Genetic and plastic variation in abdominal pigmentation in *Drosophila immigrans* along an altitudinal gradient.

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Abstract

Climatic stresses impose strong natural selection that may cause rapid phenotypic changes in body melanization with correlated changes in desiccation resistance. In the present studies, three populations of a cosmopolitan but cold adapted species, *Drosophila immigrans,* were investigated for three ecophysiological traits (abdominal melanization, desiccation resistance, and cuticular water loss) from an altitudinal gradient (600 to 2202 meters) in the Shivalik range of the Himalayan region in northern India. Laboratory experiments with different rearing temperatures (15, 21, 25, and 28°C) helped in finding relationships between varying melanization and its resulting impact on desiccation and rate of water loss. The results showed that these traits are highly plastic in response to temperature and that this pattern is also consistent with the genetic changes over the altitudinal gradients. Local climatic conditions vary significantly with altitude and exert differential selection pressures on phenotypic traits. Seasonal changes in temperature as well as humidity (*T*cv and RHcv) of sites of origin of populations help to explain observed changes in various quantitative phenotypic traits in altitudinal populations of *D. immigrans*. Present investigations suggest a role of body melanization in maintaining the thermal balance as well as water balance in altitudinal populations of *D. immigrans*.

Introduction

Insects are unique in diversity, abundance, and in conquering diverse habitats (Louw, 1993; Willmer *et al.,* 2005). Melanin patterns are involved in diverse aspects of ectothermic insect ecology (Majerus, 1998; Rajpurohit *et al*., 2008). Several field and laboratory studies have proposed that melanization patterns in ectothermic insects play a role in thermoregulation (Watt, 1968; Brakefield and Willmer, 1985; Jacobs, 1985; Goulson, 1994; de Jong *et al*., 1996; de Jong and Brakefield, 1998; Majerus, 1998); camouflage (Majerus, 1998; Cloudsley-Thompson, 1999); resistance to pathogens (Wilson *et al.,* 2001); and sexual selection (Majerus, 1998). There are a few investigations on the ecological significance of pigmentation polymorphism in *Drosophila,* whereas there is substantial heterogeneity in pigmentation patterns (color, stripes, spots etc on abdominal tergites) among species groups as well as within species subgroups in drosophilids, *e.g*., uniformly dark coloration in the obscura group; stripes and/ or spots in quinaria, cardini, and other species groups (Hollocher *et al*., 2000 a, b; Llopart *et al*., 2002; Wittkopp *et al.,* 2003; Brisson *et al.,* 2005). Pleiotropic effects of melanism that might be associated with other physiological traits (desiccation resistance and cuticular water loss) have received less attention. Brisson *et al*. (2005) reported a significant relationship of habitat type (open *versus* forest *versus* open forest) with average abdominal pigmentation phenotypes of *Drosophila polymorpha* collected from several localities in Brazil. Further available reports do not show any evident consensus on the ecological significance of pigmentation polymorphism in *Drosophila* (see True, 2003).

For ectothermic organisms like *Drosophila*, temperature is a most important factor of the environment (Morin *et al*., 1997). It has been observed that the same genotype reacts to different environments in different ways to give rise to different phenotypes. Several studies in insects concern the rate of water loss in laboratory selected strains for desiccation resistance, but such analyses have not been considered in the context of body melanization (Hoffman and Parsons, 1993; Gibbs *et al.*., 1997). Field data (de Jong and Brakefield, 1998; Ellers and Boggs, 2002) suggest that darker individuals are better adapted to colder as well as drier habitats at quite high elevational localities. By contrast, lighter phenotypes prevail in the foothills. There is ample data to support such differences on the basis of the thermal budget hypothesis, but the possible correlated effects of melanism are largely awaited. On the basis of within and between population variations, body melanization has been shown to impact desiccation resistance (Parkash *et al*., 2008a, b, c; Parkash *et al*., 2009), but the impact of growth temperatures on different physiological traits have not been considered so far. Melanic flies are expected to have tighter crosslinking of the cuticular proteins which would make it less permeable (Walter *et al*., 1991; Wittkopp *et al*., 2002). Since the ambient temperature is inversely correlated with elevation and if there is a possible pleiotropic link of pigmentation with other physiological traits (desiccation resistance and cuticular water loss), rearing populations at different growth temperatures can result in significant correlations.

In the present investigation, an attempt has been made to analyze the effect of a range of rearing temperatures on different physiological traits in altitudinal populations of *Drosophila immigrans*. I maintained the flies from lowland, mid land, and high land populations at constant temperatures (15, 21, 25, and 28°C) and compare genetic and plastic effects. Populations exhibit substantial quantitative variation in abdominal melanization and desiccation resistance within as well as between altitudinal localities. The present paper describes the results obtained for the first time in *D. immigrans* on the effect of growth temperatures on the abdominal pigmentation with correlated changes in desiccation resistance. Genetic and plastic changes in body melanization are negatively correlated with cuticular water loss. It may be inferred from the results that growth temperature indeed affects the abdominal pigmentation and suggests possible adaptive pleiotropic effects of melanization in relation to water balance in *D. immigrans*.

Material and Methods

Measures of body melanization

Pigmentation patterns were stable since emergence but were analyzed in six days old adults. There is no sexual dimorphism in the pigmentation patterns in *D. immigrans*. Estimates of abdominal pigmentation were obtained through visual inspection as described by Singh *et al.* (2009).

Desiccation resistance

For measuring desiccation resistance, after scoring segment-wise pigmentation for each isofemale line, ten individuals were isolated in a dry plastic vial closed with a plastic cap. These vials contained 4 gm of silica gel at the bottom of each vial and covered with a disc of plastic foam piece. Four such replicates were run for each isofemale line (n = 40 for males and females). The vials were inspected every hour, and the numbers of dead flies (completely immobile) were recorded. As the numbers of dead approached half, vials were inspected after every 30 minute interval until all the flies died. Such experiments were run for all the altitudinal populations of *D. immigrans*.

Water balance analyses

Rate of cuticular water loss in live flies due to short-term desiccation (2 hr to 10hr) was standardized in groups of five flies. Repeatability of this assay was ensured before analyzing populations. Both before and after desiccation, groups of five flies of one sex were weighed on a microbalance, and weight loss (expressed as the percentage of initial wet body weight) represented cuticular water loss rate

Effects of four growth temperatures for rearing the offspring

Effects of rearing temperatures (15, 21, 25, and 28°C) were analyzed in three populations (one each from low, mid, and high altitude) for body melanization, desiccation resistance, and cuticular water loss. These assays were done on mass cultures maintained at 21°C in the laboratory. Thirty pairs per population were randomly selected to oviposit in eight vials in successive 24 hr periods. Five vials were randomly assigned to one of the four growth temperatures for rearing the offspring. Six days old adults were simultaneously analyzed for the physiological traits.

Statistical analyses

For all the traits, isofemale line means (n = 10) and population means (n = 10 x 10) along with S.E. were used for illustrations and tabular data. Measures of abdominal pigmentation were regressed against altitude of origin of populations. Standardized values for each trait were used to compare slope values. For trait variability analysis, ANOVA helped in comparing F values and their percent variation contribution. The climatic data for each collection sites were obtained from climatological data book published by Indian Meteorological Department, New Delhi (Table 1). In order to find a possible link between altitudinal trait variability of physiological trait with climatic conditions (T_{cv} and RH_{cv}), simple regression analysis was attempted. The usual correlations with family means, simple regression analysis, and all other statistical and graphical operations were done with the help of Statistica software.

Table 1. Data on geographical and climatic variables for the three-altitudinally varying sites of the origin of populations of *Drosophila immigrans*.

Collection sites	Climatic variable				
(altitudes)	T_{max} (°C)	T_{ave} (°C)	T_{cv} (°C)	RH (%)	RH_{cv} (%)
Kalka (600)	22.00	15.00	15.00	68.50	3.00
Solan (1440)	16.20	11.10	19.72	55.50	6.75
Shimla (2202)	11.45	7.62	30.64	45.80	12.95

Table 2. Data on population means (± S.E.) for three physiological traits-pigmentation score, desiccation hours and % cuticular water loss/ hr (in both sexes) for three altitudinally varying populations of D. immigrans.

Population (Altitude)	Pigmentation score		Desiccation resistance		% Cuticular water loss/hr	
	Male	Female	Male	Female	Male	Female
Kalka (600)	13.70 ± 0.18	15.70 ± 0.29	11.80 ± 0.10	14.10 ± 0.12	3.06 ± 0.02	2.72 ± 0.06
Solan (1440)	20.30 ± 0.48	23.00 ± 0.15	14.80 ± 0.12	17.50 ± 0.20	2.50 ± 0.07	2.19 ± 0.03
Shimla (2202)	27.00 ± 0.18	28.40 ± 0.18	17.80 ± 0.12	20.60 ± 0.10	2.05 ± 0.02	1.65 ± 0.04

Basic data on population mean values (± S.E.) for different physiological traits are given in Table 2. There is substantial variability for all the traits in three altitudinal populations of *D. immigrans*. Desiccation resistance and body melanization demonstrate regular clinal increase along altitude whereas reverse trend occurs for rate of cuticular water loss. Females demonstrate slightly higher desiccation resistance (1.3 to 2.6 hr) and pigmentation score (1.5 to 2.5) as compared with males across populations. The cuticular water loss rate, however, was slightly but consistently higher in males as compared with females (Table 2). The overall change can be appreciated by a ratio of trait values to end populations along elevational increase, *i.e.*, except for the sum of pigmentation values which increase about two fold, for the other traits such as desiccation resistance and rate of cuticular water loss per hour; there are 1.5 fold increases across populations. Significant negative correlations of cuticular water loss/hr with pigmentation as well as desiccation resistance across populations and sexes point out possible link between these physiological traits.

Table 3. Data on slope values for variability in three physiological traits as a function of altitude of origin of populations. Data on each of the four growth temperatures was analyzed individually for comparison of altitudinal slope values.

Temperature (°C)	Pigmentation Score		Desiccation Resistance		% Cuticular water loss/ hr	
	Male	Female	Male	Female	Male	Female
15	0.0070 ± 0.0008	0.0085 ± 0.0007	0.0060 ± 0.00010	0.0070 ± 0.0004	-0.001 ± 0.00004	-0.001 ± 0.00002
21	0.0070 ± 0.0006	0.0080 ± 0.0012	0.0040 ± 0.00007	0.0050 ± 0.0003	-0.002 ± 0.00015	-0.001 ± 0.00008
25	0.0045 ± 0.0011	0.0055 ± 0.0011	0.0030 ± 0.00005	0.0040 ± 0.0002	-0.003 ± 0.00021	-0.002 ± 0.0001
28	0.0040 ± 0.0007	0.0050 ± 0.0006	0.0020 ± 0.00002	0.0020 ± 0.0001	-0.003 ± 0.0002	-0.002 ± 0.0001

Table 4. Regression analysis for altitudinal variations in three physiological traits as a function of climatic variables [coefficient of averages for winter months for temperature (Tcv) and relative humidity (RHcv)] of the site of origin of *D. immigrans* populations.

Trait	*Sex*	*Tcv*			*RHcv*		
		r	a	b***	r	a	b***
Pigmentation score	M	0.96	3.23 ± 0.98	0.78 ± 0.09	0.97	11.42 ± 0.96	1.14 ± 0.11
	F	0.96	5.34 ± 0.91	0.77 ± 0.08	0.97	13.37 ± 0.91	1.12 ± 0.11
Desiccation resistance	M	0.95	7.08 ± 0.98	0.37 ± 0.04	0.98	10.79 ± 0.28	0.54 ± 0.03
	F	0.94	8.49 ± 1.21	0.39 ± 0.05	0.97	12.52 ± 0.51	0.58 ± 0.06
% Cuticular water loss/ hr	M	-0.95	3.89 ± 0.19	-0.06 ± 0.009	-0.98	3.26 ± 0.07	-0.09 ± 0.008
	F	-0.95	3.65 ± 0.19	-0.07 ± 0.009	-0.98	2.97 ± 0.05	-0.10 ± 0.006

All values for r and b are significant at $p < 0.001$.

Impact of growth temperature on physiological traits

To find a possible link between physiological traits (abdominal melanization, desiccation resistance, and cuticular water loss), populations from contrasting elevations (low, mid, and high) were simultaneously analyzed at four different growth temperatures. Since the ambient temperature is negatively correlated with elevation and if there is a possible link between three physiological traits, rearing populations at different growth temperatures (15, 21, 25, and 28°C) can result in significant correlations. The regression analysis of each population across the thermal range resulted in slope values which vary for the three populations and evidence genotype and environment interactions or genetic reactivity of trait slope values for temperature changes (Figure 1). Abdominal melanization was always significantly higher at low growth temperature then higher growth temperature and vice versa, and there are significant correlations between physiological traits. The same data was further analyzed at a particular growth temperature and the resulting slope values

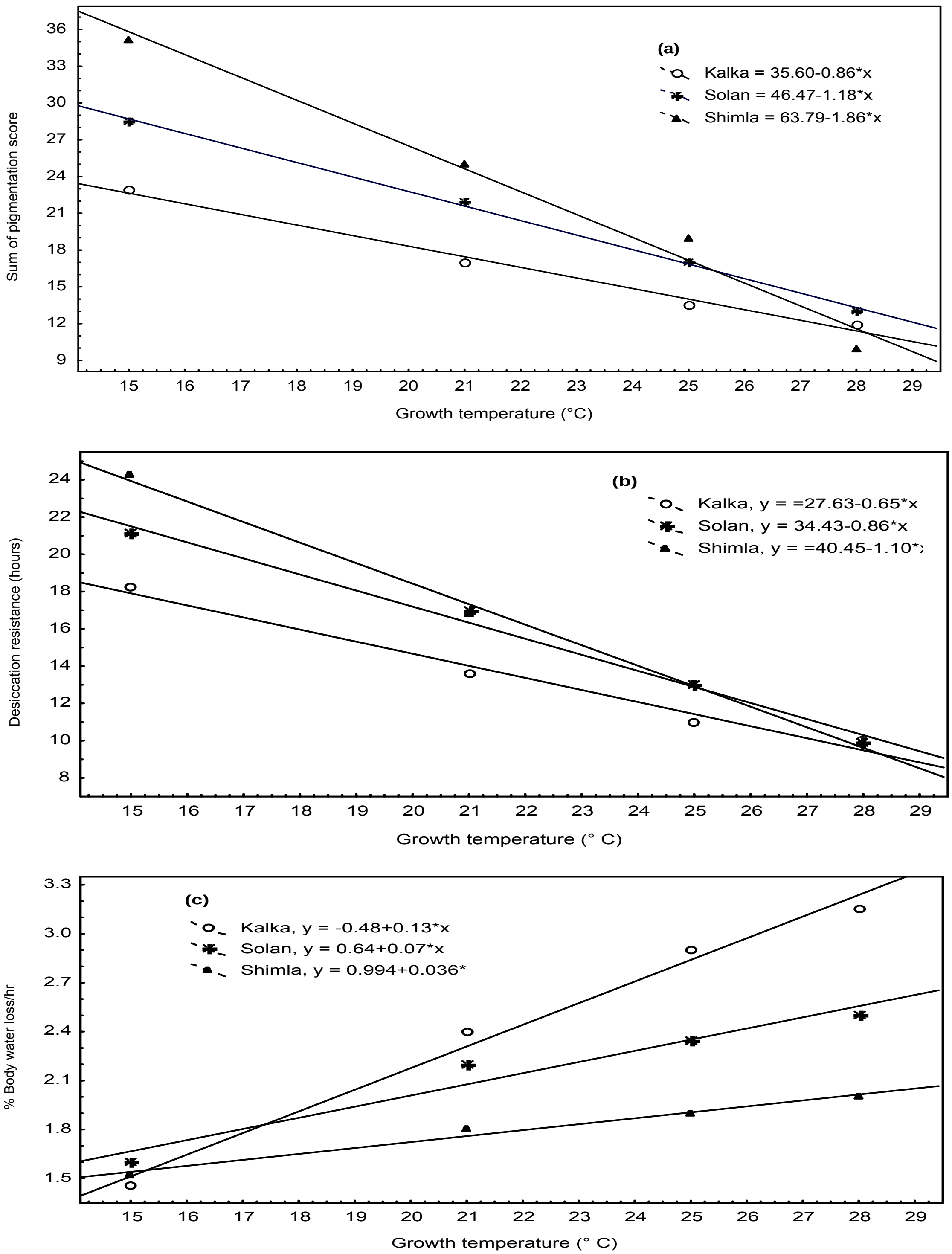

Figure 1. Regression analyses of three physiological traits (a: pigmentation score; b: desiccation resistance; c: % cuticular water loss/hr) as a function of four growth temperatures (15, 21, 25, and 28°C) in three altitudinal populations of *D. immigrans.*

differ significantly for flies reared at 15°C *versus* those grown at 28°C (Table 3). Different intercepts as well as slope values in populations grown at different temperatures result due to genetic as well as environmental effects. Data were further subjected to ANOVA in order to find effects due to populations and temperature (data not shown). As expected, effects due to growth temperatures were significant (65-77%) for all the three physiological traits. But effects due to populations, only from three contrasting altitudes, were 1.5 times higher for abdominal pigmentation (30%) as compared with desiccation and cuticular water loss (15 to 18%). The variations due to sexes was higher (~3%) for desiccation and cuticular water loss as compared with pigmentation. Since only five lines per population were analyzed, line effects as well as interaction effects were lower.

Impact of climatic variables on physiological traits

Different montane populations of *Drosophila immigrans* experience seasonal changes in temperature as well as relative humidity [*i.e.*, T_{cv} and RH_{cv} increased two- and fourfold, respectively (Table 1)]. In order to find a possible link between altitudinal trait variability of physiological traits with climatic conditions (T_{cv} and RH_{cv}), we attempted simple regression analysis (Table 4). The mean monthly coefficient of variation of temperatures (T_{cv}) and relative humidity (RH_{cv}) gave significant positive slope values for pigmentation and desiccation resistance, which were identical between the sexes of both traits, whereas reverse trend was observed for cuticular water loss. Such analysis helped in explaining the evolving altitudinal trends in physiological traits on the basis of seasonal variations in temperature and relative humidity. Thus, physiological traits in *Drosophila immigrans* respond to selection pressures and evidence climatic adaptations.

Discussion

Altitudinal gradients are characterized by rapid changes in the physical environment (Dillon and Frazier, 2006). Wild populations of diverse ectothermic insect taxa can cope with such varying climatic conditions through phenotypic plasticity, which allows a single genotype to produce different phenotypes (Parkash *et al.*, 2009). Quantitative traits (body melanization and desiccation resistance) are controlled by polygenes and vary due to interactions of environmental factors with genetic attributes (Falconer and Mackay, 1996). The insect cuticle is an important interface between physiological systems and the environmental conditions (Neville, 1975). The mechanistic basis of desiccation resistance has been reported in altitudinal as well as latitudinal populations of *Drosophila* species (Parkash *et al*., 2008a, c); however, so far there are no data on temperature effects to support such contentions. Slope values for the populations grown at four different growth temperatures (15, 21, 25, and 28 ºC) were compared in order to test genetic as well as plastic effects. Changes in slope values for quantitative traits across temperatures are expected due to plastic responses. By contrast, lack of differences in slope values would suggest the absence of plastic effects for quantitative traits. In the present studies, slope values for physiological traits (abdominal melanization, desiccation resistance, and cuticular water loss) differ significantly for flies reared at 15ºC *versus* those grown at 28ºC. Different intercepts as well as slope values in populations grown at different temperatures result due to genetic as well as environmental effects. Thus, in montane localities, phenotypic plasticity for body melanization in *D. immigrans* leads to better adaptation under colder and drier conditions.

In ectothermic insect taxa, the role of body melanization in the thermoregulation has been demonstrated in (a) field studies, *i.e.*, evidence of latitudinal clinal variation for melanics in ladybird beetle (*Adalia bipunctata*), by Brakefied and colleagues in The Netherlands (de Jong and Brakefield, 1998) and in *Colias* butterflies in the USA (Ellers and Boggs, 2002); (b) in laboratory studies by experimental manipulation of butterfly wing colour with black marker and exposing to solar radiation (Ellers and Boggs, 2004). Such evidence in favour of thermal melanism has been reviewed by Clusella-Trullas *et al.* (2007) and Majerus (1998). Such investigations on wild populations of *Drosophila* species and populations have, however, received less attention. Present results on heritable elevational increase in melanization of abdominal segments in *D. immigrans* are in agreement with the hypothesis that black body surfaces better absorb solar radiation in order to maintain optimum body temperature under colder ambient temperatures. A disadvantage of being darker is that the animal may overheat more easily, but this is often compensated by behavioral mechanism, as in the firebug (Honek, 1986). Melanic flies are expected to have tighter cross linking of the cuticular proteins which would potentially makes it less permeable (Walter *et al.*, 1991; Wittkopp *et al.*, 2002). So transpiration through the cuticle will be more where the body surface is lighter, whereas a darker body surface helps in low transpiration through the cuticle, reducing the rate of water loss and hence increasing desiccation resistance. In the present studies, there is a significant reduction in the cuticular water loss along the elevational transect, suggesting that, at high altitudes, due to lower rates of water loss, flies survive significantly longer in desiccating conditions and vice versa. Further, the association between desiccation and rates of water loss is confirmed by rearing the three contrasting populations at four different growth temperatures. It may be inferred that the rate of water loss and desiccation are associated mechanisms and along the elevational transect, increase in desiccation being a consequence of selection on melanization.

In montane habitats with increasing elevations, ectothermic organisms cope with colder and drier conditions. At low ambient temperatures, the water content of the ambient air is reduced and increased wind speeds also add to the dehydrating effects. Smaller drosophilids, having a greater surface area to volume ratio, are highly vulnerable to dehydration. Several investigations have considered interspecific differences in desiccation resistance with mechanistic link to the problems of water balance (Zachariassen, 1996; Gibbs *et al.,* 1997; Hoffman and Harshman, 1999; Addo-Bediako *et al.,* 2001). But similar studies on the intraspecific level are limited (Eckstrand and Richardson, 1981). The present study reveals that montane populations of *D. immigrans* from high altitude locations survive desiccating conditions significantly longer than the low altitude populations.

In conclusion, analysis of *D. immigrans* populations from low to high altitude localities demonstrates that phenotypic variability for quantitative traits result from genotype-environment effects. Significant genetic and plastic effects were observed by comparing slope values for flies reared at four different growth temperatures. Melanism is a likely candidate for cuticular impermeability for reducing dehydrating conditions along an altitudinal transect. The adaptive role of plastic changes described here has not been reported previously. The analysis of climatic factors has shown that seasonal variations in temperature and humidity (T_{cv} and RH_{cv}) can be responsible for maintaining genetic heterogeneity in physiological traits (melanization, desiccation resistance, and cuticular water loss) in *Drosophila immigrans* and evidence climatic adaptations. Further investigations are needed in several species and populations of drosophilids and other insect taxa in order to establish such pleiotropic or correlated effects of melanism.

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Effect of colored light on average weight in *D. melanogaster* iso-female strains.

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The object of the present experiment was to study the effect of three different colored lights (white, blue, and red) on weight in iso-female lines of *D. melanogaster*.

Experimental Procedure

One vial of *D. melanogaster* was purchased from Carolina Biological Supply Company (CBSC), North Carolina in September 2009. It was grown at room temperature (19°-21°C) using the fly medium (formula 4-24 plain) supplied by the CBSC. Several iso-female lines were derived from this strain by placing one fertile female/vial. However, only seven iso-female lines (numbered as genotype: 1, 2, 3, 4, 5, 6, and 7) were used for the experiment. The F_1's and F_2's within each strain were made. Each of the seven parental lines, the F_1's and F_2's made within each line were tested under three different colored lights (White: 25W; Blue: 25W; and Red: 25W). Eight to ten males and eight to ten females were used for the experiment under each colored light. The fly medium/vial varied from 10-12 ml. The temperature ranged from for: **white** light (20°-21°C); **blu**e light (20°-21°C); and **red** light (19°-20°C). The total number of males and females were counted, recorded, and