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Number 9

MANUAL OF RESEARCH METHODS FOR MARINE INVERTEBRATE REPRODUCTION

Issued on the occasion of the Workshop on
MARINE INVERTEBRATE REPRODUCTION
jointly organised by
the Department of Zoology, University of Madras and
the Centre of Advanced Studies in Mariculture,
Central Marine Fisheries Research Institute, Cochin
held at the University of Madras
from 25th October to 10th November 1982

The Centre of Advanced Studies in Mariculture was started in 1979 at the Central Marine Fisheries Research Institute, Cochin. This is one of the Sub-projects of the ICAR/UNDP project on 'Post-graduate agricultural education and research'. The main objective of the CAS in Mariculture is to catalyse research and education in mariculture which forms a definite means and prospective sector to augment fish production of the country. The main functions of the Centre are to :

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—make available the modern facilities, equipments and the literature ;

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—develop linkages between the Centre and other Institutions in the country and overseas ;

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Front cover : SEM picture showing surface topography of *Streptocephalus dichotomus* egg.

Manual of Research Methods for Marine Invertebrate Reproduction

EDITED BY

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ISSUED ON THE OCCASION OF THE WORKSHOP ON MARINE INVERTEBRATE REPRODUCTION JOINTLY ORGANISED BY THE DEPARTMENT OF ZOOLOGY, UNIVERSITY OF MADRAS AND THE CENTRE OF ADVANCED STUDIES IN MARICULTURE, CENTRAL MARINE FISHERIES RESEARCH INSTITUTE HELD AT THE UNIVERSITY OF MADRAS FROM 25TH OCTOBER TO 10TH NOVEMBER, 1982.

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PREFACE

The technologies of controlled reproduction, induction of spawning, sex reversal, artificial fertilisation, sterilisation and preservation of gametes are increasingly applied in aquaculture to obtain quality seed, quality fish stock and better yield. In this context, researches on different aspects of reproduction, developmental biology and physiology have assumed considerable importance besides their values in understanding of the ontogeny of the organisms. Extensive researches carried out in recent years from several laboratories in the world have not only accumulated a body of information, but also broughtforth several new concepts to our understanding of the development and reproductive behaviour of finfishes and shellfishes.

In India, directed research on reproductive physiology and biology is taken up only recently and the field is still in an infant stage. In view of its emerging importance, it is identified as an area for priority research and for expertise development in the programmes of the Centre of Advanced Studies in Mariculture at the Central Marine Fisheries Research Institute, and several programmes of research are being taken up in this field with particular reference to the reproductive behaviour of the cultivable finfishes and shellfishes.

Advances made on the frontiers of invertebrate reproduction in recent years have been significant enough to organise a national workshop and to prepare a manual on research methodologies for the study of the subject. Several histological, histochemical and biochemical methods and sophisticated instruments have been introduced in these studies making it essential that the scholars who desire to work and specialise in the field are given adequate basic information on the research methods so as to enable them to appreciate and advance research to understand the problems confronted in the field.

The present manual, the third in the series, is prepared and compiled by Dr. T. Subramoniam, Leader of the 'Unit of

Invertebrate Reproduction' of the Zoology Department of the University of Madras, Tamil Nadu. During the past decade, a team of research scholars are working on different aspects of marine invertebrate reproduction including the cultivable crustaceans such as *Scylla serrata*, *Panulirus homarus* and *Macrobrachium* spp. under his leadership. Contributing to our knowledge on the subject, the research results achieved so far in these aspects by the Unit have unfolded several new concepts in oogenesis, spermatogenesis, sperm transfer strategy, fertilization and endocrine control of reproduction and gamete formation.

I wish to express my great appreciation to Dr. T. Subramoniam and his team of Scholars, who by their dedication and interest evolved a series of tested research methods and set a theme of investigation through insight and skill on marine invertebrate reproduction. I am sure that this manual will be of immense use to the research scholars and scientists who would like to specialise in the subject and cognate fields.

This is the second workshop we are organising in close collaboration with the University of Madras. I wish to express my gratitude to Dr. M. Santappa, Vice-Chancellor, University of Madras for the keen interest evinced in such collaborative programmes and for the advice. I am also indebted to Dr. K. Ramalingam, Professor and Head of the Department of Zoology, University of Madras for productive discussions, continuous support and suggestions. I wish to thank Shri P. T. Meenakshisundaram and Shri K. Rengarajan, Scientists of the Central Marine Fisheries Research Institute for their help in the preparation of this manual.

E. G. SILAS,
Director, C.M.F.R.I.

V. NEUROENDOCRINE CONTROL OF REPRODUCTION

14

STAINING METHODS FOR NEUROSECRETORY SYSTEM IN CRUSTACEANS*

14.1. Introduction

A neurosecretory system consists of neurosecretory cells (NSCs) and a neurohaemal organ for synthesis and storage of neurohormones respectively. In the central nervous system (CNS) the distribution of NCSs is confined to the brain, thoracic ganglia, circum-oesophageal connective and eyestalk. The NSCs elaborate secretory materials which can be demonstrated by staining techniques (Gomori, 1939, 1941, 1950 ; Dogra and Tandan, 1964). Among them, Gomori's (1939) chrome-hematoxylin phloxine (CHP) and Gomori's (1950) paraldehyde fuchsin (PF) are the most common.

14.2. Principle

Chrome-hematoxylin, basic fuchsin and Victoria blue are the commonly used basic dyes for staining the NSCs. The rationale of chrome-hematoxylin phloxine and paraldehyde fuchsin staining of the neurosecretory material (NSM) is based on the affinity of these stains for the acidic groups, appearing after oxidation of NSM with oxidizing agents such as performic acid and potassium permanganate. Raabe (1980) thus found an increased neurosecretory activity after increasing the time of oxidation for the NSCs in the pars intercerebralis of insects. The specific oxidation of the NSM involves the formation of cysteic acid from both cysteine and cystine which are present in enormous quantity in the NSM of insects (Dogra and Tandan, 1964) and crustaceans (Lake, 1970). The same oxidation process also produces free aldehyde groups, to which the basic stains can bind (Gabe, 1953).

* Prepared and verified by M. Panneerselvam and T. Subramoniam, Unit of Invertebrate Reproduction, Department of Zoology, University of Madras, Madras-600 005.

14.3. Material

Brain and thoracic ganglion of the anomuran crab, *Albunea symnista*

14.4. Preparation of Tissue

1. Dissect the animal in a medium of 0.9% saline solution.
2. Remove the brain, thoracic ganglion and wash well with the saline.
3. Fix it in Bouin's fixative for 18—24 hours.
4. Dehydrate in alcohol, clear in xylol and embed in paraffin wax (m.p. 54°C—56°C).
5. Cut sections at 6 μm thickness.

14.5. Gomori's (1939) Chrome-Hematoxylin Phloxine Method [Bargmann's (1949) Modification]

14.5.1. Reagents

1. *Bouin's fluid* : *Vide* expt. No. 1.
2. *Mordant solution* : Dissolve 4 gm of chromium potassium sulphate in 100 ml of Bouin's fluid by heating gently to about 50°C.
3. *Oxidizing solution* : Add 10 ml of 2.5% potassium permanganate (2.5 gm in 100 ml distilled water) to 10 ml of 5% sulphuric acid (5 ml in 95 ml distilled water). To this add 80 ml of distilled water.
4. *Chrome-hematoxylin* : Add 50 ml of 1% hematoxylin (1 gm in 100 ml distilled water) to 50 ml of 3% chromium ammonium sulphate (3 gm in 100 ml distilled water). To this add 2 ml of 5% potassium dichromate (5 gm in 100 ml distilled water) and 1 ml of 5% sulphuric acid.
5. 1% *Oxalic acid* : Add 1 ml of oxalic acid to 99 ml of distilled water.
6. 0.5% *Acid alcohol* : Add 0.5 ml of hydrochloric acid to 99.5 ml of 70% alcohol.

7. 0.5% *Phloxine*: Dissolve 0.5 gm of phloxine in 100 ml of distilled water.
8. 5% *Phosphotungstic acid*: Dissolve 5 gm of phosphotungstic acid in 100 ml of distilled water.

14.5.2. Procedure

1. Bring sections to distilled water.
2. Immerse the sections in mordant solution for 12—24 hours at 37°C (mordant forms a link between the tissue and the stain).
3. Wash in running tap water until the sections become colourless.
4. Keep the sections in oxidizing solution for 5 minutes.
5. Bleach in 1% oxalic acid for 1 minute.
6. Wash in running tap water for 5 minutes.
7. Rinse in glass distilled water.
8. Stain in chrome-hematoxylin for 30—40 minutes at 4°C.
9. Differentiate in 0.5% acid alcohol for 30 seconds.
10. Rinse in running tap water for 2 minutes.
11. Stain in 0.5% phloxine for 2—3 minutes.
12. Transfer to 5% phosphotungstic acid for 5 minutes.
13. Wash in running tap water for 5 minutes.
14. Differentiate in 70% alcohol.
15. Dehydrate, clear in xylol and mount in DPX.

14.5.3. Result

Neurosecretory substance—deep purple.
Nuclei—purple. Background—pinkish red.

14.6. Gomori's (1950) Paraldehyde Fuchsin Method [Cameron and Steele's (1959) Modification]

14.6.1. Reagents

1. *Gomori's fluid*: Dissolve 15 gm of potassium permanganate in 50 ml of distilled water. Add to this 0.1 ml of concentrated sulphuric acid.

2. 2.5% *Sodium bisulphite* : Dissolve 2.5 gm of sodium bisulphite in 100 ml of distilled water.

3. *Paraldehyde fuchsin* : Add 1 gm of basic fuchsin to 200 ml of boiling distilled water. Boil once again for 1 minute, cool and filter. Add 2 ml each of concentrated hydrochloric acid and paraldehyde solution (100%) to the filtrate. Close the container air tight and leave it at room temperature. Wait until the maximum precipitation of fuchsin occurs at the bottom of the bottle. At this time, the solution will lose its reddish fuchsin colour. Filter the solution and discard the filtrate. Dry the precipitate on the filter paper (Temperature 57°C—59°C is suitable for this). Remove the dry crystals from the filter paper and store in a reagent bottle. Dissolve 0.25 gm of the crystal in 50 ml of 70% alcohol. The solution can be used upto 6 months.

4. *Halmi's mixture :* Dissolve 0.2 gm of light green and 1.0 gm of orange G in 100 ml of distilled water. Shake well. To this add 0.5 gm of phosphotungstic acid and 1 ml of glacial acetic acid. The stain need not be filtered.

5. 0.2% *Acetic acid :* Add 0.2 ml of acetic acid to 99.8 ml of distilled water.

14.6.2. Procedure

1. Bring sections to distilled water.
2. Oxidise in Gomori's fluid for 1 minute.
3. Rinse in 2.5% sodium bisulphite solution until all permanganate stain is removed (a few seconds).
4. Rinse in distilled water.
5. Transfer the slide through 30% to 70% alcohol.
6. Stain in the paraldehyde fuchsin solution for 2—10 minutes.
7. Quickly wipe the back of the slide and rinse in 95% alcohol.

8. Transfer the slide to a second bath of 95% alcohol for 1—5 minutes (until no more paraldehyde fuchsin comes away).
9. Bring to water through 70% and 30% alcohols.
10. Counter stain in Halmi's mixture for 20—30 seconds.
11. Wipe the back of the slide and differentiate in 95% alcohol containing 0.2% acetic acid until no more stain comes away(2—3 minutes).
12. Rinse in 95% alcohol.
13. Dip the slides in absolute alcohol (2 changes), clear in xylol and mount in DPX.

14.6.3. Results

Neurosecretory cells—deep purple, dark blue and pale violet.

14.7. Identification of Neurosecretory Cells and their Pathways in Intact Neuroendocrine Organs

14.7.1. Introduction

In crustaceans, a large number of NSCs are distributed on the surface of the brain, thoracic ganglia and optic lobe. The NSCs as well as their pathways are identified by using performic acid/Victoria blue staining method of F.D. Humberstone, modified by Dogra and Tandan (1964). This method was originally used to study the axonal pathways of the NSCs in the pars intercerebralis of insects.

14.7.2. Materials

Brain, thoracic ganglion and eyestalk of the penaeid prawn *Metapenaeus monoceros*.

14.7.3. Performic Acid/Victoria Blue Method of F.D. Humberstone [Dogra and Tandan's (1964) modification]

14.7.3.1. Reagents

1. *Performic acid*: Add 4 ml of 30% hydrogen peroxide (30 ml in 70 ml distilled water) and 0.5 ml of concentrated sulphuric acid to 40 ml of 98% formic acid (98 ml in

2 ml distilled water). Allow the mixture to stand for atleast an hour. Use preferably within 24 hours (use 100 vol. hydrogen peroxide, not more than 3 weeks after opening the bottle).

2. *Victoria blue solution*: Dissolve 0.5 gm of dextrine, 2 gm of Victoria blue RN_{275} and 4 gm of resorcin in 200 ml of distilled water. Heat the mixture to boil. While boiling briskly add 25 ml of boiling 29% ferric chloride. Boil for 3 minutes and cool. A heavy precipitate forms. Filter and dry the precipitate in an oven at 50°C. Dissolve the precipitate in 400 ml of 70% alcohol. Shake the mixture well. To this add 4 ml of concentrated hydrochloric acid and 6 gm of phenol. Use the solution after 2 weeks for better results. The stain will last for months.

14.7.3.2. PROCEDURE

1. Dissect the animal in a medium of 0.9% saline solution to expose the neuroendocrine organs.
2. Fix *in situ* using 10% formaldehyde saline (Add 9 ml of 0.9% physiological saline to 10 ml of formaldehyde) for 1—2 minutes.
3. Remove the neuroendocrine organs, wash well with the fresh fixative and keep it in the fixative for 24 hours.
4. Wash well in tap water for 2 or 3 hours.
5. Rinse in distilled water 10—20 minutes.
6. Remove the water on the surface of the tissue with filter paper.
7. Oxidize the tissue with performic acid until it becomes transparent (5 minutes or more).
8. Wipe off the excess performic acid in the tissue with filter paper.
9. Wash thoroughly in distilled water for 20—30 minutes.
10. Dehydrate in 30% and 70% alcohols.
11. Stain the organs in Victoria blue solution for 12—18 hours (duration of staining depends on size of the organ).
12. Quickly blot off excess stain with filter paper.

13. Differentiate in 70% alcohol (3 or 4 changes until the excess stain is removed).
14. Dehydrate in 90% and absolute alcohols ; clear in cedarwood oil for 2—4 hours.
15. Rinse in xylene for 2—5 minutes to remove the cedar wood oil.
16. Mount in DPX.

14.7.3.3. RESULTS

Cell body—blue or greenish blue ; Proximal portion of axons—greenish blue ; Neurosecretory pathway—light greenish blue ; Storage and release organ—blue or dark blue ; Background—unstained or faint blue.

14.8. REFERENCES

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