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ABDOMINAL PIGMENTATION VARIATIONS IN DROSOPHILA IMMIGRANS: ADAPTATION TO ALTITUDINALLY VARYING ENVIRONMENTS.

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ABSTARCT:
In the present studies, six populations of a cold adapted species, *Drosophila immigrans*, were investigated for ecophysiological traits (abdominal melanisation, desiccation resistance and cuticular water loss) and reproductive fitness related traits (copulation duration and rate of fecundity). Changes in body melanization significantly correlated with desiccation stress. Populations with increasing melanization display better reproductive success (in terms of longer duration of copulation and increased rate of egg production). Climatic conditions (temperature and humidity etc.) vary significantly along altitude and exert differential selection pressure on phenotypic traits. Tcv (seasonal thermal amplitude) of sites of origin of populations help to explain observed changes in various quantitative phenotypic traits in altitudinal populations of *D. immigrans*. Such observations are in agreement with thermal budget hypothesis and result in reproductive success under colder environments. Present investigations suggest role of body melanisation in maintaining thermal balance and reproductive success in altitudinal populations of *D. immigrans*

Keywords: *abdominal melanisation, reproductive fitness, duration of copulation,.*

1. INTRODUCTION

Insects are unique in diversity, abundance and in conquering diverse habitats including extreme types [Louw (1993); Willmer *et al. (*2005)]. Numerous investigations concern how insects cope with challenges posed by aquatic, mesic and xeric habitats [Edney (1977); Hadley (1994); Chapman (1998)]. By contrast, there are limited studies on physiological adaptations in insects living in montane habitats which pose problems related to temperature, water availability and atmospheric pressure [Mani (1968); Lee and Denlinger (1991); Willmer *et al.* (2005)].

A major concern of evolutionary physiologists is the understanding of how ecophysiological features have arisen as an outcome of natural selection [Willmer (1982); David *et al.* (1983); Willmer *et. al.* (2005)]. Several field and laboratory studies have proposed that melanisation 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)]; and resistance to pathogens [Wilson *et al.* (2001)]. 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 obscura group; stripes and/ or spots in quinaria, cardini and other species groups [Hollocher *et al.* (2000 a and b); Llopart *et al.* (2002); Wittkopp *et al.* (2003); Brisson *et al.* (2005)]. Pleiotropic effects of melanism that might be associated with desiccation resistance have received lesser attention. Brisson *et al.* (2005) reported significant relationship of habitat type (open Vs forest Vs 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 [see, True (2003)].

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 melanisation [Hoffman and Parsons (1993); Gibbs et al (1997)]. Field data [de Jong and Brakefield (1998); Ellers and Boggs (2002) suggests 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 thermal budget hypothesis but the possible correlated effects of melanism have not been considered so far. We hypothesized that melanism may help in reducing water loss and thereby conferring desiccation resistance under colder as well as drier habitats. Since the ambient temperature is negatively correlated with body melanization and if there is 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.

Another aspect on possible pleiotropic effects of melanism in diverse insects (lepidopterans and coleopterans) concerns reproductive success due to thermal melanism [de Jong *et al.* (1998b)] but such information is lacking in Drosophilids. Melanic individuals of ladybird beetle benefit from increased mating success and earlier emergence times during spring [Brakefield (1984 a & b)]. In *Adalia bipunctata* (coleopteran) and *Ephestia Kuhniella* (lepidopteran), melanics display higher mating success as compared with typicals [de Jong *et al.* (1998b); Verhoog *et al.* (1998)]. However, in view of widespread genetic variability of abdominal melanisation in diverse *Drosophila* species, data on correlated or pleiotropic effects of melanism on ecophysiological traits and fitness consequences are largely awaited. To find a possible link between body melanization and reproductive traits we hypothesized that if laboratory selected strains for higher melanization have increased reproductive fitness, and if within population differences in assorted groups of body melanization (darker and lighter phenotypes) have significant parallel changes in reproductive traits, then body melanisation may have an adaptive role in conferring reproductive success under colder and drier habitats.

In the present investigation, an attempt has been made to explore a possible link between abdominal pigmentation and other physiological traits (desiccation resistance and cuticular water loss) and a behavioral trait (reproductive success) in altitudinal populations of *Drosophila immigrans*. Populations exhibit substantial quantitative variation in abdominal melanization within as well as between altitudinal localities. The results show significant parallel increase in trait mean values as well as standard deviations for both body pigmentation and desiccation resistance as a function of altitudinal variation. Genetic and plastic changes in body melanization are negatively correlated with cuticular water loss. Longer copulation durations and higher rate of fecundity in darker individuals from higher altitudinal localities confer adaptive advantages for better survival under colder and drier environments. Present studies suggest possible adaptive pleiotropic effects of melanization in coping with water balance as well as reproductive success in *D. immigrans*.

2. MATERIAL AND METHODS

2.1 Field collections and maintenance:

Wild living adults of *D. immigrans* were collected from six altitudinal sites which were quite distant from each other and included two low altitudinal sites (600m to 761m); two mid (1211m to 1440m) and two high altitudinal localities (1939m to 2202m). All collections were made in a single trip in the months of December. From each collection site, about 60 to 75 individuals were obtained [during winter months (November to February), low to high altitudinal sites harbor more than 25% *D. immigrans* individuals] which were used to initiate 10 isofemale lines and a mass culture with 20 pairs per population. All experiments were conducted on the first and second laboratory generations of wild caught flies. The cultures were maintained at a constant growth temperature of 21°C. The use of high precision thermometers (76 mm, Immersion Zeal, U.K) helped in checking thermal fluctuations, if any. For density control, 15 well developed inseminated females from each line were placed for oviposition, into a mating chamber with a thin layer of food for 12 h and then discarded. Food plates were incubated at 21°C. After larval eclosion, 70 first instar larvae were carefully seeded, with dissecting needles, in culture vials with a high yeast *Drosophila* medium that provides sufficient nourishment during development. For all cultures, transfers were made with randomly selected forty pairs each

generation. For each population, isofemale lines were maintained as 3 to 4 replicates because experiments required simultaneous analysis of pigmentation and mortality based desiccation resistance.

Mass cultures of different populations were used to standardize experimental protocols for quantitative as well as qualitative analysis of abdominal pigmentation, desiccation resistance and experiments relating to mechanistic basis of water balance. These preliminary experiments helped in ensuring accuracy and repeatability for all subsequent experiments done simultaneously for various traits in replicated isofemale lines of each population. The methods followed in the present studies were modified from several investigators i.e. abdominal pigmentation [Martinez and Cordeiro (1970); Robertson *et al.* (1977); David *et al.* (1990); Machado *et al.* (2001)], desiccation and estimation of water balance parameters [Hoffman and Parsons, 1989; Gibbs *et al.*, 1997, Folk et *al.*, 2001)].

2.2 Measures of body melanisation:

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 the usual quantitative (i.e. calculation of pigmented area through micrometer) and qualitative method [visual inspection of the ratio of pigmented portion out of total size of each abdominal segment (2nd to 7th)]. For calculating abdominal pigmentated area, 10 pairs of six days old adult flies of both sexes per population were anaesthetized and after removing head and thorax, total abdominal tergites per fly (after removal of viscera through a ventral slit) were mounted on slides. The slides were subjected to measurement with a micrometer under strereozoom microscope (Olympus SZ 11 Japan). For each abdominal tergite (2nd to 7th) the pigmented area was calculated by measuring mid-segmental vertical elevation (height) of pigmented stripe and the total basal length of the respective abdominal segment [i.e. ½ (base x ht) x 2]. For calculating the total area of each abdominal segment, all the four sides of each abdominal segment as well as the diagonal length were measured. The sum of area of two resulting triangles represented the segment area. This was done due to unequal lengths and breadths of each abdominal segment. Since, the abdominal segments (2 to 7th) differ in size, the total area of each of these segments was transformed into relative sizes for the purpose of qualitative scoring i.e. 0.66, 0.88, 1.0, 0.88, 0.66 for 2nd to 6th segments respectively in both the sexes. However, the size of 7th segment differs between sexes and hence relative size was 0.22 for males and 0.33 for females.

Double blinded studies were conducted for the qualitative scoring of pigmentation and the criterion of high concordance between observed values was followed in order to minimize possible errors in visual inspection of the ratio of pigmented portion out of total segment size. Since the pigmented portion resembles two opposing triangles in each abdominal tergite, flies were scored laterally by taking means of mid elevation and lateral elevation for all segments. The pigmentation score out of ten for each segment was multiplied with its relative size in order to avoid biased estimates. Both methods gave a significantly high correlation ($r = 0.99$) for elevational populations of *D. immigrans* (Fig. 1). This helped in validating the qualitative method that handles live anaesthetized flies and is suitable for simultaneous analysis of other physiological traits on the same group of individuals. By contrast, the quantitative method, which is based on mounting of abdomens on the slides, cannot be used for analysis of other physiological traits, which require live individuals. Therefore, for most experiments on several physiological traits in the replicated isofemale lines per population, the qualitative method of pigmentation scoring was followed in the present studies.

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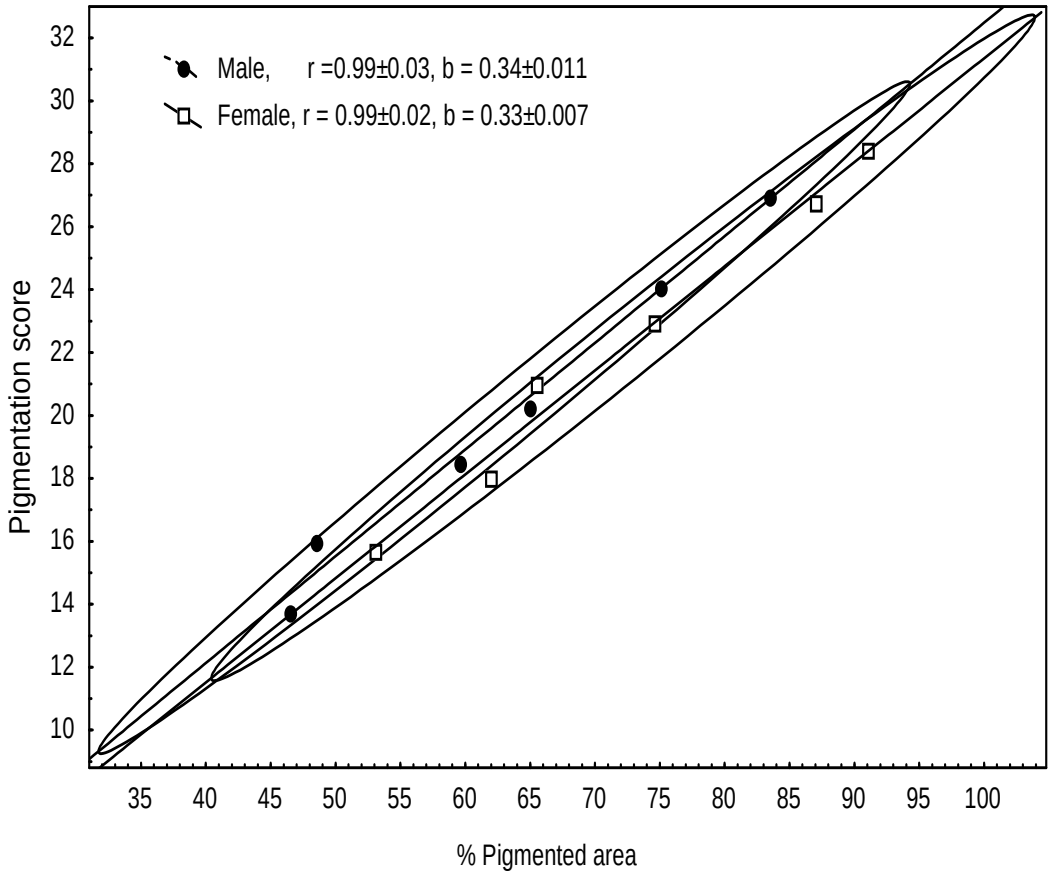

Fig 1: Correlation between pigmentation score and pigmented area of *D. immigrans* populations. For calculating the pigmented area of each fly, a ratio of the sum of pigmentation area of all the six abdominal tergites to total area of all the segments was considered. Population means of ratios were converted into percent data.

2.3 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 minutes intervals till all the flies died. Such experiments were run for all the altitudinal populations of *D. immigrans.*

2.4 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

2.5 Effects of growth temperature:

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Effects of rearing temperatures (15, 21, 25 & 28°C) were analyzed in three populations (one each from low, mid and high altitude) for 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. Two vials were randomly assigned to one of the four growth temperatures for rearing the offsprings. Six days old adults were simultaneously analyzed for the physiological traits.

2.6 Analysis of reproductive traits:

For three reproductive traits (copulation duration, ovariole number, and fecundity), five randomly chosen isofemale lines per population were analysed. All experiments were done in three to five replicates. Lines per population were again retested for their pigmentation and desiccation resistance in order to find significant deviation, if any. The randomly selected lines were in agreement with population means and deviations were not statistically significant. Copulation duration and fecundity were simultaneously analysed while data on ovariole number was obtained from the replicated isofemale lines per population. Total ovariole number (for both ovaries of 6 days old flies) was obtained by fixing the dissected ovaries in a saturated solution of potassium dichromate (n=5x5 per population). For each isofemale line, virgin females were collected and mated with virgin males in long test tubes (16 mm x 50 mm) plugged with cotton. All experiments were done in the morning hours 7 am, and in ten replicates per line. Duration of copulation was monitored with a stopwatch. Female fecundity was measured by placing a pair of virgin but 6 days old female and male in culture vials. Every two days, the flies were transferred to fresh culture vials and the eggs that had been laid were counted. Fecundity was monitored for two weeks. For each population, four replicates of five isofemale lines were used to obtain population means. Rate of fecundity was used to compare altitudinal populations varying in their ovariole number.

2.7 Statistical analyses:

For all the traits, isofemale line means (n=10) and population means (n=10x10) along with S.E. were used for illustrations and tabular data. Measures of abdominal pigmentation 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 slope values were statistically compared on the basis of 't' test. For statistical comparison, percent data was arcsine transformed. In order to find association between melanisation and desiccation resistance, trait variability (i.e. standard deviation of pooled isofemale line data per population) was investigated on the basis of regression and correlation analysis. The climatic data for each collection sites were obtained from climatological data book published by Indian Meteorological Department, New Delhi. The climatic conditions across these six altitudinal sites can be compared on the basis of changes in climatic variables per 150 meter rise in elevation (Table 1) i.e. T_{cv} and RH_{cv} increase by 1.5% and ~1.0% per 150m whereas T_{max} and $T_{average}$ decrease by 1°C and 0.7°C per 150m respectively. In order to find a possible link between altitudinal trait variability of physiological trait with climatic conditions (T_{max}, T_{min}, $T_{average}$, T_{cv}, RH and RH_{cv}) we attempted simple regression analysis. The usual correlations with family means, simple regression analysis and all other statistical and graphical operations were done with the help of Statistica software.

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

Climatic variable	Collection sites and their altitudes					
	Kalka (600)	**Mandi (761)**	**Kullu (1211)**	**Solan (1440)**	**Manali (1939)**	**Shimla (2202)**
T_{max} (°C)	22.00	21.20	17.00	16.20	14.40	11.45
T_{ave} (°C)	15.00	14.70	12.07	11.10	9.35	7.62
T_{cv} (°C)	15.00	17.44	18.30	19.72	27.55	30.64
RH (%)	68.50	65.00	57.50	55.50	48.50	45.800
RH_{cv} (%)	3.00	3.67	6.82	6.75	12.61	12.95

3. RESULTS

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 six altitudinal populations of *D. immigrans*. Desiccation resistance and body melanisation 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. However, the cuticular water loss rate 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 cutocular water loss/hr with pigmentation as well as desiccation resistance across populations and sexes point out possible link between these physiological traits. Elevational increases in desiccation resistance and rate of cuticular water loss were compared on the basis of population mean values as a function of altitude of origin of population of *D. immigrans* (Table 3). Correlation values for both desiccation resistance and rate of cuticular water loss/hr were highly significant. The slope values are quite similar between the sexes for both the traits (i.e. b=0.004±0.0002 for desiccation resistance and b=-0.001±0.00003 for rate of water loss) alongside significant levels of genetic determinant of coefficient of traits variability (R^2=0.98 for desiccation resistance and 0.99 for rate of water loss).

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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 six altitudinally varying populations of *D. immigrans.*

Population	Pigmentation score		Desiccation resistance		% Cuticular water loss/hr	
	Male	Female	Male	Female	Male	Female
Kalka	13.70±0.18	15.70±0.29	11.80±0.10	14.10±0.12	3.06±0.02	2.72±0.06
Mandi	15.30±0.49	18.00±0.30	13.00±0.20	14.30±0.20	3.00±0.01	2.67±0.07
Kullu	18.50±0.20	20.00±0.20	14.60±0.20	16.60±0.11	2.63±0.04	2.25±0.01
Solan	20.30±0.48	23.00±0.15	14.80±0.12	17.50±0.20	2.50±0.07	2.19±0.03
Manali	24.10±0.43	26.80±0.37	17.30±0.03	19.00±0.10	2.15±0.03	1.75±0.01
Shimla	27.00±0.18	28.40±0.18	17.80±0.12	20.60±0.10	2.05±0.02	1.65±0.04
Overall change	1.97	1.81	1.51	1.46	-1.50	-1.65

Table 3. Regression analysis of variability for three physiological traits as a function of altitude (in meters) of the origin of six populations of *D. immigrans*.

Trait	Sex	r (Correlation)	a (Intercept)	b (slope)	R^2
Pigmentation score	M	0.99	9.26±0.45	0.008±0.0003***	0.99
	F	0.99	11.31±0.60	0.008±0.0004***	0.98
Desiccation resistance	M	0.99	9.88±0.28	0.0037±0.0002***	0.98
	F	0.99	11.44±0.32	0.0039±0.0002***	0.98
% Cuticular water loss/ hr	M	-0.99	3.43±0.04	-0.00063±0.00003***	0.99
	F	-0.99	3.14±0.05	-0.00068±0.00003***	0.99

R^2= Coefficient of genetic determination of trait variability.

3.1 Parallel changes in melanisation and desiccation resistance:

Body melanisation in altitudinal populations of *D. immigrans* showed substantial within and between population variability demonstrated by isofemale line data (Fig. 2). Along elevational transect, trait mean values (Table 2) as well as standard deviations for body melanisation and desiccation resistance showed significant parallel increase (Fig. 3a). Genetic correlations between SD of body melanisation and desiccation hours of *D. immigrans* were highly significant (0.90-0.96) (Fig. 3b). The slope values for SD of both the traits as a function of altitude are quite similar (b~0.001) and demonstrate similar levels of trait variability.

In order to find association between abdominal melanisation and desiccation resistance, preliminary experiments indicated that wild male flies from mid altitudes (Solan), upon segregation into two groups of darker (21.8±1.0) and less darker flies (18.2±0.75) varied in their desiccation i.e. 16.0 hr Vs 13.6 hrs (data not shown). This was further confirmed by collecting male flies (n=40 to 60) in October and December from Solan i.e. there was significant phenotypic correlation between darker flies and higher desiccation (r=0.83) and also for less darker flies and lesser desiccation (r=0.78).

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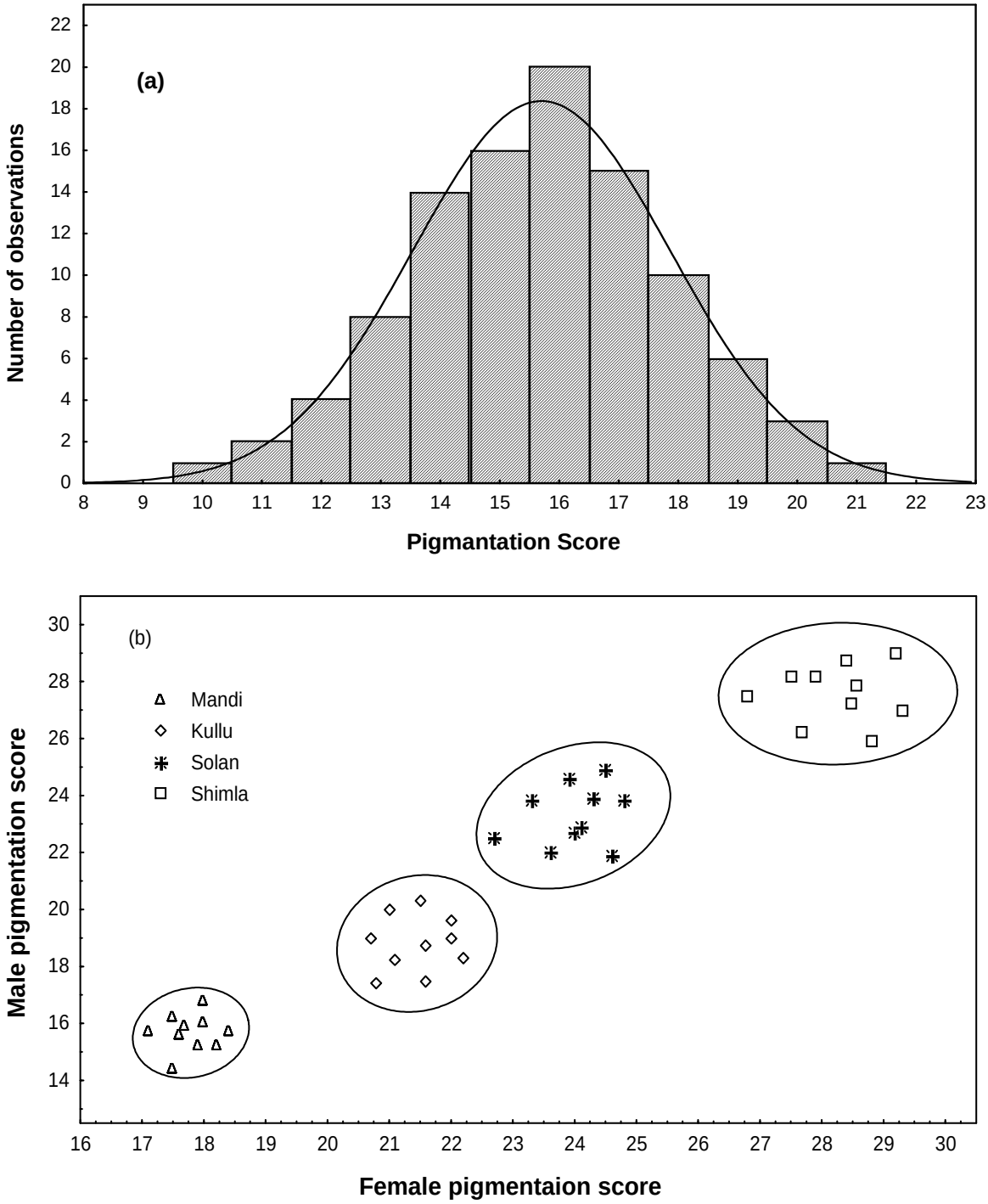

Fig 2: Histogram showing variability of overall abdominal pigmentation score per fly in laboratory population sample (a). Scatter diagram for demonstration of population variability for abdominal melanization in four altitudinal populations of *D. immigrans*. Each point indicates the mean value for 10 isofemale lines (b). Notice within and between population variability.

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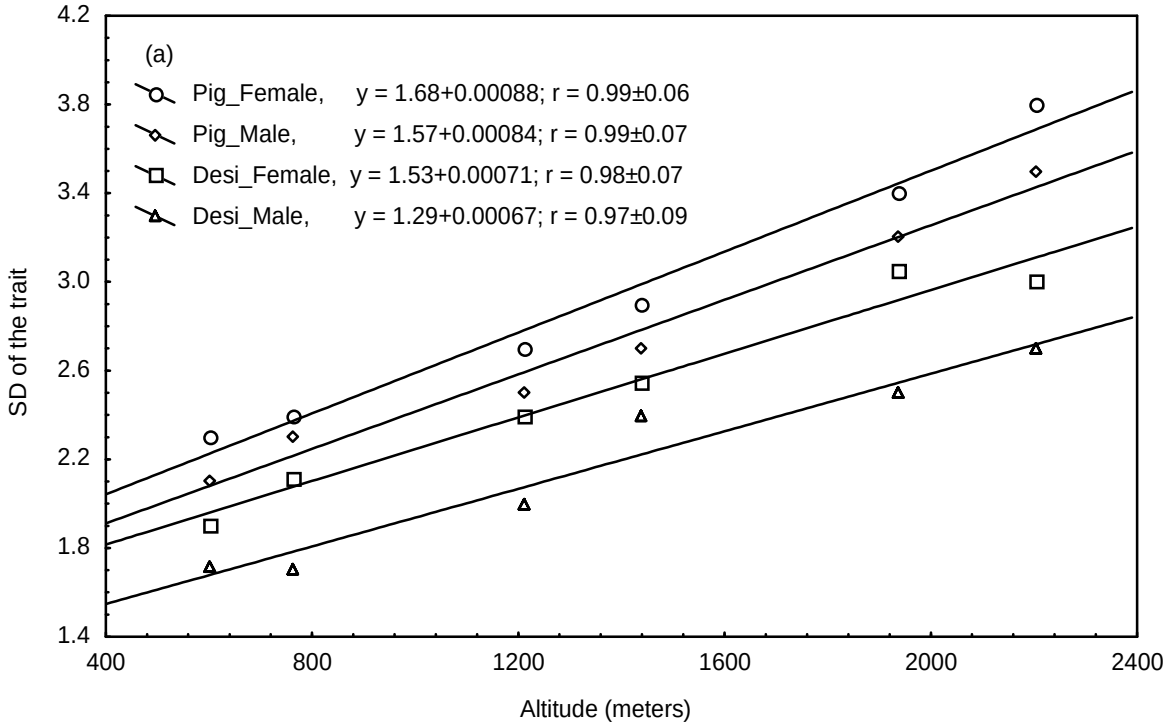

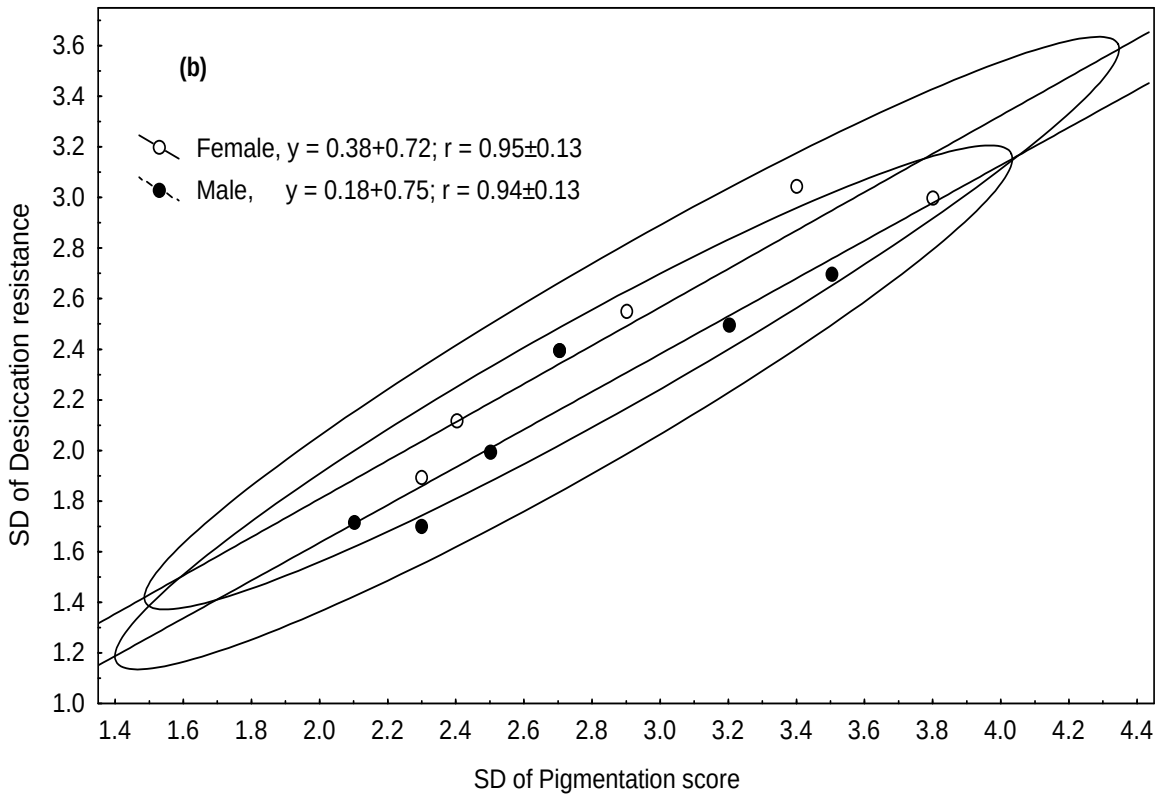

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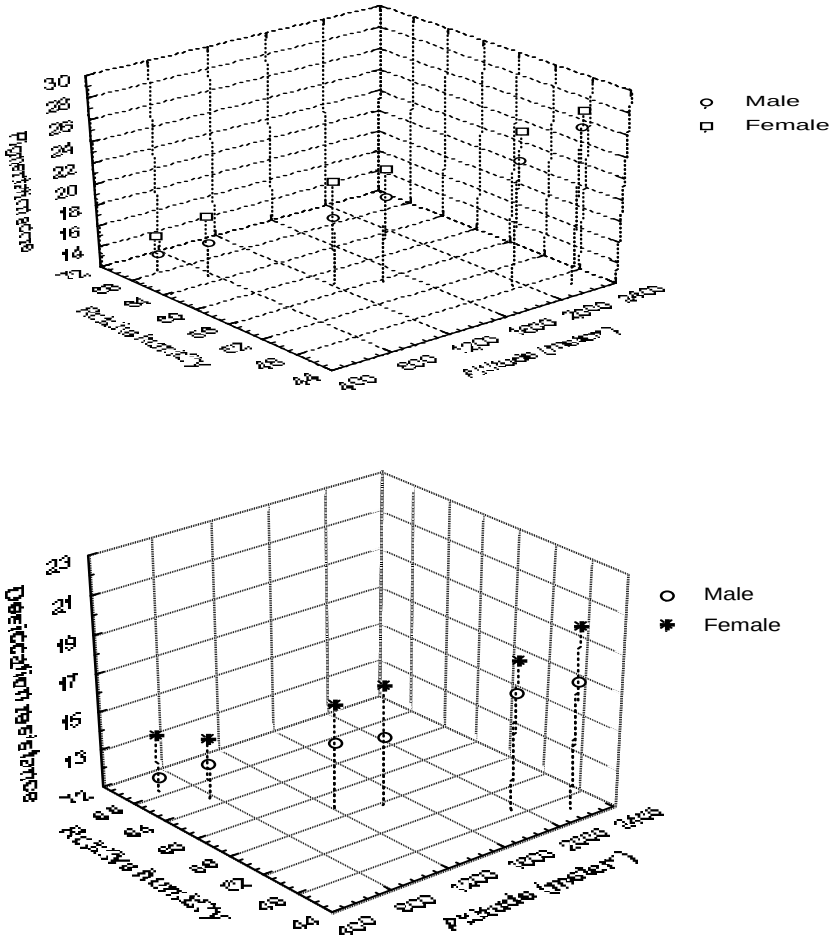
Fig 3: Regression analysis of elevational increase in trait variability (shown as S. D. = standard deviation) in both the sexes for body pigmentation and desiccation resistance (a). Covariance of S. D. for both the physiological traits demonstrates significant correlation (b). Ellipses of 90 percent probability are given.

3.2 Impact of climatic variables on desiccation resistance and rate of water loss:

In order to find a possible link between altitudinal trait variability of physiological traits with climatic conditions (T_{max}, T_{min}, $T_{average}$, T_{cv}, RH and RH_{cv}) we attempted simple regression analysis. T_{max}, T_{min}, $T_{average}$ and RH resulted in significant negative slope values for pigmentation and desiccation resistance, which were identical between sexes of both traits for the respective climatic variable whereas reverse trend was observed for cuticular water loss (data not shown). However, mean monthly coefficient of variations of temperatures (T_{cv}) and relative humidity (RH_{cv}) showed opposite trend i.e. significant positive slope values for pigmentation and desiccation resistance whereas significant negative slope values for cuticular water loss were observed. Simple regression analysis of trait variability as a function of altitude as well as RH resulted in significant slope values and negative correlation between the traits. Such inter-relationship has been illustrated in three-dimensional illustration (Fig. 4). Multiple regression analysis on the basis of one of temperature variable (T_{max}, T_{min}, $T_{average}$) and RH neither provide significant slope values nor coefficient of genetic determination of trait variability (r^2). However, T_{cv} and RH_{cv} alone (Table 4) as well as in combination gave significant results for all the three physiological traits. Such analysis helped in explaining the evolving altitudinal trends in physiological traits on the basis of seasonal variations in temperature and humidity.



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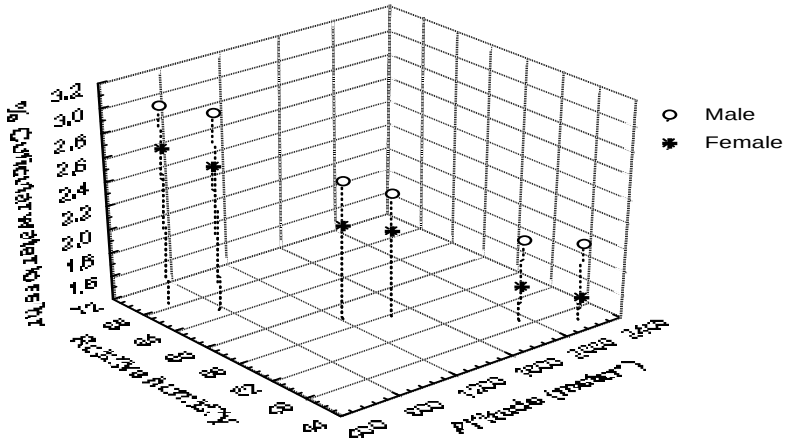

Fig 4: Three dimensional scatterplot illustrating trait variability [Pigmentation score (a); desiccation hours (b); and % cuticular water loss/hr(c)] as a simultaneous function of altitude and relative humidity of the site of origin of *D. immigrans* populations.

Table 4. Regression analysis in terms of slope (b) and R^2 (coefficient of genetic determination) 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^2	r	a	b***	R^2
Pigmentation score	M	0.96	3.23±0.98	0.78±0.09	0.92	0.97	11.42±0.96	1.14±0.11	0.94
	F	0.96	5.34±0.91	0.77±0.08	0.92	0.97	13.37±0.91	1.12±0.11	0.95
Desiccation resistance	M	0.95	7.08±0.98	0.37±0.04	0.91	0.98	10.79±0.28	0.54±0.03	0.97
	F	0.94	8.49±1.21	0.39±0.05	0.89	0.97	12.52±0.51	0.58±0.06	0.94
% Cuticular water loss/ hr	M	-0.95	3.89±0.19	-0.06±0.009	0.89	-0.98	3.26±0.07	-0.09±0.008	0.95
	F	-0.95	3.65±0.19	-0.07±0.009	0.91	-0.98	2.97±0.05	-0.10±0.006	0.98

All values for r, b and R^2 are significant at $p<0.001$.

3.3 Impact of growth temperature on physiological traits:

To find a possible link between physiological traits (abdominal melanisation, desiccation resistance and cuticular water loss), three populations from contrasting elevations (low, mid and high) were simultaneously analyzed at four different growth temperatures. Since the ambient temperature is negatively correlated with body temperature 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 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 (Fig. 5). Abdominal melanization was always significantly high at low 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 differ significantly for flies reared at 15°C versus those grown at 28°C (Table 5). Different intercepts as well as slope values in populations grown at different

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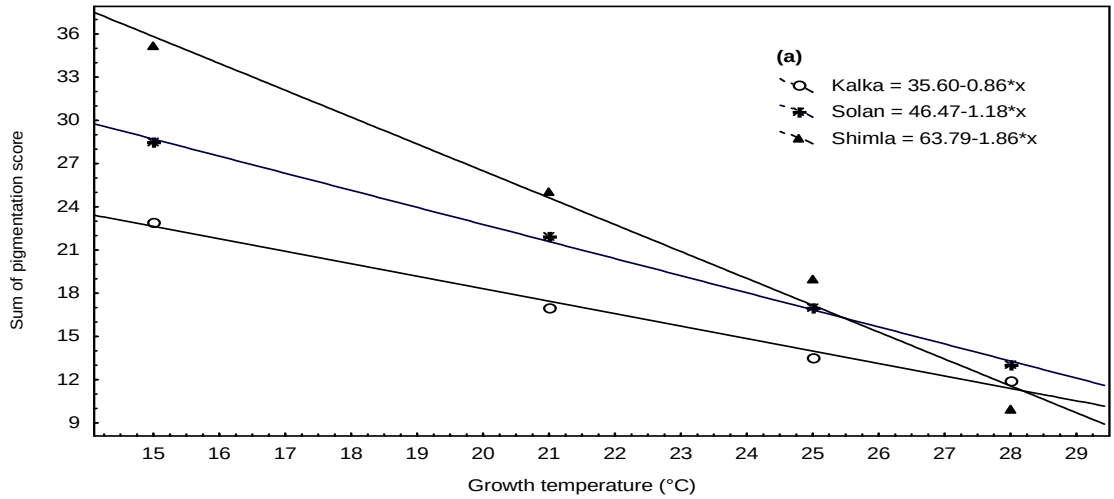
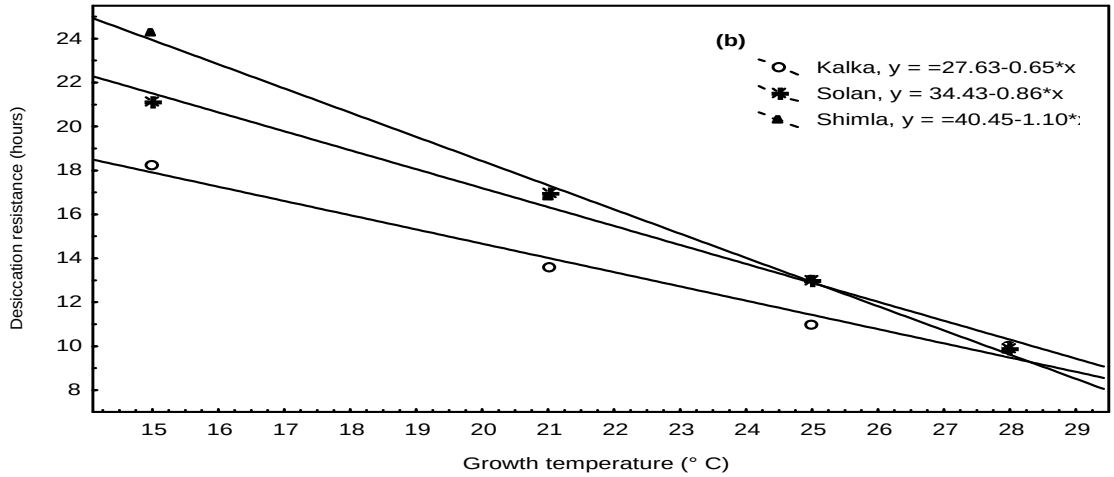
temperatures result due to genetic as well as environmental effects. Data was further subjected to ANOVA in order to find effects due to populations and temperature (Table 6). As expected, effects due to growth temperatures were significant (65-77%) for all the three physiological traits. However effects 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 populations were analyzed, line effects as well as interaction effects were lower.





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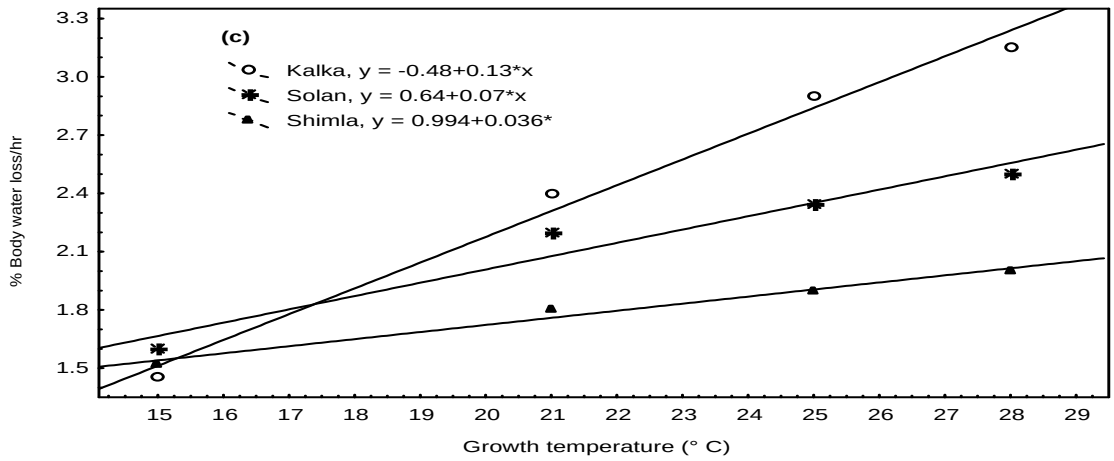

Fig 5: Regression analyses of three physiological traits (a: pigmentation score; b: desiccation resistance; c: % cuticular water loss/hr) as a function of four growth temperatures (15, 21, 25 and 28 °C) in three altitudinal populations of *D. immigrans.*

Table 5. 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

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Table 6: Data on analysis of variance (ANOVA) applied to test the variability due to populations (P), temperature (T), sex (S) and lines (L) for three physiological traits in populations grown at four growth temperatures.

Variable	d.f.	Pigmentation Score				Desiccation resistance				% Cuticular water loss/ hr			
		MS	F	P	% Var	MS	F	P	% Var	MS	F	P	% Var
P	2	11888.51	7766.9	***	29.79	4347.61	4767.44	***	18.05	752.415	86337.9	***	15.36
T	3	17333.88	11329.3	***	65.17	11634.36	12757.84	***	72.46	2519.915	289154.4	***	77.20
S	1	662.77	433.2	***	0.83	1195.14	1310.56	***	2.48	287.567	32997.6	***	2.93
L	4	39.08	25.5	***	0.20	114.57	125.63	***	0.95	0.239	27.4	***	0.009
P*T	6	427.22	279.2	***	3.21	323.56	354.81	***	4.03	54.580	6263.0	***	3.34
P*S	2	11.34	7.4	**	0.03	79.66	87.35	***	0.33	19.759	2267.3	***	0.29
P*L	8	4.63	3.03	***	0.04	25.74	30.58	***	0.46	14.222	1631.9	***	0.005
T*S	3	16.70	10.9	***	0.06	27.89	28.22	***	0.16	0.070	8.0	***	0.60
T*L	12	3.05	2.0	*	0.05	2.94	3.22	**	0.07	0.055	6.3	***	0.006
S*L	4	2.27	1.5	NS	0.01	1.11	1.21	NS	0.009	0.017	2.0	*	0.006
P*T*S	6	36.71	24.0	***	0.30	4.20	4.60	***	0.052	3.247	372.6	***	0.198
P*T*L	24	4.46	2.9	***	0.13	8.76	9.60	***	0.043	0.046	5.2	***	0.011
P*S*L	8	4.12	2.7	***	0.04	6.20	6.80	***	0.10	0.059	6.7	***	0.004
T*S*L	12	2.98	1.9	*	0.04	5.39	5.91	***	0.13	0.053	6.1	***	0.006
P*T*S*L	24	3.73	2.4	**	0.11	5.25	5.76	***	0.26	0.038	4.3	***	0.009
Error	1080	1.53	--										

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3.4 Abdominal melanisation and reproductive traits:

For analyzing fitness consequences of abdominal melanization, we analyzed ovariole number, rate of fecundity and copulation duration as a function of altitude as well as pigmentation score of respective sexes (Fig. 6). Across altitudinal populations, the overall change is about 1.5 fold for copulation duration as well as fecundity that are significantly correlated with pigmentation score (r=0.94; Fig. 6 c and d). Interestingly, the slope values for copulation duration, ovariole number and fecundity are significantly high i.e. 7 min $1000m^{-1}$ for copulation duration and 6 ovarioles $1000m^{-1}$ for ovaries, and 16 eggs $1000m^{-1}$ along elevational transect for *D. immigrans*. However the rate of fecundity has shown a slope value of 0.15 per 1000m. The basic data on ovariole number, copulation duration and fecundity from six altitudinal sites of *D. immigrans* was subjected to ANOVA (data not shown). For all the three traits, F-values were highly significant for populations, lines and interaction effects. Percent variations due to populations are 83.36% for fecundity while 51 and 54% for copulation period and ovariole numbers respectively. Line variation was lower (7%) for ovariole numbers and fecundity and quite high (28.54%) for copulation period.

Further, the association between abdominal melanization and reproductive traits was confirmed by assorting the flies into darker (H_1 and H_2) and lighter (L_1 and L_2) phenotypes from mid-altitude (Solan) populations (Table 7). Darker and lighter flies varied in their reproductive traits. The darker males (27.5±0.87) showed higher copulation duration (34.2±0.50) as compared with lighter males (13.4±0.77) which showed lower copulation duration (21.15±0.97) whereas the darker females (30.6±0.74) showed higher rate of fecundity (97.3±1.62) as compared with lighter females (17.05±0.63) which showed lesser fecundity (65.4±1.68). The ovariole number does not differ significantly among the control and selected lines. This is evident from the results that melanization is significantly correlated with reproductive traits. These results also indicate that copulation duration is a male related fitness trait whereas fecundity is female related fitness trait.

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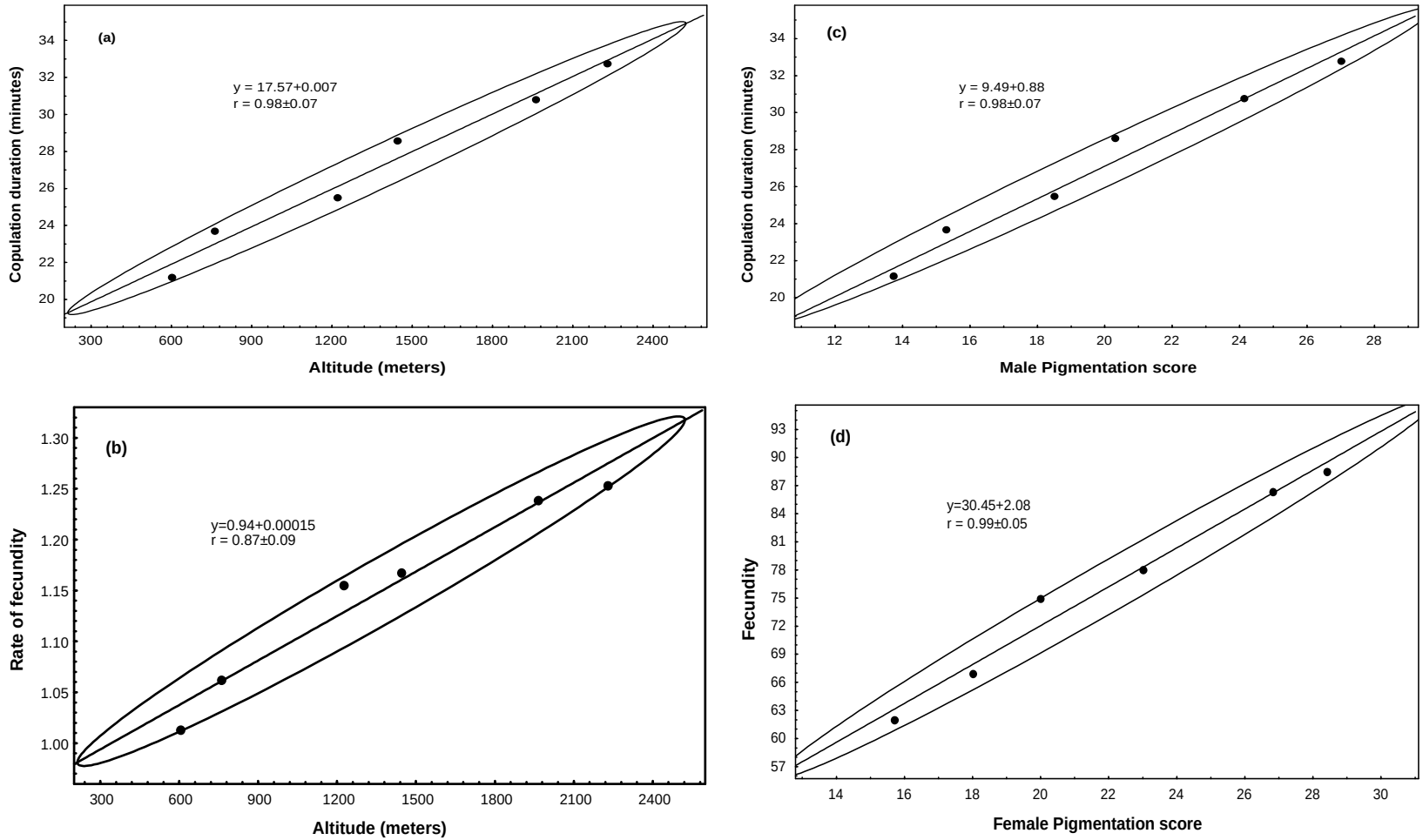

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Fig.6. Regression analysis of reproductive traits (copulation duration & fecundity) as a function of altitude (a & b); and pigmentation score (c & d). Ellipses of 90 % probability are shown.

Table 7. Data on pigmentation score and reproductive traits in control and selection lines derived from mid-altitude population of *D. immigran* from Solan (1440m).

	Pigmentation Score		Copulation duration (min)	Ovariole Number	Fecundity	Rate of fecundity
	Male	Female				
Control	21.30±0.50	23.00±0.60	28.80±0.20	65.00±0.30	80.00±0.70	1.23±0.02
H_1 (High)	26.90±0.60	30.20±0.80	33.80±0.20	66.40±0.60	96.00±1.10	1.44±0.02
H_2 (High)	28.10±0.70	31.00±0.70	34.60±0.30	65.60±0.50	98.60±0.50	1.50±0.06
L_1 (Low)	12.80±0.50	16.60±0.50	20.30±0.40	65.80±0.50	64.00±0.60	0.97±0.01
L_2 (Low)	14.00±0.40	17.50±0.40	22.00±0.25	64.60±0.40	66.80±0.90	1.03±0.02

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4. DISCUSSION:

In the present studies, altitudinal populations of *D. immigrans* demonstrate significant phenotypic variation in abdominal pigmentation within as well as between populations. The quantitative genetic basis of trait variability is evident from clinal changes in mean values as well as standard deviations. Thus, there is great deal of genetic variability for abdominal pigmentation in *D. immigrans.* Mean pigmentation scores for different populations of *D. immigrans* exhibit a clinal pattern along altitude. Because mean ambient temperature is inversely correlated with elevation, it is possible to explain the observed variation as the result of adaptation to local environments.

In ectothermic insect taxa, the role of body melanization in the thermoregulation has been demonstrated in (a) field studies i.e. evidences of latitudinal clinal variation for melanics in ladybird beetle (*Adalia bipunctata*), by Brakefied and colleagues in Netherlands [de Jong and Brakefield, (1998)[and in *Colias* butterflies [Ellers & Boggs (2002)]; (b) in laboratory studies by experimental manipulation of butterfly wing colour with black marker and exposing to solar radiation [Ellers & Boggs (2004)]. Such evidences in favour of thermal melanism have been reviewed by Clusella-Trullas *et.al.* (2007) and Majerus (1998). However, such investigations on wild populations of *Drosophila* species and populations have received less attention. Our 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 have tighter cross linking of the cuticular proteins, which potentially make it less permeable. So transpiration through cuticle will be more where the body surface is lighter whereas darker body surface helps in low transpiration through cuticle, reducing rate of water loss and hence increases 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. Our results provide evidence that the rate of water loss and desiccation are associated mechanisms and along elevational transect, increase in desiccation being a consequence of selection on melanisation.

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)]. However, similar studies on intraspecific level are limited (Eckstrand and Richardson, 1981). Present study reveals that montane populations of *D. immigrans* from high altitude locations survive desiccating conditions significantly longer than the low altitude populations.

In insects, physiologically controlled route of water loss are cuticular evaporative water loss (CEWL) and respiratory evaporative water loss (REWL). Several studies have shown that the REWL accounts for less than 10% of total water losses [Hadley (1994); Williams *et al.* (1997)]. Most investigations concern REWL rate in resting phase while losses could be more during flight or active phases. For estimation of cuticular water losses, several studies have used dead flies (after desiccation) but such data remain inconclusive due to the fact that losses are significantly high as flies approach death under desiccating conditions [Kimura *et al.* (1985); Hoffman and Parsons (1989); Graves *et al.* (1992)]. By contrast, determining water loss in live flies (through gravimetric methods or flow through respirometer) after shorter duration of exposure to desiccating conditions provides a better measure of water loss. Some investigations have reported interspecific variations in the rate of water loss in many *Drosophila* species from mesic and xeric habitats [Gibbs and Matzkin (2001)] but such data on intraspecific level has received less attention. In the present studies, desiccation resistance is significantly correlated negatively with water loss rate i.e. high altitude populations have evolved mechanism to reduce water loss under colder and drier conditions.

Insect cuticle is an important interface between physiological systems and the environmental conditions [Neville (1975)]. Numerous studies have considered the mechanistic basis of water balance under hot

and dry environmental conditions in diverse taxa of insects [Edney (1977); Louw (1993); Hadley (1994), Zachariassen (1996); Gibbs *et al.* (1997)]. Several investigations concern qualitative and quantitative changes in epicuticular lipids that have been shown to reduce cuticular water loss in ants, beetles and grasshoppers which live under xeric conditions [Hadley (1994); Gibbs (1998)]. The amount of cuticular wax varies with season i. e. in the hottest months. Wax blooms occur to reduce water loss in tenebrionid beetles [William *et al,(* 2005)]. By contrast, in *D. melanogaster*, it has been shown that significant differences in water loss can occur with little or no change in epicuticular lipids e.g. desiccation selected and control populations of *D. melanogaster* had similar amounts of epicuticular lipids [Graves *et al.* (1992); Gibbs *et al.* (1997)]. Analysis of a desiccation sensitive mutant (parched) in *D. melanogaster* demonstrated similar levels of epicuticular lipids in wild versus mutant but rate of water loss was quite high in the mutant [Kimura *et al*, (1985)]. By contrast the desert fruit fly (*D .mojevensis*) has more epicuticular lipids as compared with mesic fruit flies [Markow & Toolson (1990); Toolson *et al.* (1990)] and differences also occur in *D. preudoobscura* [Toolson & Kuper-Simbron (1989)]. In montane populations of *D. immigrans* epicuticular lipids do not differ quantitatively across populations and there is lack of correlation between water loss and epicuticular lipids [Ravi Parkash *et al.* (2008)].

Fitness consequences of melanism have been demonstrated on the basis of field as well as laboratory studies on melanics versus typicals in butterflies and beetles [Majerus (1998)]. In wild populations of ladybird beetle (*Adalia bipunctata*) from Netherlands, thermal melanism through influence on body activity results in earlier adult eclosion as well as reproduction of melanics as compared with typicals. Direct evidence of differential effects of solar radiation and thermal properties on melanics versus typicals has been demonstrated in butterflies [Roland (1982); Guppy (1986)] and in beetles [Brakefield & Willmer (1985)]. In flour moth (*Ephestia kuehniella*) the effects of genes controlling melanism resulted in significantly higher flight as well as walking activity in melanics than non-melanic genotypes [Verhoog *et al.* (1998)]. In ladybird bettle, *Coccinella septempunctata*, the melanic morphs with higher elytral pigmentation showed greater fecundity than lesser-pigmented individuals in relation with radiant heat level [Rhamhalinghan (1999)]. In *Colias eurytheme*, the artificially melanised females had a higher egg maturation rate under cold ambient temperatures [Ellers and Boggs (2004)].

Melanin synthesis and the enzymes involved are highly conserved in drosophilids as well as other insects [Kayser (1985)]. Genetic and developmental basis of pigmentation have been characterized in *Drosophila melanogaster* [Biessmann & Green (1986); Walter *et al.* (1996); Kopp *et al.* (2003)]. Mutations in some of the loci (yellow and Ddc cluster of loci which control melanisation) have shown pleiotropic effects on reproductive characters [Wright (1987); Stathtakis *et al.* (1995)]. Such mutants show a variety of modified mating behaviour (reduced courtship intensity, insufficient stimulation of the female through wing vibration and licking [Burnett and Wilson (1980); Wright (1987); Stathtakis *et al.* (1995)]. Thus, mutations in a majority of pigmentation loci affect female and/or male fertility. Pleiotropic effects of genes controlling melanization suggest that selection effects can cause incidental changes in reproductive physiology and/or mating behaviour. In the present studies, significant genetic correlations of duration of copulation as well as rate of fecundity with sum of pigmentation in altitudinal populations of *D. immigrans* support the conclusion that selection pressures affecting body melanization have correlated responses on reproductive traits. Higher duration of copulation as well as rate of fecundity constitute adaptive strategies for better survival under colder environments.

In conclusion, analysis of *D. immigrans* populations from low to high altitude localities for various physiological traits demonstrate that both desiccation and melanism have substantial genetic variability which is subjected to natural selection pressures under colder and drier conditions. Melanism is a likely candidate for cuticular impermeability for reducing water loss under increasing dehydrating conditions along an altitudinal transect. Our data is consistent with the increased desiccation being a consequence of selection on melanisation. The analysis of climatic factors have shown that seasonal variations in temperature and humidity (T_{cv} and RH_{cv}) can be responsible for maintaining genetic heterogeneity in three physiological traits related to thermal and water balance as well as reproductive traits related to fitness. 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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