CHAPTER-II

MATERIALS AND METHODS

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1 MATERIAL

A) SELECTION OF GASTROPODS

The survey of the literature indicates the variations in the neurosecretion by cerebral ganglia among the gastropod species belonging to different habitats and in species with shells and without shells, in different sub-classes like prosobranchiata, opisthobranchiata and pulmonata. But it is not known whether such a species specific differences exist in the species belonging to same habitat and in species belonging to same sub-class of the gastropoda. Hence while selecting the gastropods, the precaution was taken to collect the species belonging to same sub-class of gastropoda and for comparision, two species, one with the shell and other without the shell were selected, for the present investigation. The following is their classification-

1) Semperula maculata (Semper) -Terrestrial slug.

Phylum - Mollusca

Class - Gastropoda

Subclass - Pulmonata

Order - Stylommatophora

2) Cryptozona semirugata (Beek)- Terrestrial snail.

Phylum - Mollusca

Class - Gastropoda

Sub-class - Pulmonata

Order - Stylommatophora

These two gastropods show maximum breeding activities in the monsoon months i.e. June, July, August and September. The breeding is initiated with the onset of rainy season in first or second week of June and it ends at the cesation of rains in September or October . If the monsoon season starts in the first week of June then based on the reproductive activities, the breeding period can be divided into three phases- (I) Early breeding phase which starts in June and ends in early July, (ii) Midbreeding phase between late July and early August and (iii) Late-breeding phase-starting from late August to entire September.

Both the gastropods, during winter and summer seasons, go into aestivation period somewhere in October and remain in dormant condition till May. The aestivation period can also be divided into three phases i.e. (I) Early aestivation phase which starts immediately after the breeding season, in October and remains for one and half month till mid November. (ii) Mid-aestivation phase commenses from the middle of November to the end of March. It is the real period of aestivation. (iii) Late -aestivation phase starts in April and ends at the begining of monsoon season, when animals prepare to emerge from the dormancy period.

The phases in the breeding and aestivation periods can be summarised as follows :

a) ***BREEDING PHASES*** :

i)	Early breeding	-	Mid June to early July
ii)	Mid-breeding	-	Late July to early August
iii)	Late breeding	-	Late August to end of September

b) ***AESTIVATION PHASES*** :

i)	Early aestivation	-	Early October to mid November
ii)	Mid aestivation	-	Mid November to end of March
iii)	Late aestivation	-	Early April to early June.

Thus, these two selected gastropods satisfy all the requirements for an ideal study of the type mentioned in Introduction, chapter first.

B) SITE OF COLLECTION OF GASTROPODS :

The gastropods were collected in the gardens on the campus of Shivaji University, Kolhapur, Maharashtra, India and in the gardens, located in the extension part of Rajarampuri which is generally called as Mali Colony, adjoining to the University Campus within the periphery of three to four kilometers. Normally, these gastropods remain near humid area in the moist soil.

C) TIME OF COLLECTION OF GASTROPODS :

The gastropods were collected for a period of twelve months from August, 1993 to July ,1994, early in the morning before sun-rise. Only 100 matured slugs and snails were obtained from their natural environment in a fortnight of every month.

D) MAINTENANCE OF GASTROPODS :

Along with the freshly collected gastropods freshly from their natural environment, laboratory maintained animals were also simultaniously sacrified for the present study.Therefore, some gastropods were maintained in the laboratory throughout the year. They were kept in big plastic containers filled with the same soils from where gastropods were collected and the containers were covered with naeted cotton clothes. The laboratory maintained animals were well fed with their natural and liking foods including ground nut and canna leaves, grass, potato and rippen banana chips, small pieces of bread, etc. During breeding months, water was sprinkled over the gastropods but with the stopage of rains the sprinkling was stopped. During aestivation period natural and laboratory maintained gastropods were lethargic and remained quite. Snails sealed their operculum with white cement like secretory material.

The freshly collected and laboratory maintained gastropods were sacrificed, dissected and their cerebral ganglia, optic tentacles and ovotestis were removed during different phases of breeding and aestivation period and treated for the histological and histochemical studies described in the next part of this chapter.

2 *METHODS* :

The selected organs were cut into small pieces and they were fixed in the aqueous and alcoholic Bouins fixative, Stieve's fixative and in ice-cold CAF (Calcium-acetate-formalin 2% in 10% formalin) fixative for 24 hrs. Then, they were washed in chilled distilled water and in running tap water, dehydrated in

alcohol, cleared in xylene and embeded in paraffin. The sections were cut at 6 to 7μ. Then these sections were stained by employing histological and histochemical staining procedures.

A) HISTOLOGICAL METHODS :

I) WEIGERT'S IRON HEMATOXYLIN-EOSINE (HE1) METHOD (HUMASON, 1967) :

a) SOLUTIONS :

Solution A : Iron chloride, $FeCl_2$, 29% aqueous - 4 ml, Distilled water- 95 ml and HCl- 1 ml.

Solution B : Hematoxylin- 1 g, 95% ethyl alcohol-100 ml. Equal volumes of A and B were mixed each time. It can remain for 7-8 days

b) PROCEDURE :

1) Deparaffinize and hydrate slides to water.
2) Stain in Weigert's hematoxylin 3-5 minutes or longer.
3) Wash in running water for 5 minutes.
4) Counterstain by Eosin.
5) Dehydrate, clear and mount.

c) Results :(Nuclei-Black, Other Elements Pink)

ii) Harri's Hematoxylin -Eosin (HE2) Method (Humason, 1967) :

a) ***SOLUTION*** : 1 g hematoxylin was dissolved in 10 ml ethyl alcohol. Then 20 g potassium or ammonia alum $KAl(SO_4)_2$ 12 H_2O or NH_4 Al $(SO_4)_2$.$12H_2O$ was dissolved in 200 ml water and boiled. Hematoxylin was mixed and again boiled for 1/2 minute. 0.5 g mercuric oxide was added and cooled rapidly. In order to keep away from metalic luster and brighten nuclear structure, few drops of glacial acetic acid were added. (It remains for a month or two).

b) ***PROCEDURE*** :

1) Deparaffinize and hydrate slides to .70% alcohol
2) Lugol's solution 3 minutes and running water.
3) 5% Sodium thiosulfate ($Na_2S_2O_3$) 2-3 minutes.
4) Running water.
5) Hematoxylin stain- 1-5 minutes, running water.
6) Scott's solution 3 minutes, running water.
7) Eosin 1 minute, dehydration, clear and mount.

c) ***RESULTS : NUCLEI*** - Deep blue, cytoplasmic structures pink, rose, etc.

III) ***MALLORY'S TRIPLE STAINING (MT) METHOD (PANTIN, 1946):***

a) ***SOLUTIONS*** : Mallory I : Acid fuchsin 1 g in 100 ml D.W. ,phosphomolybdic acid- 1 g in 100 ml Distilled water,

Mallory II -Aniline blue 0.5 g ; Orange G 2 g and Distilled water 100 ml.

b) ***PROCEDURE*** :

1) Deparffinize and hydrate slides to water.

2) Stain in Mallory I for 15 seconds.

3) Rinse in distilled water for 10 or more seconds, to differentiate reds.

4) Treat with phosphomolybdic acid, 1-5 minutes

5) Rinse briefly in distilled water.

6) Stain in Mallory II for 2 minutes.

7) Rinse in distilled water.

8) Differentiate aniline blue in 90% ethyl alcohol.

9) Dehydrate in absolute alcohol, clear and mount.

c) RESULTS : Nuclei-blue, muscle and some cytoplasmic elements red to orange, nervous system lilac, collagen dark blue, mucus, connective tissue, and hyaline substance blue, chitin red, yolk yellow to orange, myelin and red blood cells yellow and orange, liver (dense cellular tissue) pink with red nuclei, bone matrix red.

B) HISTOCHEMICAL METHODS :

I) PERIODATE ACID-SCHIFF(PAS) METHOD (MC MANUS AND MOWRY, 1960) :

a) SOLUTIONS (AQUEOUS) : Periodic acid (HIO_4) 0.6 g, distilled water 100 ml,concentrated nitric acid 0.3 ml,

Schiff's reagent-sodium metabisulfite ($Na_2S_2O_5$) 0.5 g and distilled water 100 ml.

b) PROCEDURE :

1) Deparaffinize and hydrate slides to water.

2) Treat with periodic acid for 5 minutes.

3) Wash in running water 5 minutes.

4) Treat in Schiff's reagent 10 minutes.

5) Transfer through sulfite solutions, 3 changes-

1.5 -2 minutes each.

6) Wash in running water 5 minutes.

7) Counterstain , if desired.

8) Dehydrate, clear and mount.

c) RESULTS : Glycogen, starch, cellulose, mucins, colloid of thyroid, cartilage matrix, chitins, reticulum, fibrin, collagen-rose to purplish-red, fungi-red, nuclei and other tissue elements-colours of counterstain.

II) FEULGEN METHOD (HUMASON, 1967) :

a) SOLUTION : N hydrochloric acid, Schiff's reagent, Bleaching solution- 5 ml N HCl, potassium or sodium bisulfite ($K_2S_2O_5$ or $Na_2S_2O_5$) 10% aqueous 5 ml and 100 ml distilled water (Fresh bleach each time). Fast green-0.05 g and 100 ml 95% ethyl alcohol.

b) PROCEDURE :

1) Deparaffinize and hydrate slides to water

2) Rinse at room temperature in N HCl for 2 minutes.

3) Hydrolyze at 60°C in N HCl for 5-12 minutes.

4) Rinse at room temperature in N HCl and in distilled water.

5) Stain in Schiff's reagent for 2 hours in dark.

6) Drain and transfer quickly into bleaching solution 3 changes, 1.5 -2 minutes in each

7) Wash in running water for 10-15 minutes.

8) Rinse in distilled water.

9) Counterstain in fast green 10 seconds.

10) Dehydrate, clear and mount.

c) ***RESULTS*** : Thymonucleic acid-containing substances-red-violet, other tissue elements-shades of green.

III) ***ALDEHYDE-FUSCHSIN METHOD (GOMORI, 1950*** **MODIFIED FROM THE CAMERON AND STEELE, 1959) :**

a) ***SOLUTIONS*** : 0.3% potassium permanganate -0.3 g potassium permagnate 100 ml distilled water and 0.3 ml conc. H_2SO_4 2.5 g sodium bisulfite,Aldehyde fuchsin -0.25 g in 50 ml of 70% alcohol (keep at least six months).

b) ***PROCEDURE*** :

1) Deparaffinize and hydrate slides to water

2) Oxidize in permanganate solution 1 minute.

3) Rinse in distilled water.

4) Bleach in sodium bisulfite until permanganate colour is removed.

5) Wash in running water for 5 minutes.

6) Transfer to 70% alcohol for 2 minutes.

7) Stain in aldehyde fuchsin 2-10 minutes.

8) Wipe off back of slide and rinse in 95% alcohol.

9) Differentiate in 95% alcohol until no more aldehyde-fuchsin comes out of sections.

10) Dehydrate in absolute alcohol, clear and mount.

c) RESULTS : Elastin-deep purple, mast cells, chief cells of gastric mucosa,β cells of pancreas, basophils of pituitary and some kinds of mucin-purple.

IV) ALCIAN BLUE (PH 2.5) METHOD (STEDMAN, 1950 ; MODIFIED BY MOWRY, 1956; 1963) :

a) SOLUTIONS : Alcian blue-0.5 -1.0 g, distilled water 100 ml, glacial acetic acid 3 ml, filter and add thymol crystal to prevent mold.

b) PROCEDURE :

1) Deparaffinize and hydrate slides to water.

2) Rinse in 3% acetic acid for 3 minutes.

3) Stain in Alcian blue for 2 hours.

4) Rinse in distilled water, then 3% acetic acid for 3-5 minutes.

5) Wash in tap water 3 minutes.

6) Rinse in distilled water, dehydrate, clear and mount.

c) RESULTS : Acid mucopolysaccharides-blue green

V) ALCIAN BLUE (PH 1.0) METHOD (LEV AND SPICER, 1964) :

a) SOLUTIONS : 1% Alcian blue in 0.1 N HCl and other solutions as above

b) PROCEDURE :

1) After dewaxing and hydration sections were brought to distilled water

2) Stained for 30 minutes in 1% AB in 0.1 N HCl (pH 1.0)

3) Blotted on a puffless filter paper.

4) Dehydrated quickly, cleared and mounted.

c) RESULTS : Only sulfomucins stain intense blue.

VI) CHROMIUM- HEMATOXYLIN-PHLOXINE-CHP METHOD (GOMORI, 1941) :

a) SOLUTIONS : Bouin's solution -75 ml saturated aqueous picric acid, 25ml concentrated formalin and 5 ml glacial acetic acid. Potassium dichromate- sulfuric acid solution- 0.15 g potassium dichromate, 100 ml distilled water, 0.15 ml concentrated sulfuric acid. Hematoxylin solution- 0.5 g hemastoxylin in 50 ml distilled water, when dissolved add 50 ml 3% potassium chromium sulfate (chrome alum) mix well and add 2 ml 5% potassium dichromate and 2 ml N/2 sulfuric acid (about 2.5 ml /100 ml water). Allow to ripen for 48 hours,store at 0-4° C, filter before use. Phloxine B solution 0.5 g in 100 ml distilled water.

b) ***PROCEDURE*** :

1) Deparaffinize and hydrate slides to water.

2) Refix in Bouin's solution -12-24 hours.

3) Wash in running water for 5 minutes.

4) Treat with potassium dichromate-sulfuric acid 5 minutes.

5) Decolorize in Sodium bisulfite (5%) -3-5 minutes.

6) Wash in running water 5 minutes.

7) Stain in hematoxylin solution -10-15 minutes.

8) Differentiate in 1% HCl about 1 minute.

9) Wash in running water for 5 minutes.

10) Stain in phloxine for 5 minutes.

11) Rinse briefly in distilled water.

12) Treat with 5% phosphotungstic acid for1 minute.

13) Wash in running water for 5 minute, sections turn red again.

14) Differentiate in 95% alcohol.

15) Dehydrate, clean and mount.

c) ***RESULTS*** : Neurosecretory β cells-blue, α cells-red, δ cells-pink to red.

The sites of various chemical constituents of neurosecretory cells by above staining techniques were detected and their concentrations were determined by visually estimated intensity and shade of the colour. Photomicrography was carried out within a month after the preparation.