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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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- undertake collaboration programmes ; and
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*Front cover* : SEM picture showing surface topography of *Streptocephalus dichotomus* egg.

# Manual of Research Methods for Marine Invertebrate Reproduction

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ISSUED ON THE OCCASION OF THE WORKSHOP ON MARINE  
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DEPARTMENT OF ZOOLOGY, UNIVERSITY OF MADRAS AND THE  
CENTRE OF ADVANCED STUDIES IN MARICULTURE, CENTRAL  
MARINE FISHERIES RESEARCH INSTITUTE HELD AT THE UNIVERSITY  
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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 brought forth 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 culturable 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.

**IDENTIFICATION AND CHARACTERIZATION  
OF VITELLOGENIN AND LIPOVITELLIN  
OF *SCYLLA SERRATA* AND *EMERITA ASIATICA*  
USING DISC GEL ELECTROPHORESIS\***

**3.1. INTRODUCTION**

The appearance of a sex limited plasmatic protein (FSP) in the mature crabs and other higher crustaceans is now well established. As early as 1954, Frenz observed the FSP in the blood of *Carcinus maenas* during vitellogenesis. This protein is considered to be the precursor of the main yolk protein of the egg. The occurrence of FSP in the blood is a secondary sexual feature inasmuch as they indicate the specific stage of vitellogenesis during gametogenic cycle. Earlier workers characterised the chemical nature of this protein to be a very high density lipoprotein in a few crustacean species (Wallace *et al.*, 1967; Fyffe and O'Connor, 1974). Identification of vitellogenin in the blood as well as the lipovitelin of the egg is important not only for studying the mode of yolk formation but also for identification of maturity stages in the females during the breeding season.

**3.2. PRINCIPLE**

The charged biological molecules depending on the pH and suspending medium migrate to the electrodes of opposite polarity in an electrical field. Polymerization of acrylamide and cross-linking reagent methylenebisacrylamide is done in the presence of a catalyst, ammonium persulphate. Tetramethylethylenediamine (TEMED) initiates and controls polymerization. The electrophoretic mobility of the glycinate ion is very much less. However in the pH of the

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separation gel (8.9) the mobility of glycine is greater than that of the protein, and hence the buffer always runs ahead the protein molecules. Bromophenol blue, used as the marker dye, having a low molecular weight, marks the boundary for the protein molecules and runs ahead the protein. Vitellogenin and lipovitellin of crustaceans being high density glycolipoprotein complexes are well separated on the polyacrylamide gel.

Acrylamide disc gel electrophoresis has been carried out according to Davis (1964) using his original stock and buffer systems. For fractionating the proteins of haemolymph and ovary of *S. serrata* both spacer and sample gels were omitted, since good separations were obtained without spacer and sample gels (Clark, 1964). In *S. serrata*, the haemolymph resolved into one to eight slow moving fractions, three distinct medium moving fractions and three to four fast moving fractions. This pattern however changes with the stage of ovarian maturity. Subhashini and Ravindranath (1981) have reported that the resolution of fractions is better when they used spacer gel for separating the haemolymph proteins of *S. serrata*.

### 3.3 Reagents

1. *Stock monomer solution* : Dissolve 25 gm of acrylamide with 0.735 gm of N, N-methylenebisacrylamide in 100 ml of double distilled water.
2. *Small pore buffer* : Dissolve 36.6 gm of Tris with 0.23 ml of TEMED in 48 ml of 1N hydrochloric acid and make it up to 100 ml with double distilled water. To prepare 1N hydrochloric acid dilute 9 ml of hydrochloric acid to 100 ml with double distilled water.
3. *Catalyst* : Dissolve 0.14 gm of ammonium persulphate in 100 ml of double distilled water.
4. *Stock reservoir buffer (pH 8.3)* : Dissolve 28.5 gm of glycine and 6 gm of Tris in 100 ml of double distilled water and make it up to 1000 ml with double distilled water.
5. *Working reservoir buffer (pH 8.3)* : Dilute 60 ml of stock reservoir buffer to 600 ml with double distilled water.



6. *Running gel (7%)*: Mix small pore buffer, monomer, water and catalyst in the ratio of 1 : 2 : 1 : 4 (in volume). Fix vertical tubes of 70 × 5 mm size into the polymerising stand. Pour the running gel mixture into these tubes without introducing any air bubble. Place a drop of water over the gel without disturbing the gel layer. Polymerization is purely chemical and after ensuring the polymerization, remove the water layer at the end of 30 minutes.
7. *1% Amido black*: Dissolve 1 gm of amido black in 100 ml of 7% acetic acid.
8. *0.25% Coomassie brilliant blue*: Dissolve 250 mg of Coomassie brilliant blue in a solution containing methanol, water and acetic acid in the ratio of 5 : 5 : 1 (Smith, 1968).
9. *Saturated solution of oil red 'O'*: Saturated solution of oil red 'O' is prepared in 50% methanol containing 10% TCA (W/V) (Smith, 1968 ; Kannupandi and Paulpandian, 1975).
10. *Destaining solution*: 7% acetic acid (Dilute 7 ml of acetic acid to 100 ml with double distilled water).

#### 3.4. PREPARATION OF SAMPLES

Directly collect a drop of haemolymph (0.034 ml) into a pre-chilled test tube containing 2 ml of 40% sucrose, after cutting the propodus or dactylus of one appendage so as to allow free bleeding. Collect fresh samples of *S. serrata* and *Emerita* ovary in different stages of its maturation and wash it with double distilled water to remove the adhering haemolymph. Wipe off the excess water by a filter paper.

Homogenize 10 to 20 mg of ovary in 40% sucrose, centrifuge and use the clear supernatant after removing the lipid cap.

#### 3.5. PROCEDURE

1. Introduce the sample (haemolymph or ovary homogenate) in all maturation stages of ovary of *S. serrata* and *Emerita* in 40% sucrose over the gel layer. To prepare 40% sucrose dissolve 40 gm of sucrose in 100 ml of double distilled water.

2. Remove the gel tubes by giving slight lateral shakes without damaging the polymerized bottom of the gel.
3. Insert the gel tubes with the sample into the rubber grommets in the upper buffer chamber by screwing in after moistening the rubber grommets.
4. Fill the lower buffer chamber with 250 ml of working reservoir buffer and the upper buffer chamber with 250 ml of working reservoir buffer, with 2 drops of bromophenol blue.
5. Supply a constant current of 3 mA/tube until the bromophenol blue reached the gel edge. Electrophoresis was done inside the refrigerator.

### 3.6. STAINING

#### 3.6.1. Simple proteins

Stain the gels in 1% amido black or 0.25% Coomassie brilliant blue. To stain in Coomassie brilliant blue prefix the gels in 10% TCA (Dissolve 10 gm of TCA in 100 ml of distilled water) for an hour.

*Destaining* : Repeatedly wash in 7% acetic acid and store in the same solution.

#### 3.6.2. Complex proteins

##### *Lipoproteins*

Stain the gels in oil red 'O' for about 4 hours and store the gels in the same staining solution.

##### *Glycoproteins*

Leach the gels in 7% acetic acid for an hour. Wash in double distilled water for an hour. Fix the gels in 1% periodic acid for an hour. (Dissolve 1 gm of periodic acid in 100 ml of 3% acetic acid). Wash in double distilled water for an hour. Stain in Schiff's reagent for 30 minutes (For preparation of Schiff's reagent refer Expt. No. 2).

**Destaining :** Repeatedly wash in 1% sodium metabisulphite and store in the same. (Destaining reagent is prepared by dissolving 1 gm of sodium metabisulphite in 100 ml of double distilled water).

All the staining procedures should be made inside the refrigerator.

#### *Haemocyanin*

Prepare a saturated solution of dithio-oxamide (rubeanic acid) in 5 : 2 : 5 ratio of methanol, glacial acetic acid and water (Horn and Kerr, 1969).

**Destaining :** Destain and store the gels in 7% acetic acid.

### 3.7. CALCULATION OF R<sub>m</sub> VALUES

After considerable destaining make the line drawings and calculate the relative mobility (R<sub>m</sub>) of each fraction.

$$R_m = \frac{\text{Distance travelled by the protein fraction}}{\text{Distance travelled by the bromophenol blue}}$$

Find out the histochemical nature of each protein and tabulate them.

### 3.8. DENSITOMETRIC ANALYSIS

Scan the gels in a densitometer so as to quantify each protein.

### 3.9. OBSERVATIONS

i) Compare the electrophoretic mobility as well as the staining properties of different protein fractions of the blood of male and female. Also compare the blood of mature female with the proteins of ovary in different stages of maturation in *Emerita* and *Scylla*.

ii) Observe the newly appearing proteins in the blood and their homologous bands in the ovary.

iii) Compare the electrophoretic mobility and histochemical characteristics of the sex limited protein of *S. serrata* with that of *E. asiatica* and other known decapod crustaceans.

### 3.10. REFERENCES

- CLARK, J. T. 1964. Simplified disc (Polyacrylamide gel) electrophoresis. *Ann. N. Y. Acad. Sci.*, 121 : 428-436.
- DAVIS, B. J. 1964. Disc electrophoresis II Method and application to human serum proteins. *Ibid.*, 121 : 404-427.
- FRENTZ, R. 1954. Electrophorese sur papier des proteines due serum de, *Carcinus pinnatifidus*. *C. R. Acad. Sci. Paris*, 239 : 1876-1868..
- FYFFE, W. E. AND J. D. O'CONNOR, 1974. Characterization and quantification of a crustacean lipovitellin. *Comp. Biochem. Physiol.*, 47 : 851-867.
- HORN, E. C. AND M. S. KERR, 1969. The haemolymph proteins of the blue crab *Callinectes sapidus* II Hemocyanins and certain other major protein constituents. *Ibid.*, 29 : 493-508.
- KANNUPANDI, T. AND AL. PAULPANDIAN, 1975. Studies on blood and muscle protein of crabs of Porto Novo. *Bull. Dept. Mar. Sci. Univ. Cochin*, 7 : 609-622.
- SMITH, I. 1968. Acrylamide gel disc electrophoresis. Sec. 1. Techniques of disc electrophoresis. In, 'Chromatographic and electrophoretic techniques' (Ed. Smith, I.) Vol. 1, pp. 104-147. William-Heinemann Medical Books Ltd., London.
- SUBHASHINI, M. H. AND M. H. RAVINDRANATH, 1981. Electrophoretic separation of proteins. *Manual of Research Methods for Crustacean Biochemistry and Physiology*, CMFRI Special Publication No. 7, pp. 103-114.
- WALLACE, R. A., S. L. WALKER AND P. V. HAUSCKKA, 1967. Crustacean lipovitellin. Isolation and characterization of the major high density lipoprotein from the eggs of decapods. *Biochemistry*, 5 : 1582-1598.