of dashes to represent the hydrogen bonds that form between complementary base pairs (Figure 4B).
3. Use scissors to cut excess paper from the plasmid DNA (Figure 4C).
4. Use scissors to cut each strand of plasmid DNA on the paper to represent how a restriction enzyme would cut its recognition sites (Figure 4D).
5. Still using the plasmid DNA paper model, use scissors to cut the hydrogen bonds linking the two strands together in the vicinity of the restriction enzyme recognition sites (Figure 4E). I find it is important to emphasize to students that the restriction enzymes are not cutting the hydrogen bonds, but that the hydrogen bonds in the area of the restriction enzyme cuts will tend to break spontaneously. At this point students should see that the plasmid has been cut to yield two fragments: one small fragment that contains part of the polylinker and a larger fragment that contains the remainder of the plasmid. They also should be able to see clearly how cutting with *BamHI* and *XbaI* left "sticky ends".

Use the genomic DNA template (enlarged from Figure 3) for steps 6-8.

6. Use the genomic DNA template (from Figure 3) and draw lines of the genomic DNA template to represent the "cuts" to the phosphodiester bonds that *BamHI* and *XbaI* would make (Figure 4F).

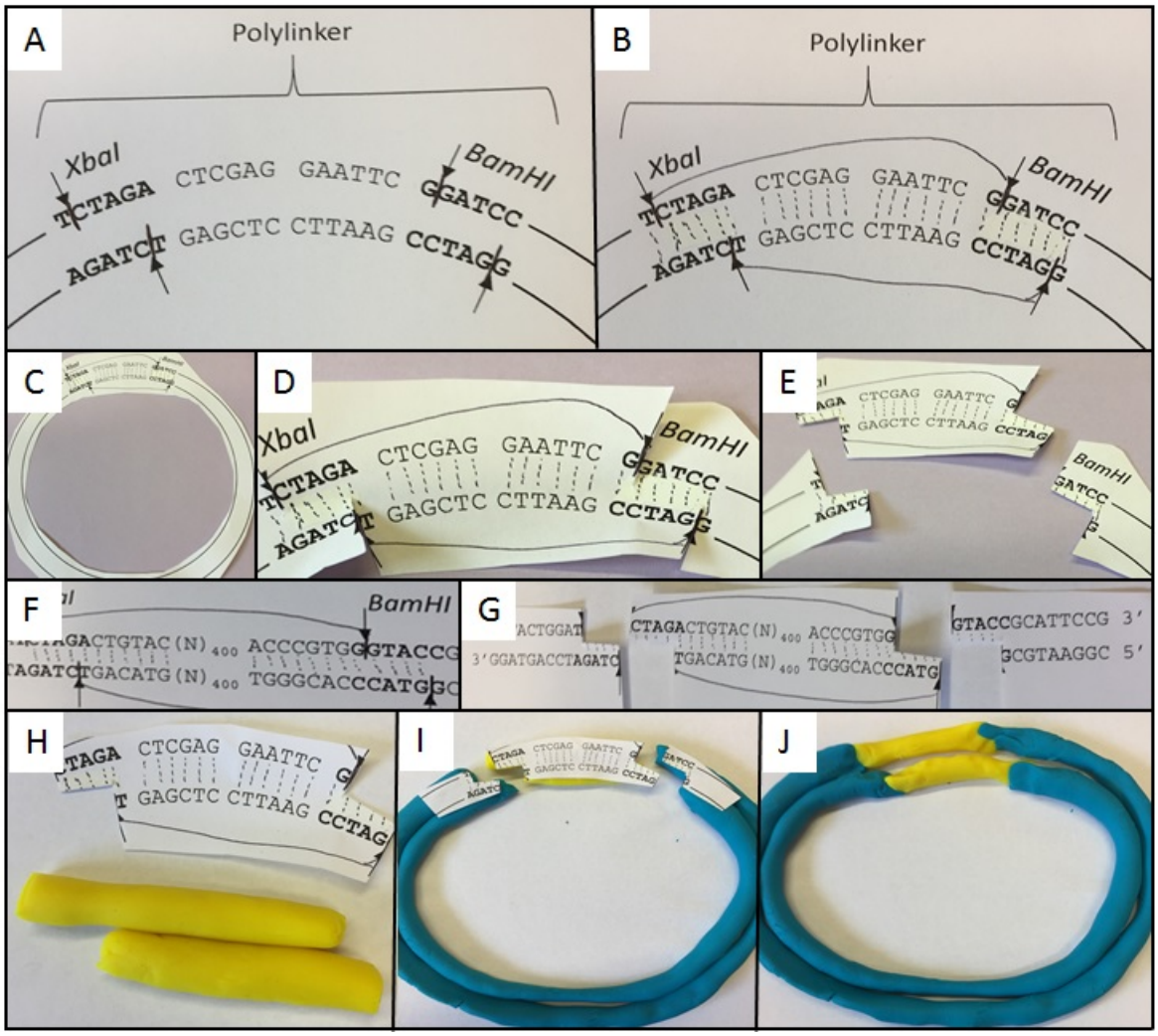


Figure 4. Pictures of the play-doh modeling exercise from start to finish.

7. Draw two lines to "link" the lines that were drawn in step 6. Also draw a series of dashes to represent the hydrogen bonds that form between complementary base pairs (Figure 4F).
8. Use scissors to cut each strand of the genomic DNA paper template to represent how *BamHI* and *XbaI* would cut their respective restriction sites. From this, three fragments should result (Figure 4G). The middle fragment contains "gene X", which in later steps will be cloned into the plasmid vector.

Use play-doh and the paper cut-outs for steps 9-12.

9. Roll out two short tubes from a single color (color 1) of play-doh and lay them side-by-side in an offset manner (Figure 4H). These two play-doh tubes should be the same size as the paper template that contains "gene X". Because of the sticky nature of play-doh, the two tubes of play-doh will adhere to one another weakly, which represents the way that DNA strands adhere to one another by hydrogen bonding between complementary, anti-parallel strands.
10. Roll out two tubes from a different color (color 2) of play-doh and lay them side to side in a circle, oriented in an offset manner so the "sticky ends" protrude from each side. Each play-doh tube represents one strand of the plasmid DNA. The play-doh tubes should be sized so that the plasmid DNA paper template will fit on top. Although the entire plasmid DNA paper template can be placed on top of the

play-doh strands, it is easier to just put the portion of the paper template with the restriction enzyme generated sticky ends on top of the play-doh (Figure 4I).

11. Place the genomic DNA paper template on top of the short tubes of the color 1 play-doh and position this play-doh/paper combination within the opening of the plasmid play-doh/paper combination (Figure 4I).
12. The final step is to allow "hydrogen bonding" to occur between the sticky end overhangs of the plasmid DNA play-doh model and the sticky end overhangs of the genomic DNA play-doh model, followed by the addition of DNA ligase. The action of DNA ligase can be simulated by pinching the plasmid play-doh model ends together with the genomic DNA play-doh model ends (Figure 4J). It is easiest to do this step if the paper cut-outs are removed from the play-doh.

Summary

I use this play-doh exercise in genetics lab the day that students start a series of hands-on experiments to use restriction enzymes to carry out a plasmid cloning experiment followed by transformation of plasmids into bacteria. This entire exercise could be performed without the play-doh by using just the paper templates. However, I prefer using play-doh because student's interest goes up at the beginning of the lab when I tell them they will be working with play-doh that day. At the end of a full day of classes (my genetics lab meets in mid-afternoon) when students are tiring, using play-doh lends an atmosphere of fun to the lab and engages students in the lab topic.

After students complete the play-doh exercise, I give them the lab worksheet questions to complete during lab time or as a take-home assignment, depending on time constraints. I have not carried out a controlled study to gather data to test the hypothesis that students taught with the play-doh exercise will do better on the lab worksheet than do students taught without the play-doh exercise. However, my impression is that since I started using this play-dog exercise, more students than in the past can accurately complete the worksheet and more students can accurately answer lab test questions about restriction enzymes and cloning.

References: Gilbert, J.K., 2005, *In*: *Visualization in Science Education* (Gilbert, J.K., ed). Dordrecht, The Netherlands: Springer; Johanson, K.E., and T.J. Watt 2015, Biochem. Mol. Biol. Educ. 43: 441-448.

Examining the impacts of inbreeding and temperature on sex ratios of *Drosophila melanogaster*: A test of the impact of anthropogenic global climate change on population and species conservation.

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It has been shown that the detrimental effects of inbreeding are enhanced under stressful environmental conditions (Bijlsma, 2000; Covell *et al*., 2019; Hoffman and Parsons, 1991; Ambruster and Reed, 2005; Frankham *et al*., 2002). These conditions include chemical stressors, crowding, and heat. Inbreeding and stress have been shown to change mating patterns in species and have an effect on viability (Schiegg *et al*., 2002). The more generations that a population is inbred, the more homozygosity and inbreeding depression the species will have (Onasch and Woodruff, 2008).

Many studies speculate how this inbreeding depression will play a role in population and species survival as global conditions become more stressful. Parmesan (2006) suggested that differences in dispersal abilities, life-history strategies, and physiological tolerances will play a role in distinguishing adaptation abilities amongst different taxa. In addition, Leigh (1981) has shown that as population size decreases, the probability of extinction of the population increases. This puts small endangered species on the forefront of viability risk due to small detrimental changes.

To examine the severity of inbreeding in a high-stress environment, we have tested the interaction of inbreeding and high temperature on sex ratios in lines of *Drosophila melanogaster* that have been inbred for

191 generations. We hypothesized that the sex ratio (mean ratio of male offspring to total offspring) would be significantly different in the test group that was exposed to high heat conditions (28°C) compared to room temperature conditions (22°C).

The inbred lines of *D. melanogaster* were obtained from crossing single brothers and sisters for 191 generations. The initial F1 crosses resulted in male and female siblings which were crossed to create the F2 generation. All subsequent generations were obtained in the same way. The flies were maintained using a cornmeal and agar-based substrate. After 191 generations, the inbreeding coefficient of each line is very close to 1, resulting in very little variation in the genetic structure of autosomes. The experimental cross consisted of C(1)DX, *y f*/Y females and w^{1118}/Y males. The w^{1118}/Y males displayed white eyes (*w*), while the females possess a compound X-chromosome and display forked bristles (*f*) and yellow body (*y*). The compound X chromosome consists of one normal X-chromosome attached to an inverted X-chromosome at a single centromere. C(1)DX is a strong balancer chromosome, meaning it aids in keeping homozygous, recessive, lethal or sterile mutations from being lost. This structure also prevents X-chromosome recombination and maintains possession of the Y chromosome in females and males, enabling this study to effectively examine the influence of temperature on each of the sexes.

The effect of increased temperatures on inbred populations was tested by storing 19 vials at room temperature (~22°C) and high temperature conditions (28°C). The number of C(1)DX, *y f*/Y females and w^{1118} male offspring were counted for each vial for up to 21 days from the set up date of the vial. The sex ratio was used to measure the effects of high temperature stress on *D. melanogaster* viability. A ratio increase would represent greater male viability while a decrease would represent increased female viability.

As shown in Figure 1, there was a significant difference in sex ratios between the room temperature and 28°C groups. A two-tail chi-squared test was used to analyze the data, resulting in a P-value of < 0.0001. The room temperature ratio was 264 males: 204 females, while the 28°C ratio changed to 256 males: 112 females.

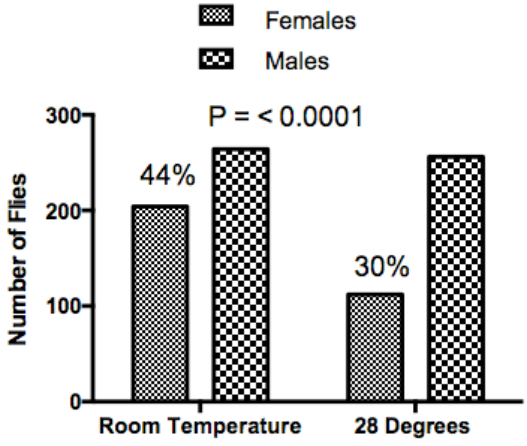

Figure 1. Significant interaction of inbreeding and high temperature (28°C) on sex ratios (P < 0.0001 by two-tailed chi-square test).

In support of the observed relationship between environmental stress and amplification of the negative effects of inbreeding on fitness, our study shows that sex ratios of inbred populations of the model organism *D. melanogaster* are affected by high temperatures. This disparity could be caused by a difference between males and females in the genes located on the X-chromosome. The females in this study possess two X-chromosomes, while the males possess one. This means that the males may be more susceptible to the effects of recessive genes, which may have led to a difference in their ability to cope with high temperatures compared to the females, as seen in Figure 1. The observed decrease in female offspring could point to the presence of a recessive gene in males that benefitted male survival at high temperatures. A species' ability to adapt to temporal variation relies on natural selection, gene flow, genetic drift, mutation, and demography. There has been much research and debate as to whether or not species and populations will be able to adapt at the pace of climate change. Some studies have seen individual species being unable to adapt quickly enough, while others have proved hopeful for the persistence of populations during environmental stress (Parmesan, 2006; Davis *et al*., 2005). It has been shown that smaller populations are more vulnerable to extinction (Leigh, 1981) and that low levels of genetic variation in small endangered species contribute to this heightened risk of extinction (O'Brien, 1994). Our study contributes to evidence that environmental stress (high temperatures) may affect the sex ratio of inbred populations. An alteration of sex

ratios can be detrimental for a population through alteration of mating patterns. If the number of females in a population decreases dramatically, reproduction rates will also decrease. Small inbred populations that are already struggling to reproduce will be affected the most by this change in sex ratios. In order to determine appropriate conservation strategies for small inbred populations, this study should be replicated on other taxa such as amphibians, birds, and mammals.

References: Armbruster, P., and D.H. Reed 2005, Heredity 95: 235-242; Bijlsma, R., J. Bundgaard, and A.C. Boerema 2000, J. Evol. Biol. 13: 502-514; Covell, E.A., D.M. Miller, M.A. Balinski, and R.C. Woodruff 2019, Dros. Inf. Serv. in press; Davis, M.B., R.G. Shaw, and J.R. Etterson 2005, Ecology 86: 1704-1714; Frankham, R., J.D. Ballou, and D.A. Briscoe 2010, *Introduction to Conservation Genetics*. Cambridge University Press, Cambridge; Hoffman A.A., and P.A. Parsons 1991, *Evolutionary Genetics and Environmental Stress*. Oxford University Press, New York; Leigh, E.G., 1981, J. Theoretical Biol. 90: 213-239; O'Brien, S.J., 1994, Proceedings of the National Academy of Sciences USA 91: 5748-5155; Onasch, K.D., and R.C. Woodruff 2008, Dros. Inf. Serv. 91: 151-155; Parmesan, C., 2006, Annu. Rev. Ecol. Evol. Syst. 37: 637-669; Schiegg, K., G. Pasinelli, J.R. Walters, and S.J. Daniels 2002, Proc. R. Soc. Lond. B 269: 1153-1159.

Symmetry of developmental cell death in wings of *Drosophila melanogaster*.

Thompson, James N., jr., Kimberly Faria, Carlos A. Delgado, Alexander W. Douglas, Nolan M. Henning, Isha Jhingan, Mary F. LaPorte, Luis E. Longoria, Jacob R. McKinney, Hunter S. Park, Sheeva Sowdagar, Willa Xie, Sairi Zhang, and Barbara Safiejko-Mroczka. Department of Biology, University of Oklahoma, Norman, OK 73068.

A series of experiments were designed in which an Experimental Genetics and Cell Biology Lab class and Independent Studies students explored genetic modifiers of developmental cell death. In earlier studies of cell death variation (Thompson, *et al*., 2015; Holy, *et al*., 2017), sequenced strains of *Drosophila melanogaster* were screened for modifiers of facet cell death in the compound eyes of *Drosophila* carrying the dominant mutation, *Bar*. In addition to identifying some key loci influencing cell death, an unexpectedly high degree of bilateral symmetry drew attention to the question, "How do structures like the two eyes in a *Bar* individual that develop during pupal metamorphosis form so symmetrically. This is surprising, given the fact that there is phenotypic variation among genetically identical individuals and the eyes are at a large distance from each other in the pupae making the coordination of their development expression difficult to explain?" Developmental variation among genetically identical individuals seems to be at odds with a high degree of symmetry within an individual. To explore how general such an observation of late-forming symmetry might be, our class has expanded the study of modifiers of cell death to the wings of *Drosophila* carrying the dominant mutation, *Beadex* (Bx^2; http://flybase.org; Figure 1).

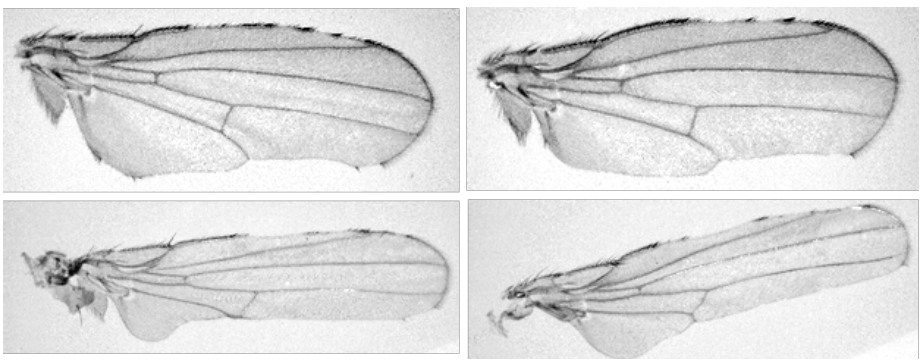

Figure 1. Representative pairs of Bx^2 wings from males heterozygous for two different sequenced genetic back-grounds showing reduced cell death (top row) and enhanced cell death (bottom row). There is a high degree of symmetry in spite of fly-to-fly variation in the degree of wing cell death.

Females carrying the X-linked allele *Beadex*[2] were crossed separately to males from sequenced strains of *D. melanogaster* developed by Trudy Mackay and her colleagues (Mackay, *et al*., 2012; DGRP, *Drosophila melanogaster* Genetic Reference Panel; Bloomington *Drosophila* Stock Center). In each such cross, F1 offspring are genetically identical, being heterozygous for the known genome of a sequenced strain and for the inbred genetic makeup of the Bx^2 standard. Both wings were removed from each F1 male adult and mounted on a microscope slide with DePeX mountant (BDH Chemicals Ltd., Poole, England). Wings were traced and quantified using the ImageJ morphometric program (Figure 2; imageJ.nih.gov) under an Olympus SZ61 microscope at 4.5× zoom and 10× eyepieces. Data measurements were replicated among students with no significant variation. There was, however, an unexpected scaling difference resulting from different computer screen sizes and the minor ways different computers were programmed. Using a small sample of wings that were measured by each student independently allowed such variation to be quantified and scaled away.

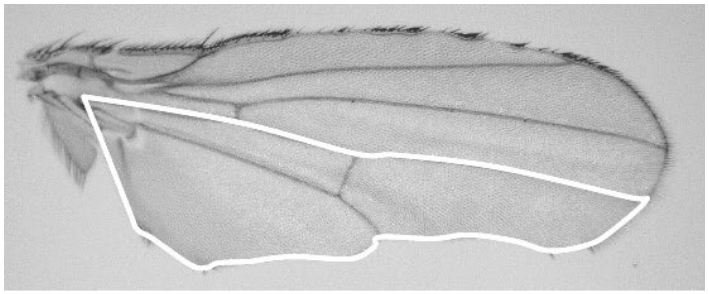

Figure 2. Wing area measurements focused on the posterior compartment of the wing, posterior to the L4 wing vein.

Areas of cell death are seen as cuts or notches along the edge of the wing, with the largest effects along the posterior margin. For this reason, our measurements now focus on the posterior margin, which is equivalent to the posterior compartment of the *Drosophila* wing. Two independent types of data are being evaluated. Symmetry of cell death expression is assayed by comparing the correspondence between the two wings from a fly. Separately, measuring genetically identical sibs from the same cross to a sequenced DGRP strain allows us to estimate environmental variation and the average genetic modifier effects of each sampled strain. Comparison of data from a series of different DGRP genomes will ultimately allow the detection of genetic regions of significant effect on wing area/cell death, marked by SNPs, using genome wide association mapping (GWAS; dgrp2.gnets.ncsu.edu). Our goal is a minimum of 100 separate genome assays. Although our dataset is not yet large enough for association mapping, the expression of symmetry within an individual is already quite evident.

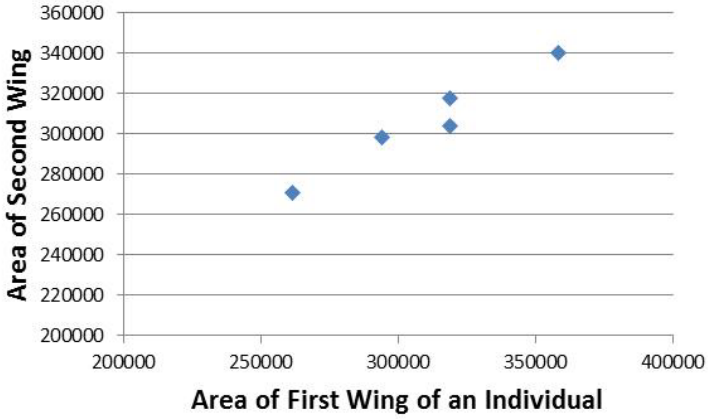

Figure 3. Degree of symmetry as seen in five pairs of wings from genetically identical individuals of the same DGRP sequenced genotype carrying the wing cell death mutation Bx^2. Amount of cell death varies but symmetry is retained. Units are pixels from ImageJ.

Symmetry can be seen in the example given in Figure 3. The high degree of symmetry between wings is seen in a correlation of 0.978 for this representative sample. That, however, highlights the remaining question. "How do sides of the body so physically distant from each other coordinate their developmental progress?" When symmetry is formed in the egg and early embryo, diffusion gradients provide the mechanism. What mechanism can yield a similar outcome in a much larger individual? That is a focus of our continuing study using the DGRP sequenced strains.

References: Holy, T., D. Tinney, J.N. Thompson, jr., T. Alford, J. Bogard, T. Bonham, A. Chang, P. Court, M. Dang, B. Davis, T. Duke, T. Dulworth, A. Ghani, H. Horning, C. Jourden, J. Kirk, M. Perkins, H.

Sowdagar, T.-N. Truong, and B. Safiejko-Mroczka 2017, Dros. Inf. Serv. 100: 167-169; Mackay, T.F.C., *et al*., 2012, Nature 482: 173-178; Thompson, J.N., jr., D. Tinney, J. Khoussine, G. Cox, J.R. Ogden, G. Audette, S. Bingabr, D. Branesky, A. Gomez, J. Lauderdale, C. Long, J. Mitchell, S. Narula, D. Nguyen, T.-V. Nguyen, D. Pons, C. Steele, G. Sutton, T. Tallman, and B. Safiejko-Mroczka 2015, Dros. Inf. Serv. 98: 162-164.

Other Reports

HISTORICAL PERSPECTIVE

It's the reason they call it a Memoir: H.J. Muller (1890-1967), Records and Recollections

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The National Academy of Sciences (NAS) Biographical Memoirs provides a starting point to learn about the life and scientific achievements of recognized researchers. Many Cornell graduates have been honored in this respect. In the field of early genetics, possibly the most cited is the work of Cornellians Harriet Creighton and Barbara McClintock (1931; McClintock, 1931) offering the cytological proof of crossing-over (Coe and Kass, 2005). While conducting research on the life and work of 1983 Nobel Laureate Barbara McClintock (Kass, 2013ff), and those researchers citing her early contributions, I was excited to learn from Hermann Joseph Muller's NAS Biographical Memoir (Carlson, 2009) that 1946 Nobel Laureate Muller had obtained his Master's degree at Cornell Medical College in 1911. Other reports, however, contradicted this claim, indicating that Muller had obtained a Master's degree from Columbia University in 1911 (Carlson, 1981; *AMS*, 1938, p. 1020; Pontecorvo, 1968). Searching for documentation at the archives of Cornell University, Cornell Medical College and Columbia University resolved this contradiction.

The search

I wrote to Muller's biographer, E.A. Carlson (email L.B.K. to E.A. Carlson, 22 March 2011), who recalled that Muller got his Master's at Cornell Medical School under J.R. Murlin. This contradicted Carlson's (1981) biographical report of a Master's for Muller at Columbia in 1911. Communications with the Cornell University Archives (Ithaca, NY) and Weill Cornell Medical College (NY, NY) provided reports that Muller had been registered as a graduate student at Cornell 1911-1912 but no degree was listed (*Cornell University Alumni Directory*, 1931). This was in agreement with a Biographical Memoir (Pontecorvo, 1968) of the Fellows of the Royal Society [London], that Muller held a teaching fellowship at Cornell Medical School 1911-1912 to teach and assist J.R. Murlin in work on creatin metabolism (physiological chemistry). Carlson also recalled a letter from Murlin to Muller which he told me had been deposited at the Cold Spring Harbor Archives (Murlin, 1948). That letter confirmed that Muller had worked with Murlin from 1911-1912, but Murlin could not recall the project and the work had never been published (Murlin, 1948). There was no mention that the work was done for a Master's degree.

The Cornell connection is strengthened by Muller's own recollection (Muller 1962, p. 6), that as a "Fellow in Physiology at the Cornell Medical College in New York City," in early 1912, he was preparing a co-authored book titled *Principles of Heredity*, only parts of which were published elsewhere. Excerpts were also reproduced in an anthology of Muller's works, *Studies in Genetics, The Selected Papers of H.J. Muller* (Muller, 1962, pp. 6-17). Muller's master's thesis, however, is not enumerated among Muller's 336 works (Muller, 1962, pp. 591-610).

Mystery solved

The Royal Society Memoir (Pontecorvo, 1968) reported that Muller held a graduate scholarship in physiology at "Columbia Medical School" under [Russell] Burton-Opitz and that he received an "M.A. in 1911, based on a thesis on the nature of the nerve impulse." The Columbia University, *Catalogue and General Announcement 1907-1908,* listed Burton-Opitz as a Member of the Columbia University Faculty and Teaching Staff. In 1902 Burton-Opitz had been appointed Adjunct Professor of Physiology in the College of Physicians and Surgeons, which was an "integral part of Columbia University" (*College of Physician and Surgeons, Announcement*, 1910). In 1910 Burton-Opitz was appointed Associate Professor of Physiology (*Catalogue of officers …*, 1912). His research laboratory was in the College of Physicians and Surgeons Building, bounded by 58th Street, Amsterdam Ave, 60th street (opposite Roosevelt Hospital), and Columbus Ave. Instruction was given at the Columbia University campus at Morningside Heights [bounded by 120th street on the North, 114th street on the south, Amsterdam Ave on the east, and Broadway on the west]. The two campuses were accessible by streetcar, or the 59th street subway station (Columbus Circle) would take the rider to the 110th street station, for a short walk to the Morningside Heights campus. In 1910 the Medical Department of Columbia University [College of Physicians and Surgeons] formed an alliance with Presbyterian Hospital, permitting ward hospital services for the "Department of Medicine and Surgery" (*College of Physician and Surgeons, Announcement*, 1910).

The Columbia University Archives resolved the contradiction of where Muller received his Master's degree by providing three documents confirming that Muller indeed was awarded his first post-graduate degree at Columbia University in 1911: 1) A copy of his Master's Essay [Thesis] is in their Archives, *The Nature of the Nerve Impulse*, Call Number: COA F11 v.39, and is bound with other titles (Muller, 1911). I have not personally examined this essay. 2) The *Columbia University Annual Commencement Program* dated June 7, 1911 records a Master of Arts for Hermann Joseph Muller (pg. 29), although his major professor is not listed. Relevant pages of the Program were scanned and sent to me from the archives on 22 August 2019. 3) The third confirmation is an entry from the Columbia University Registrar's book that lists the graduate courses Muller was enrolled in for his Master's degree during the academic year 1910 to 1911, after receiving his A.B. in 1910 (image from Columbia University Archives, received 22 August 2019). His Major and Minor subjects were recorded as Physiology, Zoology, and Biological Chemistry. The title of his Master's Essay is also entered at the bottom of the page. The date of recommendation for a Master of Arts is listed as June 1911.

Conclusions

As I demonstrated previously (Kass, 2003), memoirs and oral history interviews provide a starting place for biographical information on prominent scientists. These often lead to documents to affirm or refute the recollections recorded years after the events.

Extant contemporaneous documents have demonstrated that Muller received a Master's degree from Columbia University in 1911. Muller's Master's degree may have been awarded from the Department of Physiology in the College of Physicians and Surgeons, Columbia University, but the department and major professor are not recorded on the documents that I examined from the Columbia University Archives. His Masters' Essay [Thesis] may acknowledge his major professor and might also provide the location where the work was conducted. A visit to the Columbia University Archives to examine Muller's 1911 essay may provide this evidence. It is reasonable to assume that a student majoring in Physiology would have conducted research with the Professor of Physiology in the Department of Physiology, which was physically located in the College of Physicians and Surgeons, Columbia University. The Registrar's book shows that Muller had enrolled for two courses for his major in Physiology. The *Columbia University Bulletin of Information, College of Physicians of Surgeons Announcements 1910-1911* (1910), shows that the physiology courses that Muller enrolled in were taught by Professor Burton-Opitz.

Can I still be excited that Nobel Laureate Hermann Joseph Muller is a Cornellian? The Cornell University (Ithaca) *Alumni Directory* for 1931 states that Muller attended a graduate course from 1911-1912. Muller's Cornell University transcript (Registrar's card), held in the Cornell Archives, records that he matriculated on Sept. 30, 1911 and was admitted to the Graduate School on October 23rd 1911 to work in the Cornell University Medical College in New York City (image from Cornell University Archives, received 11 October 2019). Muller's (1946) Nobel Prize biographical sketch also states that he received a teaching

fellowship in physiology at Cornell Medical College and taught labs at Columbia University during 1911-1912. The *Merriam-Webster Dictionary* defines an alumnus as a person who *has attended* (my emphasis) or has graduated from a particular school, college, or university, and Cornell University always included Muller in its published *Alumni Directories*. I can be contented to conclude that H.J. Muller can be considered a Cornellian (an alumnus). He did *not*, however, graduate with a Master's degree from Cornell University. All his academic degrees (A.B., A.M., Ph.D.) were obtained from "Columbia University in the City of New York."

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Muller, H.J., 1962, *Studies in Genetics, The Selected Papers of H.J. Muller*. Indiana University Press, Bloomington, Indiana [A numbered list of "Works by H.J. Muller" appears on pp. 591-610; Muller's 1911 Master's Essay (Thesis) is not listed with these works].

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The Cornell University Archives, Ithaca, NY. Cornell University Library, Division of Rare and Manuscript Collections. https://rare.library.cornell.edu/collections/cuhist

60th Annual *Drosophila* Research Conference

The 60th Annual *Drosophila* Research Conference was held on 27-31 March 2019 at the Sheraton Dallas Convention Center, Dallas, Texas. The Conference Organizers were Michael Buszczak (Chair), Rachel Cox, Helmut Kramer, and Harmit Malik. The conference was sponsored by The *Drosophila* Board in association with the Genetics Society of America, 9650 Rockville Pike, Bethesda, MD 20814-3998.

Opening Remarks

Michael Buszczak: Welcome

Larry Sandler Award Talk

Laura Seeholzer (PhD, Rockefeller University)

Keynote Address

M.F. Wolfner. What's love got to do with it? Stimulating reproduction and activating eggs in *Drosophila*.

Plenary Lectures (in presentation order)

Elizabeth Gavis. Assembly and disassembly of germ plasm localized RNPs.

Bassem Hassan. The I of the fly.

Angela Douglas. The gut microbiome: the driving and driven partners of *Drosophila*.

Barbara Mellone. Interrogating centromere specification mechanisms.

Aurelio Teleman. Tissue growth and metabolic sensing: from flies to humans.

Angela DePace. Precision and plasticity in animal transcription.

J. Abrams. p53 genes and the game of transposons.

Mala Murthy. Neural mechanisms for dynamic acoustic communication.

Bernardo Carvalho. Y chromosome evolution in 400 *Drosophila* species.

Hongyan Wang. Waking up "Sleeping" neural stem cells.

Gwyneth Card. Towards a brain architecture for visual behavior selection.

Rick Fehon. Upstream regulation of Hippo signaling in epithelial cells.

The North American *Drosophila* Board

The Board's duties include: overseeing community resource centers and addressing other research and resource issues that affect the entire *Drosophila* research community. The Board also administers the finances for the annual North America *Drosophila* Research Conference and its associated awards, and it chooses the organizers and the site of the annual meeting. The Board consists of nine regional representatives and four international representatives, who serve 3-year terms. The three elected officers are President, President-Elect, and Treasurer. The three most recent Presidents continue participation on the Board as Past-President. In addition, the Board has *ex officio* members who represent *Drosophila* community resources or centers. For more information about the Board and the summaries of the annual Board meetings, see: the FlyBase web site.

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