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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

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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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THIN LAYER CHROMATOGRAPHIC SEPARATION OF LIPIDS IN OVARY, TESTIS AND GUT OF THE SEA URCHIN *SALMACIS VIRGULATA**

7.1. INTRODUCTION

Sea urchins accumulate large amounts of lipid in ovary during its reproductive cycle (Giese, 1966 ; Vivek Raja, 1980). Lipids deposited in the developing gonads may be synthesized within the oocytes or transported from the gut. A variety of lipid classes are also found to occur in the ovary, testis, gut, body wall and coelomocytes of the sea urchins (Allen, 1974 ; Vivek Raja, 1980). The present experiment is designed to separate and identify the different lipid classes present in gut, testis and ovary of the sea urchin *Salmacis virgulata* employing thin layer chromatographic method.

7.2. PRINCIPLE

Chromatography is a separation process based on differential distribution between two immiscible phases one of which moves relative to the other. The element of motion may be provided not by movement of phase but by movement within a phase, as in electromigration. In thin layer chromatography (TLC) silica gel placed on the glass plate acts as stationary phase. The mixture of compounds to be separated is placed near one end of the TLC plate and allowed to dry. Then the TLC plate is placed into a chromatographic chamber containing a relevant solvent system (mobile phase). As the solvent travels towards the far end of the plate, by adsorption and capillary action, the test mixture separates into various components. When the solvent reaches the far end of the plate, the plate is removed, rapidly dried and the spots are detected using a suitable location reagent (Stahl, 1958 ; Smith and Ersser, 1976).

* Prepared and verified by P. Vivek Raja, Department of Zoology, Govt. Arts College, Nandanam, Madras-600 035.

7.3. Materials

Immature and ripe ovary, testis and gut of sea urchin *Salmacis virgulata.*

7.4. Reagents

1. 0.9% *Sodium chloride*: Dissolve 900 mg of sodium chloride in 100 ml of distilled water
2. *Silica gel-G*
3. *Solvent—I for neutral lipid separation*: Diethyl ether—benzene—ethanol—acetic acid. Mix in the proportion of 40 : 50 : 2 : 0·2 (V/V)
4. *Solvent II for neutral lipid separation*: Diethyl ether—hexane (6 : 94 ; V/V)
5. *Solvent for phospholipids separation*: Chloroform—methanol—water (65 : 25: 4; V/V)
6. *Iodine crystals.*
7. *Standard neutral lipids.*
8. *Standard phospholipids.*

7.5. Procedure

7.5.1. Extraction of lipid (Folch *et al.*, 1957)

1. Take 500 mg of wet tissue in a homogenizer.
2. Add 10 ml of chloroform/methanol (2 : 1 V/V) solution and homogenize it well with teflon or glass homogenizer.
3. Filter the homogenate through a Whatmann No. 1 filter paper.
4. Add 2 ml of salt solution (0.9% aqueous sodium chloride) and shake well.
5. Transfer the mixture to a small separating funnel and allow it to stand overnight at 4°C.
6. A clear biphasic layer will form. The lower phase contains all the lipids. Remove the lower phase and adjust the volume to 10 ml by the addition of chloroform.

7. Take 5 ml in a watch glass and allow the solvent (chloroform) to evaporate at room temperature (preferably 50-60°C) for five hours. Then dry the same *in vacuo* over silica gel for 7 days. Weigh the lipid and calculate the percentage of lipid present in the tissue.

8. From the remaining 5 ml a known quantity (50-100μl) may be used for spotting on TLC plate using micropipette.

7.5.2. Preparation of plate

1. Mix 20 gm of silica gel-G with 40 ml of water and stir well until a fine slurry without lumps is obtained.

2. Adjust the TLC spreader to 0.3 mm thickness and pour the aqueous slurry into the spreader.

3. Draw the spreader from one end of the unit to the other end at an even rate over the glass plates (20 × 20 cm).

4. Leave the plates to dry in air for 30 minutes.

5. Keep the plates horizontally in the oven at 105°C for 1 hour.

6. Remove the plates from the oven and keep them in room temperature for 15 minutes.

7. Once the temperature of the plates reduces to the room temperature it is ready for spotting.

7.5.3. Spotting (Application of sample on the TLC plate)

For the best separation and resolution the sample dissolved in chloroform-methanol is best applied in the form of a band rather than a spot. Single spots tend to 'tail'. The band, usually spread over 2-2.5 cm can be applied dropwise with a micropipette. The solvent may need evaporating from time to time to allow more sample to be added over a small area. A stream of nitrogen gas or hair drier can be used for this purpose. A sample applicator is more convenient and has the advantage of even loading (Lake and Goodwin, 1976).

7.5.4. Neutral lipids (Freeman and West, 1966)

1. Pour 100 ml of solvent—I (Diethyl ether—benzene—ethanol—acetic acid 40 : 50 : 2 : 0.2; V/V) in the tank and close it with the lid. Leave the tank without disturbance for 30 minutes.
2. Keep the spotted plate, vertically in the tank. Care should be taken that there is about 1 cm distance between the level of irrigating solvent and spots (*i.e.* point of application of sample) on the plate.
3. Allow the irrigation solvent to run a distance of 12 cm from the spot (origin).
4. Remove the plate from the tank, allow it to dry in room temperature for 15 minutes and keep it in oven at 40°C for 5 minutes to remove the traces of acetic acid.
5. Then keep the plate in another tank containing 100 ml of solvent II (Diethyl ether—Hexane, 6 : 94 V/V).
6. Allow the solvent to run up to 15 cm from the spot.
7. Remove the plate from the tank and dry it at 60°C for 30 minutes.
8. Keep the plates in a glass chamber with iodine vapour. Clear spots will appear on the plate. Take photograph and calculate the R_f values :

$$Rf = \frac{\text{distance substance travels from the origin}}{\text{distance solvent front travels from the origin}}$$

Compare the Rf values of sample with the standard neutral lipids run in the same plate or in different plates in the identical condition.

7.5.5. Phospholipids (Allen, 1974)

1. Pour 100 ml of chloroform—methanol—water (65 : 25 : 4; V/V) in the tank and close it with the lid. Leave the tank undisturbed for 30 minutes.
2. Keep the plate in the tank and allow the irrigation solvent to run a distance of 16 cm from the spot.

3. Remove the plate from the tank and dry at 60°C for 30 minutes.
4. Keep the plate in a glass chamber with iodine vapour. Clear spots appear on the plate. Take photograph and calculate the R_f values.

7.6. Observation

Identify the neutral and phospholipid fractions by comparing the R_f values of the samples with the standard lipids, developed under the identical conditions. The carotenoid pigments exhibit yellow colour before the treatment with the iodine vapour. Hence they should be marked and identified before keeping the TLC plate in the iodine vapour. Draw the line diagram of the TLC plate showing the different lipid fractions.

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