Chapter 3

MATERIALS AND METHODS

Collections and cultures

The *Drosophila* species were collected from latitudinal (8.0 – 33 °N) as well as altitudinal sites (600 – 2262 m) of India. Seasonal collections were also done from altitudinal and northern sites. From each site 400 – 500 individuals were collected using bait trap and net sweeping method. Two types of cultures were set using these wild caught flies – (a) Isofemale lines: progeny of a single female and (b). Mass cultures: progeny of 40 – 50 pairs of individuals. All the cultures were maintained in laboratory on corn–meal-agar medium on appropriate temperatures (from 16 °C to 31 °C) according to experimental requirements. For all cultures, larval density was kept below 50 larvae per vial by limiting egg laying up-to 8 hours. Cultures were then analyzed for metric, stress related and life history related traits. Climatic data for the sites of origin of populations were obtained from Indian Institute of Tropical Meteorology (IITM; www.tropmet.res.in). Effects due to age, sex, anesthesia and ambient room temperature were controlled for all experiments. All cultures were maintained in the laboratory according to mid thermal range of a species (Fig. 1)

**Thermal Range for Development**

* • Cold adapted species (Temperate)

* • Generalist species (Temperate + tropical)

* • Warm adapted species (tropical)

**Most ectothermic organisms are only able to live within a certain temperature range**

**Fig. 1.** A schematic representation of thermal range for development of different *Drosophila* species
Culture media

The Drosophila flies were reared on Standard food medium which consists of the fixed proportion of different ingredients such as agar-agar, yeast and corn meal powder. To make the food the following ingredients are required:

- Water : 1400 ml
- Agar-agar : 15 g
- Yeast powder : 40 g
- Sugar/jaggery : 64 g
- Cornmeal powder : 72 g
  (Maize powder)

All the ingredients are mixed and cooked. After cooking the food medium fungicide (sodium paramethyl benzoate 1g and bactericide propionic acid 3ml) are added.

Metric traits

For both the sexes, wing length was measured from the thorax articulation to the tip of third longitudinal vein under Olympus SZ-11 microscope (Olympus, www.olympus.com) fitted with a micrometer. Ten adults of both the sexes from each line of eight populations were measured for thorax length from the anterior end of the thorax to the posterior end of the scutellum. Wing width was scored as the distance between distal tips of vein second and fifth. Wing area was estimated as wing length x wing width.

Melanisation

Body melanisation was measured in laboratory reared male and female individuals. Melanisation was estimated from dorsal view of the abdomen giving values ranging from 0 (no pigment) to 10 (complete darkness) for each of the six visible abdominal segments i.e. 2nd to 7th for females and 2nd to 6th for males. The flies were scored by two independent persons and a high correlation ($r > 0.96 \pm 0.02$) between two sets of observations ensured repeatability. Since the abdominal segments differ in size, relative sizes (i.e. 0.86, 0.94, 1.0, 0.88, 0.67, and 0.38 for 2nd to 7th segments, respectively, for both the species) were multiplied with segment wise pigmentation scores (Parkash et al., 2008a). The data on % melanisation were calculated as ($\Sigma$
observed melanisation scores of six abdominal segments per fly/∑ relative size of each segment x 10 per fly) x 100. The total body melanisation per fly was also estimated through Biowizard image analysis Software (Dewinter Optical Inc., www.dewinterindia.com).

![Scoring pattern for pigmentation](image)

**Figure**. Examples of phenotypic classes used to estimate pigmentation score of different abdominal segments (S1-S7) of *Drosophila* females. [Scale: 0 to 10]

**Analysis of plasticity**

Females were allowed to lay eggs at 21 °C. These eggs were transferred to different growth temperatures range for further development. Flies emerged from such eggs were examined for changes in total body color.

**Analysis of male wing spot plasticity**

For investigating variations in wing spot area (WSA) in *D. nepalensis* and *D. biarmipes*, females were allowed to lay eggs at 21 °C. Such vials were then transferred to 14, 17, 19.5 and 25 °C and the progeny were analyzed for plastic effects for WSA at these growth temperatures. To calculate wing spot area in males across different growth
temperatures (14, 17, 19.5 and 25 °C) was measured visually under stereo zoom microscope (Olympus, www.olympus.com) at 3× magnification. Wing spot area (%) per fly was also calculated through image analysis. For this purpose, the wing of each male fly at different growth temperatures was mounted on a slide which was subjected to Biowizard image analysis software - Dewinter Optical Inc. - (www.dewinterindia.com). The validity of visual method is based on highly significant correlation (r = 0.99) with data from image analysis method.

**Stress related traits**

**Desiccation resistance**

Desiccation resistance was measured as the time to lethal dehydration effect under dry air. To measure desiccation resistance, ten individual flies of each isofemale line were isolated in a dry plastic (40 × 100 mm) in which open end was covered with muslin cloth. These vials were kept on the top of another vial containing 2 g of silica gel at the bottom. Finally, this apparatus was made airtight with parafilm and kept in the desiccator chamber (Secador electronic desiccator cabinet; www.tarsons.in) which maintained ~5% relative humidity. The vials were inspected every hour and the number of dead flies (completely immobile) was recorded. The survival curves as a function of desiccation hours at experimental temperature were drawn.

**Humidity stress**

For high humidity conditions (68 ± 2% RH), ten male or female individuals of either sex or morph from each isofemale line were placed in a plastic vial containing food. Such vials were covered with a muslin cloth and were inverted on the top of another plastic vial (25 mm × 100 mm) filled with 20 ml water and this set-up was kept for 3 to 5 days. For each set-up, both the tubes were sealed together to hold them in a vertical position. The mating between ten pairs was recorded for 90 min. with a stop watch.

**Basic measures of water balance**

To estimate total body water content, 10 flies of each isofemale line were used. First, individual flies were subjected to mild anesthesia (for one min. with ether)
followed by weighing on Sartorius microbalance (Model-CPA26P; 0.001 mg precision). These flies were reweighed after drying for 24 h at 60°C. Total body water content was estimated as the difference between mass before and after drying at 60°C. Dehydration tolerance (i.e. the ability to sustain greater loss of body water before succumbing to death under desiccation stress) was calculated by the formula (wet body mass − body mass at death)/ (wet body mass − dry body mass) × 100 (Gibbs et al., 1997). For this, we estimated loss of total body water in living flies under desiccation stress (~ 5 % RH) until death of the flies by gravimetric method i.e. weighing each fly before and after loss of body water at death.

Further, the rate of water loss (mg hr⁻¹) was analyzed on ten individuals of each isofemale line by giving short term (8 hr) desiccation stress. Flies were weighed on a sartorius microbalance to the nearest 0.001 mg (CPA26P, www.sartorius.com) both before and after desiccation, and the cuticular water loss was calculated as mg hr⁻¹: (initial body weight − body weight after 8 hr. desiccation stress) / initial body weight x 8.

**Desiccation acclimation**

To measure pretreatment duration, 10 larvae / adult individuals of each replicate were subjected to desiccation stress at ~0-5% relative humidity. The initial body water content in each replicate group was recorded. The time period in which larvae / flies lost ~15–17% body water was considered as the pre-treatment time duration, followed by a subsequent recovery, such individuals were subjected to desiccation stress until death in order to test the increased desiccation resistance due to acclimation. Thus, absolute acclimation capacity (increased desiccation survival hours) was calculated by subtracting the desiccation resistance (h) of non-acclimated (control) from desiccation resistance (h) of acclimated individuals. Control and treatment experiments were run simultaneously under identical experimental conditions.

**Hemolymph content**

Each individual adult fly was placed on a paper towel followed by air drying for 2 minutes. The dry individual was carefully pinned to a microdissection dish at its anterior and posterior ends with microdissection pins, and a narrow incision was made through the cuticle with a third pin while observing through a stereo-zoom microscope (SZ-61; www.olympus.com). The leaking extractable hemolymph was absorbed with an
absorbent tissue moistened with an isotonic saline solution (Folk et al., 2001). Hemolymph content was estimated as reduction in mass following hemolymph blotting (Cohen et al., 1986; Hadley, 1994). Tissue water was estimated after subtracting exsanguinated mass before and after drying. From the same data, hemolymph water content by subtracting tissue water from total body water content was also calculated.

**Epicuticular lipids**

For estimation of cuticular lipid mass per fly, individual flies in 10 replicates per population were dried overnight at 60°C to obtain constant dry mass, that is, devoid of body water. Such dried flies were kept in high performance liquid chromatography (HPLC)-grade hexane for 1 h. Thereafter, the flies were removed from the solvent and were again dried at room temperature and finally reweighed. (The Sartorius microbalance CPA26P with precision up to 0.001 mg ensured accuracy: www.sartorius.com). For each individual fly, cuticular lipid mass in mg was estimated per unit surface area (surface area scales to 2/3 power of the wet body mass) as: difference between initial dry weight and dry weight after solvent treatment/ initial dry weight × surface area (where area was expressed in cm² and wet body mass in mg) recovery time in minutes was subtracted out of 100 to denote relative resistance level to cold stress. This helped in comparing the data on resistance to heat and cold stress. Life history related traits

**Analysis of energy metabolites**

**Estimation of trehalose and glycogen**

For trehalose and glycogen content estimation, 10 adult flies of each isofemale line were homogenized in an ultrasonic homogenizer (Labsonic® M; www.sartorius.com) with 300µl Na₂CO₃ and incubated at 95°C for 2 hours to denature proteins. An aqueous solution of 150 µl acetic acid (1M) and 600 µl sodium acetate (0.2M) was mixed with the homogenate. Thereafter, the homogenate was centrifuged (Fresco 21, Thermo-Fisher Scientific, Pittsburgh, USA) at 12000r.p.m. (9660 × g) for 10 minutes. This homogenate was used for independent estimations of trehalose and glycogen as given below.

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

For estimation of glycogen, a 50 µl aliquot was incubated with 500 µl Aspergillus niger glucoamylase solution (8.7 U/ml in 200mM of acetate buffer) for 2 hours at 40 °C with constant agitation and the suspension was centrifuged at 4000r.p.m. (1073 × g) for 5 minutes. It mainly hydrolyzed alpha-(1,4) and alpha-(1,6) glycosyl linkages and was suited for breakdown of glycogen. Glucose concentration was determined with 20 µl of supernatant from the suspension and added with 170 µl of a mixture of G6-DPH (0.9 U/ml); ATP (1.6mM); and NADP (1.25mM) in triethanolamine hydrochloride buffer (380mM TEA–HCl and 5.5mM of MgSO4}) and 10 µl of Hexokinase solution (32.5 U/ml in 3.2M ammonium sulphate buffer), and absorbance was measured at 340nm.

**Protein assay**

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

**Body Lipids**

Individual flies of both the sexes were placed in 2 ml Eppendorf tubes (www.tarson.in) were initially dried in an oven at 60 °C for 48h and then homogenized in ether for 2h in a centrifuge (200 rpm) at 37 °C so as to extract body lipids. Thereafter, the ether with dissolved body lipids was discarded. Two more washings of 6h each with ether
were given for the remaining dry mass of each individual fly so as to ensure removal of any lipid residues. Finally, the solvent was removed and individuals were again dried at 60°C for 24h and reweighed. Lipid content was calculated per individual by subtracting the lipid free dry mass from initial dry mass per fly. The method for determining the total lipid content was adapted from Marron et al., (2003).

**Life history traits**

For different sets of experimental flies, observations were made on the courting behavior (orientation, wing flapping of male and female response behavior); mating latency (time in minutes from introduction of virgin male and female fly to the initiation of copulation); copulation duration (time from initiation of mounted pair to detachment of mated couple flies). The total of these three components of mating behavior are referred to as mating propensity or mating success. Five days old sexually mature one virgin male and one virgin female fly were introduced in the Tarsons plastic food vials (37 × 100 mm) stoppered with cotton plug. All possible types of mating were recorded for 90 minutes with a stop watch (Fig. 2). All experiments were performed on seven day old flies.

**Behavior of male courtship element**

**No courtship** – the male does not perform any behavior

**Wing waving** – while oriented towards the female, the male extends both wings laterally and waves them up and down.

**Facing** – the male stand in front of the female facing her. He may keep his wing extended or touch the sides of the females.

**Following** – the male follows the female or circles to her rear.

**Circling** – the male circles around the female.

**Head under wings** – the males stands behind the females with his head under her wings. At this position he extends his wings laterally until they are 90° from the body axis. He may fan them at a slow speed.

**Wing vibration** – male may vibrate their wings in front of female, to attract female.

**Copulation** – the male curls his abdomen downwards, mount the female and
attempts to copulate.

**Different mating experiments:**

1. **No-Choice**
   - Dark ♀ X Spotted ♂
   - Dark ♀ X Spotless ♂
   - Light ♀ X Spotted ♂
   - Light ♀ X Spotless ♂

2. **Female Choice**
   - Dark ♀ X Spotted ♂
   - Dark ♀ X Spotless ♂
   - Spotted ♀ X Light ♂
   - Spotted ♀ X Spotless ♂
   - Light ♀ X Spotted ♂
   - Light ♀ X Spotless ♂

3. **Male Choice**
   - Spotted ♂ X Light ♀
   - Spotless ♂ X Dark ♀
   - Spotted ♀ X Light ♂
   - Spotless ♀ X Dark ♂

**Fig. 2.** Schematic representation of all possible matings

**Behavior of female courtship element**

- **No courtship** – the female is not courted and she does not wave her wings.
- **Wing waving** – the female extend both wing laterally and waves them in a rowing motion
- **Slashing** – the females rises up on her rear legs at the male with her forlegs.
- **Decamping** – the female leave the vicinity of the male by jumping or flying.
- **Walking** – the female walks while being courted
- **Preening** – the females preens her body part, while standing
- **Standing** – the female is motionless
- **Copulation** – the flies copulate

**Fecundity**

For estimating fecundity, one virgin female and one male were kept in a breeding chamber for 12 hours mating and thereafter the male was removed. The eggs laid on the food placed at the bottom of the oviposition chamber were counted daily. The flies were
transferred to fresh food vials everyday and the number of eggs laid by each female after 24 hours was recorded. This was continued for 15 successive days (7th to 21st) as this period coincided with highest egg production.

**Egg-to-adult viability**

Egg-to-adult viability was measured by monitoring adult emergence from the eggs. Emergences of adults were considered over the period of two to three weeks according to their growth temperatures.

**Climatic data analysis**

Ectothermic insects including *Drosophila* species adapt to varying climatic conditions. The investigated localities are highly variable in latitude and altitude as well as there are significant seasonal variations as we move from south to north. On the Indian subcontinent, thermal variations are linked with changes in relative humidity along latitude as well as altitude. Several localities in northern part show lower temperature and relative humidity. By contrast in southern peninsular region, low altitude localities are characterized by 27–28 °C temperature and high humidity (70–80 %). These differences in environmental conditions can put selection pressure on various morphological and physiological traits in drosophilids, because phenotype = genotype + environment. Thus, climatic adaptations and natural selection are directly or indirectly governed by surrounding environment. Climatic data for some of the collection sites are shown in Table 1.

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<th>Sites/Altitude (meters)</th>
<th>Lat °N</th>
<th>Tmax</th>
<th>Tmin</th>
<th>Tavg</th>
<th>Tcv</th>
<th>% RH</th>
<th>RHcv</th>
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Statistical analysis

*Mean* = Sum of all the observations in a sample divided by their number.

\[ x \bar{} = \frac{\sum x}{N} \]

*Standard Deviation* = Root mean square of the deviations from the arithmetic mean.

\[ \text{S.D (}\sigma) = \sqrt{\frac{\sum d^2}{N}} \]

*Standard Error* = Measure of mean difference b/w sample estimate of mean and the population parameter i.e., it is the measure of uncontrolled variation present in a sample.

\[ \text{SE} = \sqrt{\text{SD (}\sigma)/N} \]

*Variance* = Average of the squared deviation from the mean or it is the square of the standard deviation.

\[ \text{Variance} = \sigma^2 \]

*Coefficient of variation* = Ratio of standard deviation of a sample to its mean, expressed in percentage, does not have any unit

\[ \text{CV} = \frac{\text{SD (}\sigma)}{x \bar{}} \times 100 \]

Comparisons of two means

*Student's t-test*: It is used when sample size is small and observations were paired.

*Z-test*: It is used when sample size is large.

*Regression coefficient* = It measures the degree of dependence of one variable on the other (s).

\[ byx = \frac{\text{cov.}(xy)}{vx} \]

*Correlation coefficient*

Correlation refers to the degree and direction of association b/w two or more than two variables.

\[ r_{xy} = \frac{\text{Cov} (x,y)}{\sqrt{[ (vx) (vy) ]}} \]

Significance of correlation is tested with the help of table value at (n-2) degrees of freedom.

Comparison of multiple means

ANCOVA (*Analysis of covariance*)
Trait variability between populations was analyzed through ANCOVA with body size as a covariate and percent of total variance explained by each variable was calculated.

**ANOVA (Analysis of variance)**

It is the statistical procedure which separates or splits the total variation into different components. It helps in partitioning of phenotypic variation into genotypic and environmental components. ANOVA is used when multiple sample cases are involved. To test for differences among the means of the populations by examining the amount of variation within each of these samples, relative to the amount of variation between the samples. (a) One way ANOVA (b) Two way ANOVA & (c) Three way ANOVA

Statistica software (Statistica 5.0) was used for calculations and illustrations.