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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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—improve the quality of post-graduate education in mariculture ;

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

—enhance the competance of professional staff ;

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

—undertake collaboration programmes ; and

—organise seminars and workshops.

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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 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.

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SEROLOGICAL IDENTIFICATION OF VITELLOGENIN AND LIPOVITELLIN IN *SCYLLA SERRATA* AND *EMERITA ASIATICA* USING IMMUNO ELECTROPHORESIS*

4.1. INTRODUCTION

Vitellogenin is the blood protein precursor of lipovitellin, the main yolk protein. Many crustacean workers have used electrophoresis to detect the vitellogenin and lipovitellin in the blood and ovary respectively. However, authenticity of identical relative mobilities in homologizing vitellogenin with lipovitellin has been sometimes questioned because fluctuating current, buffer strength and gel composition may lead to variations in the relative mobility of the same component. It is therefore necessary to confirm the results of electrophoresis by serological investigations. Immunoelectrophoresis has been tried for studying the relationship between lipovitellin and vitellogenin in a few crustacean forms (Kerr, 1969 ; Croisille *et al.*, 1970 ; Ezhilarasi, 1982). Many of the crustacean blood proteins are immunogenic and produce antibodies in the mammalian blood. Therefore, cross reaction of these antibodies with the suspected identical proteins will produce specific precipitation arcs thus enabling real comparison. In addition, this method is advantageous in tracing the origin of vitellogenin into the extraovarian sources, even if the concentration of such precursors is very low.

4.2. PRINCIPLE

Antibodies to vitellogenin and lipovitellin are raised by active immunization by injecting the antigen directly to albino rabbits. Antigen-antibody reaction is carried out in the gelified medium either in agarose/agarose or polyacrylamide/agarose and bands of precipitation form wherever an antibody and its corresponding

* Prepared and verified by S. Ezhilarasi and T. Subramoniam, unit of Invertebrate Reproduction, Department of Zoology, University of Madras-600 005.

antigen meet at the optimal proportion. Both vitellogenin and lipovitellin of *S. serrata* are immunogenic in that, they give rise to antibodies that will specifically react with them. When these soluble antigen and antibody diffuse towards each other in a gel, a precipitin line is formed at their place of meeting and form an impermeable barrier to the antigen and antibody that has formed it. This is permeable to all other substances that are not concerned with that precipitate in question. However, this barrier persists only as long as some of the forming ingredients are present in the gel on either side of it.

4.3. PROCEDURE

4.3.1. Preparation of antiserum

4.3.1.1. *Rabbit anti-lipovitellin antiserum*

1. Homogenize 500 mg of freshly laid eggs of *S. serrata* and *E. asiatica* (0.0167 gm of protein) in *Carcinus maenas* Ringer solution (Smith and Ratcliffe, 1978). To prepare *C. maenas* Ringer solution dissolve 33.7 gm sodium chloride, 0.94 gm pottassium chloride, 2.83 gm calcium chloride, 5.38 gm magnesium chloride, 0.193 gm disodium hydrogen phosphate and 6.060 gm Tris (hydroxymethyl) methylamine in 42.5 ml of 1 M hydrochloric acid and make it upto 1 litre. The pH is adjusted to 7.4.

2. Centrifuge at 5,000 g and decant the supernatant without the lipid cap. This clear supernatant will serve as the source of antigen.

3. Mix 1 ml of antigen and an equal volume of Freund's complete adjuvant (Difco).

4. Inject this mixture subcutaneously on 1st, 14th, and 21st days.

5. Third injection is a booster injection having double the quantity of antigen.

4.3.1.2. *Rabbit anti-haemolymph containing female specific protein (FSP) antiserum*

1. Collect 2 ml of haemolymph containing FSP (0.9074 gm protein) from *S. serrata* in late IVth stage or early Vth stage of ovarian maturation. Add this to a mixture containing 1 ml

of 12.5% sodium citrate and 1ml of *C. maenas* Ringer solution (To prepare sodium citrate solution dissolve 12.5 gms of it in 100 ml of double distilled water).

2. Mix 2 ml of haemolymph with 2 ml of Freund's complete adjuvant and inject into the rabbits subcutaneously as described above.

4.3.1.3. *Rabbit anti-control antiserum*

Prepare the carrier medium by mixing 0.67 ml of *C. maenas* Ringer with 0.33 ml of 12.5% sodium citrate. Mix 1 ml of this with 1 ml of Freund's complete adjuvant and inject subcutaneously.

4.3.2. Collection of antiserum

1. Collect rabbit blood by puncturing ear vein on 27th day. Collect blood in the same way on 34th day also, into a sterilized boiling test tube.

2. Plug the tubes with cotton and leave it overnight in the refrigerator slantingly.

3. Collect the separated serum into sterilized screw cap vials and store in refrigerator.

4.3.3. Purification of antiserum

1. Mix 1 ml of antiserum with 1 ml of freshly collected male haemolymph in *C. maenas* Ringer.

2. Centrifuge at 6,780 g for 10 minutes to precipitate the reaction products formed by the interaction between the common antigens of male *S. serrata* and *E. asiatica* haemolymph and antibodies of female haemolymph containing FSP antiserum.

3. Mix the supernatant with 1 ml of male haemolymph repeatedly after each centrifugation, till no visible precipitate was formed (Fyffe and O'Connor, 1974).

4.3.4. Preparation of agarose gels

1. Prepare 1 litre of 0.015 M Tris-barbital buffer at pH 8.8 using a glass electrode pH meter.

2. Boil 1 gm of agarose in 100 ml of 0.015 M Tris-barbital buffer at pH 8.8 with 0.1 mg of methiolate. Methiolate avoids bacterial growth.

3. Evenly spread 2 ml of this mixture on a clean microscopic slide and allow to solidify.

4. Make a central trough and two side wells prior to the application of antigen.

4.3.5. Preparation of Polyacrylamide gels

(*vide* Expt. No. 3.)

4.3.6. Separation of antigen proteins by agarose gel electrophoresis (Fig. 1)

1. Remove the slice of agarose on the well and deposit haemolymph collectd in 40% sucrose or ovary homogenized in 40% sucrose (haemolymph and the ovary of *S. serrata* and *E. asiatica* are collected from different stages of ovarian maturation).
2. Before application mix the samples with the marking dye, bromophenol blue.
3. Fill the electrophoretic tank with 0.06 M Tris barbital buffer at pH 8.8. Place agarose smeared glass slides in the tank.

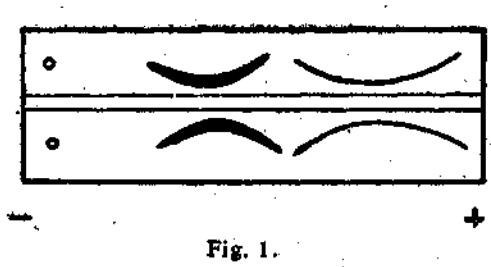

Fig. 1.

Immunoelectrophoresis: Both fractionation and diffusion of antigen is carried out in agarose: *Antigen*: Female matured *S. Serrata* haemolymph containing FSP. *Antibody*: Rabbit anti FSP antiserum.

Note the thick precipitate arc formed against FSP. Thin precipitin arc corresponds to haemocyanin fractions. (Slow moving fractions are not represented).

4. Prepare two paper wicks with Whatman No. 1 filter paper and place it on the two edges of the glass plate dipping into the buffer solution, so that the gel is connected to the buffer by the paper wicks.
5. Supply 1.6 mA/slide till the marker dye reaches the anodic end of the slide.
6. After the completion of electrophoresis switch off the current, remove the paper wick and slide.
7. Remove the agarose gel in the centre to have a longitudinal groove and procure appropriate immuno serum into it.
8. Incubate the slides in a humid chamber to allow immunodiffusion at 34°C for 24 hours.

4.3.7. Separation of antigen proteins by polyacrylamide gel electrophoresis

(*vide* Expt. No. 3 ; Fig. 2).

4.3.8. Transfer method (Fyffe and O'Connor, 1974)

1. Transfer the polyacrylamide gels containing the resolved antigenic proteins onto a slide.
2. Mix 0.5 ml of antiserum with two ml of agarose-buffer mixture and pour it around polyacrylamide gel.
3. Incubate the slides in a humid chamber to allow immunodiffusion at 34°C for 24 hours.
4. Note the precipitin arcs developed as white curved lines after the incubation period (Fig. 3).
5. Wash the gels with 1% saline for about 16 to 20 hours with frequent changes of saline and wash finally in double distilled water for an hour to remove saline.
6. Place the gel slides horizontally and filter paper strips over the gel to absorb excess water in the gel. After drying, filter paper comes out of the gel of its own.
7. Stain the gels with 0.1% amido black (W/V) for 10 minutes and destain them in 2% acetic acid till the gel becomes transparent and the arcs are clearly visible.

Fig. 3.

Polyacrylamide disc fractionation of *S. serrata* haemolymph and ovarian proteins.

1. Male *S. serrata* haemolymph. 2. Female immature *S. serrata* haemolymph. 3. Female maturing *S. serrata* haemolymph. 4. Female mature *S. serrata* haemolymph. Arrow indicates the appearance of female specific protein (FSP) in late stage IV and early stage V. 5. Mature ovarian proteins of *S. serrata*. Note the homologous proteins in the ovary and haemolymph.

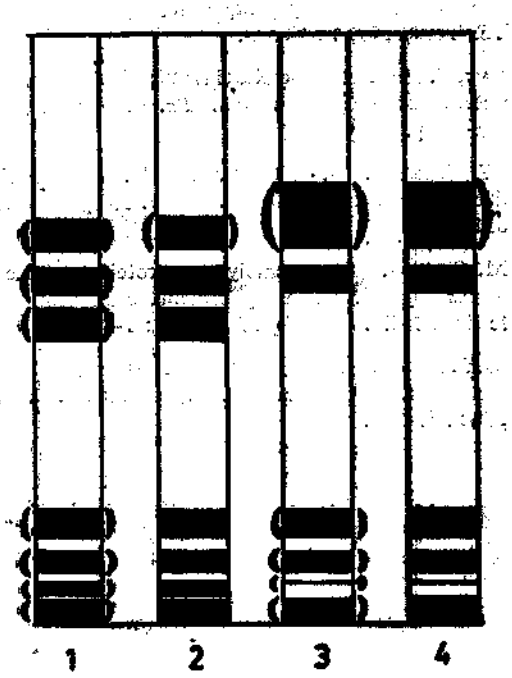

Fig. 3.

Immunoelectrophoresis: Transfer method. Antigens separated on polyacrylamide is diffused through agarose containing antiserum.

1. Female *S. serrata* containing FSP × Rabbit anti haemolymph containing FSP antiserum. 2. Female *S. serrata* containing FSP × Rabbit antilipovitellin antiserum. 3. Freshly laid egg proteins of *S. serrata* × Rabbit anti haemolymph containing FSP antiserum. 4. Freshly laid egg proteins of *S. serrata* × Rabbit anti-lipovitellin antiserum. (Slow moving fractions are not represented as they are not immunogenic).

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