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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.

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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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FERTILIZATION AND EARLY DEVELOPMENT IN SEA URCHIN*

24.1. INTRODUCTION

Echinoderms have served more extensively than any other group of animals for the investigation of basic problems of fertilization and early development. It was with sea urchins that Hertwig (1875) first effectively demonstrated the principal features of fertilization : the incorporation of the sperm into the egg and the fusion of sperm pronucleus with egg pronucleus. Sea urchins are exclusively marine organisms and the fertilization is external. Due to the semitransparent nature of the egg and less yolk content, the process of fertilization and early development can easily be observed in the living condition. In addition, the fertilization and development in sea urchin could be accomplished in the laboratory under relatively simple conditions.

In sea urchins, the cleavage is complete and equal, hence it is called *equal holoblastic cleavage*. During the process of cleavage the successive cleavage planes cut straight through the egg, at right angles to one another and the resultant blastomeres become symmetrically disposed around the polar axis. When the egg is viewed from either pole, the blastomeres are found to be arranged in a radially symmetrical form. This pattern of cleavage is called as *radial cleavage*.

24.1. PRINCIPLE

As soon as the sperm enters the egg, the vitelline membrane rises to become the fertilization membrane. A few minutes after sperm entry, the sperm head begins to swell, and changes into the sperm pronucleus. At the same time the middle piece breaks

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down and releases the centriole, around which egg cytoplasm forms the sperm monaster. The centriole stays close to the sperm pronucleus and lies at the center of the monaster which becomes larger as its rays elongate. When the ray tips reach the egg pronucleus, it suddenly moves toward the monaster centre and the sperm pronucleus. The two pronuclei remain in contact and unite to form a single nucleus, the synkarion. After syngamy the egg may divide synchronously ten times. During cleavage the cells become arranged in the form of a hollow sphere, the blastula. Embryos escape from the fertilization membrane after the tenth cleavage. The blastula pass through gastrulation, prism stage, pluteus stage, late pluteus stage, metamorphosis and young sea urchin to reach the adult stage (Okazaki, 1975).

24.3. Materials

Adult sea urchins (males and females).

24.4. Maintaining Adult Sea Urchins in the Laboratory

Sea urchins may be collected from the shore at low tide or by diving or dredging. They can easily be maintained in the laboratory. Most of them adapt well to running sea water tanks. They can also be maintained in the regular fish aquaria at room temperature. To avoid spawning in the aquaria the temperature should be kept from 15 to 20°C. Aeration is essential and up to a point, increased aeration improves the urchin's general health. When the animals are kept in the aquaria for long term maintenance they should not be over crowded. One volume of sea urchin requires 400 volume of sea water in the rearing tank. Sea urchins can be fed with large algae from the ocean, lettuce, frozen shrimp, hard boiled egg yolk and trout food (Hinegardner, 1975).

24.5. Collection of Gametes from the Animal

Vide Expt. No. 22.

24.6. Fertilization and Early Development

24.6.1. Procedure

1. When the shedding is complete the egg suspension should be filtered by a nylon mesh with opening of 100-150 μm

wide and discard the debris. The filtered eggs are allowed to settle in sea water, kept in the beaker.

2. Remove the excess sea water from the beaker and add fresh sea water. Repeat this twice to wash the eggs completely.
3. Eggs that do not settle rapidly are probably non-fertilizable. Therefore, discard them. Use separate set of pipettes for eggs and sperms.
4. Check the motility of sperm under microscope. Dilute one drop of 'dry' sperm in 10 ml of sea water and then add one or two drops of this mixture to 200 ml sea water containing fresh eggs in a finger bowl.
5. Stir the suspension of gametes. Remove the excess sperm from the finger bowl containing fertilized eggs by decanting the supernatant and replacing it with fresh sea water.
6. Transfer a few fertilized eggs on to a cavity slide for continuous observation of the formation of fertilization membrane and early development.

24.6.2. Observation

Observe the following cleavage stages: 2, 4, 8, and 16 cell stage. Time the series of development, using the chart (Table 1).

Record the cleavage planes during these stages and make sketches. Observe the size of blastomeres and the nature of their arrangement. Observe the blastula stage, hatching and ciliary movements, late blastula, early gastrula, late gastrula, prism stage, pluteus with two arm and pluteus with four arms.

24.7. Fertilization and Early Development of Sea Urchin in Different Temperature

24.7.1. Introduction

In the room temperature, male and female pro-nuclei fuse 10-20 minutes after fertilizafion, and first cleavage occurs after 30 minutes (Vivek Raja, personal observation). Early sea urchin

TABLE 1. Time sequences in development of sea urchin *Salmacis virgulata* at °C

Stage	Time after fertilisation in hours and minutes
Fertilization	
Fertilization membrane	
Two cell stage	
Four cell stage	
Eight cell stage	
Sixteen cell stage	
Thirty two cell stage	
Sixty four cell stage	
128 cell stage	
Early blastula stage	
Late blastula stage	
Early gastrula stage	
Late gastrula stage	
Prism stage	
Pluteus with two arms	
Pluteus with four arms	

development has a Q_{10} of about 2 for many species. That is, lowering the temperature by 10°C doubles development time (Hinegardner, 1975).

24.7.2. Procedure

Repeat the fertilization experiment at 18°C, 28°C and 38°C and record the results in Table 2.

24.7.3. Observation

Record the data on the Table 2 and find out the Q_{10} values.

Formula for $Q_{10} = \left(\frac{K_1}{K_2}\right)^{\frac{t_1 - t_2}{10}}$

where K_1 & K_2 are rate of activity in t_1 and t_2 respectively, t_1 & t_2 are temperatures.

TABLE 2

Stages	Time after fertilization at 18°C	Time after fertilization at 28°C	Time after fertilization at 38°C

24.8. Fertilization and Early Development of Sea Urchin in Different Salinities of Sea Water

24.8.1. Introduction

Salinity is an important factor controlling the reproductive and developmental processes of marine invertebrates. In the present experiment the effect of salinity on the early developmental stage of sea urchin is described.

24.8.2. Procedure

Repeat fertilization experiment in 50%, 75% 100%and 125% sea water at room temperature and record the results in Table 3.

24.8.3. Observation

Table 3. *Sea water salinity* (‰) *Temperatures* (°C)

Stages	Time after fertilization in hours and minutes			
	50% SW	75% SW	100% SW (Normal SW)	125% SW

24.9. Fertilization and Early Development of Sea Urchin in Different Concentrations of Chemical Inhibitors

24.9.1. Introduction

When sea urchin eggs are treated with chemical inhibitors, cleavage is usually delayed and mitosis may be morphologically abnormal (Rustard, 1975). The effects of drugs on echinoderm egg development have been summarized by Karnovsky and Simmel (1963). The effects of chemicals can be specific or general, as in the colcemid-microtubule interaction that blocks the cells before pronuclear fusion or at metaphase (Zimmerman and Zimmerman, 1967). Others may act as respiratory inhibitors that interrupt the mitotic cycle at any point (Epel, 1963). Chromosomal abnormalities are known to result from the application of the compounds that specifically bind to DNA such as actinomycin D (Kiefer *et al.*, 1969).

24.9.2. Procedure

Repeat the fertilization experiment in different concentrations of chloremphenical in sea water i.e. 0.01%, 0.001%, and 0.0001%. Record the results in the table 4 and compare the results with that of normal development.

TABLE 4. *Salinity* (‰) *Temperature* (°C)

Stages	Time after fertilization in hours and minutes		
	0.01% chlorem-phenical in SW	0.001% chlo-remphenical in SW	0.0001% chlo-remphenical in SW

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