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Genotoxic effects of fungicide *Zineb* – testing on *Drosophila melanogaster*.

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The sex-linked recessive lethal test for mutagenicity in *Drosophila melanogaster* was carried out with the fungicide *Zineb*. The test procedure provides the information on the mutagenic effects on 800 loci on the X-chromosome at the all germ line stages. The results presented here show that this fungicide induces recessive lethal mutations on X-chromosome of *Drosophila melanogaster*; therefore, it is the agent which can disturb ecological equilibrium in natural populations of insects.

Key words: *Drosophila melanogaster*, SLRL test, genotoxicity, fungicide *Zineb*

Introduction

Thiocarbamates (thiurames, etc.) are chemical compounds used for eradication of phytopathogenic and saprophytic fungi, bacteria and viruses, which cause various plant diseases. These chemicals act in different ways. Fungistatics prevent development of fungi, while antisporulants prevent formation of reproductive organs.

According to chemical composition fungicides are classified into organic, more frequently used and inorganic. They can also be classified as systemic and unsystemic; systemic fungicides are present in all parts of plants after application, and in that way they offer reliable protection for a certain period. Although unsystemic ones offer shorter protection, because they degrade faster under the sunlight and rainfalls, they are in wider use (Jovičić, 1975).

The active substances can be composed of organic compound of mercury, carbamates, copper compounds, inorganic and organic compounds of sulfur, derivatives of guanidine, etc. The presence of metallic ions in greater quantity in cells has toxic effect and causes various disturbances. Eradication of fungus diseases is based on consumption of metals (Jovičić, 1975). Mechanism of action of fungicides is twofold: phenols, amines and ketones prevent mitosis, while carbamates, copper sulfates and other compounds act on metabolism of fungi.

Frequently used thiocarbamates and thiurames are: *Ziram*, *Zineb*, *Tiram*, *Metiram* and others. *Zineb* (zinc ethylene bisdithiocarbamate) belongs to thiocarbamate group and has the widest application in protection of tobaccos, vegetables and fruits. Synonyms of this preparation are: acticupril-special, albran and aspor. Empirical formula is $C_4H_6N_2S_4Zn$.

Mutagenicity of various compounds widely used in agriculture can be tested through assays performed on different organisms, both *in vitro* and *in vivo* (Zimonjićetal, 1990). Among those, the sex-linked recessive lethal test (SLRL) with *Drosophila melanogaster* detects the occurrence of both point mutations and small deletions in the male germ line. This test is an *in vivo* assay able to screen for mutations at about 800 loci on the X-chromosome which represents about 80% of all X-chromosomal loci and approximately one-fifth of the entire *D. melanogaster* genome.

Materials and Methods

SLRL test was done with laboratory stocks of *Drosophila melanogaster* (obtained from Umea Stock center, Sweden). One is *Canton-S*, whose individuals have normal phenotype (wild type),

while Basc line flies are characterized with individuals homozygous for a balancer X-chromosome which carries two genetic markers: *Bar (B)* which produces a narrow eye shape in homo- and hemizygous conditions and a kidney shaped eye when heterozygous in females. Eyes restricted to a narrow vertical bar of 80 ± facets appear in males and 70 ± facets in homozygous females. A heterozygous female has an intermediate number of facets (360 ±) between homozygous females (70 ±) and wild-type (780 ±). The character can be regarded as partially dominant; *white-apricot* (w^a) – changes the red eye color into a light orange and is expressed only in homozygous females and hemizygous males; *scute (sc)* – recessive mutation that reduces the number of thoracic bristles. This mutation is linked with the long inversion on X-chromosome, which is necessary for suppression of crossing-over that could change existing gene combinations on treated chromosome (Zimonjić *et al*., 1990).

Although according to Hayes (1982) acute oral dose of toxicity of *Zineb* for rats is LD_{50} (mg/kg) = 5.200 mg/kg, its toxicity for adult individuals of *Drosophila melanogaster* has not been observed. For that reason the 5% solution of investigated substance was used.

Three day old *Canton-S* males were starved in empty bottles for 5 hours prior to treatment, and then transferred and fed in bottles with filter paper soaked with a solution of *Zineb* for 24 hours. After another 24 hours of recovery on standard medium, each male was mated individually to three *Basc* females, in 30 bottles, which made I brood. After two days, males were transferred to the new vials with three virgins of the *Basc* line (II brood), and after three days males were transferred again to the fresh vials with three *Basc* virgins (III brood). These males stayed with females three days and were removed afterwards. Females were left for five days to lay eggs, and then removed. The solvent 1% sucrose served as the negative control, while 0.025M ethyl-methane sulfonate (EMS) was the positive control (Lewis and Bacher, 1968; Stamenković – Radak *et al*., 1986).

After F_1 emerged, brother-sister matings were allowed for several days and 10 females from each vial were placed individually into the new vials. Each vial will give the progeny of one treated X-chromosome.

In F_2 the phenotypes were scored according to the eye color and shape. Absence of the wild type males indicated the presence of recessive lethal induced by the test substance.

The stocks were maintained and all experiments were done under optimal conditions (t = 25^{o}C, relative humidity = 60%, 12/12 hours of light/dark regime) on a standard nutritive medium for *Drosophila* (cornflour, yeast, agar, sugar and nipagin to prevent mold and infection).

Total number of treated X-chromosomes is equal to the sum of lethal and nonlethal cultures, and frequency of sex-linked recessive lethal was calculated by the ratio of number of lethal to total number of treated X-chromosomes. Testing of significance of difference in percentage of lethals was done by testing for big independent samples (testing of difference between proportions – Petz, 1985).

Results and Discussion

With accelerated development of industry and agriculture it became clear that toxity and genotoxicity are not synonyms, so in that time numerous chemical compounds were tested (Ames *et al*., 1975). With development of chemical industry and application of new scientific farming methods, the numerous groups of chemical materials of wide application have been used as pesticides that serve for eradication of weed, plant pests, and causes of plant diseases. They may contain active substances, such as alkylating agents, bromides and chlorides, which have the ability of induction of mutations in different organisms, including humans (Zimonjić *et al*., 1990). Chemical mutagens can be specific for sex, species, and phase of cell cycle, so that extensive investigations are necessary to determine at the field the chemical mutagenesis.

Zineb is an organic fungicide from the thiocarbamate group, which after some time, depending on climatic conditions, disintegrates to elementary zinc. It uses for eradication of wheat rust on grapevine, potatoes, hops, tobacco and tomato, than for eradication of rust disease and cause of spots on different farm and truck farm cultures. Also, it is used for eradication of sooty scabs on apples and pears, as well as porosity of leaves, monilia on peach, plum, cherry and apricot.

By short test for detection of mutagenicity in *Drosophila melanogaster in vivo* conditions we have found out mutagenic effect of investigated fungicide *Zineb*. In concentration of 5% it induces sex-linked recessive lethal mutations on the X-chromosome of *Drosophila melanogaster* males which were treated acutely with this fungicide (Table 1). The frequency of germinative mutations induced by this fungicide is significantly higher than the frequency of mutations induced by the known mutagen (EMS-positive control). The obtained results show that spermatid cell line (brood II) is especially sensitive to the influence of this fungicide.

Table 1. Frequencies of SLRL mutations after treatment of *Drosophila melanogaster* males with *ZINEB.*

	EMS (positive control)	SUCROSE (negative control)	ZINEB (test group)	$t_{z/s}$	$t_{e/s}$
I brood ∑	278	315	208		
N_o of lethals	27	5	24	4.29***	4.25***
% of lethals	9.71	1.59	11.54		
II brood ∑	277	269	187		
N_o of lethals	39	5	46	6.97***	5.45***
% of lethals	14.08	1.86	24.60		
III brood ∑	279	252	253		
N_o of lethals	18	6	21	2.99***	2.39**
% of lethals	6.45	2.38	8.30		
I+II+III ∑	834	836	648		
N_o of lethals	84	16	91	8.42***	7.16***
% of lethals	10.07	1.91	14.04		

** $p < 0.01$
*** $p < 0.001$

The obtained results are not in accordance with the expected based on some previously known data. Vasudev and Krishnaurthy (1980) have investigated mutagenic effect of fungicide *Mancoceb* from dithiocarbamates group, and after treatment of *Drosophila melanogaster* males, mutagenic effect of this fungicide has not been established. Frequency of autosomal and sex-linked recessive lethal mutations in descendants of these males was not significantly different. Contrary to these data, in mouse, a significant increase of aberration frequency has been established after treatment with this fungicide (Khan and Sinha, 1996). Detailed review of investigation of genotoxic effects of *Mancozeb* could be found in paper of Gandhi and Snedecer (2000).

It is well known that the zinc is one of the microelements which is present in human organism in traces, because it is necessary for normal function of pituarity, insulin and gonadotropin hormones. Similarly, participation of zinc is important in building some enzymes: carbonic anhydrase and glycin dipeptidase, as well as in activation of some enzymes of intermediary metabolism (Jovičić, 1975). The recent data show existence of so-called "finger" proteins that have series of "zinc finger", and represent structure in which zinc ions bind to a small group of amino acid. Some consider that the "finger" proteins are founders of transcriptional factors, which help function of RNA polymerase. Such transcriptional factors are identified in *Drosophila melanogaster*, and they are significant for embryonic development of this species (Lewin, 2000).

The results of our experiments show that the tested fungicide *Zineb* that contains zinc and which is used for protection of fruit, vegetables and tobacco in order to suppress phytopathogenic and saprophytic fungi, induces recessive, lethal X-linked mutations in all germ cell lines (in premeiotic - spermatocytes as well as in postmeiotic - spermatids and spermatozoids). Although the concentration of fungicide applied in agriculture (0.2-0.4%) is much lower that tested (5%), uncontrolled usage of this fungicide can disturb ecological and genetical equilibrium in natural populations of insects.

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Norm of reaction or norm of an individual.

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The expression “norm of reaction” is composed of two words: the “norm” and the “reaction”. The term ‘norm’ in genetics is defined as the behavior and survival of a phenotype or of a genotype for a trait in question in an environment. It could be in terms of interaction between a genotype and the environment (genotype-environmental interaction) or the behavior of a human being under a certain environmental condition.

In order to understand the mechanism of genotype-environmental interaction, we need to have the basic knowledge of three important elements: phenotype, genotype, and the environment.

Phenotype: an observable morphological trait or a hormonal and physiological process resulting in an observable trait of an individual. It is always expressed in words such as yellow or white for color of a flower.

Genotype: the genetic constitution for a trait of an individual. It is always represented by a combination of letters such as YY or Yy or yy. In this example, the letters Y and y denote dominant and recessive alleles respectively for a trait in question.

Environment: the place where a phenotype of an individual organism survives.

In addition to the three terms (phenotype, genotype and the environment) cited above I will introduce a new fourth term called an “**individual”** and that can be referred to as a genotype, a phenotype, a human-being or to an environment. Later, I will discuss in brief and also justify why I include the word “individual”.

The word “reaction” means the final outcome of mixing two entities, specifically chemicals, together under a certain environment. In case of human being when I consider an individual or a

group of individuals or human society in a locality or population as a whole and ask the question as to whether an individual's statement is the outcome of the chemical reaction occurred within his body. In this case, the individual does not demonstrate the involvement of any type of chemical reaction. However, such an outcome does involve a combination of an individual's emotional, genetic, hormonal and physiological reflection and such internally occurred phenomenon (within an individual's body) one does not observe. In such cases, however, what one really notices is the expression of feelings in terms of "behavior" of an individual and that, later on, can be expressed at the level of a human population or a society. In this case, an individual shows his feelings facially or expresses behaviorally. Thus, in case of a human individual, the word reaction does not fit appropriately and correctly in the scientific terminology. On the contrary, however, the word the expression of feelings in terms of "behavior" certainly does fit better to explain the question.

Several studies have been carried out on the norm of reaction in plants and animals. Such studies, however, provide the information on the effect of genotype, environment and the genotype-environment interaction from selection and evolutionary significance. (For example: on *Drosophila* by Gupta and Lewontin, 1982; on mice by Bateman, 1971; on grasses by Breese, 1969). In all the studies made on either with plants or animals, we observe only the interaction effect of a genotype with its environment *not* that of the reaction effect. Each of these studies is, however, based upon an individual genotype tested under a set of different environments, and uses the word 'interaction' and not the word 'reaction'.

For population and developmental geneticists, norm of reaction of a genotype is the final outcome for a morphological trait resulting from a genotype tested in different environments. That is to say that how a phenotype is developed from a given genotype when tested in more than one environment (or the "norm of reaction" of a genotype as the array of phenotype that will be developed by the genotype over an array of environments, Gupta and Lewontin, 1982). For this kind of study, two or more environments are required. For our purposes, the study in two environments is not enough to represent the data in graphic form as it will always provide a straight line even if there is a genotype-environment interaction effect. Such information turns out to be incomplete and wrong if the best genotype needs to be selected under a certain environment. However, three different environments are considered to be the optimal to represent the data in graphic form to demonstrate the genotype-environment interaction effect for two or more genotypes in question. For example, if we consider the study of two different genotypes tested in three different environments, we can have only five different models to show the effect of genotype, environment and the genotype-environment interaction, and that, in fact, is the *core* key for identifying and choosing the *best individual* genotype for selection purposes (see Gupta, 1978).

Thus, the norm of an individual implies the behavior or final outcome for a trait of a genotype or of a phenotype (an individual human being) tested under a set of different environments. To cite an example, let me consider the instructional capacity of an individual (human being) in producing the quality of students as one observable phenotypic trait, and an education system as the environment at the academic institution. An individual phenotype could not accomplish much in one environment because of the politics of that institution. However, when that individual is placed in a different environment where the emphasis is on academics and not on personal and group politics, he/she accomplishes a great deal. Such an example clearly reflects and demonstrates the significant impact of an environmental interaction (group of individuals in that institution) on an individual's performance. In other words, there is a relationship between an environment and the performance of an individual. Such relationship can be positive or negative depending upon the interest of that institution from academic or group view point. That is to say, how the selection of an individual for his academic capability is reflected from one environment to another. Also, how an educational-environmental system could prove to be from excellent to worse for a capable individual phenotype

depending upon whether a significant importance has been given or not to the education in producing good quality of students.

Finally, from the facts and statements cited above, I draw conclusion in brief, that the *norm* is based completely on the *scale* of an *individual* and comparing that individual with others (whether it is a genotype or a phenotype) tested under a set of environments to identify which environment is the best for that individual or which individual is the best under a certain environment and then select that environment for that individual genotype or phenotype. On the contrary, an individual human being (phenotype) can also select the best institution (environment) where he can accommodate himself and express better academically. Here, as there is no indication of the involvement of the word *reaction* and I, therefore, recommend its exclusion from the terminology of the norm of reaction and inclusion of the word "individual". That is to say, the norm of reaction should be termed as the **norm of an individual** and not the norm of reaction. Likewise, one can use the terms norm of a genotype or norm of a phenotype without including the word "reaction".

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First record of *Drosophila flexa* in the state of Santa Catarina, southern Brazil.

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The subgenus *Siphlodora* of *Drosophila* includes just two species: *D. sigmoides* Loew, 1872, from Nearctic Region, and *D. flexa* Loew, 1866, from Neotropical Region. The former is restricted to the United States, associated with the grass *Tripsacum dactyloides* L., whereas the latter breeds on maize (*Zea mays* L.). Vilela and Bächli (2000) hypothesize that *D. flexa* was probably restricted at first to Mexico, Guatemala and Honduras, the habitat of the ancestors of maize, and would have expanded its distribution following the spreading of modern maize cultivation. Nevertheless, as the cultivated maize is just around 8,000 years old, it is probable that this fly uses also a wild relative of maize as a breeding site, although this is an aspect of its biology still to be elucidated. According to those authors, *D. flexa* is found nowadays from Mexico to Brazil and Argentina, including Guatemala, El Salvador, Nicaragua, Costa Rica, Panama, Colombia, Venezuela, Ecuador, Peru, and Bolivia. In Brazil, it had already been recorded in the states of Rio Grande do Norte, Goiás, Rio de Janeiro, São Paulo and Paraná. The southernmost locality in which its distribution has been established was, previously, the province of Salta, northern Argentina (24º46'S; 65º28'W).

Santa Catarina Island, municipality of Florianópolis (27º39'S; 48º31'W), lies on a humid subtropical area and shows a great variety of environments. Notwithstanding the great amount of collections of drosophilids that has been carried out in the island since the beginning of the 1990's (De Toni and Hofmann, 1995; and unpublished data), no *D. flexa* individual was collected until July

2002, when one single male specimen was captured in Itacorubi mangrove forest. Thereafter, other individuals were caught. Altogether, 14 specimens have already been collected, in six localities of the island, as seen in Table 1.

Table 1. Individuals of *D. flexa* collected in Santa Catarina Island.

Locality	Date	Number of individuals
Itacorubi	July/2002	1
(mangrove forest)	October/2003	1
Campus of UFSC	February/2003	1
(urban zone)	May/2003	1
Florianópolis downtown	February/2003	2
(urban zone)	August/2003	1
Morro da Cruz (urban zone)	August/2003	1
Ratones	January/2004	4
(mangrove forest)	April/2004	1
Tavares (mangrove forest)	April/2004	1

This fly is, therefore, very rare in the Santa Catarina Island collections. The only 14 individuals collected were found in six localities where the total of drosophilids analysed reaches, altogether, 281,078 flies. This fact is, however, very probably an outcome of a biased method of collection. The flies were captured using banana-baited traps (similar to Tidon and Sene, 1988), and this collection method has only occasionally been effective in attracting this species. Contrasting to this, Vilela and Bächli (2000) have collected 291 individuals aspirated from tassels and ears of maize in only three collections. Similarly, 376 imagines emerged from a total of 16 maize tassels, in Santa Isabel, state of São Paulo, Brazil.

It is important to pay attention to the types of environments in which *D. flexa* has been recorded in Santa Catarina Island. Until now, all the individuals were found in urban zones (Florianópolis downtown, *campus* of UFSC and Morro da Cruz) and mangrove forests (Itacorubi, Ratones and Tavares). These environments are relatively open and, perhaps are closer to the cultivated maize, the only breeding site known for this species until now, or to another putative wild host plant. Interestingly, *D. flexa* has never been found inside the Atlantic Rain Forest, despite several studies on this environment in Santa Catarina Island (De Toni and Hofmann, 1995; De Toni *et al.*, 2001; unpublished data). Incidentally, this is probably the reason why *D. flexa* went by undetected in the state of Santa Catarina before, since field works in areas of open vegetation have begun just very recently.

So, the present paper reports, for the first time, the occurrence of *D. flexa* in the state of Santa Catarina, southern Brazil, and widens its geographical range southwards, down to Santa Catarina Island. This record agrees with Vilela and Bächli (2000), who suggest that *D. flexa* would be present in many regions of America where maize is also present, and its apparent absence in some areas might be mostly due to improper collections.

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Effect of light-dark regimes on the deviant sex-ratio in *Drosophila rajasekari*.

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Introduction

Mendelian laws are pertinent to all principles of population genetics, and in most populations an equilibrium is maintained in the sex-ratio. In *Drosophila*, the deviation from the expected 1:1 sex-ratio is prevalent in a few species symptomatic of the rare non-Mendelian form of segregation (Tao *et al.*, 2001). The major mechanisms postulated for modification of sex-ratios are prezygotic selection including meiotic drive, dependence of viability selection on zygote genotype, and the interaction of larval genotypes influencing egg to adult viability (Curtsinger, 1984). A deviant sex-ratio is an important evolutionary phenomenon in sexually reproducing species, and it has strong ecological implications like the unrestrained increase of individuals of one sex, which could result in a population bottleneck (Dermitzakis *et al.*, 2000) and augment the risk of species extinction (Hamilton, 1967).

Occurrences of the deviant sex-ratio in *Drosophila* due to the meiotic drive of X chromosomes have been discussed (James and Jaenike, 1990). The sex-ratio bias was attributed to a male killing agent in Brazilian strains of *D. melanogaster*; however, in this case, males did not transmit this trait to their offspring (Montenegro *et al.*, 2000). The varying expression of sex-ratio was perceptible in *D. mediopunctata* where aging in males was found to enhance the gender bias towards females (De Carvalho and Klackzo, 1992). In *D. simulans*, disparity in the relative numbers of functional X and Y bearing sperm or the elimination of Y bearing sperm resulted in the sex-ratio distortion (Montchamp-Moreau and Joly, 1997). An extreme meiotic drive was shown to give rise to exceptionally skewed sex proportions in *D. mediopunctata* (Varandas *et al.*, 1997). Moreover, an increase in the density of natural or cage populations of *Drosophila* led to a decrease in the number of females, which was interpreted as the density-dependent selection of sex (Grechanyi and Pogodaeva, 1996).

Here we report the deviant sex-ratio in *D. rajasekari* where the females significantly outnumbered males in natural populations captured in the Ahmednagar College campus, Ahmednagar. This species is found in tropical and subtropical regions under diverse ecological conditions (Joshi, 1999). Most of the earlier reports on deviant sex-ratios in *Drosophila* have emphasized the genetic analysis of distortion and suppression of sex chromosomes (Dermitzakis *et al.*, 2000; Montchamp-Moreau and Cazemajor, 2002). However, the effects of abiotic factors such as light-dark cycles, photoperiod, and temperature on the biased sex-ratio seem not to be investigated. In the present study, the deviant sex-ratio was investigated under four light-dark regimes. It was observed that artificial light dark cycles as well as the natural light-dark cycles resulted in the deviant sex-ratio, while the aperiodic environments of continuous light or continuous darkness at a constant temperature suppressed it.

Materials and Methods

The routine capturing and sexing of adults of *D. rajasekari* revealed a sex ratio bias in the natural populations of the Ahmednagar College campus. This casual observation was investigated in

the field and laboratory in detail. Ten groups of flies were trapped at random from the same area and sexed to confirm the sex-ratio bias. The laboratory populations of *D. rajasekari* originated from seven gravid females captured on the Ahmednagar College campus. Populations were raised as a mass culture and maintained on the standard culture medium at 27 $\pm$ 0.5ºC and about 60% relative humidity in climate controlled cubicles.

The deviant sex-ratio was investigated under three light-dark regimes at 27 $\pm$ 0.5º C in the laboratory: 12 h of white light at 450 lux and 12 h of complete darkness (LD 12:12), continuous light at 450 lux (LL) and, continuous darkness (DD). The gender ratio was also determined in flies maintained in the natural light dark and temperature cycles (NLD) in outdoor cages kept in the animal house near our laboratory. The temperature fluctuated from 19ºC to 37ºC, and the light intensity of about 12 h photoperiod varied from about 10 lux in the morning or evening to about 64,000 lux at noon and less than 1 lux at night.

In each cycle, the sex ratio was determined in the progeny of ten groups (freshly eclosed, 10 males and 10 females per group) of flies. Each group was introduced in a culture bottle (vol. 500 ml) and allowed to breed. The parents were transferred to fresh bottles when the F1 pupae appeared. The newly eclosed F1 flies were sexed and counted every day. This schedule was continued until all the stocks were exhausted and resulted in counting of all the viable offsprings with little or no mortality in the adult stage. One-way Analysis of Variance (ANOVA) was carried out to determine the significant effect of various light-dark cycles on the sex-ratio.

Results

The effect of light-dark regimes on the deviant sex-ratio was investigated in the laboratory reared populations as well as in the natural population of *D. rajasekari* and the results are summarized in Figure 1. It was observed that the populations reared in LD 12:12 cycles at constant temperature (LD 12:12) and in the natural light-dark cycle (NLD) at fluctuating temperature and light intensity exhibited the deviant sex-ratio. Similarly, the natural populations (NP) captured in the Ahmednagar College Campus also showed the sex-ratio bias. However, the bias was not observed in the populations maintained under aperiodic regimes of continuous light (LL) and continuous darkness (DD) at a constant temperature of 27ºC. The males were significantly less than the females in populations maintained in LD 12:12 cycles ($F_{1,18} = 4.97$, $p < 0.05$) and in NLD cycles ($F_{1,18} = 15.41$, $p < 0.05$); a similar trend was observed in the natural populations ($F_{1,18} = 664.0$, $p < 0.05$). The males, however, were not significantly different from the females in the populations maintained in LL ($F_{1,18} = 0.29$, $p > 0.05$) and DD ($F_{1,18} = 0.28$, $p > 0.05$).

Discussion

These results demonstrate the presence non-Mendelian segregation in the natural and laboratory reared populations of *D. rajasekari*. It was shown that the non-Mendelian segregation in *Drosophila* was the result of the genetic variation in the natural populations of sexually reproducing species (Curtsinger, 1984). The infrequency of non-Mendelian segregation in natural populations reflects not only its transience as an evolutionary phenomenon, but also its rarity as a biological phenomenon (Tao *et al.*, 2001). In the present study, the deviant sex-ratio was observed in the natural populations of *D. rajasekari* hence, the contributory factors that could simulate the sex-ratio bias such as the differences between males and females in their individual response to baits, location, weather, or time of collection are ruled out. These results are in agreement with those discussed by James and Jaenike (1990) who investigated the female favored bias in the populations of *D. testacea* inhabitating natural breeding sites.

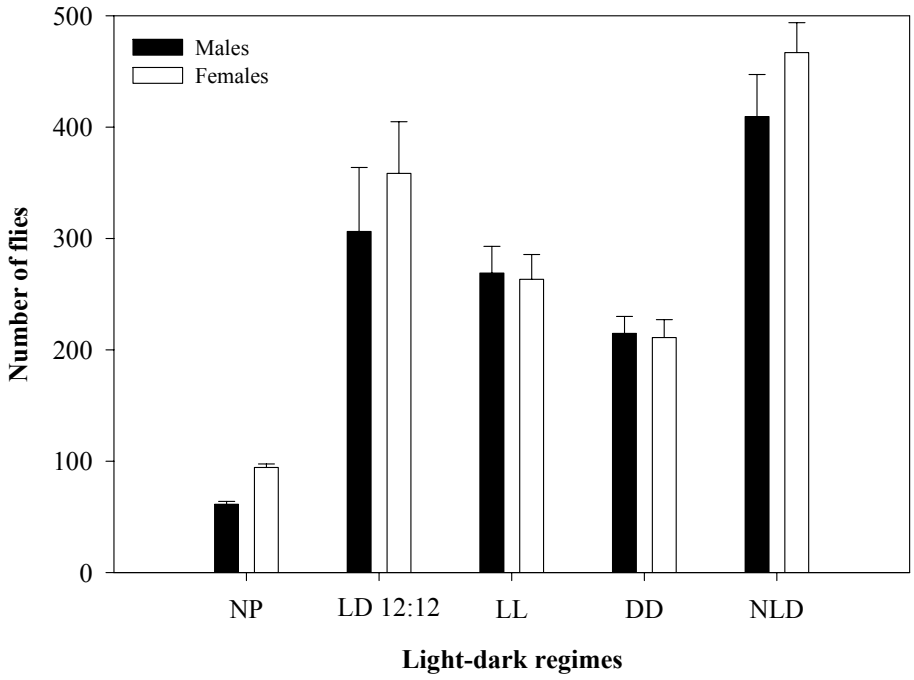

Figure 1. The males and females (mean ± SD, N = 10 groups) of *D. rajasekari* reared in artificial light-dark cycles of LD 12:12 (LD 12:12), continuous light (LL), continuous darkness (DD), natural light-dark cycles (NLD) and the natural population (NP) captured in the field.

Most of the earlier reports on deviant sex-ratios were attributed to the meiotic drive of sex chromosomes. Other plausible assumptions for the biased estimates of sex-ratio in *Drosophila* include gamete dysfunction during spermatogenesis (Hartl *et al.*, 1967; Policansky and Ellison, 1970; Matthews, 1981) or nondysjunction of the X chromosome due to heterochromatin deficiency (Mc Kee, 1984). Transposition events by the transposable genetic elements that generate chromosomal 'hotspots' could also inadvertently alter the sex-ratio in *Drosophila* (Engels and Preston, 1981).

Our results entail the principle of natural selection and are ecologically important, since the predominance of females over males provides a greater potential for population growth in the natural habitats (James and Jaenike, 1990). Mechanisms of evolutionary biology are likely to be significant for the deviant sex ratio in *D. rajasekari*, since this subtropical species evolved under entirely different environmental conditions than the temperate species of *Drosophila* exhibiting a gender bias. In the laboratory reared strains, both types of light dark cycles: the artificial and the natural LD cycles retained the gender bias whereas, aperiodic environments of LL and DD at a constant temperature suppressed it. Analogous to our findings, earlier reports of age and temperature dependent segregation also exemplified that the gender bias was affected by various physiological variables (Sandler and Hiraizumi, 1961; Faulhaber, 1967; Hamilton, 1967; Hiraizumi and Watanabe, 1969; Chanter and Owen, 1972; Lyttle, 1977; Janzen 1994). The cause of bias suppression under aperiodic regimes is to be ascertained.

Although the sex-ratio evolution is a prominent countervailing phenomenon in evolutionary biology, information of genetic variation on which the selection presumably acts is limited. The classical Fisherian principle predicts convergence to the Mendelian proportions irrespective of the sex determining system. This was confirmed in *D. mediopunctata* with the accretion of drive suppressors facilitating the sex-ratio equilibrium; however, the predicted trajectory of 1:1 was anticipated only after 330 generations or 29 years (Carvalho *et al.*, 1998). The biological implications are clear; unisexual extinctions could occur in such species due to the lack of genetic variation or the inherent slowness of Fishers principle (in absence of sex chromosome effects). Similarly, the deviant sex-ratio within the natural populations of the subtropical species *D. rajasekari* could render them susceptible to stronger shifts in the sex-ratio and invariably accentuate the threat of extinction.

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Recombination mapping of P[lacW] transposons.

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We have continued the previously reported project of generating a set of P[lacW] transposon insertions with known recombination map positions in order to facilitate the mapping and characterization of other mutations that lack cytogenetic map assignments (Marcus, 2003). With the help of undergraduate students in introductory Genetics labs, we have expanded the previous list to chromosome arms 2L, 2R, and 3L (Tables 1-3). Here we report a total of 61 P[lacW] transposon inserts on these chromosome arms for which both cytogenetic and recombination map data are available. Of these, 17 are new reports based on our crosses, while the remainder of lines are from insertions with previously reported recombination map positions.

Third chromosome map positions were determined by crossing P[lacW] strains obtained from the Drosophila Stock Center in Bloomington, Indiana, to a standard mapping strain w^{1118}; h^1 ri^1 e^s.

Table 1. Map positions of *P[lacW]* transposon insertions on chromosome arm 2L. Underlined entries indicate markers that had already been assigned meiotic map positions at the beginning of this study. These data, and cytogenetic data for the markers used in this study were obtained from Flybase (2004). SE is the standard error of each of the recombination frequency calculations, and N is the number of flies scored to calculate the map positions.

Mutation	Cytogenetic Position	Meiotic Map	SE	N
P[lacW]kis^{k13416}	021B07-08	0		
P[lacW]ex^{k12913}	021C03-04	0.1		
P[lacW]S^{k09530}	021E01-03	1.3		
P[lacW]Mad^{k00237}	023D03-04	7.7		
P[lacW]for^{k04703}	024A01-02	10		
P[lacW]l(2)k08617^{k08617}	024C01-02	16		
P[lacW]ed^{k01102}	024D03-04	11		
P[lacW]tkv^{k16713}	025D01-02	16		
P[lacW]vri^{k05901}	025D04-05	17		
P[lacW]raw^{k01021}	029F01-02	19		
P[lacW]ab^{k02807}	032E01-02	44		
b^{1}	034D5	48.5		
P[lacW]Su(H)^{k07904}	035B08-09	50.5		
P[lacW]esg$^{B7\text{-}2\text{-}22}$	035C03	59.3	2.63	139
P[lacW]Mhc^{k10423}	036B01-02	52/54.7		
P[lacW]dl^{k10816}	036D01-03	52.9		
P[lacW]Catsup^{k05424}	037B08-12	53.9		
P[lacW]Ddc^{k02104}	037C01-02	53.9+		
P[lacW]spi^{s3547}	037F01-02	54		
P[lacW]neb^{k05702}	038B01-02	54.5		
P[lacW]dia^{k07135}	038E05-06	65	3.37	84
P[lacW]bur^{k07130}	039B01-02	55.7		
P[lacW]tsh$^{A3\text{-}2\text{-}66}$	039E-040A	55.8		

The F1 from this cross were crossed back to the mapping strain and the backcross progeny were scored for eye color and mapping strain phenotypes. The h^1 phenotype was not scored as reported previously because it was difficult to score with the student-grade microscopes available to us for use in the Genetics Labs (Marcus, 2003). Second chromosome map positions were determined by mapping P[lacW] strains to a second chromosome mapping strain w^{1118}; al^1 b^1 c^1 sp^1. Again, the F1 from this cross were crossed back to the mapping strain and the progeny were scored. Only b^1 c^1 were used as visible markers, again because of limitations of the microscopes. It should be noted that the w^{1118}; al^1 b^1 c^1 sp^1 mapping strain does poorly on instant medium from a variety of sources (Carolina Biological, Connecticut Valley, Ward's Biological), which has complicated the generation of data for the second chromosome in our laboratory. Student data were compared to data generated in parallel by the authors and the clearly aberrant data were discarded.

Meiotic map distances were calculated from the closest visible marker on the mapping strain, and Maximum Likelihood standard errors for each meiotic recombination map distance were calculated according to Weir (2003). Cytogenetic map positions for all insertions discussed here and recombination map locations for insertions with previously published map positions were obtained

from FlyBase (2004). In most cases, 95% confidence intervals for the meiotic map positions (Snedecor and Cochran, 1989) of each locus from our data overlap with the map location predicted by FlyBase (2004). The observed disparities may be due to viability differences among genotypes, to the large distances between some inserts and the visible markers used for mapping (Haldane, 1919), or to sampling errors caused by the small sample sizes of backcross progeny for some of our lines.

Table 2. Map positions of *P[lacW]* transposon insertions on chromosome arm 2R.

Mutation	Cytogenetic Position	Meiotic Map	SE	N
P[lacW]EcR^{k06210}	042A10-12	40.8	5.23	83
P[lacW]jing^{k03404}	042B01-03	73.5	4.51	92
P[lacW]cos^{k16101}	043B01-02	57		
P[lacW]lin19^{k01207}	043F01-02	74.7	3.6	149
P[lacW]dap^{k07309}	046B01-02	60.9	2.98	142
P[lacW]shn^{k00401}	047D05-06	62		
P[lacW]wal^{k14026}	048C01-02	59		
P[lacW]bic^{k10712}	049D05-06	64		
P[lacW]sca^{A2-6}	049D3	66.7		
P[lacW]Psc^{k07834}	049E06-07	67		
P[lacW]Su(z)2^{k06344}	049E06-07	67		
P[lacW]Nrk^{k14301}	049F07-08	71.7	5.08	69
P[lacW]arr^{k08131}	050A09-10	66		
P[lacW]drk^{k02401}	050A12-14	70		
P[lacW]fl(2)d^{k16105}	050C04-05	70.1		
P[lacW]Cp1^{k15606}	050C17-19	72.8	4.22	103
P[lacW]mam^{k02214}	050C22-23	70.3		
c^{1}	052D03-07	75.5		
P[lacW]Dek^{k09907}	053D12-14	99.5	5.37	63
P[lacW]POSH^{k15815}	054C07-08	79.4	3.96	136
P[lacW]grh^{s2140}	054F01-02	86		
P[lacW]thr^{k07805b}	054F04-05	86		
P[lacW]Pcl^{s1859}	055B05-06	84		
P[lacW]fj^{9-II}	055C1	81.5		
P[lacW]mus209^{k00704}	056F10-11	92.3		
P[lacW]rig^{k07917}	057A05-06	106.4	4.92	88
P[lacW]mei-W68^{k05603}	058D08-10	94		
P[lacW]px^{k08316}	058E01-02	100.5		
P[lacW]blw^{k00212}	059A01-03	90.7	7.36	45
P[lacW]bs^{k07909}	060C07-08	107.3		
P[lacW]Kr^{k05826}	060F03-05	107.6		

Collectively, the data presented here, along with data previously reported for P[lacW] insertions for chromosome arm 3R (Marcus, 2003) represent a “kit” of transposon stocks with known cytogenetic and recombination map positions that can be used as a starting point for mapping mutations (and determining locations on both maps simultaneously) anywhere on either of the two major autosomes of *Drosophila melanogaster*.

Table 3. Map positions of *P[lacW]* transposon insertions on chromosome arm 3L.

Mutation	Cytogenetic Position	Meiotic Map	SE	N
P[lacW]Hsp83^{j5C2}	063B07-08	5		
P[lacW]RpL14^{1}	066D8	17.9	4.97	83
P[lacW]aay^{S042314}	067B1-10	20.7	4.02	119
P[lacW]RpS12^{s2783}	069F05-06	15.6	5.18	80
P[lacW]trn^{S064117}	070A	16.5	4.21	119
P[lacW]l(3)j2E11^{j2E11}	070C05-06	40.9		
P[lacW]th^{j5C8}	072D01-02	43.2		
P[lacW]SsRbeta^{s1939}	072D08-09	39.9	1.93	173
ri^{1}	077E03	46.8		
P[lacW]skd^{L7062}	078A02-03	51		
e^{s}	093D01	70.7		

References: FlyBase, 2004, *The* Drosophila *genetic database* (http://flybase.bio.indiana.edu); Haldane, J.B.S., 1919, J. Genet. 8: 299-309; Marcus, J.M., 2003, Dros. Inf. Serv. 86: 168-171; Snedecor, G.W., and W.G. Cochran 1989, *Statistical Methods*, Iowa State University Press, Ames, Iowa, 8th Edition; Weir, B.S., 1996, *Genetic Data Analysis II: Methods for Discrete Population Genetic Data*, Sinauer, Sunderland, Mass, 2nd Edition.

Confirming the ancestral allele of *exuperantia2* (*exu2*) of *Drosophila pseudoobscura*.

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A recent study of DNA sequence polymorphism in *Drosophila pseudoobscura* reported the action of natural selection on a new allele of the *exuperantia2 (exu2)* gene. Here, we confirm that this allele is novel to *D. pseudoobscura* compared to its sibling species *D. persimilis* and their outgroup *D. miranda*. We surveyed multiple lines of *D. persimilis* through RFLP and DNA sequence assays and failed to detect the novel allele. Hence, the ancestral allele found in the outgroup species, *D. miranda*, is indeed fixed in *D. persimilis*.

The study of the DNA sequence variation in the *Drosophila pseudoobscura* species group has aided genetic studies of adaptation and speciation. Recently, Yi and Charlesworth (2003) identified an unusual pattern of polymorphism in the *exuperantia2* (*exu2*) locus in *D. pseudoobscura.* Two single nucleotide polymorphisms within sympatric populations of *D. pseudoobscura* are in complete linkage disequilibrium (Yi and Charlesworth, 2003). Yi and Charlesworth (2003) proposed that one of these two alleles of *exu2* detected in *D. pseudoobscura* is novel, as it was not found in *D. persimilis* or *D. miranda* sequences. *D. miranda* diverged from *D. pseudoobscura* 2,000,000 years ago, and *D. persimilis* diverged from *D. pseudoobscura* 500,000 years ago. One of the polymorphisms within the novel allele changes an amino acid within this gene from proline to

histidine. The authors suggested that this change and the subsequent spread of the novel allele were driven by natural selection.

Here, we confirm the findings of these authors by eliminating two potential concerns. First, Yi and Charlesworth (2003) surveyed one line of *D. persimilis*: this species, like *D. pseudoobscura*, may actually possess both alleles, suggesting the second allele has been maintained for a long time. Second, other studies have shown extensive gene exchange between *D. pseudoobscura* and *D. persimilis* (Wang *et al.*, 1997; Machado *et al.*, 2002; Hey and Nielsen, 2004), so the novel allele may have spread into *D. persimilis*. Here, we evaluate these possibilities by surveying multiple strains of *D. persimilis* for the presence of the novel allele using an RFLP assay. We assayed nine *D. persimilis* inbred lines; five from Mt. St. Helena, California, four from Mather, CA; and two *D. pseudoobscura* strains from Mt. St. Helena, CA and James Reserve, CA. We extracted DNA using the protocol of Gloor and Engels (1992). The *exu2* gene was amplified using a 50 µl PCR with a primer designed from the *D. persimilis* sequence provided by S. Yi. 10µl of the PCR product was added to a restriction enzyme digest using *Hha I* and ran the digestion reaction with 2 % agarose gel electrophoresis for the RFLP assay. To verify the *D. persimilis* sequence found by S. Yi, we repeated the PCR and visualized it on a 2% agarose gel. We extracted the PCR product from the gel using a QIAquick Gel Extraction kit (Qiagen). This extracted DNA was sequenced in both directions.

Our RFLP assay of *exu2* in *D. persimilis* and *D. pseudoobscura* corroborated the authors' assertion that the novel allele is specific to *D. pseudoobscura*. All nine lines of *D. persimilis* contained the same ancestral allele. We submitted our sequence of the *D. persimilis* strain to GenBank/ EMBL as accession number AY725999.

We conclude that the interpretation of Yi and Charlesworth (2002) was correct in that the histidine-bearing allele of *exu2* is novel to *D. pseudoobscura*. Although gene flow has been documented between *D. persimilis* and *D. pseudoobscura*, we failed to detect the novel allele within *D. persimilis*. *exu2* is located on the XL chromosome arm, which is inverted between these species. This region fails to introgress between these species (Machado et al 2002), likely because of its association with reproductive isolation (Noor et al 2001).

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Switch genes in tumor formation.

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Summary

The analysis of the genetics of a multigenic defect *Drosophila* tumor cell line revealed the existence of oncogenes, tumor suppressor genes, and proliferative genes. Those genes add to tumor formation by mutation. Without being mutant in the tumor, switch genes interact in tumors: mutations in *trans* are lethal for tumors and not lethal for wild type. *Antennapedia, Ultrabithorax, bicoid* and *hunchback* are switch genes and belong to RNA-polymerase II transcription factors. Involved alleles thereof change recombination frequencies. An extract of Amanita phalloides, containing alpha-amanitin, an inhibitor of RNA-polymerase II, is able to reduce tumor formation.

Introduction

In humans, tumors can occur in any tissue. It is generally accepted that a tumor originates from cells that are mutant in one or several genes. A tumor grows; thus, the addition of a mutation that causes cells to proliferate is most important in the process. Several mutations are known that dispose a risk to develop a tumor. Anyhow, in addition to this predisposition at least one somatic, initiative mutation is necessary to lead to manifestation of a primary tumor. The first mutation is a proliferative mutation, leading to additional cell divisions; the cell loses growth control. Additional, promotive, mutations are necessary to lead into progressive, metastatic behavior. During the process of successive mutations, tumor cells lose their original differentiation. Additionally due to a defect in the program for apoptosis/cell death, resistance to chemotherapy occurs. Usually tumor cells lose part of their genome. In contrast to tumor cells, normal cells would not proliferate uncontrolled, would stop cell growth when in contact with other cells (contact inhibition), would keep their genome integrity, would age, would not grow in other tissue but die by apoptosis. In general, every tumor carries its own set of mutations. In a novel approach, a genetic model has been developed to analyze general features of tumor related genes.

Drosophila tumors are genetically determined, opening a way to a genetic and cellular model for tumor formation. In *Drosophila* the formation of a brain tumor is due to several mutations. The tumor forming strain Malignant Brain Tumor (MBT) has been subject to extensive genetic and cellular analysis (Riede, 1996, 2001). By analyzing the genes being responsible for tumor formation, several gene classes could be identified.

Tumor suppressor genes are genes that add to tumor formation when their function is absent. Both copies of the gene on the homologous chromosomes have to be defective. Thus a recessive or loss of function behavior of the gene is expected. *tolloid*^{MBT} has been identified as the tumor suppressor gene in MBT. When at least one *tolloid*$^{+}$ is present, the brain tumor is not malignant, thus intact tumor suppression leads to a reduced malignancy of the cells. *tolloid*$^{+}$ addition does not stop overgrowth of cells, thus tumor suppression does not interfere with the mutation that initiates tumor growth, the proliferative mutation. *tolloid*^{MBT} alone does not lead to cell proliferation. Thus, tumor suppression is not the cause of cell proliferation.

Oncogenes show a dominant effect, gain of function, and enhance the tumor formation process. One copy of a gene defect is sufficient to add to tumor growth of cells. *Toll* has been identified as an Oncogene. One copy of mutant *Toll* is sufficient to enhance malignancy of the brain

tumor. A mutant Oncogene alone does not lead to proliferation. Thus, Oncogene action is independent from cell proliferation. Like tumor suppressor genes, Oncogenes add to the differentiation of the cells, the proliferative aspect remains unaffected.

Proliferative genes mutations are responsible for the tumor growth of cells (Riede 1997, 2000, 2003). They influence the chromatin structure, leaving unpaired chromosome regions. Reduction in the frequency of meiotic recombination is observed in distinct regions; other regions show increased recombination frequencies. Replication is allowed. The genes are involved in DNA copy number control. One mutant proliferative gene alone is able to induce tumor formation, to break the restriction of the cell cycle. By mutation of a proliferative gene, resistance to chemotherapeutic drugs occurs. They map to different loci on different chromosomes; about 10 interacting loci on each chromosome can be found. Their molecular structure is complex; different transcripts in both directions overlap. The genes interfere with each other in a quantitative matter.

Tumor therapy with molecular tools requires central targets. Defining a molecular tool for tumor therapy is difficult in complex genetic structures and genes, on the level of proliferative genes. To define better targets, genes that induce lethality in *trans* for a variety of tumors were identified. They are not mutant in tumors and are defined to be switch genes: a mutation in a switch gene is lethal for a tumor in *trans*, but permissive for wild type. They might serve as targets for novel drugs.

Results and Discussion

Table 1. *Antp* ^{Aus} was crossed over the *Antp*-alleles and deficiencies (Df) indicated, in both directions (Tearle and Nüsslein-Volhard, 1987, Lindsley and Zimm, 1997). The only gene that is covered by all deficiencies is *Antp*. Other alleles of genes in the ANTC complement *Antp* ^{Aus}. *stern*^{88} is a proliferative allele with several interference points, it does not complement all *efendi* alleles in trans, had been mapped like *Antp*^{Aus} to 56 cM on the third chromosome, but could so far not be identified as known gene. *stern*^{88} does not complement *Antp* ^{Aus}, but complements all deficiencies of the region. Whereas *Antp* ^{Aus} complements *Antp* $^{73b+R40}$, a partial reversion of the phenotype of *Antp* ^{73b}, resulting in recessive lethality, *stern*^{88} is affected by this mutation. The reversion in *Antp* $^{73b+R40}$ is coupled to the ANTC. Recombinations, based on 200 single events tested, between *Antp* ^{73} and *Antp* $^{73b+R40}$ do not occur.

Antp alleles			
Allele	Mutation	*Antp* ^{Aus}	*stern*^{88}
Antp ^{B}	inversion	-	+
Antp ^{73b}	inversion	-	+
Antp $^{73b+R40}$	inversion + reversion	+	-
Antp ^{4B}	?	-	+
Df(3R)Scr	84A1;84B1	-	+
Df(3R)Ns	84B1;84D	-	+
Df(3R)Antp	84A6;84D13	-	+
Df(3R)4SCB	84A6:84B2	-	+

Antennapedia (Antp) *is a switch gene*: EMS mutagenesis and screening for mutations lethal over MBT lead to a variety of proliferative alleles of different genes, like *Aus, stern, mali, amanda, merlin,* or *hexe* (Riede, 1997). *Aus*^{9} showed embryonic lethality as a homozygote and lethality in *trans* over a variety of proliferative mutations on different loci (*merlin*^{14}, *stern*^{88}, *drache*^{47}, MBT, FAT-GD, DLG-1, and LGL-1). This qualifies the mutant gene as a switch gene. The mutation of *Aus* influences the state of the cell, induced by proliferative mutations, in *trans*. The change of *Aus* switches this fate, leading to lethality.

Aus^{9} is a dominant proliferative allele. Heterozygotes over wild type induce melanotic tumors; however, adults are viable and fertile. It induces large unpaired chromosome regions on the third chromosome. The allele induces increased frequency of recombination between *ri* and *e*. Here it is identified as *Antp* allele (Table 1). It had been mapped to 56 cM.of the third chromosome, does not complement alleles of *Antp* or deficiencies comprising *Antp,* thus *Aus*^{9} is renamed to *Antp*^{Aus}. It complements all other genes of the Antennapedia Complex (ANTC).

Table 2. ANTC alleles were crossed over *Antp Aus* and the series of proliferative alleles, in both directions. Complementation of the mutations occur (+) or lethality of the F1 generation is indicated (-). Proliferative genes or mutants that interfere are given in brackets. *pb, zen, Scr,* and *DfD* show no strong interaction, *ftz.* and *bcd* interact with proliferative mutations. Several alleles of *bcd* were crossed over proliferative mutations. Interaction depends on the allele tested. Strongest interaction is obtained with allele [E2], a deletion of 260 bp deleting a homeodomain. Alleles of *bcd:*[E1], strong allele, deletion of 2482-2650 plus TA insert, resulting in frameshift, 55 aminoacids replace aminoacids 156-494, including the homeodomain. [E2], strong allele, 260 basepairs delete the homeodomain. [E3], intermediate allele, 2406C-T transition result in 131Ser-Leu exchange[E4], intermediate allele, 2393C-T transition result in 127Leu-Phe exchange. [E5], weak allele, 2804C-T transition result in 264stop. [E6], 2388-2420 deleted, resulting in deletion of aminoacids 125-135. [GB], strong allele, 2486C-T transition resulting in 158stop. [085], 2564C-T transition resulting in 154stop, at the end of the homeodomain. [2-13], 3885T-A transition, resulting in 453Leu-His exchange. [111], 2788C-T transition, resulting in 262stop (Frohnhöfer and Nüsslein-Volhard, 1986, Tearle and Nüsslein-Volhard 1987, Struhl *et al*., 1989).

ANTC alleles in proliferation

Gene	Allele	Proliferative alleles
pb		+
zen	W36	+
Scr	XT130	+
Scr	7F28	+
ftz	9H34	- (*hexe*^{77})
ftz	7b	- (DLG-1)
DfD		+
bcd	E1	+
bcd	E2	- (*stern*^{88}*, efendi* alleles)
bcd	E3	+
bcd	E4	- (*stern*^{88}*, efendi* alleles)
bcd	E5	- (*stern*^{88}*, efendi* alleles)
bcd	085	+
bcd	GB	+
bcd	2-13	+
bcd	23-16	*stern*^{88}*, efendi* alleles)
bcd	33-5	(*stern*^{88}*, efendi* alleles)
bcd	111	(DLG-1)

Antp maps to 47.5 of the third chromosome. The difference in the map position of *Antp*^{Aus} and other *Antp* alleles could originate from the somatic pairing deficiency and the differences in recombination frequencies, induced by proliferative genes. *Antp*^{Aus} had been shown to induce lethality over a large variety of tumor forming genomes. *Antp*^{B}, *Antp*^{73b}, and *Antp*^{4B} show similar effects. They are lethal over *efendi* alleles and DLG-1.

The *Antp* locus was initially recognized by dominant gain-of-function alleles. These transform the antenna of the adult into a leg. Most of the dominant lesions are associated with recessive lethality and gross chromosome arrangements, which fall in the interval between the distal and proximal promoters. The *Antp* transcription unit lies at the distal end of the ANTC, it is transcribed from two different promoters, with splice variantion and about 100kb long. It encodes a homeobox in exon 8, a DNA binding motif.

Antp^{Aus} is a proliferative mutation; thus, the gene is not only involved in the differentiation process during development, but might determine the numbers of cell divisions.

Ultrabithorax (Ubx) *is a switch gene*: The bithorax complex (BXC) is a gene cluster that functions to assign unique identities to body segments. Three functionally integrated regions, *Ultrabithorax (Ubx), abdominal-A (abdA) and Abdominal-B (Abd-B)* appear as complementation groups. *Antp* expression is dependent on the expression of *Ubx*. BXC alleles were crossed over proliferative alleles. *abdA*^{M1}, *AbdB*^{M5} and *Ubx*^{79} complement all proliferative alleles tested. *Ubx*^{C1} shows strong interaction with proliferative alleles. It is lethal over *stern*^{88} and *efendi* –alleles. *Ubx*^{C1} is a 100 kb deletion in BXC producing a hybrid protein *abdA-Ubx*, with the homeobox of *Ubx* (Rowe and Akam, 1988).

bicoid (bcd) *is a switch gene*: ANTC harbors several homeotic loci, like *proboscipedia (pb), Sex combs reduced (Scr*), and *bcd*. Several alleles of the loci have been crossed to proliferative mutations (Table 2). Less involved are *pb, zen (zerknuellt), Scr*, and *Deformed (DfD).* They show no strong interaction. *fushi tarazu (ftz)* and *bcd* interact with proliferative mutations. Several alleles of *bcd* were crossed over proliferative mutations. Interaction depends on the allele tested. Strongest interaction is obtained with allele [E2], a deletion of 260 bp including the homeodomain. [E2] is lethal over a variety of proliferative mutations, including *stern*^{88}*, efendi*-alleles, *hexe*^{77}*, mali*^{MN},

amanda[19], LGL-1, DLG-1 and BRAT. This qualifies *bcd* as a switch-gene: specific alleles thereof can act in *trans* and induce lethality to tumor forming gene constellations. Hereby the homeodomain appears to be important.

Maternal-effect lethal mutations of *bcd* show defective head and thorax development. The mRNA appears in the anterior extremity of the egg. The transcript is 2.6 kb and codes for a homeobox containing protein. *bcd* protein forms a gradient along the egg and except during mitosis is concentrated in nuclei.

Table 3. Alleles were crossed over the series of proliferative alleles. Dependent on the allele in *trans* lethality occurs. [14F], class I-allele (embryos without cephalic fold or prothorax segments, cell death after 6 hours), instead aminoacid 256 stop. [FB], class I-allele, deletion of 10 nucleotides, resulting in frameshift at aminoacid 150. [7M48] class I-allele, stop before the first Zn-finger domain. [7L06] class II-allele (retains prothorax segments) 286Trp-Asn exchange in first Zn finger domain. [349] aminoacid 331Met-Ile exchange in first Zn-finger domain. [9K57] class V-allele (individual segment deformations and gaps), stop between first and second Zn-finger domain. [9Q17], class I allele, aminoacid 215 stop, before first Zn-finger domain. [14F21], class I allele, aminoacid 256 stop, before first Zn-finger domain. [IIU], class III-allele (retains labial segments) aminoacid 608 stop, before second Zn-finger domain. [9K49], class V allele aminoacid 347Arg-U in C-box at the end of first Zn-finger domain. (Jürgens *et al*., 1984, Sommer *et al*., 1992, Lindsley and Zimm, 1997, Tearle and Nüsslein-Volhard 1987, Lehmann and Nüsslein-Volhard, 1987).

hb alleles in proliferation

Allele	Proliferative alleles
14F	+
FB	+
7M48	+
7L06	+
G1	- (*Antp* ^{Aus})
349	- (*merlin*^{14})
9K57	- (*efendi* alleles, LGL-1, DLG-1, *Antp* ^{Aus})
9Q17	- (*efendi* alleles)
14F21	- (*efendi* alleles)
IIU	- (*efendi* alleles)
9K49	- (*efendi* alleles)

hunchback (hb) *is a switch gene*: In the egg, the mother sets independent systems: the anteroposterior gradient of *bcd*, a posterior system effected by the *nanos (nos)* gene, and a terminal system for defining the head, depending on *torso (tor). nos* blocks the translation of *hb* and *bcd*. Alleles of the genes were crossed over proliferative alleles. *tor*^{R1}, *nos*^{53}, *nos*^{6}, and *nos*^{17} did not show interactions. Several alleles of *hb* interfere with proliferative mutations, strongest allele herein is [9K57] (Table 3). It codes for a truncated protein, that lost the second of the Zn-finger domains. The interaction of *hb* with proliferative alleles in *trans* is specific: some strong class I-alleles interfere and some do not. On the other hand weak alleles show strong interference. Therefore, the effect is not due to combination of strong lethal effects, but due to specific interactions.

Homozygotes for *null* alleles of *hb* are embryonic lethals of the gap type. The gene is transcribed in five overlapping transcripts in two classes. Its protein contains two DNA binding Zn-finger domains of the C2H2 type and acts as a transcriptional activator or repressor. *bcd* binds to five sites upstream of *hb*, three binding sites are sufficient for transcription activation. *hb* binds to the promoter region of *Ubx* as repressor and activates *Antp*-expression (Lindsley and Zimm 1997; Sommer *et al*., 1997).

Polycomb (Pc) *and* trithorax (trx): *Pc* may be considered as a negative regulator of the BXC and ANT Complexes. Genes of the *Pc* group are responsible for maintaining the inactive expression state of homeotic genes. *trx* is required throughout development to maintain expression of homeotic genes and seems to be involved in the positive regulation of the BXC and ANT Complexes (Zink and Paro, 1995; Ingham, 1998). Mutations in the human homologue of *trx*, *All-1*, result in severe leucemias (Cimino *et al*., 1991). *Pc*^{9M21}, *Pc*^{XT09}, *trx*^{E2} and *trx*^{1} reveal low interactive potential with proliferative alleles. Interaction is found with *trx* ^{B11}: disadvantage occurs for DLG-1.

Recombination frequencies: Proliferative mutations and tumor formation are coupled to somatic pairing defects of homologous chromosomes, indicating a regional change of chromatin structure. Changes in recombination behavior, reduction in several regions and enhanced frequencies in other regions, reflect this phenotype. Switch gene alleles were analyzed for recombination between *ri* and *e*. Ubx^{79} hinders any recombination in this chromosome region. bcd^{E2}, trx^{B11}, Pc^{9M2}, $Antp^{B4}$, $Antp^{73b}$, $Antp^{73b+R40}$, and $Antp^{B}$ all increase the recombination frequency in this region by about 50%. Thus, the switch genes act on the same level like proliferative genes, in the chromatin structure – changing recombination frequencies.

alpha-Amanitin: In molecular terms, all switch genes bind to DNA with specific motifs and act as transcription factors of RNA-polymerase II. In humans the same class of genes is involved: Hox genes are upregulated in leukemias and are thought to be centrally involved in the tumor formation process (Rozovskaia *et al*., 2001; Hudlebush *et al*., 2004).

Amanita phalloides contains alpha-amanitin, a drug inhibiting RNA-polymerase II. The question arises, whether inhibition of RNA-polymerase II could influence proliferative alleles. Therefore, animals were grown on medium containing alpha-amanitin. Without alpha-amanitin, homozygous $efendi^{289}$ die 20 days old in larval stage three with large melanotic tumor masses. Growth on medium with alpha-amanitin reduces the tumor masses significantly. In addition, viability is increased: the lifespan is elongated to 30 days. 30% of the homozygotes reach the pupal stage. Thus, reduction of the action of RNA polymerase II by alpha-amanitin significantly reduces tumor formation and increases viability of animals containing a proliferative mutation.

Materials and Methods

All proliferative alleles and crosses have been described (Riede 1997). *Pc* and *trx* strains were a gift from Renato Paro; all other flies were obtained from the Tübingen stock center.

Amanita phalloides contains alpha-amanitin, a drug inhibiting RNA-polymerase II. Fungi were collected in Überlingen/Germany in September 2002. According to the Homöopathisches Arzneimittelbuch, ethanolic exctraction was performed. The extract was diluted 1:10,000 and applied to standard fly medium 1:1. This mixture should contain alpha-amanitin at a concentration of about 10^{10} molecules per milliliter. Animals grew at 22°C. Parent flies were left for egg position for three days. The F1 generation was analyzed.

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Isolation of new alleles of *ifm(2)RU* mutants by EMS mutagenesis.

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Introduction

Mutation in any of the genes that regulate the development of an organism causes at large lethality, and in many instances, viable "mutants" are formed depending on how vital the gene involved is. The submissiveness of specific genes discovered in the fruit fly *Drosophila melanogaster* that route the differentiation and development of the tissues and organs, to genetic and molecular techniques makes it one of the most favorable systems to study the functions of genes and gene products (Adams *et al*., 2000; Kornberg and Krasnow, 2000). In almost all stages of life of most of the animals, muscles play a very important role for its survival. The indirect flight muscles (IFMs) of the fruit fly has been one of the most accessible and extensively worked systems to study development (Nongthomba and Ramachandra, 1999).

The mechanisms underlying myogenesis have been extensively worked out in many of the model organisms. In the fly, muscle development takes place twice during its life cycle, once during embryogenesis, when the muscles of the larvae are formed and for the second time, when the muscles of the adult are formed during pupal development (Bate, 1990; Fernandes, *et al*., 1991). Ethylmethanesulfonate (EMS) mutagenesis and subsequent screening under polarized lights resulted in the isolation of sixteen lines of flies with defects in the IFMs. Complement analysis of these lines showed that they fell into eight complementation groups of which one was semidominant and the remaining were viable recessive. These mutations have been named *indirect flight muscle (2) RU1*, (semidominant line), *indirect flight muscle (2) RU2* to *indirect flight muscle (2) RU8*, recessive viable lines sequentially. They have been mapped onto the 2nd chromosome using the deficiency lines (Nongthomba and Ramachandra, 1999). The preliminary investigation showed that they have defined role in myogenesis. However, prediction of the function of a gene or gene product is very difficult in the absence of multiple alleles (Nongthomba and Ramachandra, 1999). In this regard, we undertook mutagenesis to generate stronger alleles. Here we report the newly induced mutations and the isolated stronger alleles for the previously generated *ifm(2)RU* mutations.

Materials and Methods

Fly stocks

Drosophila melanogaster flies of strain Canton-S were used as wild type for control studies. All fly lines used are as described in Lindsley and Zimm (1992), unless otherwise specified. The fly lines were obtained from the National Drosophila Stock Center, Mysore, India.

All fly stocks were cultured in half pint milk bottles using cream of wheat agar media and the experimental crosses were carried out in glass vials at 25ºC.

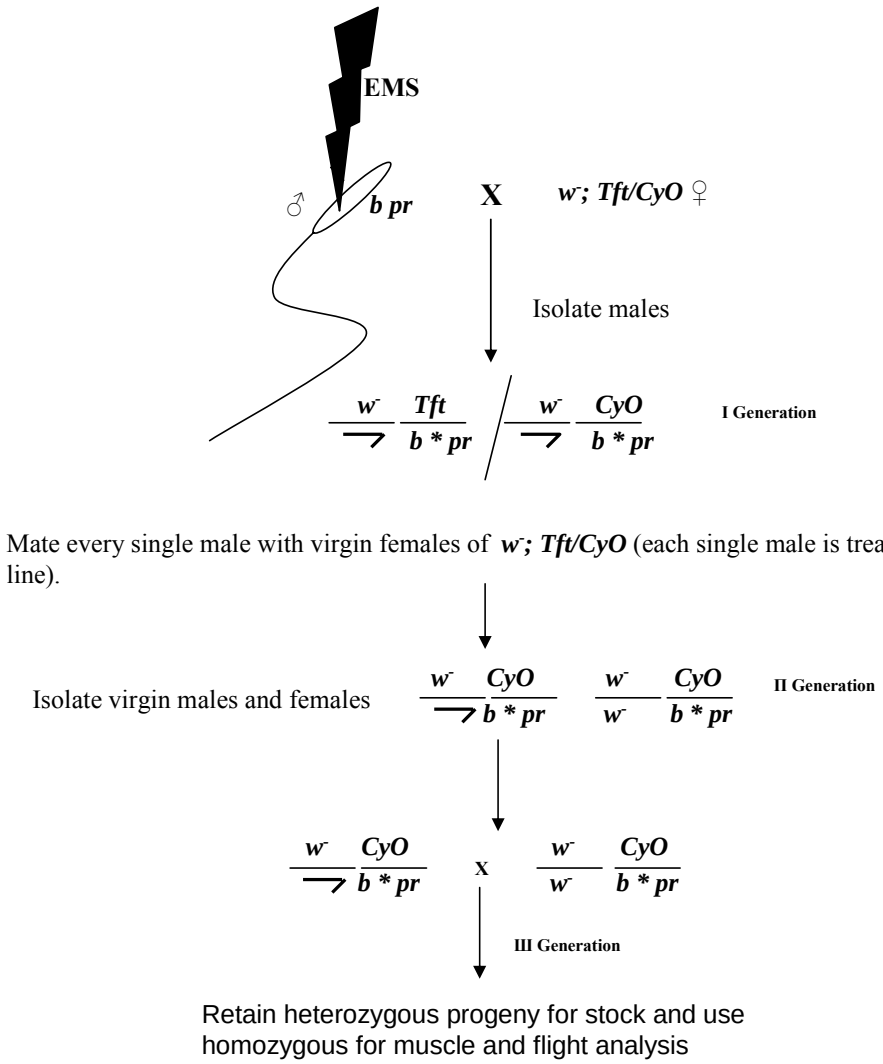

Figure 1. Schematic representation of the ethylmethanesulfonate (EMS) mutagenesis to generate viable recessive second chromosome mutations involved in the development of indirect flight muscles. Males of *b, pr* were aged for 2 days and administered EMS by transferring into a feeding chamber after a brief period of starvation. EMS was administered using a syringe by discharging 5 ml of solution. This was sufficient to wet the filter paper and leave a small puddle below. The treated flies were transferred into a food vial and left to recover. These were then mated with 2 day old virgin females of the genotype *w-; Tft/CyO*. The treated males were transferred into new vials every alternate day and were provided with virgin females aged for 2 days. This was continued till the male flies were 12 days old. The resultant male progeny from this cross was isolated and back crosses were conducted (treating every single male as an individual line) with the virgins having the maternal genotype *w-; Tft/CyO*. The resultant progeny from this cross was selected by the presence of (*CyO*) the balancer chromosome. Isolated virgin females and unmated males were then crossed *inter se*. The resultant homozygotes from this cross were screened for muscle defects and the heterozygous progeny was retained as stock.

EMS mutagenesis

Adult males of the genotype *b, pr* were mutagenised by treating with EMS following the protocol of Grigliatti (1976) with slight modifications. In brief, the male flies were isolated after eclosion prior to mating and were aged for a period of two days. The flies were starved for a period of 2-3 hours prior to feeding EMS at a final concentration of 25 mM in 1% sucrose. After about four hours, the flies were aspirated into a fresh food media bottle and allowed to recover for a day. The flies were then crossed *en masse* to virgin females of *w; Tft/Cyo;* +/+ strain. Next day, the same males were remated with another batch of virgin females. This was continued till the males were 12 days old. Subsequent crosses were done as described in Figure 1. The homozygous progeny of the third generation was screened for muscle defects and the heterozygous progeny was retained as stock.

Table 1. Data of the mutagenesis is presented in this table. A total of 300 males were administered with EMS and the crosses were constructed as given in figure1. The generation wise data of flies obtained and the analysis is given here. In the first generation seven hundred and twenty flies (males) were obtained. Of these 394 were sterile and did not produce any offspring. The remaining were fertile and gave viable progeny. The next stage was of sib mating crossing the heterozygous progeny *interse*. In the second generation obtained 19 lines were sterile. The remaining lines were analyzed for muscle defects. Of the 55 lines which produced viable homozygous progeny, twenty six lines showed muscle defects; of which seventeen showed strong penetrance and phenotype expressivity. These lines were chosen for complementation with the *ifm(2)RU* mutants. The figures within the parentheses indicate percentage of expressivity and penetrance.

Generation	1st	2nd	3rd
♂ Flies obtained	720		
Sterile lines	394	19	
Lines that gave viable homozygotes			55
Lines with muscle defects			26
No of lines chosen for complementation			17 (>90%)

Flight Testing

The flight test was performed, as described by (Cripps *et al*., 1999), upon 1- to 2-day-old flies with minor modifications. Briefly, flies were released inside a clear plastic box, of dimensions 30 cm × 30 cm × 45 cm (L × B × H), illuminated from the top. Flies were scored for whether they flew "Upward" toward the light (U), "Horizontally" (H), "Downward", but still with some little flight ability (D) or "Not-at-all" (N). The lines that failed to complement flight ability of the *ifm(2)RU1, ifm(2)RU2* and *ifm(2)RU3* phenotypes in trans were analyzed for the muscle phenotype under polarized light.

Direct screening under polarized lights

The IFMs were analyzed by preparing the thoracic whole mounts as described by Fyrberg, *et al*. (1994). Hemisections of the thorax were taken from adult flies after flash freezing them in liquid nitrogen. The thoraces and the hemisections were processed by dehydrating in a series of alcohol grades starting from 50% to absolute passing through 60, 70, 80 and 90%, for a minimum of 30 minutes and were then cleared in methyl salicylate. The cleared sections were mounted in DPX and covered with a cover glass and observed under polarized light optics. Based on the severity of the muscle phenotypes and similarities with the previously isolated mutants, fly lines were chosen for complementation.

Complementation analysis

The analysis was carried out as given in Figure 2. The lines which failed to complement the *ifm(2)RU* phenotypes were treated as alleles (Table 2).

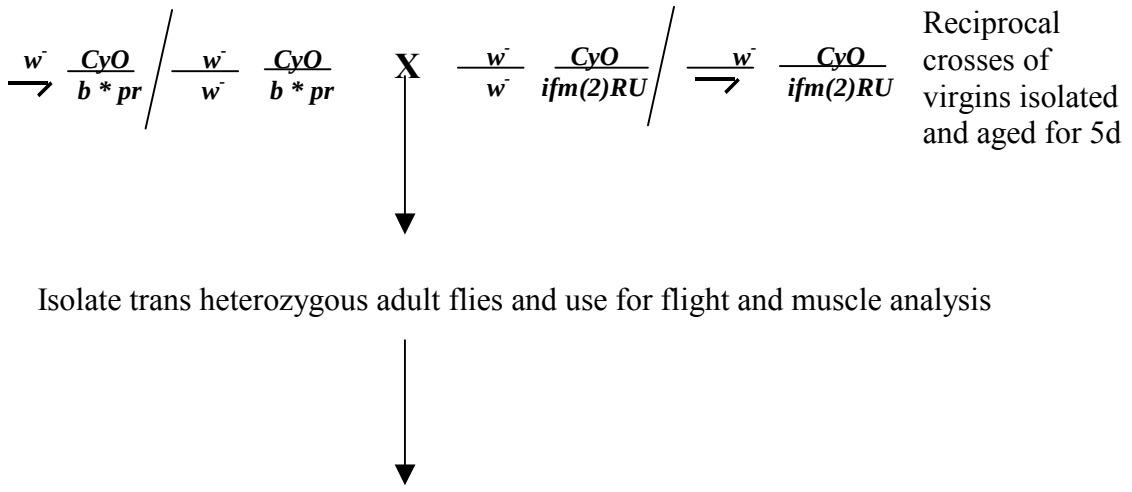

Figure 2. Schematic representation of the complementation crosses to isolate alleles of the previously isolated *ifm(2)RU* mutations. Virgin flies carrying the mutation in trans with the balancer chromosome were mated to virgin flies of *ifm(2)RU* mutations reciprocally. All resultant progeny of this cross which did not bear the balancer chromosome were analyzed for flight and muscle defects. The lines which failed to complement the mutants *ifm(2)RU* phenotypes were treated as alleles.

Table 2. Complementation crosses between the previously isolated alleles of *ifm(2)RU1, ifm(2)RU2* and *ifm(2)RU3* and the newly generated fly lines by EMS mutagenesis showing consistent defects in indirect flight muscles. 1, 2 and 3 designates *ifm(2)RU1, ifm(2)RU2* and *ifm(2)RU3* respectively. The new allele numbers are given as superscripts for *ifm(2)RU1*(1), *ifm(2)RU2* (2) and *ifm(2)RU3*(3). The new mutations are named as *ifm (2) RS 9- 14* sequentially and are represented in the table by RS9- RS14. The complementation crosses were carried out between the lines themselves and with *ifm(2)RU1, ifm(2)RU2* and *ifm(2)RU3*. In the table + indicates complementation of the mutant phenotype and - indicates failure to complement. The lines that fail to complement the *ifm(2)RU* mutations are treated as alleles of the respective *ifm(2)RU* mutations

	1	**2**	**3**	**RS9**	**RS10**	**3^{8}**	**RS11**	**2^{10}**	**2^{11}**	**2^{12}**	**2^{13}**	**1^{2}**	**RS12**	**RS13**	**1^{3}**	**1^{4}**	**3^{9}**	**2^{14}**	**2^{15}**	**RS14**
RS9	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RS10	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3^{8}	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+
RS11	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
2^{10}	+	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	-	-	+
2^{11}	+	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	-	-	+
2^{12}	+	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	-	-	+
2^{13}	+	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	-	-	+
1^{2}	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	+	+
RS12	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Rs13	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
1^{3}	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	+	+
1^{4}	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+	+	+	+
3^{9}	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+
2^{14}	+	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	-	-	+
2^{15}	+	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	-	-	+
RS14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-

Results and Discussion

The bulk of the thorax in the fruit fly, *Drosophila melanogaster*, is made up of two sets of muscles termed as the Indirect Flight Muscles (IFMs), the Dorsal Longitudinal Muscles (DLMs), and the Dorsoventral Muscles (DVMs). The IFMs aid flight indirectly by changing the shape of the thorax (Crossely, 1978). A large number of genes involved in myogenesis have been identified to date and of these, three genes identified recently, namely *Dumbfounded* (*duf*) (Gomez *et al*., 2000), *sticks and stones* (*sns*) (Bour *et al*., 2000; Baylies and Michelson, 2001), and *myoblasts incompetent* (*minc*) (Gomez *et al*., 2002), which are involved in myogenesis. Although different mutations and alleles of *ifm(2)RU* have been reported, not much work to know their role in myogenesis was possible, because of lack of stronger alleles. Here we report the generation of new stronger alleles which will be helpful to understand the possible role of *ifm(2)RU* mutants in IFM development.

The summary of the EMS mutagenesis is presented in Table 1. Of the 55 lines which produced viable homozygous progeny in the third generation, 26 lines showed muscle defects. Of these, 17 showed strong penetrance and expressivity (> 90%), which were chosen for complementation with the *ifm(2)RU* mutants. Of the 17 lines, 3, 6 and 2 lines fail to complement *ifm(2)RU1, ifm(2)RU2* and *ifm(2)RU3*, respectively (Table 2). Accordingly the new alleles (Table 3) are named as *indirect flight muscle (2) RU1*$^{2\text{-}4}$, *indirect flight muscle (2) RU2*$^{10\text{-}15}$ and *indirect flight muscle (2) RU3*$^{8\text{-}9}$.

Table 3. Description of *ifm(2)RU* mutations and its alleles isolated earlier and the newly isolated stronger alleles for those mutations. The screen identifies a total of eleven mutations to be allelic to the previously isolated mutations. They have been assigned the allele number in continuation with the previously assigned numbers. Original ifm(2)RU1 mutation failed to complement 3 lines and hence these 3 are new alleles of the mutation and have been assigned numbers from 2 to 4 considering the original mutant to be first allele. The new alleles have penetrance and expressivity greater than 90%. The new mutations which complemented the *ifm(2)RU* mutations are also described in this table and these are named as *ifm(2)RS* mutations and the numbers are assigned in continuation with the mutations isolated earlier.

ifm(2)RU mutations			New mutations isolated and to be characterized	
Existing	New alleles	% Expressivity	Symbols	% Expressivity
ifm(2)RU1	ifm(2)RU1^{2}	93		
	ifm(2)RU1^{3}	96	ifm(2)RS9	(93)
	ifm(2)RU1^{4}	95	ifm(2)RS10	(94)
ifm(2)RU2^{1}			ifm(2)RS11	(90)
ifm(2)RU2^{2}	ifm(2)RU2^{10}	92	ifm(2)RS12	(91)
ifm(2)RU2^{3}	ifm(2)RU2^{11}	94	ifm(2)RS13	(95)
ifm(2)RU2^{4}	ifm(2)RU2^{12}	95	ifm(2)RS14	(94)
ifm(2)RU2^{5}	ifm(2)RU2^{13}	94		
ifm(2)RU2^{6}	ifm(2)RU2^{14}	96		
ifm(2)RU2^{7}	ifm(2)RU2^{15}	96		
ifm(2)RU2^{8}				
ifm(2)RU2^{9}				
ifm(2)RU3^{1}				
ifm(2)RU3^{2}	ifm(2)RU3^{8}	96		
ifm(2)RU3^{3}	ifm(2)RU3^{9}	97		
ifm(2)RU3^{4}				
ifm(2)RU3l^{5}				
ifm(2)RU3l^{6}				
ifm(2)RU3l^{7}				

The three new alleles of *ifm(2)RU1* (Table 3) have defective DLMs with considerable thinning of the fibers. The fibers disrupt in the posterior region of the thorax and appear to be loosely bundled at the posterior region and the attachment is totally absent in many of the preparations; however, the consistency is that the posterior region shows degeneration. All six fibers of the DLMS are formed, in a few specimens one or two fibers are missing thereby

reducing the fiber number to five or four, respectively. DVMs show slight thinning; however, otherwise they seem to appear normal.

The six alleles of *ifm(2)RU2* (Table 3) show diffused DLMs, the fibers appear to be stumped and brittle in a few cases, there is consistent thinning of the fibers and have reduced volume, and the thoracic cavity appears to harbor a large vacuole within. There seems to be consistent posterior degeneration of the fibers and defective fibril assembly. The fibers appear to be formed of diffused fibrils, and this gives it a spongy appearance. In a few instances the fibers appear to be 'wiggly', comparable to the DLMs of $Act88F^{KM88}$ (Nongthomba *et al*., 2004). DVMs are nearly normal and show slight thinning. The jump muscle also shows thinning but is otherwise normal in all aspects.

ifm(2)RU3 alleles show drastic phenotype of the DLMs while the DVMs are not affected. There are only three or four or five fibers and never six fibers of DLMs. The fibers are reduced in thickness and appear to be 'wiggly'. There is considerable posterior degeneration and defective attachment to the thoracic wall, anterior region shows slighter defects when attachment is considered.

The complementation analysis also resulted in the isolation of six lines that complemented the *ifm(2)RU1*, *ifm(2)RU2* and *ifm(2)RU3* mutations. They have been named as *indirect flight muscle (2) RS9* to *indirect flight muscle (2) RS15* sequentially.

A clear picture of the molecular interactions of all the genes involved in IFM development has not yet arisen, though a large amount of genes are identified and its functions interpreted.

The alleles and the new mutations isolated from the present study are the novel stocks, further characterization of which will give an insight into their possible role in IFM development.

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Effect of water on larval pupation site preference in a few species of *Drosophila.*

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Drosophila larvae exhibit behavior such as burrowing, skipping, feeding, and pupation site preference (PSP). At the end of larval phase, mature third instar larvae form puparia, which

incorporate the skin to the solid surface. The larval PSP is an important event in *Drosophila* preadult development, because the place selected by the larva can have decisive influence on their subsequent survival as pupae (Sameoto and Miller, 1968). The site chosen for pupariation by a larvae is a component of behavior (Grossfield, 1971).

The effect of abiotic and biotic factors such as moisture, light, temperature, density, sex, larval developmental time, selection for high and low pupation height and its genetic control on PSP in different species of *Drosophila* has been studied (Sameoto and Miller, 1968; Sokal *et al.*, 1960; Mensua, 1967; Markow, 1979; Ringo and Wood, 1983; Bauer, 1984; Bauer and Sokolowski, 1985; Schnebel and Grossfield, 1986; Casares and Carracedo, 1987; Singh and Pandey, 1991; Pandey and Singh, 1993; Singh and Pandey, 1993a, b). Most of the studies are made by measuring the pupation height. The pupation height measured is the distance a larva moves and pupates above the surface of the food medium. Wherein the larvae pupated in/on media was not considered and taken as zero.

The PSP is also studied by counting the number of larvae pupated at each site viz; medium, glass and cotton (Barker, 1971; Manousis, 1985; Shirk *et al.*, 1988; Shivanna *et al.*, 1996; Shivanna and Ramesh, 1997; Vandal *et al.*, 2003). These studies reported that most of the species such as *D. immigrans, D. rubida, D. pararubida, D. nasuta* subgroup species, *D. simulans*, *D. yakuba, D. mauritiana, D. malerkotliana* and *D. bipectinata* pupated maximum in/on media. These studies were made using standard food medium and it has been related to the quantity of larval salivary gland protein. The larval PSP has not been studied in modified (increased water) food medium in these species. An attempt was made to study the effect of water in the medium on larval PSP in different species of *Drosophila* which are inhabiting at different sites.

For the present investigation closely related sibling species, *D. melanogaster*, *D. simulans, D. yakuba* and *D. mauritiana* belong to the *melanogaster* subgroup species, *D. ananassae, D. bipectinata, D. malerkotliana* and *D. rajasekari* are closely related sympatric species belonging to the *ananassae* subgroup of the *melanogaster* species group and *D. virilis, D. hydei* and *D. novamexicana* belong to the *virilis* group, were taken to study the effect of water in the medium on the larval PSP (Singh and Pandey, 1991; Bock and Wheeler, 1972; Ashburner, 1989; Ranganath *et al.*, 1985; Ehrman, 1978). Standard culture medium prepared according to the procedure described by Shivanna *et al.*, (1996) was used as control. To study the effect of water the culture medium was modified by adding 50 ml to 200 ml water keeping the food media constant 100 ml. Totally four sets of modified media were prepared and the ratio of the media and water varies from control 1:0 and modified media 1:0.50 to 1:2.0.

In order to maintain uniformity with regard to the density and age of the larvae the eggs were collected every six hours using modified technique of Delcour described by Ramachandra and Ranganath (1988) and allowed to hatch. First instar larvae (50) were removed and seeded carefully in control and modified culture medium vials and stoppered with cotton plugs. Ten replicates were carried out for each experiment. These cultures were raised at constant temperature 22 ± 1º C with RH 80%. The percentage of pupation was calculated on the basis of the number of larvae pupated at different sites viz; cotton, glass, and medium.

Figure 1 reveals that the maximum PSP both in control and modified medium of *D. melanogaster*, *D. ananassae*, *D. virilis*, *D. hydei,* and *D. novamexicana* is on glass, *D. simulans, D. yakuba, D. mauritiana*, *D. bipectinata,* and *D. malerkotliana* is in/on media and *D. rajasekari* is on cotton. There is no maximum change in percentage of PSP in any species analysed. In modified medium *D. melanogaster*, *D. ananassae*, *D. hydei,* and *D. novamexicana* pupate 2.6%, 12%, 5%, and 2% higher on glass than control, whereas *D. virilis* pupates 7% less on glass than the control. *D. simulans*, *D. yakuba* and *D. mauritiana* pupate 5%, 11%, and 1.3% less on media than control. *D. bipectinata* and *D. malerkotliana* pupate 9% and 19% higher on media than control. *D. rajasekari* pupates 2% less on cotton than control; it remains same at 200% increased water content. The cotton

preference is zero in *D. simulans, D. virilis, D. hydei,* and *D. novamexicana,* less than 1% in *D. melanogaster* (except control), *D. ananassae, D. bipectinata,* and *D. malerkotliana*. *D. mauritiana* pupates less than 1% and *D. yakuba* pupates 2% higher on cotton compared to control.

The percentage of larval mortality varies between species. The highest mortality was found in *D. novamexicana* 15.8%, *D. ananassae* 12.3%, *D. yakuba* 11.4%, *D. virilis* 9.7% and *D. rajasekari* 9.5% with increase of 200% water in the media and followed by *D. hydei* 13.2% and *D. mauritiana* 8.6% increase of 100% water in the medium. The larval mortality rate was found to be less than 5.4% in *D. melanogaster*, *D. bipectinata*, *D. malerkotliana* and *D. simulans.*

The larvae of *D. melanogaster, D. ananassae, D. hydei, D. virilis,* and *D. novamexicana* were moved (100%, 91%, 74%, 90%, and 78%, respectively) away from the food medium and pupated on the glass wall. *D. rajasekari* moved more than 80%-88% from the medium and pupated 60% on cotton and 18% on glass. Whereas in other species, *D. simulans, D. yakuba, D. mauritiana, D. rajasekari, D. bipectinata,* and *D. malerkotliana* the larvae were not moved (93.8%, 92.9%, 60-65%, 40-48%, 93.6%, and 93%, respectively) and pupated on medium. The larvae belongs to different species are shifted about 10% from their normal (control) pupation site preference and about 90% of the larvae remained without any change in their PSP in modified medium. The present study reveals the absence of water effect on larval PSP and supports the opinions of Backendrof and Kafatos (1976), Manousis (1985), Shirk *et al.* (1988), and Shivanna *et al.* (1996).

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A new front for a global invasive Drosophilid: The colonization of the Northern-Western desert of Egypt by *Zaprionus indianus* Gupta, 1970.

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The Afrotropical fruitfly *Zaprionus indianus* Gupta, 1970 (Diptera: Drosophilidae) is now widely extending its geographic distribution beyond its boundaries in the Afrotropical zones to the Palearctic region and the New World (Chassagnard and Tsacas, 1993). Successfully, it invaded India (Gupta, 1970; Karan *et al.*, 2000), Saudi Arabia (Amoudi *et al.*, 1991), and, of late, Brazil (Vilela, 1999; Tidon *et al.*, 2003) and Uruguay (Goni *et al.*, 2002). In Brazil, it was considered as a serious pest of fig production (Vilela, 1999).

A recent survey in the oases of the Northern-Western Desert of Egypt (May-October 2003) recorded its presence for the first time in the country along with other drosophilid species. Flies were collected using fermenting fruit baits from three isolated localities (Alexandria, Bawiti, and Siwa) about 330 km apart, and their frequencies are given in Table 1. *Z. indianus* was the most widespread species and was found in the three localities. Furthermore, it was the unique species present in Bawiti. Alexandria had the richest fauna in species, mainly due to its wet climate and marginality with the agricultural Nile Delta environment. The different distribution of the two introduced species of *Zaprionus* suggests that each of them had a different trajectory in colonizing Egypt. *Z. tuberculatus* is thought to originate from Israel where it became a well established species (Korol *et al.*, 2000), while *Z. indianus* may be introduced from Saudi Arabia via fruit trade or during the annual Muslim pilgrimage season.

Table 1. Survey of Drosophilid fauna in the Northern-Western Desert of Egypt.

Species	Sex	Alexandria	Bawiti	Siwa
D. busckii	♂	---	---	---
	♀	2	---	---
D. hydei	♂	---	---	---
	♀	1	---	---
D. melanogaster	♂	12	---	7
	♀	6	---	4
D. pseudoobscura	♂	---	---	---
	♀	2	---	---
D. simulans	♂	62	---	51
	♀	33	---	55
Z. indianus	♂	22	18	21
	♀	22	21	32
Z. tuberculatus	♂	76	---	---
	♀	64	---	---

In fact, the present survey does not reflect the accurate distribution of the different species. This may be attributed to the following: (1) these localities were not extensively collected over, (2) the collections were made at regular intervals, thus they do not reflect seasonal fluctuation, and (3) because of certain difficulties, there are still many localities in the Northern-Western Desert that need to be examined.

However, the astonishing result of this survey is that *Z. indianus* is now being able to adapt to hyper-arid desert environment, a situation not faced before by the species while invading tropical zones in India and Brazil. Besides, the oasis of Taif, from where Amoudi *et al.* (1991) had collected *Z. indianus* in Saudi Arabia, is known with its cold and temperate climate due to its high altitude.

Another specificity of this new front for *Z. indianus* is that oases, like islands, provide a simplified system in which to tease apart some of population genetics processes, due to their isolated nature (*i.e*., limiting the number of migrants) and finite habitat area (*i.e*., small population size).

The colonization of India, Middle East, and the New World by *Zaprionus indianus*, resembles that of North America by *Drosophila subobscura* three decades ago, which was considered by Ayala *et al.* (1989) 'a grand experiment in evolution'. Experiments in the University of Sao Paolo (Brazil), the Centre National de Recherche Scientifique (France), and in the University of Alexandria (Egypt) are now in progress to explore population genetics, ecological preferences and competitive ability of *Z. indianus*, using molecular, cytogenetic, and quantitative techniques. Many evolutionists can treasure now this global and rich opportunity.

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Evaluating the mode of reinforcement in *Drosophila pseudoobscura*: Discrimination enhancement vs. preference evolution.

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Introduction

Reinforcement is the process by which natural selection increases premating reproductive isolation (*e.g*., mating discrimination) to prevent maladaptive hybridization. For example, if two species have overlapping geographic ranges, and if these species do not mate exclusively with conspecifics, sterile hybrids may be produced. Producing sterile hybrids imposes a cost on these species, and any variation allowing preferential mating with conspecifics will be favored by natural selection in the regions of geographic overlap. This leads to a pattern of "reproductive character displacement": individuals derived from populations of overlap between species (sympatry) will exhibit strong mating discrimination while those from other populations (allopatry) may exhibit weaker mating discrimination.

This process has been documented in a wide range of taxa (see reviews in Howard, 1993; Noor, 1999; Servedio and Noor, 2004). Less clear, however, is how selection reduces hybridization. Some theoretical models (*e.g*., Lande, 1981; Liou and Price, 1994) posited that reinforcement occurs by divergence of the distribution of female preferences (see Figure 1). Females exhibiting preferences for extreme traits that are only present in males of one species are favored, so the entire female preference distribution shifts in populations of geographic overlap. Concomitant with this, males

exhibiting extreme traits are also favored, and the distribution of male traits is expected to coevolve in parallel (*e.g.*, Ritchie, 1996). We call this scenario "preference evolution."

An alternative scenario is that females increase discrimination through reducing the range of characters with which they're willing to mate (Kelly and Noor, 1996). The outcome of this process would be a reduction in overlap of female preferences between the two species, followed by a concomitant reduction in overlap of male traits. We call this scenario "discrimination enhancement."

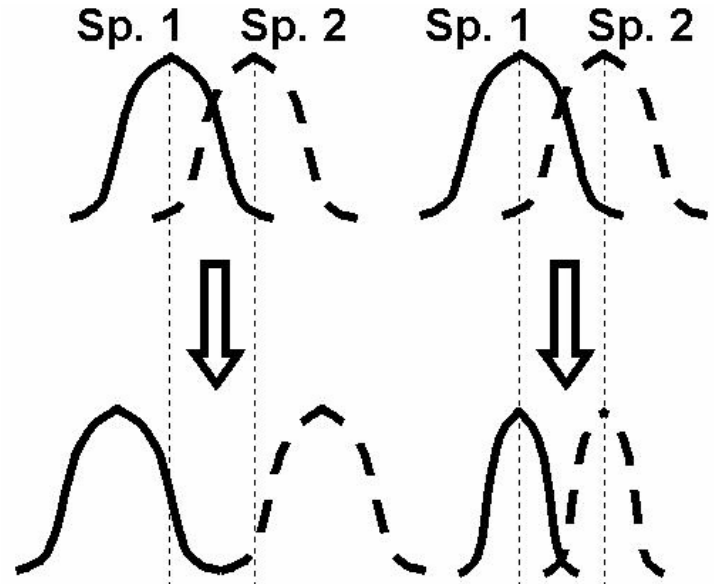

Figure 1. Sympatric species female preference function change under preference evolution (left), with a shift in the distribution of preferences, vs. discrimination enhancement (right), with a narrowing in the breadth of female preferences.

Both scenarios reduce the overlap of male traits and female preferences in hybridizing populations, hence causing reinforcement. However, the target of selection differs in the two: in preference evolution, the primary change is a directional shift in the distribution of female preferences; while in discrimination enhancement, the primary change is a reduction in the variance or breadth of such a distribution. Distinguishing these hypotheses can have a great impact on determining the likelihood of speciation by reinforcement.

Several predictions distinguish these models. First, the preference evolution hypothesis predicts partial sexual isolation among populations WITHIN species, as divergence in the distribution of female preferences should only occur in populations that co-occur with heterospecifics. Discrimination enhancement makes no such prediction, as most males and females in the separated populations within species should be phenotypically and behaviorally similar. Second, preference evolution predicts that heterospecific females should prefer males from nonoverlapping populations relative to those from overlapping populations. In contrast, discrimination enhancement predicts no difference in how heterospecific females perceive males from different populations, as again, they should be phenotypically similar.

We examine these hypotheses in the context of reinforcement in *Drosophila pseudoobscura* and *D. persimilis*. These species overlap in western North America, hybridize rarely, and exhibit reproductive character displacement in female preference (Noor, 1995). Consistent with the discrimination enhancement model, Anderson and Ehrman (1969) observed no mating discrimination among populations of *D. pseudoobscura*. Here, we test the second prediction of discrimination enhancement: whether *D. persimilis* females prefer *D. pseudoobscura* males from nonoverlapping (allopatric) populations. Consistent with the discrimination enhancement model, we find that they do not.

Materials and Methods

Flies used in the mating experiments were reared at 20 ± 1°C, 85% relative humidity, 12:12 hour light:dark cycle, on standard sugar/agar/yeast medium. Bottles were cleared of adults before

incubator lights came on and virgin adults collected less than seven hours later under CO_2 anesthesia. Virgin flies were separated by sex and stored in vials containing food for seven days. One day prior to mating, males were separated and stored in vials individually to reduce crowding mediated courtship inhibition (Noor, 1997). On the eighth day, single females were aspirated into vials with single males for mating observations.

We performed the experiment using inbred *D. pseudoobscura* lines and using outbred F_1 progeny from crosses between inbred *D. pseudoobscura* lines. The *D. persimilis* line used was collected in Mount St. Helena (MSH), California, in 1993. The inbred *D. pseudoobscura* lines used were Mather, California number 17 (collected 1997) and Flagstaff, Arizona (collected 1993). The *D. pseudoobscura* lines crossed for the outbred experiments were Flagstaff lines 6 and 14 (collected 2001) and Mount St. Helena lines 12 and 17 (collected 2001). The California lines are from areas of species coexistence (sympatry), while *D. pseudoobscura* is found alone in Arizona (allopatry).

We first confirmed the pattern of reproductive character displacement in female *D. pseudoobscura*; we examined mate preferences of Arizona vs. California *D. pseudoobscura* females when paired with *D. persimilis* males. We anticipate that the females derived from California would exhibit the greater reluctance to mate with *D. persimilis* males (Noor, 1995). Flies were paired singly in food vials and observed for 10 minutes after onset of male courtship for mating (no-choice mating design). For our test of the discrimination enhancement model, we paired *D. persimilis* females with *D. pseudoobscura* males singly (no-choice mating design) and observed them for 10 minutes after onset of male courtship. Statistical analyses used Fisher's exact tests as executed on StatView®.

Results

Consistent with reproductive character displacement in female preferences, *D. pseudoobscura* females derived from sympatric populations in California were more reluctant than *D. pseudoobscura* females from Arizona to mate with *D. persimilis* males (Table 1). This was true both for the inbred and the outbred lines tested. In contrast, *D. persimilis* females exhibited no preference for *D. pseudoobscura* males from Arizona vs. California populations.

Table 1. No-choice mating experiment results involving crosses between *D. persimilis* (per) and *D. pseudoobscura* (ps).

Female	Male	% Mated	N	p
ps Mather 17	per MSH 1993	11.3	106	<0.0001
ps Flagstaff 1993	per MSH 1993	41.5	106	
ps MSH 12 x 7	per MSH 1993	12.0	100	0.0279
ps Flagstaff 6 x 14	per MSH 1993	25.0	100	
per MSH 1993	ps Mather 17	51.9	106	0.680
per MSH 1993	ps Flagstaff 1993	55.7	106	
per MSH 1993	ps MSH 12 x 7	37.0	100	0.314
per MSH 1993	ps Flagstaff 6 x 14	45.0	100	

Discussion

Using mate preference experiments with *Drosophila pseudoobscura* and *D. persimilis*, we present an explicit test of two models of speciation by reinforcement: preference evolution vs. discrimination enhancement. Both models predict that females derived from populations co-occurring with heterospecifics will exhibit greater mate discrimination than females derived from populations where no heterospecifics exist (reproductive character displacement). The preference evolution model further predicts that females should prefer to mate with heterospecific males from populations where conspecific males do not occur over heterospecific males from populations where conspecific males do occur. The discrimination enhancement makes no such prediction.

Our results are consistent with the discrimination enhancement model of reinforcement: while we did detect the signature of reproductive character displacement, we failed to observe a preference by females for heterospecific males from allopatric populations. Other data on this species pair also fail to provide evidence for the other prediction of preference evolution: that some weak mating discrimination should be observed against individuals from other populations (Anderson and Ehrman, 1969).

Discrimination enhancement may be a common mode by which reinforcement occurs. For example, Butlin (1993) showed that, in the brown planthopper *Nilaparvata lugens*, there was greater variation in the width of female preference functions than in mean female preference. Hence, if natural selection were to reduce overlap in female preferences between two species, it would likely do so through increasing discrimination rather than shifting the mean female preference.

Reinforcement was once a controversial mode of speciation, but empirical studies have provided evidence for its existence and theoretical studies have suggested specific conditions under which it may be particularly likely (see reviews in Noor, 1999; Servedio and Noor, 2003; Coyne and Orr, 2004). As the discrimination enhancement model was suggested as a likely means in which it could occur (Kelly and Noor, 1996), and as we provide empirical data consistent with this model in the *D. pseudoobscura* group, it merits further empirical investigation and confirmation in other taxa.

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Nitric oxide signaling disruption is associated with the maintenance of an undifferentiated status in imaginal tissues of *D. melanogaster*.

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Abstract

Imaginal discs development in *Drosophila* requires a tight coordination between tissue patterning/differentiation and cell proliferation/cell cycle progression. In these tissues, the control of cell proliferation involves the concerted action of many Tumor Suppressor Genes (TSG), whose loss gives rise to overgrown larval epithelial structures. Two phenotypic classes of TSG mutations are known in *Drosophila*, showing hyperplastic *vs.* neoplastic overgrowth.

Nitric Oxide (NO), a versatile second messenger produced by the Nitric Oxide Synthase (NOS)

enzyme, is reversibly able to suppress DNA synthesis and cell division. In developing larvae, the antiproliferative action of NO is crucial in controlling the balance between cell proliferation and cell differentiation and, eventually, the shape and size of adult appendages.

Here we investigate NOS activity in hyperplastic and neoplastic tumor suppressor mutants and observe that early third instar discs from both hyper- and neoplastic larvae do not show noticeable impairments in NOS activity with respect to the wild type.

In late discs, NOS activity increases in wt and hyperplastic individuals, while it is completely absent in neoplastic mutants. This evidence suggests that the loss of NO signaling is required in neoplastic imaginal disc development for the maintenance of an undifferentiated status.

Introduction

In *Drosophila*, several TSG, whose mutation causes overgrowth of imaginal discs epithelia, have been so far identified (Watson *et al.*, 1994). The TSG fall in two main phenotypic classes: HYPERPLASTIC MUTANTS, in which overgrowth occurs retaining both the fundamental epithelial structure and the ability to differentiate; NEOPLASTIC MUTANTS, displaying hyperproliferation associated with dramatical histological changes, such as loss of cell architecture, autonomous growth and metastatic potential (Gateff, 1994). Among TSG, *discs overgrown (dco), fat (ft), large tumor suppressor (lats)* and *hyperplastic discs (hyd)* belong to the hyperplastic class, while *lethal giant larvae (lgl)* belongs to the neoplastic class.

The *dco* gene encodes a Casein kinase type I (CKI) that has been shown to phosphorylate β-Catenin and Gli1 *Drosophila* homologues Armadillo and Cubitus interruptus, targeting them for degradation (Price and Kalderon, 2002; Yanagawa *et al.,* 2002). Armadillo and Cubitus interruptus are oncogenic proteins belonging respectively to the Wnt and Hedgehog pathways, notoriously implicated in tumorigenesis both in humans and *Drosophila* (Pasca di Magliano and Hebrok, 2003; Moon *et al.*, 2002). *ft* encodes a transmembrane protocadherin that we have recently associated to EGFR-mediated proliferation control in imaginal discs (Garoia *et al.*, 2005); *lats* is a cell-cycle regulator that acts by sequestering the CycA kinase partner Cdc2 during early mitosis so slowing G1/S entry (Turenchalk *et al.*, 1999) and *hyd* encodes a HECT domain E3 ubiquitin ligase that is likely to act by targeting Cubitus interruptus for proteolysis (Lee *et al.*, 2002).

The *lgl* gene, whose loss induces neoplastic, metastatic lesions in developing imaginal tissues (Woodhouse *et al.*, 1998), encodes a cytoskeleton-associated protein which participates in the establishment of epithelium apical-basal polarity (Humbert *et al.*, 2003).

In clonal analysis experiments it was observed that, while hyperplastic cells grow faster than wild type twins and form benign tumors in the adult structures where clones have been induced, neoplastic cells grow slowly and are outcompeted during development from surrounding wild type cells (Bilder, 2004). It is likely that these two classes of TSG follow different genetic routes in controlling cell proliferation, whatever molecule they encode.

The Nitric Oxide Synthase (NOS) is a macromolecular enzymatic complex that synthesizes Nitric Oxide (NO) and citrulline starting from arginine, NADPH and molecular oxygen. In *Drosophila* one gene has been identified, named *dnos*, that is constitutively expressed and shows a Ca^{2+}/Calmodulin dependent activity (Regulski and Tully, 1995). This *locus* gives rise to almost eight different transcripts, four of which are translated into proteins; the dNOS1 is the prominent active form of NOS in *Drosophila* (Enikolopov *et al.*, 1999). NO is a short-range radicalic gas showing a very brief half-life, easily diffusible through membranes, and for these intrinsic features a role in many intercellular signaling pathways has been proposed and then largely demonstrated (Bredt and Snyder, 1994; Nathan and Xie, 1994). It has been shown that NO donors such as S-Nitroso-

AcetylPenicillamine (SNAP) can suppress DNA synthesis and cellular division in mammalian cultured cells and *Drosophila* embryos (Garg and Hassid, 1989; Kwon *et al.*, 1991; Wingrove and O'Farrell, 1999); this suppression is reached either through the inhibition of pRB phosphorylation - an event required for pRB release from E2F and consequent transcription of genes inducing S phase entry - (Ishida *et al.*, 1997) or through the activation of the $p21^{waf}$ promoter, a cyclin-dependent kinases inhibitor (Poluha *et al.*, 1997). In *Drosophila*, experiments of inhibition and overexpression of NOS demonstrated a direct role of NO in the imaginal discs developmental control and in the adult structures morphogenesis (Kuzin *et al.*, 1996). Flies without NOS activity show overgrown wings and legs, while flies with an ectopic expression of NOS induced at the beginning of the third larval instar show a severe reduction of these structures (Kuzin *et al.*, 1996).

Furthermore, recent studies performed in the *Drosophila* developing eye demonstrate that dNOS interacts with the pRb pathway to control the ommatidia number (Kuzin *et al.*, 2000), and a screening in insect cell cultures revealed that NO is able to activate the transcription of several genes involved in cell cycle control (Hemish *et al.*, 2003). In the light of all this evidence, it appeared particularly interesting to investigate the role of NO in modulating cell proliferation and differentiation in different TSG mutant backgrounds.

Experimental Procedures

Fly stocks and depositions

The following fly stocks were used in this work: *Canton S* (wild type strain); *dco^3/TM6B, Tb* (Zilian *et al.*, 1999); *$lats^{P2}$/TM6b, Hu, Tb* (Justice *et al.*, 1995); *ft^4/In (2L,R) Gla, Bc* (Bryant *et al.*, 1988); *hyd^{37}/TM6b, Hu, Tb* (Mansfield *et al.*, 1994), and *l(2)gl^4/In (2L,R) Gla, Bc* (Gateff and Schneiderman, 1969). Flies were reared at 25°C on a standard medium; heterozygous flies were allowed to lay for 2 hours, then eggs were collected and transferred onto new dishes in the number of 100/dish. Homozygous larvae were selected for the absence of *Tb* or *Bc* larval markers associated with *TM6b* and *In (2L,R) Gla, Bc* balancer chromosomes respectively.

NADPH diaphorasic colorimetric assay

Homozygous larvae were harvested at the beginning of the third instar and at the end of larval development for all strains except *lgl*, whose thoracic discs at the extreme end of the larval life result in a large bulk in which a reliable reaction is not feasible; for this mutant, 216 hours AEL larvae were utilized. Larvae were dissected in RPMI at 4°C, imaginal discs were fixed for 30 minutes in paraformaldehyde 4% in Phosphate Buffer Saline (PBS) 1× pH 7.4 and permeabilized for 1 hour (wild type and hyperplastic) or 3 hours (neoplastic) at 4°C in PBS 1×/Triton 0.1% (PBT). The colorimetric assay was performed incubating discs for 1 hour at 37°C in PBT pH 7.4 with 0.1 mM Nitroblue Tetrazolium (NBT) and 0.5 mM NADPH. Discs were washed several times in PBT and mounted on glasses. The images were captured with an Axiocam digital camera mounted on a Zeiss optical microscope.

Results and Discussion

The NADPH-diaphorase enzyme colocalizes with NOS and the histochemical reaction that detects its activity is widely used to display NOS activity, also in insects tissues (Muller and Buchner, 1993). Through this method it has been demonstrated that in third instar wild type imaginal discs there

is a strong correlation between the increasing of the NOS activity and the slow-down of the proliferation rate (Kuzin *et al.*, 1996). We thus performed preliminary studies on the dNOS activity in imaginal wing discs from wild type *(Canton S), dco, fat, lats, hyd* and *lgl* larvae using the NADPH-diaphorasic colorimetric assay.

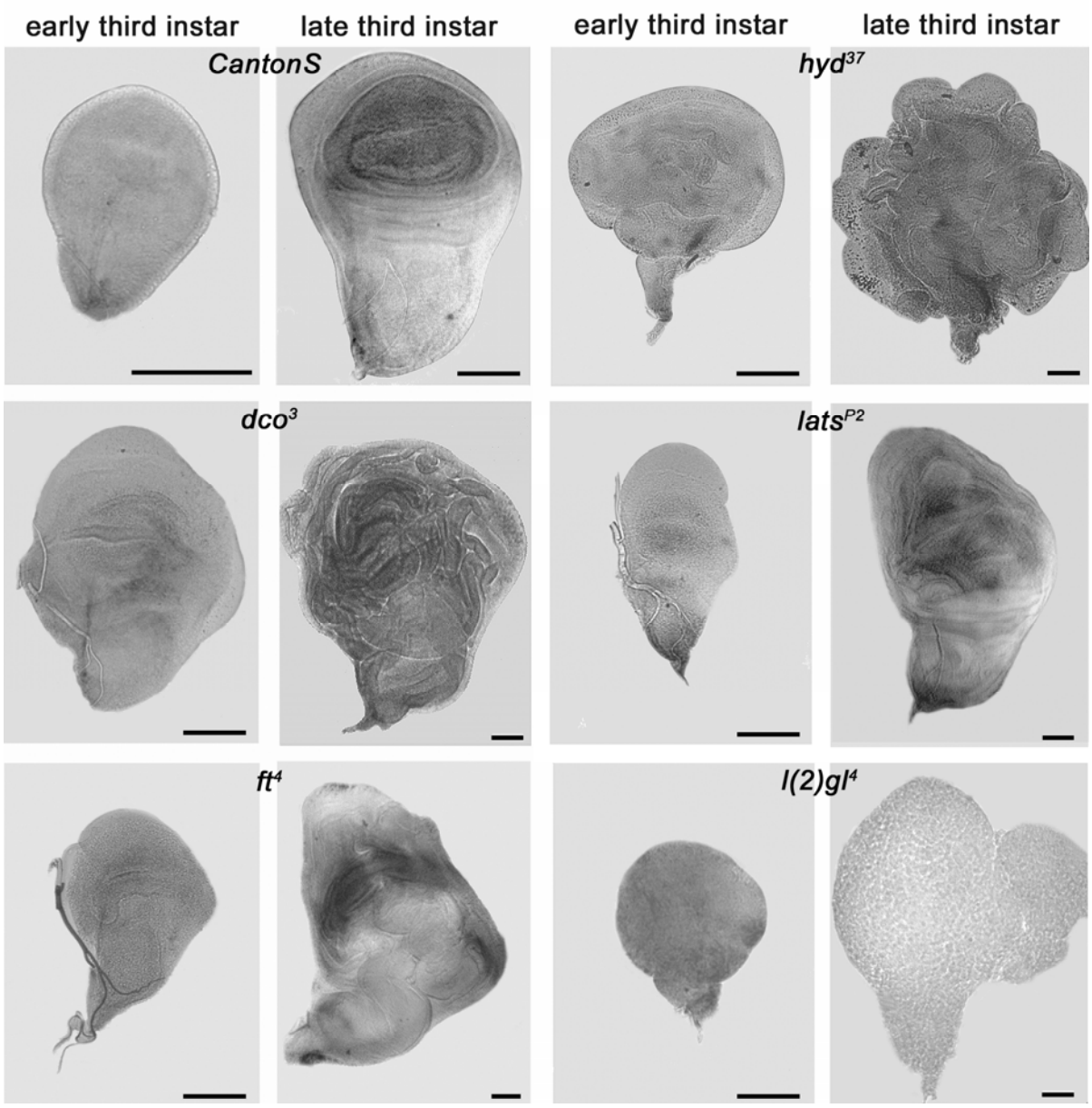

Figure 1. Panel showing NOS activity in early and late third instar wing discs. The panel shows a wild type *Canton S* control, hyperplastic mutants (hyd^{37}, dco^{3}, $lats^{P2}$, ft^{4}) and the $l(2)gl^{4}$ neoplastic mutant. The black bar represents the relative size of the discs.

At 120 hours after egg laying (AEL) the wt larvae pupariate, while the TSG mutants show a delayed larval development, at the end of which the hyperplastic animals almost complete pupal development, while the neoplastic individuals die in the shape of large, bloated larvae. An important

feature of the *lgl* neoplastic discs is that at 120 hours AEL it already shows an abnormal shape, but several differentiation markers still show a wild type localization; later in development, cell populations intermix and the correct segregation is no longer observed (Agrawal *et al.*, 1995).

Our results show that the pattern of NOS activity in all the TSG mutants is comparable to that of the wild type, showing in time an accumulation correlatable with the slow-down of the proliferation rate and the consequent onset of differentiation (see Figure 1). This result was expected, because in these individuals the proliferation control is impaired but still present; increased proliferation alone does not indeed prevent terminal differentiation.

The results obtained in the *lgl* neoplastic background are instead quite different; we observed that, with larval development, the *lgl* overgrown discs show a complete absence of NOS activity (see Figure 1) that persists till larval death (data not shown).

NOS activity so seems to be strictly associated with the onset of differentiation; in *lgl* mutants, where terminal differentiation never occurs, NO signaling is irreversibly switched off at the moment in which cells fail to respond to positional clues. In this mutant, NOS drop could hence be a consequence rather than a cause of the neoplastic growth, and this could in turn provide cells with new properties that maintain the undifferentiated status. On the contrary, the presence of NOS activity in the early *lgl* imaginal tissues suggests that NO signaling is not involved in the onset of the neoplastic phenotype.

Aside from confirming the essential role of NOS activity in the differentiation of *Drosophila* developing imaginal tissues, our evidence strongly suggests that lack of NO could be required for the maintenance of an undifferentiated status in the neoplastic imaginal tissues, supporting the progression towards malignancy.

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Proving an old prediction: The sex comb rotates at 16 to 24 hours after pupariation.

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The sex comb is a row of thick, dark bristles (resembling a hair comb) on the forelegs of adult *Drosophila melanogaster* males. Forty-two years ago, the renowned geneticist Chiyoko Tokunaga reported indirect evidence that the sex comb must rotate at some time during development (Tokunaga, 1962). We have now directly determined when this rotation occurs.

We used a *scabrous-Gal4* driver to express green fluorescent protein (GFP) in nascent bristle cells (a.k.a. sensory organ precursors or SOPs) via *UAS-GFP*. Virgin *y w; sca-Gal4/CyO* females were crossed with males carrying an α-tubulin *UAS-GFP* construct (Bloomington Stock Center #7374). F_1 white prepupae were collected and humidified at 25˚C until they reached the desired age. We tried other UAS constructs (nuclear, cytoplasmic, and cell-surface GFP), but none was as effective in delineating SOPs as the α-tubulin marker.

The opaque pupal case precludes observation of the leg epidermis. For pupae older than 12 hAP (hours after pupariation) the case can be entirely removed without any bleeding since by this stage a transparent pupal cuticle has been secreted that covers the fragile epidermis (Poodry, 1980). Wild-type pupae treated thusly survive to eclosion (Held, 1992). To our surprise, however, >95% of the *sca-Gal4; UAS-GFP* pupae died within hours after removal of the case. Death was not due to the insertion site of the #7374 P-element because it also occurred with #1521, nor was it due to fragility since the cuticle remained intact, nor was it rescuable by (1) asepsis, (2) fungicides, or (3) application of a thin coat of oil. Its cause remains unknown.

This lethality prevented us from monitoring cell movements in individual living pupae with time-lapse microscopy. Instead, we pieced together the sequence of events using cohorts of pupae aged for varying lengths of time after collection as white prepupae—namely 16, 17, 18, 19, 21, 22, 24, 26, and 28 hAP. We enriched the frequency of males to 90% by selecting the thinnest 20% of pupae. (Male pupae tend to be thinner than females, though distributions overlap.) Half the F_1 expressed GFP (those inheriting *sca-Gal4*); half did not (those with *CyO*).

Pupae were dissected by the method of Held (1992), placed ventral-side down, and aligned side-by-side (8 per row) in petri dishes whose bottom was replaced by a glass coverslip. Each pupa was rolled ~30˚ so that the inner (anteroventral) surface of its right foreleg could be seen using an inverted (Olympus IX70 epifluorescence) microscope. Forelegs of fluorescent pupae were examined (within a half hour of dissection) at 250× and 500× magnification. A film of water intervened between the coverslip and the pupae, and dishes were kept humidified. We also tried using immersion oil instead of water at the interface, but the resolution was poorer.

Each leg had to be photographed at both magnifications because the higher magnification afforded better acuity, but its field was too narrow to discern the orientation of the tarsus, which was essential to assess the angle of the sex comb. For each leg, we rotated the low-power picture in Adobe Photoshop™ so that the tarsus was vertical and then rotated the high-power picture to the same extent. Angular assessments were aided by using the "central bristle" (Hannah-Alava, 1958) as

a reference point. On the forelegs of adult males, the central bristle is located in the middle of a field of trichomes (hence its name). Tokunaga concluded that this SOP must initially be part of the sex comb. The deviations of the marked cell clones in genetic mosaics convinced her that the sex comb

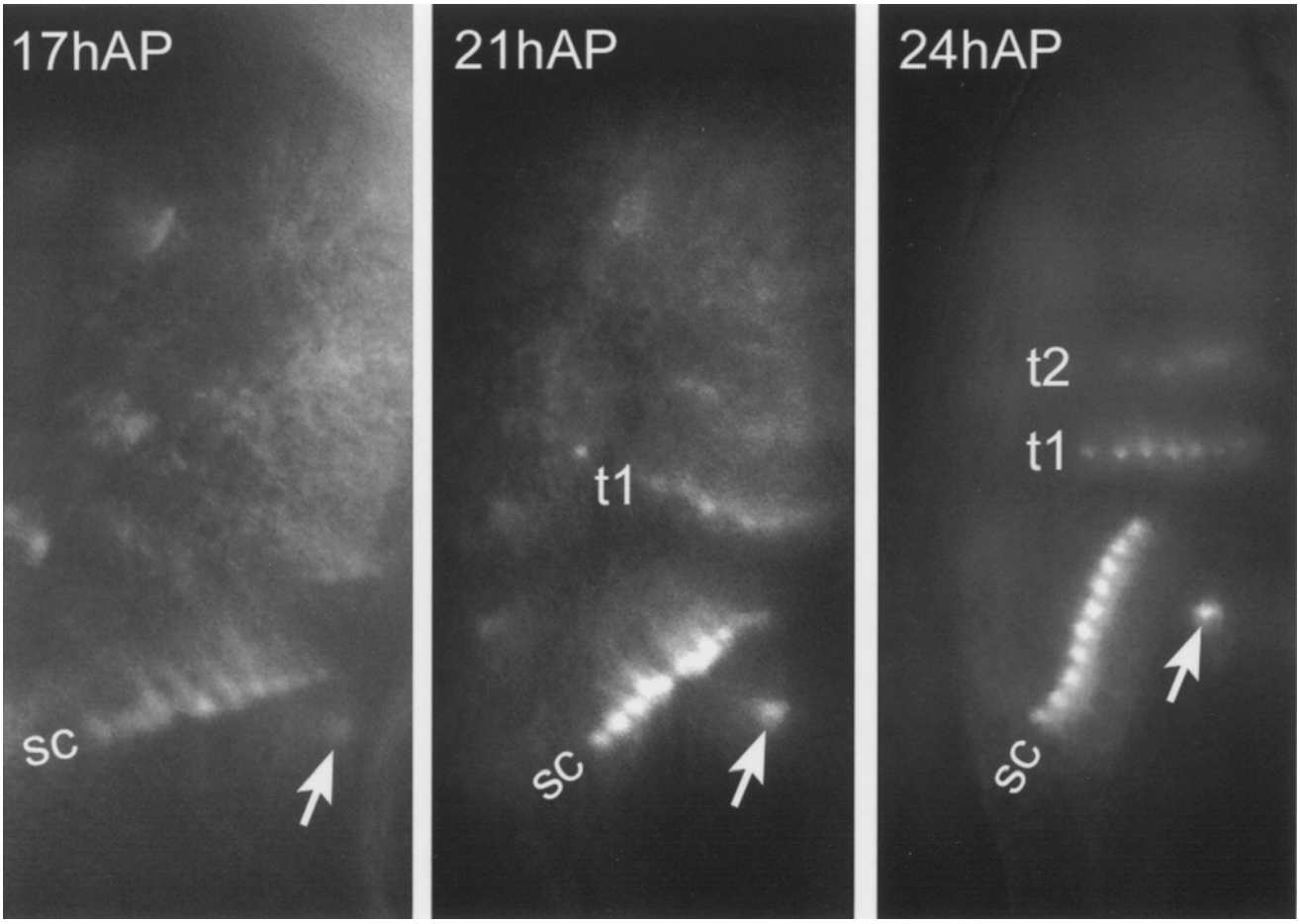

Figure 1. Anteroventral surfaces of right foreleg basitarsi of *sca-Gal4; UAS-GFP* male pupae at 17, 21, and 24 hAP. Basitarsi are oriented with proximal at the top and distal at the bottom, dorsal to the left and ventral to the right. White spots are SOPs. During this period the sex comb (*sc*) appears to pivot from a transverse (~3 o'clock) to a longitudinal (~1 o'clock) orientation. All pictures are at the same magnification. (The mature sex comb is ~60 microns long.) The arrow in each panel points to the SOP that will become the central bristle. The GFP signal intensifies in a distal-to-proximal pattern—with the sex comb shining before the next most distal transverse row (*t1*), and that row shining in turn before its neighbor (*t2*). SOPs tend to be in straighter rows on older legs, and sporadic gaps (*e.g.*, between the 4th & 5th SOP in the 17 hAP pupa and between the 5th & 6th SOP in the 21 hAP pupa) become less frequent. The S-shape of the sex comb in the 24 hAP specimen is common but not universal since other pupae at the same age have their SOPs in a straight line. The number of sex comb SOPs in these legs appears to be 10 (17 hAP pupa), 9 (21 hAP pupa), and 11 (24 hAP pupa). In a control sample of thin pupae that were allowed to complete development, the number of sex comb bristles averaged 10.0, with a range from 8 to 12 bristles (N = 60 adult forelegs). The number of sex comb SOPs is known to become fixed at 14 hAP (Belote and Baker, 1982) when normalized to the 25˚C rate.

must arise as a transverse row that then pivots to become longitudinal, leaving behind its ventralmost SOP, which becomes the central bristle (Tokunaga, 1962).

Consistent with Tokunaga's predictions, we found that the sex comb does indeed arise as a transverse row. This row has clearly pivoted away from the central bristle SOP by 17 hAP (Figure 1), and some rotation is evident in pupae as young as 16 hAP. (Males cannot be discerned from females in the tarsus unless the sex comb has separated from the central bristle SOP.) Attempts to examine pupae before 16 hAP were hampered by faintness of the GFP marker, which only begins to be expressed (via *scabrous*) in sex comb SOPs around this time.

In older cohorts the sex comb has a steeper angle and greater distance from the central bristle SOP, reaching its final (~longitudinal) orientation by ~24 hAP. By this stage, the GFP marker is not only apparent in the sex comb but also in two distal rows of SOPs that remain transverse ("t1" and "t2" in Figure 1). This distal-to-proximal sequence of differentiation has been documented before (Graves and Schubiger, 1981; Held and Bryant, 1984; Held, 1990). Interestingly, the central bristle SOP itself evidently migrates proximally as the comb turns.

Remaining mysteries concern the cellular basis of the movements (Held, 2002). To what extent do mitoses behind the row push it forward? How do cells interchange partners? Do any junctions remain intact as they do so? How do sex comb SOPs stay aligned as the row pivots?

Acknowledgments: This article is dedicated to the memory of Dr. Chiyoko Tokunaga, who patiently trained me (L.I.H.) in fly genetics when I was a graduate student in the lab of Dr. John Gerhart. The *y w; sca-Gal4/CyO* stock was kindly provided by Susan Younger (Y.N. Jan lab). Thanks also to the Department of Biology Imaging Facility for use of the microscope equipment.

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CpC methylation is present in *Drosophila melanogaster* and undergoes changes during its life cycle.

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Summary

DNA methylation plays an important role in regulation of gene expression in eukaryotes. 5-methyl-cytosine (5mC) is the most commonly found methylated base in the genome of eukaryotes. Due to the low level of 5mC in *Drosophila melanogaster*, the presence of 5mC in the *Drosophila* genome has been controversial. However, there have been conclusive reports confirming presence of 5mC in *Drosophila melanogaster*. Our results show that 5mC is present in the genome and that its pattern varies during different stages in the life cycle.

Methylation of DNA is a heritable epigenetic phenomenon that occurs in many organisms like bacteria, plants, and mammals (Adams, 1990). It involves addition of a methyl group to carbon 5 of a cytosine ring. It is a post-replicative modification brought about by DNA methyltransferases. 5mC gives hydrophobicity to the DNA molecule. It protrudes out of the major groove of DNA. It is known to influence DNA:protein interactions and DNA conformation. Normally, the conversion from B to Z form of DNA takes place under high salt conditions. In the presence of 5mC, this B to Z transition occurs even under physiological salt concentrations (Moller and Nordheim, 1981). In mammals**,** it has been implicated in several important processes like transcriptional repression (Razin and Riggs, 1980)**,** parental imprinting, X chromosome inactivation, development of neoplasia/cancer and ageing (Riggs, 1975; Holliday and Pugh, 1975; Singal and Ginder, 1999). The majority of methylation in mammals is found at CpG dinucleotides (Seigfried and Cedar, 1997) and involves several DNA methyltransferases and associated factors (Kunert and Marhold, 2003). Over the past decade there has been a tremendous boost in the research involving DNA methylation in eukaryotes due to its increasingly important role in regulation of the genome. However, the status of DNA methylation in *Drosophila sp.* was controversial in spite of strong evidence supporting the presence of 5-methyl-cytosine in the *Drosophila* genome (Achwal and Chandra, 1982; Achwal *et al.*, 1983; Achwal *et al.*, 1984; Ashburner, 1989). Now, it is fairly accepted that *Drosophila* DNA does contain methylated cytosines. Introduction of hspCAT gene in *Drosophila* cells demonstrated that the machinery for methylation-mediated suppression is present in *Drosophila melanogaster* (Deogbagkar *et al.*, 1997). Moreover, unlike mammals, *Drosophila* DNA methylation is established and maintained by a single Dmnt2 enzyme (Kunert and Marhold, 2003). Methylation in *Drosophila* is found largely at non-CpG sites (Lyko *et al.*, 2000). The frequency of methylation is extremely low (one cytosine methylated in every 12000-20000 nucleotides, Achwal *et al.*, 1984). It is shown to be concentrated in CpT and CpA dinucleotides (Lyko *et al.*, 2000; Gowher *et al.*, 2000). Our results establish the presence of a distinct restriction enzyme digestion pattern in the DNA of *Drosophila* larva, pupa, and adult that strongly supports the presence of CpC methylation in these stages.

Genomic DNA was isolated from larva, pupa, and adult flies of *D. melanogaster* ORK5. Samples were crushed in liquid nitrogen and homogenized on ice in $T_{10}E_{10}$ (10 mM Tris-Cl, pH 8.0; 10 mM EDTA, pH 8.0) and NP-40. DNA was extracted from the nuclear pellet using phenol:chloroform method (Deobagkar *et al.*, 1984). Quality of DNA was checked on a 0.8% agarose gel.

Larva, pupa, and adult DNA (5 μg each) was digested with *Msp*I and *Hae*III, separately, for 3.5 hours and run on a 1.2% agarose gel at 150 volts. The gel was stained with ethidium bromide and observed.

RESTRICTION ENZYME	**CUTTING SITE**	**5mC FOR INHIBITION (*)**
MspI	CCGG	C*CGG
HaeIII	GGCC	GGC*C

*Hpa*II and *Msp*I are isoschizomers and have been extensively used for analysis of CpG methylation. Digestion of larval, pupal, and adult DNA with *Hpa*II does not show any difference in the relative *Msp*I/*Hpa*II digestion pattern. *Msp*I is, however, sensitive to CpC methylation in the CCGG sequence. It can be clearly seen upon examination of the *Msp*I digestion pattern in the larva, pupa, and adult that there is a small amount of high molecular weight DNA in these samples remaining undigested that indicates the presence of 5mC in the restriction enzyme cleavage site,

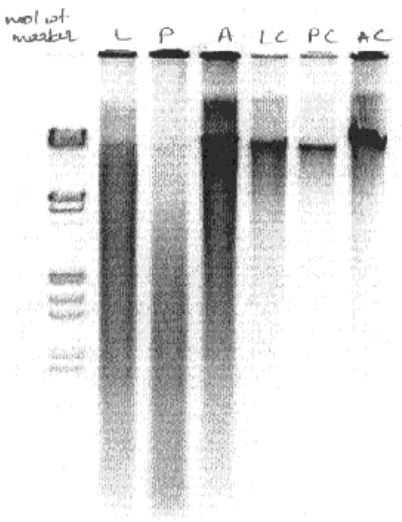

Figure 1. Digestion of larva, pupa, and adult DNA with *Msp*I. Lane 2: larva digest, 3: pupa digest; 4: adult digest; 5,6,7: larva, pupa, adult controls, respectively.

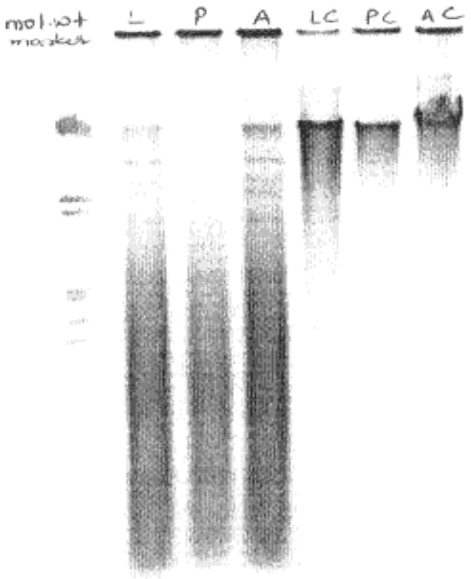

Figure 2. Digestion of larva, pupa, and adult DNA with *Hae*III. Lane 2: larva digest, 3: pupa digest; 4: adult digest; 5,6,7: larva, pupa, adult controls, respectively.

CCGG. Pupal DNA, however, shows considerably more digestion of DNA suggesting that methylation of cytosines in CpC in the CCGG sites undergoes changes.

The *Hae*III restriction enzyme digestion also reveals a similar pattern in adult and larva showing a band of high molecular weight DNA remaining undigested. Digestion of pupal DNA, again, does not show this undigested band indicating presence of CpC methylation in adult and larva at restriction site, GGCC, and not in pupa. The genomic DNA sequence organization is not expected to change in these stages.

Thus, our results demonstrate the presence of CpC methylation in *Drosophila melanogaster*. Moreover, there is a difference in the methylation pattern in the three developmental stages viz. larva, pupa, and adult. Earlier reports indicate that DNA methylation in *Drosophila* is mostly seen during embryonic development (Lyko *et al.*, 2000). It would be of interest to examine possible implications of these changes in the methylation pattern.

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Loss of *Thor*, the single 4E-BP gene of *Drosophila*, does not result in lethality.

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Thor was first identified in a genetic screen in *Drosophila* designed to detect genes that are up-regulated by bacterial infection (Rodriguez *et al.*, 1996). In this screen, we identified *Thor* and subsequently characterized the molecular identity and mutant phenotype (Bernal and Kimbrell, 2000). *Thor* is a member of the *4E-binding protein* (*4E-BP*) family, which in mammals has been defined as critical in the pathway that controls initiation of translation through eukaryotic initiation factor 4E (eIF4E).

The role of 4E-BP derives from its binding and sequestration of eIF4E. In general terms, when 4E-BP is bound to eIF4E, eIF4E cannot form a complex with mRNA, resulting in blockage of translation. When 4E-BP is phosphorylated, eIF4E is released, resulting in allowance of initiation of translation. For a review of translation mechanisms, see Poulin and Sonenberg (2003).

Thor transcription is apparent in many tissues, and after bacterial infection, the level of *Thor* transcripts is increased, but not *eIF4E* transcripts (Bernal and Kimbrell, 2000). The *Thor* promoter has canonical NFkB and associated GATA recognition sequences that have been shown to be essential for immune induction. Other sequences commonly found for *Drosophila* immune response genes are also present, including forkhead homologs/hepatocyte nuclear factors, and in addition interferon related regulatory sequences (Bernal and Kimbrell, 2000). The original *Thor* strain is an enhancer trap mutation of *Thor*, with the P-element insertion 5' of the start of transcription and resulting in a hypomorphic mutation (Bernal and Kimbrell, 2000). This strain and other mutants were tested for survival after injection with bacteria. Some bacterial types had little or no effect on survival whereas others strongly reduced survival capacity. Of the four bacterial strains tested, the survival correlated with *Thor* mutants being less resistant to gram-positive bacteria. These results provided the first evidence that any gene in this translation initiation pathway is immune induced, and further that mutation of the *Thor* component of the pathway results in an immune compromised state (Bernal and Kimbrell, 2000).

Since the studies of Bernal and Kimbrell (2000), induction of *Thor* by bacteria has been confirmed by microarray analysis (*e.g.*, De Gregorio *et al.*, 2001) and extended to induction by fungus (Roxstrom-Lindquist *et al.*, 2004; Schoenfeld and Kimbrell, unpublished) and the parasite *Octosporea muscaedomesticae* (Roxstrom-Lindquist *et al.*, 2004). Roxstrom-Lindquist *et al.* (2004) found a reduction of *Thor* for *Serratia marcescens*, a gram-negative bacterium, perhaps correlating with the phenotype of *Thor* mutants being more resistant to gram-negative bacteria. In addition, *Thor* has been found to be involved in a wide variety of processes. For example, microarray analysis shows induction of *Thor* under both starvation and sugar conditions, consistent with *Thor* acting as a cell growth signal (Zinke *et al.*, 2002). *Thor* has also been found to be involved in insulin signaling, and more specifically as a target of FOXO, a forkhead family transcription factor (Junger *et al.*, 2003). Insulin signaling and FOXO are important for a variety of functions, including regulation of lifespan (Tu *et al.*, 2002; Hwangbo *et al.,* 2004).

We have produced a *Thor* null mutation by mobilization of the original P-element insertion at the *Thor* locus (Figure 1). Homozygous null flies are viable with no obvious morphological defects, and have the same phenotype with respect to bacterial survival as the hypomorph (Bernal and

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                                        Forward Primer →
 781 tgcattaaac aataacaaat gaccaaagat aaacaagagc tcaaggcgag aagcccctca
null                          ..... .......... .......... ..........

 841 aaacagcccc ccaccatcgc tacacccctt atcatctaaa acctccgagg tttgcaggct
null .......... .......... .......... .......... .......... ..........

 901 gcaacaaaaa taaaaacaat aggggaatat attagaatac ggcaataaca acaagaacca
null .......... .......... .......... .......... .......... ..........

 961 gccggtttgt cataaactac gcagaaaata caccaaataa aaacaaaaaa cttcagccga
null .......... .......... .......... .......... .......... ..........

1021 ccaagcagaa taatcaggca agagagcagg cgaaagagcg agaagagagc gagtaagagt
null .......... .......... .......... .......... .......... ..........

1081 aagaatgggg gggtataaat agagccacac tccgtaatac cgccagcaat cgctcagcga
null .......... .......... ......g... .......... .......xxx xxxxx

1141 acagccaacg gtgaaacaca tagcagccac acaagctcta tagctgatac aagcaacgaa
1201 atacaaacaa cgcagtttgt gtaaacaatc aaattgtcgt agccatatcg agtgtgctta
                                                        START
1261 cacgtccagc ggaaagtttt cgaaacccat ccaatcaatc agctaagATG TCCGCTTCGC
1321 CCACCGCCCG TCAAGCCATC ACCCAGGCCC TGCCCATGAT CACCAGGAAG GTTGTCATCT
1381 CGGATCCGAT CCAGATGCCC GAGGTGTACT CCTCGACGCC CGGCGGAACC CTCTACTCCA
1441 CCACTCCTGG AGGTAAGTTT CGAAAAGTGG AAGATGAGAC TACATCGAGT ATTATTAAGT
1501 TCCTTCCGGA AAGTGGAGTG ATTTTCTTAT CATCAGATAT GATATCAAGA TGAAATTAAT
1561 CAACATATTT CGAAAACATA AACAAGTATA CCGTTTCTTT CCTTTGCTCA TACAACAAAC
1621 CAGCTTTTGT TTGAGTTGTG ATACATTATT TTTGTAAAAC TCACTCACCA TTTTTTGGCT
1681 GATGAAACTG GGCCATCTCG CCGAGTCAGT CAGTTGGAAG ATAATCCCGA GCAATGTTCC
1741 ACAGTTCGTG ACTCAAAGTA GCGCGGCAAA AAAGCGTTTA CTCAGCTGGC TTATCAGTGG
1801 GGAACTTTGA ATACCTTTCT GATGCAACGC AAACCACATT GACTAATCAA AATTTCCGCT
1861 TTCCTAAAAT TCCAGGCACC AAACTTATCT ACGAGCGGGC TTTCATGAAG AATCTCCGTG
1921 GCTCCCCATT GAGCCAAACT CCGCCGTCCA ACGTGCCCAG TTGCTTGCTG AGGGGCACTC
1981 CGCGTACTCC CTTCCGCAAG TGCGTGCCCG TCCCCACGGA ACTGATCAAG CAGACCAAGT
                                                        STOP
2041 CGCTGAAGAT CGAGGACCAG GAACAGTTCC AACTGGATCT Gtaggggggtg tggcgtgtac
2101 accgccatgc agcaactgcc aaatccaact agttcttaga tgcacccact aaaaccgatc
2161 ttataagctt agtgtaccca ttaactagat gctaagttct tagttcttcc accttgtttg
2221 ttctctcggt catattgacc ccgttacctt gtaaaatcgt agatcttaag taaaatgcaa
2281 taaaaaaaag gcacaacaat atttaaaaga gaaccacacc ttccattggg attcagtagc
2341 tgccaattta caattataaa ttttaattta tgtttgagaa attcagtgcc aaattacttt
2401 ctttatcggc aattcaacct tggcaggcca cgcgttatgt gatagaacac tttccacttc
2461 accttgagtc agtttcggtt tagtagtatt ttcggcagtt tgttgaactg tttgaactgt
2521 ttacgaagaa atttagcgaa agggggcttt ttgggggggcg gtacacatgt tagacagctg
2581 gaaataccgg ccggcttttt agttagccag tcagtcagtg gccccagcag cgcgtgtccc

2641 acttattgca aagatatgca tacatattta taatgtatcc aaaaaccggc aaacaaacgc
null               xxxxxx xxxxxxxxxx xxxxxxxxxx catgatgaaa taacatatca

2701 cacaaaaaaa tagatcttta agaaactgcg cagttgggaa acgacctcgc ctttggcccc
null gatcatg... .......... .......... .......... .......... ..........

2761 cgccccgccc tttaccattt ttcttatata tacaaaagta gtgtgctgag cacatgttcg
null .......... .......... .......... .......... .......... ..........

2821 ttcagataga ccgatccctt gtggtccaac aactacatcc gactacactc gactaatccc
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```
null ......... ......... ......... ......... ......... .........

2881 ggcacgttcc agacccattt gaatattgcg catgaataat tacctaatcg caaaaagtgt
null ......... ......... ......... ......... ......... .........

2941 ttgctcgaca aagatttagc atcgcagtgc aaaaattcga ccgcacgaga tagtgcaaat
null ......... ......... ......... ......... ......... .........

          ← Reverse Primer
3001 agtggatccc agatgcttac agagt
null ......... ......... .....
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Figure 1. Sequence comparison of the wild type *Thor* locus and the *Thor* null mutation. The *Thor* start and stop codons are shown in bold. Between these, the coding region is underlined and the single intron is italicized. The numbering of the bases corresponds to GenBank Accession AF244353, beginning with base 781. Dots (·) indicate identity between the wild type and null sequence. x indicates ambiguity in the precise position of deletion in the *Thor* null mutation. The highlighted bases match P-element end sequences. The positions corresponding to the forward and reverse primers used to diagnose (PCR fragment size of wild type versus null) and analyze (determine DNA sequence) the null sequence are underlined. The primer sequences used are: forward primer 5'-AGATAAACAAGAGCTCAAGGCG-3', and reverse primer 5'-ACTCTGTAAGCATCTGGG ATCC-3'.

Kimbrell, 2000). *Thor* null was expected to cause lethality, particularly as *Thor* has been confirmed as the only *4E-BP* gene in *Drosophila* (Adams *et al*., 2000) and is involved in many processes. At least an obvious phenotype would be expected, in particular a growth change, as, for example, overexpression of eIF4E in mammalian cells results in malignant transformation that is rescuable by overexpression of 4E-BP (see Clemens, 2004). However, loss of a variety of genes often has no obvious phenotype. In this regard, it is important to point out that *Drosophila* FOXO, also a single copy gene, is viable, has no overgrowth phenotype, but does show hypersensitivity to oxidative stress (Junger *et al.*, 2003).

The *Thor* null strain is an important tool to decipher the complexities of the pathways involving *Thor*, and we have provided this strain for use by a variety of laboratories for this purpose. Given the advances in understanding the involvement of translation in malignant transformation (Clemens, 2004), forkhead family transcription factors in the immune system (Coffer and Burgering, 2004), and hormonal regulation of aging (Tatar, 2004), these studies by various laboratories are beginning to decipher the specific roles of *Thor*. In keeping with this, we have determined that *Thor* mutants have reduced lifespans (Cline and Kimbrell, unpublished), thus providing one of several findings that are helping to bring together the diverse areas of *Thor* involvement.

References: Adams, M.D. *et al*., 2000, Science 287: 2185-2203; Bernal, A., and D.A. Kimbrell 2000, PNAS USA 97: 6019-6024; Clemens, M.J., 2004, Oncogene 23: 3180-3188; Coffer, P.J., and B.M. Burgering 2004, Nat. Rev. Immunol. 4: 889-899; De Gregorio, E., P.T, Spellman, G.M. Rubin, and B. Lemaitre 2001, PNAS 98: 12590-12595; Hwangbo, D.S., B. Gersham, M.P. Tu, M. Palmer, and M. Tatar 2004, Nature 429: 562-566; Junger, M.A., F. Rintelen, H. Stocker, J.D. Wasserman, M. Vegh, T. Radimerski, M.E. Greenberg, and E. Hafen 2003 J. Biol. 2: 20.1-20.17; Poulin, F., and N. Sonenberg 2003, In: *Translation Mechanisms*, (Lapointe, J., and L. Brakier-Gingras, eds.). pp. 280-297, Landes Bioscience, Austin, TX; Rodriguez, A., Z. Zhou, M.L. Tang, S. Meller, J. Chen, H. Bellen, and D.A. Kimbrell 1996, Genetics 143: 929-940; Roxstrom-Lindquist, K., O. Terenius, and I. Faye 2004, EMBO Rep. 5: 207-212; Tatar, M., 2004, Exp.

Gerontol. 39: 1745-1750; Tu, M.P., D. Epstein, and M. Tatar 2002, Aging Cell 1: 75-80; Zinke, I., C.S. Schutz, J.D. Katzenberger, M. Bauer, and M. Pankratz 2002, EMBO J. 21: 6161-6173.

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