CHAPTER 5

Materials and Methods

5.1 Materials

Simulium (*Nevermannia*) *praelargum* "IIIL-1.2" Thapa et al., 2014
Simulium (*Nevermannia*) *praelargum* "IL", Thapa et al., 2017
Simulium (*Montisimulium*) *dattai*, Takaoka and Samboon, 2008 and *Simulium* (*Gomphostilbia*) *williei*, Takaoka et al., 2010 constituted the material for the purpose of study.

5.2 Collection of the larvae

The Simuliid larvae were collected from different streams, trickles and other free flowing natural water bodies (Fig. 1a, 1b, 1c, 1d) of Darjeeling Hill areas. The larvae were found to be attached with pebbles, twigs, submerged vegetations and also in some other debris such as plastic materials, polyethylene sheets etc.

5.3 Collection Sites

The entire specimens for this research work were collected from out skirt areas of Darjeeling town standing at an altitude of 2,354 m. The larvae were collected from small to medium sized streams from two sites in Dali around 4 kms south from Darjeeling town with Latitude of 27.023253°N and Longitude of 88.250685°E. The streams were named as stream 1 and stream 2. One stream from Happy Valley 1

km north from Darjeeling town with 27.051023°N of Latitude and 88.279866°E of Longitude, one from Sonada area approximately 17 km south from Darjeeling town with 26.935997°N of Latitude and 88.248367°E of Longitude, one from Bokshi Jhora, 4 km south from Darjeeling town with 27.019296°N Latitude and 88.252788°E of Longitude and one from Gandhi Road with Latitude of 27.018187° N and Longitude of 88.254247°E. The water current in all the sources were mild to moderate. The width of the streams varied from 12 to 15 cm with the depth of about 2 to 3 cm. The temperature of the water varied from 13°C - 16°C. The collection sites were of different types. Some portions of the water body were exposed to sunlight whereas some others were shaded with different vegetations. Out of the four species worked out, the three species of the larvae collected for this study viz, *Simulium* (*Nevermannia*) *praelargum* "IL", *Simulium* (*Gomphostilbia*) *williei* and *Simulium* (*Montisimulium*) *dattai* were found together in almost all the mentioned collections sites and were associated with other species like, *Simulium* (*Nevermannia*) *praelargum*-st, *Simulium* (*Gomphostilbia*) *sachini*, *Simulium* (*Gomphostilbia*) *darjeelingense*, *Simulium* (*Gomphostilbia*) *asakoe* and *Simulium (Montisimulium) ghoomense* but were sometime but rarely associated with *Simulium* (*Gomphostilbia*) *gracile* and *Simulium* (*Gomphostilbia*) *purii*. Whereas *Simulium* (*Nevermannia*) *praelargum* "IIIL-1.2"and *Simulium* (*Nevermannia*) *praelargum*-st were found together in Happy valley and Sonada site. All the association of the mixed population were period selective.

FIGURE 2: Last-instar larva

5.4 Period of Collection

Collection period started annually from the month of May to November. The collections of the specimens were made during the period 2008 - 2016. The collection of the larval population was affected due to disturbances in the environmental conditions and disruptive rains and monsoon.

5.5 Fixation of the larvae

The *Simulium* larvae (larva-Fig. 2) were collected live and fixed in the field after soaking them in a blotting paper. The matured larvae were plunged into freshly prepared aceto-ethanol 1:3 (Glacial acetic acid: ethyl alcohol). Only mature larvae were collected identified on the basis of well developed respiratory histoblast. The larvae were then transported to the laboratory.

5.6 Preservation of the larvae

The larvae were then transferred to fresh fixative with 3 – 4 changes and then kept in the freeze at - 4° centigrade for immediate use and at -20° for prolonged use.

5.7 Identification of the larvae

All the identification has been done by the author mainly on the basis of the number of filaments and their pattern in the respiratory histoblast, the larval gills; size and shape of the post -genal cleft; head patterns along with other identifying distinctive characters as described by the authors through published papers. The population

FIGURE 3: Salivary glands

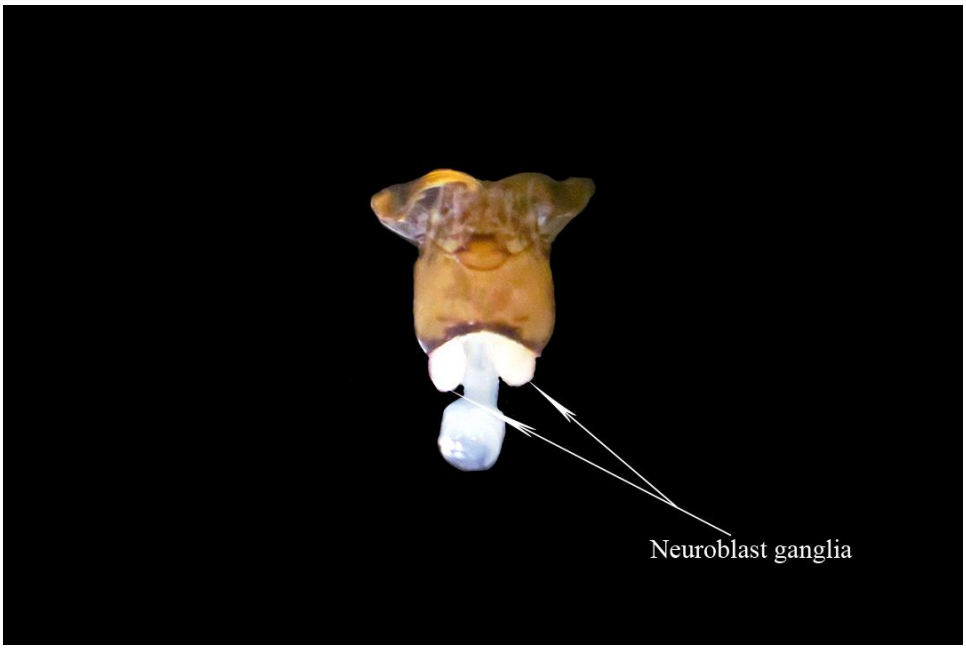


FIGURE 4: Neuroblast ganglia

FIGURE 5a: Yashika binocular microscope

of collected specimens of individual species was separately preserved with proper collection indices.

5.8 Extraction of salivary glands, neuroblast ganglia and ovary

The dissection of the larvae for salivary glands (Fig. 3) differs in case of live and fixed specimens. In case of live larvae the head and the tail regions is pulled apart in a 0.67% of NaCl where the salivary glands float. In case of prefixed and preserved specimens the larvae are dissected ventrally with the help of fine needles that make the incision of the skin. The salivary gland which occupies approximately 2/3rd of the body cavity is easily recognizable. The salivary glands have long stem with a sharp U-shaped curvature at the basal end that has a bulged ends (Fig. 3). The dissections were made in 95% ethanol under a binocular microscope (Yashika Japan – Fig. 5a).

The paired Neuroblast ganglia (Fig. 4) were dissected from the base of the head region that appeared as small rounded bodies. Dissections were made in 95% ethanol under the binocular microscope (Yashika Japan – Fig. 5a). Sometimes 0.25% of Colchicine was used for the mitotic plates; however, the mitotic plates without Colchicine pretreatment also offered clear and prominent mitotic chromosomal plates. The treatment with Colchicine posed a serious threat to the survival of the larvae. The time required for Colchicine pretreatment is between 2 to 3 hours.

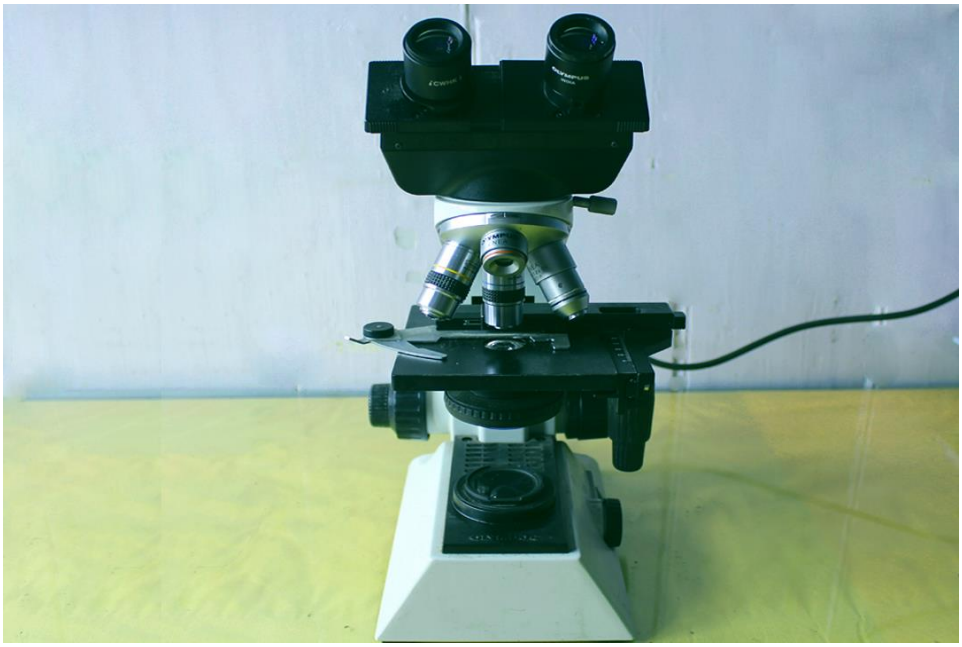


FIGURE 5b: Olympus **CH 20i compound microscope**

FIGURE 6: Camera

The rudimentary ovaries have provided the best source for mitotic plates. Ovaries found to be located at the base of lower abdomen embedded within fat bodies.

5.9 Preparation of temporary slides

The glands were then stained in a cavity slide with 2% Lacto-Propionic- Orcein for about 20-25 minutes (2grams of Orcein powder dissolved in a solution containing 25ml of lactic and 25 ml of Propionic acid mixed in 50 ml of distilled water).

Adequately stained salivary glands were then dissected for epithelial layer of the gland. Jelly like substance of the glands was rejected. The epithelial layer of the glands were then squashed under a cover glass in 50 % lactic acid or 50 % mixture of lactic and propionic acid with requisite thumb pressure. The neural glands were also squashed in the same manner as that of salivary glands. The slides were sealed with DPX mountant. Observations were made under the compound light binocular microscope (Olympus CH 20i) (Fig. 5b).

5.10 Observations

Nuclei with considerably well spread plates of polytene chromosomes were selected and photographed with a digital camera (Canon Power shot SX 150, 14.1 megapixels)(Fig. 6). The photographs of the polytene chromosomes were transferred to computers. The polytene chromosome maps were then constructed using computer software -Adobe Photoshop CS. The constructed

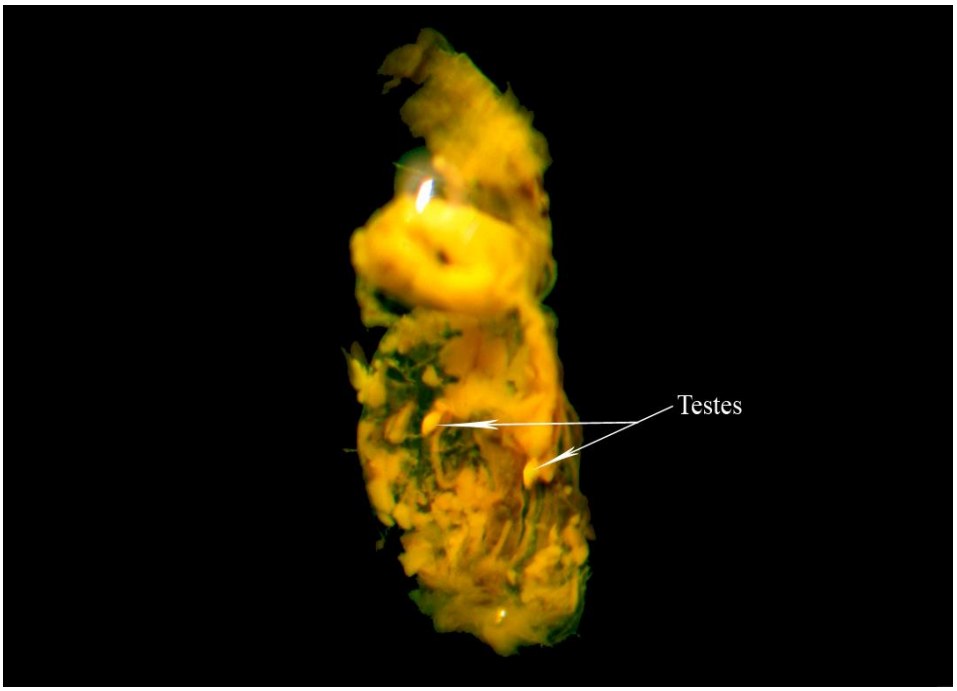
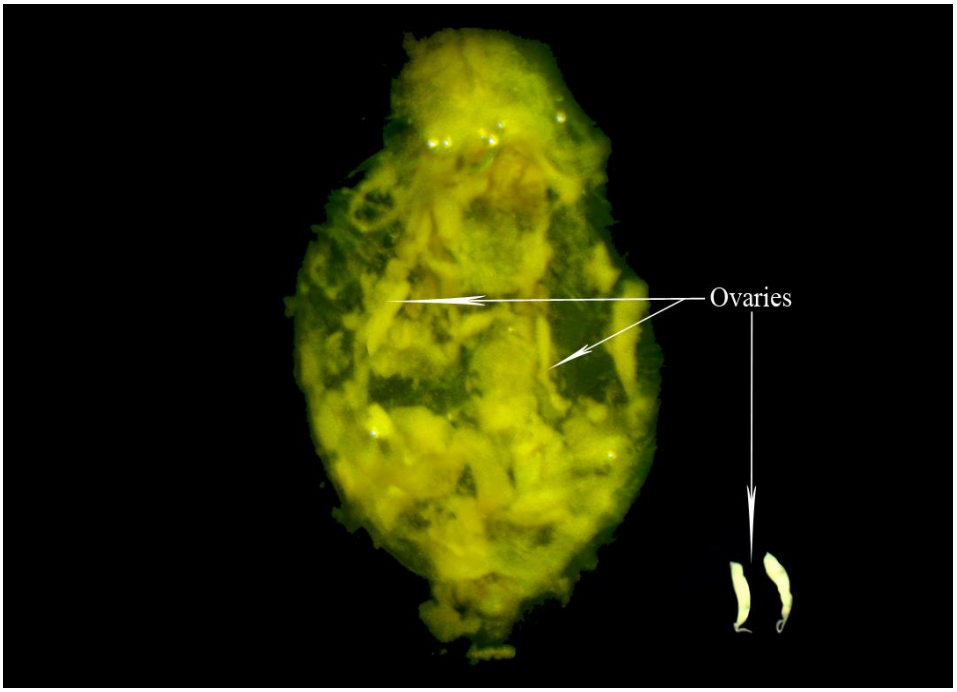


FIGURE 7a: Testes



FIGUREE 7b: Ovaries

maps were compared with available standard maps in respect to species maps.

All the species under observation exhibited n=3 haploid sets of chromosomes. The three chromosomes were distinct in respect of length. The chromosomes are numbered I, II and III according to their lengths. The longest being the Chromosome I and shortest the chromosome III, middle one being chromosome II.

5.11 Sex identification of the larvae

The larvae have rudimentary gonads. The larval ovaries and the testes remain embedded in the fat body at the abdominal region. The ovaries are elongated where the testes are oval (Fig. 7a, 7b). The male and the female larvae are identified likewise.

5.12 Measurement of Polytene Chromosomes

Relatively well spread and condensed polytene chromosomal plates were selected for the purpose of measurement of the chromosomal complements. Camera lucida (Prism type) was used to draw the outline of the Chromosomes. The drawing of the chromosomes was measured conventionally with the help of a flexible wire, whose length in turn was measured with the help of graduated scale.

Polytene Chromosome arms in 10 nuclei from each of 10 larvae were measured to determine their percentage total complement length (%TCL) and the polytene chromosome maps were divided

into 100 sections assigning number of sections to each arm under equivalent to the value of percentage of length. The conventions and nomenclature of Rothfels et al., 1978 were followed to label the chromosome maps.

For mitotic measurement ocular micrometer on calibration with the stage micrometer under oil immersion magnification (10x100X) was used to measure the relative lengths of the mitotic chromosomes in μ. Standard error of the mean (SE) was calculated conventionally.

5.13 Classification of the chromosomes

Chromosomes were classified as metacentric (m) (arm ratio 1-1.7) and submetacentric (sm) (arm ratio 1.7-3) basing on the position of the centromere and the arm ratio between short arm and the long arm of the chromosomes following conventional method proposed by Levan et al., 1964. Nomenclatures of different parts of the polytene chromosomes have been done following the method proposed by Rothfels et al., 1978.