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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 :

—provide adequate facilities to carry out research of excellence in mariculture/coastal aquaculture ;

—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.

IV. BIOCHEMISTRY OF SEMINAL SECRETIONS

12

BIOCHEMICAL ANALYSES OF SEMINAL PLASMA AND SPERMATOPHORES OF *SCYLLA SERRATA**

12.1 Introduction

A volume of information is available on the nature, origin and role of seminal plasma in mammals (Mann, 1964 ; Hafez, 1976). Among invertebrates such information is limited to insects and echinoderms only. Cirripedes are the only crustacean group that has received sufficient attention on the biochemistry of seminal plasma (Barnes and Blackstock, 1974). Decapods, including many economically important crustacean species, have not received any attention with regard to seminal biochemistry. Hence, the commercially important brachyuran crab, *S. serrata* has been chosen for the analysis of important chemical components of seminal secretion. As the crabs of bigger size range are found to contain more quantity of seminal secretion in the mid vas deferens (Uma, 1982), only the crabs in the size range of 14-16.5 cm are used.

12.2 Method of Collection of Seminal Plasma and Spermatophores

1. Separate the mid vas deferens from the reproductive system of crabs (14-16.5 cm) and keep them in a Petri-dish.
2. As the seminal contents coagulate immediately, pipette out the luminal contents using a micropipette into a centrifuge tube.
3. Centrifuge the contents for 5 minutes at 3000 rpm at 4°C to separate the seminal plasma and spermatophores. The

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

spermatophores will sediment along with a small quantity of seminal fluid.

4. To separate and wash the spermatophores, mix the bottom sediment with distilled water and centrifuge for 3—5 minutes at 3000 rpm.

12.3. Estimation of Protein (Lowry *et al.*, 1951)

12.3.1. Principle

The carbamyl groups of protein react with the copper ion of the alkali and when this complex reacts with phosphomolybdic acid of Folin reagent get reduced with tyrosine and tryptophan.

12.3.2. Reagents

1. 80% *Ethanol* : Dilute 80 ml ethanol with 20 ml of double distilled water.
2. 0.1 *N Sodium hydroxide* : 400 mg of sodium hydroxide in 100 ml distilled water.
3. 1 *N Sodium hydroxide* : 4 gms of sodium hydroxide in 100 ml of double distilled water.
4. *Solution A* : 2 gm of sodium carbonate in 100 ml of 0.1 *N* sodium hydroxide.
5. *Solution B* : 500 mg of copper sulphate in 1% sodium tartrate. (1 gm of sodium tartrate in 100 ml of double distilled water).
6. *Solution C*: Mix 50 ml of solution A with 1 ml of solution B and treat that as alkaline copper solution.
7. *Folin-ciocalteu phenol* : Mix 1 ml of Folin phenol with 1 ml of double distilled water.
8. *Standard* : Dissolve 1 mg of bovine serum albumin in 10 ml of 1 *N* sodium hydroxide and make up to 100 ml in a standard flask.

12.3.4. Procedure

1. Take 0.1 ml of seminal plasma and add 1 ml of 80% ethanol to precipitate the protein.

2. Centrifuge for 5 minutes at 4000 rpm to get a clear supernatant.
3. Dissolve the precipitate in 1*N* sodium hydroxide and make up to 10 ml with the same.
4. Take 1 ml from this and treat with 5 ml of solution C for 10 minutes.
5. Add 0.5 ml of Folin-ciocalteu reagent.
6. Read the colour intensity after 20 minutes at 700 nm.
7. Standard—From the standard take 1 ml.
8. Blank—Take 1 ml of 1*N* sodium hydroxide.

12.3.5. Calculation

$$\frac{\text{O.D. of unknown}}{\text{O.D. of known}} \times \mu g \text{ of standard} = \mu g \text{ of protein in ml of seminal plasma}$$

12.4. Estimation of Carbohydrate (Roe, 1955)

12.4.1. Principle

Sulphuric acid hydrolyses the di-and oligosaccharides into monosaccharides and converts the monosaccharides into furfural or furfural derivatives, which react with anthrone and produces a complex coloured product.

12.4.2. Reagents

1. 80% *ethanol*
2. *Anthrone reagent* : Dissolve 50 mg of anthrone powder in 100 ml of 66% sulphuric acid, to this add 1 gm of thiourea to stabilize the colour.
3. *Standard* : Dissolve 1 mg of glucose in 10 ml saturated benzoic acid.

12.4.3. Procedure

1. Take 1 ml of the sample, add 1 ml 80% ethanol ; centrifuge it. Take 0.5 ml of clear supernatant and add 5 ml of

anthrone. Keep the tubes in the boiling water bath for 15 minutes.

2. Bring the tubes to the dark to protect from the light.
3. Record the absorbancies at 620 nm.
4. Standard—Take 0.5 ml of standard.
5. Blank—Take 0.5 ml of 80% ethanol.

12.4.4. Calculation

$$\frac{\text{O.D.of unknown}}{\text{O.D. of known}} \times \mu g \text{ of standard} = \mu g \text{ of carbohydrate in ml seminal plasma}$$

12.5. Estimation of Lipid

12.5.1. Principle

The quantitative determination of lipid by sulphophosphovanillin method depends on the reaction of lipids extracted from the sample using chloroform - methanol, with sulphuric acid, phosphoric acid and vanillin to give a red complex.

12.5.2. Reagents

1. *Chloroform : methanol* (2 : 1) : Mix 20 ml of chloroform with 10 ml of methanol.
2. 0.9% *sodium chloride* : Dissolve 900 mg of sodium chloride in 100 ml of distilled water.
3. *Phosphovanillin reagent* : Add 800 ml of orthophosphoric acid to 200 ml of distilled water. Dissolve 2 gm of vanillin in this solution.
4. *Standard* : Dissolve 8 mg of cholesterol in 4 ml of chloroform : methanol (2 : 1).

12.5.3. Procedure

1. Take 1 ml of seminal plasma and extract the lipid following the method of Folch *et al.* (1957).
2. To 1 ml of seminal plasma, add 1 ml of methanol and 2 ml of chloroform and to which add again 2 ml of chloroform methanol (2 : 1 V/V). Mix thoroughly.

3. To this add 0.2 volume of 0.9% sodium chloride solution. Pour this into a separating funnel and mix it. Allow it to stand for few hours.
4. Separate the lower phase into a clean tube. Make up the volume of the lower phase to the original quantity of chloroform added before.
5. To estimate the lipid quantity (Barnes and Blackstock, 1973) measure 0.5 ml of extract into a clean test tube. Allow it to dry in a vacuum desiccator over silica gel; dissolve in 0.5 ml of concentrated sulphuric acid. Mix well. Plug the tubes with non-absorbant cotton wool. Place in a boiling water bath for 10 minutes. Cool the tubes to room temperature.
6. Take 0.2 ml of this acid digest in a separate tube; add 5 ml of vanillin reagent. Mix well and allow to stand for half an hour. Measure the developed colour at 520 nm.
7. Standard—Take 0.2 ml of standard.
8. Blank—Take 0.2 ml of chloroform, allow it to evaporate.

12.5.4. Calculation

$$\frac{\text{O.D. of unknown}}{\text{O.D. of known}} \times \mu g \text{ of standard} = \mu g \text{ of lipid in ml seminal plasma}$$

12.6. Estimation of Ascorbic Acid (Roe, 1961)

12.6.1. Principle

The ascorbic acid is converted to dehydro-ascorbic acid by shaking with Norit and this is coupled with 2—4 dinitrophenyl hydrazine in the presence of thiourea as a mild reducing agent. Sulphuric acid then converts the dinitrophenyl hydrozone into a red compound which is analysed colorimetrically.

12.6.2. Reagents

1. *6% TCA*: Dissolve 6 gm of TCA in 100 ml of distilled water.
2. *4% TCA*: Dissolve 4 gm of TCA in 100 ml of distilled water.

3. *Acid washed Norit:* Place 200 gm of Norit in a large flask and add 1 litre of 10% HCl. Heat to boiling; then filter with suction. Remove the cake of Norit to a large litre of double distilled water, stir thoroughly, and filter. Repeat this procedure once. Dry the Norit overnight in an oven at 100—120°C.

4. *Dinitrophenyl hydrazine—Thiourea reagent*: Dissolve 2 gm of 2, 4—dinitrophenyl hydrazine in 100 ml 9N H_2SO_4 (3 parts of H_2O to 1 part of concentrated H_2SO_4). Add 4 gm of reagent grade thiourea, shake occasionally. This reagent should be freshly prepared. It should be checked for the presence of active reducing agent. To do this, place 2 ml of 1% $HgCl_2$ in a test tube and add the reagent drop wise. The addition of 3—5 drops will produce a copious precipitate of $HgCl_2$ if adequate thiourea is present.

5. 85% *sulphuric acid*: Add 85 ml of sulphuric acid to 15 ml distilled water slowly.

6. *Standard*: 1 mg of ascorbic acid in 10 ml of 4% TCA. Take 1 ml from this and dilute to 6 ml.

12.6.3. Procedure

1. To 1 ml of pooled seminal plasma, add 6 ml of 6% TCA. Stir thoroughly.
2. Centrifuge to get a clear supernatant.
3. Add 300 mg of acid washed Norit to the supernatnat. Shake vigorously and filter.
4. To 2 ml of filtrate, add 0.5 ml of dinitrophenyl hydrazine.
5. Incubate the tubes for 3 hours at 37°C after stoppering them. Cool the tubes by keeping them in the ice water bath.
6. Add 2.5 ml of 85% sulphuric acid drop by drop to avoid sudden rise in temperature. Shake the tubes thoroughly.
7. After half an hour bring the tubes to room temperature.

8. Record the absorbancy of the colour developed at 540 nm.
9. Control : For each sample use 2 ml of filtrate without dinitrophenyl hydrazine.

12.6.4. Calculation

$$\frac{\text{O.D. of unknown}}{\text{O.D. of known}} \times \mu\text{g of standard} = \mu\text{g of ascorbic acid in ml of seminal plasma.}$$

12.7. Separation of Free Aminoacids Using Two Dimensional Descending Paper Chromatography

12.7.1. Principle

The stationary phase acts as adsorptive force which binds with substances by hydrogen bonding or Vander waals force or ionic exchange. Non polar solvent in the mobile phase acts as a driving force and partitioning force.

12.7.2. Reagents

1. 80% *ethanol* :
2. *Solvent I* : Prepare by mixing butanol 120 ml ; acetic acid 30 ml and distilled water 50 ml.
3. *Solvent II* : Dissolve 160 gm of phenol in 40 ml of distilled water and to this add 1 ml of ammonia. 0.2% *ninhydrin solution*: Dissolve 200 mg of ninhydrin in 100 ml of acetone.

12.7.3. Procedure

1. Add 1 ml of 80% ethanol to 0.3 ml of seminal plasma.
2. Centrifuge and separate the supernatant. Again add 0.5 ml of 80% ethanol to the supernatant in order to check whether the supernatant is free from protein.
3. Centrifuge again to see whether there is any precipitate.
4. Add 3 volumes of chloroform to the clear supernatant.
5. Pipette out the aqueous phase alone into a cleaned dried small vials.

6. Spot the sample following the method of Smith (1968) on Whatmann No. 1 chromatographic paper and allow to run in the solvent system I, for the first direction and for the second direction use solvent system II.
7. Dry the paper and dip it in the 0.2% ninhydrin solution to locate the spots.
8. Again dry the paper in air and in oven at 105°C for 10—15 minutes.
9. Mark all the spots. Determine the Rf value and identify the spots by comparing with authentic standards.

12.8. References

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