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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.

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)

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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.

I. OOGENESIS AND ORGANIZATION OF THE OVARY

1

A HISTOLOGICAL CLASSIFICATION OF THE DEVELOPMENTAL STAGES OF CRUSTACEAN OOCYTE*

1.1. Introduction

Oogenesis is a dynamic process comprising i) a generative (proliferative) and ii) a vegetative (growth) phase. The generative phase refers to the mitotic multiplication of the primary oogonial cell (=gonocytes) into the secondary oogonial cell that transforms to primary oocyte. These events normally occur in the germinal zone (= germarium) of the ovary. The primary oocyte with a diploid number of chromosomes enters into the prophase of meiotic division. However, the meiotic divisions are arrested at the pachytene stage and the ooplasm starts accumulating yolk materials. This process is referred to as the vegetative phase and is normally completed in the growth zone (= vitellarium) of the ovary. The remaining stages of meiotic divisions are then quickly completed before or after the ovulation.

The morphological and functional characteristics of the oogonium and oocyte are given below :

Oogonia :

The nucleus is very prominent and basophilic. Nucleolus, not distinguishable. The cytoplasm is in the form of a thin rim and lacks stainable material. The primary and secondary oogonial cells are not distinguishable under light microscope.

Primary Oocytes :

Previtellogenesis : Nucleus is transformed into a germinal vesicle. A prominent basophilic nucleolus is evident. Baso-

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

philic granules (nucleolar extrusion bodies) are seen inside the nucleus and in the perinuclear region. Yolk materials are not detectable.

Vitellogenesis : Represents the period of rapid accumulation of yolk materials. The yolk is composed of yolk granules and yolk globules.

In general the decapod crustacean ovary undergoes changes in its coloration during maturation. This is due to the presence of carotenoid pigments linked to the main yolk protein. Therefore, the intensification of color is an index of the accumulation of the yolk protein. Based on color change as well as external morphology, the ovary is divisible into several stages. However, a corresponding histological examination of all stages should be made before finalizing the ovarian stages. The classification of the growing oocytes into the previtellogenic and vitellogenic stages are rather arbitrary as there is often overlapping of these two processes. Therefore this experiment is designed to make a correct assessment of the various ovarian stages both by external morphology and direct histological observations using an ocypod crab *Ocypoda platytarsis* (Milne Edwards).

1.2. Material

Ocypod crab *Ocypoda platytarsis* in different stages of ovarian maturation.

1.3. Procedure

1.3.1. Morphological observations on the ovary

Take the female ocypod crab, remove the carapace, pick out the 'H' shaped ovary and find out the stages based on the criteria given below :

Immature

Stage I: Ovary colorless, thin and flimsy. Restricted only to cephalothoracic region. The ovary is hidden in the hepatopancreatic tissue.

Vitellogenesis-I :

Stage II : Ovary light yellow, transparent, posterior arms slightly extend to abdomen and are unequal.

Stage III : Ovary yellowish orange and flexible. The anterior arms extend and end near the gill chamber.

Stage IV : Ovary light orange, translucent, bulged and covered by transparent connective tissue layer.

Vitrellogenesis-II

Stage V : Ovary orange and lobulated, opaque in nature, occupies the entire haemocoel.

Stage VI : Ovary deep orange, lobulated, oocytes are not very compact.

Stage VII : Ovary spent and colorless, flaccid, larger than immature and the arms extend upto abdomen.

1.3.2. Preparation of the paraffin sections of ovary

Fixation : Fixation helps preserving the structural integrity of intact animal, cells or tissue. Bouin's fixative (Saturated aqueous picric acid 75 ml; Formalin 25 ml; Glacial acetic acid 5 ml) is mainly used for early vitellogenic ovaries, whereas Ciaccio's fluid (Formalin 20 ml. ; 5% aqueous potassium dichromate 80 ml. and glacial acetic acid 5 ml) is recommended for late vitellogenic ovaries in which lipid yolk is enormous (Chou, 1957).

1. Fix the early vitellogenic ovaries in Bouin's fixative and the late vitellogenic ovaries in Ciaccio's fluid.
2. After 24 hours of fixation, wash the ovarian tissues repeatedly in running tap water until the yellow color of the Bouin's fluid is removed.
3. After washing the vitellogenic ovaries fixed in Ciaccio's fixative, soak the material in 3% potassium dichromate for 24 hours at room tempeature and transfer the same to a saturated potassium dichromate solution and incubate at 37°C for one week.

4. The ovary is then dehydrated in a series of alcohol from 30% to 100%.
5. Clear the ovary either in xylene or methyl salicylate. Take care to avoid the material becoming brittle.
6. Transfer the transparent ovary into the molten wax (melting point 52-54°C) already kept in the oven.
7. After complete infiltration, make blocks of the ovary in fresh molten wax.
8. Cut sections at 6-8 μm in a rotary microtome.
9. Take a clean dry slide and apply a drop of Mayer's glycerol albumen adhesive, a combination of fresh egg white and glycerol (1 : 1).
10. Spread the sections over the slide with the help of hot plate.
11. Dewax the sections in xylene and hydrate the slides in series of alcohol from 100% to 30% and then in distilled water.
12. Stain the slides in Ehrlich's haematoxylin and counter stain in 1% aqueous eosin (Bancroft and Stevens, 1977).
13. Dehydrate the slides through alcohol series and mount in DPX.

1.4. Observation

1. Observe the placement of germinal zone in the immature ovary. Distinguish the oogonial and follicle cells in the germinal zone based on the shape and tinctorial properties of the cells.
2. Observe the behaviour of follicle cells in different stages of vitellogenesis.
3. Observe the changes in the nuclear morphology and the ooplasmic content.
4. Observe the stages in the oosorption of relict oocytes and recuperation of ovary after ovulation.

1.5. Micrometric Measurement of Oocytes in Different Stages of ovary

Size increase of the oocyte is a function of oogenesis, and hence micrometric measurements of oocytes in different stages of maturation will provide an important criteria for classifying the oocytes.

1.5.1. Procedure

1. Since oocytes deviate strongly from a spherical shape, measure the longest and shortest axes of oocyte diameter using ocular micrometer (Gonor, 1973).
2. Calculate the area of oocytes using the formula πr^2
3. Plot the values of oocyte area against percentage of oocytes in the ovarian stages classified as above, in the form of histogram (Laulier and Demeusy, 1974).

1.5.2. Inference

The histogram (Fig.1) represents data on the gradual size increase of oocyte during ovarian maturation in the ocypod, *Ocypoda platytarsis.* The presence of different size classes in each ovarian stage suggests that there is intermittant spawning of eggs within a particular breeding season.

1.6. Observation on Oocyte Maturation of the Ocypod Crab Using Cryocut Sections of Unfixed Ovary

The cryocut was originally devised by Linderstrom-Lang and Mogenson (1938) for quantitative cytochemical investigations. In this technique unfixed frozen tissues are used for enzyme and lipid histochemistry. For immediate observation of cytological details in the ovary, cryocut sections are employed in the present experiment.

The cryostat is a well insulated chamber equipped with a rotary microtome. The temperature of the chamber is automatically maintained at a low temperature, between —5°C and —30°C.

TABLE 1. *Area and the percentage of different stages of oocytes (OC ; PVO ; VO) in different ovarian stages of* Ocypoda platytarsis

Stage with colour of the ovary	Oogonial cells		Previtellogenic oocytes		Vitellogenic oocytes	
	Area in mm^2	Percentage	Area in mm^2	Percentage	Area in mm^2	Percentage
Light yellow (St. II) ..	0.265	57.00	1.658	30.00	4.78	13.00
Yellowish orange (St. III) ..	—	—	2.030	70.59	5.95	29.41
Light orange (St. IV) ..	—	—	1.714	36.36	9.355	63.64
Orange (St. V) ..	—	—	1.170	22.58	12.234	77.42
Deep orange (St. VI) ..	—	—	1.145	13.30	24.185	86.70

OC — Oogonial cells
PVO — Previtellogenic oocytes
V — Vitellogenic oocytes.

Area in $mm^2 = \pi r^2$; $r = \frac{r_1 + r_2}{2}$

r_1 — radius of long axis of oocyte

r_2 — radius of short axis of oocyte

$$\text{Percentage of cells} = \frac{\text{Number of individual stage oocytes}}{\text{Total number of different stages of oocytes}} \times 100.$$

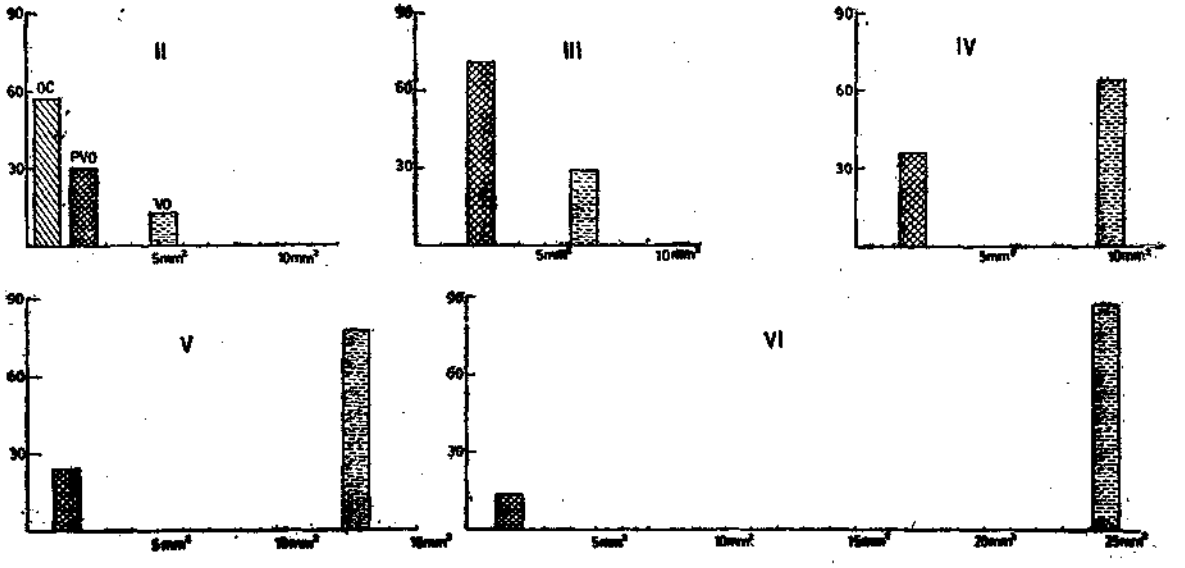

Fig. 1

Growth sequence of female gametes during gametogenic cycle of *Ocypoda platytarsis*.

Abscissa-Area of stages of oocytes in mm²; Ordinate-Percentage of different stages of oocytes in ovarian stages; II-IV—Substage a, b and c of vitellogenesis I; V-VI—Substage a and b of vitellogenesis-II.

1.6.1. Procedure

Block making

1. Adjust the temperature of the cabinet to —20°C.
2. Use 40% sucrose or distilled water as freezing agent for fixing the material on the tissue holder or object disc.
3. Excise a small bit of fresh ovary and place in the tissue holder and add 40% sucrose solution drop by drop.
4. Place the tissue holder in the heat sink, which by possessing high thermal conductivity, draws the heat from the ovarian tissue. By this process, the tissue remains in a frozen condition.

Section cutting

1. Use 120 mm microtome knife and fix in correct angle.
2. To rapidly advance the ovary towards the knife, use the crank on the fast feed knob in the clockwise direction.
3. For fine adjustments of ovary in position, use the knurled knob portion of the control.
4. Adjust the black set screw knob on the backside of the thickness scale to cut sections at 8 to 10 μm.
5. Take sections by rotating the hand which yield single separate sections. Collect the sections in an embryo cup or transfer directly to glass slide using fine camel hair brush.
6. Stain in haematoxylin and eosin and observe the cytological details of all stages of ovary under microscope.

1.7. References

Bancroft, J. D. and A. Stevens, 1977. *Theory and practice of histochemical techniques.* Churchill Livingstone, New York pp. 436.

Chou, J. T. Y. 1957. The cytoplasmic inclusions of the neurones of *Helix aspersa* and *Limnaea stagnalis*. *Quart. J. microsc. Sci.* **98**: 47-58.

GONOR, J. J. 1973. Reproductive cycles in oregon populations of the echinoid, *Strongylocentrotus purpuratus* (Stimpson). I. Annual gonad growth and ovarian gametogenetic cycles. *J. Exp. Mar. Biol. Ecol.*, **12**: 45-64,

LAULIER, M. AND N. DEMEUSY 1974. Etude histologique du fonctionnement ovarien au cours d'une maturation de ponte chez le crabe *Carcinus maenas* L. (Crustacé Decapode). *Cah. Biol. Mar.*, **15**: 343-350.

LINDERSTROM-LANG, K. AND K. R. MOGENSON, 1938. Enzymic histochemistry XXXI. Histological control of histochemical investigations. *C. R. Trav. Lab. Carlsberg. Ser. Chim.*, **23**: 27,