CMFRI SPECIAL PUBLICATION
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.

Under the programmes of the Centre, post-graduate courses leading to M.Sc. (Mariculture) and Ph.D. are offered in collaboration with the University of Cochin since 1980.

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.

(LIMITED CIRCULATION)

Published by: E. G. SILAS
Director
Central Marine Fisheries
Research Institute
Post Box No. 1912
Cochin 682 018

PRINTED IN INDIA
AT THE DIOCESAN PRESS, MADRAS-7—1982 C3626

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.

6

ESTIMATION OF CAROTENOIDS IN THE OVARY OF THE EDIBLE CRAB *SCYLLA SERRATA**

6.1. INTRODUCTION

Carotenoids, the yellow-red pigments found in plants and animals are C_{40} H_{56} compounds with 8 polyene isoprenoid residues, aliphatic or alicyclic in structure and with many conjugated double bonds. β-carotene is the starting point for the range of carotenoids in the animals. These are primordial plant pigments evolved along with the chlorophyll system acting as an accessory light-harvesting system not known to be synthesised by animal tissues. Entering animals exclusively through food, β-carotenes may be oxidized in the tissues into ketocarotenoids (Cheeseman *et al.*, 1967) best exemplified in the ovarian tissue of decapods. The normal pathway is β-carotene——→isocryptoxanthin——→echinenone——→canthaxanthin——→astaxanthin. In the Brachyura, the carotenoids may also be deposited in protein complexes in the integument or may be dissolved in lipids in the ovary and hepatopancreas or form carotenoglycolipoprotein complexes in the hemolymph (Zagalsky *et al.*, 1967). In *Eupagurus bernhardus* and *Clibanarius erythropus*, astaxanthin-protein complexes are found in the exoskeleton (Goodwin, 1952). In *Emerita analoga*, Gilchrist and Lee (1972) found a differential distribution of carotenoids in the different coloured eggs. *Eg*, Orange eggs (stage 1) have lipocarotenoprotein complexes while brown eggs (stage 2) have carotenoprotein complexes.

The function of carotenoids in decapod systems is as varied as the number of forms in which they are present. The unenolised keto groups of carotenoids are essential to complex formation. Experiments of Zagalsky *et al.* (1967) have shown carotenoids

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* Prepared and verified by Sudha Varadarajan, Zoological Survey of India, Madras-600 004.

to act as a powerful lock to the tertiary and quarternary structure. Carotenoprotein complexes protect tissues against photodamage as well as lipid peroxidation (Mathews-Roth *et al.*, 1974). Carotenes can act as substitutes to vitamin A in invertebrate tissues. Ceccaldi and Martin (1969) suggested that carotenoids can act as carriers for other macromolecules from hepatopancreas to ovary. The tail fin-pattern in the penaeid post-larvae of the Madras brackish waters, provides a reliable criterion for identification of the post-larval stages (Muthu, 1978).

6.2. Principle

Carotenoids, being lipids containing in solution coloured hydrocarbons, are soluble in light petroleum, ether, benzene, chloroform, carbon-tetrachloride, alcohol and acetone. Suspension of carotenoid- containing tissue in any of these solvents extracts carotenoids. Bound carotenoids are released from complexes by addition of protein denaturing agents followed by a chosen lipid solvent. The extract is diluted to a known volume before measuring the maximum absorption in that solvent for β-carotene (*Eg.* absorption at 450 nm in chloroform).

6.3. Material

1 gm of tissue in every developmental stage of ovary, egg and corresponding hepatopancreatic tissue.

6.4. Reagents (Olson, 1979)

1. *Anhydrous sodium sulphate*, reagent grade.
2. *Chloroform* stabilized with 0.75% absolute ethanol,
3. *Crystalline β-carotene* (E-Merck).

6.5. Procedure

6.5.1. Sample Storage

Samples should be quickly removed from the animals, placed in small glass vails closed with teflon stoppers or with screw-caps lined with aluminium foil, frozen at -20°C and stored until analysis. Samples should be analysed within a week after storage.

6.5.2. Sample Extraction (Olson, 1979)

Quickly weigh 1 gm of tissue on a tared piece of aluminium foil to the nearest 10 mg, place in a 10 ml screw cap clean glass vial. Add 2.5 gm of anhydrous sodium sulphate. Gently mash the sample with a glass rod against the side of the vial until it is reasonably well-mixed with the sodium sulphate. Cover with 5 ml of chloroform. Seal the glass vial and place at 0°C overnight (8-24 hours). The chloroform should form a clear 1-2 cm layer above the caked residue. Prepare a blank in a similar manner.

6.5.3. Spectrophotometric Analysis

Take an aliquot of 0.3 ml of the chloroform extract and dilute to a volume of 3 ml with ethanol. Treat the blank in a similar manner. Transfer to 1 cm cuvette (4 ml capacity) and read absorption in a spectrophotometer at 290, 350, 380, 450, 475 and 500 nm. Plot the readings on a graph.

6.5.4. Calculation

The total carotenoid content is calculated as μg carotenoids gm tissue :

$$\frac{\text{Absorption at 450 nm} \times \text{dilution factor}}{0.25 \times \text{sample wt. (gm)}}$$

Dilution factor in this experiment : 50
0.25 = Extinction coefficient.

6.5.5. Comments on Procedure (Olson, 1979)

Mashing should not be done too well, the mixture of sample and sodium sulphate should never be ground to a fine powder because

a) aerosol from the powder poses a health problem, and

b) the chloroform layer may become cloudy with fine powdering interfering in assaying.

Use manual pipettes for all transfers. Do not use ground glass stoppered vials as they leak even if taped. Use only screw-cap vials and seal tubes hermetically to avoid loss of solvent during the 8-24 hr. extraction period.

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