STUDIES ON SOME ASPECTS OF THE REPRODUCTIVE PHYSIOLOGY OF THE FEMALE GREY MULLET *MUGIL CEPHALUS* L.

THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE
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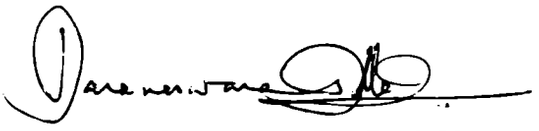
CERTIFICATE

This is to certify that the thesis entitled **"STUDIES ON SOME ASPECTS OF THE REPRODUCTIVE PHYSIOLOGY OF THE FEMALE GREY MULLET** MUGIL CEPHALUS **L."** is the bonafide record of the research work carried out by Shri. **A. GOPALAKRISHNAN** under my guidance and supervision in the Centre of Advanced Studies in Mariculture, Central Marine Fisheries Research Institute, and that no part thereof has been presented for the award of any other Degree.



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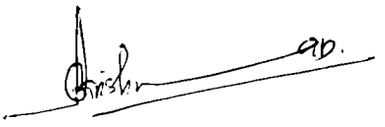
Cochin - 682 031,
January 1991.

DECLARATION

I hereby declare that this thesis entitled **"STUDIES ON SOME ASPECTS OF THE REPRODUCTIVE PHYSIOLOGY OF THE FEMALE GREY MULLET *MUGIL CEPHALUS L."*** has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition.



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CONTENTS

** ** **

PREFACE

Aquaculture is the dynamic pursuit of production of organisms from water a process analogous to agriculture on land. The field of aquaculture is an emerging bioindustry, based upon the culture and husbandry of economically utilizable aquatic organisms. Of late, there has been a global upsurge for aquaculture, the main reasons for which include the requirement of protein source for the increasing world population, the decision by various world nations to increase the fish yield by developing unutilized or partially utilized water bodies and depletion of natural stock which is evident in recent years due to excessive exploitation.

In the development of aquaculture, finfishes have been given prime importance, and among cultivable finfishes, mullets form one of the most important groups which can be cultured in different ecosystems such as coastal seawater, estuaries, lagoons, brackishwater bodies and even in freshwater. The grey mullet, Mugil cephalus is one of the fastest growing and highly delicious table fish which is extremely tolerant to wide ranges of salinity and temperature and which occupies the lowest position in the food chain. This species is widely distributed and very popular world-wide as a cultivable fish. Historically, the fishery of mullets was described in the records of the ancient Greeks and Romans. The farming of these fishes has also been practised for centuries in different parts of the world. In India, mullet is farmed from ancient times in Kerala, West Bengal, Tamil

Nadu and Goa, and even now they form an important constituent in the catches of the traditional brackishwater fishing operations in these areas. Appreciable advances have been made in recent past in research leading to the development of culture techniques of this group. With a view of the significant characteristics of mullets as cultivable fish, the aquaculturists and scientists are evincing keen interest in developing this group into a full-fledged source of culture, not merely by the present status, but also by the promise of even greater significance in future.

The Mugilidae have the greatest potential for becoming the most important supplier of aquatic animal protein for the mankind. However, this potential can only be realised by the large-scale culture activities. The availability of vast areas of coastal water bodies suitable for culture, project the immense prospects for large scale farming of mullets in India. For the purpose of mass culture programme of mullets, seeds are the pre-requisite and the culture of M. cephalus remains contingent on the availability of juveniles from the wild. They are collected from brackishwater areas, shallows of estuaries and tidal creeks. The fluctuating and capricious nature of the seed supply of this species in the last decade has prompted investigations into artificial spawning of brood stock in captivity and mass propagation of the larvae; considerable information is available on the procedures adopted in these lines. However, there is a paucity of detailed and indepth information on certain aspects of the reproductive physiology of M. cephalus, which is one of the reasons for the lag in the large sale expansion of the culture of this species. In India too, except for a few successful attempts on the artificial breeding of M. cephalus carried out by the erstwhile Central Inland Fisheries Research Institute (1962),

no further development has been achieved in this field till date.

Control of reproduction of the candidate species is one of the most important aspects of aquaculture management. Reproductive control has multiple significance in that, it helps the quality seed production and genetic improvement of the stock on the one hand, and the production of monosex population as preferred on the other. In order to proceed with the artificial means of reproduction and to produce good quality eggs, the aquaculturists have to be fully aware of the gonadal maturation stages, spawning season and the nutritional status of the breeders a priori, after and at the time of spawning. Moreover, an indepth knowledge of the biology, physiology and biochemistry of the fishes that are to be cultured is an essential prerequisite, in order to confine them in most suitable environmental conditions and to nourish them with appropriate quality feed in time and required quantity. This, in turn, can efficiently enhance the growth of stock with the resultant increase in over all fish production. Further, the stress such as the mobilisation of the biochemical components, due to the phenomena like maturation, migration and spawning, which most of the fishes undergo during their life cycle has a profound effect on the composition of fish body. So the fish chemistry assumes significance and a special approach to this branch of science is essential for developing aquaculture practices.

It is against this background that the present study has been taken up on the reproductive physiology of the female grey mullet, M. cephalus.

The thesis is presented in seven chapters. The First Chapter presents a review of relevant works done in the same field and other important investigations on mullets, in order to bring an awareness of the present status of our knowledge on the subject and also to stress the importance of such study on finfishes for coastal aquaculture operations on scientific lines.

The Second Chapter presents the material and methods employed in the collection of specimens, collection of blood from specimens, reproductive biology, histology, histochemistry, electrophoresis and biochemical analyses.

Chapter Three covers various aspects of the breeding biology. The organisation and structure of female reproductive system of M. cephalus is described in detail based on the maturity processes, variation in the gonadosomatic index (GSI), the condition factor (K) and hepatosomatic index (HSI) during the breeding cycle. The oocyte size - frequency profiles were constructed at various developmental stages. The fecundity has been estimated and the values were correlated with the total length, total body weight and total gonad weight of the fish.

In Chapter Four, histological (light microscopic and transmission electron microscopic) picture of oocyte development, ovarian atresia and postovulatory follicles are detailed. Various types of yolk inclusions formed during vitellogenesis and the cortical alveolar layer were identified. A number of distinct developmental stages were delineated and the histological observations related to the macroscopic ovary maturity stages. The cyclic

histological changes of hepatocytes were also studied and results presented.

The histochemistry of oocytes and hepatocytes are presented in Chapter Five. Histochemical reactions of proteins, carbohydrates, lipids, nucleic acids, and enzymes (alkaline phosphatase, acid phosphatase, succinate dehydrogenase and 3β HSD) were studied and results presented.

The female specific serum protein(s) (FSSP) or vitellogenin (Vtg) which appear(s) in the serum of maturing and mature fishes is regarded as the immediate precursor of the egg yolk proteins. Polyacrylamide gel electrophoresis of egg proteins and serum of immature, maturing, mature, ripe and spent females and mature male were carried out; the details of which are treated in Chapter Six of the thesis.

Biochemical composition of fishes, which is an indicator of their nutritive value has been subject to variations depending on season, food intake, breeding and migration. In the present study, variations in the major biochemical parameters namely, moisture, proteins, lipids, carbohydrates cholesterol, carotenoid, ash, calcium and iron in four tissues viz. muscle, liver, ovary and bloodserum of M. cephalus have been analysed at different maturity stages. The analysis of variance (ANOVA) - two way with interaction was carried out for each biochemical parameter to test significant changes (i) between different tissues at various stages of maturity and (ii) between different stages of maturity in various tissues. The results of these studies form the topic of Chapter Seven.

An Executive Summary of the results of investigation is presented in the final section of the thesis followed by a detailed list of References on the subject matter.

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CHAPTER I

INTRODUCTION

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INTRODUCTION

The grey mullet species belonging to the family Mugilidae (order Mugiliformes) is one of the widely distributed group of fishes. There are about 282 nominal species under the family Mugilidae, of which fourteen genera and sixty four species has been recognized (Thomson, 1981). Although, the taxonomical interest on mullets dates back to the time of Linnaeus in the 18th Century, the identity and taxonomic status of the different species have been a subject of controversy due to their wide geographical distribution and the local variations in the morphological features. The criteria employed for identifying the genera and species are also adopted differently by different workers. The morphological features used for the distinction of mullet species include mouth parts (Schultz, 1946), dentition and facial characteristics (Thomson, 1954), otoliths (Erman, 1960), adipose eye lid, lips, nostrils, pyloric caeca (Hotta & Tung, 1966; Luther, 1975) and vertebral column (Luther, 1975). Attempts were also made to distinguish the species and subspecies biochemically using electrophoresis. Variations in the zymograms of muscle proteins of different mullets were observed by Herzberg & Pasteur (1975) while Sulya *et al.* (1961), Gunter *et al.* (1961), Hongskul (1970) and Senkevich & Kulikova (1970) observed differences in serum proteins of *M. cephalus* and related grey mullets. Eye-lens protein patterns were studied to distinguish different populations of mullets by Peterson & Shehadeh (1971b) and Reddy (1977). Thus, despite

the several accounts, discussions and reviews available at present on the taxonomy of the family (Schultz, 1946; Thomson, 1954, 1966, 1981), the status of the few genera and species under this group still remains to be established. However, several keys are available for the identification of the adult species from different regions of the world (Thomson, 1954; Ebeling, 1957; Ben-Tuvia, 1975; Reddy, 1977; FAO, 1974, 1983).

Thomson (1966), while reviewing the geographic distribution of mullets which inhabit the tropics and subtropics, with some species occurring in warm temperate zones and a few penetrating to the cold temperate waters (eg. Liza provensalis), observed ten genera with forty nine species distributed in the Indo-Pacific region, two genera and six species in the Northeast Atlantic, three genera and nine species in the Southeast Atlantic, two genera and seven species in the West Atlantic and three genera and five species in the East Pacific. Of all the mullets, Mugil cephalus is most abundantly distributed, and extends geographically between latitudes 42° North and 42° South in the coastal waters (Nash & Shehadeh, 1980).

Mullets inhabit different ecosystems such as inshore sea, estuaries, brackishwater and freshwater areas. The typical freshwater species include the genera Trachystoma, Agonostomus, Rhinomugil and Sicamugil and the species Liza abu. Mugil cephalus is found to be the most tolerant among the mullets, tolerating a wide range of salinity which has been recorded as from trace to 113‰ (Odum, 1970). Although, information on the temperature tolerance of mullets is scanty, M. cephalus is reported

not to inhabit waters below 16°C (Thomson, 1963, 1966; Mohanty, 1973). Several of the authors consider this species as diadromous.

Several studies are available on the biology of grey mullets. The most important ones, to mention a few, are by Thomson (1963, 1966), Hickling (1970), Oren (1971), Wallace (1975), Marquez (1975), Brulhet (1975), De Silva & Wijeyaratne (1977), De Silva (1980). The age and growth of mullets were studied mainly on the basis of interpretation of annuli observed in the scales and otoliths (Jacot, 1920; Wimpenny, 1932; Kesteven, 1942, 1953; Pillay, 1951; Broadhead, 1958; Grant & Spain, 1975; Chubb et al., 1981). The available information on this aspect were reviewed by Thomson (1963, 1966), Brusle (1981a) and Quignard & Farrugio (1981), which showed wide disparity and disagreement on the age and growth rate of different mullets. Anderson (1958) and Broadhead (1958) showed that M. cephalus attains a length of 160mm standard length at the end of first year and generally females grew faster than males. However, growth rate of mullets is reported to be faster in tropics. The age at first maturity of M. cephalus which is widely accepted by many authors is two years for males and three years for females. The smallest size at maturity have been recorded in fishes from warm water region and the largest fishes from cold water areas (Brusle, 1981a), although this point has been refuted (Rangaswamy, 1972b; Sulochanamma et al., 1981). Thomson (1966) in a comprehensive review, summarized the existing knowledge on the habitat, biology, reproduction, population structure, behaviour, migration and fishery of mullets, upto that time. This was followed by annotated bibliographies of grey mullets by Pillay (1972) and Alvarez-Lajonchere (1974)

and an excellent edition on the "Aquaculture of Grey Mullets" by Oren (1981), wherein aspects such as taxonomy, reproduction, age and growth, food and feeding, energy metabolism, artificial propagation, parasites and diseases and aquaculture methods of grey mullets were dealt with.

Sexuality and reproductive biology of grey mullets have been reviewed by Thomson (1963, 1966) and Brusle (1981a). The structure and development of the gonads of M. cephalus were described by Stenger (1959). The histological pictures of gonadal development of different mullet species have been given by Abraham (1963), Abraham et al. (1966, 1968), Kuo et al. (1974a), Brusle (1980a,b, 1981c, 1983a, 1985), Brusle & Brusle (1975, 1978a,b) and recently by Elizabeth (1987). Majority of mullets are known to prefer brackishwater for growth and seawater for breeding. The seaward migration for spawning purpose has been previously recorded by Breder (1940), Broadhead (1953), Dekhnik (1953), Arnold & Thompson (1958), Jhingran (1958, 1959), Patnaik (1962), Fitch (1972), Wallace (1975), Whitfield & Blaber (1978), Blaber (1987) and Whitfield (1990). However, Shireman (1975) believed, M. cephalus spawn in freshwater, while Breder (1940),Jacob & Krishnamurthy (1948), John (1955) and Kurian (1975) opined that they spawn in estuaries, tidal creeks or in brackishwater areas. Kesteven (1953) reported the breeding of this species in the surf of Australian waters. Breeding of this species in offshore grounds at varying depths has also been recorded (Finucane et al., 1978). Information on the pattern of sex-ratio distribution spawning frequency and seasons of mullets by different workers are often inconsistent and controversial (Brusle, 1981a).

The fecundity in mullets has been found to vary greatly and depends on the species, its size, region and period (Chan & Chua, 1980; Alvarez-Lajonchere, 1982). In M. cephalus, the estimates of egg production range from one million to fourteen million. Mugilid eggs are pelagic and relatively small. The eggs and larvae of mullets are found abundantly in coastal waters (Panikkar & Nair, 1945; Basu, 1946; Nair, 1946, 1952, 1957; Pakrasi & Alikunhi, 1952; Kuthalingam, 1961; Thomson, 1963, 1966; De Silva & Perera, 1976; Whitfield & Blaber, 1978; De Silva, 1980). Anderson (1957, 1958), Thomson (1963), Yashouv & Berner - Samsonov (1970) and Zismann (1981) have discussed the various characters for identification of eggs, fry and fingerlings of different mullet species. Blaber (1987) discussed about the factors, affecting the recruitment and survival of mugilids in estuaries and coastal waters.

The food and feeding habits of grey mullets in natural and artificial habitats have been investigated by several workers and reviewed by Chacko & Venkataraman (1944), Pillay (1953), Thomson (1963, 1966), Odum (1968,1970) Hickling (1970), Zissmann et al. (1975), Marais (1980) and Brusle (1981b). In juvenile stages, mullets feed on a wide variety of organisms of both plant and animal origin. Diatoms form the main component of the plant material taken in by the juveniles, while planktonic and benthic organisms such as copepods, ostracods amphipods, isopods and zoea larvae also constitute other major components of their food taken at this stage (Odum, 1970). Adult mullets feed on all available food, although they are generally considered as herbivores, mainly feeding on algae and detritus. Thus, feeding at the lowest trophic level, mullets play a significant role in the flow of

energy in the ecosystem. The effect of different salinities on feeding and food conversion in mullets have been described by De Silva & Perera (1976), De Silva & Wijeyaratne (1977) and Paulraj & Kiron (1987).

Le Grande & Fitzsimons (1976) studied the karyology of M. cephalus (2n = 48) and M. curema (2n = 24). Contributions on the proximate composition and energy content in the fillets, frozen and processed roe of mullets have been reviewed by Ghosh & Guha (1934), Krzynowek (1984), Gooch et al., (1987), Kinsella (1987) Shcherbina et al. (1988) and Chiou et al. (1989).

Paperna & Overstreet (1981) reviewed the parasitic infections and diseases occurring in mullets and the public health aspects of mullets, as toxicants to men. Mullets are susceptible to various bacterial and fungal diseases. Many protozoan parasites, copepods, isopods, flat worms, nematodes and leeches are found parasitising on mullets and often cause mortalities, particularly in farming systems.

In capture fishery, mullets form a group of considerable importance. The world landings of mullets during the year 1986 was estimated at 0.17 million tonnes (about 0.2% total world fish catch), and among the mullets, M. cephalus contributed about 41% (FAO, 1986). The countries where mullets are regularly landed are Australia, Bangladesh, Bulgaria, Burma, China, Egypt, Ethiopia, France, Hawaii, Hong Kong, India, Israel, Italy, Japan, Mauritius, Philippines, Portugal, South Africa, Spain, Taiwan, Thailand, Turkey, UK, USA, USSR and Yogoslavia. In India, mullets constitute about

0.4% of the total marine fish landings and more than 25% of the mullet catch in India comes from Gujarat. The production from Kerala backwaters forms about 11% of its total annual landings (Chhaya et al., 1979; Jhingran, 1985). Various methods of capture of grey mullets were discussed by Ben-Yami & Grofit (1981).

For centuries, the farming of mullets has been in vogue as a traditional practice in the Mediterrnean region, Southeast Asia, Taiwan, Japan and Hawaii in the lagoons, creeks, swamps and ponds. Following a global awareness on the potentialities of large scale production of mullets, particularly of M. cephalus, among the cultivable marine and brackishwater finfishes in developing aquaculture, a vast body of knowledge has been accumulated on the cultivation of these fishes during the past three decades. Efforts were directed not only to improve traditional farming practices (as the "Valli culture" method followed in Italy), but also to introduce intensive culture practices to enhance their production. Different species of mullets were introduced to countries like USSR, Egypt and Israel, which boosted the aquaculture production in these countries to the present level. Intensive efforts were also made in several southeast and far-east countries towards semi-intensive and/or intensive culture, either in monoculture system or in polyculture along with compatible species.

The different studies carried out on broodstock development,pituitary hormones, induced spawning, egg and larval rearing, cryopreservation of gametes, larval nutrition, nursery management and culture techniques were reviewed by Tang (1964), Kuo et al. (1974a,b) Kuo & Nash (1975), Nash &

Shehadeh (1980), Nash & Koningsberger (1981) and Lee & Tamaru (1988). The credit of the first successful induced spawning of M. cephalus in the world goes to the team of scientists in CIFRI, who have experimentally bred this fish in capitivity in Chilka Lake (CIFRI, 1962; Chaudhuri et al., 1977), although it has been stated that Tang (1964) and Yashouv (1969) made attempts on this line (Nash et al., 1974; Nash & Shehadeh, 1980).

Majority of the Indian works on Mugilidae pertain to their biology and fishery resources. The important contributions include those of Pillay (1947, 1948, 1951, 1954, 1962a,b), Sarojini (1951, 1953, 1954, 1957, 1958), Devasundaram (1952), Chidambaram & Kurian (1952), Pakrasi & Alikunhi (1952), Qasim & Qayyam (1961), Patnaik (1962), Kowtal (1967), Thakur (1970), Ranganathan & Natarajan (1970), Rangaswamy (1972b, 1976), Singh (1972), Luther (1968), Kurian (1975), Sunny (1975), Narayanan (1974), Das (1977a,b,c), Surendrababu & Neelakantan (1983) Sathyashree et al. (1981), Kurup & Samuel (1983) and Krishnakumar et al. (1986). In India, mullet is farmed from time immemorial in the traditional low-lying fields near the estuaries, deltaic areas, brackishwater ponds and paddy fields in Kerala, West Bengal, Goa, Tamil Nadu and Karnataka. Research on intensive culture began in the 1920s when rearing experiments with young mullet were conducted in fish farms in Tamil Nadu. In 1940s further aspects like poly-culture and acclimation of mullets were tried (Mookerjee et al., 1946). Artificial breeding experiments on M. cephalus were successful in 1961 (CIFRI, 1962). Later, Sebastian & Nair (1973, 1975) discussed the attempts made on artificial breeding of Liza macrolepis at Cochin, James et al. (1983) at Mandapam and Kowtal & Gupta (1986) in Chilka Lake. Hora (1938),

Singh (1972) and Sugunan & Vinci (1981) reported the breeding of Rhinomugil (Mugil) corsula in freshwater.

Investigations on the physiology and biochemistry of mullets from India are limited. Devanesan & Chacko (1943) and Job & Chacko (1947) reported the acclimation of mullet from saltwater to freshwater, while Kuthalingam (1959) and Mohanty (1973) studied the temperature tolerance levels of mullets. Various aspects of oxygen consumption and tolerance and energy metabolism of different mullet species were studied by Kutty (1969, 1981), Kutty & Mohamed (1975) and Ushadevi (1987). Other Indian works include morphohistology of the pituitary gland of mullets (Narayan, 1983); influence of environmental factors on the survival of mullet eggs and larvae (Pillay, 1984); biochemical genetics of mullets (Parag, 1984; Mary Mathews, 1985; Ravi, 1986); spermatogenesis and cryopreservation in M. cephalus and L. parsia (Elizabeth, 1987); biochemistry of oogenesis in L. parsia (Muthukaruppan, 1987) and nutritional requirements and enzymology of L. parsia (Kiron, 1989; Palanisamy, 1989). Hybridisation experiments were carried out between M. speigleri and M. subviridis by Kowtal & Gupta (1989) and between L. parsia and L. marolepis by Krishnan & George (1985).

Based on the brief review of literature in the foregoing section on mullets from different parts of the world and from India, it is inferred that,although sufficient knowledge is available at present on their reproductive biology, information on the development and maturation of the gonad at cellular level and the intrinsic aspects of oogenesis are lacking. With a

view to fill up the lacuna of information on the above aspects and for understanding the detailed processes involved in the oogenesis, which is an essential pre-requisite for any successful breeding programme as well as genetic improvement of stock, the present study on oogenesis of Mugil cephalus along with the biochemical aspects of the reproduction has been taken up and the results of investigations presented and discussed in the present thesis.

Description of Species:

Order Mugiliformes

Family Mugilidae

Mugil cephalus Linnaeus 1758

Synonyms: Mugil oûr Forskal 1775; Mugil japonicus Schlegel 1846; Mugil oeur Klunzinger 1870; Mugil galapagensis Ebeling 1961.

Vernacular names: Flat head mullet, Jumping mullet, Black mullet, Leaping mullet.

Distinctive characteristics: Body robust; head much flattened dorsally; snout blunt; fatty adipose tissue covering most of the eye; mouth rather small, terminal and inferior; lips thin; lower lip with a high symphysial knob; hind end of the upper jaw reaching vertical from anterior rim of eye; preorbital bone slender, unnotched, fitting only half the space between lip and eye;premaxilla protrusible; lower third of upperlip without crenulations

or papillae; teeth-labial, 1-6 rows of upper teeth, 1-4 rows in lower lip, outer rows of teeth uncuspid, inner rows usually bicuspid; hind tip of maxilla not curved below the tip of premaxilla; scales feebly ctenoid; lateral line absent; pyloric caecae two.

Fin formula:- B VI; D_1IV; D_2 I,8; P 15; V I,5; A III,8; C 15; L. lat. 42-44; L. tr. 14.

First dorsal fin origin nearer to snout tip than to caudal fin base; second dorsal fin origin in front of vertical through mid point of anal fin base; pectoral fin 66-74% of head length; pelvic fins abdominal; caudal fin moderately forked; modified (axillary) scales present below first dorsal and pectoral fins; pectoral axillary scale long, 33 to 36% of pectoral fin length.

Colour:- Olive green on back; silvery on sides, shading to white below; six or seven distinct horizontal brown bands down flanks; a dark purple or dark blue blotch at the base of pectoral fin; margins of dorsal and caudal fins dusky; pelvic fin yellowish; snout tip black (Fig. 1).

Maximum size of recorded: Females - 90cm; Males - 60 cm.

Distribution (India): The species supports a capture fishery of considerable importance in Chilka Lake, Pulicat Lake, Mahanadi and Godavari estuaries, Gulf of Mannar, Palk Bay, Vembanad and Kayamkulam lakes; rare in Bengal; forms aggregation during spawning time; spawns in sea.

Fishing Gear & Utilization: Caught especially in gill nets, cast nets, stake nets, dip (lift) nets, barrier nets and beach seines; Marketed fresh and

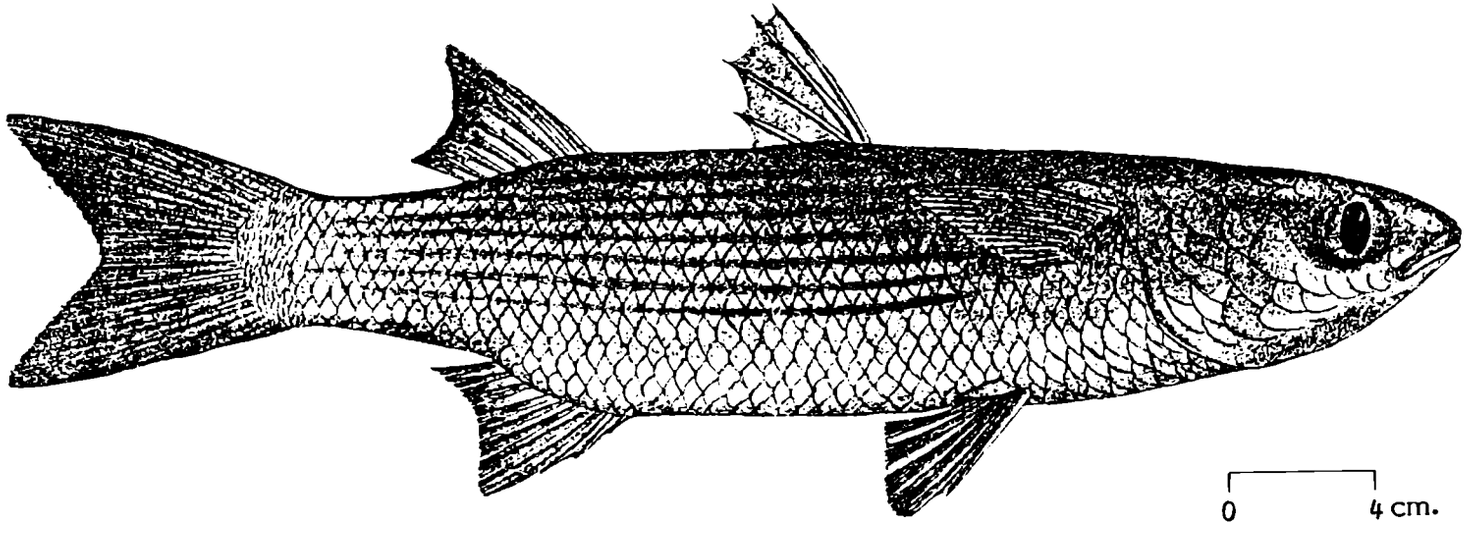


Fig. 1. *Mugil cephalus* Linnaeus, 1758

salted; Fish roe highly priced.

Culture Prospects: Euryhaline; eurythermal; fast growing; good market value; feeds on small algae, diatoms, foraminifera, zooplankters & other organic matter (both living & detrital) taken with sand or mud; no natural reproduction in ponds; artificial breeding experiments successful.

CHAPTER II

MATERIAL & METHODS

CHAPTER II

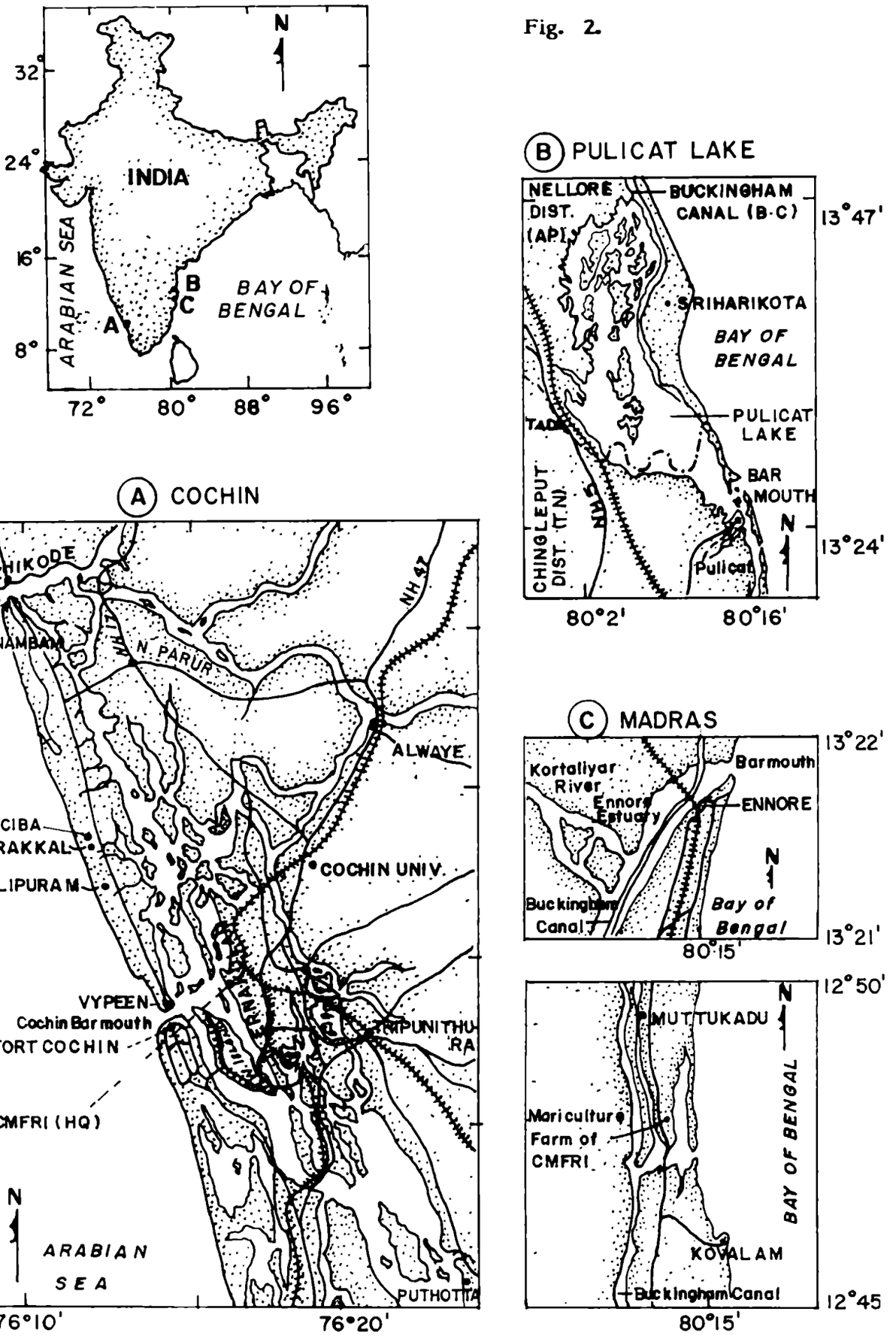
MATERIAL AND METHODS

The present work was carried out at the Central Marine Fisheries Research Institute (CMFRI - ICAR), Cochin, under the Centre of Advanced Studies (CAS) in Mariculture programme, for a period of thirty months from February 1986 to July 1988.

2.1. Collection of Specimens

Live specimens of female Mugil cephalus were regularly collected from the landing of Chinese dipnets operated near the barmouth areas of Cochin and Azhikode (Plate I, II; Fig. 2). Specimens were also collected from the brackishwater fishfarms of MATSYAFED at Katikunnu (Puthotta), Malipuram (Vypeen Island) and from the ponds of Narakkal Research Centre of Central Institute of Brackishwater Aquaculture (CIBA-ICAR), using dragnets cast nets and gill nets. Detailed investigations were carried out on the morphological histological, histochemical and biochemical changes taking place in the fish during the process of ovarian development. Collection of specimens were also made from the Pulicat Lake, Ennore Creek and the Muthukkadu fishfarm of CMFRI, along Tamil Nadu coast during December 1987 to March 1988 and the fish samples from here were used only to study the histological and histochemical changes taking place in the ovary during different stages of maturity. Mugil cephalus having a total length

Fig. 2.



MAPS SHOWING THE SITES OF SPECIMEN COLLECTION

PLATE I

a. The Cochin barmouth from where the samples were collected.

b. Chinese dipnet - An important gear in mullet fishery.

PLATE II

a. Mullet haul from Chinese dipnets.

b. Fully ripe ovaries of Mugil cephalus
(Note: The entire body cavity filled by the gonads).

of more than 300 mm alone were selected for study as sex could be visually differentiated only at this stage.

During sampling, water samples were collected to estimate environmental parameters such as surface water temperature, dissolved oxygen and salinity. In the laboratory, the dissolved oxygen was determined by Winkler's method and the salinity by the titration method (Strickland & Parsons, 1968).

2.2. Collection of blood from live specimens

The blood samples were collected from live specimens at the collection site iteself by cardiac puncture method. Live fish collected were restrained gently by applying slow physical pressure on it. The operculum was gently lifted up and the position of the heart was located. The heart was punctured using a clean dry syringe of 10 ml capacity equipped with a hypodermic needle (no.19 or 21), and the blood was slowly drawn into it. The needle was then removed from the syringe and the blood was expelled slowly into clean, dry, labelled glass stoppered centrifuge tubes. The tubes were then sealed and stored in ice. Usually around 10-15 ml of blood was collected from a single large specimen. Care was taken to avoid contamination and haemolysis of serum. The fish were tagged and then transported to the laboratory along with the blood samples in an ice box containing ice for further analysis.

2.3. Reproductive Biology

The specimens were sorted out in the laboratory and grouped according to size. They were then cleaned with freshwater and the excess

water on the body was blotted out. Each fish was then weighed upto the nearest milligram (mg) using a sensitive balance for their total wet body weight. They were then measured upto the nearest millimeter (mm) for their total length (from tip of the snout to the tip of the caudal fin) and standard length (from the tip of the snout to the end of the caudal peduncle) using a fish measuring board. The specimens were then cut open and the gonads examined. Males if any, were discarded and only females were taken for further analysis. The gonads were assigned to five different maturity stages as suggested by Qasim (1973), Kuo et al. (1974a), Kurup & Samuel (1983) and Muthukaruppan (1987) based on the colour of the ovary (Graham, 1924), macroscopic appearance such as shape and size in relation to the body cavity (Bowers, 1954), extent of yolk formation and microscopic structure such as ova diameter measurements (Clark, 1934).

2.3.1. Gonadosomatic Index (GSI): The gonadosomatic idex (GSI) for each fish was calculated using the formula of June (1953) and Yuen (1955).

$$GSI = \frac{\text{Wet weight of the ovaries}}{\text{Total body weight of fish}} \times 100$$

The range and average values of GSI were determined for each maturity stage.

2.3.2. Condition Factor "K": The 'ponderal index' or 'condition factor', 'K' for each fish was calculated using the formula suggested by Clark (1934):

$$K = \frac{W}{L^3} \times 100$$

Where, W = Total weight of fish in grams and

L = Total length of fish in cm.

The range and average values of "K" were determined in each maturity stage.

2.3.3. Oocyte size - frequency profiles: Oocyte diameter measurements were taken from ovaries belonging to various developmental stages and oocyte size - frequency profiles were constructed, with a view to trace the development of ova from immature stage to ripe condition (Clark, 1934; Prabhu, 1956; Greeley et al., 1987). A representative piece from the freshly collected ovary was gently teased on a clean dry glass slide along with a large drop of a solution of 1% formalin in 0.6% NaCl (Shehadeh et al., 1973b). The diameter of the egg was measured along the horizontal axis by using an ocular micrometer (ERMA, Japan) which was calibrated using a stage micrometer (Each ocular division was found to be equal to 0.0131 mm at a magnification of 100 x). The measurements were classified into 50 /um class intervals and the prominent mode and the largest oocytes diameter (LOD) were measured for each maturity stage. Specific area in the ovary was not chosen to collect the eggs, as the mullet oocytes are known to develop in synchrony (Shehadeh et al., 1973b; Greeley et al., 1987; Muthukaruppan, 1987).

2.3.4. Fecundity: The potential fecundity or the number of eggs available to be spawned in a single breeding season, was estimated for fish in stage

IV (ripe) of ovary development when the recruitment of oocytes into vitellogenesis ceased. These estimates were based on subsampling of unbiased samples of ovaries from gravid fish collected during the peak spawning period, as recommended by Bagenal & Braum (1978).

Subsamples weighing about 200 mg were taken from the anterior, middle and posterior regions of the bilobed pre-weighed ripe ovary and placed on a glass slide with a drop of 1% formalin in 0.6% NaCl. The individual eggs were separated and the number of yolk eggs were counted under a dissection microscope. The fecundity of the fish was determined using the formula:

$$F = \frac{nG}{g}$$

where, F = Fecundity,

n = number of eggs in the subsample,

G = Total weight of paired ovaries in grams and

g = weight of the subsample in grams.

The relationship between the fecundity (F) and the total length (L), fecundity and total body weight (W), and fecundity and total gonad weight (G) of the fish were determined using regression equations.

2.3.5. Hepatosomatic Index: The hepatosomatic index (HSI) was calculated using the formula suggested by Crupkin et al. (1988).

$$HSI = \frac{\text{Wet weight of whole liver in grams}}{\text{Total weight of fish in grams}} \times 100$$

The range and average values of liver index at each maturity stage were calculated.

2.4. Histology

2.4.1. Light Microscopic Studies: The histological changes taking place in the ovaries of Mugil cephalus during various stages of maturity were studied. Sample pieces of ovary (5 mm thick) were taken from freshly killed specimens and fixed in 10% neutral buffered formalin, or Bouin's fixative or Smith's dichromate fixative for 20-24 hours. The tissue was then thoroughly washed with running freshwater for 6-7 hours and then stored in 70% ethyl alcohol until further processing. A code number was given for each tissue and its details recorded. The tissues were dehydrated in graded alcohol series by following the standard procedure (Weesner, 1960). These tissues were then cleared with chloroform for 3-4 hours, impregnated with and embedded in paraffin wax containing ceresin (Glaxo, melting point 58-60°C).

Different processing techniques were employed on yolky oocytes before sectioning to get better results. The thick outer membrane, zona radiata of the oocytes made the penetration of wax into the yolk difficult. Routine wax embedding method resulted in the wrinkling and collapse of this membrane. Effective sectioning was finally achieved by modifying the double embedding method adopted by Khoo (1979). Yolk laden oocytes were quickly dehydrated and placed in 2% celloidin solution in 50% ethanol-ether mixture for five hours in the refrigerator. They

were then cleared using chloroform for three hours and finally infiltrated with paraffin.

The prepared blocks were trimmed, catalogued and stored in labelled polythene bags. Sections of 4-6 µm thickness were cut manually using a rotary microtome (Weswox Optik model M.T.-1090 A). Mayer's egg albumen -glycerol (1:1 v/v) (Gray, 1973) was used as the adhesive for fixing the paraffin ribbon with sections, on clean, dry glass slides. The slides containing the spread ribbons were then incubated overnight at 40°C on a slide warmer. The sections were deparaffinised, hydrated and stained with Ehrlich's haematoxylin (Weesner, 1960), Harri's haematoxylin (Preece, 1972), Heidenhain's (1896) iron haematoxylin (Weesner, 1960) and eosin as the counterstain. Sections were also stained using Mallory's triple stain (Mallory, 1944). DPX was used as the mounting medium for all the slides.

The diameter measurements of oocytes (mean of long and short axes), nuclei, yolk nucleus, lipid droplets, and yolk globules width of zona radiata and granulosa were made from the sections prepared from preserved gonads using an ocular micrometer which was calibrated with the stage micrometer (ERMA, Japan). The number of nucleoli per nucleus of an oocyte was also counted. Approximately 50 oocytes were measured from each of the randomly selected set of slides of ovaries of mullet belonging to different maturity stages, captured during different seasons. Counts and measurements were made on all oocytes in which the section passed through the nucleus except in the late tertiary yolk globule or subsequent stages when the nucleus migrated to the animal pole, broke down (GVBD)

and disintegrated. The sections were observed and photographed using a binocular compound microscope ('Microstar', American Opticals, U.S.A) with a camera unit. Appropriate projection eye piece was used and the photographs were taken using 24 x 36 mm ORWO NP 22 (125 ASA, Panchromatic), black and white negative film. The prints were taken on soft, glossy, single weight contrast paper and enlarged to the required size.

To observe the histological changes in the liver cells following gonadal maturation, liver samples taken from fresh specimens were fixed and processed as in the case of immature oocytes. The sections of 4-5 /um thickness were stained with Ehrlich's haematoxylin and eosin. The cytoplasmic and nuclear details were observed and photographed.

2.4.2. Ultrastructure Studies (Transmission Electron Microscopy-TEM): The ultrastructural details of the oocytes and the hepatic cells of Mugil cephalus during different maturity stages were studied with the help of the transmission electron microscope (TEM), at the Regional Station of the Central Plantation Crops Research Institute (CPCRI-ICAR) at Kayamkulam, Kerala. The procedure adopted is presented in the form of a Flow Chart.

2.5. **Histochemistry**

Standard histochemical techniques suggested by Pearse (1968, 1972), Barbara et al. (1979) and Subramonium (1982) were followed to study the distribution of specific types of proteins, carbohydrates and lipids in the ovary of M. cephalus. Tests were also carried out to detect the changes taking place in the patterns of RNA; DNA; enzymes like acid

Fine Structure (TEM) of ovarian and hepatic cells of *Mugil cephalus* L. Flowchart of procedure followed *

TISSUE SAMPLES
(Ovarian and hepatic cells from live specimens; Size : 4 mm thick)

↓

FIXATION
(At collection site - in ice-cold 5 % (0.1 M) glutaraldehyde solution, buffered to pH 7.3 using 0.1 M cacodylate buffer)

↓

Transportation to the laboratory in ice and refrigeration in fixative for 5 hours

↓

TRIMMING
(Tissue size : rectangular pieces of 2 mm thick)

↓

REFIXATION
(In chilled glutaraldehyde solution; 5 hours under refrigeration)

↓

VACUUM INFILTRATION
(In fresh fixative for one hour, followed by repeated washing in ice-cold cacodylate buffer)

↓

POST - FIXATION
(In freshly prepared 1 % osmium tetroxide in 0.1 M cacodylate buffer; 2 hours at 4 ° C)

↓

WASHING & REFRIGERATION
(In cacodylate buffer, 5 times, 15 minutes each. Followed by refrigeration of tissues overnight in fresh buffer)

↓

STAINING
(In fresh 0.5 % aqueous uranyl acetate - 3 hours under refrigeration followed by repeated washing in double distilled water)

↓

DEHYDRATION
(Through graded alcohol series 30 %, 50 %, 70 %, 95 % and 100 % - 15 minutes each, at 4 ° C followed by 2 changes in pure acetone - 10 minutes each, at room temperature)

↓

INFILTRATION
(In 2 1 acetone - Spurr's ** resin (1 hour), 1 1 acetone - Spurr's resin (1 hour), 1 : 2 acetone - Spurr's resin (1 hour) and in 100 % Spurr's resin (overnight under refrigeration))

↓

EMBEDDING
(In fresh resin taken in plastic capsules - one hour at room temperature followed by incubation at 70 ° C for 48 hours)

↑

TRIMMING
(Resin blocks were trimmed using a glass knife fitted to an ultramicrotome - LKB 2128, Ultrotome IV, Bromma, Sweden)

↑

SECTIONING (FOR SEMITHIN SECTIONS)
(Using ultramicrotome with a new glass knife)

↑

Semithin sections, 1 μ m thick

↑

STAINING (Humphrey & Pittman, 1974)
(Methylene blue - Azure II / Basic fuchsine technique)

↑

Observation under a binocular compound microscope to locate desired regions

↑

PHOTOGRAPHY (LIGHT MICROSCOPY)
(Semithin sections ; 'Microstar' (American Opticals, USA) binocular compound microscope with camera unit; various magnifications; ORWO NP22 (125 ASA, Panchromatic, 24 x 36 mm) black and white negative film; soft, glossy, single weight contrast printing paper)

↑

Further trimming of block heads

↑

SECTIONING (FOR ULTRATHIN SECTIONS)
(Using fresh glass knives)

↑

Ultrathin sections - (Silver interference colour, 600 - 700 ° A)

(Ultrathin ribbon - floating on distilled water taken in a small plastic boat - Wrinkles on the ribbon, removed by exposing them carefully to chloroform vapours - Sections collected on the dull or matty surface of copper or nickel grids prewashed in acetone (300 mesh size, 3.00 mm dia., LADD, USA); Grids with sections dried in air for few seconds)

↑

STAINING (Hayat, 1970)
(Fresh, filtered 2 % uranyl acetate in 50 % ethanol (15min.); Rinse in glass distilled water (3 times) – Fresh, filtered 0.4 % lead citrate in 0.1 N NaOH (5 - 10 min.) – 0.02 N NaOH (5 sec.)

↑

SCREENING & PHOTOGRAPHY
(CARL ZEISS EM 109 R Transmission Electron Microscope; Current 80 KW; Various magnifications; Agfa Ortho25 negative film; High contrast, glossy printing paper)

*** References : - Hayat (1970), Hawkes & Stehr (1980), Weakley (1981), Papathanassiou & King (1984), Dawes (1988)**

**** Spurr's resin - 'firm standard' : ratio - ERL 10 g; DER 6 g; NSA 26 g and DMAE 0.4 g (Spurr, 1969)**

phosphatase; alkaline phosphatase; 3 β - hydroxysteroid dehydrogenase; succinate dehydrogenase and metals like calcium and iron (Table 1).

Neutral buffered formalin, Baker's formol-calcium, Carnoy's solution, Smith's dichromate fixative and ethyl alcohol were used as fixatives. Routine processing techniques were followed and tissues were embedded in paraffin for all the tests except for lipids and enzymes. Fresh tissue squashes and cryocut sections of both fresh and fixed tissues embedded in gelatin, were used for detecting lipids and enzymes. Few semithin sections of oocytes fixed in 5% glutaraldehyde (pH 7.3) and embedded in Spurr's resin were also used to detect lipids.

Gelatin embedding provided a good support and effectively avoided shattering of loosely arranged yolky oocytes while sectioning with cryostat. Pieces of fresh, unfixed tissues or samples fixed in ice cold 10% neutral buffered formalin or formol-calcium were first infiltered with 6% gelatin in double distilled water at 37°C for 5 hours. Fixed samples were thoroughly washed in running water before putting in gelatin. The tissues were then transferred to 12% gelatin for 5 hours at 37°C. They were embedded in fresh 12% gelatin and placed in refrigerator until the blocks were firm. The blocks were trimmed using a sharp razor blade and then transferred to ice cold 10% neutral buffered formalin to harden and later kept overnight in the refrigerator. The hardened blocks were washed with water (Barbara et al., 1979) and sections of 10-15 μm thickness were cut with a cryocut (Histostat-American Opticals, model 975C) at -20°C. The sections were picked up singly on albuminized slides and processed for various staining

Table 1 : List of histochemical techniques carried out to study the chemical composition of oocytes and hepatic cells of *Mugil cephalus* during different stages of gonadal development.

TESTS @	FIXATIVES	SECTIONS	REACTIVE GROUPS	COLOUR
PROTEINS				
**Mercuric Bromophenol Blue (HgBPB) (Bonhag, 1955)	NBF; BFC	Paraffin	Proteins	Deep Blue
Aqueous Bromophenol Blue (ABB) (Pearse, 1968)	NBF; BFC	Paraffin	Basic proteins	Blue
Toludine Blue (Pearse, 1968)	NBF; BFC	Paraffin; Frozen	Acidic group	Purple
Ninhydrin-Schiff (Yasuma & Ichikawa, 1953)	C; F	Paraffin; Frozen	Amino group	Pink
Ferric ferricyanide (Chevremont & Frederic, 1943)	NBF; F; S	Paraffin; Frozen	-SH group	Prussian Blue
Performic acid-Alcian Blue (Adams & Sloper, 1955)	NBF	Paraffin	-SS group	Dark Blue
Thioglycollate Ferric ferricyanide (Adams, 1956)	NBF	Paraffin	-SS group	Prussian Blue
Sakaguchi's Reaction (Baker, 1947)	NBF; F; S	Paraffin; Frozen	Arginine	Orange red
Pauly's reaction (Reaven & Cox, 1963)	NBF	Paraffin	Histidine	Orange red
Millon's test (Baker, 1956)	NBF; F	Paraffin; Frozen	Tyrosine	Red or Pink
p-Dimethyl amino-benzaldehyde nitrite (DMAB) (Adams, 1957)	F	Frozen	Tryptophan	Deep blue
CARBOHYDRATES				
**Periodic acid-Schiff (PAS) (McManus, 1948)	NBF; C	Paraffin	1,2, glycol groups	Magenta
**Best's Carmine (Best, 1906)	C; A	Paraffin	Glycogen	Red
Toludine Blue at pH 1.09, 3.09, & 4.19 (Pearse, 1968)	NBF	Paraffin	Sulphated AMP	Blue
Toludine Blue at pH 1.99 (Pearse, 1968)	NBF	Paraffin	Phosphated AMP	Blue
Toludine Blue at pH 7.0 (Pearse, 1968)	NBF	Paraffin	Carboxylated AMP	Blue
Alcian Blue-Critical Electrolyte Concentration (CEC) (Steedman, 1950)	NBF	Paraffin	Acidic Sulphomucin	Blue
Alcian Blue 1% (pH 2.5) (Steedman, 1950)	NBF	Paraffin	Weakly acidic sulphomucin	Blue
Benzidine Reaction (Bracco & Curti, 1953)	NBF	Paraffin	Sulphated AMP	Deep Blue
LIPIDS				
**Sudan Black 'B' (Mc Manus, 1946)	BFC; F; G	Frozen; Semithin	Lipids	Bluish black
1% Nile Blue at 60°C (Cain, 1947)	BFC; F	Frozen	Neutral lipids	Red or Pink
			Acidic lipids	Blue
**Acid Haematin (Baker, 1946)	BFC; F	Frozen	Phospholipids	Bluish black
Nile Blue with conc. H_2SO_4 (Menschik, 1953)	BFC; F	Frozen	Phospholipids	Blue
**Oil red 'O' (Lillie, 1944)	BFC; F; G	Frozen; Semithin	Neutral lipids	Red
UV-Schiff Reaction (Belt & Hayes, 1956)	BFC; F	Frozen	Unsat. lipids	Magenta
Sudan Black 'B' with acetic acid (Ackerman, 1952)	BFC; F	Frozen	Masked lipids	Bluish black
Schultz's (1924) Method	BFC; F	Frozen	Cholesterol	Reddish violet turning green
NUCLEIC ACIDS &BASIC PROTEINS OF NUCLEUS				
**Feulgen Reaction (Feulgen & Rossenbeck, 1924)	C	Paraffin	DNA	Reddish purple
**Methyl Green - Pyronin (Kurnick, 1955)	C; F	Paraffin; Frozen	DNA	Green
			RNA	Bright red
Methyl Green - Toludine Blue (Gurr, 1955)	C	Paraffin	DNA	Green
			RNA	Blue
Alkaline Fast Green (Albert & Geschwind, 1953)	NBF; C	Paraffin	Histones & Protamines	Bright green
ENZYMES				
**Calcium Cobalt method (Gomori, 1939)	NBF; F	Paraffin; Frozen	Alkaline phosphatase	Blackish brown
**Standard Coupling Azodye method (with Fast Garnet GBC) (Grogg & Pearse, 1952)	F	Frozen	Acid phosphatase	Reddish brown
Nachlas *et al.* (1957) Test (with Tetra Nitro BT)	F	Frozen	Succinate dehydrogenase(SD)	Dark brown
Wattenberg's, (1958) Test (with Nitro BT)	F	Frozen	Δ^5 3β Hydroxysteroid dehydrogenase(3βHSD)	Purple
METALS				
Alizarin Red 'S' (Dahl, 1952)	A	Paraffin	Calcium deposits	Brick red
Perl's (modified) method (Gomori, 1936)	NBF; BFC	Paraffin	Iron deposits	Prussian Blue

A Ethyl alcohol as fixative
BFC Baker's Formol-Calcium fixative
C Carnoy's fixative
F Frozen tissue fresh, unfixed or fixed in Cold BFC/NBF and embedded in 12% gelatin.
G Semithin sections from glutaraldehyde fixed tissues, embedded in Spurr's resin.
NBF Neutral Buffered formalin (10%)
S Smith's dichromate fixative
@ Not referred in original. Cross reference from Pearse (1968, 1972), Van Den Hurk (1973) and Barbara *et al.* (1979).
** Hepatic cells at different stages of ovarian maturation were subjected only to these histochemical tests.

techniques. The stained sections were mounted in gelatin-glycerine mountant and observed under microscope.

Mercuric bromophenol blue and Periodic Acid - Schiff (PAS) tests were used as general staining methods for proteins and carbohydrates respectively. Sudan black-'B' was used as the general stain to detect lipids. Alkaline phosphatase activity was demonstrated according to the method of Gomori (1939)*. The substrate was 3% w/v sodium β-glycerophosphate, dissolved in 2% aqueous sodium diethyl barbiturate, the final pH being 9.3. Magnesium ions activated the reaction and the incubation time was one hour at 37°C. The method of Grogg & Pearse (1952)* was adopted to detect the presence of acid phosphatase. The incubation medium contained the substrate sodium α-naphthyl phosphate, dissolved in 0.1M veronal acetate buffer (pH 5.0); polyvinyl pyrrolidone and Fast Garnet GBC Salt. The sections were incubated in the above medium for 30-60 minutes, at 37°C. Succinate dehydrogenase (SD) was demonstrated after Nachlas et al. (1957)*, using disodium succinate as the substrate at pH 7.4. Tetra Nitro Blue Tetrazolium (TNBT) was the final electron acceptor. The incubation time was 30 minutes at 37°C. Wattenberg's (1958)* method was adopted to demonstrate the activity of 3 β-hydroxysteroid dehydrogenase (3 β HSD). The substrate, dehydroepiandrosterone (DHA) was dissolved in acetone (5 mg/ml) with NAD^+ as cofactor and Nitro Blue Tetrazolium (NBT) as the final electron acceptor. The solution was buffered, using 0.2M Tris (pH 8.3) and the sections were incubated for 45 minutes at 37°C.

The presence of each reactive group was confirmed simultaneously by subjecting the control sections to blocking procedures for specific groups

* *Originals not referred; Cross reference from Pearse (1968,1972).*

Table 2 : Blocking reactions and extraction procedures of specific reactive groups.

	Methods	Reactions
Proteins	Deamination	Removal of amino group
	Methylation	Removal of acidic group
	Mercaptide	Blocking of -SH group
	Iodination	Blocking of -OH group
	40% formaldehyde	Blocking of tryptophan
	Thioglycollate reduction	Blocking of -SS group.
Carbo-hydrates	Schiff alone	No oxidation of 1,2 glycols; presence of other free aldehydes.
	Acetylation	Blocking of 1,2 glycols
	Deacetylation	Removal of acetylation
	Diastase treatment	Removal of glycogen.
	Trypsin digestion	Removal of protein - carbo-hydrate complexes.
	Methylation	Removal of acidic groups
Lipids	Chloroform - methanol extraction	Removal of lipids
	Pyridine extraction	Removal of phospholipids.
Nucleic Acis	Hot perchloric acid extraction	Removal of DNA & RNA
	Ribonuclease treatment	Removal of RNA
Enzymes	Incubation of sections without substrates	No enzymatic reaction
Metals	Strong acid (HNO_3) treatment	Removal of calcium
	EDTA treatment	Removal of calcium

(Table 2). For enzymes, control reactions were carried out by incubating the sections in the respective media in the absence of substrate.

The hepatic tissue samples collected from fish in various stages of ovarian development were also subjected to histochemical tests (Table 1). Blocking procedures were also carried out for each reactive group.

The results were interpreted by subdividing the oocytes at different maturity stages into different regions. Reactions of various cytoplasmic inclusions like yolk granules, lipid droplets, yolk nucleus etc. were noted down. In the case of liver cells, cytoplasmic and nuclear details and chemical nature of cytoplasmic inclusions were investigated. The following arbitrary symbols were used to score the intensity of histochemical staining in each case: -(no reaction); + (weak); ++ (moderate); +++ (strong); ++++(highly intense) reaction. The absence of a cell type or a cytoplasmic inclusion in a particular stage was indicated by (*). The tests were performed in a specific sequence, from the general ones to the more specific types.

2.6. **Electrophoresis**

Samples of serum and ovary were collected from M. cephalus in different maturity stages and analysed electrophoretically to identify the female specific protein (FSSP) or vitellogenin in serum and the egg yolk proteins in oocytes. The serum sample of a mature female fish was compared with that of a mature male fish with normal human serum as the standard.

The polyacrylamide gel disc electrophoretic (PAGE) method of Davis (1964), as given by Subhashini & Ravindranath (1981) and Prathibha (1984) was followed with necessary modifications.

2.6.1. Preparation of tissue extract: Blood samples transported to the laboratory, in ice from the collection site were allowed to clot naturally at room temperature for about two hours. The supernatant was centrifuged at 3,000 rpm for 20 minutes and the clear straw coloured serum was stored at -20°C in clean, dry, labelled screw-capped vials until required, but always used within five hours of preparation. Samples showing traces of haemolysis were discarded.

For analysis, 0.2 ml of serum was diluted with 1 ml of ice cold double distilled water (the appropriate serum: double distilled water ratio was determined after standardisation).

Egg proteins were extracted, following the method of Hara et al. (1980), with little modification. The appropriate tissue weight for extraction was determined after standardisation and 200 mg ovary/ml of solvent gave best resolution of bands. During the extracting of proteins from eggs, the outer ovarian membrane was removed and the weighed sample was washed in double distilled water. It was then thoroughly homogenized in cold with 1 ml of ice-cold double distilled water (pH 7.0) using a mechanical homogenizer. The contents were centrifuged at 10,000 rpm for 30 minutes at 4°C in a refrigerated super speed centrifuge (SORVALL RC-5B). The supernatant was collected and filtered through a clean filter paper (Whatman No.41). This filtrate was again centrifuged at 10,000 rpm for 30 minutes

at 4°C and the clear upper layer was collected and stored at -20°C, until further analysis. The repeated centrifugation effectively removed lipid droplets, pigments and all debris which otherwise obliterated the ovarian sample analysis. Various other solvents like Tris-glycine buffer (pH 8.3), Tris-Boric acid-EDTA buffer (pH 9.0), 40% sucrose in double distilled water (pH 5.3), and 0.9% sodium chloride in double distilled water (pH 7.3) used as extracting media for the proteins, resulted in poor resolution.

2.6.2. Volume of the sample loaded:

The optimum volume of sample to be loaded was found to be 80 /ul/tube for ovary (containing 800-950 /ug protein) and 60 /ul/tube for serum (protein content 800-900 /ug), which gave best band resolution.

2.6.3. Apparatus used: The cylindrical perspex tanks manufactured by Dalal & Co. with facilities to run 12 tubes at a time were used. A steady current was maintained with the electrophoretic power pack manufactured by Biochem, Madurai. Glass tubes of uniform length (75 mm) and diameter (5 mm inner dia), with three equidistant markings of 5 mm from the upper edge, were used for setting gels.

2.6.4. Preparation of reagents:

Separating gel concentration: Separating or running gels of concentrations ranging from 5% to 10% were prepared by varying the amount of acrylamide and bis acrylamide, following the method of Bo Gahne et al. (1977). The 7% acrylamide gel concentration with 2% bis acrylamide showed the best resolved bands with protein fractions distributed all along

the length of the gel, which was prepared by thoroughly mixing the following solutions:

40% acrylamide stock solution in double distilled water	3.5 ml
2.1% stock solution of methylene bis acrylamide in double distilled water	2.0 ml
Double distilled water	4.5 ml
Small pore buffer (pH 8.9) (To 36.6 g Tris in 48 ml IN Hcl, added 0.23 ml of TEMED(N, N, N', N ' Tetramethyl ethylene diamine) and made upto 100 ml with double distilled water)	5.0 ml
0.14% Ammonium per sulphate in double distilled water (prepared afresh)	5 ml.

(All the above solutions were prepared and stored in refrigerator in amber coloured bottles and brought to room temperature before use. The pH of small pore buffer was adjusted using IN HCl or 1% Tris solution).

Spacer gel: The spacer gel or the stacking gel was prepared by mixing the following solutions:

Large pore buffer (pH 6.7) (5.98 g of Tris (hydroxymethyl) was dissolved in little double distilled water and to this, added 0.46 ml of TEMED and 48 ml of IN HCl. Mixed well and made upto 100 ml with double distilled water)	1 ml
Monomer solution (3%) (Dissolved 20 g acrylamide and 2.5 g methylene bis acrylamide in 100 ml double distilled water)	2 ml
4 mg of riboflavin dissolved in 100 ml double distilled water	1 ml
40 g Sucrose dissolved in 100 ml double distilled water (40% solution)	4 ml

(All the above reagents were refrigerated separately in amber coloured bottles and thawed before use. The pH of large pore buffer was adjusted using IN HCl or 1% Tris solution)

Tank Buffer: Stock solution of tank buffer was prepared by dissolving 6 g Tris and 28.8 g glycine in little double distilled water and making it upto 1,000 ml. The pH of the buffer was adjusted to 8.8 with a solution of 10% Tris in double distilled water.

To prepare working solution of tank buffer, 60 ml of the stock solution was diluted to 600 ml with double distilled water.

Marker (Tracking) dye: Working solution of marker dye or the indicator solution (bromophenol blue) was prepared afresh by diluting the stock solution using 40% w/v sucrose, in the following proportion:

Stock solution (0.1% aqueous) of Bromophenol Blue	0.5 ml
40% sucrose solution	4.5 ml

2.6.5. Procedure: Clean dry gel tubes were fixed tightly in the gel setting stand. The separating gel mixture (7% acrylamide and 2% bisacrylamide) was prepared and carefully poured into the gel tubes along its sides using either a wide-mouthed dropper or a 10 ml syringe, upto the 15mm marking (lowest) in the tube. Care was taken to avoid trapping of air bubbles in the gel. Few drops of water were added carefully over the gel using 1 ml syringe with a needle (No.22) attached to it to avoid meniscus formation

at the gel surface. The tubes were left undisturbed until gelation was complete (10 minutes). The overlaying layer of water was carefully removed by inserting small rolls of blotting paper into the gel tubes.

The spacer gel mixture was prepared as described earlier and it was carefully layered over the separating gel, upto the 10 mm (middle) mark. Drops of double distilled water were added over the solution with care and the tubes were then placed under a fluorescent lamp for photopolymerisation (20-30 minutes). The overlaying water was removed using blotting paper wicks.

The tissue extracts (80 µl/tube for ovary and 60 µl/tube for serum) were loaded carefully over the gel using a digital finnpipette (Labsystems, Finland). Few drops of diluted marker dye (bromophenol blue) were added over the sample in each tube using finnpipette and gently mixed with sample. The remaining space in the gel tubes were carefully filled up to the brim with dilute tank buffer.

The tubes were removed from the stand and inserted into the grommets of the upper tank. Drops of dilute buffer were suspended from the lower end of the gel tubes to avoid trapping of air bubbles. About 300 ml of the tank buffer was added to the lower tank and the upper tank with tubes was placed over this. Buffer was carefully added to the upper tank without disturbing the samples in the tubes and then covered with the lid. The whole unit was placed inside the refrigerator and connected to the powerpack. A steady current of 1mA/tube was supplied till the marker dye crossed the spacer gel after which the current was increased

to 3mA/tube until the bromophenol blue migrated almost to the end of the tubes.

The current was switched off, the tubes were taken out and placed serially in a petridish containing double distilled water. The gels were removed by inserting a thin syringe needle between the gel and wall of the tube and carefully discharging a jet of water from the syringe while rotating the tube. The gels were then treated with appropriate stains.

2.6.6. Staining: Various staining techniques and destaining methods adopted are presented in the Table 3. Gels were finally stored in 7% v/v acetic acid in labelled test tubes.

2.6.7. Relative fraction (R_f) values and electropherograms: The total length of separating gel, distance travelled by the marker dye in the separating gel and the various distances migrated by the different protein fractions were measured. The relative mobility (R_m) or the relative fraction (R_f) value of each band was calculated using the following formula:

$$\text{Relative fraction } (R_f) \text{ value} = \frac{\text{Distance travelled by the protein fraction}}{\text{Distance travelled by marker dye (bromophenol blue)}}$$

Separate electropherograms were drawn based on these values.

The electropherograms were broadly classified into 3 zones namely the upper, the middle and the lower zones starting from the cathodal region. Each protein fraction was numbered serially with the fastest migrating protein fraction as the first number and the slowest fraction the last number.

TABLE 3: Details of staining techniques adopted in the electrophoretic study of Mugil cephalus L.

TESTS @	Fixation & time	Staining time	Destaining solution & Duration	Colour of Bands
PROTEIN STAINING				
0.25% Kenacid Blue or Coomassie Brilliant Blue in methanol, water and acetic acid (5:5:1 ratio) (Davis, 1964)	10% TCA for 30 minutes	30 minutes in dark	Methanol, water and acetic acid (5:5:1 ratio) for 30 minutes	Blue
POLYSACCHARIDE STAINING				
Periodic Acid-Schiff (PAS) test, (Gordon, 1980).	12.5% TCA for 30 minutes	One hour in 1% periodic acid in 3% acetic acid; one hour wash in double distilled water; 3 hours in Schiff's reagent in dark, under refrigeration.	1% aqueous sodium metabisulphite followed by repeated washing in 7% acetic acid.	Magenta
LIPOPROTEIN STAINING				
Oil red-'O' saturated solution in 50% methanol containing 10% TCA (Davis, 1964).		3 hours at 60°C		Reddish Orange
CALCIUM STAINING				
Alizarin red-'S' (pH 6.5) test. (Dahl, 1952)		20 minutes	Repeated washing in double distilled water, rinse in acid ethanol (10^{-3}M HCl in 95% OH) until background is clear.	Deep reddish orange.
IRON STAINING				
Perl's (modified) method (Gomori, 1936) 10% potassium ferrocyanide and 20% HCl, Mixed afresh (in the ratio 1:1).		Two changes in 30-40 minutes.	Repeated washing in double distilled water.	Prussian Blue.

* Incubation of gels in TCA prior to staining was avoided as the staining methods are pH specific.

@ Not referred in original; Cross reference from Pearse (1968,1972), Gordon (1980), Subhashini & Ravindranath (1981) and Prathibha (1984).

The chemical nature of each protein fraction was found out from the different staining reactions and the results were tabulated.

2.7. **Biochemical Analyses**

Moisture, total proteins, total carbohydrates, total lipids, total cholesterol, total carotenoids and ash contents in the muscle, liver and gonad tissues of Mugil cephalus, were estimated during different stages of ovarian maturation. The serum was also analysed for all the above biochemical parameters except for the moisture and ash contents. Total calcium and iron levels were estimated in serum and ovary during different stages of maturation.

The white muscle for estimation was taken from the region, just below the first dorsal fin. Care was taken not to include any skeletal parts. Samples of ovary were taken from the anterior, central and the posterior regions. All the tissue samples were accurately weighed to the nearest milligram with an electronic balance (Mettler PC 440, Switzerland). When adequate amount of tissues were not available from a single specimen, tissues from different specimens belonging to the same stage of maturity and size group were pooled together to make a sample for analysis. A portion of all the tissues was kept apart for the moisture content analysis and the rest were kept in labelled polythene bags and frozen at -20°C, for further analysis. In the case of liver samples, all estimations were carried out in fresh conditions.

Serum was separated from the blood samples as in the case of electrophoretic analysis (Page 24). This was stored at -20°C, in clean dry labelled screw capped vials until further analysis. Care was taken to avoid contamination and haemolysis. When enough blood was not available from a single fish, blood samples of 2-3 fishes of similar maturity stage and size group were pooled and then centrifuged.

For all the estimations extra pure of "AnalaR" grade chemicals only were used.

The optical density of the colour developed for total proteins, lipids, carbohydrates, cholesterol and carotenoids were measured using a senior spectrophotometer (ECIL G6865D), with the samples taken in silica cuvettes. Standard graphs were plotted with the concentration of each biochemical parameter in different dilutions of the working standard solution, in the X-axis and the optical density in the Y-axis. The concentration of different parameters in the samples were calculated (in mg%) by comparing the optical density (O.D.) obtained for the sample with the values in the standard graph and also using the following formula:

Concentration in mg/100 mg wet tissue =

$$\frac{\text{(O.D.of the sample - O.D.of the blank)}}{\text{(O.D.of the standard - O.D.of the blank)}} \times \text{Concentration of standard} \times \frac{100}{\text{Weight of sample in mg.}}$$

Concentration in mg/100 ml blood serum =

$$\frac{\text{(O.D.of the sample - O.D.of the blank)}}{\text{O.D.of the standard - O.D.of the blank)}} \times \text{Concentration of standard} \times \frac{100}{\text{Volume of sample in ml.}}$$

The calcium and iron contents in the ovary and blood serum were estimated using Perkin-Elmer 2380 atomic absorption spectrophotometer, following standard methods (Fernandez & Kahn, 1971). The concentration of these metals in the samples were calculated using the following formula:

$$\text{Metal, } \mu\text{g/g dry tissue} = \frac{(\text{Concentration of metal (in ppm) in the sample} - \text{concentration of metal in the blank})}{(\text{Dry weight of the sample taken in mg})} \times \text{Dilution factor} \times 1{,}000$$

$$\text{Metal, } \mu\text{g/ml serum} = \frac{(\text{Concentration of metal (in ppm) in the sample} - \text{concentration of metal in the blank})}{(\text{Volume of the undiluted sample in ml})} \times \text{Dilution factor}$$

$$\text{where, Dilution factor} = \frac{(\text{Volume of diluted sample in ml})}{(\text{Volume of aliquot taken for dilution in ml})}$$

2.7.1. Moisture content: The tissue samples were cleaned and the water adhering to them was removed using a blotting paper. The wet weight of the tissues were taken accurately and the samples were gradually dehydrated to constant weight in a hot air oven at 70°C. The moisture content was then calculated gravimetrically as the difference in the wet weight and the dry weight of the tissue and was expressed as percentage of wet weight.

The dried samples were powdered in a mortar, transferred to labelled polythene bags and stored in a desiccator to estimate the ash, calcium and iron contents.

2.7.2. Total proteins: The Folin - Ciocalteu Phenol method of Lowry et al. (1951) was adopted for the estimation of total proteins in the tissues.

Wet tissues samples (muscle, liver and ovary), each weighing 25 mg were thoroughly homogenized with 1 ml of deproteinising agent (10% trichloroacetic acid) by keeping the tubes in ice. In the case of blood serum samples, 0.1 ml of serum was mixed well with 1 ml of deproteinising agent. All the samples were centrifuged for 20 minutes at 3,000 rpm. The supernatant obtained in the individual tubes was used for the estimation of total carbohydrate. The protein precipitate in each tube was dissolved in 5 ml 1 N NaOH and to 1 ml of this solution, freshly prepared 5 ml alkaline mixture (50 ml of 2% $Na_2 CO_3$ in 0.1 N NaOH + 1 ml of 0.5% $CuSO_4$, $5H_2O$ in 1% sodium tartrate) was added and kept at room temperature for 10 minutes. After 10 minutes, 0.5 ml of 1 N Folin - Ciocalteu's reagent (diluted the 2N stock solution with double distilled water) was added and mixed rapidly.

A standard stock solution was prepared using bovine serum albumin crystals at a concentration of 25 mg/5 ml 1 N NaOH. Different dilutions in the range 0.25 - 2.5 mg/ml were prepared from this stock solution, and the alkaline mixture and Folin-phenol reagent were added as in the case of tissue samples. A blank was prepared with 1 ml 1 N NaOH and treated the same way as above.

All the test tubes were kept for 30 minutes at room temperature and the optical density of the blue colour developed was measured against the blank at 660 nm.

2.7.3. Total carbohydrates: The phenol sulphuric acid method of Dubois et al. (1956) was followed to estimate the total carbohydrates in the samples.

The supernatant obtained during protein estimation procedure was used for the analysis. To 1 ml of the supernatant of muscle, ovary and blood serum, taken separately, 1 ml of 5% phenol (5.5 ml of 90% liquid phenol added to 94.5 ml water) was added and mixed well. In the case of liver tissue, 0.5 ml of the supernatant was made upto 1 ml with saturated solution of benzoic acid in double distilled water and to this solution, 1 ml of 5% phenol solution was added. One ml of concentrated sulphuric acid was added rapidly and carefully to each tube and mixed well using a cyclomixer.

A standard stock solution was prepared using D-glucose (concentration-20 mg/100 ml saturated solution of benzoic acid). Different dilutions of the working solution with the concentration of glucose ranging from 10-100 µg/ml were prepared and the procedure adopted for the tissue was followed. A blank solution with 2 ml of 5% phenol was prepared and the above procedure followed.

All the tubes were kept for 30 minutes at 30°C and the optical density of the orange colour developed was measured at a wavelength of 490 nm.

2.7.4. Total Lipids: The total lipids were quantitatively determined by sulphophosphovanillin method of Barnes & Blackstock (1973).

About 10 mg of muscle and ovary and 5 mg liver samples were separately homogenized well in 1 ml of chloroform methanol (2:1 v/v) and kept overnight at 4°C for complete extraction. 0.1 ml of serum was thoroughly mixed with 1 ml 2:1 v/v chloroform ; methanol and left overnight in the refrigerator. The mixture taken in glass stoppered centrifuge tubes were then centrifuged for 15 minutes at 3,000 rpm and the clear supernatant containing all lipids was transferred to clean, dry glass tubes. 0.5 ml of this lipid extract of all the tissues were taken separately in clean glass tubes and dried in vacuo over silica gel in a desiccator. To each dried sample, 0.5 ml of concentrated sulphuric acid was added and shaken well. The tubes were then plugged with non-absorbent cotton wool and heated at 100°C in a boiling water bath exactly for 10 minutes. The tubes were rapidly cooled to room temperature under running tap water. To 0.1 ml of this acid digest, 2.5 ml of phosphovanillin reagent was added and mixed well on a cyclomixer

Stock solution was prepared afresh by dissolving 80 mg of cholesterol in 100 ml of chloroform methanol (2:1 v/v) mixture (equivalent to 100 mg of total lipid in 100 ml (2:1 v/v) chloroformmethanol mixture). Working solutions of different concentrations were prepared from the stock solution in the range 50-500 µg/0.5 ml and the procedure adopted for the tissue samples was followed. 0.5 ml of 2:1 v/v chloroform:methanol mixture was treated as blank.

All tubes were kept at room temperature for 30 minutes. The intensity of the pinkish red colour developed was measured against blank at 520 nm.

2.7.5. Total cholesterol: Total cholesterol was determined by Henly's (1957) method as given by Varley (1976).

Wet tissue samples (muscle, liver and ovary) of 25 mg each were weighed accurately and homogenized well with 10 ml of ferric chloride - acetic acid reagent (0.05% solution of $FeCl_3$, $6H_2O$ in glacial acetic acid) in ice and left overnight in refrigerator for the proteins to flocculate. For the blood sample, 0.1 ml serum was mixed well with 10 ml of ferric chloride-acetic acid reagent and left overnight in the refrigerator. All the samples were centrifuged for 20 minutes at 3,000 rpm and 5 ml of the supernatant from each tube was transferred to clean dry glass tubes. 3 ml of concentrated sulphuric acid was added to all the tubes and thoroughly mixed on a cyclomixer.

Stock solution was prepared by dissolving 100 mg of cholesterol in 100 ml of glacial acetic acid to get a concentration of 1 mg/ml. Working solution was prepared afresh by mixing 1 ml of stock solution with 24 ml ferric chloride-acetic acid reagent. Different dilutions of this solution with the concentration of cholesterol ranging 20-200 /ug/5 ml were taken and treated with 3 ml of concentrated sulphuric acid as in the case of tissue samples. 5 ml of ferric chloride-acetic acid reagent was treated as the blank solution.

All the tubes were allowed to stand for 30 minutes at room temperature. The intensity of the reddish brown colour developed was measured against the blank at 560 nm.

2.7.6. Ash content: A preweighed amount of oven dried powdered tissue sample was ignited in a silica crucible for 5 hours at 600°C in a muffle furnace, till all the organic matter was burnt out leaving no carbon residue. The ignited content was weighed and the difference in weight gave the ash content of the tissue. The percentage ash content of the tissue was calculated as follows:

$$\text{Ash mg\%} = \frac{\text{Ash weight}}{\text{Dry weight of tissue}} \times 100$$

2.7.7. Total carotenoids: The method described by Olson (1979) was adopted to estimate the total amount of carotenoids in different tissues and blood serum.

About 1 g of the fresh tissue (muscle, liver and ovary) was weighed on a small piece of aluminium foil and placed in labelled clean dry screw capped 10 ml vials, containing 2.5 g of anhydrous sodium sulphate. The tissues were gently mashed using a glass rod. 5 ml of chloroform was added to each vial to extract all the carotenoids from the sample. For the blood samples, 1 ml of the serum was mixed well with 5 ml of chloroform in a clean screw capped vial. All the vials were sealed and kept overnight at 0°C in the dark. The blank contained only 5 ml of chloroform.

An aliquot of 0.3 ml chloroform extract from each vial was diluted to 3 ml with absolute ethanol. The blank was also treated in the similar manner. The optical density was read at 380, 450, 475 and 500 nm. The wavelength with maximum absorption (450 nm) was used for calculation.

The total carotenoid content in /ug carotenoid/g in the muscle, liver and ovary were calculated as follows:

$$\text{Total carotenoids } /\text{ug/g} = \frac{\text{Absorption at 450 nm x dilution factor}}{0.25 \text{ x sample weight in gram.}}$$

Here, dilution factor = 50

extinction coefficient = 0.25

In the case of serum, total carotenoids, as /ug/ml serum was calculated as follows:

$$\text{Total carotenoids } /\text{ug/ml} = \frac{\text{Absorption at 450 nm x dilution factor (50)}}{0.25 \text{ x sample volume (ml)}}$$

2.7.8. Total calcium: Oven dried powdered ovary samples weighing 100 mg each and 0.1 ml of serum samples taken separately in clean dry digestion tubes were digested with 2 ml extrapure nitric acid and 1ml perchloric acid at 150°C for two hours in the digestion chamber, until a clear solution was formed. The digested samples were then diluted to 5 ml with 0.1% (w/v) lanthanum chloride solution in deionized water.

To prepare the stock solution of calcium, 1.249 g of calcium carbonate, predried at 120°C for four hours and cooled in a desiccator was dissolved in 50 ml of deionized water. To this, 10 ml of extrapure hydrochloric acid was added dropwise and then diluted to 1000 ml with deionized water to get final concentration of 500 ppm. Different calibration standards (1 ppm, 2 ppm....) were prepared afresh by diluting the stock

with 0.1% lanthanum chloride solution. 0.1% lanthanum chloride solution was treated as the blank.

The samples, standard and blank were analysed in Atomic absorption Spectrophotometer (AAS) at a wavelength of 422.7 nm using air-acetylene flame.

2.7.9. Iron content: Oven dried powdered ovary samples weighing 100 mg and 0.1 ml serum taken separately in clean dry tubes were digested with 2 ml of extrapure nitric acid and 1 ml of perchloric acid at 150°C for two hours in the digestion chamber, until a clear solution was formed. The digested samples were then diluted to 5 ml with deionized water.

The stock solution was prepared by dissolving 1 g iron wire in 50 ml of 50% nitric acid. This was diluted to 1000 ml with deionized water to get a final concentration of 1000 mg/l. Different calibration standards were prepared afresh by diluting the stock standard solution with deionized water. Deionized water (0.1 ml) was treated as the blank solution.

All the solutions were analysed is AAS at a wavelength of 248.3 nm using air-acetylene flame.

2.7.10. Statistical analysis: All the biochemical analyses were carried out in triplicates and repeated atleast ten times. The values were pooled up and the mean and standard deviation were calculated. The analysis of variance was calculated for each parameter to test any significant variation (1) between the different maturity stages in each tissue, and (2) between the tissues in each stage of maturity except for calcium and iron estimations.

CHAPTER III

REPRODUCTIVE BIOLOGY

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REPRODUCTIVE BIOLOGY

The success or failure of a species in any environment largely depends on its spawning potential. To overcome the various physical and biological hazards in the environment and to attain successful recruitment, fishes have a high reproductive potential. Information on the various aspects of reproductive mechanism and breeding biology of fishes is therefore an essential pre-requisite for the successful management of both capture and culture fisheries.

Contributions on the breeding biology of teleosts are chiefly those by Clark (1934), June (1953), Yuen (1955), Radhakrishnan (1957), Swarup (1959, 1961), Pollard (1972), Sobhana & Nair (1974), Siddiqui et al. (1976), Guraya et al. (1977), Davis (1977, 1982, 1984, 1985), Htun-Han (1978a,b), Ritakumari & Nair (1978), James & Baragi (1980), Delahunty & De Vlaming (1980a), Jacob & Nair (1983), Aida et al. (1984), Miller (1984), Cyrus & Blaber (1984), Pankhurst et al. (1987), Schaefer (1987), Crupkin et al. (1988), Mayer et al. (1988,1990), Jayasankar (1989) and Lal (personal communication).

Prabhu (1956) gave a detailed account on the maturation of eggs and spawning habits of some marine teleosts. Qasim (1973) reviewed the studies on breeding biology of marine teleosts from Indian waters.

De Vlaming et al. (1982) and Cone (1989) examined the validity of gonado-somatic index (GSI) and condition factor as valid indices of gonadal activity in teleosts, while Erickson et al. (1985) suggested the use of the relative gonadal index (RGI) for quantification of reproductive condition. A body length-related measure of eye size (eye index) was developed as an index of sexual maturity in eels by Pankhurst (1982). Miller (1984) used the term 'tokology' for the study of both the mechanics and profitability of reproduction (reproductive biology) in living organisms. Bagarinao & Chua (1986) studied the relationship between the egg size and larval size among teleosts. Witthames & Walker (1987) developed an automated method for counting and sizing fish eggs and Shoesmith (1990) compared the different methods for estimating mean fecundity.

The existing knowledge on the reproduction of Mugil cephalus and other mullet species from different parts of the world is reviewed in the introductory part (Chapter One) of the thesis. But it was observed that information on various aspects of breeding biology of M. cephalus in Indian waters other than seasonal spawning time and migration is fragmentary eventhough the fish is considered as one of the most important species for aquaculture in India (Patnaik, 1962; Rangaswamy, 1972; Das 1977b; and Sulochanamma et al., 1981). Further, most of the results evinced considerable degree of geographical variation. The available literature on the reproduction of this species from Kerala waters have also not provided details such as the dynamics of oocyte development, ovarian recrudescence, fecundity and changes in GSI, HSI (Kurian, 1960, 1975; Sunny, 1975; Elizabeth, 1987; Lalmohan & Nandakumaran, 1987).

RESULTS

Organisation and structure of female reproductive system

Mugil cephalus exhibits gonochorism and external distinguishing sexual characters are not visible. Generally males are smaller and slender than females. The abdomen of the female fish becomes flabby during the spawning period due to enormous increase in size of the ovaries.

The ovaries of M. cephalus are paired cylindrical compact structures lying in the body cavity ventral to the kidney. They are attached to the dorsal coelomic wall by a thin membranous mesovarium. Both the lobes of ovary are closely opposed to each other throughout their length and are separable. Posteriorly the two ovaries are fused together to form a common, short and muscular oviduct, opening to the exterior through a common urinogenital aperture immediately behind the anus. The anterior free ends of the ovaries are relatively more broader and rounder than the posterior end. The left lobe is 11-15% smaller than the right lobe. The right lobe showed a maximum length of 20-25 cm in the ripe condition.

In M. cephalus, the ovaries are of cystovarian type. Each lobe is covered by a thin and transparent peritoneal layer (serosa) beneath which lies a thick elastic tunica albuginea comprising of connective tissue, smooth muscles and blood vessels. These two layers together constitute the ovarian wall, the posterior extension of which forms the oviduct. Inner to the

ovarian wall, lies the inner ovarian (germinal) epithelium which projects into the lumen of the ovary (ovocoel) along with the tunica albuginea to form finger like ovigerous folds or ovigerous lamellae. Oocytes belonging to different developmental stages lie in these folds in a loose connective tissue called stroma. All the above layers are clearly observed only in immature and post-spawned ovaries, as the ovarian wall in the ripe stage becomes thin and highly stretched owing to the pressure exerted by large yolk-laden oocytes.

Classification of maturity stages of Ovary

Juvenile M. cephalus does not exhibit internal differentiation of sex. At the size of 25-30 cm (total length), the females can be distinguished from males by the presence of pinkish, translucent, ribbon-like strands of gonadal tissue, occupying one-fourth of the posterior part of the body cavity. In males, the testes make their appearance as two colourless, thin, thread-like strands, occupying the lower half of the body cavity.

Based on the size, shape, colour and texture of the ovaries and microscopic structure of the ova, five arbitrary stages of maturity have been identified.

Stage I (Immature): The ovaries appear as two pinkish translucent and jelly like structures united at the posterior end, measuring about 6 to 7 cm in length and less than 1 cm in width; entire gonad occupies about one fourth of the body cavity of the fish; oocytes under magnification

appear irregular, transparent with a central nucleus and without yolk; diameter of the oocytes ranges from 20 /um to 170 /um with mode at 70 /um.

Stage II (Maturing virgins and Recovering spent): Each ovarian lobe at this stage becomes cylindrical and appears slightly yellowish; the two lobes are unequal in length and the right lobe is longer than the left by 1-2 cm; they measure about 8-10 cm in length and 1.0-1.5 cm in width and occupy about half of the body cavity; maturing ova are distinguishable to the naked eye; under microscope, they appear white to pale yellow in colour, granular and still not yet fully rounded, central portion of which is darker; the oocyte diameter ranges from 170 /um to 250 /um with mode at 200 /um. Few atretic follicles still present in recovering spents. (Stage II is often subdivided into maturing virgins and recovering spents, which cannot be distinguished macroscopically, but histologically a recovering spent ovary is characterized by having a thicker ovary wall and often containing residual atretic oocytes).

Stage III (Ripening or Mature): Pair of distinctly yellowish and cylindrical ovarian lobes; the length of ovary varies 11-13 cm, the width 1.5-2.5 cm; the right lobe shows

a maximum length of 14 cm; the entire gonad occupies three fourths of the body cavity, which are almost fully packed with yolky oocytes; the ovarian wall appears as very thin, distended and almost transparent ; ova are yellowish, granular, round, yolk - laden and appear as dark bodies under the microscope, the diameter of which ranges from 250 /um to 650 /um with mode at 500 /um.

Stage IV (Ripe): The massive yellowish-orange or golden yellowish ovaries, fully packed with ripe eggs, fill the entire body cavity with numerous blood vesseels ramifying over their surface; they measure about 18-20 cm in length and 5-6 cm in width; almost all the eggs are round, loosely arranged and under microscope they are transparent and with a single large oil drop inside; some ripe ova are visible to the exterior at the vent region; the diameter of ova ranges from 650-900 /um with a mode at 810 /um.

Stage V (Spent): The highly shrunken and collapsed ovaries occupy approximately half of the body cavity; they appear blood shot, translucent, flaccid and loosely packed with primary oocytes; blood vessels prominent over the surface; majority of the oocytes are microscopic, transparent

and belonging to the immature stock with diameter ranging from 30 to 200 /um (mode at 50 /um); a few opaque, large, disintegrated yolky oocytes are also observed as scattered which undergo the process of resorption; each lobe of ovary measures about 9 cm in length and 1.5 cm in width.

Gonadosomatic Index (GSI) (Table-4; Fig.3)

The mean GSI was found to be 0.55 in stage I which showed a gradual increase to 9.44 in stage III. The highest value of GSI, 19.4 ± 2.04 was observed in stage IV followed by a steep decline in the spent stage (stage V) with a mean value of 4.25.

Condition Factor 'K' (Table-4; Fig.4)

The mean value of 'K' was lowest in stage I (0.90), which gradually increased reaching its peak (1.35) in ripe (stage IV) condition. In stage V, 'K' came down to 0.94, showing that, the condition factor is influenced by the breeding cycle.

Progression of Ova towards maturity (Table-5; Fig.5)

With a view to trace the development of ova from the immature stage to the ripe condition, oocyte size - frequency profiles were constructed at various developmental stages of M. cephalus. The ova diameter measure-

Table 4: Gonadosomatic Index (GSI), condition factor 'K' and Hepatosomatic Index (HSI) at different maturity stages,in female Mugil cephalus.

Maturity stages	GSI (%)	Condition Factor 'K'	HSI (%)
Stage I	0.55 ± 0.19	0.90 ± 0.04	0.99 ± 0.15
Stage II	2.20 ± 1.03	0.96 ± 0.02	1.41 ± 0.12
Stage III	9.44 ± 1.82	1.10 ± 0.07	2.21 ± 0.21
Stage IV	19.40 ± 2.04	1.35 ± 0.05	1.58 ± 0.32
Stage V	4.25 ± 0.95	0.94 ± 0.03	1.44 ± 0.09

All values are mean ± standard deviation.

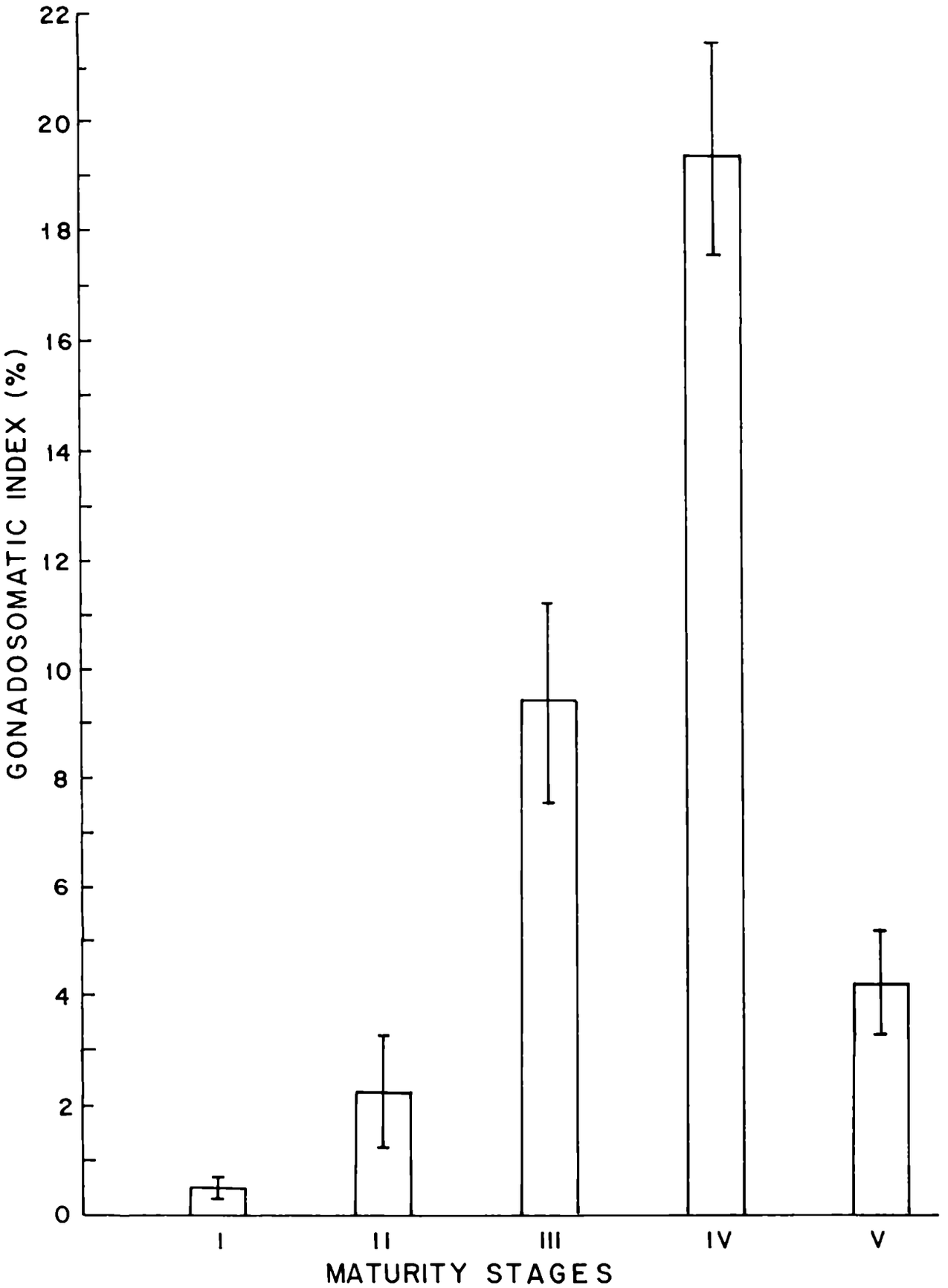


Fig. 3. Variation in gonadosomatic index (GSI) at different maturity stages in female Mugil cephalus. Vertical lines represent standard deviations

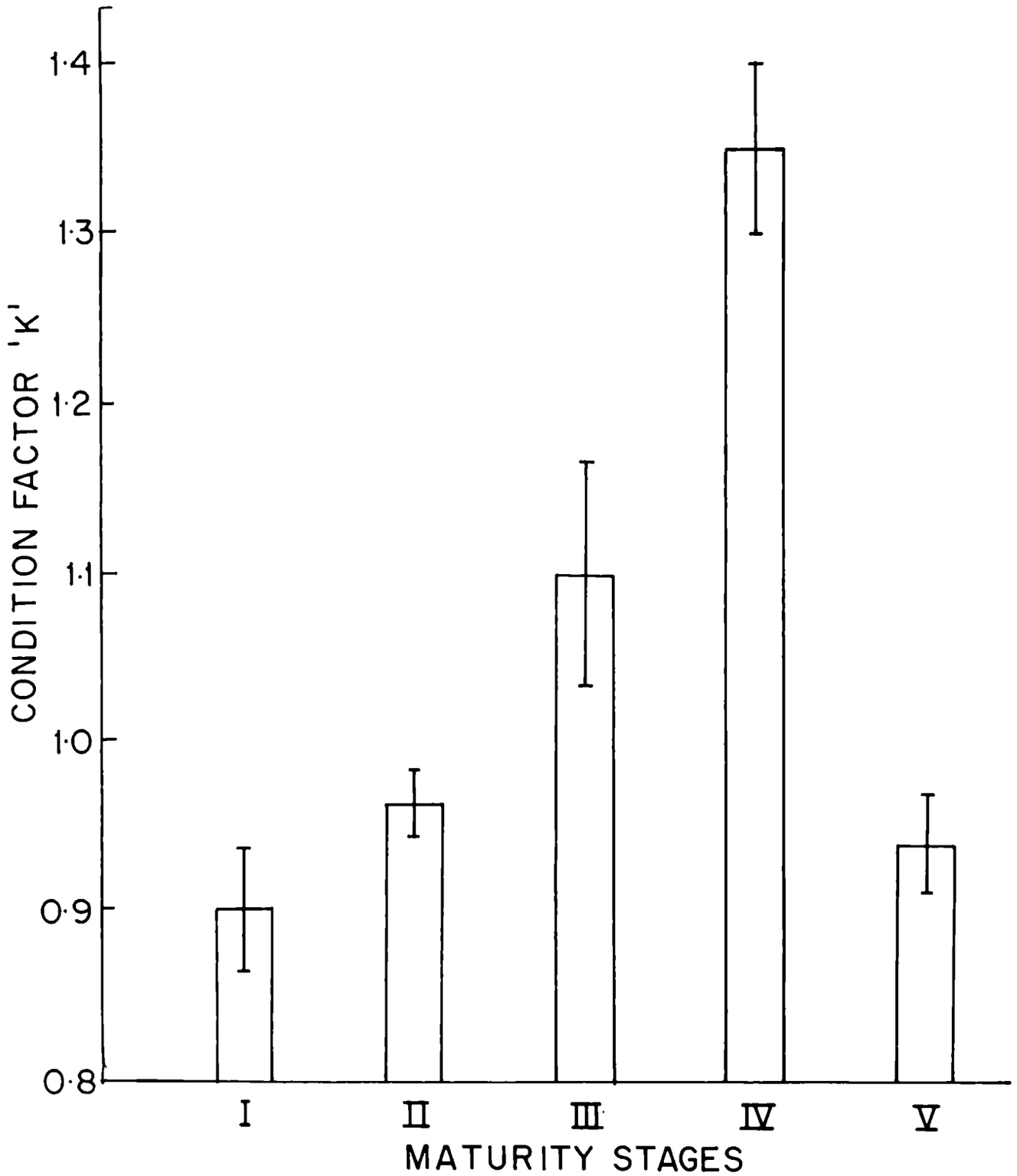


Fig. 4. Variations in the condition factor (K) at different maturity stages in female Mugil cephalus. Vertical lines represent standard deviations

ments were grouped into 50 /um intervals.

Stage I had only immature oocytes ranging their diameter from 20 /um to 170 /um. A prominent mode was observed at 70 /um. In stage II, a gradual growth of oocytes was discernible with the largest oocyte diameter (LOD) of 250 /um, having a mode at 200 /um and there was only one batch of oocytes, observed apart from the immature stock. A further progression of ova was obvious in stage III with the developing batch of eggs forming a distinct mode at 500 /um and with a range of 250-650 /um in diameter. Ripe (stage IV) ovary showed a clear and well differentiated group of eggs with a mode at 810 /um and the LOD 890 /um. However, irrespective of the stage of maturity, an immature reserve stock of oocytes was always observed in ovaries belonging to all stages of maturity. In stages III and IV, the peaks of ripening and ripe oocytes remained well separated from these immature reserve oocytes.

In stage V (spent), the immature stock of oocytes (30-200 /um size), with a maximum percentage of 50 /um oocytes were left along with a few residual eggs and empty follicles of the released ova.

Almost all oocytes when grown to a diameter of 250 /um entered the phase of exogenous vitellogenesis. Yolky oocytes at a size of 650 /um reached the minimum prematuration size.

Fecundity (Fig. 6)

The fecundity was estimated in 35 specimens of M. cephalus

Table 5: Oocyte diameter percentage-frequency at various maturity stages of Mugil cephalus L.

Oocyte diameter range (/um)	Percentage of Oocytes				
	STAGE I	STAGE II	STAGE III	STAGE IV	STAGE V
1 - 50	26.2	10.5	8.3	6.4	29.8
51 100	58.4	11.3	5.7	3.8	40.0
101 150	12.6	17.4	2.6	2.0	19.5
151 - 200	2.8	40.6	1.3	1.2	4.7
201 250		20.2	1.0	-	1.0
251 300			4.0	-	
301 350			6.8	-	
351 - 400			6.5	-	
401 - 450			10.2	-	
451 500			26.2	-	
501 - 550			13.1	1.2	
551 - 600			10.3	1.6	1.0
601 - 650			4.0	5.2	3.0
651 700				12.3	1.0
701 750				14.2	
751 800				23.1	
801 850				24.4	
851 900				4.6	
Oocyte Diameter Mode	70 /um	200 /um	500 /um	810 /um	50 /um
Largest Oocyte Diameter (LOD)	170 /um	250 /um	650 /um	890 /um	200 /um

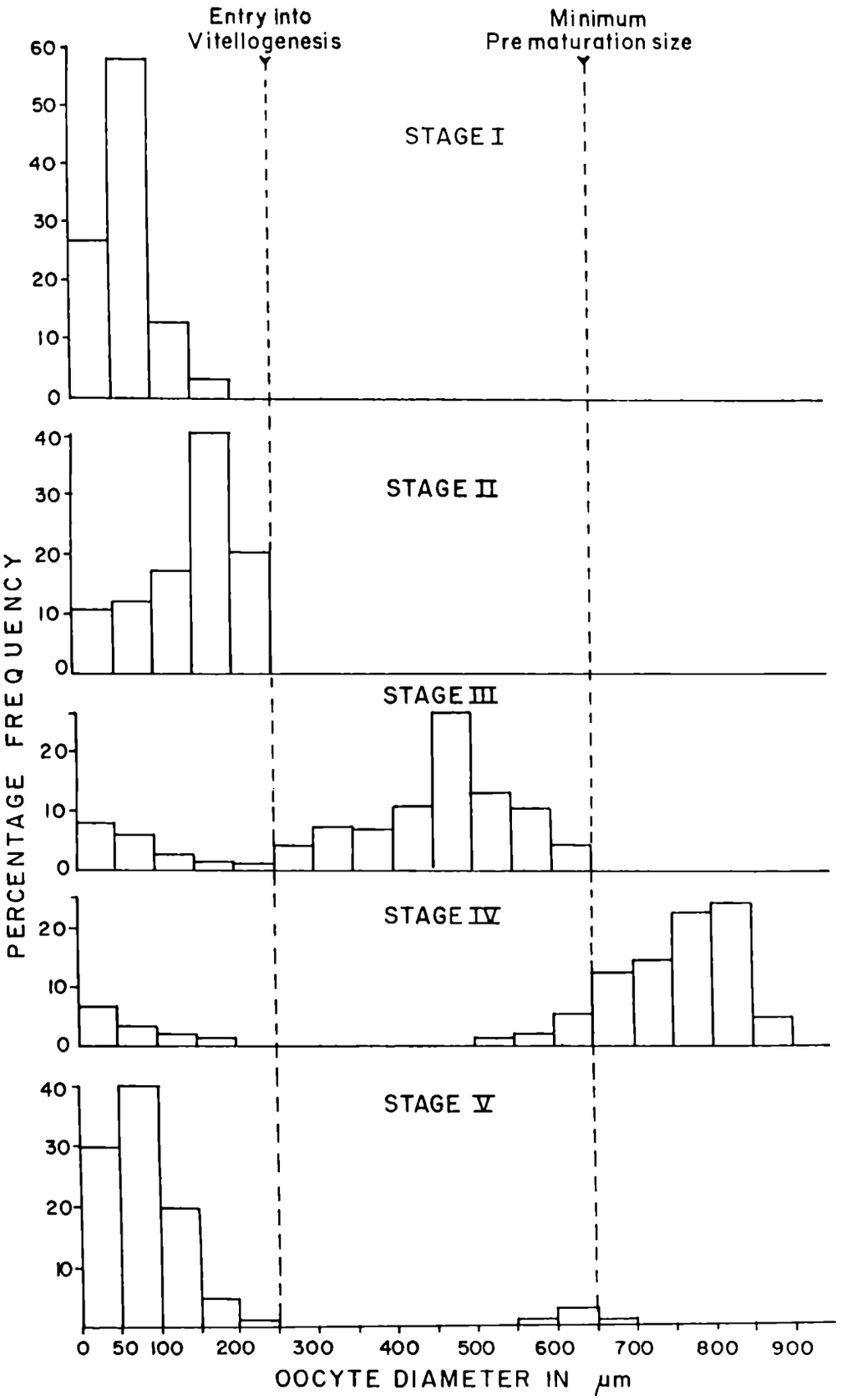


Fig. 5. Oocyte diameter - frequency profiles corresponding to stages of ovarian recrudescence in Mugil cephalus. Dashed line on left represents the transition between previtellogenic and vitellogenic oocytes; dashed line on the right delineates the minimum prematuration oocyte size.

with ripe (stage IV) ovaries. The number of ripe ova was found to vary from 5.27 lakhs to 35.6 lakhs in fish ranging in total lengths from 430 to 680 mm, whole body weight between 900 g and 3,200 g and total ovary weight ranging from 170 to 590 g. Fecundity was related to the total length of fish, total body weight and total gonad weight, using regression equations.

The relationship between fecundity (F) and total length of fish (L) was found to be curvilinear, as follows:

$$\text{Log } F = -8.4289 + 3.6027 \log L$$

$$F \text{ (in lakhs)} = 3.726 \times 10^{-9} L^{3.6027}$$

$$r = 0.789$$

Fecundity (F) was found to be linearly related to the total body weight (W), as follows:

$$\log F = -2.6385 + 1.2145 \log W$$

$$F \text{ (in lakhs)} = 0.0023\ W^{1.2145}$$

$$r = 0.874$$

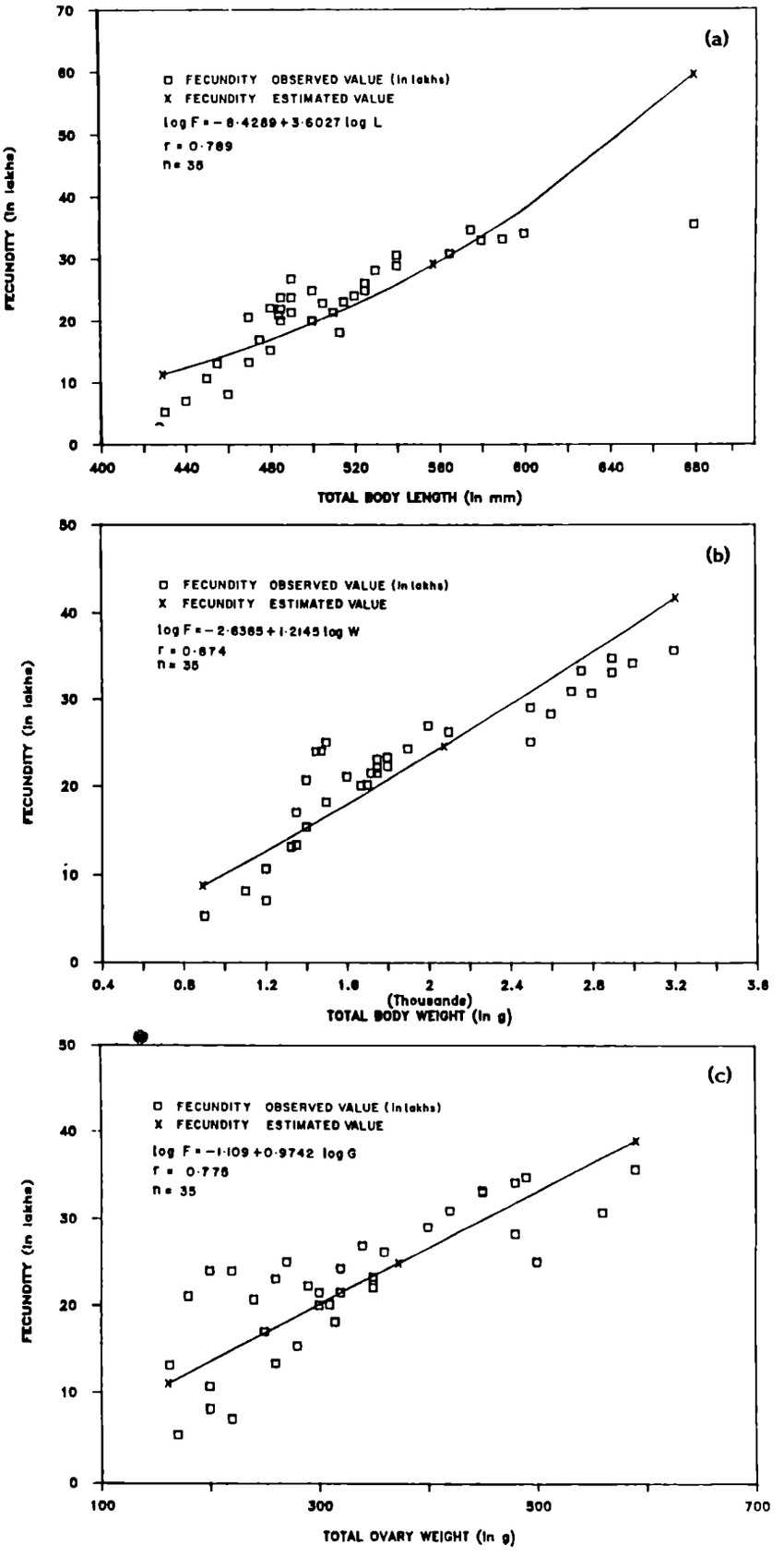


Fig. 6. The relationship of the potential annual fecundity of Mugil cephalus from Cochin to (a) total body length, (b) total body weight and (c) gonad weight.

The regression of fecundity on the total gonad weight (G) was found to be linear, as follows:

log F	= −1.1094 + 0.9742 log G
F (in lakhs)	$0.07773\ G^{0.9742}$
r	0.776

The coefficient of correlation (r) was found to be significant in all cases.

Hepatosomatic Index (HSI) (Table-4; Fig. 7)

The dark reddish-brown coloured liver (paler in colour in mature and ripe specimens) in female Mugil cephalus, which is placed rather obliquely under the stomach is composed of three incompletely divided lobes. A thin-walled and oblong gall bladder lies in the lower margin of the right lobe and the bile duct from it opens into the duodenum behind the pyloric caeca.

The mean value of HSI (percentage liver weight in relation to total body weight) was lowest (0.99) in stage I, which showed a gradual increase in the following stages and reached its peak in stage III, showing a value of 2.21 ± 0.21. In ripe stage, the HSI showed a decline with a mean value of 1.58 and a further decrease to 1.44 ± 0.09 in the spent stage.

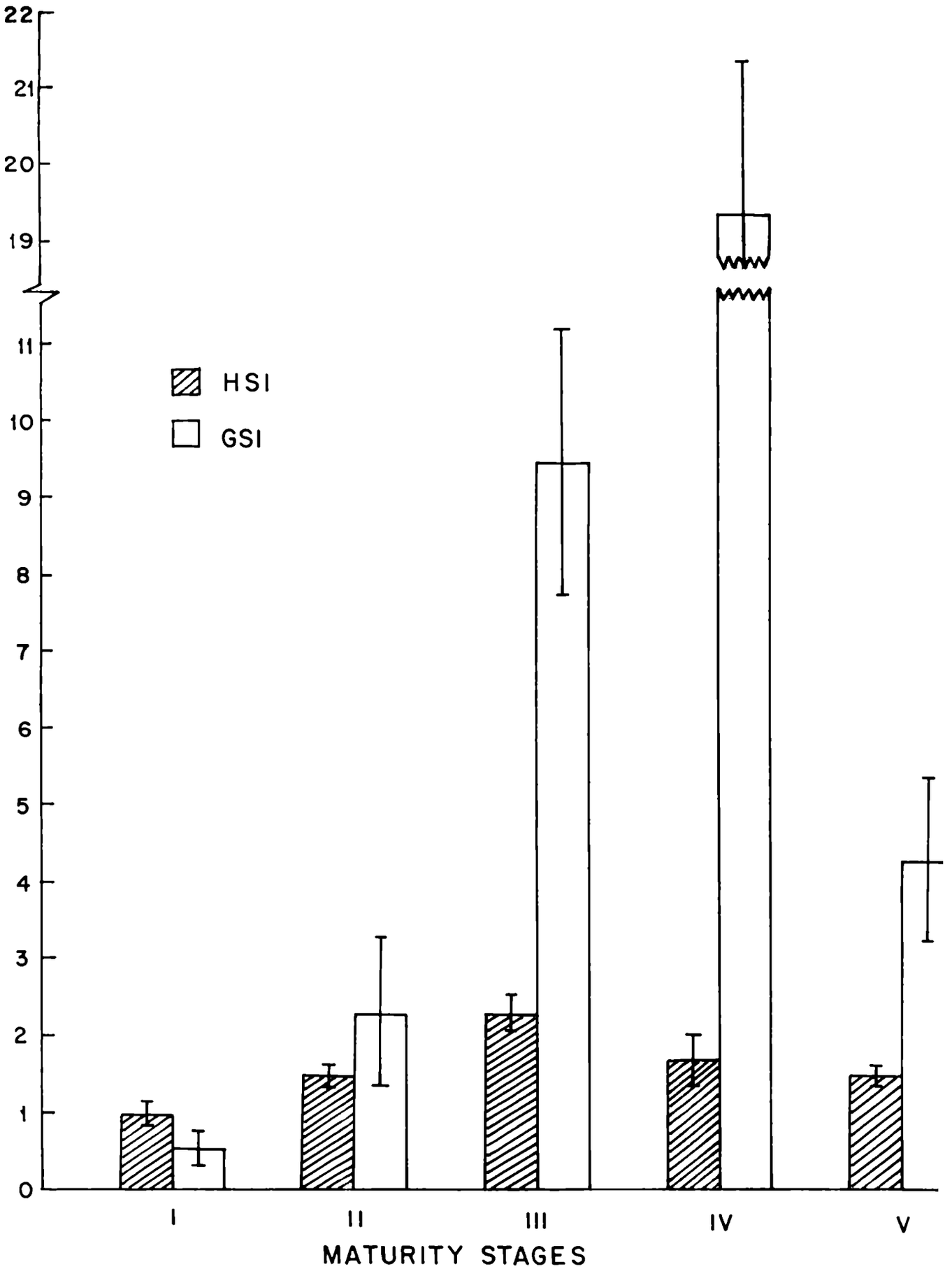


Fig. 7. Variation in the hepatosomatic index (HSI) and gonadosomatic index (GSI) different stages of ovarian maturation in female Mugil cephalus. Vertical lines indicate standard deviations.

DISCUSSION

The teleosts are predominantly dioecious (gonochorists), but hermaphroditism, sex reversal and gynogenesis occur in some species. Although this complexity of reproduction in teleosts is reflected in a wide range of gonadal structures, basically, the morphology of germ cells and various somatic cell elements constituting the gonadal tissue are similar (Nagahama, 1983). The complementary tasks of the gonads of teleosts are to produce fertilizable gametes necessary for successful procreation and the pituitary dependent synthesis and secretion of a variety of steroid hormones, which regulate the development of germ cells.

During embryonic development in elasmobranchs, ganoids, lungfishes and all tetrapods, the ovaries arise from the cortex and the testes from the medulla of the peritoneal wall. But in teleosts and cyclostomes, the entire gonad (i.e. ovary and testis) develops from single primordia - originates directly from the peritoneal epithelium and corresponds to only the cortex of other vertebrates. There is evidently no contribution from the interrenal (mesonephric) blastema (Hoar & Randall, 1969). It is assumed that the lack of cortical and medullary organisation in the embryonic gonad may account for the more widespread occurrence of intersexuality among the cyclostomes and teleosts.

Mullets are heterosexual fishes and gonochorism is the general rule (Brusle, 1981a), but some cases of abnormal hermaphrodites or intersexual mullets have been recorded (Kesteven, 1942; Stenger, 1959; Thomson, 1966; Brusle & Brusle, 1975 and Brusle, 1981a). However, in the present study such abnormalities were not encountered.

According to Brusle (1981a) and Muthukaruppan (1987), the hollow, paired ovaries of mullets can be classified as "cystovarian type" which has coelom-derived ovarian lumen (ovocoel) continuous with the oviduct. Ripe oocytes which are liberated into the ovocoel move to the oviduct. In oviparous teleosts, the ovarian cavity has been regarded as merely a "stock room" in which ovulated eggs are kept temporarily until they are spawned (Nagahama, 1983). In M. cephalus, the oviduct is formed by the posterior extension of the ovarian wall and not derived from the Mullerian duct as in many teleosts (Wake, 1985). The left ovarian lobe was found to be 11-15% smaller than the right one in M. cephalus in the present study. El Maghraby et al. (1974), Brusle (1981a) and Muthukaruppan (1987) also have observed that the right ovarian lobe is 12.8 15.0% larger than the left one in mullets. The circular smooth muscle cells in the ovarian wall of M. cephalus are probably important in the egg release from the ovary, as Begovac & Wallace (1987) have suggested the formation of a contraction wave in the ovarian wall of pipefish during spawning, which in effect squeezes the eggs out of the ovocoel.

Juvenile M. cephalus does not exhibit internal differentiation of sex. In the present study, discrimination of female sex became possible

in fishes having a total length of 25-30 cm, which is in agreement with the observation on M. cephalus from Israel (Abraham, 1963). According to him, the sexes of M. cephalus could be distinguished, when the fishes were about 28 cm long and 6-7 months of age.

Different workers have adopted different maturity schemes (scales) to quantify ovarian maturation in mullets as reviewed earlier by Qasim (1973), Brusle (1981a) and Elizabeth (1987). The five-point maturity scale, followed in the present study to define stages of sexual maturity in M. cephalus has been developed by Qasim (1973). This particular ovarian classification was found to be most ideal for the tropical total spawners and the different stages were distinguished taking all morphological and histological features into consideration. A similar scale was also used in mullets, by Kuo et al. (1974a), Kurup and Samuel (1983) and Muthukaruppan (1987).

Reproductive cycles are characterized by pronounced variations in gonadal size, which is dependent upon the body size. The gonadosomatic index (GSI) eliminates the effect of body size of fish on gonadal weight (De Vlaming et al., 1982; Erickson et al., 1985). The GSI increases with the progressive development of the gonads in M. cephalus until ripe stage and since then the index declined sharply in spent fishes. Maximum value of GSI (21.4%) was recorded in the ripe stages, which was possibly due to large deposits of yolk in the oocytes. The GSI or 'RGS' - 'rapport gonosomatique' (Brusle, 1981a) values in the present case compare well with those reported for M. cephalus earlier (Morovic, 1963; Abraham et al., 1966; Yashouv, 1969; Miller, 1971; Shehadeh et al., 1973; Brulhet, 1975;

Grant & Spain, 1975; Lalmohan & Nandakumaran, 1987). The GSI is also useful to measure experimental maturation and artificially induced spawning after hormone treatments (Shehadeh & Ellis, 1970). The heaviest gonad weighed 300 g in the present study, from a ripe fish having a total body weight of 1,400 g and total length, 500 mm. Kesteven (1942) recorded a maximum weight for ovary of 230 g in M. cephalus and Brulhet (1975) 566 g in M. cephalus ashentensis.

In the present study, maximum values of GSI in M. cephalus were recorded during the months of June, July and early August (Southwest monsoon peeriod) in Cochin waters, when most of the ovaries were in ripe condition. This finding clearly indicates that the spawning season of this species is from June to middle of August in Cochin, which is further supported by the sharp fall in GSI values and the occurrence of spent females and abundance of small fry in backwaters from August onwards. Pillay (1948), Qasim (1973), Kurian (1975), Sebastian & Nair (1975) Sunny (1975), Lalmohan & Nandakumaran (1987) and Elizabeth (1987) have observed that mullets breed during the southwest monsoon period along Kerala coast, whereas Jacob & Krishnamurthy (1948), Sarojini (1951, 1957, 1958), Patnaik (1962), Luther (1963), Kowtal (1967), Rangaswamy (1972b), Chaudhuri et al. (1977), Sathyashree et al. (1981) and Sulochanamma et al. (1981) have recorded the breeding of mullets during and after the northeast monsoon season along the east coast of India. This variation in spawning period may be due to the availability favourable climatic conditions which differs at different localities. However, most of the species are found to spawn during periods of low or declining temperature, salinity and photoperiod (Blaber, 1987; Elizabeth, 1987).

Kuo et al. (1974b) observed that a constant low temperature of 21°C and reduced photoperiod (6 hours light and 18 hours darkness) stimulated vitellogenesis within 40-62 days in M. cephalus. But, many researchers have observed that the gametogenic changes take place independent of the photoperiodic rhythm in tropics (Qasim, 1973; Siddiqui et al. 1976; Lam, 1983; Elizabeth, 1987) and variations in temperature play an important role. Blaber (1987) reported that, in Southeastern Africa, mullets spawn during rainy periods in the sea in the vicinity of estuarine mouths, and the high turbidity in the estuaries and near-shore areas acts as the important cue for the recruitment of postlarval mullets to riverine systems. De Silva & Perera (1976) found that optimal conditions for the growth of young fry of M. cephalus occur at salinities of 10-20‰. Heavy southwest monsoon showers (from June to August) bring all these congenial factors together: low surface water temperature (average 27°C), low salinity in backwaters (10-15 ppt); high turbidity; high dissolved oxygen content (8 ml/l) (Table-6; Fig. 8) and high productivity (Elizabeth, 1987; Lalmohan & Nandakumaran, 1987) along the Cochin coast which might ultimately result in the successful spawning of M. cephalus during this period. Similar situation may occur during the active northeast monsoon season along the east coast of India. The duration of spermatogenic activity is longer than the ovarian maturity in mullets. This is to assure fertilization of whole eggs (Brusle, 1981a). Oozing males (M. cephalus) were caught few months or weeks before the onset of ovarian ripeness and after the cessation of egg-lying (Thieberger-Abraham, 1967; Elizabeth, 1987).

Conflicting information exists on the natural spawning locations of grey mullets. Spawning in nature has not been unequivocally observed,

Table 6: Monthly variation in surface water temperature, salinity and dissolved oxygen at Cochin barmouth during the period of study.

Month	Mean surface water temperature °C	Mean salinity (ppt)	Mean dissolved oxygen (ml/litre)
1986			
January	29.5	31.20	3.11
February	31.0	31.00	3.78
March	32.0	33.50	4.00
April	31.0	32.44	4.12
May	31.8	34.82	3.76
June	30.1	30.21	4.84
July	28.5	16.63	7.02
August	29.0	10.10	6.43
September	31.0	21.28	5.51
October	30.0	20.35	6.39
November	29.8	22.12	5.24
December	30.2	29.99	3.83
1987			
January	28.0	33.29	3.25
February	31.5	31.20	3.60
March	32.0	33.00	3.80
April	31.6	31.45	3.66
May	32.0	29.45	2.20
June	28.5	10.00	3.05
July	26.3	16.05	5.42
August	26.5	28.50	8.05
September	26.5	29.66	6.11
October	28.5	19.36	5.38
November	29.5	20.30	4.95
December	29.0	29.48	3.85
1988			
January	27.0	30.25	3.34
February	30.0	31.50	3.90
March	32.0	35.14	3.92
April	31.0	32.38	4.05
May	32.2	35.00	2.97
June	28.0	28.00	3.25
July	27.5	19.81	4.02

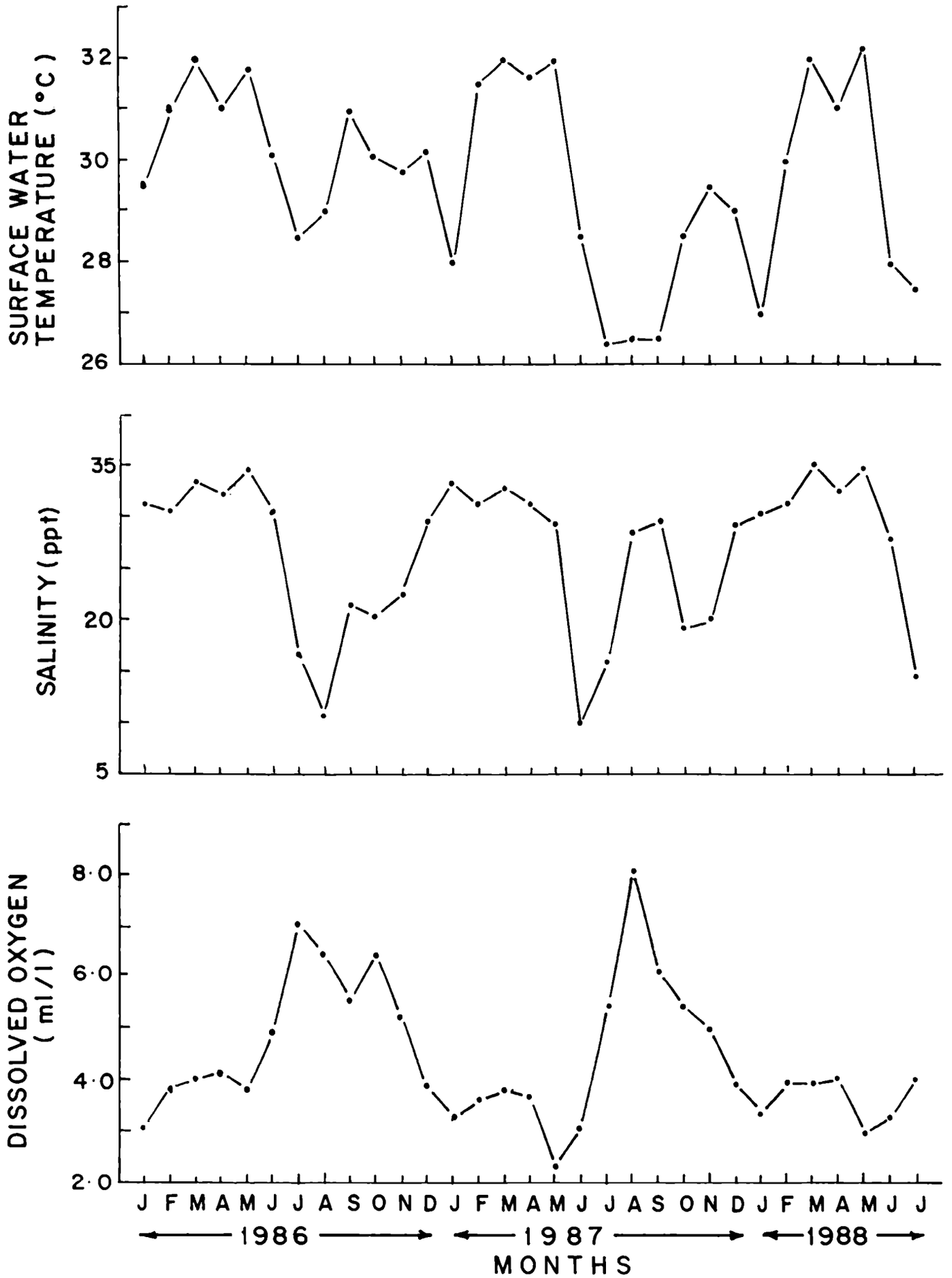


Fig. 8. Monthly variation in the surface water temperature, salinity and dissolved oxygen at Cochin barmouth region during the period of study.

but by inference, is generally thought be in the sea (whether in close vicinity to the sea coast or off-shore is still controversial). Breder (1940), Thong (1969a), Kowtal (1967), Rangaswamy (1972b),Grant & Spain (1975), Sathyashree et al. (1981), Sulochanamma et al. (1981), Blaber (1987) and Whitfield (1990) reported marine inshore spawning of mullets. Indications of breeding in deeper seas, far off the coast were presented by Arnold & Thompson (1958), Fitch (1972), Gopalakrishnan (1974) and Finucane et al. (1978). But Kurian (1975) has reported that the mullets spawn in backwaters. Spawning in freshwater seems impossible, except in the case of the Indian freshwater mullet, Rhinomugil corsula, which is reported to breed in rivers and enclosed waters (Kurian, 1975; Sugunan & Vinci, 1981).

Running ripe females and oozing males were collected near the barmouth at Cochin, Azheekode and Pulicat Lake, during the breeding season. The histological pictures revealed the ripeness of eggs collected from these specimens, such as coalesced yolk, presence of a single large oil droplet, absence of germinal vesicle (Chapter 3; Plate VII). These factors suggest the possibility of M. cephalus spawning inside the backwater system at Cochin. But relatively late incursion (from August onwards) of spent fish and querimana (mullet fry of length 20-25 mm; Anderson (1958) estimated 30-40 days for larvae to reach this size) into the backwaters indicates the spawning of M. cephalus in the littoral or sublittoral waters, as suggested by Grant & Spain (1975) from Queensland. This is further supported by the decreased salinity levels (10-15‰) in Cochin backwaters during the breeding season, as the eggs of M. cephalus cannot survive salinities $< 28‰$ for more than 24 hours (Sylvester et al., 1975).

In the present study, many specimens caught from the brackishwater fish farm (salinity 10-12 ppt) at Puthotta, far away from Cochin barmouth, showed yolky atretic ovaries during spawning period. Eckstein & Eylath (1970) have shown the occurrence of very high concentration of a most potent androgen, 11-ketotestosterone, in the ovaries of M. capito, confined to freshwater and suggested that the high levels of this androgen (very low levels in normal, migrating fish) inhibited the release of ovulating hormone(s), which caused ovular atresia in this fish. Probably, the same reason can be attributed to the high incidence of atresia observed in the present study in M. cephalus which are confined to brackishwater ponds.

The coefficient of condition or the condition factor or the ponderal index (K) has been used in fishery science to express the relative robustness of the fish, in age and growth studies, to measure the effect of environmental improvement and the attainment of sexual maturity and spawning (Luther, 1963). The fluctuation observed in the condition factor of fishes have been attributed to changes in ecological (absence or lack of food) or physiological conditions (sexual maturity) (Blanco-Racedo, 1983). The ponderal index also varies depending on the amount of food in the gut and the fatness of the body (Rangaswamy, 1972). But with the increase in age, there is a lower level of condition throughout the seasonal cycle consequent upon the increased metabolic strain of spawning (Radhakrishnan, 1957). The condition factor may also give an indication of size at first maturity in fishes (Cone, 1989). The ponderal index of mullets has been studied by Sarojini (1957, 1958), Luther (1963), Thong (1969a), Rangaswamy (1972b), Landret (1975), Chan & Chua (1980), Sathyashree et al. (1981),

Blanco-Racedo (1983) and Elizabeth (1987), but only some of the results indicate the influence of gonadal maturation on the value of 'K'. Hence, the present study was aimed at estimating the variations in Fulton's condition factor (K), if any, with respect to the maturity cycle in female M. cephalus. The results indicated a gradual increase in 'K' value upto the ripe stage followed by a significant drop in the spent condition. The rise in condition coefficient might be due to the occurrence of ripe gonads and the drop due to the loss of gonadial products due to spawning. Radhakrishnan (1957) in Sillago sihama, Chan & Chua (1980) in L. subviridis, Sathyashree et al. (1981) in Osteomugil speigleri, and Blanco-Racedo (1983) in Mugil incilis have observed similar variations in condition factor. However, Elizabeth (1987) in male M. cephalus and Luther (1963) in the juveniles of M. cephalus and L. macrolepis did not observe any specific pattern of change in ponderal index. During maturation, the increase in weight of the ovary is quite higher than that of the testis and the growth in length is more important than the weight gain in juvenile fishes, therefore condition factor can hardly be considered as an index of well being of male fish and juveniles (Thong, 1969a; Blanco-Racedo, 1983). The variations in condition factor with respect to length offer help in determining the size of fish at first maturity (Sarojini, 1957, 1958; Chan & Chua, 1980; Brusle, 1981a). At Cochin, male and female M. cephalus attain sexual maturity in the size of 380 mm and 430 mm (total length) respectively and in Pulicat Lake, 376 mm for males and 425 mm for females (Rangaswamy 1972).

Most workers agree that individual female mullet spawns only once a year. All the ripe oocytes are liberated out at the same time and the spawning is considered to the non-intermittent (Sarojini, 1958; Luther, 1963; Sathyashree et al., 1981; Sulochanamma et al.,1981; Chubb et al.,1981; Muthukaruppan, 1987; Greeley et al., 1987). But, some have observed a prolonged breeding season in mullets and release of eggs in two or more batches (Sarojini, 1957; Chan & Chua, 1980; Sugunan & Vinci, 1981; Surendrababu & Neelakantan, 1983). The oocyte size - frequency profiles of this study support the assumption that only one distinct batch of ripe eggs with a mode at 810 /um is clearly demarked from the group of immature eggs in a season and this single clutch is eventually spawned in its entirety; thus confirming the fact that M. cephalus has a group synchronous type (Synchronisme par group) ovary. In such an ovary, a single clutch of mature oocytes forms the major share during the breeding season; along with this, coexists an apparently small asynchronous pool of oogonia and previtellogenic oocytes (only 13.4% of the total number of eggs in the present case) as the replenishing stock.

The annual fecundity of M. cephalus has been reported to be from 1.2×10^6 to 3.6×10^6 by most authors, although estimates ranged from as low as 0.5×10^6 to as high as 14×10^6 (Alvarez-Lajonchere, 1982). Shehadeh et al. (1973) quoted a fecundity of 648 ova per gram body weight of three year old fish, while Nash et al. (1974) reported 849 eggs per gram for older individuals. In the present study, the fecundity of M. cephalus in Cochin was found to range from 0.5×10^6 to 3.5×10^6 The fecundity of fish varies with age, length and weight. Correlation

with body length has been most widely used, as length is reckoned to be a better measure of size, since a fish will not shrink, but can lose weight (Chan & Chua, 1980). In M. cephalus, the relationship between fecundity and length has been found to be curvilinear, as in most of the fishes. The exponential value 3.6027 in the present case, is within the accepted range of 2.34 5.28 for most species of fish (Chan & Chua, 1980) and indicates that fecundity increases at a rate greater than cube of the length. The linear relationship between fecundity, body weight and ovary weight indicate that in M. cephalus, the number of eggs in the ovaries increased progressively in proportion to these two variables. According to Brusle (1981a), increase in growth rate of females and the accompanying increase in their fecundity are considered as an adaptive response to improvement in the living conditions of populations.

The range of egg production in the present investigation was much higher than that observed in the same species from Goan waters. Das (1977b) recorded a low fecundity in M. cephalus along Goa coast ranging from 43,000 to 5,13,000 ova in specimens varying in length from 140 mm to 315 mm (5.27 lakhs to 35.6 lakhs ova in the present study). Ovarian maturation of M. cephalus at a length of 140 mm has never been recorded earlier in the world (Brusle, 1981a). In Cochin, all females even at the size of 300-315 mm (total length) were either immature or juveniles and specimens with yolky oocytes were encountered only at lengths above 400 mm. The report from Goa coast might be an example of precociously maturing population of M. cephalus as was also observed in Lates calcarifer, in Gulf of Carpentaria in Australia (Davis, 1984). But further

investigations alone will prove the validity of this conclusion

Among mullets, highest prolificity occurs in M. cephalus (Brusle, 1981a; Alvarez-Lajonchere, 1982). The high fecundity observed may be due to the large size of the species, size of eggs which are small and attainment of first sexual maturity at a greater size and age (Chan & Chua, 1980); thereby M. cephalus may have more accommodative capacity for increased oocyte production. Moreover annual spawners like M. cephalus might have a high fecundity compared to other smaller mullets which are multiple spawners. The higher ova production by this species is also an adaptation to compensate for the losses incurred during the life stages prior to entering the estuary (Whitfield, 1990). However, the larval survival rate in nature is yet to be documented.

Gonadal recrudescence in female fishes involves an accumulation of lipid and protein stores within the developing oocytes. These stores may come from visceral organs, body muscles and ingested food, if fish feeds during recrudescence (Shulman, 1974). Therefore, changes in weight in some visceral organs such as the liver may be inversely correlated with oocyte development in some species. The hepatosomatic index (HSI) or the liversomatic index (LSI) is a way of measuring the energy retained in the tissue. It is also used to obtain information about the metabolic state of fish, which store fat in the liver (Shulman, 1974). This expression appears to be a good indicator of food intake (Crupkin et al., 1988). HSI is an appropriate expression of liver size, as it does not change over the range of body weights, unlike the liver weight (Delahunty & De Vlaming, 1980).

The HSI values in the present investigation gradually increased upto third stage of maturity, and then decreased in subsequent stages. The highest value of HSI in stage III suggests the peak synthesis of vitellogenin (the yolk protein precursor) by the hepatocytes. The low HSI level in ripe condition indicates the serious depletion of liver reserves during yolk formation, and in spent stage due to the exhaustion as a result of spawning and migration. The ultrastructural, histochemical and biochemical studies in M. cephalus (Chapters IV, V and VII of the thesis) present firm support to the above inference. Muthukaruppan (1987), in Liza parsia, Crupkin et al. (1988) in M. hubbsi, Delahunty & De Vlaming (1980) in C. auratus and Htun-Han (1978b) in Limanda limanda have recorded similar observations as in M. cephalus. An enlargement of liver and its cells, and several cytoplasmic changes in hepatocytes indicative of vitellogenin synthesis in response to exogenous or endogenous oestrogen have been reported in many teleosts (Peute et al., 1978; Guraya, 1986).

CHAPTER IV

HISTOLOGY OF OOCYTES AND HEPATOCYTES

CHAPTER IV

HISTOLOGY OF OOCYTES AND HEPATOCYTES

Reproductive activities in most animals undergo cyclic rhythms. The patterns of these changes in the gonads are characteristic for each species. One of the convenient methods of determining the reproductive cycle including the spawning period of a fish, is to study the seasonal developmental changes in the gonads through both macroscopic (morphometric) and microscopic observations. Macroscopic examination alone has its limitations, as it is difficult to distinguish macroscopically, the maturing virgins from recovering spents, presence of atretic follicles; and most important is the actual developmental stages of growing oocytes themselves may not be determined. For these reasons, to understand the reproductive mechanism of any fish, a detailed histological study has become necessary.

Investigations on the seasonal changes in the oocytes of teleosts (light microscopy) are many, among which the more relevant to the present study include those of Yamamoto (1955a,b, 1956a,b,c, 1963), Chopra (1958), Sathyanesan (1960, 1961), Yamamoto & Yamazaki (1961), Venugopalan (1962) Malone & Hisaoka (1963), Yamamoto et al. (1965), Rajalakshmi (1966), Dutt (1967), Lambert (1970a,b), Pollard (1972), Aravindan & Padmanabhan (1972a,b), Te Winkel (1972), Beams & Kessel (1973), Khoo (1975, 1979), Guraya et al. (1977), Ritakumari & Padmanabhan (1976), Varghese (1976), Sathyanesan & Joy (1976), Davis (1977, 1982, 1985), Riehl (1977a), Sobhana

& Nair (1977), Verma (1977), Htun-Han (1978a), Guraya & Kaur (1979, 1982), Ritakumari & Nair (1979a,b) Shackley & King (1979), Pankhurst (1982), Cyrus & Blaber (1984), Hunter & Macewicz (1985), Emel'yanova (1985), Makeeva & Saha (1985), Selman et al. (1986, 1988), Abu-Hakima (1987), Begovac & Wallace (1987), Pankhurst et al. (1987), Schaefer (1987), Mayer et al. (1988), Van der Merwe et al. (1988), Jayasankar (1989) Coello & Grimm (1990), Sahu et al. (1990) and Lal (personal communication).

Transmission electron microscopy (TEM) has allowed a better description of cytological and nuclear processes such as yolk accumulation;formation of yolk nucleus, egg membranes, lipid droplets and cortical alveoli, while the micropyle and surface structure of eggs has been analysed with the help of scanning electron microscope (SEM). Contributions on these lines in the near-past include those of Flugel (1964a,b,c), Yamamoto & Onozato (1965), Yamamoto & Oota (1967), Beams & Kessel (1973), Riehl (1977b), Szollozi et al. (1978), Van den Hurk & Peute (1979, 1985), Lang (1981a,b), Ohta & Teranishi (1982), Brusle (1983b, 1987), Wu & Sun (1983), Selman & Wallace (1982,1983), Kessel et al. (1985) and Lal (Personal communication). The functional morphology of teleost gonads has been dealt with by Nagahama (1983), while Wallace & Selman (1981), Wallace (1985) and Wallace et al. (1987) have studied cellular and dynamic aspects of vitellogenesis and oocyte growth in teleosts. Guraya (1986) in his excellent monograph, has provided an integrated account of cellular and molecular aspects of oocyte growth, maturation, ovulation and atresia in teleosts while, West (1990) reviewed the methods of assessing ovarian development in fishes.

The morphology of liver in teleosts differs considerably with sex and sexual activity as the organ synthesizes and secretes vitellogenin (Vtg- the yolk protein precursor) under the triggering action of estrogens. Reports describing light microscopic as well as ultrastructural changes of hepatocytes during maturation, include those by Ishii & Yamamoto (1970), Weis (1972), Aida et al. (1973b), Welsch & Storch (1973), Yamamoto & Egami (1974), Varghese (1976), Van der Gaag et al. (1977), Peute et al. (1978), Olivereau & Olivereau (1979), Van Bohemen et al. (1981), Nunomura et al. (1983) Eurell & Haensly (1982), Avila (1986) and Leatherland & Sonstegard (1988).

Many descriptions on the histology of spermatogenesis and oogenesis of different species of mullets have been recorded. But inconsistencies in the use of several stage designations, varying and somewhat confusing terminology have made the cellular events during oogenesis, difficult to understand. Also, the reports fail to explain the ultrastructural details of oogenesis, ovarian atresia and histo-morphology of hepatocytes, especially in M. cephalus. The present histological study of oocyte development in M. cephalus is intended to, firstly define an appropriate staging series for oogenesis and ovarian atresia and secondly, relate the histological observations to the macroscopic ovary maturity stages. The histological changes of hepatocytes, during oogenesis are also documented.

Terminology used to describe oogenesis (Table-7)

As several diverse and rather perplexing key terms are in use to describe the oocyte development, the terminology used in the present study is clarified along with some of the analogous terms. It is now generally

Table 7 : Terminology used in this study to describe oogenesis and analogous terms used by other authors.

Terminology used and abbreviations	Analogous terminology
Oogenesis (the entire process of development of the ripe egg from oogonia through to ovulation. Raven, 1961; Guraya, 1986)	Oogenesis *per se* (meiotic transformation of oogonia into primary or previtellogenic oocytes. Tokarz, 1978; Wallace & Selman, 1981)
Mesovarium (MSM), Serosa (SA), Tunica albuginea (TA), Stroma (SM), Ovigerous lamellae (OGL), Germinal epithelium (GEM), Ovocoel (OVC), Oolemma (OL) (Ritakumari & Nair, 1979 a)	
Primary growth phase (PGP) or Previtellogenesis; Previtellogenic or Primary oocytes (PVO) (Raven, 1961; Wallace & Selman, 1981)	First growth phase (Khoo, 1979), Nuclear transformation or Amphitene, Juvenile phase, Protoplasmic growth, Period of slow growth (Meyyen, 1939)
Resting oocytes (RO) (Bowers & Holliday, 1961 *)	Reserve fund oocytes (Hickling, 1935)
Secondary growth phase (SGP) or Vitellogenesis (Raven, 1961; Khoo, 1979), Vitellogenic (Secondary) oocytes (VO).	Period of rapid growth, Trophoplasmic growth (Meyyen, 1939)
Zona radiata (ZR) (Raven, 1961; Khoo, 1979; Mayer *et al.*, 1988)	Chorion (Wallace & Selman, 1981), Zona pellucida (Guraya, 1986), Vitelline envelope (membrane) (Selman & Wallace, 1983), Primary envelope (Anderson, 1967 *), Cortex radiata (Gotting, 1961 *), Tunica propria (Zhitenev *et al.*, 1974)
Zona radiata interna (ZRI), Zona radiata externa (ZRE) (Mayer *et al.*, 1988)	Inner zona pellucida, Outer (proper) zona pellucida or glycocalyx (Brusle', 1985)
Follicular layer (FL), Granulosa (follicle) cells (GC), Thecal cells (TC), Collagen filled spaces (CLS), Special thecal cells (STC) or Interstitial gland cells (IGC) (Abraham *et al.*, 1984; Guraya, 1986; Sahu *et al.*, 1990)	
Basal lamina (BL) (Brusle' 1985)	Basement membrane or membrane propria folliculi (Flugel, 1964b)
Nuage (NG), Cement (C), Mitochondrial aggregates (M) (Andre', 1962 *; Cle'rot, 1976 *; Brusle' & Brusle', 1978)	Marker of germ cells (Hogan, 1973 *), Germinal dense bodies (Satoh & Egami, 1973 *), Granular masses/bodies (Azevedo, 1976 *), Chromatoid bodies (Schjeide *et al.*, 1972 *), Nuclear-like material (Kanobdee, 1975 *)
Yolk nucleus (YN) (Hubbard, 1894 *; Abraham *et al.*, 1966)	Perinuclear body, Balbiani's vitelline body, Idiosome, Circumnuclear ring, Juxtanuclear complex (Stenger, 1959; Raven, 1961; Cle'rot, 1976 *, Eme'lyanova, 1985)
Lipid droplets (LD) (Mayer *et al.*, 1988)	Oil (fat) drops/vesicles (Yamamoto *et al.*, 1965), Non-staining/primary yolk (Cyrus & Blaber, 1984), Vacuolation (Selyukov, 1986 *), L_1, L_2 & L_3 bodies (Dutt & Govindan, 1967), Yolk vesicles (Kuo *et al.*, 1974a)
Yolk globules (YG) (Yamamoto, 1956a; Guraya, 1986)	Exogenous (heterosynthetic) yolk (Wallace, 1985), Yolk granules (Mayer *et al.*, 1988), Red-staining/secondary yolk (Cyrus & Blaber, 1984), Yolk platelets (Pollard, 1972), Deutoplasmic components (Malone & Hisaoka, 1963)
Cortical alveoli (CA) (Guraya, 1986; Selman *et al.*, 1986)	Intravesicular (autosynthetic) yolk (Guraya, 1986), Yolk vesicles (Yamamoto, 1955a,b; Khoo, 1979), Cortical vesicles (Kobayashi, 1985 *)
Migratory nucleus stage (MGN) (Guraya, 1986)	Subperipheral stage
Ripe oocyte (Ovum) (RO)	Hyaline oocyte (Pankhurst *et al.*, 1987)
Postovulatory follicle (POF) or Corpus luteum (Guraya & Kaur, 1979)	Corpus luteum of ovulation (Khoo, 1975)
Atretic follicles (AF) or Corpus atreticum (Lambert, 1970a)	Corpus luteum of atresia or Preovulatory corpus luteum (Guraya, 1986)

* Original not referred; Cross reference from Brusle' (1985), Guraya (1986), Mayer *et al.* (1988).

recognized that oogenesis occurs in four distinct phases 1) The primary growth phase (PGP) which is gonadotropin-independent, involves oogonial proliferation by mitotic division, meiotic transformation of oogonia into primary oocytes and growth of primary oocytes concomitant with nuclear changes (Tokarz, 1978; Khoo, 1979); 2) the secondary growth phase (SGP) which is gonadotropin-dependent, involves deposition of yolk viz. vitellogenesis and is characterized by rapid oocyte growth; 3) oocyte maturation - which involves resumption of meiosis, germinal vesicle breakdown, coalescence of yolk and hydration of oocytes to form the ripe egg, the mechanisms of which are still not fully understood; 4) ovulation - the process by which the eggs after the completion of first meiotic division are released into the ovarian or peritoneal cavity, which involves a combination of factors including prostaglandins, corticosteroids, catecholamines and proteolytic enzymes.

RESULTS

A. HISTOLOGY OF OOCYTE DEVELOPMENT

The classification of main stages of oogenesis followed in the present study is based on the developmental sequence laid by Yamamoto & Yamazaki (1961), Guraya (1986) and Mayer et al. (1988) for different teleosts. The tinctorial affinities of ooplasmic components are given in Table-8.

TABLE 8: Tinctorial affinities of ooplasmic components of Mugil cephalus L.

STAINING	Oogonia	OOCYTES									Follicular layer	Atretic Yolky Oocytes
		Oolemma	Zona Radiata	OOPLASM					Nucleus	Nucleoli		
				Ground Cytoplasm	Yolk Nucleus	Lipid * Droplets	Yolk Globules	Cortical Alviolar Layer				
Ehrlich's Haematoxylin & Eosin (Weesner, 1960)	Pale Violet	Pale Pink	Pink	Deep Blue	Dark Violet		Red	Blue	Pale Violet	Bright Pink	Pale Red	Red
Heidenhain's Iron Haematoxylin (Weesner, 1960)	Faint Grey or no reaction	Pale Yellowish Brown	Pale Yellowish Brown	Pale Bluish Black	Black		Deep Bluish Black	Pale Yellowish Brown	Pale Grey	Black	Pale Yellowish Brown	Deep Black
Mallory's Triple (Mallory, 1944)	Orange	Purple	Red	Blue	Deep Purple		Brick Red	Pale Blue	Pink	Bright Orange	Blue	Reddish Orange
Methylene Blue - Azure II & Basic Fuchsin (Humphrey & Pittman, 1974)	Faint Blue or no reaction	Purple	Blue	Violet	Dark Blue		Blue	Not Clear	Blue	Bright Red	Pink	Blue

* Lipid bodies appear as blank spaces in histological preparations.

Primary Growth Phase (PGP):

Oogonia (Plate IIIa; Vd)

a) Morphology The oogonia appear as oval or rounded cells, having almost regular outline and with a diameter of 10-16 /um. Although they are observed singly, the cells tend to occur in nests (4-6 cells together) in the stroma of ovigerous folds.

b) Cytology

Nuclear characteristics:- The large ovoid or roughly spherical nucleus (7-8 /um in diameter) is situated almost centrally and has a regular outline. The nucleus-to-cell ratio is about 0.50 to 0.60. The nucleus contains one to three prominent nucleoli (> 2 /um in diameter) which are roughly spherical and electron-dense. The nuclear envelope is nearly smooth; the chromatin appears rather granular and dispersed in the nucleoplasm, but small dense clumps are found associated with the inner side of the nuclear membrane.

Cytoplasmic characteristics:- The oogonial cytoplasm is less basophilic and contains mitochondria, free ribosomes and scant endoplasmic reticulum; Golgi complex is not distinctly seen. Mitochondria are generally round (0.6 - 1.0 /um diameter) with a rather dense matrix. Aggregates of electron dense "nuage" material are frequently observed in the cytoplasm and near to the nuclear membrane. The electron dense "cement" material is surrounded by mitochondrial aggregates (Plate IIIa).

PLATE III

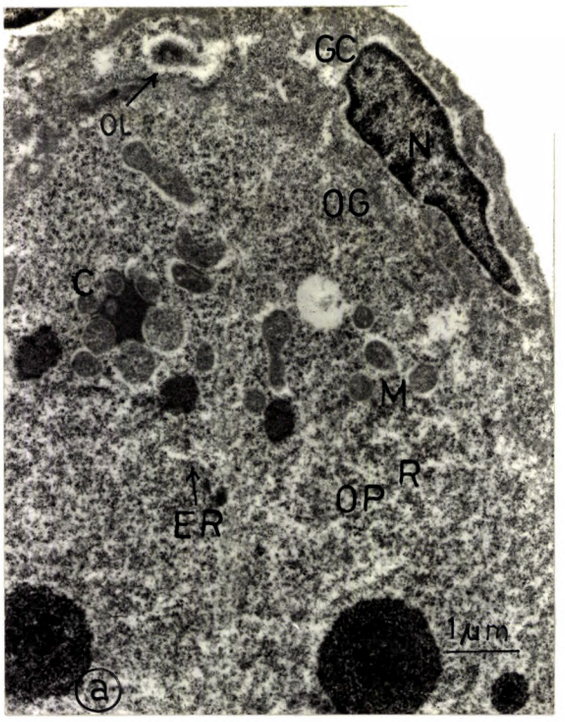
a. Electronmicrograph of an oogonium (OG). Cement (C), mitochondria (M), nuage (NG) and ribosomes (R) are present in the cytoplasm. Also note a follicle (granulosa) cell (GC) with an elongated nucleus (N). ER, endoplasmic reticulum; OL, ooplasm. X 9,560.

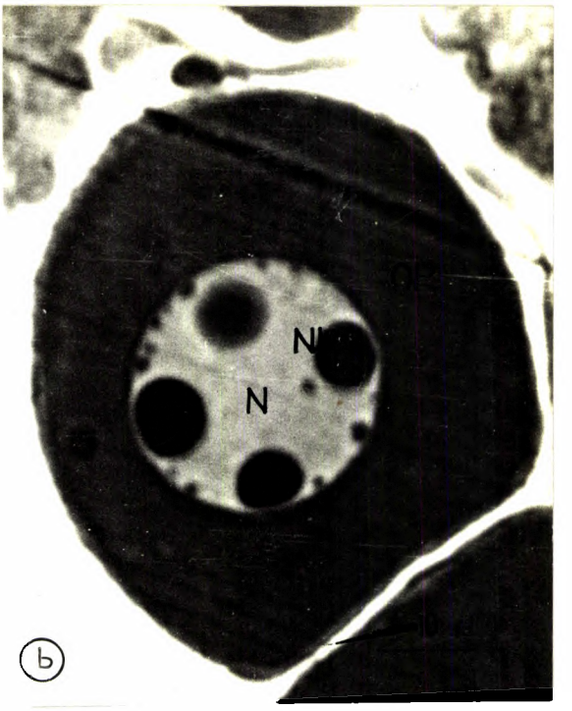
b. Lightmicrograph of a chromatin nucleolar oocyte illustrating highly basophilic ooplasm (OP) and the large round nucleus (N) containing nucleoli (NL). X 1,812.

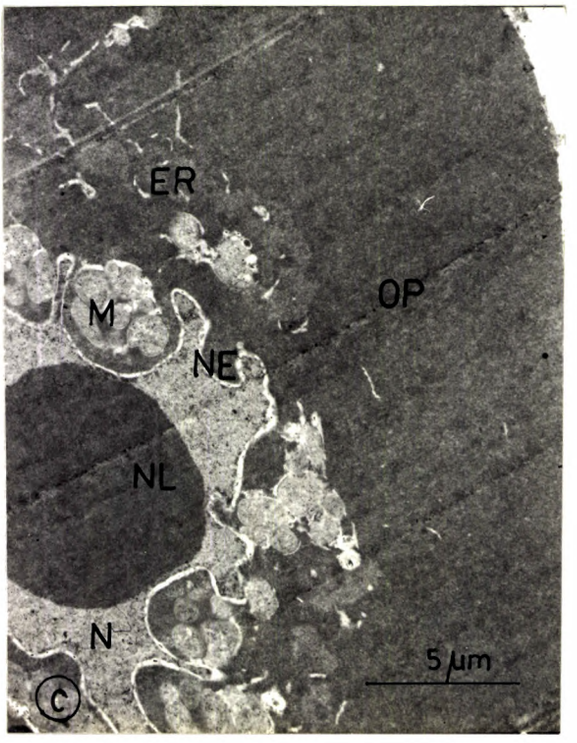
c. Electronmicrograph of a chromatin nucleolar oocyte. Note a highly electron-dense ooplasm (OP), homogeneous nucleolus (NL), the crown of mitochondria (M) and the highly undulating nuclear envelope (NE). Nucleoplasm is homogeneous. N, nucleus; ER, endoplasmic reticulum. X 4,141.

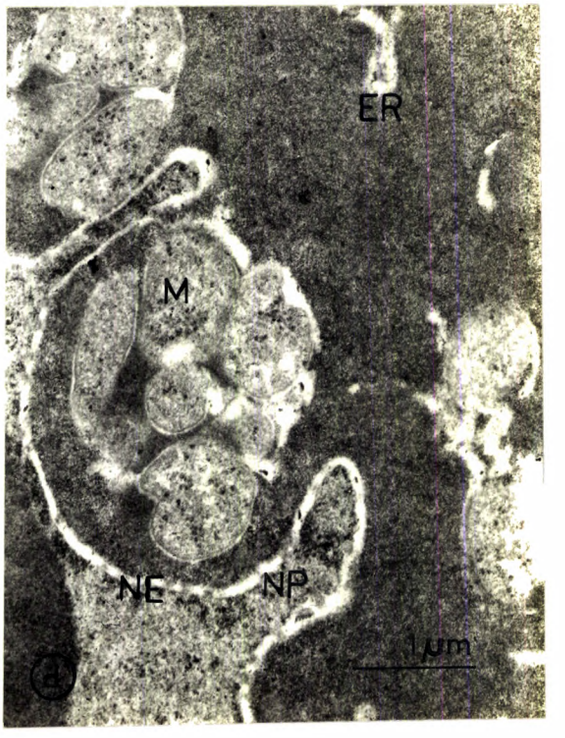
d. The undulating nuclear envelope (NE) consists of two parallel membranes and shows typical nuclear pores (NP). M, mitochondrial aggregates; ER, endoplasmic reticulum. X 16,056.

PLATE III









Follicular layer:- The oogonia are already invested with few flat, irregular follicle cells (granulosa) (2-3 /um in height) with regular outlines. Their large, elongate nucleus (1-2 /um high and 4 /um long) which is oriented parallel to the oogonial surface, exhibits condensation of chromatin close to the nuclear envelope and scattered in the nucleoplasm (Plate IIIa). The cytoplasm is more electron-dense than that of oogonia because of abundant ribosomes. Endoplasmic reticulum and mitochondria are scarce. There is no evidence of intercellular bridges or desmosomes. A distinct thecal cell layer is not visible in the follicular layer.

Chromatin Nucleolus stage (Plate IIIb,c,d)

a) Morphololgy Round or oval cells measuring 20-50 /um and having an almost regular outline. The oocytes are arranged in finger-like lamellar structures (ovigerous lamellae) projecting into the ovocoel.

b) Cytology

Nuclear characteristics:- The roughly spherical, large (10-15 /um dia), and slightly eccentrically located nucleus is well developed and occupies greater part of the cell; the nucleus/cell ratio is 0.45 (hereafter termed as N/C). The fine structure revealed the highly wavy or undulating nature of nuclear envelope and waves are accentuated into digitations infront of the area where cytoplasmic organellaes are packed. The nuclear envelope consists of two parallel membranes, separated by a perinuclear cisterna, approximate ly 10-20 nm in width. Numerous nuclear pores ($\approx$ 80-100 nm in diameter) interrupt the nuclear envelope, around the margins of which

both the membranes of the envelope fuse (Plate III d). The nucleoplasm is homogeneous and clear, with peripherally situated, strongly basophilic, larger (5-6 /um) nucleoli (2-3 in number), which are characterized by dense fibrillar material. In some cells, one of the nucleoli is larger and differs from rest by its staining properties and indefinite outlines. Few clumps of chromatin lie along the inner membrane in the nucleoplasm.

Cytoplasmic characteristics:- The thin layer of cytoplasm is strongly basophilic and more electron-dense. Ribosomes are numerous and thickly packed in the cytoplasm. Mitochondrial aggregates are conspicuously arranged near the nuclear envelope. In some places ribosomes (small black granules $\approx$ 20 nm) are present in the mitochondrial matrix, often situated close to the cristae. Few concentric profiles of smooth endoplasmic reticulum are observed near to the mitochondria. Dictyosomes are scarce. The nuage has become less striking and seen as scattered small dense patches close to nuclear envelope.

Follicular layer:- The follicle or granulosa cells did not show any noticeable change from the oogonial stage.

Early Perinucleolus stage (Plate IVa,b,c)

a) Morphology The round or oval or polygonal oocytes, measuring 40-70 /um are arranged in ovigerous lamellae.

b) Cytology

Nuclear characteristics:- The large, spherical (25-30 /um)

PLATE IV

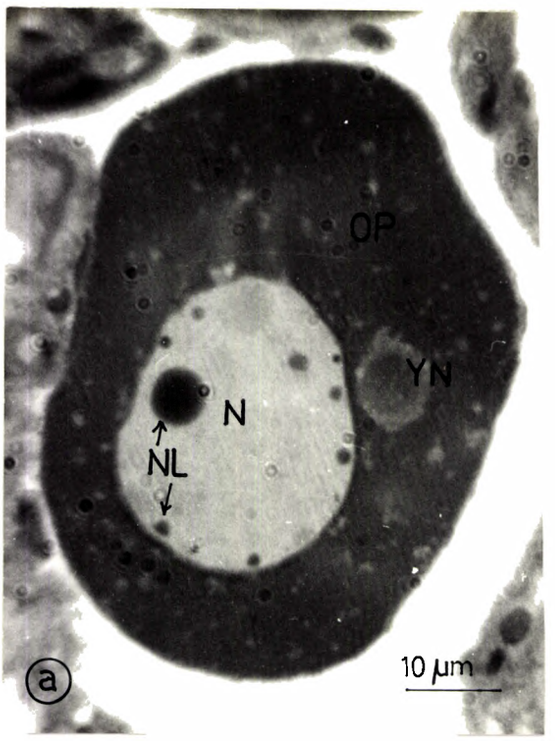
a. Semithin section of a perinucleolar oocyte, containing many small nucleoli (NL) and a prominent spherical yolk nucleus (YN). The ooplasm (OP) is highly basophilic. Also note one large nucleolus (NL). N, nucleus. X 1,280.

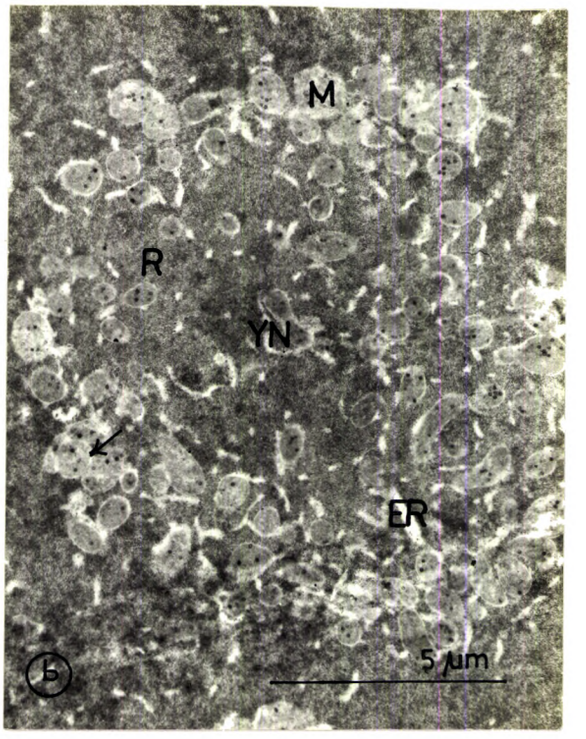
b. Electronmicrograph of a yolk nucleus (YN). It is composed of numerous round or oval mitochondra (M), ribosomes (R) and tubules of endoplasmic reticulum (ER). Also note ribosomes(⟶) in mitochondrial matrix. X 6,535.

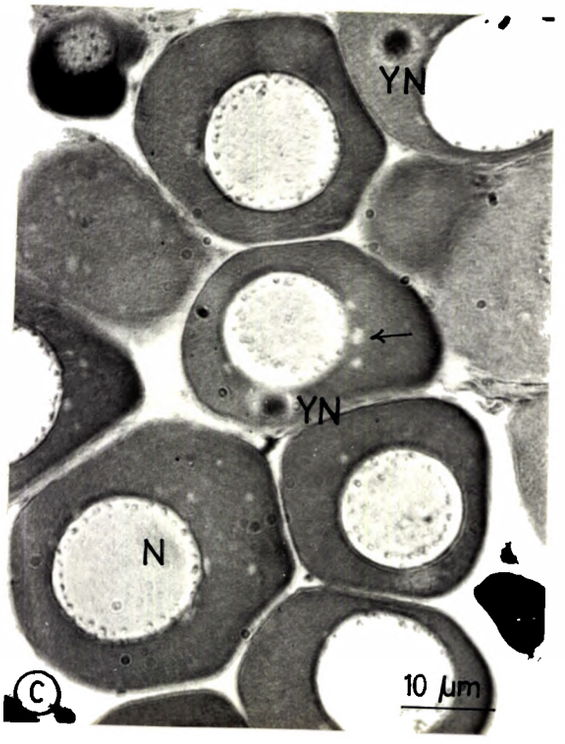
c. Section through an ovigerous lamellae of an immature ovary, showing perinucleolar oocytes containing conspicuous yolk nucleus (YN) and minute lipid droplets (⟶). N, nucleus. X 1,246.

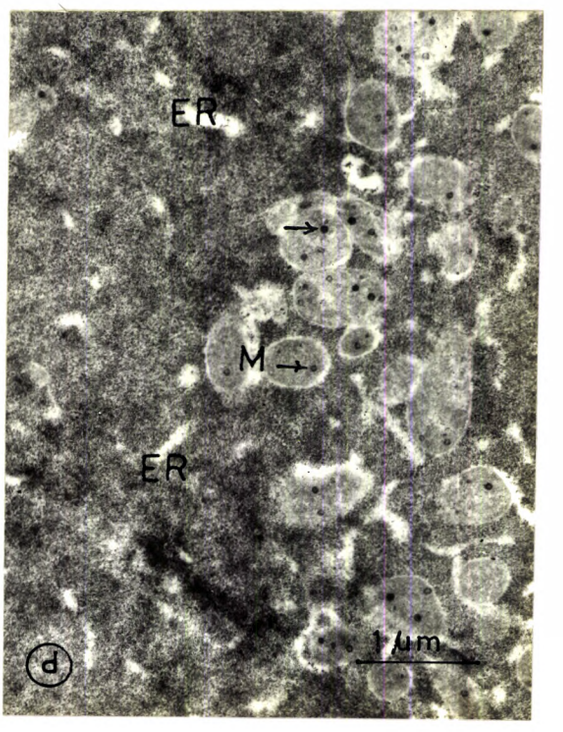
d. Electronmicrograph of the peripheral ooplasm of late perinucleolar oocyte, showing dispersed mitochondria (M) and endoplasmic reticulum (ER), after the disintegration of the yolk nucleus. ⟶ , ribosomes in mitochondrial matrix. X 16,055.

PLATE IV









centrally located nucleus now referred to as "germinal vesicle" (N/C 0.40 - 0.50) shows an almost smooth outline. It contains about 8-17 small, round (3-6 /um), highly basophilic and electron-dense nucleoli, in the peripheral nucleoplasm, close to the inner nuclear membrane.

Cytoplasmic characteristics The ooplasm is homogenous and highly basophilic; its electron density continues to increase owing to the very great number of ribosomes. The membrane structures consist of some endoplasmic reticular profiles; mitochondria are observed near the nuclear membrane as well as scattered in the ooplasm. In many larger oocytes (70 /um diameter), a spherical or oval (5-8 /um dia), deeply staining, basophilic, compact yolk nucleus (YN) is observed in the juxtanuclear ooplasm (Plate IVa,b). (In the light microscope the yolk nucleus generally appears surrounded by a light area, which may be the result of a fixation artefact due to the contraction of YN materials). Ultrastructure reveals, the YN is composed of numerous mitochondria (round or oval; 0.6 to 1.0 /um dia), ribosomes and tubules of endoplasmic reticulum (40-50 nm dia) (Plate IVb). Ribosomes are still visible inside the mitochondria. Small lipid droplets (5-10 /um in diameter) are detected in the ooplasm.

Follicular layer:- A thin basal lamina (90-100 cm thick) lies closely on the granulosa cells. A layer of thecal cells associates with the follicle cells external to the basal lamina. They are flat cells with an elongate nucleus oriented parallel to the basal lamina; the nucleus shows condensations of chromatin. The thin layer of cytoplasm contains few membranous organelles, ribosomes and many microfilaments.

Late Perinucleolus stage (Plate IVd)

a) Morphology The round oocytes in this stage measure 70-120 /um.

b) Cytology

Nuclear characteristics:- The nucleus increases in size to a mean diameter of 40 /um (35-45 /um), (N/C 0.38 - 0.50) and stains more lightly. The nucleoli increase in number to an average of 21 (range 12-28) per section and become more peripheral in position. The nucleoplasm becomes light due to decrease in number of the distributed granules. The nuclear envelope runs rather smoothly, occasionally ruptured by nuclear pores.

Cytoplasmic characteristics:- The cytoplasm is still basophilic and homogeneous in appearance. The basophilic YN migrates within the ooplasm; in older oocytes (100-120 /um), it is situated near the periphery than in younger ones and lies parallel to the cortex of oocytes. The structure of YN is fairly constant in all the cells. It finally loses its identity and merges with the peripheral ooplasm. Concomitant with the migration of YN, the ooplasm becomes granular and the granulation is first observed in the juxta-nuclear area, which expands centrifugally and eventually reaches the boundary of oocytes. Under electron microscope, the striking feature of increasingly dense cytoplasm is the general dispersion of both mitochondria and reticulum. Mitochondria are scattered from the perinuclear to the cortical area, most of them begin to develop an elongated shape (0.3-2.0 /um long and 0.3-0.4 /um wide) with a clear matrix and many cristae; the ribosmes associated with the cristae are not observed now (Plate IVd; Vb).

Many small patches of nuage remain tangential to the nuclear envelope (Plate VIa). At the end of this stage, the oolemma folds and short, curved microvilli protrude intermittently from the surface of the oocyte.

Follicular layer:- The follicle cells do not yet show microvilli as does the oolemma. Thecal cells are well developed which form the continuous outer-most layer of the oocyte (Plate V a). Other marked changes are not observed in the cells of follicular layer.

Thus during PGP, while the nucleus-to-cell ratio decreases as the oocyte volume increases, the absolute size of the nucleus increases and multiple nucleoli appear. On attaining their maximum size (100 - 120 /um), the late perinucleolus stage oocytes remain inactive for long periods until they are recruited into the secondary growth phase (vitellogenesis). For this reason, they have been termed as "resting oocytes", the chromosomes of which are arrested (during late chromatin nucleolus stage or early perinucleolus stage) at the prophase (diplotene stage) of the first meiotic division.

Secondary Growth Phase (SGP)

Early Lipid Droplet Stage (Plate Va,b,c)

a) Morphology The oocytes show a diameter of 110-160 /um in this stage.

b) Cytology

Nuclear characteristics:- The N/C is about 0.40. The nucleus

PLATE V

a. Electronmicrograph of ooplasm (OP) of early lipid droplet oocyte. Some lipid droplets (LD) appear as blank spaces, while lipids are not lost in some others during processing. The ooplasm contains numerous mitochondria (M). The zona radiata (ZR) starts forming. ⟶ basal lamina; GC, follicle cell; TC, thecal cell; ⇉ space between the follicle cell and the zona radiata. X 4,100.

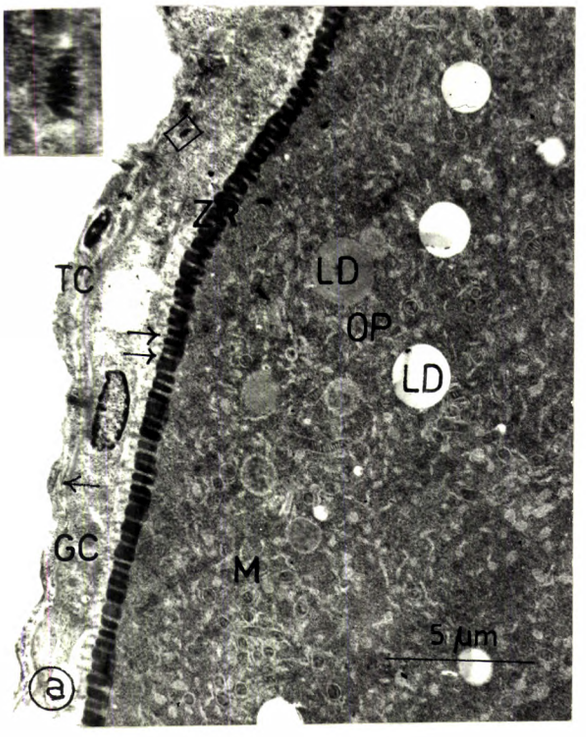
Inset:- Magnified view of a desmosome between the granulosa and thecal cells. Finger shaped tonofibrils are clearly seen. X 18,000.

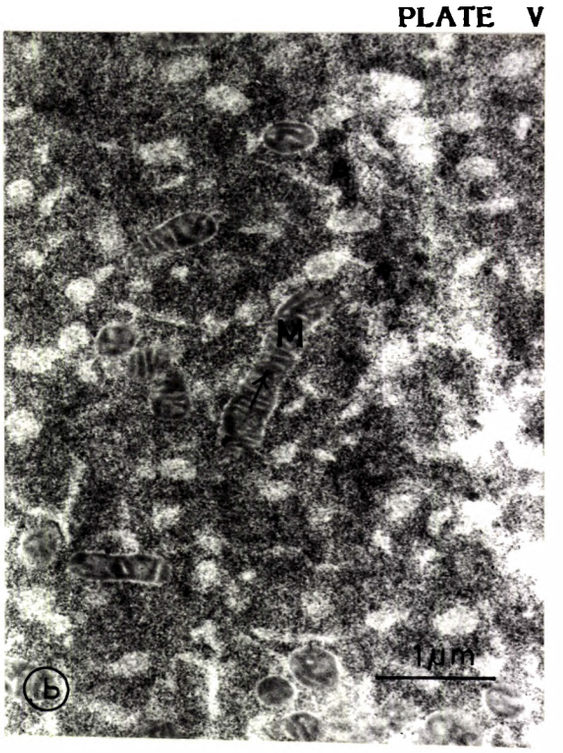
b. The change in shape of mitochondria (M) of oocytes is evident in the electron microscope, during early lipid droplet stage; they assume an elongated shape with a clear matrix and many cristae (⟶). The ribosomes in the matrix are absent now. X 16,056.

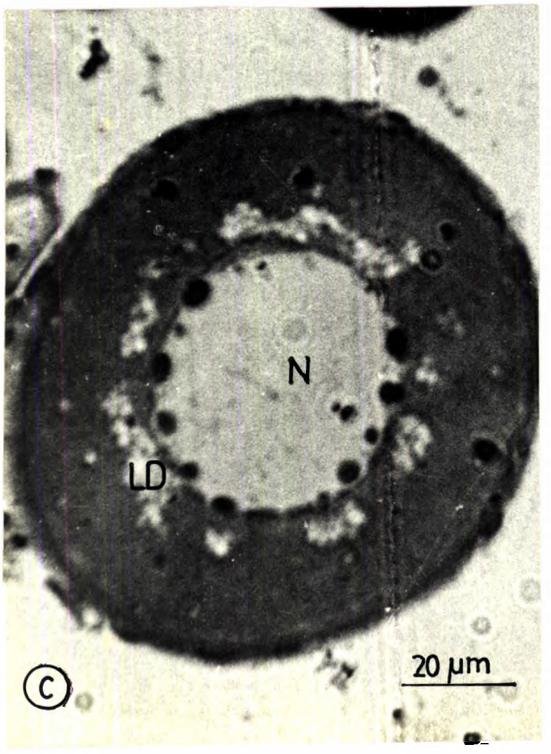
c Semithin section of an early lipid droplet oocyte. LD, lipid droplet, N, nucleus. X 760.

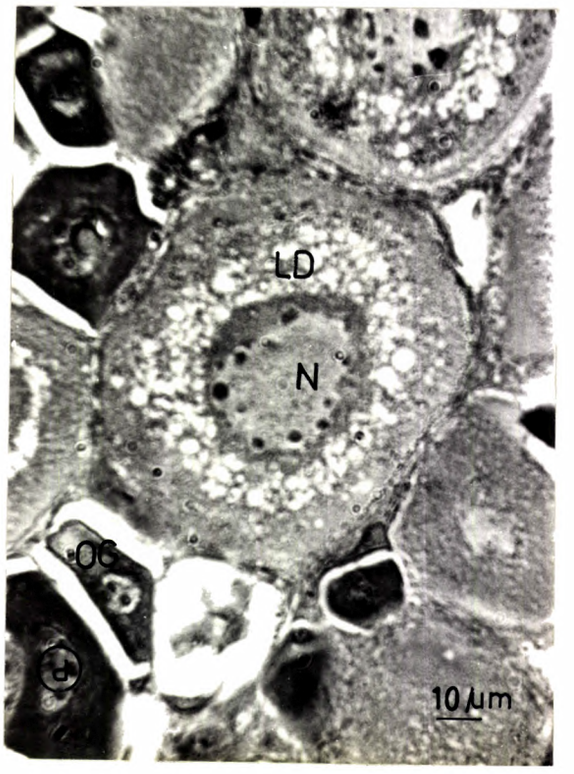
d. Light micrograph of a late lipid droplet oocyte. LD, lipid droplets; OG, oogonia; N, nucleus. X 545.

PLATE V









has become less basophilic and its outline irregular and wavy. The number and size of the nucleoli is as in the previous stage. Other striking changes are not observed in the nucleus from the previous stage.

Cytoplasmic characteristics:- The major event that marks the beginning of this stage is the appearance of a clear zone of lipid droplets in the ooplasm near the nucleus. These colourless round or oval vacuoles (Sudan Black 'B' and Oil Red 'O' positive) which are not membrane-bordered measuring 1-5 /um, continue to accrue and increase in size. The ooplasm is granular and less basophilic, the organelles (mitochondria, ER and ribosomes) increase in number and are scattered throughout the ooplasm. Elongated mitochondria appear similar to the ones in the previous stage. Small clusters of nuage are still located near the nuclear envelope. The oocyte microvillar processes become elaborate and are observed throughout the oocyte surface (≈1.0 /um long and 0.3 /um wide). The zona radiata (ZR) appears in oocytes of approximately 140 /um diameter. In the light microscope, the ZR is first observed as a thin acidophilic band between the oocytes and overlying follicle cells. Ultrastructurally, a homogeneous electron dense material (0.35 /um thick) located between the oocyte microvilli constitutes the early formed external zona radiata (ZRE). The amount of zona material increases so that in oocytes of 160 /um, the thickness of ZRE is 0.6 /um and a second more darker layer - zona radiata interna (ZRI) subjacent to the ZRE is secreted by the oocyte, having a thickness of 0.1 /um. As the ZR develops pushing follicle cells away from the oocyte membrane, the oocyte and the follicle microvilli increase in length and both pass through the zona, which is perforated (Plate Va; VIb).

Follicular layer:- In light microscope, the follicular layer is observed as a thin, lightly basophilic layer (2.5 - 3.5 /um) external to the acidophilic ZR, the minute nuclei of which are highly basophilic. At the ultrastructural level, the granulosa show short microvilli, few of them begi n to interdigitate with the oocyte processes. They remain squamous in shape (≈ 2.0 /um wide) and contain more number of mitochondria, ribosomes, ER, few dictyosomes and blank spaces (1.5-2.0 /um in diameter) left by lipid droplets (Plate Va; VIc). The cells are separated from the zona radiata by a 0.4-0.5 /um wide interspace. Thecal cells are well developed; desmosomes are observed along the basal lamina (≈ 0.3 /um thick and 0.5-0.6 /um long); which contain many dark, finger-like tonofibrils (Plate Va inset).

Late Lipid Droplet stage (Plate Vd; VIa,b)

a) Morphology The oocytes in this stage measure 160-210 /um.

b) Cytology

Nuclear characteristics:- The N/C is about 0.40. The nuclear membrane becomes very convoluted. The nucleoli maintain their peripheral position, but many of them become smaller (2-3 /um in diameter) and less regular in shape.

Cytoplasmic characteristics:- The lipid droplets have increased in number and size (8-15 /um in diameter) and they now form a discrete zone in the inner and middle ooplasm, a few of them are seen in the peripheral ooplasm also. Mitochondria and ER are dispersed between the lipid droplets

as well as in the cortical region. The ground ooplasm is still granular, but less basophilic. The acidophilic ZR is thickened further reaching a width of 1.7-2.0 /um. Its bipartite nature is very evident in the electron microscope. The ZRI is thickened rapidly (which becomes less darker) than the ZRE, the formed having a thickness of 1.3 /um and the latter 0.7 /um in this stage. Oocyte microvilli are now about 3.0 /um long; they traverse the pore channels of ZR, protruding from its free end towards the follicle cells. The pore channels of ZR appear round in cross section, containing microvilli of oocyte and follicle cells.

Follicular layer:- The follicle changes from a flattened layer to one containing more or less cuboidal granulosa cells having a mean width of 4 /um (Plate VIc). The cytoplasm contains more number of organalles. Mitochondria 0.2 /um in diameter have a dense matrix, some of them having tubular or lamellar cristae. A large number of free ribosomes were scattered throughout the cytoplasm. Endoplasmic reticulum showed dilation of vesicular cisternae. The voluminous oval-shaped nucleus exhibits condensations of chromatin close to nuclear envelope as well as scattered in the nucleoplasm The nuclear envelope shows many nuclear pores. Follicular microvilli are still shorter; only a few of them penetrate into the pore channels. The basal lamina and thecal cells are similar to those observed in the previous stages.

Primary Yolk Globule Stage

a) Morphology The oocytes in this stage measure 200-400 /um.

PLATE VI

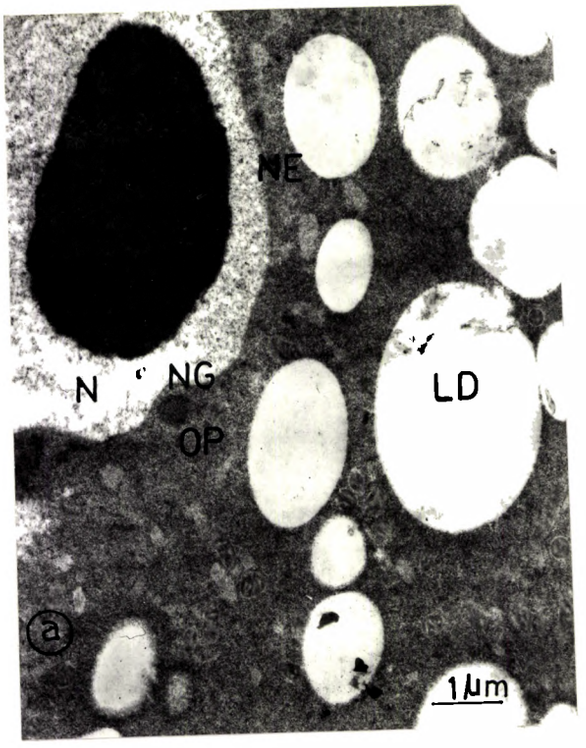
a. Electron micrograph of juxtanuclear ooplasm (OP) of a lipid droplet stage oocyte. LD, lipid droplets; N, nucleus; NE, nuclear envelope NG, nuage; NL, nucleolus. X 9,560.

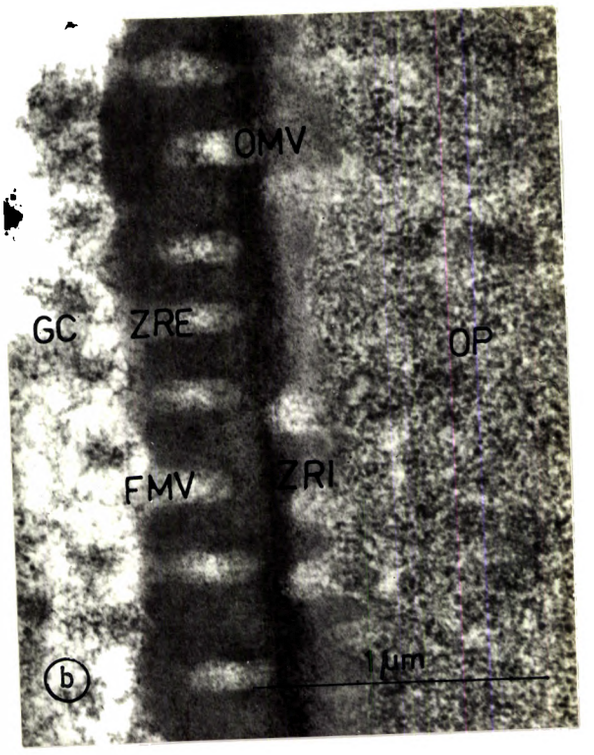
b. Formation of zona radiata (ZR) - an electronmicrograph. FMV, follicular microvilli; GC, follicle cell; OMV, oocyte microvilli; OP, ooplasm; ZRE, zona radiata externa; ZRI, zona radiata interna. X 40,000.

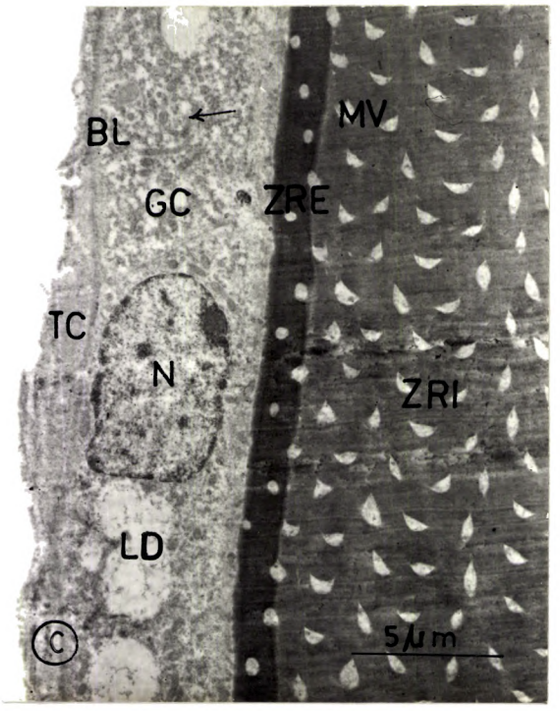
c. Fully differentiated zona radiata in yolk globular oocyte: ultra-structural details. BL, basal lamina; GC, granulosa (follicle) cell; LD, lipid droplets; ⟶ mitochondria; MV, microvilli; N, nucleus of follicle cell, (also note pores in nuclear membrane); TC, thecal cell; ZRE, zona radiata externa; ZRI, zona radiata interna. X 4,000.

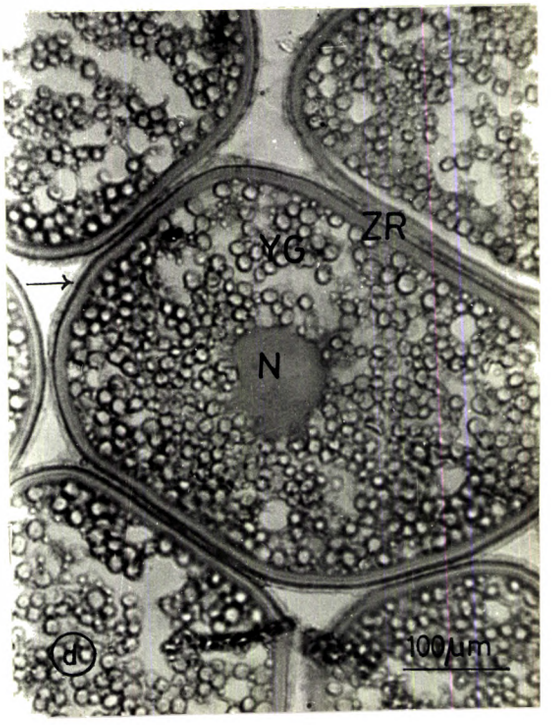
d. Lightmicrograph of a secondary yolk globular oocyte. ⟶ follicular layer, N, nucleus; YG, yolk globules; ZR, zona radiata X 650.

PLATE VI


NE
N
NG
OP
LD
a
1µm


OMV
GC
ZRE
OP
FMV
ZRI
b
1µm


BL
MV
GC
ZRE
TC
N
ZRI
LD
c
5µm


ZR
YG
N
d
100µm

b) Cytology

Nuclear characteristics:- the N/C is about 0.35. The nucleus becomes more irregular in outline and the peripheral nucleoli also become smaller and more irregular in shape. Few lampbrush chromosomes are visible in the nucleus.

Cytoplasmic characteristics:- The major change observed in the ooplasm in comparison to the earlier stage is the appearance of minute acidophilic yolk globules (2-4 /um in diameter) in the peripheral cytoplasm. At the ultrastructure level, the yolk globules are electron-dense, membrane-limited and homogeneous structures (Plate VIIa). Lipid droplets have enlarged (5-25 /um in diameter) and scattered throughout the ooplasm. The ZR showing radial striations in the light microscope is now having an average width of 5 /um (3-7 /um). The ZRE has the mean thickness of 0.8 /um and the ZRI, 4.5 /um thick.

Follicular layer:- The follicular wall is now about 7-8 /um thick; the cuboidal granulosa cells measuring 6 /um and the thecal cells almost 2 /um in height. The granulosa layer contains many small lipid droplets as in the previous stage. Special thecal cells are not observed.

Secondary Yolk Globule Stage (Plate VIb,c,d).

a) Morphology The round oocytes in this stage measure 400-550 /um.

b) Cytology

Nuclear characteristics:- The N/C is about 0.25-0.30. The

large central nucleus still exhibits a very convoluted form with 4-14 small nucleoli in the periphery.

Cytoplasmic characteristics:- Accumulation of yolk globules proceeds rapidly. The yolk globules have multiplied and increased in size (5-10 /um in diameter) and become distributed throughout the cytoplasm. In the electron microscope, the pinocytic vesicles which appear between the bases of the microvilli, are limited by a membrane and contain an electron-dense material. The lipid droplets have further enlarged (10-40 /um) through coalescence and now occupy a discrete zone in the middle and inner ooplasm. A thin basophilic layer of cortical alveoli is observed at the cortex, immediately beneath the ZR (Plate XIId). The ZRE is 1.0 /um wide while the ZRI shows 7-8 /um thickness.

Follicular layer:- The granulosa, thecal cells and the basement membrane do not show any noticeable change from that of the previous stage. The width of the layer is 6-8 /um and it does not contain any special thecal cells.

Tertiary Yolk Globule Stage (Plates VIc; VIIa,b)

a) Morphology: The diameter of oocytes at this stage ranges from 550 to 650 /um.

b) Cytology

Nuclear characteristics:- The highly notched nucleus is still visible

in the centre of the oocyte with 6-7 tiny, peripherally situated nucleoli, but some nucleoli are scattered randomly in the nucleus. The N/C is about 0.25.

Cytoplasmic characteristics:- The ooplasm is now tightly packed with the acidophilic yolk globules (7-15 /um in diameter); but unlike in many teleosts, the yolk globules do not exhibit zonation (Plate VIIa). The lipid droplets continue to increase in size (70-120 /um in diameter) with one another while their number in the ooplasm reduces and the larger ones lie near the nucleus. The thin layer of basophilic ooplasm (cortical alveoli) is still visible in the cortex of oocytes. The zona radiata increases further in thickness, measuring 12.5 /um. The ZRE is 1.3 /um wide and the ZRI is 11.2 /um in thickness. Ultrathin sections show increased distance between oocyte microvilli (1.3 /um apart) in ZR and in cross sections the pore-channels appear sickle-shaped or semicircular (instead of round) especially in ZRI - both due to stretching of the ZR during growth.

Follicular layer:- The follicular layer is about 6-8 /um thick in this stage also. The cytoplasm of cuboidal granulosa cells (4-6 /um high) contains lipid droplets, mitochondria, ER and few ribosomes. The thecal layer (1-2 /um thick) remains as a thin envelope of connective tissue, outside the basal lamina, containing flattened cells with oval nuclei; microfilaments are observed in its cytoplasm (Plate VIc; VIIIa).

At the end of the tertiary yolk granule stage, when the sequestration of yolk material has been curtailed, the oocytes reach a critical size which

PLATE VII

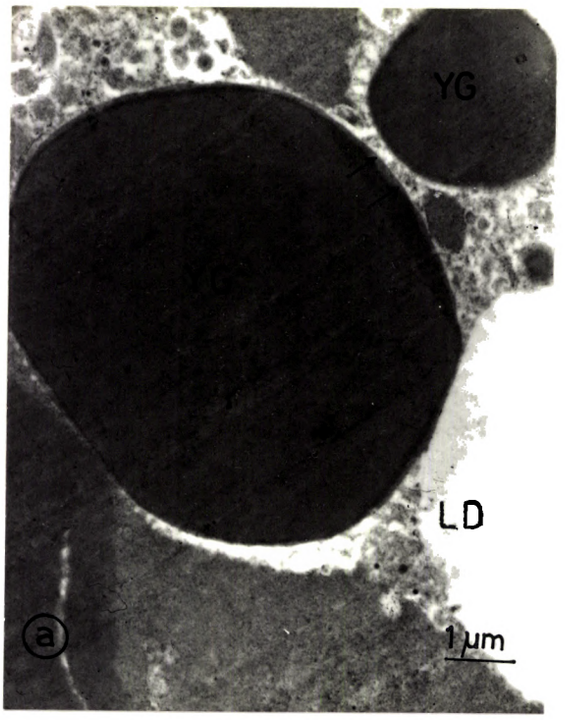
a. Ultrastructure of protein yolk globule. LD, lipid droplet; YG, yolk globule; ⟶ limiting membrane. X 9,600.

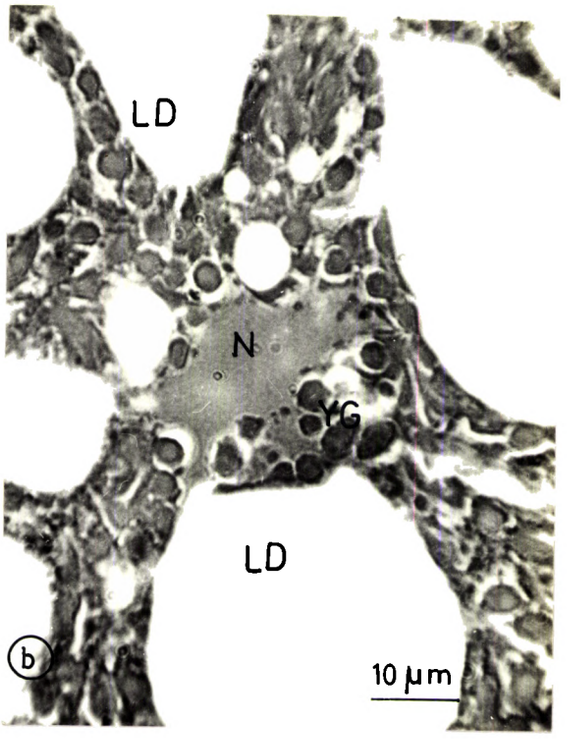
b. Light micrograph of a tertiary yolk globular oocyte showing highly notched nucleus (N), a discrete zone of large lipid droplets (LD) in the inner ooplasm and yolk globules (YG). X 1,180.

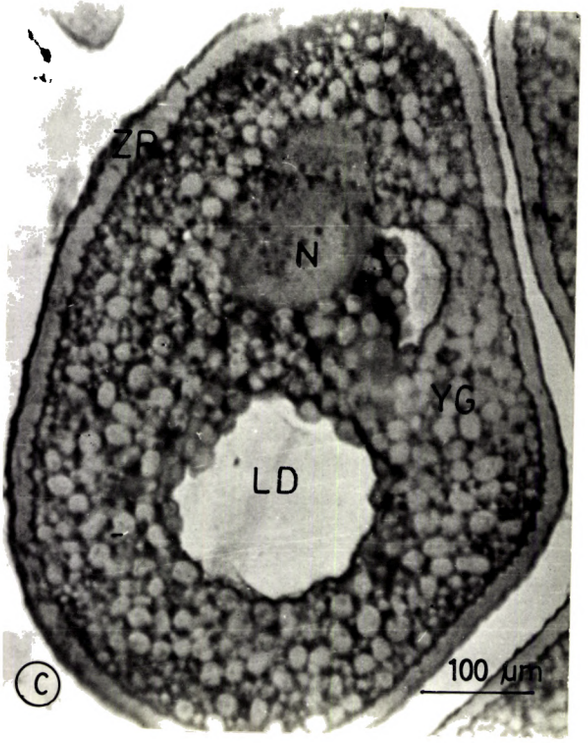
c. Light micrograph of an oocyte belonging to migratory nucleus stage. LD, large oil droplet; N, migrating nucleus; YG, yolk globules; ZR, zona radiata. X 610.

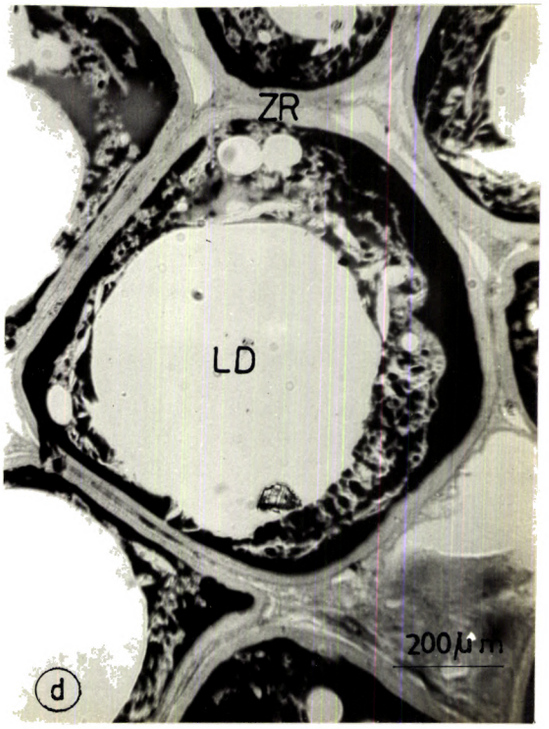
d. Ripe oocyte - a lightmicrograph. Iron haematoxylin staining. LD, single large oil drop; Y, coalesced yolk; ZR, zona radiata. X 460.

PLATE VII









is species-specific and now termed "post-vitellogenic oocytes"(600-650 /um). They either resume meiosis in response to the appropriate hormonal stimulus (i.e. undergo maturation) or are resorbed (oocyte atresia).

Oocyte Maturation

Oocyte maturation involves multiple biochemical and physical changes, the mechanisms of which are still not fully understood, but the primary event is the resumption of meiosis which had been arrested at the perinucleolus stage and subsequent dissolution of the nucleus (Germinal Vesicle Break Down GVBD). The protein yolk globules and lipid droplets start to coalesce; the follicle epithelium thins and stretches while the ZR remains prominent. The oocyte rapidly increases its volume due to hydration and prior to ovulation (now an egg) its contents appear homogeneous and translucent. The following are the two stages of oocytes during the process of maturation.

Migratory Nucleus Stage (Plate VIIc)

a) Morphology: The oocytes have diameter range of 650-750 /um.

b) Cytology

Nuclear characteristics:- The nucleus is progressively displaced towards the animal pole. Scattered in the nucleoplasm, 6-8 small nucleoli are still visible. The N/C is about 0.20. After the migration of the nucleus to the periphery, the nuclear envelope ruptures (germinal vesicle break down-GVBD) and the contents are dissolved in the ooplasm (pre-ripening stage).

Cytoplasmic characteristics:- The coalescence of yolk globules and lipid droplets takes place. The yolk globules form a homogenous mass in the ooplasm. Lipid droplets also increase in size and the largest one in the centre measures 262 /um. The cortical alveolar layer is not very clear. The zona radiata measures 12-13 /um as in the previous stage, but the micropyle is not observed. It loses its striated appearance in light microscope. The microvilli of oocyte as well as that of follicle cells are withdrawn.

Follicular layer:- The follicular layer becomes stretched due to the increase in cell volume and forms wide intercellular spaces. At some places, the layer gets detached from the oocyte surface. The follicular microvilli are withdrawn.

Ripe Egg Stage (Plate VIId)

a) Morphology The translucent, brilliant yellowish-orange (in fresh condition) ripe eggs reach diameters of 800 /um (formalin-fixed material) which are now separated from the follicle epithelium.

b) Cytology

Cytoplasmic characteristics:- Complete fusion of the yolk globules is seen together with an overall increase in the oocyte translucency. Its eosinophilic nature is reduced. The single large oil droplet in the centre of the egg measures 300-320 /um in diameter. The ZR measures 14 /um (ZRE,1.5 /um thick and ZRI, 12.5 /um); cortical alveolar layer is not observed clearly.

Follicular layer:- The layer contracts which results in the disruption and collapse of the follicular fabric and finally the release of the ripe eggs from it.

Ovulation

Ripe eggs after the completion of first meiosis division are released into the ovarian cavity (ovulation) by rupturing the follicular wall and finally to outside (spawning).

Follicular (Oocyte) Atresia

Follicular atresia is a degenerative process by which the residual oocytes in various phases of development and differentiation and the empty follicles, retained after ovulation are lost from the ovary. Two types of degenerating follicles occur in the spent ovaries of teleosts. The first one is derived from the empty follicles (granulosa and thecal layers) after ovulation the postovulatory follicle (POF) or corpus luteum which collapses, becomes pycnotic followed by rapid resorption. Following the final spawning, a small but significant number of (2-10%) vitellogenic and postvitellogenic oocytes which fail to undergo maturation/ovulation are retained in the ovary. Subsequently they degenerate and are resorbed, which form the second type, namely the atretic follicles (AFs) or the corpora atretica. Very rarely few atretic previtellogenic oocytes also have been observed. The morpho-histological changes of both types of follicles in the ovary are investigated. The nomenclature and general characteristics used for the atretic stages follow those of Davis (1977) in the case of corpora atretica

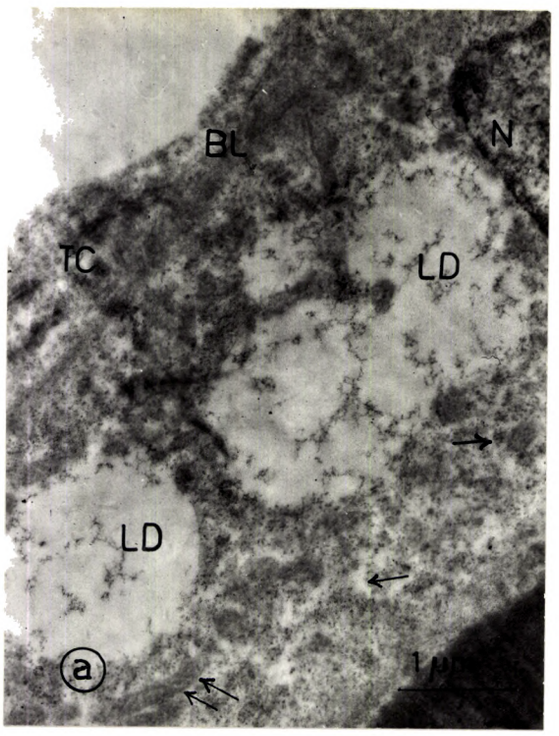
PLATE VIII

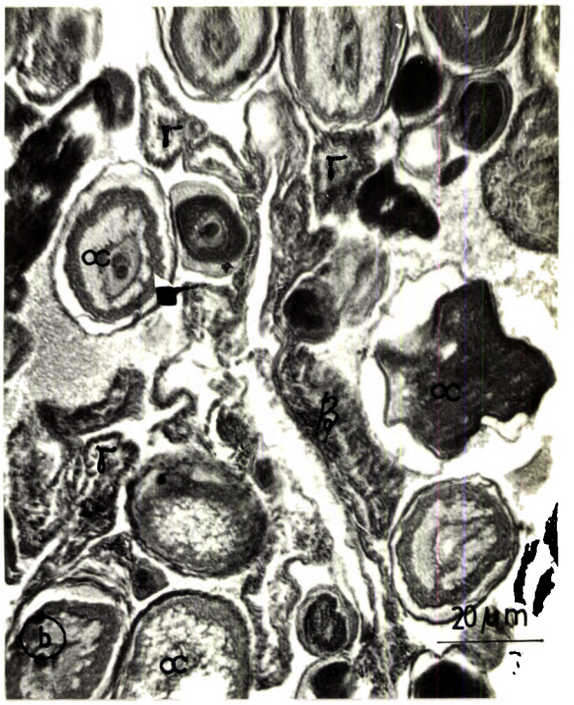
a. Fine structure of the granulosa cell of a normal vitellogenic oocyte. Note the empty spaces left by lipid droplets (LD). BL, basal lamina; →, smooth endoplasmic reticulum; mitochondria; N, nucleus, TC, thecal cell. X 16,225.

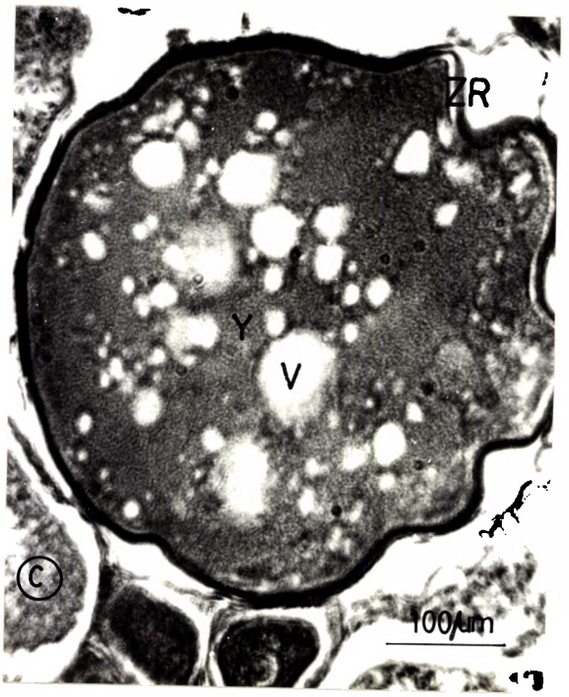
b. Light micrograph of atretic oocytes. Majority of the follicles are in α -stage of atresia, while some others are in β and γ stages. X 680.

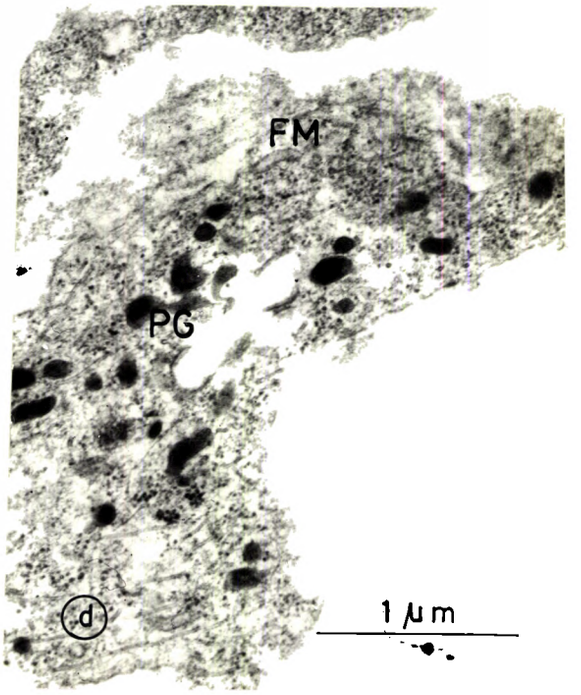
c. A vitellogenic oocyte in α -stage atresia. Note shrinkage of the oocyte, folding of zona radiata (ZR), disintegration of yolk globules (Y), and many vacuoles (V) in the ooplasm. X 1,560.

d. Fine structure of δ -stage atresia. A lightly staining fibrous mass (FM) is left behind, containing dark yellowish brown pigments (PG). X 27,040.


BL
N
TC
LD
LD
a
1 µm


r
r
α
α
β
r
α
b
α
20 µm


ZR
Y
V
c
100µm


FM
PG
d
1 µm

and of Lambert (1970a) for postovulatory follicles.

Corpus atreticum (Atretic follicle) (Plate VIIIb,c,d).

α Stage With the onset of atresia, the entire oocyte shrinks causing a wide space between the follicular layer and the ZR. The hypertrophy of the granulosa occurs the cells change from a squamous to a columnar layer. The ZR develops folds and cracks in several places, becomes uneven in thickness and loses its striations. It begins to erode and the yolk globules along its peripery liquifies. The lipid droplets often coalesce into a central mass. The ooplasm contains disintegrating mitochondria. The nucleus of the oocytes (if present) becomes shrunken irregular and disorganized and finally disintegrates. The yolk globules lose its globular nature and vacuoles (10-20 μm diameter) and cracks are observed in the acidophilic granular ooplasm. The atretic previtellogenic oocytes (very rarely observed) also show similar degenerative changes. The disintegration of their nucleus and ooplasmic components occur first.

β Stage The ZR has completely disintegrated and the yolk in the periphery of atretic oocyte continues to liquify and pass into the granulosa in streams of droplets. The central portion of the oocyte still contains a crumbled mass of yolk. The hypertrophied granulosa cells invade the atretic oocytes, upon the rupture of the ZR. They show small lipid droplets (seen as empty vacuoles) inside the cytoplasm. Their nucleus appears oval or round and highly basophilic. The ultrastructure of cytoplasm of granulosa revealed few microvilli, many rough endoplasmic reticulum, oval

or round mitochondria and small, round acidophilic, electron-dense homogeneous materials (similar to yolk globules). The thecal cells are extensively folded with many slender cell processes penetrating the connective tissue space and surrounding bundles of collagen fibres. Their cytoplasm contains many smooth-walled, clear, small vesicles. The basal lamina becomes tortous and no firm attachment exists between it and the cells on either side. Numerous blood capillaries and vessels are observed between the atretic follicles, especially in the thecal layer; the leucocytes are found proliferating or invading the degenerating oocyte.

γ Stage the structure becomes greatly reduced in size. The degenerating oocyte become taut with the phagocytic granulosa cells, which also aggregate interior of the oocyte. Blood cells are also seen in large numbers close to the granulosa layer, possibly helping resorption. Finally the oocyte is resorbed completely and all that remains is the granulosa cells, surrounded by a layer of thecal cells, which fill the small cavity the "atrium" formerly occupied by the degenerated oocyte. A light - yellow coloured flocculent material is often observed, encapsulated by a layer of granulosa and thecal cells. The nucleus (2-3 μm in diameter) of the granulosa cells becomes pycnotic; the cytoplasm contains many empty vacuoles, few spherical eosinophilic electron-dense material, small mitochondria and rough endoplasmic reticulum. The thecal layer appears to be riddled by the phagocytic vesicles (containing collagen fibres) which make nearly contact with each other leaving only a small cytoplasmic strand between each vesicles. The smooth-walled small vesicles are often seen in close proximity of the large phagocytic vesicles and even fusion between the

two can be seen occasionally.

δ Stage This is the final stage of atresia in the ovary of M. cephalus. The atretic follicle shrinks further; more number of the nuclei in granulosa become pycnotic and irregular. The flocculent material is now in the form of finely granular, dark yellow-brown pigments which is still visible in between the granulosa cells (Plate VIIId). This stage persists for long periods. Finally the phagocytic granulosa cells and the thecal layer degenerate, leaving behind a lightly staining fibrous mass containing yellow-brown granules surrounded by stromal elements and few blood vessels. These structures also become histologically indistinguishable after some time.

Corpus luteum (Postovulatory follicle) (Plate IXa,b,c; Xa,b,c)

Four distinct phases in histogenesis can be observed.

Stage-A Postovulatory follicles (POFs) are characterized by a large follicular lumen (atrium) formerly occupied by the oocyte. The POFs are formed of two layers an outer thecal layer and the inner hypertropied granulosa layer. The ultrastructure of granulosa revealed few oval or round mitochondria, rough ER, tiny granules and a basophilic round nucleus in the electron-lucent, acidophilic cytoplasm. These cells are loosely attached with the thecal layer and some collapse into the follicular lumen. Thin thecal layers contain blood vessels and fibroblasts. In electron microscope, thecal cells are extensively folded with many slender cell processes penetrating the connective tissue space and surrounding bundles of collagen fibres. Their cytoplasm contains many small, smooth-walled, clear vesicles

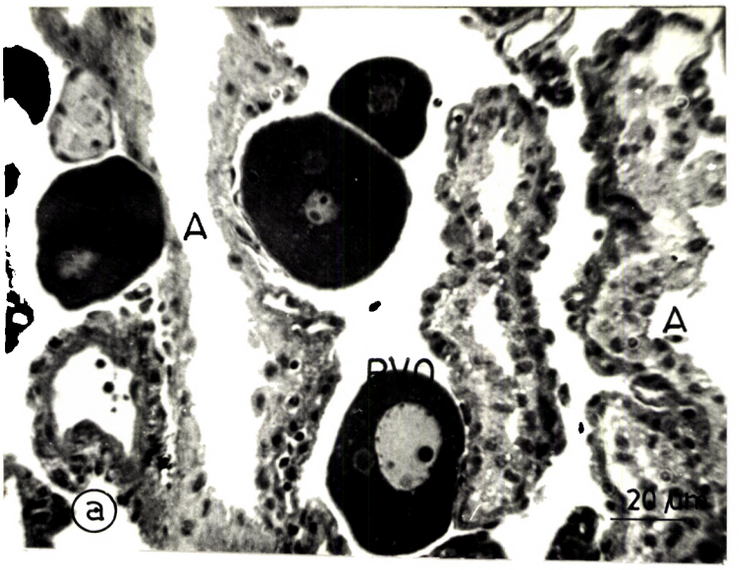
PLATE IX

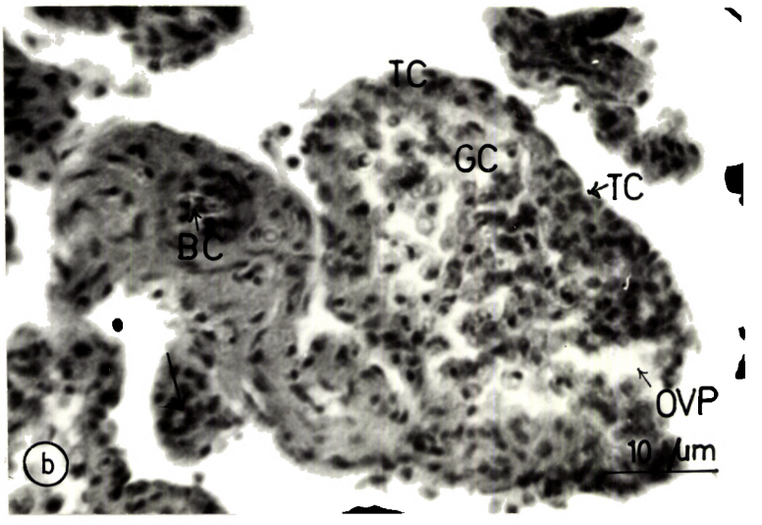
a. Postovulatory follicles belonging to stage-A (A) along with non-atretic previtellogenic oocytes (PVO) X 1,050.

b. Light micrograph of a stage-B corpus luteum in M. cephalus ovary. GC, hypertrophied granulosa cell; BC, blood cells; OVP, ovulation pore; TC, thecal cells. X 1,700.

c. Postovulatory follicles belonging to stages B (B) and C(C). BC, blood cells; GC, hypertrophied granulosa cells; PVO, previtellogenic oocyte; TC, thecal cells. X 1,050.

PLATE IX





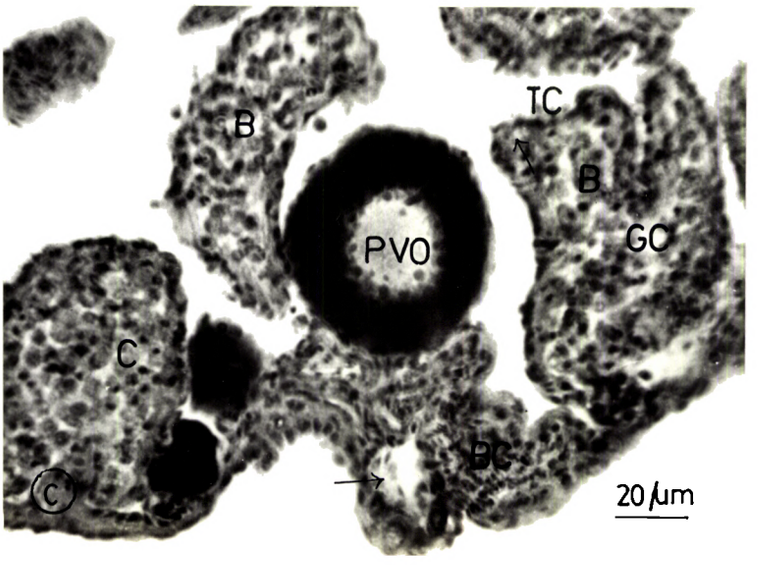


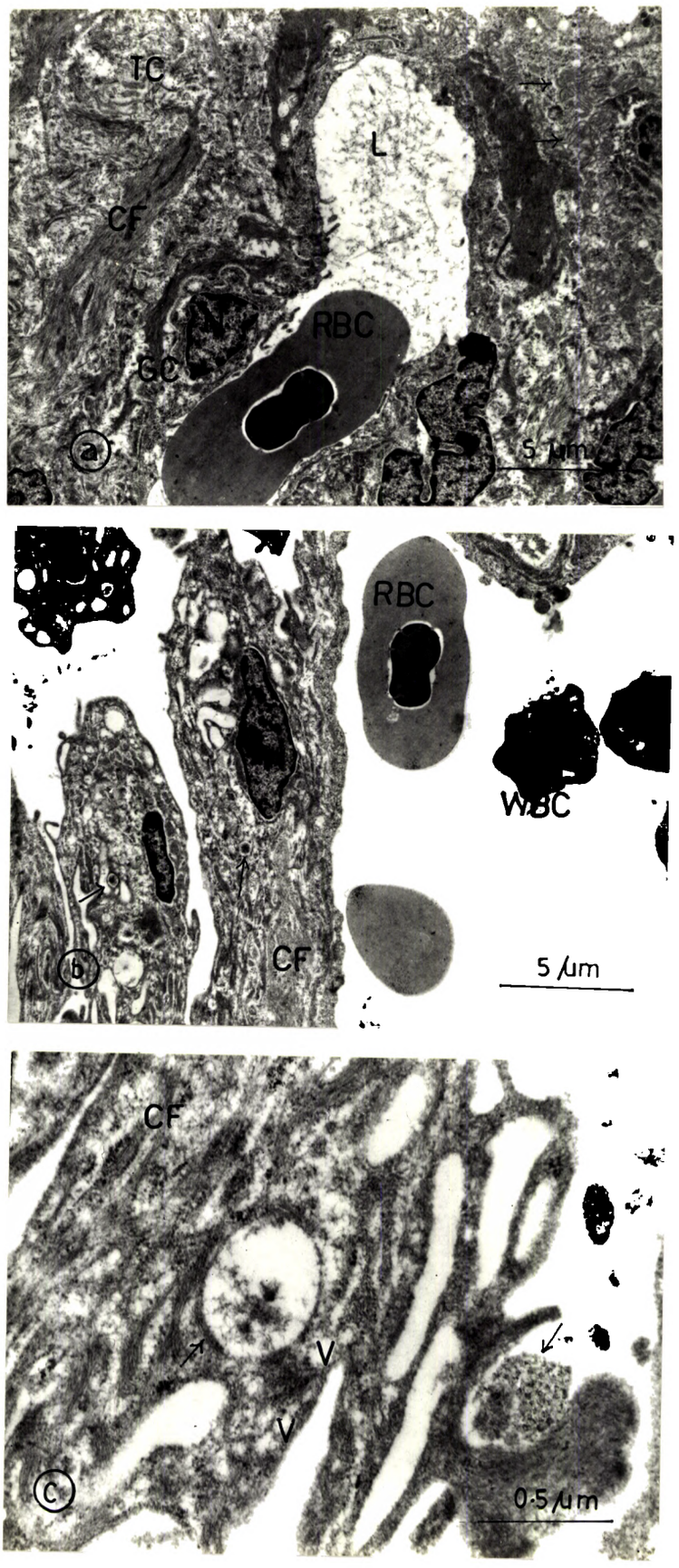
PLATE X

a. Electron micrograph of a stage-B postovulatory follicle. CF, collagen fibres; GC, granulosa cell; L, lumen of POF; ⟶, degenerating mitochondria; N, nucleus of granulosa cell; RBC, red blood cell; TC, thecal cell. X 4,100.

b. Ultrastructure of the thecal cells of stage-B postovulatory follicle. CF, collagen fibres; N, nucleus; RBC, red blood cell; WBC, white blood cell; ⟶ phagocytic vacuoles. X 4,100.

c. Fine structure of a portion of postovulatory thecal cell (stage C) showing phagocytic vesicles containing collagen (⟶) and small vesicles (V). Bundles of collagen (CF) are still observed, scattered in the cytoplasm. X 40,780.

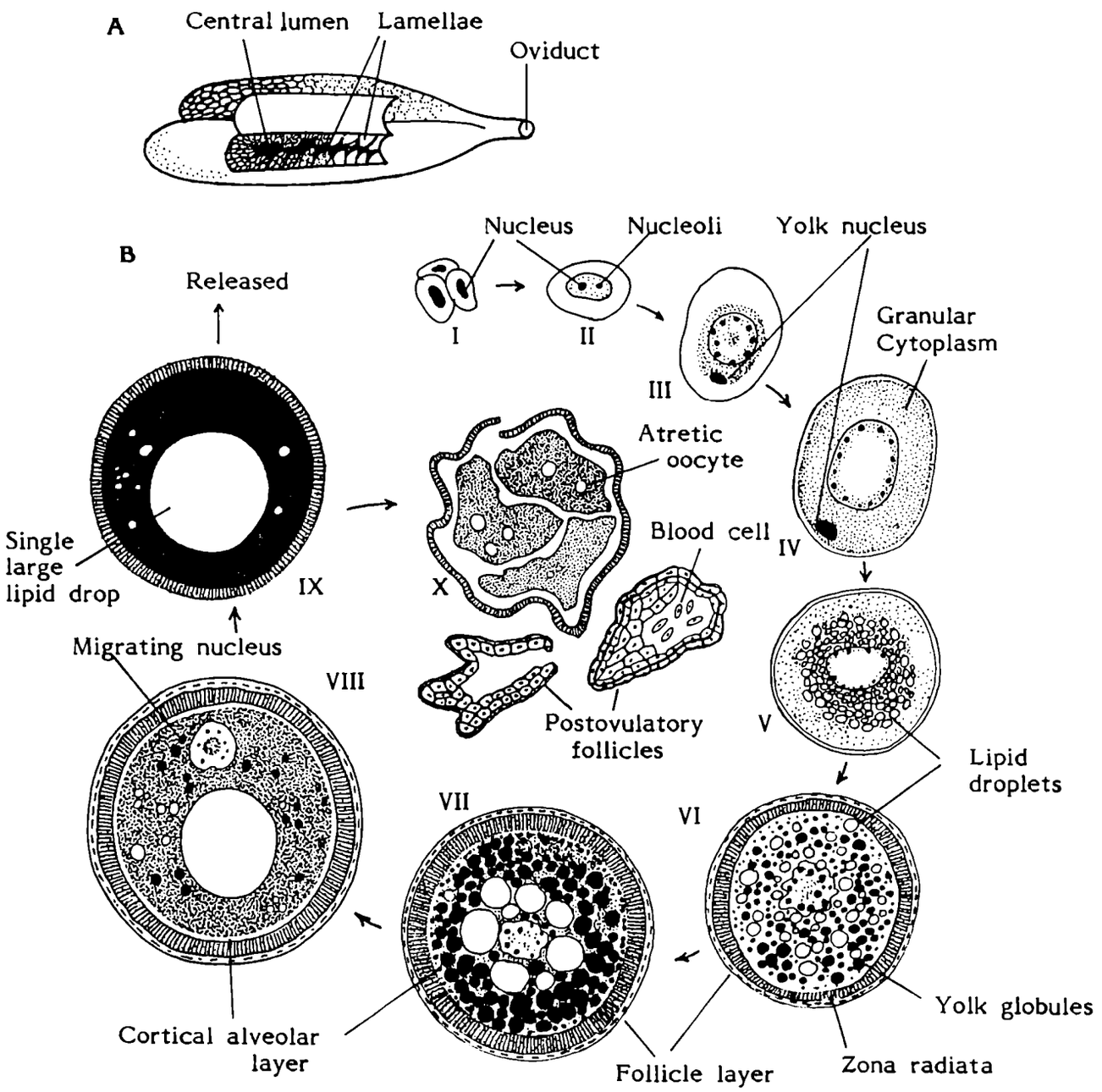
PLATE X



and a highly basophilic nucleus. The basal lamina is wavy and loses connection with the cells on either side. In many sections, an opening or a crack in the follicle epithelium is observed, which may be the region through which the egg is liberated (ovulation pore).

Stage- B The follicular lumen becomes greatly reduced in size or closes completely as a result of the continuous shrinkage of the follicle. The follicle epithelium forms villus-like projections which are irregularly placed in the lumen which are not uniform in thickness. The granulosa cells are further hypertrophied and their cytoplasm is homogeneously eosinophilic. Several of these cells however, contain larger granules which contain an accumulation of coarse rER, disintegrating mitochondria; the contents are gradually dissolved and resorbed. The nuclei of many granulosa cells have become pycnotic. More and more granulosa cells shift towards the lumen where they form patches. The striking difference of the granulosa cells of POF from that of the γ-stage atretic follicles is the absence of yellow flocculent material in the former. The thecal layer in the electron microscope appears to be riddled by phagocytic vesicles containing collagen fibres; small, smooth-walled vesicles are seen in close proximity of the large phagocytic vesicles. More number of blood cells and blood vessels are observed in the thecal layer. However, interstitial gland cells (IGC) or special thecal cells (STC) are not observed. The ovulation pores have become smaller with rounded margins, but are still open.

Stage-C Degeneration of corpus luteum advances; the POFs are much



I Oogonia
II Chromatin nucleolus stage
III Early perinucleolus stage
IV Late perinucleolus stage
V Lipid droplet stage
VI Early yolk globule stage
VII Late yolk globule stage
VIII Migratory nucleus stage
IX Ripe Ovum
X Spent/Atresia

Fig.9. Schematic representation of the ovaries (A) and (B) sequential stages of oocyte development in Mugil cephalus L.

smaller than those of the previous groups and decrease in number. The lumen is completely obliterated by granulosa cells and the POF thus becomes a multilayered structure. The ovulation pores have been closed. The nuclei of most of the granulosa cells become pycnotic. The histological characteristics of the thecal layer remain the same as that of the previous stage. Some granulosa cells are separated from one another. The vascularity in the thecal layer is greatly reduced during this stage.

Stage-D The postovulatory follicle is reduced to a very small structure. The granulosa and the thecal layer finally degenerate leaving behind a fibrous structure, which also becomes indistinguishable from the ovarian stroma after sometime.

A schematic drawing (Fig. 9) highlights oocyte development in M. cephalus and summarizes the cytological changes during specific stages.

Shrinkage due to processing

Shrinkage of oocytes was detectable due to fixation and dehydration during histological processing. The size of oocytes after processing was 12-13% less than that of the fresh egg diameter.

Percentage-distribution of oocyte types in relation to reproductive phases in gonads (Table-9; Fig.10).

For each of the five morphological maturity phases of ovary, percentage-distribution of various types of oocytes (based on histological picture) was estimated. In stage I ovary, early perinucleolar oocytes formed the single largest group (50%); while in stage II, early lipid droplet oocytes

Table 9: Distribution of various oocyte types, based on histological picture during different maturity stages of Mugil cephalus L.

Oocyte types	Percentage of Oocytes				
	STAGE I	STAGE II	STAGE III	STAGE IV	STAGE V
Oogonia	15	5	2	2	20
Chromatin nucleolus	30	7	5	3	30
Early perinulceolus	50	13	10	3	17
Late perinucleolus	5	25	3	-	-
Early lipid droplet	-	40	5	-	-
Late lipid droplet	-	10	10	2	-
Primary yolk globule	-	-	25	-	-
Secondary yolk globule	-	-	30	-	-
Tertiary yolk globule		-	10	10	-
Migratory nucleus	-		-	15	-
Ripe ova	-		-	65	-
Residual oocytes	-	-	-	-	5
Empty follicles		-	-	-	28

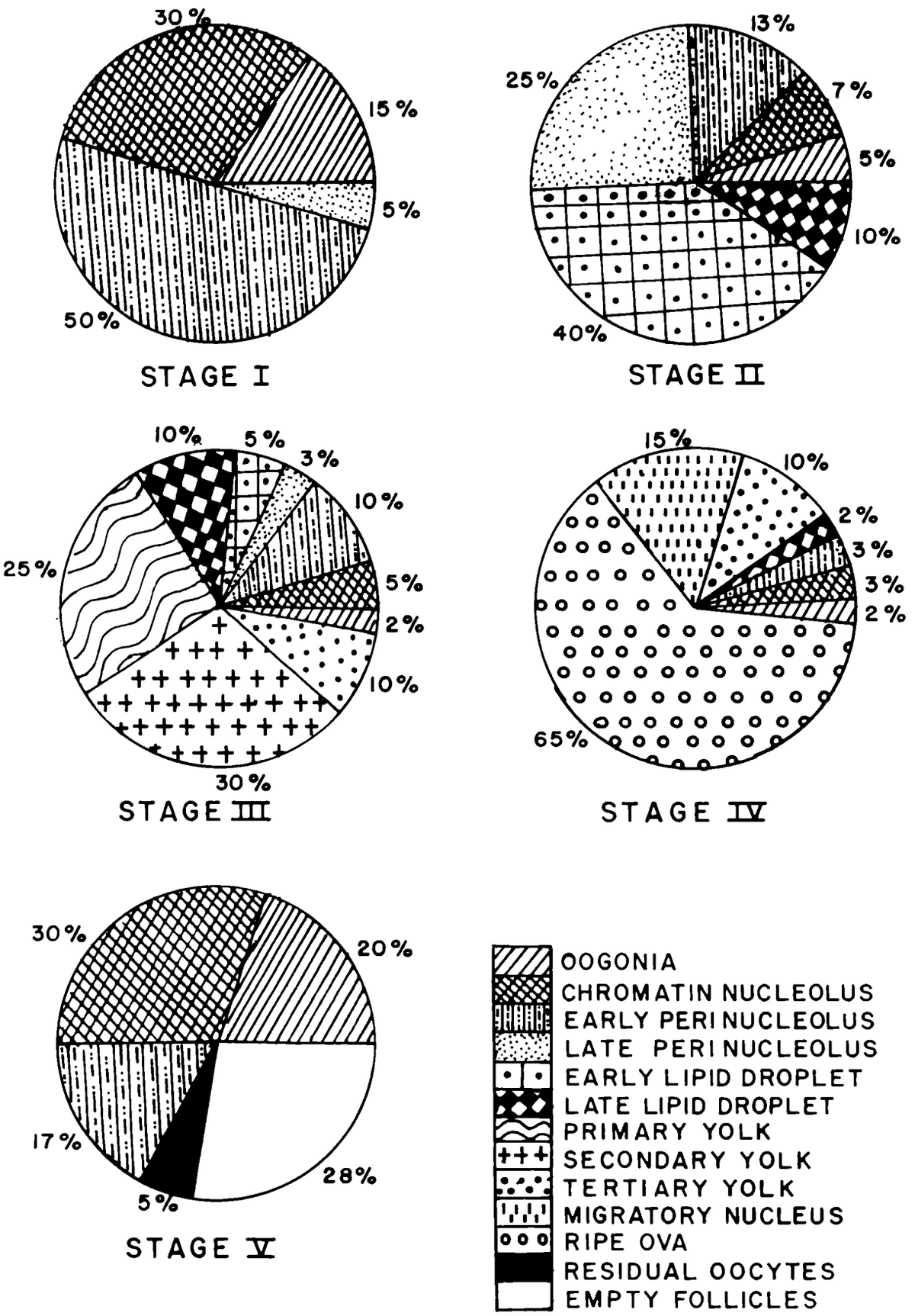


Fig. 10. Distribution of various oocyte types, based on the histological picture during different maturity stages of Mugil cephalus L.

were abundant (40%). Tertiary yolk globular oocytes formed 30% in stage III ovary and the ripe oocytes had a share of 65% in stage IV gonad. In spent stage, majority of oocytes (30%) belonged to chromatin nucleolus stage, while the empty follicles had a proportion of 28% of the whole clutch.

B. HISTOLOGICAL CHANGES IN THE HEPATOCYTES

The hepatocyte population consists of cells with a rather basophilic and electron-dense cytoplasm, containing flat cisternae of the rough endoplasmic reticulum. The bile canaliculi are not only intercellular but also intracellular; the microvilli of the latter are short (Plate XIc).

Stage I (Immature) (Plate XIa)

The feebly basophilic cytoplasm of hepatocytes from immature females contains moderately developed rough endoplasmic reticulum (r ER). Glycogen deposits, RNA and lipid droplets are sparsely distributed. Mitochondria are small and oval or slightly elongated (1 /um long). Some of them contain ribosomes (mitoribosomes) inside the matrix, situated close to cristae. The round or oval nucleus shows condensation of chromatin in the centre as well as along its margins.

Stage II (Maturing)

The hepatocytes in this stage contain a large amount of glycogen and numerous stacked profiles of rough endoplasmic reticulum and lipid droplets (4-7 /um in diameter), many of which have one or more adjoining

PLATE XI

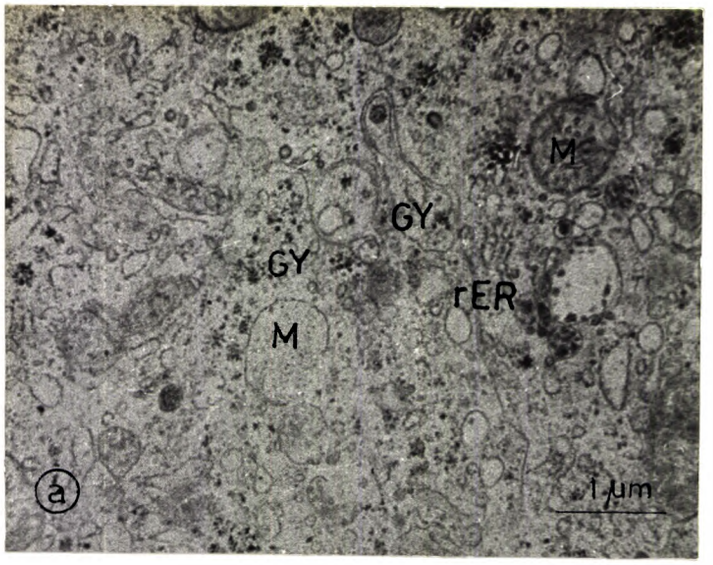
a. Electronmicrograph of a hepatocyte from an immature female M. cephalus. Glycogen granules (GY), mitochondria (M) and lipid droplets (LD) are seen in the cytoplasm. Rough endoplasmic reticulum (rER) is undeveloped. x 15,000.

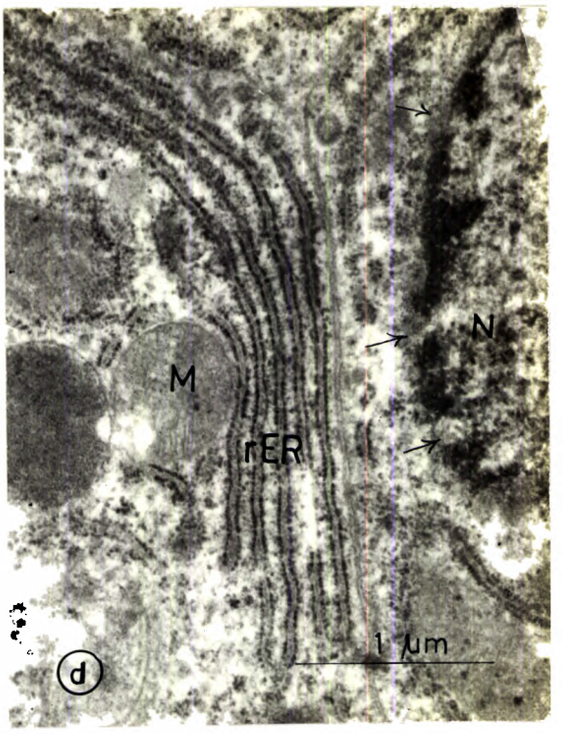
b. Fine structure of the liver cell of mature female mullet. Note the cytoplasm packed with rough endoplasmic reticulum (rER). The nucleus (N) contains many nuclear pores (⟶). M, enlarged mitochondria. X 15,000.

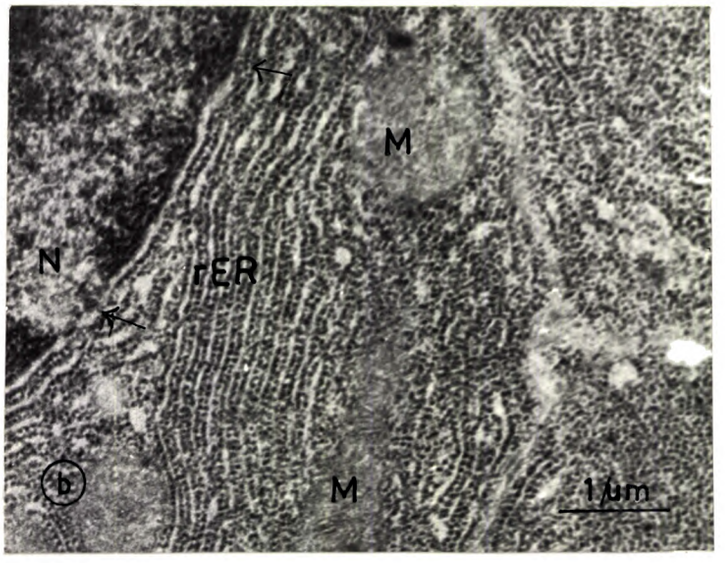
c. Ultrastructure of the hepatocyte of a ripe female mullet. Rough endoplasmic reticulum (rER), lipid droplets (LD) and glycogen (GY) are sparse in the cytoplasm. BCL, bile canaliculi with microvilli, DB, electron dense bodies (small and large); M, mitochondria, X 9,560.

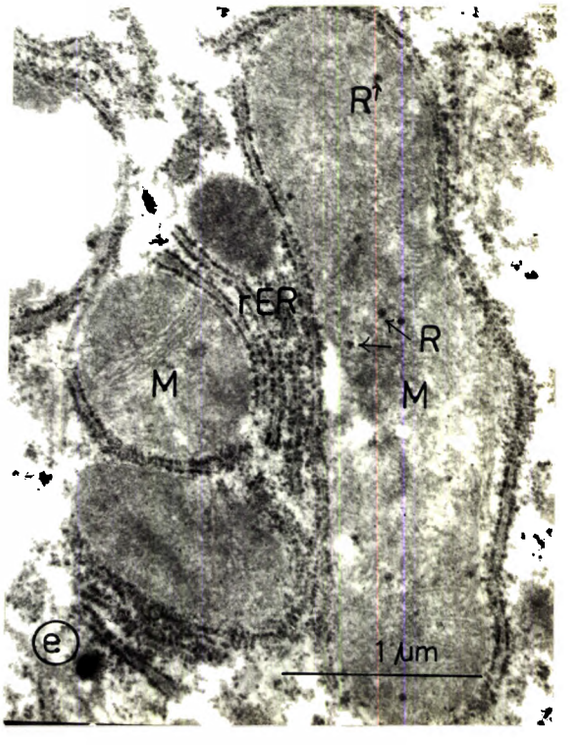
d & e. Hepatocytes from a mature female M. cephalus. Rough endoplasmic reticulum (rER) are numerous and concentrated in laminar stacks. Enlarged mitochondria (M) are in contact with rER. N, nucleus; →nuclear pores; R, ribosomes inside mitochondria. Both X 27,040.

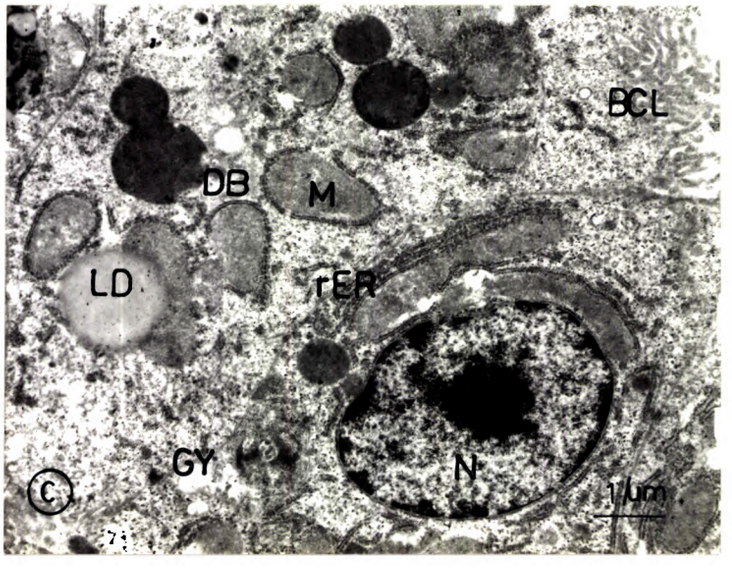
PLATE XI


M
GY
GY
rER
M
a
1 µm


N
M
rER
d
1 µm


M
N
rER
b
M
1 µm


R
rER
R
M
M
e
1 µm


BCL
DB
M
LD
rER
GY
N
c
1 µm

mitochondria. There are small electron-dense bodies scattered in the cytoplasm. The nucleus enlarges; the nuclear membrane is traversed by many nuclear pores.

Stage III (Mature) (Plate XIb,d)

The hepatocytes hypertrophy; in the light microscope, their cytoplasm appears highly basophilic with an enlarged nucleus containing 2-3 nucleoli. The fine-structure of hepatocytes reveals, the cytoplasm is thickly packed with profiles of rough endoplasmic reticulum. Glycogen deposits are no longer observed. The lipid droplets have become small (2-3 /um in diameter) and less frequent. The mitochondria become highly enlarged and measure upto 6 /um and most of them are surrounded by rough endoplasmic reticulum (Plate XId,e). Their cristae are well developed and many ribosomes are associated with them. Electron dense bodies are absent.

Stage IV (Ripe/Spawning) (Plate XIc)

During this stage the hepatocytes, their nuclei and the basophilia of the cytoplasm are reduced. The rER and the mitochondria are in regression. The rER consists mainly of individual cisternae (no stacks), distributed throughout the cytoplasm. Smooth endoplasmic reticulum (sER) has rarely been noticed. The mitochondria, oval or slightly elongated, measures 1-2 /um in length. Small lipid droplets, glycogen deposits and electron dense bodies (1-2 /um) are scattered in the cytoplasm.

Stage V (Spent)

The ultrastructure of the hepatocytes of spent female fish appears same as that of the ripe stage, except for the little increase in glycogen deposits in the hepatic cells of post-spawned fish.

DISCUSSION

A. HISTOLOGY OF OOGENESIS

Cytological evidence of sexual differentiation begins in M. cephalus at sizes over 20-25 cm (total length). Stenger (1959) has given an account of "germ-cells" in the "protogonial masses" (primordial germ cells) of 20mm fry of M. cephalus. The primordial germ cells (PGC) of M. cephalus are ovoid and larger (9-13 /um in diameter) than the somatic cells. Fine structure of PGC showed the presence of "germ cell marker" - the electron dense round "nuage" and intermitochondrial "cement" material in the cytoplasm (Brusle, 1980a). The PGC differ ultrastructurally from the gonia in having a highly electron-dense cytoplasm and a very irregular nuclear and cellular outlines. It is now generally accepted that the bipotential PGC in teleosts originate extragonadally and migrate to the gonadal region as in higher vertebrates (Nagahama, 1983). The highly undulating cellular out line of PGC may probably be indicating the amoeboid moments during migration (Brusle & Brusle, 1978b). The gonia (both spermatogonia and oogonia) are larger than the PGC (10-15 /um in size) in M. cephalus and characterized by a more regular outline of nuclear and plasma membranes and a less electron-dense cytoplasm.

The oogonia, which represent the stem-cell population, giving rise to oocytes are found in ovaries of M. cephalus in all stages of maturation. They are distinguished from the spermatogonia by the following main features more regular cellular outline, less extensive endoplasmic reticulum, almost centrally located spherical nucleus with a regular envelope and a larger electron dense nucleus.

The dynamics of early oogenesis in M. cephalus are, in general, in agreement with previous observations, on other teleosts (Yamamoto & Onozato, 1965; Brusle, 1980b; Begovac & Wallace, 1988). The present results provide evidence for the cytological changes of great interest during the first fundamental steps of oogenesis, which are little known than the terminal development of eggs. The early differentiation of an oogonium into a primary oocyte in M. cephalus is characterized by the highly undulating or wavy nuclear envelope, which increases the surface area of nucleus. A progressive increase in the diameter of the nucleolus was observed in accordance with the growth of early oocytes, which was followed by an increase in the number of nucleoli. The nucleolus was constituted only by a fibrillar component. The ooplasm of early previtellogenic (chromatin-nucleolus) oocytes became highly electron-dense, basophilic and RNA positive. These observations provide evidence of nucleogenesis and high nuceolar activity such as synthesis of RNA. The transcription for rDNA and ribosomal RNA synthetic activity in the oocyte nucleoli of vertebrates are known to be located in the fibrillar centre (Wallace, 1985). Biochemical investigations in teleosts have shown that the previtellogenic oocytes mostly synthesize 5S RNA and transfer RNA, which are stored in the form of RNP

particles (42S and 8S) in the ooplasm (Brusle, 1980b). The RNP particles may be responsible for the increased electron density of M. cephalus oocytes. The chromosomes in the chromatin nucleolar oocytes are in the early meiotic prophase and later they develop a lampbrush appearance and show a very fine structure. The presence of lamp brush chromosomes also indicates a high rate of RNA and protein synthesis in the oocytes. The chromosomes of the late chromatin nucleolar oocytes became arrested in late diplotene of first meiotic prophase and shortly after this, their ribosomal genes are amplified (Wallace et al., 1987). The appearance of multiple nucleoli in the perinucleolar oocytes of M. cephalus may be attributed to this gene amplication.

Metabolic activity of early previtellogenic oocytes is further evidenced by the presence of the electron-dense "nuage". This material is considered as an universal component of germ cells (Brusle, 1980b). In M. cephalus, the nuage observed from oogonial stages, increases during the previtellogenic growth in a pattern of small patches close to the outer side and especially in the depressions of the undulating nuclear envelope. The sequential cytological observations show that the increase in clumps of "nuage" and "cement" preceeds the appearance of new mitochondria. These ribonucleoprotein-rich bodies are suggested to play a prominent role in the mitochondriogenesis (Brusle, 1980b). The electron micrographs in the present study have shown that the mitochondria in the ooplasm undergo changes in their distribution, amount, shape, size and internal structure during oocyte growth. In the oogonia and the oocytes at the beginning

of cytoplasmic growth, the mitochondria were round and clustered around the electron-dense intermitochondrial "cement" material near the nucleus and they contained ribosomes in their matrix (mitoribosomes). As the development of oocyte proceeded, the mitochondria increased in number and became distributed uniformly in the ooplasm. They become elongated with well developed tubular cristae and are devoid of mitoribosomes. Similar changes are observed in the oocytes of Liza auratus (Brusle, 1980b) and many other teleosts (reviewed by Guraya, 1986). These alterations in mitochondrial density and shape are indicative of metabotic changes during oocyte growth. The mitoribosomes may help the mitochondria of early previtellogenic oocytes in performing certain biosynthetic or anabolic functions i.e.synthesis of certain hydrophobic proteolipids (De Robertis & De Robertis, 1981) for the ooplasm. During early oogenesis, in M. cephalus, the endoplasmic reticulum profiles and the Golgi complex are infrequent while structures like annulate lamellae are totally absent.

A prominent feature of the perinuclear stage is the formation of a rather amorphous "yolk nucleus" within the ooplasm. The yolk nucleus (Balbiani's vitelline body) in the oocytes of M. cephalus is composed of numerous round or oval mitochondria, ribosomes and tubules of endoplasmic reticulum, as observed in many other teleosts (reviewed by Guraya, 1986); but appears to lack annulate lamellae , multivesicular bodies, lipid bodies and Golgi complex. Various histochemical tests conducted during the present study have revealed that, this basophilic structure is rich in RNA, proteins and enzymes like alkaline phosphatase and succinate dehydrogenase and

contains traces of lipids. However such a structure has not been observed in mullets, Liza auratus and Chelon labrosus by Brusle (1980b, 1985). The functional significance of the yolk nucleus is not fully understood, but it is presumed that yolk nucleus is intimately involved in the metabolic activities related to the formation, multiplication, accumulation and distribution of cytoplasmic organelles and inclusions, which are needed within the oocyte prior to yolk formation (Guraya, 1986).

By the end of the primary oocyte growth (previtellogenesis) in M. cephalus, the oocyte has grown from a diameter of 10-15 µm to a diameter of 100-120 µm. Thus approximately a thousand-fold increase in volume has taken place, primarily due to the elaboration of the normal cytoplasmic organelles and accumulation of huge amounts of cytoplasmic RNA and proteins. It is important to emphasize however, that the ovaries containing oocytes only in primary growth stages are still relatively small (GSI < 1) and are generally perceived as immature.

Histological and histochemical studies carried out during the present investigation show that three types of inclusions are formed during vitellogenesis in M. cephalus. These three types of inclusions viz. lipid droplets, protein yolk globules and cortical alveoli, differ distinctly in their morphology, staining properties and chemical nature and they are deposited sequentially although considerable overlap occurs.

The first type of yolk inclusion to accumulate in the developing oocytes of M. cephalus, is the lipid yolk (triglycerides) in the form of distinct lipid droplets, the appearance of which can be considered to mark

the start of endogenous vitellogenesis (Shackley & King, 1977). In M. cephalus oocytes, they first appear in the perinuclear cytoplasm, but their origin has yet to be fully understood. It has been opined that these lipid bodies arise either from dictyosomes or mitochondria in the ooplasm. Shackley & King (1977) suggested, the syntheis of lipid droplets is probably endogenous (occurring in the perinuclear ooplasm) but in the present study no organelle could be detected, responsible for the origin of lipid droplets. During oocyte maturation, these lipid droplets coalesce and form a single large oil drop. The lipid inclusion may keep the eggs buoyant. Further studies are needed to determine the actual functional significance of lipid droplets during oocyte growth is M. cephalus.

Protein yolk accumulation (yolk globule stage) occurs after, and concomitant to lipid yolk accumulation in M. cephalus. Protein yolk (glyco-lipophosphoprotein) is distinguished as discrete, membrane-limited, electron-dense, acidophilic, fluid-filled yolk globules in M. cephalus, rather than orthorhombic crystalline platelets as observed in some freshwater fishes (Lange et al., 1983). Small yolk globules first appear in the cortical region of the oocytes and later they fill the entire ooplasm in the form of larger globules. In many teleosts, the smaller yolk globules before becoming larger (mature) ones, pass through certain transitional stages and undergo further chemical processing. Lysosomes and multivesicular bodies are detected during the transitional stages (Begovac & Wallace, 1988). However, in M. cephalus no such intermediate stage(s) could be identified; in electron microscope, the yolk globules of various sizes were structurally similar and never showed any zonation. This is in agreement with the observations

of Brusle (1985) in other two mullets, Chelon labrosus and Liza aurata. But during the hydration of oocyte, the protein globules fuse and form a continuous mass of yolk, as a result of which, the ripe eggs become transparent. It is now generally accepted that the protein yolk is hepatically produced (exogenous in origin), the yolk precursor having been identified as the female-specific serum glycolipophosphoprotein complex, vitellogenin (Vtg) (Guraya, 1986). The Vtg is specifically sequestered through clathrin-coated micropinocytic vesicles, under gonadotropin contol by the developing oocyte (Wallace, 1985). The present biochemical, electrophoretic and ultra-structural studies support the exogenous origin of protein yolk in M. cephalus.

The third and quantitatively minor type of inclusion is the cortical alveoli (carbohydrate yolk or yolk vesicles), which release their contents into the perivitelline space during cortical reaction. For this reason, they cannot be considered as yolk in strict sense. This inclusion appears as a narrow zone below the zona radiata after both lipid yolk and protein yolk formation have started in M. cephalus; whereas in majority of teleost species, the cortical alveoli formation occurs prior to both lipid and protein yolk formation (Khoo, 1979; Guraya, 1986). Histochemical studies have indicated that the cortical alveoli contain mucopolysaccharides in M. cephalus which are now believed to be synthesized endogenously under the control of gonadotropins (Guraya, 1986). But the cortical alveoli in this fish are far less pronounced than that of many freshwater species. Similar results have been recorded from other marine spawning fishes like G. filamentosus (Cyrus & Blaber, 1984), D. labrax (Mayer et al., 1988) and L. calcarifer

(Lal, personal communication). A comparison of oogenesis of M. cephalus with that of freshwater spawning mullet R. corsula, may reveal whether the formation, structure and composition of cortical alveoli vary in different environments.

The formation and development of bipartite zona radiata (ZR) were similar as in Liza aurata and Chelon labrosus, as observed by Brusle (1985), except that the arc-shaped fibre arrangement of inner zona radiata (ZRI) was absent in M. cephalus. This thick ZR gives a mechanical protection to the oocyte and later to the embryo of mullets and its plasticity allows the increase in size of the oocyte without preventing nutrition (Brusle, 1985).

The fine structure of the follicular layer revealed some of its modifications during oocyte growth in M. cephalus. During yolk synthesis, the granulosa cells become higher, develop follicular microvilli and contain many sudanophilic lipid droplets, mitochondria (having tubular cristae) and smooth ER. A strong 3 β HSD activity is noticed in the follicular layer at the same time. These results point out the possible steroidogenic role of granulosa in this fish, as reported in other teleosts (Hoar & Nagahama, 1978). However, ultrastructurally, no steroidogenic activity was noticed in the granulosa of mullets, Liza aurata and Chelon labrosus and involvement of special thecal cells (thecal gland cells) has been suggested in these fishes eventhough not proved (Brusle, 1985). It is unknown why results differ in the closely related species. In M. cephalus, special thecal cells were not detected during oogenesis. The thecal cells, appearing at the end of

primary growth phase also did not show any structural modification and they had only few cytoplasmic organelle. However in vitro experiments have indicated, both thecal and granulosa cells are necessary (two-cell model) for the biosynthesis of steroids in amago salmon (Nagahama, 1983); this may not be applicable to M. cephalus. In Liza aurata and Chelon labrosus, numerous membrane-bordered pinocytic vesicles and rough ER have been observed in the cytoplasm of granulosa, suggesting these cells are involved in the synthesis of protein or transport of yolk from the blood to the developing oocytes (Brusle, 1985), as shown in many teleosts (Guraya, 1986). Abraham et al. (1984) have demonstrated that the protein (yolk) transport occurs in teleosts from perifollicular capillaries to the oocyte surface via. patent intercellular channels of follicular epithelium, but not through the granulosa cells. Absence of pinocytic vesicles in the granulosa of M. cephalus, supports the suggestion mentioned above. Desmosomes are observed between the granulosa and thecal cells in M. cephalus, rather than between adjacent follicle cells. In contrast, specialized junctions were lacking in the follicular layer of Liza aurata and Chelon labrosus (Brusle, 1985).

The oocyte maturation in M. cephalus is initiated with the migration of the germinal vesicle to the periphery of the oocyte and its subsequent breakdown (GVBD). These changes in the oocytes are the indications of resumption of meiosis I and the liberation of first polar body (Guraya, 1986). The remaining haploid set of chromosomes enters second meiotic metaphase and the meiosis is arrested once again and the egg becomes

"mature" (Wallace et al., 1987). Another noticeable change during this period was the increase in size of the oocyte due to hydration. This rapid water intake is characteristic of many marine teleosts, and as a consequence of this, the eggs are rendered buoyant in seawater (Wallace, 1985). This process is accompanied by the coalescence of protein yolk globules to form a uniform mass and the ripe eggs look translucent. The lipid droplets also fuse together and a large single oil drop was found. In teleost oocytes, all these maturational changes are induced by the maturation hormone, 17∝ 20β-DHP (Guraya, 1986). Scanning electron micrographs have shown that, in mullets, the micropyle is represented only by a very small and shallow spherical depression of the oocyte surface, which cannot be considered as a micropylar funnel (Mikodina, 1987). This may be the reason for not detecting this surface structure of M. cephalus egg by routine histological preparation in the present study.

Atresia of oocytes was not widespread in the ovary of M. cephalus. In post-spawned ovaries, the atretic oocytes formed only 5% of the total population. The histological picture of the resorbing oocytes in M. cephalus, clearly indicates that they do not have any steroidogenic capacity. The acidophilic, electron-dense material seen in the hypertropied granulosa of γ -stage ovary may be the engulfed yolk globule particles undergoing degeneration, as was also observed in C. gariepinus by Van den Hurk and Peute (1985). The yellowish-brown pigments formed during the final stages of regression are ascribed to a relative deficiency of lysosomes in lipid digestion. These residual bodies formed due to the incomplete

lysis, principally consist of complex lipids which are finally converted to the lipofuscin (Lang, 1981a). The lipofuscin granules were reported to be absent in the atretic follicles of Mugil capito (Abraham, 1963).

The postovulatory follicles (POF) (corpora lutea) are characteristic of spent ovary. In M. cephalus, the empty follicles form 28% of the total population in the post-spawned ovary. They undergo a series of morphological changes in the present fish, leading first to its evolution and then its involution. The hypertrophied granulosa cells are very transient in the present teleost, as after attaining their full differentiation, they begin to degenerate; the thecal layer also phagocytoze and later hydrolize the adjacent collagen bundles, by their cellular processes. The functional significance of the postovulatory follicles in teleosts is still unknown. But they have clearly shown to develop the histological and histochemical features of steroid-secreting cells in some teleosts (Guruya & Kaur, 1979). In POFs of M. cephalus, ultrastructural and histochemical results did not show any steroidogenic potency. Khoo (1975) postulated that some of the corpora luteal cells have differentiated into oogonia in gold fish. Ultrastructurally, no such differentiation of POFs was visible in M. cephalus. On the other hand, a replenishing stock of immature oocytes was always present in the ovary of this fish, even in ripe and spent stages. These potent oogonia and primary oocytes were found to escape the atretic changes and they were supposed

to be derived from germinal epithelium. The postovulatory follicles of some teleosts exhibit enlarged interstitial gland cells of thecal origin, the exact function of which is not known (Guraya, 1986). No such cell could be distinguished in corpora lutea of M. cephalus during the present study. In conclusion, it can be stated that the corpora lutea of M. cephalus are only degenerating empty follicles similar to the corpora atretica and have no endocrine function.

B. HISTOLOGY OF HEPATOCYTES

The liver of M. cephalus, contains intra and intercellular bile canaliculi (BC). The intracellular canaliculi can be interpreted as ramifications of the intercellular BC, facilitating transport from the cytoplasm to the lumen of the canaliculi (Peute et al. 1978). The hepatocytes of grey mullet contain relatively few smooth ER, small amounts of glycogen and large amounts of lipid as in medaka and zebrafish (Yamamoto & Egami, 1974; Peute et al. 1978). The changes in glycogen and lipid contents in the hepatocytes of the female M. cephalus during the reproductive cycle, however do not allow a strict classification of liver cells into glycogen rich or lipid rich varities as proposed by Welsch & Storch (1973). The presence of ribosomes in the mitochondrial matrix (mitoribosomes) has been demonstrated in the hepatocytes of zebrafish also by Peute et al. (1978). These mitoribosomes help mitochondria in performing certain biosynthetic or anabolic functions, i.e. synthesis of certain hydrophobic proteolipids (De Robertis & De Robertis, 1981).

The ultrastructural study showed, the liver cells have an activated state of protein synthesis during maturation and that their morphological changes during annual reproductive cycle are related to the appearance of the yolk protein precursor vitellogenin (Vtg) in the blood serum. This indicates the possible role of this organ in the synthesis of yolk proteins. In stage III (mature), the hypertrophied hepatocytes show an enlargement of the nucleus and mitochondria, proliferation and accumulation of abundant rER and numerous ribosomes. The hepatosomatic index (HSI) reaches its peak value in stage III. The liver cells show a highly intense histochemical reaction for proteins and RNA in this stage; the biochemical analysis also reveals the highest value of total proteins in liver at the same stage. In stage III, the electropherogram of blood serum shows a prominent vitellogenin (Vtg) band and the ovary exhibits majority of yolky (high GSI) oocytes. After spawning, the Vtg band is no more observed in the serum and the hepatocytes now become small and show only moderately developed rER and ribosomes. Peute et al. (1978) in zebrafish and Van Bohemen et al. (1981) in Salmo gairdneri suggested that, ultrastructural changes in hepatocytes which coincide with special phases of ovarian activity are indicative of vitellogenin synthesis in liver. Steroids,especially 17 β estradiol may trigger this process (Ng & Idler, 1983; Fostier et al., 1983). In the present study, the period of increased activity of the liver cells is also the period of maximal activity of the enzyme 3 β HSD in the ovarian follicle epithelium. This enzyme (3 β HSD) has a key position in the synthesis of steroid hormones and teleost ovaries are able to synthesize 17 β-oestradiol (Lambert et al.,1971). Sexual dimorphism is recognized in the liver cells of mature fish (Aida et al., 1973b). The histological changes in the hepato-

cytes of males are far less pronounced than that of females during gonadal maturation. However, Aida et al. (1973b) and Olivereau & Olivereau (1979) observed changes in the liver cells of estrogen treated, males and gonadectomized females similar to that in mature females. Autoradiographic and immunofluorescence experiments also indicated that liver cells were responsible for the synthesis and secretion of vitellogenin, stimulated by estrogen (Plack & Fraser, 1971; Shackley & King, 1979; Hara, 1987).

The present ultrastructural examination did not reveal any Vtg granules in the cytoplasm of hepatocytes and it is therefore doubtful whether the liver cells are involved in the storage of Vtg. However, it is possible that, the formation of electron-dense bodies (as seen in stage IV liver, in the present case) is related to Vtg secretion. The results of Idler & Campbell (1980) and Copeland et al. (1986) who reported low liver Vtg levels in trout, which had high blood levels; of Van Bohemen et al. (1981, 1982) who could not detect Vtg in liver even after treatment of the trout with 17 β-oestradiol, and of Selman & Wallace (1983) who noted that the hepatocytes of Fundulus heteroclitus contained few secretory granules eventhough they are secreting copious amounts of serum proteins; all support the view that Vtg is not stored in liver to any appreciable degree but rather it is secreted rapidly after synthesis. The disappearance of glycogen and lipid droplets at the beginning of exogenous vitellogenesis as observed in the present study probably reflect the metabolic demands of the hepatocyte during the period of intensive Vtg synthesis.

CHAPTER V

HISTOCHEMISTRY OF OOCYTES AND HEPATOCYTES

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HISTOCHEMISTRY OF OOCYTES AND HEPATOCYTES

Histochemical techniques provide qualitative information about the chemical nature, function and characterization of tissues, cells, organelles and other cellular inclusions without disturbing their normal structural organisation. Cytochemical studies on the ovarian tissue of teleosts have been carried out earlier by Chaudhary (1952), Yamamoto (1955a,b; 1956a,b,c), Chopra (1958), Stahl&Leray (1961), Venugopalan (1962), Guraya (1965, 1986), Malone & Hisaoka (1963), Dutt (1967), Dutt & Govindan (1967), Lambert (1970a,b), Te Winkel (1972), Donato & Contini (1974a,b), Varghese (1976), Riehl (1977a), Verma (1977), Shackley & King (1977, 1978, 1979), Saidapur (1978), Khoo (1979), Guraya & Kaur (1982), Emelyanova (1985), Gutierrez et al. (1985), Thomas & Sathyanesan (1985), Van den Hurk & Peute (1985), Selman et al. (1986, 1988), Mayer et al. (1988) and Jayasankar (1989).

Histoenzymological methods are often used to locate the site of synthesis of certain compounds and also to demonstrate areas of intense metabolic activity in cells. Enzymes like Δ^5 3β HSD, alkaline phosphatase, succinate dehydrogenase (SD), acid phosphatase, G6PD, α-glycerophosphate dehydrogenase, UDP-glucose dehydrogenase (UDPGD), NADH-diaphorase, 3β HSD, 17β HSD, isocitrate dehydrogenase, lactate dehydrogenase, malate dehydrogenase, non-specific esterase, lipase, adenylate cyclase and E-600

resistant esterase were detected in the teleost ovary by Venugopalan (1962), Lambert (1970a,b), Lambert et al. (1971), Livni (1971), Van den Hurk (1973), Lambert & Van Oordt (1974), Hoar & Nagahama (1978), Lang (1981a,b), Shanbhag & Nadkarni (1981), Fostier et al. (1983), Ali et al. (1987), Goldemberg et al. (1987) and Van den Hurk et al. (1987).

Relatively very little effort has been made to study the gonadal histochemistry of mullets. Stahl & Leray (1961) studied the cytochemistry of the zona radiata of the oocytes of Mugil cephalus and M. capito. Abraham et al. (1966) classified the yolk inclusions of five species of mullets based on primary histochemical techniques. The cytochemical nature of the yolk nucleus in the oocytes of M. cephalus was studied by Abraham et al. (1968). Eckstein & Eylath (1968) studied steroidogenesis in the testes of M. cephalus while the histochemistry of dehydrogenases in the ovary of M. capito was studied by Blanc-Livni et al. (1969) and Livni (1971). Donato & Contini (1974a,b) carried out histochemical studies on protein and lipid yolk bodies of Mugil chelo while Elizabeth (1987) studied the histochemistry of testicular tissue of M. cephalus and Liza parsia.

In teleosts, hepatic cells being the site of synthesis of vitellogenin, undergo considerable changes in their chemical composition during and after yolk synthesis. Histochemical investigation on the cyclic changes of liver tissue enables one to unveil the role of this organ in vitellogenesis. The only results obtained in this line are those of Sarkar & Deb (1965), Welsch & Storch (1973), Aida et al. (1973b), Varghese (1976), Peute et

al. (1978) and Nunomura et al. (1983).

Histochemical reactions of proteins, carbohydrates, lipids, nucleic acids, enzymes and metals in the ovarian and hepatic cells of M. cephalus are studied and results presented.

RESULTS

A. OOCYTES

Proteins (Table-10)

The cytoplasm of previtellogenic oocytes (Plate XIIa), zona radiata and nucleoli stained strongly for proteins, while the nucleus gave only a weak reaction in all stages. The yolk nucleus and yolk globules stained intensely, but the oolemma and the follicular layer showed only moderate staining for proteins.

Yolk globules indicated a very intense reaction and the yolk nucleus and nucleus a moderate reaction for basic proteins. The ooplasm showed a gradual increase in staining intensity for the acidic groups of proteins. The nuclei in the follicular layer showed a strong reaction for acidic groups while the yolk globules give a negative reaction to it. The nucleus, nucleoli and yolk nucleus stained moderately with toludine blue.

Yolk globules, zona radiata and nucleoli stained strongly and the cytoplasm moderately for amino group.

TABLE 10: Histochemical reactions of Proteins in the ovarian tissue of Mugil cephalus.

Histochemical tests for Proteins	Oolemma	Zona Radiata	Ooplasm						Nucleus	Nucleoli	Follicular layer	Post Ovul. Follicles	Corpus Atreticum
			Matrix (CN & EPN)	Matrix (LPN & LD)	Yolk Nucleus	Lipid Droplets	Yolk Globules	Cortical Alveolar layer *					
Mercuric Bromophenol Blue (Proteins - General)	++	++	+++	+++	++++		++++		+	+++	++	++	+++
Aq.Bromophenol Blue (Basic Proteins)	+		+	+	++		++++		++	+	+	+	+++
Deamination													
Toludine Blue (Acidic group)	+		++	+++	++				++	++	+	+	
Methylation													
Ninhydrin-Schiff (Amino group)	+	+++	++	++	+		+++		+	+++	+	+	+++
Deamination													
Ferric-ferricyanide (-SH group)	++	++	+++	++	++		++		+	++	+	+	++
Mercaptide													
Performic Acid-Alcian Blue (-SS group)	+	+	+	+			+				+	+	+
Thioglycollate Ferric-ferricyanide (-SS group)	+	+	+	+			+				+	+	+
Thioglycollate reduction													
Sakaguchi's reaction (Arginine)	+	++	+	+	+		+			+	+	+	+
Deamination											-		
Pauly's reaction (Histidine)			+	+			+		+				+
Deamination													
Millon's test (Tyrosine)	+	+++	+	+			+++						+
Iodination													
DMAB - nitrite (Tryptophan)							+				-		+
40% formaldehyde													

(no reaction); + (weak); ++ (moderate); +++ (strong); ++++ (highly intense reaction).

CN - Chromatin nucleolus stage; EPN/LPN - Early and late perinucleolus stages; LD - Lipid droplet stage.

* Protein staining reactions of cortical alveolar layer not clearly observed due to numerous large protein yolk globules.

PLATE XII

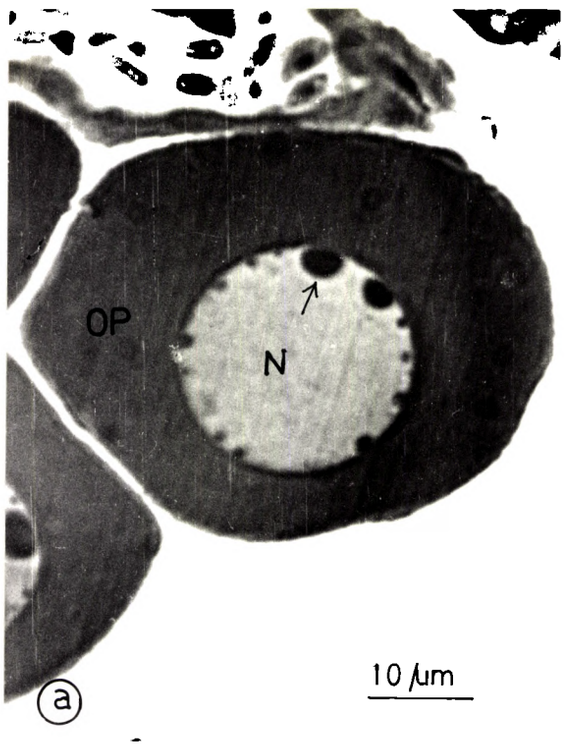
a. Perinulceolar oocyte stained with mercuric bromophenol blue for proteins. OP, ooplasm; N, nucleus; ⟶ nucleolus. X 1,400.

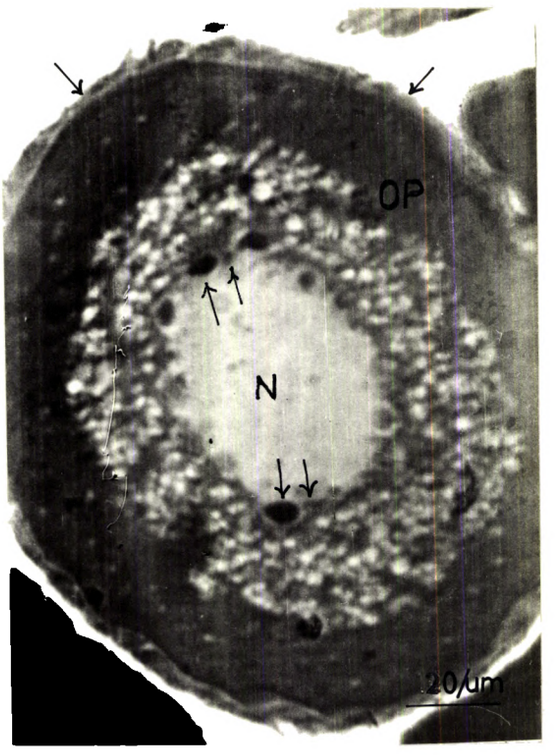
b. Ferric ferricyamide reaction for -SH group. The ooplasm (OP) and the nucleoli (⟹) of the lipid droplet stage oocyte show strong -SH positive reaction while the follicular layer (⟶) reacted moderately. N, nucleus. X 1,250.

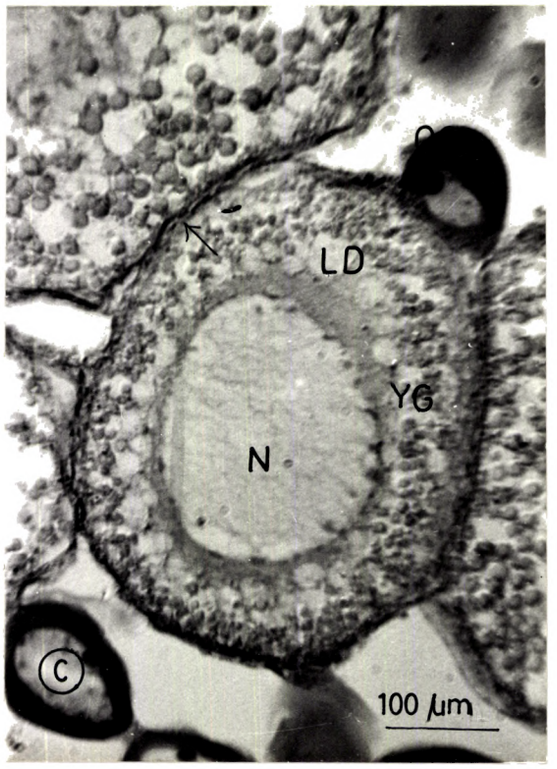
c. Best's carmine test for glycogen. The yolk globules (YG), follicular layer (⟶) and zona radiata of the primary yolk globular oocyte show a moderate reaction, while the oogonial (OG) cytoplasm show a strong glycogen positive activity. The nucleus (N) and the juxta-nuclear lipid droplets (LD) of the oocyte show a negative reaction. X 1,100.

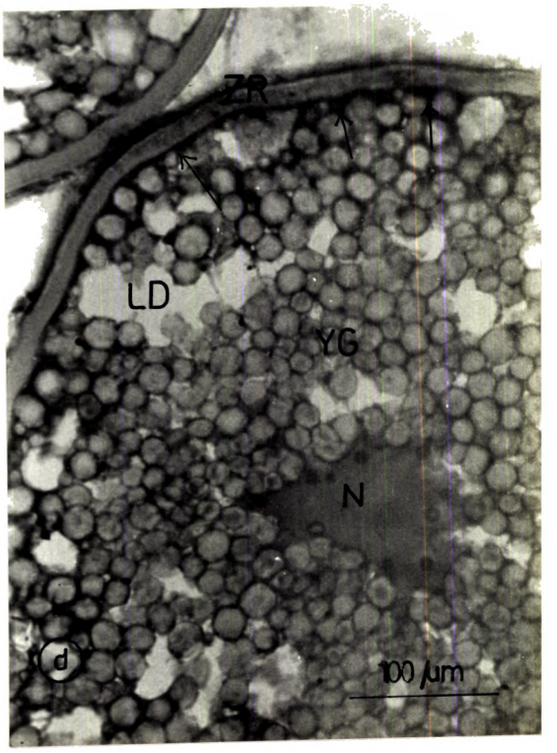
d. Secondary yolk globule stage oocyte, stained for polysaccharides with periodic acid - Schiff's (PAS) reaction. Note a strong positive reaction (⟶) below the zona radiata (ZR). LD, lipid droplet; N, nucleus; YG, yolk globules. X 3,500.

PLATE XII









The cytoplasm of previtellogenic oocytes showed a strong reaction to -SH group which decreased in the advanced stages (Plate XIIb). The yolk nucleus, nucleoli, oolemma, yolk globules and zona radiata stained moderately for -SH group.

Yolk globules, zona radiata, ooplasm and follicular layer showed a faint reaction for disulphide (-SS)group .

Traces of arginine was detected in the zona radiata, yolk nucleus, yolk globules, ooplasm, nucleoli and the follicle cell nucleus. A weak reaction for histidine was observed in the yolk globules, ooplasm and nucleus. A strong tyrosine positive reaction was observed in yolk globules and zona radiata. The yolk globules also showed a faint reaction for tryptophan.

The cortical alveolar layer would also stain positively for proteins, but it was difficult to determine with any certainty due to the presence of numerous large protein yolk globules. Unlike many teleosts, the yolk globules in M. cephalus did not exhibit any zonation or change in staining reaction from the stage of their deposition in oocytes upto the ripe condition.

Carbohydrates (Table-11)

The oolemma and zona radiata stained strongly with Periodic Acid Schiff (PAS) indicating some polysaccharides in them. The PAS positivity showed a considerable decrease after pre-treatment, both with diastase and trypsin indicating the presence of glycogen and glycoproteins in them.

TABLE 11: Histochemical reactions of Carbohydrates in the ovarian tissue of Mugil cephalus.

Histochemical tests for Carbohydrates	Oolemma	Zona Radiata	Ooplasm					Nucleus	Nucleoli	Follicular layer	Post Ovul. Follicles	Corpus Atreticum
			Matrix	Yolk Nucleus	Lipid Droplets	Yolk Globules	Cortical alveolar layer					
Schiff's reagent alone												
PAS (1,2 glycol groups)	+++	+++	+	+		+	+++			+	++	++
Acetylation												
Deacetylation	++	++	+	+		+	++			+	+	+
Chloroform-methanol	+++	+++	+	+		+	+++			+	++	++
Diastase							+++				+	+
Trypsin			+	+			+++			+	++	+
Best's Carmine (Glycogen)	++	++	++	++		++				++	+	++
Diastase												
Toludine Blue-pH 1.09,												
3.09 & 4.19 (Sulphated AMP)			+	++					++	+		
Methylation			+	++					++	+		
Ribonuclease												
Toludine Blue pH 1.99 (Phos.AMP)			++	++					++	+		
Methylation			++	++					++	+		
Ribonuclease												
Toludine Blue-pH 7.00 (Carbo.AMP)			+++	+++				+	+++	++	+	+
Methylation			++	+++				+	+++	++	+	+
Ribonuclease												
Alcian Blue-CEC (Acidic Sulphomucin)												
1% Alcian Blue-pH 2.5,												
(Weakly acidic sulphomucin)												
Benzidine Reaction (Sulphated AMP)												

(no reaction); + (weak); ++ (moderate); +++ (strong); ++++ (highly intense reaction).

The cortical alveolar layer showed a strong reaction with PAS (Plate XIId), which was not lost even after pre-treatment with diastase, trypsin or chloro-form-methanol indicating that 1, 2 glycol groups of mucopolysaccharides were responsible for the staining reaction in the untreated sections and absence of glycogen and lipids. The cytoplasm of primary oocytes, yolk nucleus the protein yolk globules and the follicular layer also showed a weak reaction with PAS (negative after diastase) suggesting the presence of traces of glycogen in them and glycoproteins in yolk globules (negative after trypsin treatment).

With Best's carmine, a moderate reaction was observed in cytoplasm of pre-vitellogenic oocytes, yolk nucleus, oolemma, zona radiata, follicular layer and yolk globules (negative after diastase) (Plate XIIc) indicating the presence of glycogen in all the above structures.

The cytoplasm of previtellogenic oocytes, yolk nucleus and nucleoli exhibited metachromasia with toludine blue (at pH 1.09, 3.09, 4.19, 1.99 and 7.0). But loss of metachromasia in sections pre-treated with ribonuclease and persistance of the colour in methylated control sections indicated the presence of RNA, not acidmucopolysaccharides. Weakly acidic and acidic sulphomucins were also absent in various structures or inclusions of oocytes (negative with alcianblue and benzidine reactions).

Lipids (Table-12)

The lipid droplets in all stages and those seen in follicular layer,

TABLE 12: Histochemical reactions of Lipids in the ovarian tissue of Mugil cephalus.

Histochemical tests for Lipids	Oolemma	Zona Radiata	Ooplasm					Nucleus	Nucleoli	Lipid Droplets in follicular layer	Post Ovul. Follicles	Corpus Atreticum
			Matrix	Yolk Nucleus	Lipid Droplets	Yolk Globules	Cortical alveolar layer					
Sudan Black-'B' (Lipids)				+	++++	+++			+++	+++	+++	+++
Chloroform-methanol extraction					-							
Pyridine extraction					++							
1% Nile Blue at 60°C (Neutral lipids)				+	++++					+++	+++	+
(Acidic lipids)						+++			++			++
Chloroform-methanol extraction												
Pyridine extraction					++					-	+	
Acid Haematin (Phospholipids)						+++			++			++
Pyridine extraction												
Nile Blue with Conc. H_2SO_4 (Phospholipids)						+++			++			++
Pyridine extraction												
Chloroform-methanol extraction												
Oil Red-'O' (Neutral lipids)				+	++++	++				+++	+++	++
Chloroform-methanol extraction												
U.V.-Schiff Reaction (Unsat.lipids)						+++			++	+++		++
Pyridine extraction					-							
Sudan Black-'B' with acetic acid (Masked lipids)	++		++	+	++++	+++			+++	+++	+++	+++
Pyridine extraction					+					+	+	
Schultz's reaction (Cholesterol)										+++		-
Chloroform-methanol extraction												

(no reaction); + (weak); ++ (moderate); +++ (strong); ++++ (highly intense reaction).

PLATE XIII

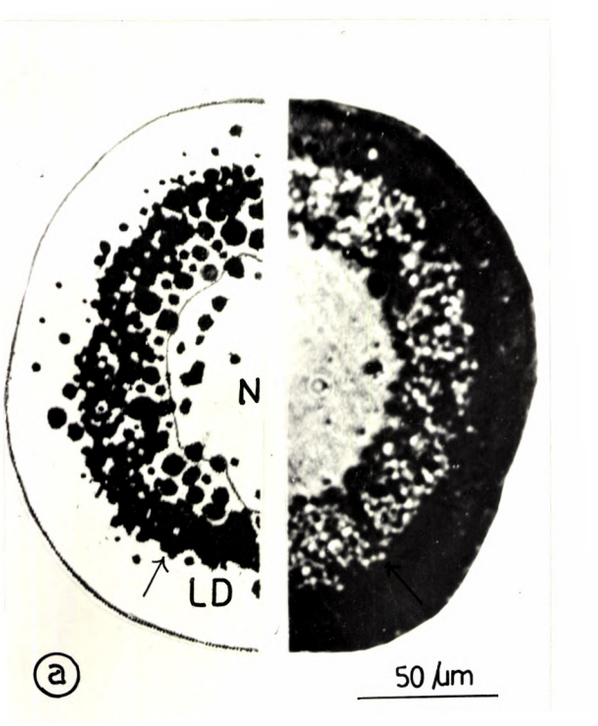
a. Two halves of a lipid droplet stage oocyte. The ooplasm of the left half shows highly sudanophilic lipid droplets (LD) in the perinuclear zone, while the same droplets appear as blank spaces (⟶) in the routine histological preparation as in the right half. X 1,000.

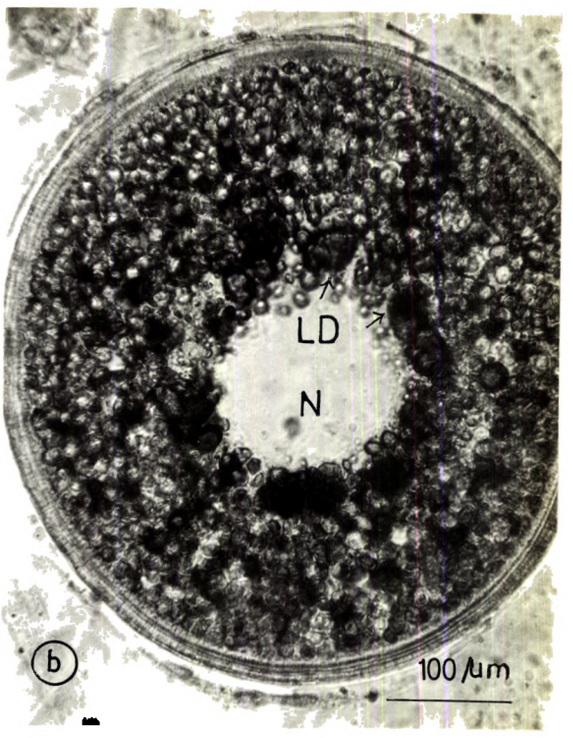
b. Cryocut section of a yolk globule stage oocyte, stained with Oil red - 'O'. The lipid droplets (LD) stain dark orange red for neutral lipids. The yolk globules also show a moderate reaction. N, nucleus. X 1,275.

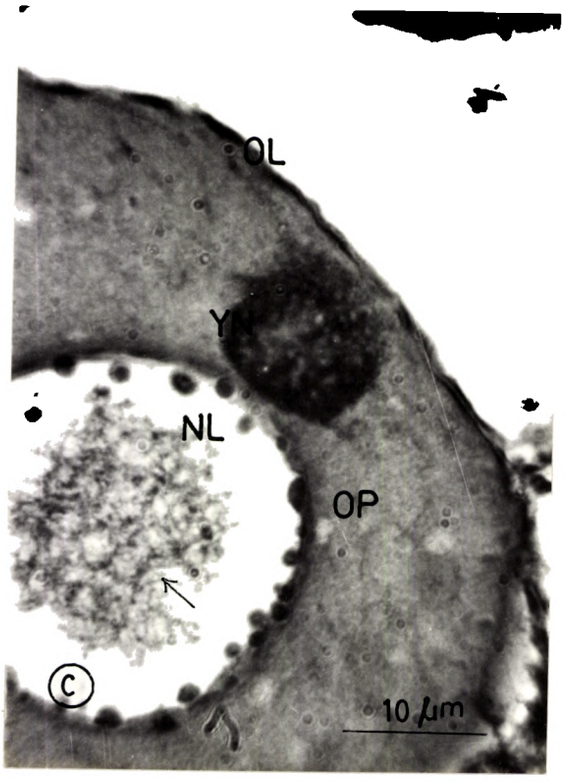
c. Perinucleolar oocyte, stained with methyl green - pyronin for DNA and RNA. The yolk nucleus (YN) and the nucleolus (NL) are strongly RNA positive, while the chromatin net work (⟶) in the nucleus (N) shows a positive result for DNA. The ooplasm (OP) and the oolemma (OL) reacted moderately for RNA. X 3,185.

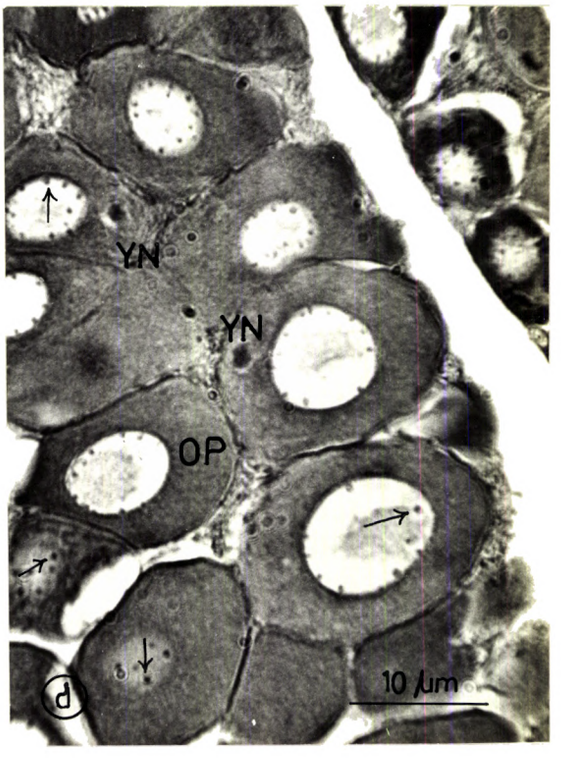
d. Alkaline phosphatase activity in the perinucleolar oocytes: calcium - cobalt method. Apart from a strong activity in the yolk nucleus (YN) and the nucleolus (⟶), a moderate reaction is observed in the ooplasm (OP). X 550.

PLATE XIII









postovulatory follicles and atretic oocytes showed highly intense reaction with Sudan Black-'B' indicating the presence of lipids (Plate XIIIa). The nucleoli and yolkglobules showed a strong sudanophilia, while the yolk nucleus showed only a weak reaction.

The lipid droplets of all stages contained neutral lipids as they developed a deep pink colour with 1% nile blue. But the yolk globules and the nucleoli showed positive reaction for acidic lipids with nile blue.

The yolk globules and nucleoli were markedly positive to acid haematin (AH) indicating rich amounts of phospholipids in these structures. But the lipid droplets were AH negative. Nile blue with conc. sulphuric acid confirmed the results obtained for phospholipids by AH.

With Oil red-'O', the lipid droplets reacted intensely showing the rich amounts of neutral lipids in them (Plate XIIIb). The yolk globules and yolk nucleus showed only a moderate reaction with Oil red-'O'.

The lipid droplets in the follicular layer and the yolk globules reacted strongly with U.V.-Schiff indicating rich amounts of unsaturated lipids in them. But the lipid bodies in oocytes and postovulatory follicles were negative to U.V.-Schiff reaction.

The cytoplasm of oocytes and the oolemma revealed traces of masked lipids with Ackerman's (1952) method.

A strong positive reaction was observed for cholesterol in the

follicular layer. But the lipid bodies both in ooplasm and the postovulatory follicles showed a cholesterol negative reaction.

Nucleic Acids & Basic proteins of Nucleus (Table-13).

The nucleus of oocytes took a faint reddish purple colour and the pycnotic nuclei of follicle layer, a deep red colour in Feulgen's test indicating the DNA content in it.

The cytoplasm of previtellogenic oocytes, yolk nucleus and nucleoli reacted strongly either with pyronin or toludine blue indicating rich amounts of RNA in them (Plate XIIIc). The oocytes of stage II ovary indicated a progressive increase in the cytoplasmic RNA content after the disappearance of yolk nucleus. The oocyte nucleus reacted weakly and the follicle cell nucleus strongly for DNA with methyl green.

The nucleus, nucleoli, yolk nucleus, ooplasm and nuclei in the follicular layer showed a moderate reaction for the nuclear proteins like protamines and histones with alkaline fast-green FCF. Amino acids like arginine and lysine also give a positive reaction with alkaline fast-green. Hence arginine containing oolemma and zona radiata were weakly positive to this dye.

Enzymes (Table-13)

The reaction for alkaline phosphatase was weak in the cytoplasm of previtellogenic oocytes. The staining intensity showed an increase in

TABLE 13: Histochemical reactions of Nucleic acids, Nuclear proteins, Enzymes and Metals in the ovarian tissue of Mugil cephalus.

Histochemical Tests	Oolemma	Zona Radiata	Ooplasm						Nucleus	Nucleoli	Follicular layer	Post Ovul. Follicles *	Corpus Atreticum
			Matrix (CN & EPN)	Matrix (LPN & LD)	Yolk Nucleus	Lipid Droplets	Yolk Globules	Cortical Alveolar layer					
NUCLEIC ACIDS & BASIC PROTEINS OF NUCLEUS													
Feulgen reaction (DNA)									+		+++	+++	
Hot perchloric acid treatment													
Methyl Green-Pyronin (DNA)									+		+++	+++	
(RNA)			+++	++++	+++					+++	+	+	
Hot perchloric acid treatment													
Ribonuclease treatment									+		++	++	
Methyl Green-Toludine Blue (DNA)									+		+++	+++	
(RNA)			+++	++++	+++					+++	+	+	
Hot perchloric acid treatment													
Ribonuclease treatment									+		++	++	
Alkaline Fast Green (Histones & Protamines)	+	+	+	+	++				+	++	++	++	
ENZYMES													
Calcium-Cobalt (Alkaline phosphatase)			+	++	+++		+			+++	+++		
Incubation without substrate													
Coupling Azodye method (Acid phosphatase)			+	+								+++	+++
Incubation without substrate													
Nachlas et al. method (SD)			++	++	++++						+		
Incubation without substrate													
Wattenberg's test (3βHSD)											+++		
Incubation without substrate													
METALS													
Alizarin Red-'S' (Calcium)	+	+	++	++	+		+++			+	+	+	+
Strong acid treatment													
EDTA treatment													
Perl's modified method (Iron)													

(no reaction); + (weak); ++ (moderate); +++ (strong); ++++ (highly intense reaction).

CN - Chromatin nucleolus stage; EPN/LPN - Early and late perinucleolus stages; LD - Lipid droplet stage.

* In follicular layer and post-ovulatory follicles reactions for DNA & basic proteins were answered by nuclei and for RNA, the layer as a whole.

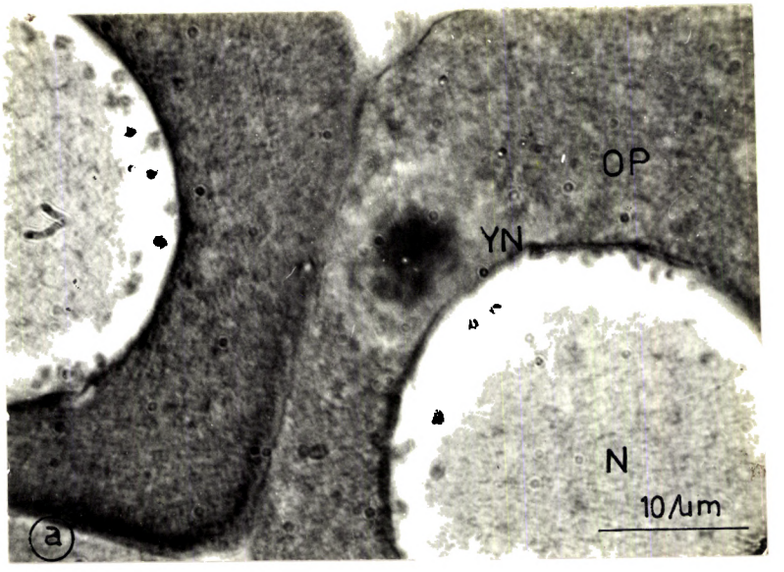
PLATE XIV

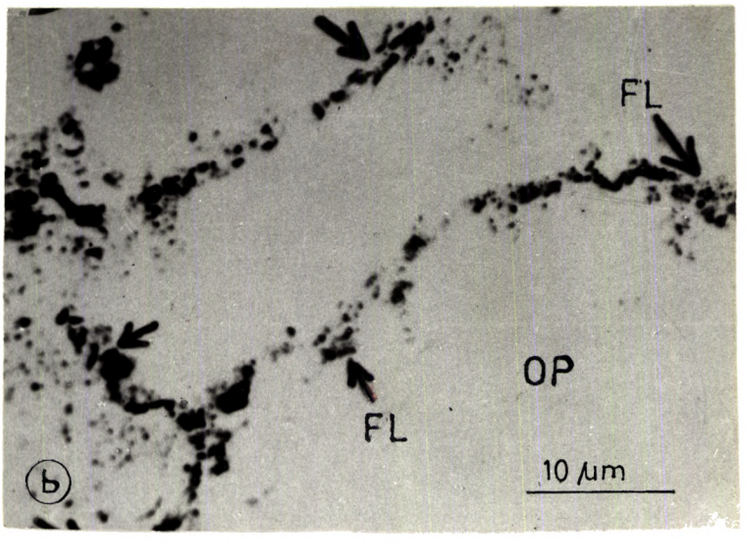
a. Succinate dehydrogenase activity in the perinucleolar oocytes: Nachlas *et al.* test. Note the moderate reaction in the ooplasm (OP) and the strong activity in the yolk nucleus (YN). N, nucleus. X 3,180.

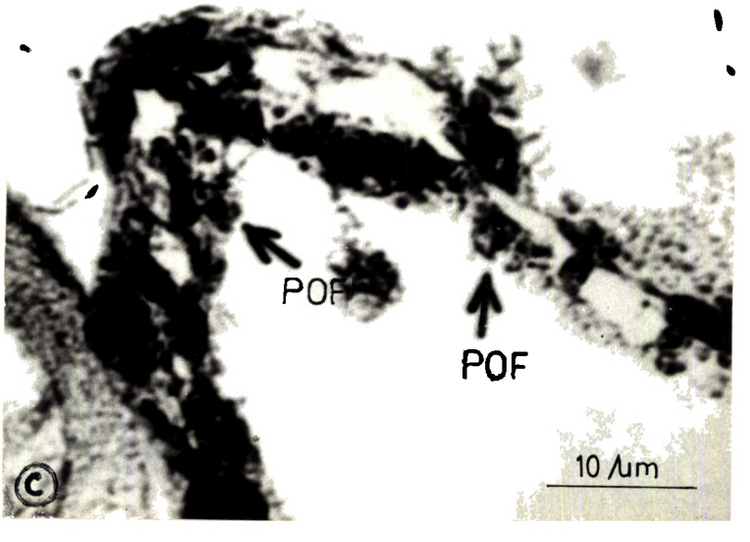
b. Wattenberg's test for 3 β HSD. A strong reaction in the follicular layer (FL) of primary yolk globule stage oocyte. OP, ooplasm. X 3,035.

c. Standard coupling azodye method to detect acid phosphatase. A strong activity is visible in the postovulatory follicles (POF) of spent ovary. X 3,060.

PLATE XIV







the succeeding stages. The yolk nucleus, nucleoli and the follicular layer were strikingly positive to this enzyme (Plate XIIId). Numerous blood cells seen in the spent and atretic ovary showed a strong enzyme activity, but the empty follicles and atretic oocytes were not stained.

A strong acid phosphatase activity was noticed in the empty follicles (Plate. XIVc) and the peripheral cytoplasm of atretic oocytes. The enzyme gave only a feeble reaction in the cytoplasm of young and maturing oocytes.

Succinate dehydrogenase (SD) is known to be localized in the mitochondria. A positive granulation of formazan was noted in the cytoplasm of primary oocytes with an intense reaction in the yolk nucleus (Plate XIVa). No enzyme activity was detected in the advanced and resorbing oocytes.

Δ^5 3 β hydroxysteroid dehydrogenase (Δ^5 3 β HSD) activity was detected during vitellogenesis. Selective deposition of formazan end-product was restricted to the follicular layer of vitellogenic oocytes (Plate XIVb). Activity of this enzyme was not detected in the postovulatory and atretic follicles.

Metals (Table-13)

The yolk globules showed a strong calcium positive reaction (Plate XVa). The cytoplasm of primary oocytes showed a moderate reaction, while the oolemma, zona radiata, nucleoli and yolk nucleus stained weakly for calcium.

With Perl's modified method, presence of iron was not detected in oocytes. The reason may be that, this method is not sensitive to detect minute quantities of iron present in the ovarian tissue.

B. **HEPATOCYTES** (Table-14)

Proteins

The hepatic parenchyma showed moderate reaction for proteins in stages I, IV and V, while the staining intensity was strong in stage II and highly intense in stage III.

Carbohydrates

The hepatocytes showed a strong reaction in stages I, IV and V with Periodic Acid-Schiff (PAS) reagent. The staining intensity was highest in stage II and moderate in stage III. A negative reaction was observed when sections were pre-treated with diastase, suggesting that the reaction was due to glycogen in the hepatocytes.

With Best's carmine (for glycogen), the hepatic cells showed similar results as with PAS (as above). Control sections treated with diastase failed to take carmine indicating the absence of glycogen.

Lipids

The hepatocytes belonging to stage II exhibited a highly intense

TABLE 14: Histochemical changes in the hepatic cells following ovarian maturation in Mugil cephalus L.

HISTOCHEMICAL TESTS	MATURITY STAGES				
	STAGE I	STAGE II	STAGE III	STAGE IV	STAGE V
PROTEINS					
Mercuric Bromophenol Blue (Proteins)	++	+++	++++	++	++
Deamination					
CARBOHYDRATES					
Periodic Acid-Schiff (1,2 glycol group)	+++	++++	++	+++	+++
Acetylation					
Deacetylation	++	+++	++	++	++
Chloroform-methanol	+++	++++	++	+++	+++
Diastase					
Best's carmine (Glycogen)	+++	++++	++	+++	+++
Diastase					
LIPIDS					
Sudan Black 'B' (Lipids)	++	++++	+++	++	++
Chloroform-methanol					
Pyridine extraction					
Acid Haematin (Phospholipids)	++	++	+++	++	++
Pyridine extraction					
Oil red 'O' (Neutral lipids)	++	++++	+++	++	++
Chloroform-methanol					
NUCLEIC ACIDS					
Fuelgen reaction (DNA)*	++	+++	+++	++	++
Hot perchloric acid treatment					
Methylgreen-Pyronin (DNA)*	++	+++	+++	++	++
(RNA)	++	+++	++++	++	++
Hot perchloric acid treatment					
Ribonuclease treatment					
ENZYMES					
Calcium Cobalt (Alkaline phosphatase)	++	+++	+++	++	++
Incubation without substrate					
Coupling azodye method(Acid phosphatase)	+	+	+	++	++
Incubation without substrate					

- (no reaction); + (weak); ++ (moderate); +++ (strong); ++++ (highly intense reaction).
* Reactions for DNA were answered by the nucleus of hepatic cells.

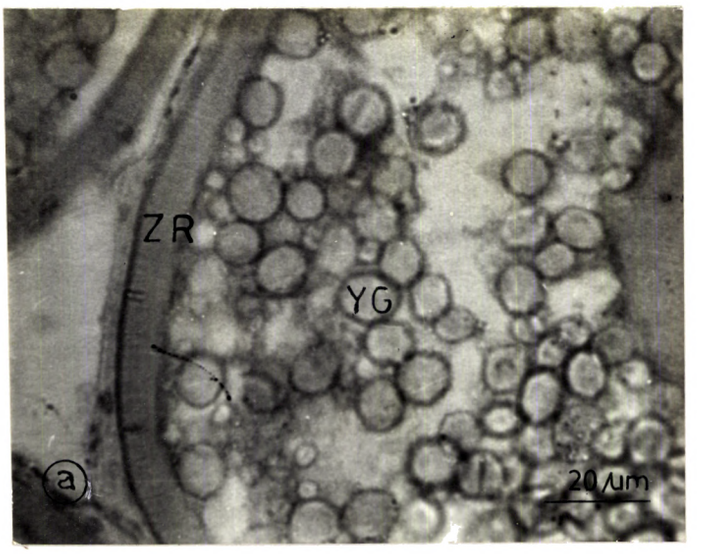
PLATE XV

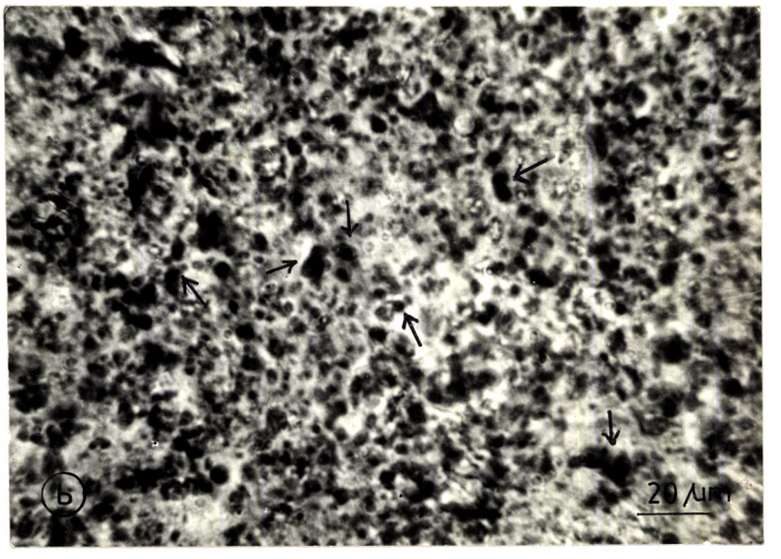
a. Alizarin red - 'S' method for calcium. The yolk globules (YG) reacted strongly, while the zona radiata (ZR) showed only a moderate reaction for calcium. X 3,195.

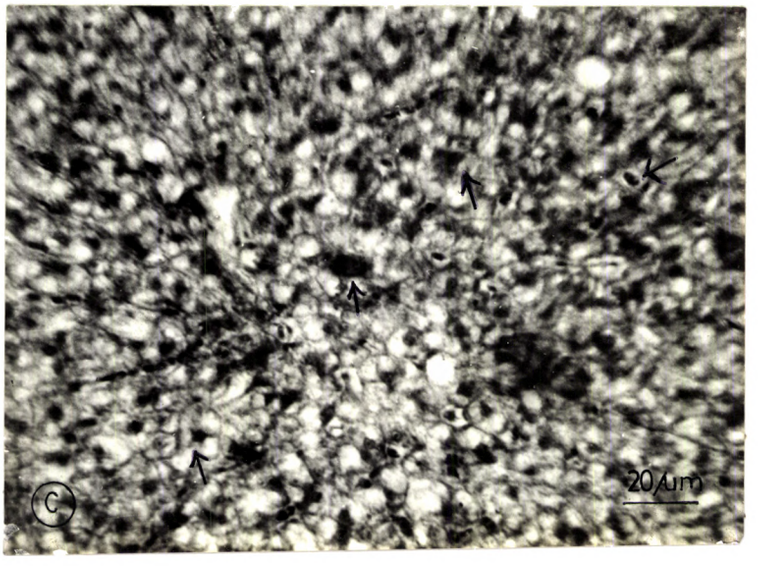
b. Hepatocytes belonging to stage II, stained with Sudan black - 'B' for lipids. Note numerous lipids droplets and intense reaction (⟶). X 1,100.

c. Hepatocytes belonging to stage IV, stained with Sudan black - 'B'. Reaction is moderate with a few number of lipid droplets (⟶). X 1,100.

PLATE XV







reaction with Oil red–'O' and Sudan black-'B' for neutral lipids (Plate XVb). The cells contained numerous large oil droplets in this stage. In rest of the stages, the reaction was moderate and less number of oil droplets (also smaller in size) were detected (Plate XVc).

With acid haematin, the staining intensity for phospholipids was moderate in the cytoplasm of hepatocytes in stages I, II, IV and V, while in stage III a strong reaction was observed.

Nucleic acids

The nucleus of hepatocytes belonging to stages I, IV and V exhibited a moderate reaction for DNA both with Feulgen and methyl green. The reaction was strong in the nucleus of hepatic cells belonging to stages II and III.

A highly intense reaction for RNA was observed in the cytoplasm and nucleolus of hepatocytes in stage III. The reaction was strong in stage II while stages I, IV and V showed only moderate reaction for RNA.

Enzymes

A strong alkaline phosphatase activity was noticed in the cytoplasm of hepatocytes (especially along the periphery of cells) and in the blood sinusoids in stages II and III. The staining intensity was moderate in all other stages.

Acid phosphatase activity was generally weak in the hepatocytes and blood sinusoids of liver, but in stages IV and V, a moderate activity

of this enzyme was observed.

DISCUSSION

A. HISTOCHEMISTRY OF OOCYTES

The histochemical findings revealed the chemical nature of oocytes and the different types of inclusions formed during vitellogenesis. This study has also offered help to locate the areas of steroidogenesis and draw conclusion on the possible functions of the postovulatory and atretic follicles in the ovary of M. cephalus.

Nucleus

Weak reaction of the oocyte nucleus for DNA (Feulgen & Methyl green) was significantly noticed in the present investigation. The faint/ negative results for DNA may be explained by the fact that chromosomes in the lampbrush stage are largely despiralized and show a very fine structure so that the cytochemical demonstration of DNA becomes impossible (Guraya, 1986). In many teleost species, intranuclear lipid bodies are observed (Guraya 1986). However, the oocyte nucleus of M. cephalus is devoid of any lipid bodies.

Nucleoli

The nucleoli contained rich quantities of RNA, proteins and $-NH_2$ group; small quantities of phospholipids, basic nuclear proteins, -SH group proteins and traces of arginine. It also showed a strong alkaline phosphatase activity especially during previtellogenic stages. The

nucleoli are the site of synthesis of RNA and they play an important role in the relay of information via. RNA from the nucleus to the cytoplasm in teleost oogenesis (Guraya, 1986) and hence strong staining reaction for RNA by the oocyte nucleoli. Traces of arginine in the nucleoli may be due to the presence of nuclear basic proteins (histones and protamines), which contain appreciable amounts of this dibasic amino acid.

Yolk nucleus

The basophilic yolk nucleus (Balbiani's vitelline body) gave strong histochemical reactions for RNA, proteins (both acidic and basic groups) nuclear basic proteins and enzymes such as succinate dehydrogenase and alkaline phosphatase. It also showed traces of -SH group proteins, lysine, arginine, glycogen and neutral lipids. Abundant ribosomes, observed in the electronmicrographs of the yolk nucleus (Chapter IV of this thesis) supports the RNA positivity of this structure. In the present study, the yolk nucleus of M. cephalus is found to be an assembly of mitochondria, ribosomes and tubules of endoplasmic reticulum (Chapter IV of this thesis), which indicates the intense metabolic activity of this ooplasmic structure and this may be attributed to a strong reaction for succinate dehydrogenase and alkaline phosphatase by this basophilic body. Succinate dehydrogenase is known to be localized in the mitochondria (Livni, 1971). Venugopalan (1962) and Livni (1971) have described the presence of the above mentioned enzymes in the yolk nucleus of teleosts. The yolk nucleus acts as the centre of metabolic activities related to the formation, multiplication and accumulation of other organelles which are finally distributed to the ooplasm

before the deposition of yolk (Guraya, 1986). Arginine and -SH group proteins were detected in the yolk nucleus of O. striatus (Venugopalan, 1962). In many teleosts, the yolk nucleus has been reported to contain phospholipids (Guraya, 1986). But, the yolk nucleus of M. cephalus showed a faint reaction only for neutral lipids and thus in agreement with the observations of Vengugopalan (1962) in O. striatus and Dutt (1967) in A. scandens. The indication of traces of calcium in the yolk nucleus (as shown by its weak Alizarin Red-'S' positivereaction) can be confirmed only by further studies.

Ground cytoplasm (Matrix)

The cytochemical details of the ooplasm could be detected only in the previtellogenic and lipid droplet stage oocytes. In the subsequent stages, the ground cytoplasm was reduced and it oocupied the interstices of the large deutoplasmic inclusions, in the form of a very thin reticulum. The intense colouration with mercuric bromophenol blue indicates a high synthesis of proteins in the ooplasm of previtellogenic oocytes. The marked elevation in the staining reaction for RNA and acidic proteins by the ooplasm, harmonizes the progressive increase of ribosomes in the primary oocytes (revealed by ultrastructure studies as presented in Chapter IV of this thesis). The occurrence of -SH group, tyrosine and basic amino acids like lysine, arginine and histidine in the ooplasm of M. cephalus as in the present case, was also reported in the oocytes of other teleosts (Venugopalan, 1962; Gutierrez et al., 1985). Other than the lipid droplets, the ground ooplasm in M. cephalus contained only meager amounts of lipids as revealed by the Sudan black-'B' with acetic acid test. A slight increase in the activity

of succinate dehydrogenase and alkaline phosphatase in the matrix, following the disappearance of yolk nucleus may be attributed to the dispersal of essential cytoplasmic components to the ooplasm by the yolk nucleus. The lysosomal components may be responsible for giving a very faint acid phosphatase reaction in the ooplasm.

Ooplasmic inclusions during vitellogenesis

In M. cephalus, three types of inclusions viz. lipid droplets, protein yolk globules and cortical alveoli, formed during vitellogenesis, differ distinctly in their morphology, chemical nature and staining properties and they are deposited sequentially although considerable overlap occurs.

The first type of inclusion to accumulate in the developing oocytes of M. cephalus is lipid droplets. These lipid inclusions contained only neutral lipids (triglycerides) at different stages of oocyte development. Unlike many other fishes, their chemical composition was not altered and they never exhibited any zonation. Phospholipids were totally absent. In teleosts, fatty (lipid) yolk consists mainly of neutral lipids (Guraya, 1986), but many have shown the presence of phospholipids also (Venugopalan, 1962; Dutt & Govindan, 1967; Donato & Contini, 1974b; Riehl, 1977a; Verma, 1977). However, the existence of lipid droplets has not been reported in the oocytes and many teleosts like Carassius auratus (Khoo, 1979), Solea senegalensis (Gutierrez et al., 1985), Labeo capensis (Van der Merwe et al., 1988).

Protein yolk accumulation (yolk globules) occurs after and concomitant to lipid yolk accumulation. The cytochemical studies have shown

that these fluid-filled yolk globules in the eggs of M. cephalus consist mainly of proteins (basic proteins) and glycolipoproteins. The lipid component includes predominantly phospholipids and small quantities of neutral lipids (triglycerides). These results corroborate with the findings of Venugopalan (1962), Malone & Hisaoka (1963), Donato & Contini (1974,a,b), Riehl (1977a), Verma (1977) and Khoo (1979), in different teleosts. The electrophoretic and biochemical investigations of yolk proteins of M. cephalus also support this observation. Osmium tetroxide is a useful lipid stain as it stabilizes unsaturated lipids (e.g. phospholipids) by means of cross-linking adjacent molecules to form stable diesters (Hayat, 1970) and thus preventing their dissolution during dehydration with alcohols. The yolk globules of M. cephalus eggs are highly positive to this post-fixation agent in electron microscopic preparations, thus reiterating that, they contain rich amounts of phospholipids. The yolk globules in the present study showed a strong reaction also for lysine ($-NH_2$ group), tyrosine and -SH group proteins, but found to contain only traces of -SS group proteins, alkaline phosphatase, arginine, histidine and tryptophan. However in Mugil chelo, the yolk globules are rich in tryptophan, arginine, tyrosine and -SS group and poor in -SH groups, unlike in M. cephalus; thus exhibiting species difference in yolk composition (Donato & Contini, 1974a). The yolk globules in M. cephalus did not show any zonation and undergo any considerable chemical transformation from its initial constitution, unlike in M. chelo (Donato & Contini, 1974a) and several other teleosts (Venugopalan, 1962; Dutt, 1967; Riehl, 1977a; Khoo, 1979; Guraya, 1986; Begovac & Wallace, 1988). The calcium-positive reaction, shown by the yolk globules of M. cephalus may be attributed to their affinity for this metal.

The third and quantitatively minor type of inclusion is the cortical alveoli, which appear after both lipid yolk and protein yolk formation have started. H istochemical studies indicate that the cortical alveoli contain mucopolysaccharides or glycoproteins (Khoo, 1979; Guraya, 1986; Selman et al., 1986), which are believed to be synthesized endogenously (Guraya, 1986). In agreement with this, the cortical alveoli in M. cephalus oocytes indicate the presence of mucopolysacchrides. The cortical alveoli in this fish did not appear as discrete vesicles, but as a highly PAS- positive narrow zone, just beneath the zona radiata. The large protein yolk globules which filled almost the entire ooplasm did not permit to determine, whether the cortical alveoli of M. cephalus eggs were composed of glycoprotein conjugates. Yamamoto (1955a,b; 1956a,b,c) opined that the chemical nature of polysaccharides in the cortical alveoli varies according to the habitat of fishes. According to him, the yolk vesicles (cortical alveoli) of marine teleosts contain neutral polysaccharides and those of freshwater fishes are composed of acid mucopolysacharides. Although this classification was refuted by many (Dutt, 1967; Dutt & Inoue, 1980; Guraya, 1986; Selman et al., 1986), the present study showed the absence of acidic group in the cortical alveoli of the eggs of M. cephalus which spawns in the littoral areas.

Oolemma

Histochemically, the thin, flimsy, outermost membrane of oocytes the oolemma is highly rich in protein and carbohydrate. It also contains traces of -SH group proteins, arginine, lysine and tyrosine and moderate quantities of glycogen and lipids. The staining characters of this membrane

in M. cephalus could no more be detected, when the acellular zona radiata started developing.

Zona radiata

The fine structure pictures of the zona radiata (ZR) of M. cephalus revealed that it was composed of two layers (Chapter IV). But using light microscopic cytochemical techniques, the bipartite nature of ZR could not be distinguished, as the entire structure got uniformly stained. The zona material of M. cephalus in the present study consisted mainly of proteins and polysaccharides. Lipids were totally absent. Amino acids like tyrosine and lysine were present in rich amounts and glycogen, arginine and cysteine in moderate quantities. Stahl & Leray (1961), Verma (1977), Khoo (1979), Gutierrez et al. (1985), Guraya (1986) and Mayer et al. (1988) have observed that, in teleosts the zona radiata is composed of carbohydrate-protein matrix. Acidic mucopolysaccharides (AMP) are localized along the outer ZR of many freshwater teleosts, having adhesive eggs. AMP help the eggs to stick to the substratum (Guraya, 1986). The zona radiata of free-floating eggs of M. cephalus was devoid of acidmucopolysaccharides.

Follicular layer

The most striking observation was the strong activity of enzyme 3 β hydroxysteroid dehydrogenase (3 β HSD) in the follicular layer of the vitellogenic (yolk globular) oocytes. Fine, sudanophilic, neutral lipid droplets present in the follicle layer of these oocytes, showed a strong cholesterol positive reaction. A strong alkaline phosphatase activity was also observed in the layer, but the succinate dehydrogenase reaction was feeble. The

activity of acid phosphatase was not detected during the present study.

The enzyme 3 β HSD which catalyses the oxidation of dehydroepiandrosterone (DHA a C_{19}, 3 β hydroxysteroid) to androstenedione (C_{19}, 3 k etosteroid) is known to play a significant role in the fish steroid synthesis (Van den Hurk, 1973; Guraya, 1986). The visualization of this steroid dehydrogenase together with sudanophilic or cholesterol positive fine lipid droplets is generally believed to indicate the site of cellular steroidogenesis, especially in female fish (Lambert, 1970a). The results of the present study clearly suggest the involvement of the follicular layer surrounding the vitellogenic oocytes, in active steroid synthesis, in M. cephalus. In male fish, another enzyme (17 β HSD),which converts androstenedione to testosterone is detected in cells which are involved in the active biosynthesis or metabolism of male sex steroids (Elizabeth, 1987). The presence of succinate dehydrogenase (SD) also to a certain degree strengthen the opinion about the steroid synthesizing function of the follicle layer as was also observed in many other teleosts (Van den Hurk, 1973). SD is related to the oxidative phosphorylation; this enzyme has been frequently detected in mitochondria. In cells, that synthesize steroids, enlarged mitochondria with dialated tubular cristae, are often encountered (Guraya, 1986). The cells which are involved in the transport mechanism stain strongly for alkaline phosphatase, which suggests that this enzyme is engaged in tras-membrane transport, possibly enabling an influx of substances into cells (Livni, 1971). Thus, the marked enzymatic activity in the follicular layer of yolky oocytes in the present study may be due to the intake of large quantities of exogenous yolk for

the developing oocytes through these enveloping cells. Another enzyme 20 β HSD, essential for the synthesis of maturation hormone (progestogen 17α, 20 β dihydroxy 4-pregnen-3-one (17α, 20β DHP), from 17α hydroxy-progesterone)has been detected in the follicle (granulosa) layer of teleosts during the phase of ovum maturation (Nagahama, 1983). The biochemical aspects of steroid production in mullets, have been dealt with by Eckstein & Eylath (1968, 1970), Eylath& Eckstein (1969), Eckstein & Azoury (1979, 1981), Azoury & Eckstein (1980) and Elizabeth (1987).

In many teleosts, some enlarged (hypertrophied) cells in the thecal layer of ovary, known as the special thecal cells (interstitial cells) (identical to the Leydig (interstitial) cells in testis) have been identified as steroidogenic cells (Lambert & Van Oordt, 1974; Shanbhag & Nadkarni, 1981; Guraya & Kaur, 1982; Nagahama, 1983). But in the ovary of M. cephalus, special thecal cells were not identified either histochemically or ultrastructurally. The activity of 3 β HSD was rather uniformly spread throughout the follicle layer, which is in agreement with the observations of Livni (1971) in Mugil capito. Ultrastructurally, the granulosa cells of follicular layer exhibited some characteristics indicative of steroidogenesis, but not the thecal cells. But, the major cellular site for steroidogenesis in the teleost ovary is still controversial. However recent in vitro experiments in amago salmon (O. rhodurus) indicated that both thecal and granulosa layers are necessary for 17 β oestradiol and 17α 20 β-DHP production and a two-cell type model has been proposed for teleosts (Nagahama, 1983).

Postovulatory follicle & Corpus atreticum

Strong acid phosphatase activity was observed in the postovulatory follicle (POF) and corpus atreticum (AF) of M. cephalus ovary at different stages of resorption. Lambert (1970a) have opined that, presence of lysosomal enzymes like acid phosphatase, E-600 resistant esterase and non-specific esterase in the POF and AF of fish ovary points out the active degeneration process going on in these structures. Although numerous sudanophilic, neutral lipid droplets were seen both in POF and AF, enzymes indicating the active cell metabolism or biosynthesis, like 3β HSD, SD, alkaline phosphatase could not be demonstrated in these ovarian structures. These results together with the ultrastructural observation (Chapter IV) clearly suggests that the POF and AF in the ovary of M. cephalus do not have any steroidogenic or endocrine role unlike the corpora lutea of mammals; they are merely large yolky eggs (AF) and empty follicle layer (POF), undergoing the process of degeneration and resorption.

The degenerating protein yolk globules in the AF of M. cephalus ovary showed similar staining reaction as that of yolk globules (protein-polysaccharidellpid complex) of normal oocytes. The lipid droplets in AF too remained as neutral lipids (triglycerides). As the atresia advanced, these structures are taken up by the hypertrophied granulosa cells. The round, acidophilic, electron-dense homogeneous material in the granulosa, as observed in the present case, might represent the degenerating protein yolk materials, which are finally resorbed, except their lipid fraction (Van den Hurk & Peute, 1985). Insufficient quantity of the enzyme lipase in the lysosomes of fish usually results in partial lysis of the lipid materials

in atretic follicle (Lambert, 1970a). As opined by him, the appearance of sudanophilic yellow-brown pigment in γ and δ stages may be the result of this incomplete breakdown of lipids and they form the so-called "residual bodies", containing lipofuscin granules.

B. HISTOCHEMISTRY OF HEPATOCYTES

A steady increase of proteins, RNA, DNA, glycogen and alkaline phosphatase activity upto stage III and a subsequent decline, observed in the hepatocytes during the present study indicate the activated state of liver during vitellogenesis. These observations agree with the reports of earlier workers in other teleosts (Welsch & Storch, 1973; Peute et al., 1978; Emmersen et al., 1979). In the present investigation, the increased activity of hepatocytes coincides with a strong 3 β HSD positivity in the ovarian follicular layer. This enzyme (3 β HSD) plays an important role in the fish steroid synthesis (Guraya, 1986). Vitellogenesis in teleost liver, is triggered by steroids especially 17 β oestradiol (Ng & Idler, 1983). The increase in the staining reaction for glycogen in stage IV liver suggests gluconeogenesis,to meet the energy requirement during migration and spawning as mullets usually do not feed during spawning migration (Miller, 1971). Supporting this, a peak activity of glycogen metabolizing enzyme - phosphorylase and a high concentration of glycogen have been recorded in the liver of female flounder during spawning migration (Petersen & Emmersen, 1977). Acid phosphatase activity was generally weak in hepatocytes of M. cephalus, except a slight increase in the ripe and postspawning fishes. Welsch & Storch

(1973) have reported, the lysosomes occur sparsely and the activity of lysosomal enzymes is generally weak in teleost hepatocytes. The little rise of acid phosphatase activity in stages IV and V hepatocytes is presumed to be due to the degenerative changes taking place in the exhausted liver.

CHAPTER VI

ELECTROPHORESIS OF BLOOD SERUM AND OVARY

CHAPTER VI

ELECTROPHORESIS OF BLOOD SERUM AND OVARY

Intraspecific variations in the blood serum proteins of animal species occur as a result of their genetic constitution and the influence of physiological and environmental factors which they encounter. Investigations on fish plasma or serum proteins in relation to sexual differences have been carried out using polyacrylamide, agar and starch gel electrophoresis and by immunochemical methods. Harris (1974) and Perrier et al. (1974) reported the presence of a dense-staining new component in the serum of mature lamprey and some teleosts. Vanstone & Ho (1961) identified a slow migrating fraction called "serum vitellin" in the serum of maturing female coho salmon (O. kisutch) which was absent in the plasma of males, immature, spawning and spent females. Krauel & Ridgway (1963) using immunodiffusion method, detected a complex antigen called as "Sm antigen" in the sera of maturing female red salmon (O. nerka),while Utter & Ridgway (1967) reported the existence of a protein known as "HM factor" in the sera of maturing females and estrogen treated males of a few teleosts. Aida et al. (1973a) named similar protein bands in the maturing female Ayu (Plecoglossus altivelis) as "female specific plasma proteins" (FSPP).

Recently the term "vitellogenin" (Vtg), coined by Pan et al. (1969) in insects, has become the generally accepted functional name for the female specific serum protein(s) in fishes and other oviparous vertebrates,

which is regarded as the immediate precursor of egg yolk proteins. Isolation and characterisation of Vtg and estimation of its level in the serum of teleosts were carried out by Emmersen & Emmersen (1976), Emmersen & Petersen (1976), Hara (1976, 1987), Shackley & King (1978), Emmersen et al. (1979), LeMenn (1979), Masurekar & Pai (1979), Tata & Smith (1979), De Vlaming et al. (1980), Nath & Sundararaj (1981), Hara et al. (1980, 1983, 1987), Nunomura et al. (1983), Selman & Wallace (1983), Ng & Idler (1983), Tinsley (1985), Ando (1986a) and Copeland et al. (1986). Gordon et al. (1984) identified a female specific protein (Vg), using immunochemical methods in the skin mucus of premature coho salmon, while Matsubara et al. (1985) purified a coelomic fluid specific protein (CFSP) from ovulated Oncorhynchus keta.

Many investigators have attempted to study the changes occurring in the protein patterns of teleost ovary during its development and to identify, purify and characterize the vitellogenin – derived yolk proteins (lipovitellin and phosvitin) in a variety of fishes (Mano & Lipmann, 1966; Jared & Wallace 1968; Market & Vanstone, 1971; Shackley & King, 1978; DeVlaming et al., 1980; Hara et al., 1980, 1987; Mukhopadhyay & Bose, 1982; Naurial & Singh, 1985; Shigeura & Haschemeyer, 1985; Wallace & Begovac, 1985; Wallace & Selman, 1985; Ando, 1986; Greeley et al., 1986, 1987; Hara, 1987 and Riazi et al., 1988).

Electrophoretic studies carried out in Mugilidae deal only with biochemical systematics, genetics and isozyme patterns (Sulya et al., 1961; Hongskul, 1970; Senkevich & Kulikova, 1970; Peterson & Shehadeh, 1971a,b;

Herzberg & Pasteur, 1975; Reddy, 1977; Parag, 1984; Mary Mathews, 1985; Ravi, 1986; Vijayakumar (personal communication). The identification and characterization of vitellogenin and yolk proteins of mullets have not been done yet. In view of the close relationship of fish Vtg with egg yolk formation, an attempt has been made in the present study to observe and record the changes in protein patterns of blood serum and ovary of M. cephalus during various maturity stages.

RESULTS

A. BLOOD SERUM (Table-15, Fig. 11)

General Proteins

A total of 21 protein bands, their R_f values ranging from 0.005 to 0.956 with varying thickness and staining intensity were detected in the blood serum of female fish. In the serum of stage II fish, a strong band (No.17) having a low R_f value (0.118) appeared in zone I of the gel. This broad and intensely staining band, which was more prominent in stage III, became diffused in the ripe (stage IV) condition. No band having the same R_f value and staining intensity was detected in the sera of fish belonging to stages I and V. Other protein fractions however did not show any significant variation during different maturity stages.

Glyco-, Lipo- and Calcium-binding proteins (Fig. 12)

The serum contained seven glycoprotein bands, all of them having the same R_f value as that of general proteins (Nos. 4, 7, 8, 9, 10, 17 and 21). Similarly seven fractions had lipoprotein character (Nos. 4, 7,

TABLE 15: Number of protein fractions, their relative fraction (R_f) values and staining intensities in the blood serum of female Mugil cephalus L. during different maturity stages.

Zone	Band No.	R_f Value	MATURITY STAGES																			
			STAGE I				STAGE II				STAGE III				STAGE IV				STAGE V			
			General proteins	Glyco-proteins	Lipo-proteins	Calcium bind.proteins	General proteins	Glyco-proteins	Lipo-proteins	Calcium bind.proteins	General proteins	Glyco-proteins	Lipo-proteins	Calcium bind.proteins	General proteins	Glyco-proteins	Lipo-proteins	Calcium bind.proteins	General proteins	Glyco-proteins	Lipo-proteins	Calcium bind.proteins
ZONE III (6 - 11 cm)	1	0.956	++				++				++				++				++			
	2	0.909	+				+				+				+				+			
	3	0.873	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-
	4	0.800	++++	++	++	++	++++	++	++	++	++++	++	++	++	++++	++	++	++	++++	++	++	++
	5	0.727	+				+				+				+				+			
	6	0.682	++	-			++	-	-	-	++		-	-	++	-	-	-	++	-	-	-
	7	0.618	++++	++	++	++	++++	++	++	++	++++	++	++	++	++++	++	++	++	++++	++	++	++
ZONE II (3 - 6 cm)	8	0.545	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+		
	9	0.455	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
	10	0.382	++++	++++			++++	++++			++++	++++			++++	++++			++++	++++		
	11	0.327	+				+				+				+				+			
	12	0.300	+		-	-	+				+		-		+		-		+			
	13	0.272	++++		++	++	++++		++	++	++++		++	++	++++		++	++	++++		++	++
	14	0.236	+				+				+				+				+			
ZONE I (0 - 3 cm)	15	0.200	+		-		+		-		+		-		+		-		+		-	
	16	0.163	++++		++		++++	-	++	-	++++	-	++		++++		++	-	++++		++	
	17	0.118	-				++++	++++	++++	++++	++++	++++	++++	++++	++	++	++	++	-			
	18	0.065	+				+				+				+				+			
	19	0.036	+				+				+				+				+			
	20	0.018	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-		
	21	0.005	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++

\+ Narrow and sharp bands

++ Broad and diffuse bands

+++ Broad and lightly-stained bands

++++ Broad and intensely-stained bands

Absence of bands

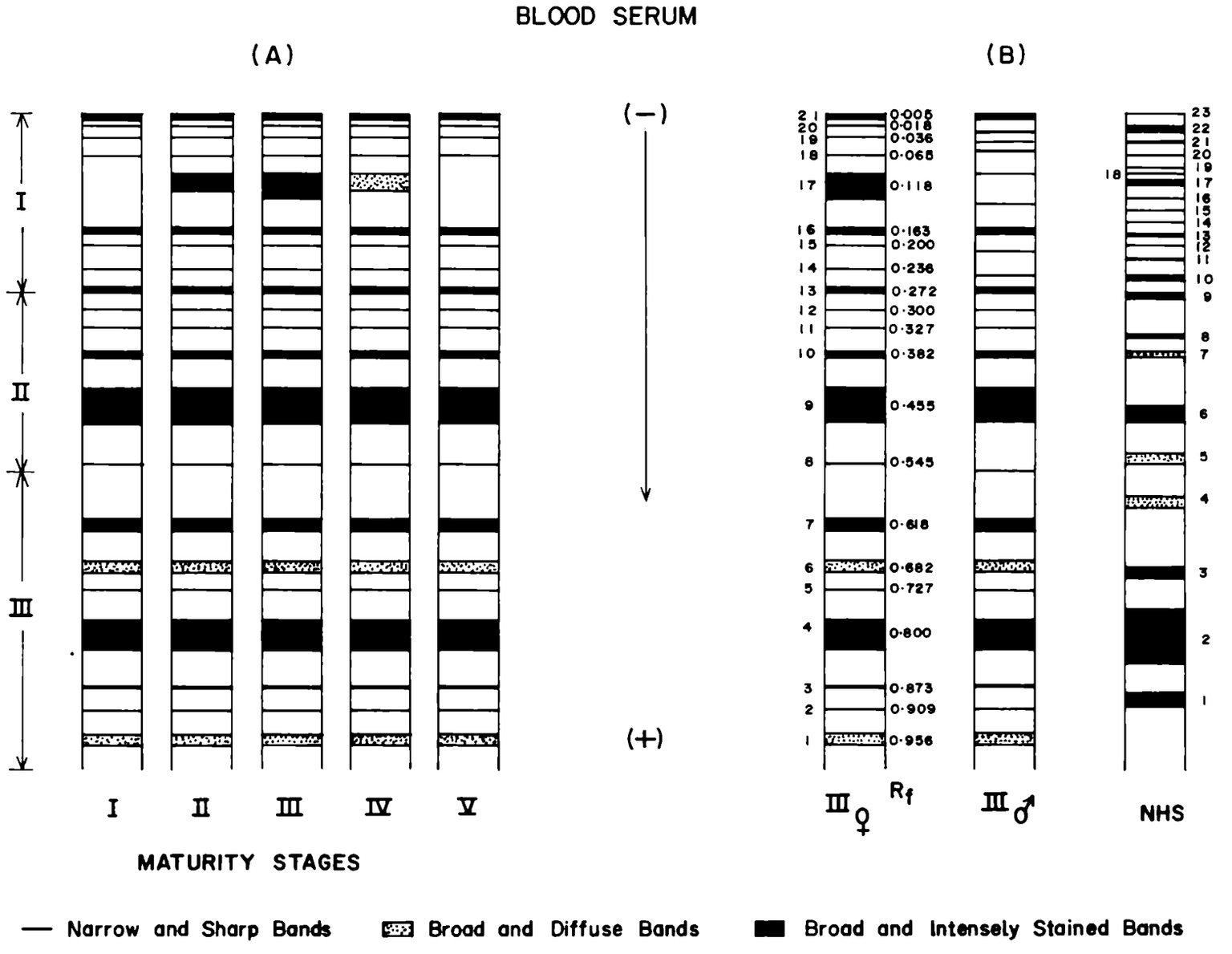


Fig. 11 A - Comparative electropherograms of blood serum protein patterns at different maturity stages of female Mugil cephalus L.

B - Blood serum protein patterns of mature female and male Mugil cephalus compared with normal human serum (NHS).

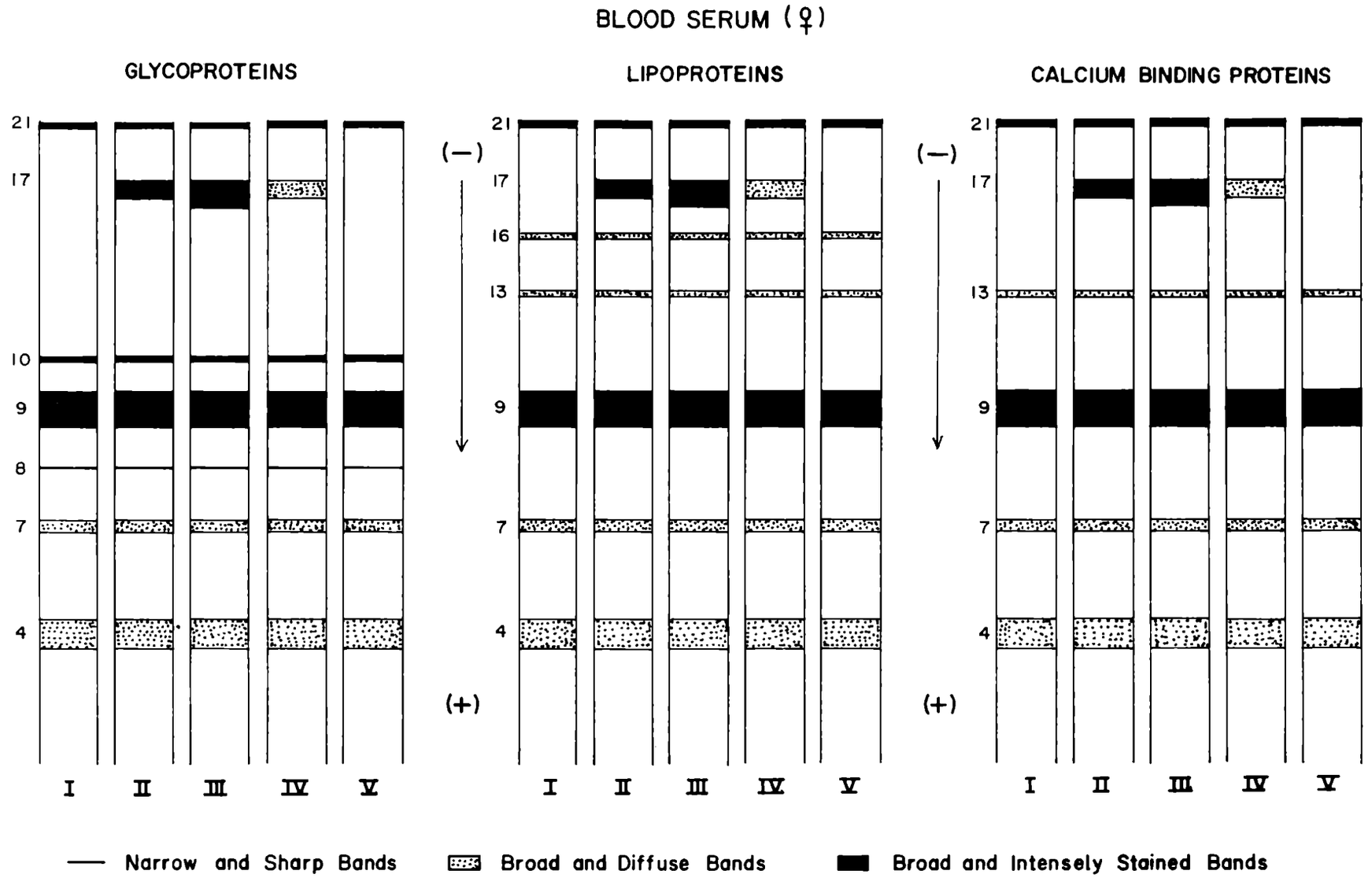


Fig. 12 Comparative electropherograms of glyco, lipo and calcium binding protein patterns in the blood serum during different maturity stages of female Mugil cephalus L.

9, 13, 16, 17 and 21) and six fractions stained for calcium (Nos.4, 7, 9, 13, 17 and 21). Therefore, the bands 4, 7, 9, 17 and 21 have been considered as calcium containing glycolipoprotein complexes.

Fraction 17 showed an intense reaction for glycoproteins, lipoproteins and calcium in stages II and III, while in stage IV, the band became diffuse and only a faint reaction was observed.

Iron binding proteins

Iron binding protein fraction(s) could not be detected with Perl's method, as destaining of the gel was never attained in various solvents.

Sexual differences in serum protein patterns (Fig. 11)

The serum of maturing and mature female mullet showed a strongly staining component in zone I of gel (No.17, R_f value 0.118) where as in mature males no such component was observed in that region.

Albumin and Prealbumin bands (Fig. 11 & 12)

Irrespective of sex and maturity stage, the mullet serum contained four fast moving fractions in the albumin zone (Nos. 1, 2, 3 and 4 in zone III of gel). One intensely staining thick band (No.4) among these, took lipid, glycoprotein and calcium stains also. After comparing with the corresponding bands in normal human serum (run in similar conditions as that of mullet serum) these bands may be considered as albumin or albumin like proteins and pre-albumins.

B. **OVARY** (Table-16; fig.13)

General Proteins

A comparison of the pattern of proteins of ovary showed 18 bands at different stages of maturity (R_f value range from 0.00 to 0.842), which differed in thickness and staining intensity. In stage II, two broad and diffusely staining new bands (No. 13 & 15) were detected in the cathodal (zone I) region of gel, which became very distinct and intensely staining in stages III and IV. These bands, having an R_f value of 0.175 and 0.100 respectively, disappeared totally in stage V (spent).

Glyco-, Lipo- and Calcium-binding proteins (Table-16; Fig. 14).

The fractions that stained for glyco-and lipoproteins and calcium showed the same R_f values as that of general protein bands. The ovary contained eight glycoprotein bands (Nos. 3, 4, 6, 13, 14, 15, 16 and 18) and six bands showing lipoprotein character (Nos. 3, 8, 9, 13, 15 and 18). Similarly eight bands took stain for calcium (Nos. 3, 8, 9, 13, 15, 16, 17 and 18). So the bands 3, 13, 15 and 18 have been considered as calcium containing glycolipoprotein complexes.

In stage II, fraction No. 13 and 15 showed a weak reaction, but the staining intensity of these bands became intense in stages III and IV. Similar bands were not detected in stages I and V.

Iron containing proteins

Due to the failure in the destaining of gels in Perl's method,

TABLE 16: Number of protein fractions, their relative fraction (R_f) values and staining intensities in the ovary of Mugil cephalus L. during different maturity stages.

Zone	Band No.	R_f Value	OVARY STAGES																			
			STAGE I				STAGE II				STAGE III				STAGE IV				STAGE V			
			General proteins	Glyco-proteins	Lipo-proteins	Calcium bind.proteins	General proteins	Glyco-proteins	Lipo-proteins	Calcium bind.proteins	General proteins	Glyco-proteins	Lipo-proteins	Calcium bind.proteins	General proteins	Glyco-proteins	Lipo-proteins	Calcium bind.proteins	General proteins	Glyco-proteins	Lipo-proteins	Calcium bind.proteins
ZONE III (6 - 11 cm)	1	0.842	++				++				++				++				++			
	2	0.742	++	-		-	++	-	-	-	++	-	-		++	-		-	++	-		
	3	0.675	++++	++++	++	++	++++	++++	++	++	++++	++++	++	++	++++	++++	++	++	++++	++++	++	++
	4	0.625	++	+++			++	+++			++	+++			++	+++			++	+++		
	5	0.567	+				+				+				+				+			
ZONE II (3 - 6 cm)	6	0.475	++	++++			++	++++			++	++++			++	++++			++	++++		
	7	0.417	+		-		+		-	-	+		-	-	+		-	-	+			
	8	0.383	+++		++	++	+++		++	++	+++		++	++	+++		++	++	++		++	++
	9	0.334	+++		++	++	+++		++	++	+++		++	++	+++		++	++	++		++	++
	10	0.300	+				+				+				+				+			
ZONE I (0 - 3 cm)	11	0.217	+				+				+				+				+			
	12	0.200	++++				++++			-	++++	-	-		++++	-		-	++++	-		
	13	0.175	-				++	++	++	+++	++++	++++	++++	++++	++++	++++	++++	++++	-	-		
	14	0.133	+	+			+	+	-	-	+	+	-	-	+	+		-	+	+		
	15	0.100	-	-			++	++	++	+++	++++	++++	++++	++++	++++	++++	++++	++++	-	-		
	16	0.067	+	+		+	+	+		+	+	+		+	+	+		+	+	+		+
	17	0.033	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+
	18	0.000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

\+ Narrow and sharp bands

\++ Broad and diffuse bands

\+++ Broad and lightly-stained bands

\++++ Broad and intensely-stained bands

Absence of bands

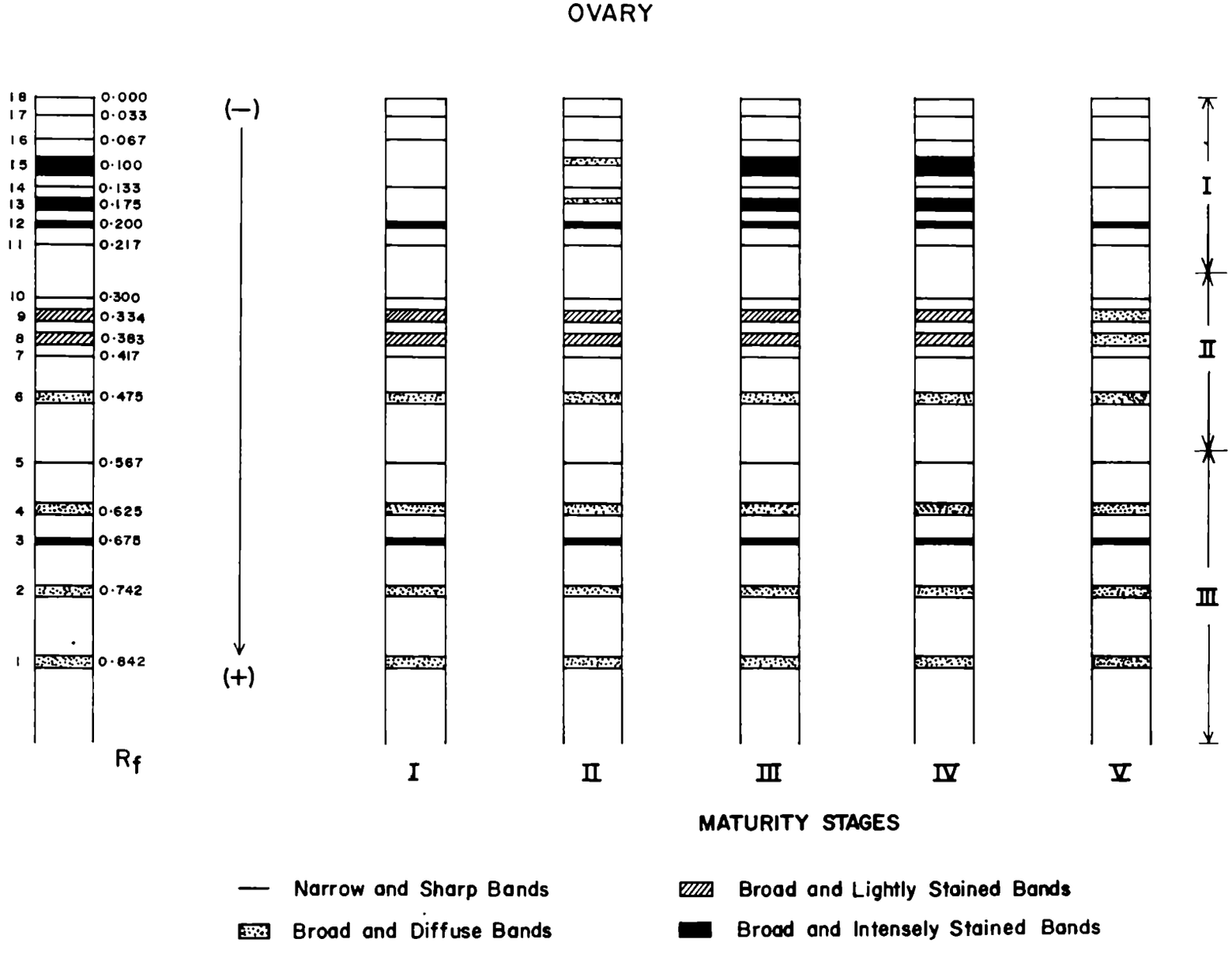


Fig. 13 Comparative electropherograms of ovary protein patterns at different stages of maturity in Mugil cephalus L.

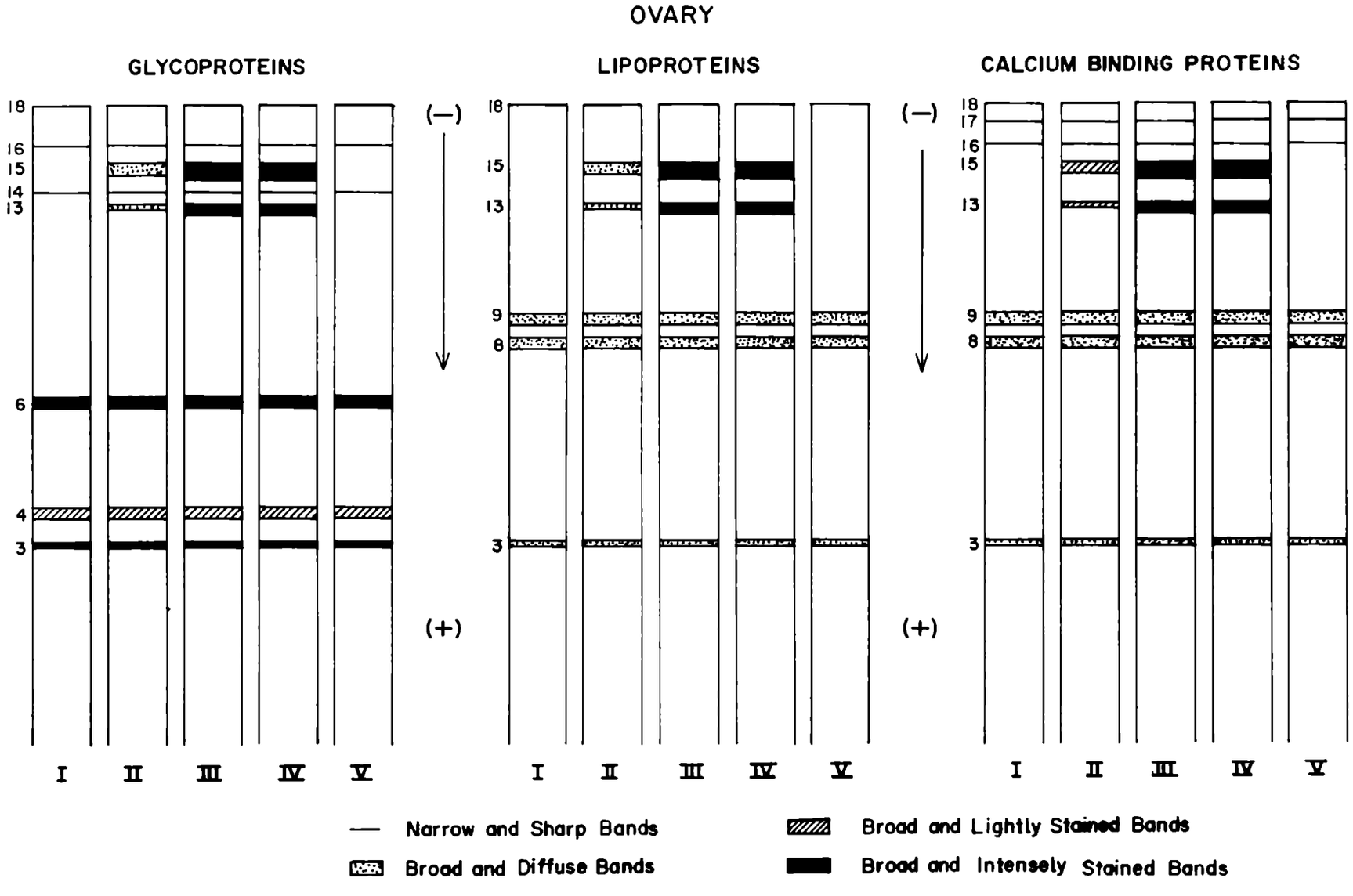


Fig. 14 Comparative electropherograms of glyco, lipo and calcium binding protein patterns in the ovary during different maturity stages of Mugil cephalus L

iron binding protein fraction(s) could not be detected.

DISCUSSION

Studies on the protein vitellogenin (Vtg) in oviparous vertebrates have revealed some of its characteristic properties, such as (1) Vtg appears in the serum of the female during vitellogenesis, (2) can be induced in male and immature female sera by the administration of estrogen and or a pituitary extract, (3) it has a characteristic capability of binding calcium, iron, copper and magnesium as a glycolipophosphoprotein complex, (4) it may be a possible precursor of egg yolk proteins - lipovitellin and phosvitin, (5) Vtg might be closely related to the transport of carotenoids (as carotenoglycolipoprotein complex) from somatic sources to developing oocytes and (6) It has a molecular weight of 350,000 - 600,000 daltons and consists of two identical polypeptide chains (Ando, 1986a; Hara, 1987).

In the present study, the prominent slow-moving protein band, which appeared in the β-globulin region of sera of maturing and mature female M. cephalus (R_f value 0.118; fraction No.17) is considered as the native Vtg, as it satisfied many of the above cited properties:

It is a female specific protein and appeared only during the period of vitellogenesis.

It contained lipid and carbohydrates as revealed by different staining reactions.

It showed calcium-binding capacity. The affinity of Vtg for calcium was proved by its intense staining reaction with Alizarin Red-'S' and the increase in total calcium levels in serum concomitant with the appearance of Vtg. The high calcium content of Vtg promotes the solubility of this protein and much of the Vtg - bound calcium may be incorporated into the oocytes to meet the needs during embryonic development. Total plasma calcium has frequently been used as an indirect index of exogenous vitellogenesis in fishes (Tinsley, 1985). Maximum levels of serum iron were also encountered when Vtg was conspicuous in the serum but, Perl's staining reaction (Pearse, 1972) may not be sensitive to the very low iron concentrations in serum (1.2-2.4 μg/ml) and ovary (10-25 μg/g) of M. cephalus. Normally, iron transport is carried out by transferrin in serum (Hara, 1987). But during yolk synthesis, Vtg plays this role too and transfers iron from somatic tissues to the developing oocytes (Hara, 1987).

This protein fraction (Vtg) in serum shows similar staining reactions (polysaccharides, lipids, protein and calcium) like the two prominent egg protein bands of vitellogenic oocytes. Using immunoelectrophoresis, autoradiography and electron microscopy, Vtg has been proved as the precursor of egg proteins in teleosts (De Vlaming et al., 1980; Copeland et al., 1986; Guraya, 1986).

Eventhough the carotenoid carrying property of Vtg could not be proved during the present study, the total serum carotenoid levels markedly increased during vitellogenesis and decreased following the disappearance of Vtg from blood. Similarly yolky oocytes showed higher levels of total carotenoids than the immature ones.

Multiple molecular weight forms of native Vtg have been reported in some teleosts (Aida, 1973a; De vlaming et al., 1980); but within the sensitivity limits of the staining, only one band was detected in the present case, as reported in Anguilla anguilla (Hara et al., 1980). More extensive studies with the aid of SDS and immunoelectrophoresis and radioimmunoassay are needed to characterize the Vtg of M. cephalus.

Electrophoresis of liver extracts is likely to show that, there is a phase during reproductive development when liver contains appreciable amounts of Vtg, while little has yet been secreted into the blood. Interpretation of such results is complicated by the presence of blood in the liver homogenate, especially as the liver is highly vascularized. But livers of teleosts are reported not to store Vtg, but rather secrete it rapidly after synthesis (Idler & Campbell, 1980; Copeland et al., 1986). The electron micrographs prepared during the present observation did not reveal any Vtg granules in the hepatocytes, thus supporting the view mentioned above.

Sulya et al. (1961), Perrier et al. (1974) and Hara (1987) have observed analbuminemia in some teleosts and elasmobranchs and opined that the reason for the absence of albumin fractions in serum is unknown.

However, Harris (1974) and Masurekar & Pai (1979) showed the blood sera of two cyprinid fishes, L. leuciscus and Cyprinus carpio respectively contained albumin and prealbumin fractions. The largest and fast moving fraction in the present study (No.4; R_f value 0.80) may be considered as the albumin band of M. cephalus, as it has a corresponding mobility and staining intensity with the established albumin component in the human serum electropherogram (No.2; R_f value 0.782). The other three less prominent but faster fractions (No.1, 2 & 3) are presumed to be the prealbumins.

It has been suggested that teleost Vtg(s) are selectively sequestered from the serum by the developing oocytes and proteolytically cleaved at alternate and/or more numerous sites during their conversion to different smaller yolk protein fractions (De Vlaming, et al., 1980). The constituents of egg yolk include the lipovitellin, characterized as a lipoprotein, containing very low amounts of protein phosphorus (molecular weight 200,000 to 300,000, having two subunits ranging Mr 100,000 - 110,000 and 19,000-25,000 daltons); phosvitin, a highly phosphorylated acidic protein with large amounts (more than 50%) serine residues (Mr 10,000-43,000); and a β component (mol. wt. 20,000-30,000), which is a simple serum protein (Guraya, 1986; Riazi et al., 1988).

In the vitellogenic oocytes of M. cephalus, proteins as large as native Vtg were not observed. Instead, two slow-migrating prominent yolk protein bands were detected in the zone III of the gel, which were glycolipoproteins and which showed affinity for calcium (No. 13 & 15; R_f values 0.175 & 0.100 respectively). These fractions can be considered as the

two components of lipovitellin. Greeley et al. (1987) recorded the two large lipovitellin subunits (Mr 104,000 & 90,000) to be the major yolk proteins in the oocytes of grey mullet.

Phosvitin and β component bands could not be detected in the present investigation. Phosvitins are very less concentrated or sometimes even completely absent from the yolk preparations of some marine fish eggs (Ng & Idler, 1983; Guraya, 1986). If present in egg extracts, they stain poorly with Coomassie blue or Kenacid blue (Greeley et al., 1986). In marine pelagic eggs, traces of phosvitin may provide energy for oocyte hydration and the developing eggs are likely to obtain their required phosphorus for metabolic purpose from the environment (Ng & Idler, 1983; Craik & Harvey, 1984a). The β component occurs in the egg extracts of only very few freshwater teleosts like trouts (Guraya, 1986).

The precise mechanism of how Vtg can be taken into oocytes via. the associated follicle epithelium is still unknown. In order to elucidate this, further studies on some special proteins or enzymes which can act as a receptor of Vtg are necessary. Otherwise, it is quite difficult to explain why and how such amounts of Vtg (or its related egg yolk proteins). can accumulate specifically in the oocyte.

CHAPTER VII

BIOCHEMICAL CHANGES DURING OOGENESIS

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Biochemical composition of fishes which is an indicator of their nutritive value is subject to variations depending on season, food intake, breeding and migration. Attainment of gonadal maturity marks a change in the growth pattern of fish, resulting from the "reproductive drain" of materials meant for somatic growth to the gonads. Proximate composition analysis of fish during gonadal development can also disclose the energy banks of various nutrients in the body and trace out the pathway through which they are mobilized to the gonad. From aquaculture point of view, a thorough understanding of the biochemical composition of the parent fish keeps us informed about their suitability to face the natural phenomenon of procreation and that of eggs helps us to assess their quality and the ensuing condition of the emerging young ones.

Variations in the biochemical composition of different tissues of teleosts during pre-spawning, spawning and post-spawning periods have been observed with special reference to moisture, protein, lipid, carbohydrates cholesterol, ash, carotenoids, DNA, RNA, protein bound phosphorus, gonadotropins and gonadal steroids by Idler & Bitners (1958, 1959), Venugopalan (1962), Loginova (1967), Crozier (1970), Love (1970, 1980) Tripathi & Meur (1972), Varghese (1976), Emmersen & Emmersen (1976), Petersen & Emmersen (1977), Larsson & Fange (1977), Kapur & Toor (1978), Medford & Mackay

(1978), Delahunty & DeVlaming (1980a,b), Hille (1982), Mikhail et al. (1982), Miki et al. (1982, 1983) Craik & Harvey (1984a,b,c,d), Hardy et al. (1984), Craik (1985), Ando (1986a,b), Ando & Hatano (1986), Diwan & Krishnan (1986), Black & Skinner (1987), Das et al. (1987), Cornish & Veith (1987), Kobayashi et al. (1988) and Das & Satpathy (1989). The tactics and energetics of reproduction in teleosts have been dealt by Love (1970, 1980), Iles (1974), Reay (1984) and Wootton (1985).

Metals such as calcium, magnesium, iron and copper play a prominent role in vitellogenesis. Teleost vitellogenin shows a strong calcium and iron binding activity and much of the vitellogenin – bound metals is incorporated into oocytes and reserved for embryogeny. Total plasma calcium and phosphoprotein levels are compared as indirect indices of exogenous vitellogenesis in teleosts (Tinsley, 1985). An increase in the metal levels in serum and gonads, concomitant with maturation has been reported in many teleosts by Sulya et al. (1960), Robertson et al. (1961), Oguri & Takada (1967), Yamane & Yamada (1977), Fletcher & King (1978), Sharma & Saxena (1979), Shackley et al. (1981), Craik & Harvey (1984a,b,c), Hardy et al. (1984), Tinsley (1985), Bjornsson & Deftos (1985), Bjornsson & Haux (1985), Bjornsson et al. (1986).

Despite the voluminous literature that have accumulated on the proximate composition of various groups of fishes, contributions on similar aspects in mullets are few except that of processed roe and fillets. Protein, fat water and ash contents were established in M. cephalus, Liza dumerili Liza ramada, Liza richardsoni, Liza saliens and Liza tricuspidens

by El-Saby (1934), Jowett & Davies (1938) Marais & Erasmus (1977), Haschemeyer et al. (1979) and Thomas (1984). Dwivedi & Menezes Maria (1975) observed variations in serum protein levels in M. cephalus and M. parsia, while Starr et al. (1957), Sulya et al. (1960), Blanc-Livni & Abraham (1970), Cameron (1970), Spieler et al. (1976), Peterson & Shehadeh (1971a), Nordlie & Whittier (1983) and Fouchreau-Peron et al. (1987) have discussed changes in serum components of mullets in different environments. The physico-chemical properties of ripe, unspawned eggs of Rhinomugil corsula were analysed by Hasan & Jafri (1964), while water and ion balance in the hydrating oocytes of M. cephalus was studied by Watanabe & Kuo (1986). But, studies relating the body composition with that of spawning habits of mullets are not much accomplished. The only results obtained in this line are those of Miller (1971) and Dindo & McGregor (1981) in the serum of M. cephalus, Kuo (quoted by Nash & Shehadeh, 1980) in the eggs of M. cephalus; Elizabeth (1987) in male M. cephalus and Muthukaruppan (1987) in Liza parsia.

In the present study, the biochemicalcomposition of four tissues viz., muscle, liver, ovary and blood serum of M. cephalus has been studied. Seven major parameters such as the moisture, proteins, lipids, carbohydrates, cholesterol, carotenoid and ash have been estimated in relation to the maturation of gonad. The concentration of total calcium and iron in serum and ovary has also been determined at different maturity stages.

RESULTS

PROXIMATE COMPOSITION ANALYSES

1. **Muscle** (Table-17; Fig. 15)

Moisture: Moisture content ranged between 71.32 to 79.31 mg% with the low value recorded in stage II and the high value in stage V.

Total proteins: Protein content was highest in stage II (24.38%) and the lowest in stage IV (15.99%)

Total lipids: Lipid content showed a range between 0.92% and 3.73%. The minimum value was recorded in stage II and the maximum in stage III.

Total carbohydrates: Carbohydrate concentration was comparatively less in the muscle, with a range of 0.17% in stage I to 0.38% in stage II.

Total cholesterol: Cholesterol level in the muscle did not show any significant variation. The values exhibited a narrow range of 0.43% to 0.49% The minimum level was obtained in stage I and the maximum in stages II, IV and V.

Ash: Ash content ranged betweeen 1.11% (stage V) and 1.86% (stage II) with a steady decline from the highest level to the lowest.

Total carotenoids: Carotenoid level was at its maximum in stage II (8.05 /ug/g), while the minimum value of 1.25 /ug/g was recorded in stage IV.

Table 17: Variations in biochemical composition in the **muscle** of female Mugil cephalus L.

Stages	Moisture (%)	Total Proteins(%)	Total Lipids (%)	Total Carbo-hydrates (%)	Total Cholesterol	Ash (%)	Total Carotenoids (µg/g)
Stage I	72.74 ± 3.02	19.85 ± 2.26	1.18 ± 0.23	0.17 ± 0.02	0.43 ± 0.02	1.34 ± 0.06	6.66 ± 0.23
Stage II	71.32 ± 4.23	24.38 ± 2.55	0.92 ± 0.22	0.38 ± 0.04	0.49 ± 0.03	1.86 ± 0.25	8.05 ± 0.18
Stage III	74.47 ± 2.35	16.25 ± 1.87	3.73 ± 1.34	0.22 ± 0.04	0.44 ± 0.07	1.19 ± 0.12	3.43 ± 0.12
Stage IV	78.10 ± 1.84	15.99 ± 1.68	2.34 ± 0.52	0.24 ± 0.05	0.49 ± 0.02	1.15 ± 0.01	1.25 ± 0.30
Stage V	79.31 ± 1.79	16.86 ± 1.91	1.65 ± 0.57	0.22 ± 0.03	0.49 ± 0.01	1.11 ± 0.10	1.57 ± 0.08

All values are mean ± standard deviation.

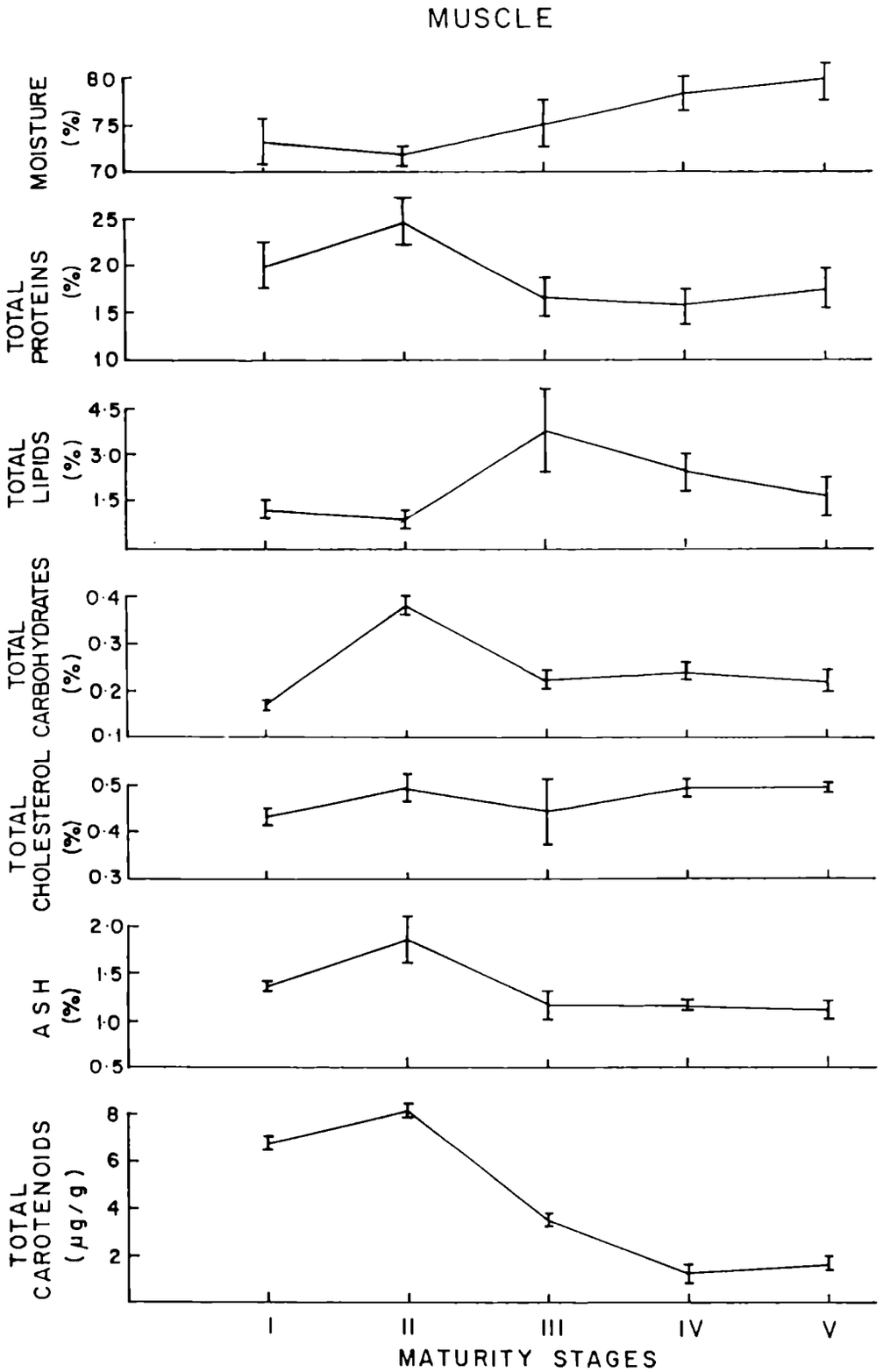

Fig. 15. Variations in biochemical composition in the muscle of female Mugil cephalus during ovarian development. Vertical lines represent standard deviations.

2. **Liver** (Table-18; Fig.16)

Moisture: Moisture content in liver ranged from 66.85% (stage III) to 74.31% (stage V). The level showed an alternate increasing and decreasing pattern.

Total proteins: The maximum concentration of protein was observed in stage III (18.88%) and the minimum in stage IV (8.87%).

Total lipids: Lipid level was maximum (10.10%) in stage II, which then gradually decreased to a minimum value of 5.32% in stage IV.

Total carbohydrates: Carbohydrate content showed its highest level in liver, of all the tissues. From the low value of 0.99% in stage III, a steep increase to the maximum value of 4.06% in stage IV was observed.

Total cholesterol: Cholesterol content showed a pattern of alternate increase and decrease. The maximum value was recorded in stage I (2.58%) and the minimum in stage II (1.20%).

Ash: Ash content had a range of 0.94% in stage IV to 2.07% in stage III.

Total carotenoids: The concentration of carotenoids in hepatic tissue was maximum in stage II (15.70 μg/g), which then gradually reduced to a minimum value of 10.70 μg/g in stage V.

3. **Ovary** (Table-19; Fig. 17)

Moisture: Moisture content was found to be highly fluctuating in the ovary. The value showed a decrease from the maximum of 76.79% in stage I

Table 18: Variations in biochemical composition in the **liver** of female Mugil cephalus L.

Stages	Moisture (%)	Total Proteins (%)	Total Lipids(%)	Total Carbo-hydrates (%)	Total Chole-sterol (%)	Ash (%)	Total Caro-tenoids (/ug/g)
Stage I	70.16 ± 4.88	16.79 ± 2.80	6.08 ± 2.01	2.15 ± 0.50	2.58 ± 0.70	1.66 ± 0.47	12.79 ± 1.67
Stage II	70.37 ± 4.10	15.59 ± 2.55	10.10 ± 1.87	1.73 ± 0.22	1.20 ± 0.18	1.13 ± 0.25	15.70 ± 4.58
Stage III	66.85 ± 2.18	18.88 ± 1.11	8.08 ± 0.70	0.99 ± 0.15	1.47 ± 0.34	2.07 ± 0.18	15.14 ± 4.42
Stage IV	72.42 ± 3.00	8.87 ± 1.36	5.32 ± 0.51	4.06 ± 0.44	1.24 ± 0.14	0.94 ± 0.05	11.70 ± 0.81
Stage V	74.31 ± 3.70	11.08 ± 1.38	7.57 ± 0.85	1.58 ± 0.19	1.36 ± 0.15	1.00 ± 0.07	10.70 ± 0.81

All values are mean ± standard deviation.

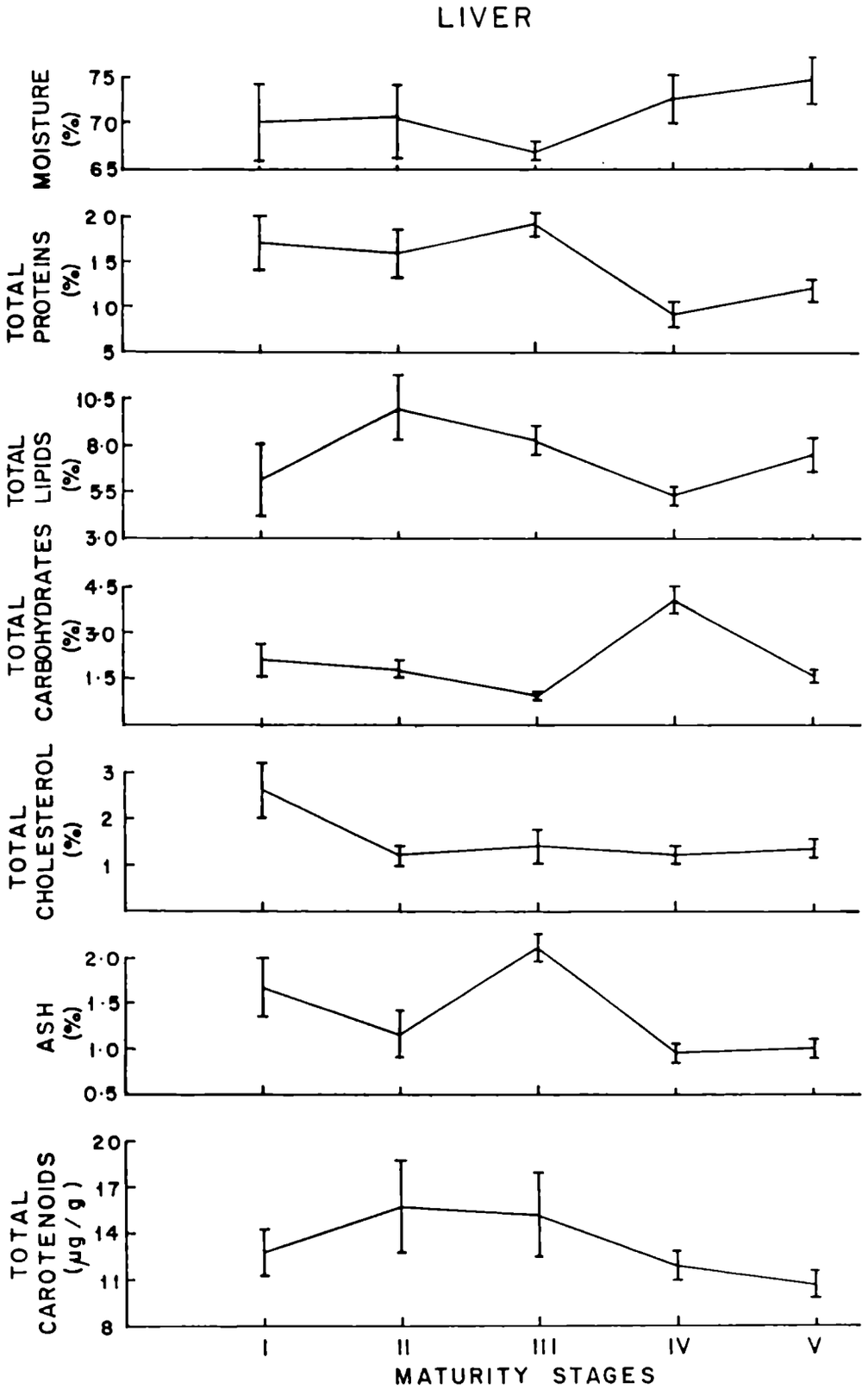

Fig. 16. Variations in biochemical composition in the liver of female Mugil cephalus during ovarian development. Vertical lines represent standard deviations.

Table 19: Variations in biochemical composition in the **ovary** of Mugil cephalus L.

Stages	Moisture (%)	Total Proteins(%)	Total Lipids (%)	Total Carbo-hydrates(%)	Total Chole-sterol (%)	Ash (%)	Total Caro-tenoids (µg/g)
Stage I	76.79 ± 4.68	14.62 ± 0.48	4.48 ± 0.65	0.40 ± 0.05	0.48 ± 0.08	1.71 ± 0.20	13.40 ± 2.05
Stage II	73.61 ± 5.70	15.71 ± 0.78	6.16 ± 1.06	0.91 ± 0.16	0.62 ± 0.10	1.93 ± 0.09	18.08 ± 5.90
Stage III	58.24 ± 4.20	23.13 ± 1.66	13.92 ± 2.49	0.74 ± 0.02	1.38 ± 0.16	2.15 ± 0.12	71.30 ± 7.81
Stage IV	63.80 ± 10.33	26.19 ± 2.47	16.05 ± 1.53	0.72 ± 0.02	1.54 ± 0.16	2.46 ± 0.10	79.00 ± 6.16
Stage V	73.99 ± 2.93	15.66 ± 1.37	6.19 ± 1.19	0.58 ± 0.19	0.67 ± 0.12	1.80 ± 0.35	20.43 ± 4.76

All values are mean ± standard deviation.

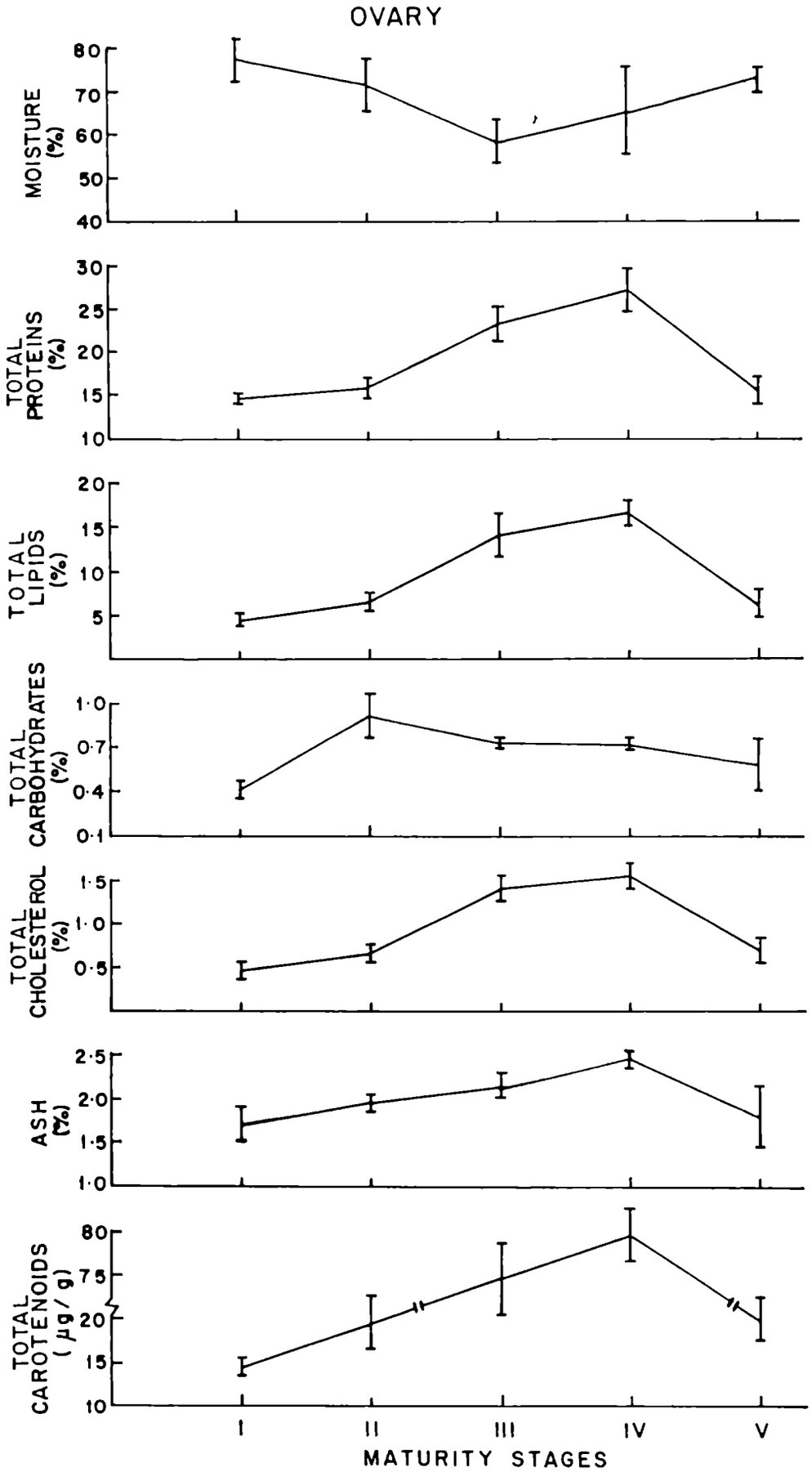

Fig. 17. Variations in biochemical composition in the ovary of Mugil cephalus at different maturity stages. Vertical lines represent standard deviations.

to a minimum of 58.24% in stage III

Total proteins: The range of protein concentration was 14.62% to 26.19% with the maximum value in stage IV and the minimum in stage I.

Total lipids: Following the same pattern of proteins, the lipid level in gonad showed an increase from the minimum value of 4.48% in stage I to a maximum of 16.05% in stage IV and the rate of increase was remarkably high.

Total carbohydrates: Carbohydrate content was maximum in stage II (0.91%) and the minimum in stage I (0.40%).

Total cholesterol: Cholesterol level indicated a constant increase from 0.48% in stage I to 1.54% in stage IV.

Ash: a steady increase in ash content was observed from stage I (1.71%) to stage IV (2.46%), followed by a decrease in stage V (1.80%).

Total carotenoids: A remarkable increase was observed in the carotenoid content from 13.40 /ug/g in stage I to 79.00 /ug/g in stage IV. The value dropped to 20 .43 /ug/g in stage V.

4. **Blood serum** (Table-20; Fig. 18)

Total proteins: Protein content in the serum was found to vary between 6.73 g/100 ml (stage I) and 10.08 g/100 ml (stage III).

Table 20: Variations in biochemical composition in the **blood serum** of female Mugil cephalus L.

Stages	Total Proteins (g/100 ml)	Total Lipids (g/100ml)	Total Carbohydrates (mg/100 ml)	Total Cholesterol (mg/100 ml)	Total Carotenoids (µg/ml)
Stage I	6.73 ± 0.63	0.86 ± 0.050	57.82 ± 7.42	288.00 ± 35.96	3.25 ± 0.79
Stage II	9.40 ± 0.73	1.67 ± 0.057	70.0 ± 4.99	553.69 ± 67.53	4.82 ± 0.90
Stage III	10.08 ± 0.97	1.51 ± 0.158	85.0 ± 12.34	320.65 ± 22.61	8.58 ± 2.15
Stage IV	9.24 ± 0.81	1.52 ± 0.043	60.88 ± 7.26	400.10 ± 22.15	8.20 ± 0.66
Stage V	8.09 ± 0.71	1.25 ± 0.081	51.99 ± 3.91	283.22 ± 34.21	7.38 ± 0.70

All values are mean ± standard deviation.

Calculated F Value	68.68	309.20	60.20	191.89	87.43
Remarks	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001

Table value for $F_{(4,75)}$ = 5% level, 2.49; 1% level, 3.58; 0.1% level, 5.18.

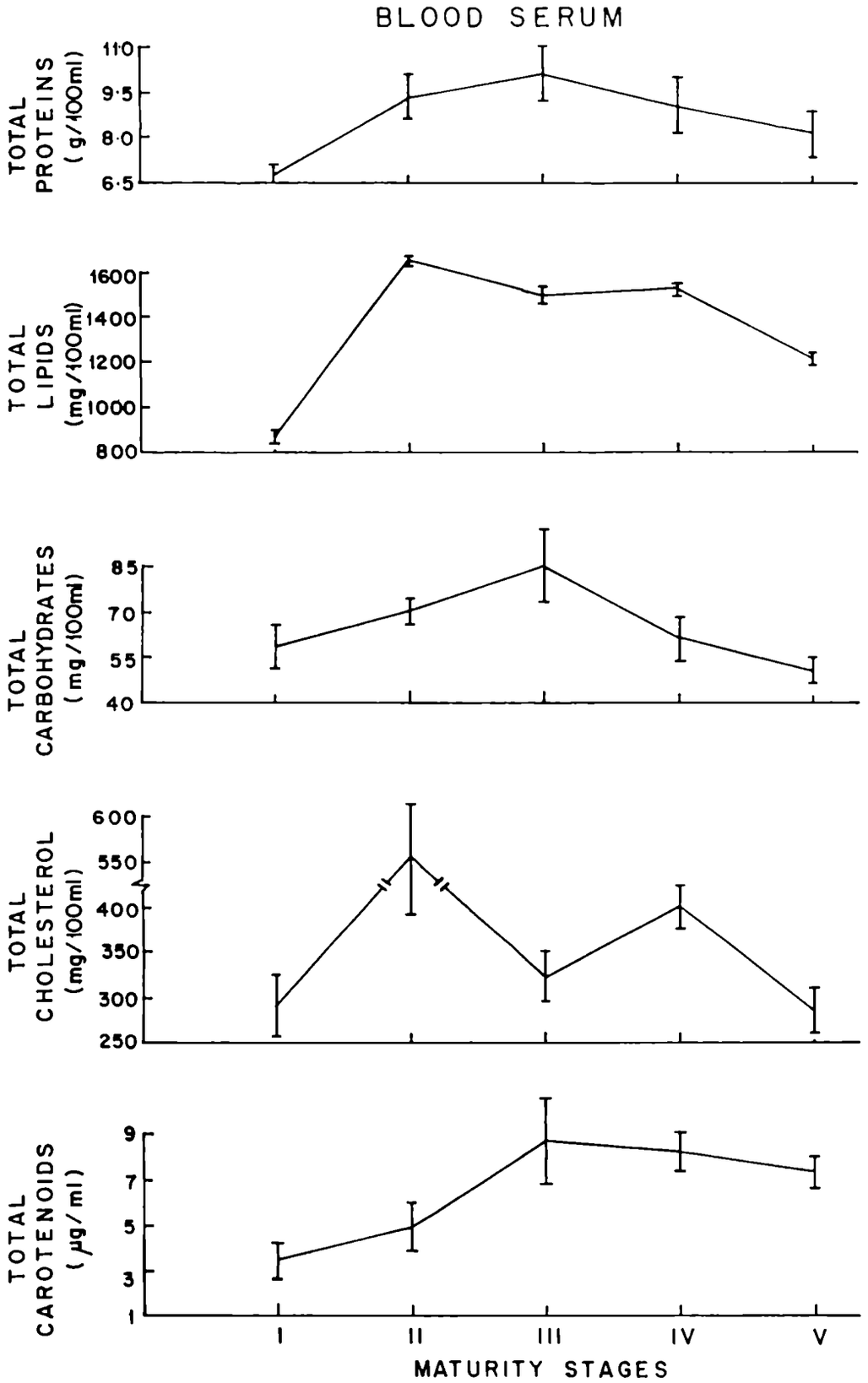

Fig. 18. Variations in biochemical composition in the blood serum of female Mugil cephalus during ovarian development. Vertical lines represent standard deviations.

Total lipids: The lipid content indicated an alternating pattern of increase and decrease in different stages. The peak value was observed in stage II (1.67 g/100 ml) and the minimum level in stage I(0.86 g/100 ml).

Total carbohydrates: Carbohydrate level in serum was maximum in stage III (85 mg/100 ml) and minimum in stage V (51.99 mg/100 ml).

Total cholesterol: Cholesterol level in blood serum followed the same pattern of serum lipids, showing an alternate increase and decrease in concentration. The maximum level of 553.69 mg/100 ml was recorded in stage II while the lowest value recorded was in stage V (283.22 mg/100 ml).

Total carotenoids: Serum carotenoid level showed an increase from the minimum value of 3.25 μg/ml in stage I to a maximum value of 8.58 μg/ml in stage III, followed by a gradual decrease to 7.38 μg/ml in the fifth stage.

Statistical analysis (Table-21)

The analysis of variance (ANOVA) was carried out for each biochemical parameter in muscle, liver and ovary to test significant changes (i) between different tissues at various stages of maturity and (ii) between different stages of maturity in various tissues. Since the interaction between the two sources of variation (tissue and stage) was significant at 0.1% level, the main effects were tested against interaction. The results indicate significant variation of all parameters between tissues at 5% level and of carbohydrate at 1% level. But between stages, only protein, lipid and carotenoid showed significant variation at 5% level.

Table 21: Result of the Analysis of Variance of biochemical parameters between maturity stages and tissues in Mugil cephalus L.

Parameters	Source of variation	Calculated F values	Remarks
Moisture	Between Tissues Between Stages Interaction	4.65 1.04 50.41	$P < 0.05$ NS $P < 0.001$
Protein	Between Tissues Between Stages Interaction	4.86 5.72 115.79	$P < 0.05$ $P < 0.05$ $P < 0.001$
Lipid	Between Tissues Between Stages Interaction	6.95 3.93 136.76	$P < 0.05$ $P < 0.05$ $P < 0.001$
Carbo-hydrate	Between Tissues Between Stages Interaction	10.648 0.958 210.540	$P < 0.01$ NS $P < 0.001$
Cholesterol	Between Tissues Between Stages Interaction	5.90 0.395 82.35	$P < 0.05$ NS $P < 0.001$
Ash	Between Tissues Between Stages Interaction	4.79 0.921 25.15	$P < 0.05$ NS $P < 0.001$
Carotenoid	Between Tissues Between Stages Interaction	4.948 3.87 352.13	$P < 0.05$ $P < 0.05$ $P < 0.001$

NS Not significiant.

Degrees of freedom (df)for tissues = 2; stages = 4 and interaction = 8.

Table value for F: $F_{(2,8)}$ = 5% level, 4.46; 1% level, 8.65; 0.1% level, 18.49.

$F_{(4,8)}$ = 5% level, 3.84; 1% level, 7.01; 0.1% level, 14.39.

The variation in the composition of blood serum at different stages of maturity was investigated by a one-way analysis of variance, applied separately for each biochemical component. The result showed significant variation of all parameters ($P < 0.001$) between different maturity stages.

ESTIMATION OF METALS (Calcium & Iron) (Table-22; Fig.19)

Blood serum

Calcium and iron in blood serum exhibited a similar trend in variation. The minimum value for total calcium in serum was recorded as 95 /ug/ml in stage I and the maximum of 246 /ug/ml in stage III. Serum iron reached its peak level of 2.41 /ug/ml in the third stage, while in stage I, a minimum value of 1.20 /ug/ml was recorded.

2. **Ovary**

Total calcium content in the ovary showed a gradual increase from 951 /ug/g (dry weight) in stage I to 2135 /ug/g (dry weight) in fourth stage. Iron concentration in the ovary followed the same pathway of calcium. The level showed a range from 10.5 /ug/g (dry weight) in stage I to 24.8 /ug/g (dry weight) in stage IV.

STATISTICAL ANALYSIS (Table-22)

One-way analysis of variance, applied separately for the two metals in blood serum and ovary indicate significant variation of these elements at 0.1% level between different maturity stages in both the tissues.

Table 22: Variations in total calcium and iron contents in the blood serum and ovary of Mugil cephalus at different stages of maturity.

Stages	BLOOD SERUM		OVARY	
	Total Calcium (µg/ml)	Total Iron (µg/ml)	Total Calcium (µg/g dry weight)	Total Iron (µg/g dry weight)
Stage I	95.0 ± 8.20	1.20 ± 0.34	951.0 ± 18.41	10.5 ± 0.50
Stage II	133.0 ± 10.17	1.84 ± 0.51	1443.5 ± 21.6	15.5 ± 2.50
Stage III	246.0 ± 16.20	2.41 ± 0.20	1889.0 ± 38.5	20.75 ± 1.75
Stage IV	235.0 ± 20.22	2.37 ± 0.14	2135.0 ± 30.2	24.8 ± 1.50
Stage V	129.0 ± 6.37	1.57 ± 0.48	1059.0 ± 21.04	18.25 ± 3.20

Each entry is mean ± standard deviation.

Calculated F value	329.64	20.71	4303.86	80.97
Remarks	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.001$

Table value for $F_{(4,25)}$ = 5% level, 2.76; 1% level, 4.18; 0.01% level, 6.49 and $T_{(25)}$ (5%) = 1.708.

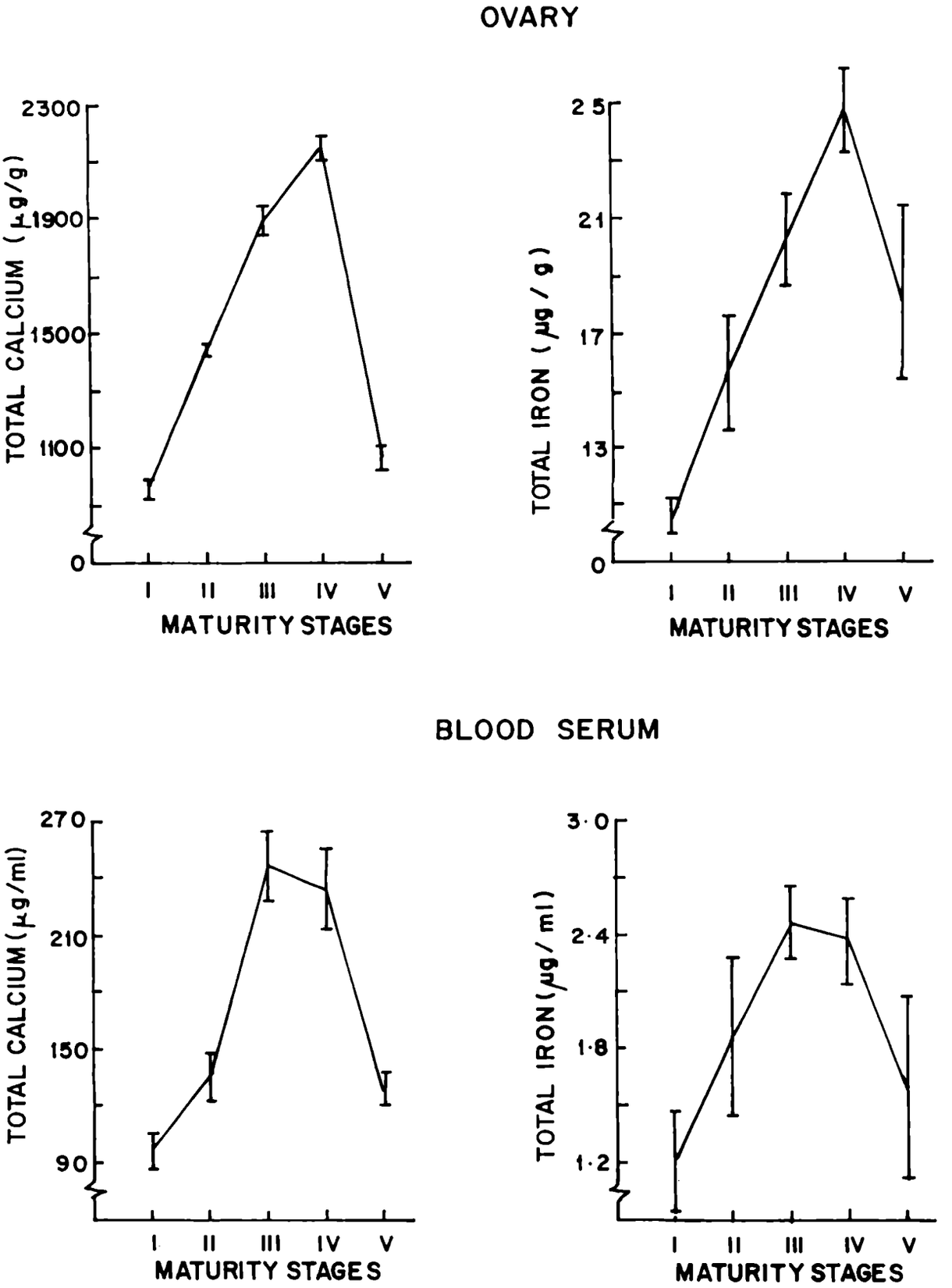

Fig. 19. Variations in calcium and iron contents in the ovary (dry weight) and blood serum in Mugil cephalus at different stages of gonadal development. Vertical lines indicate standard deviations.

DISCUSSION

Reproductive function in fishes involves three important aspects, namely production of gametes, development of secondary sexual characters and exhibition of reproductive behaviour (Miller, 1984). Studies are available on the endogenous factors that control activation of gametogenesis and on exogenous factors which actually determine when the endogenous factors become functional (Wootton, 1985). Maturation of gonads in fishes is accompanied by profound changes in the chemistry of fish, due to the translocation of material and energy mainly from the somatic sources (e.g. muscle, liver) to gonads with an overall purpose of making the eggs self-sufficient. The spawning cycle in teleosts has been demonstrated to evince major alterations in the blood chemistry also (Peterson & Shehadeh, 1971a).

Moisture

Moisture plays a decisive role in several biochemical functions, in view of its ionic properties and its association with other constituents. Its fluctuation in the body tissues, besides being influenced by environmental factors, osmotic properties of the cells and other profound physiological activities serves as an indicator of accumulation or decline of metabolic components in tissues or cells (Marais & Erasmus, 1977; Elizabeth, 1987). Studies on the proximate composition, conducted in the past have indicated that about 75-80% of body weight of mullets is formed of water (Mukundan et al., 1981; Elizabeth, 1987; Gooch et al., 1987; Muthukaruppan, 1987). The moisture content in the body muscles of M. cephalus, observed during

the present study was also between 72-80%.

The moisture level in muscle tissue of M. cephalus evinced an initial decrease from Stage I to the stage II, thereafter increased steadily in the forthcoming stages. This increase from the third stage as well as the simultaneous decrease of protein, carbohydrate, lipid and carotenoid components in the muscle and the rise of these in the ovaries indicate the depletion of these components from the muscle and their replacement by water. This pattern of variation also show that, translocation of components in the muscle tissue of the species, starts only after the stage I and that until then, the body resources are actually being built up.

The moisture content in the liver tissue of M. cephalus is more or less steady upto the stage II of maturity, but in the stage III it showed a sudden decline, suggesting the building up of resources in this tissue. An increase in moisture level was noticed only after this stage, coinciding with the depletion of other biochemical components from the liver and building up of the generative tissues in the gonad.

Water content in the gonad showed a wider range of variation. A drastic decrease in moisture content was observed upto the stage III but an increasing trend is discernible afterwards. The rise in moisture content of gonads in the ripe stage, however, is not accompanied by any significant depletion in the chemical composition of tissues, but rather an increase in these components was observed which is in agreement with the previous findings (Kapur & Toor, 1978; Craik & Harvey, 1984a, 1987;

Greeley et al., 1986; Watanabe & Kuo, 1986; Elizabeth, 1987). In testes, the increase in water content in the final stages of maturation facilitates the formation of free running milt during spawning (Elizabeth, 1987). In marine fishes with pelagic eggs as in M. cephalus, oocyte hydration and hence a pronounced increase in the osmotic pressure and volume of eggs is an adaptation, making the eggs buoyant which leads to wide dispersal of floating eggs by water currents and this feature is related to the high fecundity of these species; the protein phosphate (phosvitins) in the yolk are utilized for the intake of water (Craik & Harvey, 1984a). The utilization of phosvitins before fertilization in the life cycle constitutes an extreme example of "heterochrony" (Craik & Harvey, 1984a). The high influx of K^+ ions into the oocytes during maturation has been suggested as the major effector causing the oocyte hydration through increasing the osmotic potential of the oocyte (Mc Pherson et al., 1989). In some teleost eggs, yolk proteolysis also contributes a share in effecting oocyte hydration (Wallace, 1985).

Total Proteins

Love (1970), Emmersen & Emmersen (1976), Medford & Mackay (1978), Masurekar & Pai (1979) and Wootton (1985) have shown that the protein synthesized and accumulated in the liver and muscle during prematuration period is utilized for gamete formation in addition to the growth of the fish. Further, Love (1970) suggested that certain aminoacids from the muscle may be mobilized for the production of sex products. Most often, the amino acids provided through food intake might not be sufficient to fulfil the requirement imposed by spawning. It has also been

found that certain essential amino acids for this purpose are not adequately supplied (Love, 1970). Love(1979) and Masurekar & Pai (1979), commenting on the increase of collagen content during spawning in fishes, have opined that much of the gonad tissue is built from protein drawn from the musculature and the amino acids which are not needed by the gonad are converted into collagen and deposited in the skin, jaw extension and in the humped back of the salmon species.

In the present study, the protein in the muscle and liver is found at relatively higher levels in immature and maturing stages. The muscle tissue indicates a sharp rise in protein level from the stage I to stage II, but in the case of liver, a slight fall in total protein content was observed in stage II, followed by an increase in the stage III. As the maturation of the gonad advanced, protein level decreased in both these tissues, while it showed a steady increase in ovaries. This pattern and distribution of protein level suggests that, protein gets accumulated in the somatic tissues in the immature and maturing stages and subsequently gets translocated to the ovaries as oogenesis progresses. The protein content of the liver in the present study appears drained in the early stages of gonad development. The increase in total proteins in the hepatic tissue in the mature stage can be attributed to the synthesis of the yolk proteins vitellogenin (Vtg) by the liver cells, which has already been proved histologically and histochemically in the present study. Striped mullet, like salmon exhibits complete abstinence from food during spawning migration (Kesteven, 1942; Erman, 1959; Miller, 1971; De Silva & Perera, 1976), due to the paucity of abdominal space and high pressure on the alimentary canal as a result

of much enlarged ripe gonads (Dasgupta & Sircar, 1987). A heavy premigratory feeding in M. cephalus has been reported by Anderson (1957) and Dindo & Mac Gregor (1981). The peak level of total muscle protein in stage II can be attributed to this voracious feeding of fish before migration.

In the spent stage, there is an obvious fall in the protein level in the gonad, while a slight increase is noted in the liver and muscle. It is surmised that the decline in the protein in gonads is due to the strains imposed by the extrusion of eggs and the still low protein levels in muscle can be due to the enormous tissue breakdown.

The total protein level in the blood serum showed a gradual increase from stage I to stage III and then declined. This observation coincides with the appearance of the yolk protein precursor (Vitellogenin-Vtg) band in zymogram of blood serum in stage II, which becomes stronger in stage III and then diminishes in the ripe stage. Vtg is glycolipophosphoprotein in nature in most of the teleosts (Hara, 1987). Hence, it is presumed that the increase in total serum proteins, noted in stages II and III is due to the presence of Vtg in blood, in these stages. Similar rise in the total serum proteins and phosphoproteins has been suggested in different teleosts (Robertson et al., 1961; Miller, 1971; Emmersen & Emmersen, 1976; Sharma & Saxena, 1979; Van Bohemen et al., 1981; Hille, 1982; Tinsley, 1985, Hara, 1987). The total plasma protein value, as suggested by Dwivedi & Maria (1975) in Mugil cephalus comes to 9.1 g% and in Mugil parsia,

8.2g%. The total protein content in blood serum of M. cephalus in the present study also falls between 6.73 to 10.08%.

Total Lipids

During gonadal maturation and spawning, the lipid material of the fish, is utilized primarily for three purposes, namely (1) as an immediate endogenous source of energy for sustaining the increased muscular activity during the spawning migration of the fish. (2) for the synthesis of generative materials (eggs and sperms) and yolk deposition, and (3) for the synthesis of steroid hormones. Fish, in contrast to mammals employ lipids rather than carbohydrates as their major energy source (Black & Skinner, 1987). The main sites of storage of lipids in the fish body are the subcutaneous connective tissue, liver, skeletal muscles, mesentry and interosseus tissue. Of these sites, the lipids from the liver has been reported to be the first to evince mobilisation during gonadal maturation in fishes (Shulman, 1974). Idler & Bitners (1959) observed that in O. nerka, 8% of body lipids was transferred to the ovary during spawning migration.

In the present study, a steady increase in the lipid content in the ovary of M. cephalus from the stage I to stage IV, with the corresponding decline in lipid levels of the liver and muscle, indicate that there is a positive correlation between the two processes. Though the results show that the muscle lipids tend to get depleted earlier than the liver lipids, the maximum drain has been from the lipid reserve of the liver, as it shows a sharp decline from 10.1% to 5.3%. The increase in total lipid content of the liver from 6.1% in the first stage to 10.1% in the

second stage is indicative of an assimilatory phase of fat depots during the preparatory stage, while, the subsequent decline could be due to its utilization for the various physiological processes related to maturation. Similar observations were also made by Elizabeth (1987) and Muthukaruppan (1987) in other mullets and Marais & Erasmus (1977) in Liza dumerili. The low muscle lipid level in spent stage in the present study indicates its utilization in spawning activity.

The lipid content of the ovary in the present study increased four times (on the basis of a per 100 g wet weight of the ovary) in the ripe condition when compared with that during the preparatory phase. Similar records were made by Das et al. (1987) in C. punctatus and Muthukaruppan (1987) in L. parsia. An inverse relationship is discernible between moisture and lipid contents in the ovary of M. cephalus in the present study.

Another significant feature observed in the lipid level in M. cephalus is the wide variation of this component among the tissues. While the gonad lipid level showed a high gradient (5-16%), a corresponding withdrawal in quantity is not reflected in other tissues; their lipid levels ranged 1-10%. The high lipid content in the ovary can be due to the endogenous lipid droplets (triglycerides) present in the oocytes of mullets in addition to the lipid rich exogenous yolk proteins. Further investigations are needed to unveil more details of lipid deposition in tissues and their translocation to gonads during gametogenesis in fish.

The accumulation of wax esters has been demonstrated in the eggs of M. cephalus (Sen & Schlenk, 1964; Iyengar & Schlenk, 1967). Wax esters form 70% of the total ovarian lipid in this fish. The source of wax esters in the ovaries of M. cephalus may be the dietary fatty acids. However Wiegand (1982, cited by Guraya, 1986) has suggested that some ovarian wax ester components may be synthesized endogenously, but the site of synthesis is unknown. Wax esters are reduced to alcohols after its uptake by the ovary. However, the significance of the storage and functions of the alcohol portion of wax esters in teleost vitellogenesis, are not yet understood.

Fishes are grouped as "fat" fishes (those store fat in muscle) and "lean" fishes (those store fat in liver) (Love, 1975). In the present study, M. cephalus with a hepatic lipid content at a range of 5-10%, which is much higher than the muscle fat (1-3%) can be included under "lean" fishes.

The lipid content of the serum seems to evince changing pattern in different stages, and this appears to relate well with the period of female gonadal enlargement and with the spawning season of the species. It is inferred that the elevated levels of serum total lipids in stage II is the result of active premigratory feeding and concomitant transport of fat to the deposition sites. The fall in total serum lipids in stage III and simultaneous increase in muscular lipid content indicate that the deposition of lipid occurs rapidly prior to migration. The increase in total lipids in the ripe fish may be explained on the basis of mobilisation of fat from depots

to the gonads. The overall deprivation of energy resources in the spent fish is reflected in the serum of M. cephalus; the total lipid in the serum at this stage is very low. The present observation agrees with those made by Miller (1971), Dindo & Mac Gregor (1981) and Elizabeth (1987) in striped mullet and L. parsia.

Total Carbohydrates

Shulman (1974) suggested fat as the main source of energy instead of carbohydrate in all cases of large scale functions in fish and much of the energy comes from gluconeogenesis. But, as carbohydrate is readily mobilized, highly reducible and liberates energy under anaerobic conditions, it can be treated as an important source of energy in many fishes. The carbohydrate stored in the form of glycogen contributes to 0.5-2% of the body composition of fish (Love, 1970). The glycogen content in various tissues of the body of fish varies with seasons and stages of gonadal maturation (Tripathi & Meur, 1972; Varghese, 1976; Petersen & Emmersen, 1977, Dargupta & Sircar, 1987).

In the present study, the total carbohydrate content with a narrow range of 0.1-0.4% in the muscle is not showing any definite trend, except a rise in stage II which is possibly due to the premigratory feeding. The level declined in the third stage and did not show further oscillation in subsequent stages. However, in the ovary of M. cephalus, the carbohydrate content was found to increase only upto stage II of maturity, after which there was a decrease. This might indicate that the depletion observed

in the muscle is not entirely for deposition in the gonad, but a part of the carbohydrate may be utilized for some other energy demanding activities such as spawning and migration. Similar observations have been made by Elizabeth (1987) in male M. cephalus and L. parsia. Tripathi & Meur (1972) suggested that glycogen is not stored, but more readily utilized in females, when the fish almost starves during the maturation phase. The liver in M. cephalus had a comparatively high carbohydrate content (0.99-4.06%) and it decreased from stage I to stage III, with a corresponding increase in its level in the blood serum, further substantiating the evidence of secretion of vitellogenin by the hepatocytes, which contains appreciable amounts of glycogen. The marked rise in hepatic carbohydrate content in stage IV observed in the present study suggests the synthesis of carbohydrates especially from other sources (gluconeogenesis) to meet the energy requirement during migration and spawning.

In the ovary of M. cephalus, a decrease in level of carbohydrates is noticeable from stage III onwards which is supported by weak histochemical reactions of oocytes. A possible explanation would be that, when fat and protein have accumulated in the gonad,the role of carbohydrates becomes negligible. The continuous decrease of this biochemical component in the ovary, even after the deposition of glycogen containing yolk proteins, also indicates that the carbohydrates already present in oocytes are utilized for metabolic activities. Similar observations are made by Kuo (quoted by Nash & Shehadeh, 1980) and Muthukaruppan (1987) in mullets.

In the blood serum of M. cephalus, the total carbohydrates increased upto the third stage, followed by a gradual decrease upto the spent

condition. The increase in the serum carbohydrate content, might be due to the active premigratory feeding behaviour of mullets. But in stage III, the highest value of serum carbohydrate observed might be as a result of the appearance of the glycogen containing Vtg in the serum. The low level of serum carbohydrates in spent stage as observed in the present study, has been reported in other teleosts also (Petersen & Emmersen, 1977).

The results of the present study thus supports the view that carbohydrates are not stored in fish, but lipids play a significant role as energy reserve in aquatic animals because the former is in very low concentrations in tissues and that its role in mobilisation of energy during maturation and spawning may be negligible.

Total Cholesterol

Cholesterol forms the most prominent sterol in the vertebrate cells, from which the common precursor for both androgens and estrogens, namely pregnenolone is synthesized. The estimate of the cholesterol content thus gives an indication of the steroidogenic activity of the cells. Cholesterol content in the fish body depends on sexual development, maturation and the dietary level (Love, 1970). Dindo & Mac Gregor (1981), studying the seasonal variation of serum total lipids and cholesterol in striped mullet, suggest their possible deposition and subsequent mobilisation associated with the reproductive cycle. In the present study, a gradual accumulation of cholesterol in the ovary was obseerved from stage I

through stage IV, followed by a decrease in the stage V. Simultaneously, a decrease in cholesterol level is obvious in the liver. In muscle, on the other hand, with an insignificant level of 0.43-0.49%, cholesterol does not appear to play any decisive role in germ building. El Sayed et al. (1986) in T. nilotica and S. auratus and Muthukaruppan (1987) in Liza parsia have suggested a drain of liver cholesterol with a corresponding accumulation in the ovary during maturation, as observed in the present case. Diwan & Krishnan (1986) found, cholesterol levels to increase upto stage III of maturity and then to decrease at a later stage in gonad of E. suratensis. Nauriyal & Singh (1985) in P. chilinoides recorded high levels of cholesterol in the gonad at the end of the maturing phase. These evidences support the result obtained in the present study, where cholesterol levels increase with maturity in the gonad. High levels of cholesterol in gonads act as a reservoir to meet the demand of maturing ovary (for synthesis of hormones such as 17 β - oestradiol and 17α, 20 β - dihydroxy, 4-pregnen – 3 one) and increase in steroidogenesis decreased the levels (Larsson & Fange, 1977). The elevated cholesterol level in the ovary during the spawning phase in this species could be due to decreased conversion of cholesterol into gonadal steroids, when vitellogenesis is completed.

The serum total cholesterol level in M. cephalus followed a similar pattern as that of serum lipids, showing an alternate rise and fall. The premise that striped mullet were actively feeding and transporting fats for storage prior to migration and gonadal development, is supported by the highest level of serum cholesterol in stage II. Increased serum cholesterol is associated primarily with dietary lipid transport in the form of very

low-density and high-density lipoproteins (Dindo & Mac Gregor, 1981). But, Miller (1971) has opined that the rise in serum cholesterol in stage II could be as a result of its redistribution from other tissues to the maturing gonads. A fall in blood cholesterol level in stage III, followed by a slight elevation in stage IV in M. cephalus can be attributed to the corresponding increase and decrease of steroid biosynthesis in the ovaries. The lowest value of serum cholesterol in spent stage indicates a state of energy (lipid) deprivation in M. cephalus. The reports of Farrell & Munt (1983), that 91-96% of the fat reserves in salmon are utilized for migration and spawning, corroborate the present finding in M. cephalus.

It is also interesting to note the intraspecific variation of serum cholesterol in M. cephalus. Miller (1971) reported a total serum cholesterol value of 398 mg% and Dindo & Mac Gregor (1981) 354 mg% for M. cephalus from Alabama, U.S.A, which are considerably lower than the maximum mean value for females (553 mg%) recorded in the present study, but agreeing with the observations (560 mg%) of Sulya et al. (1960) from Gulf of Mexico. The lipid content and composition in teleosts depend mainly on food and environmental conditions (Mikhail et al., 1982) and the difference in cholesterol levels in the present study may be attributed to the wide variations in these factors between the two habitats.

Compared to the higher vertebrates, M. cephalus show hyperlipemia and hypercholesterolemia. Such a condition seems to be physiologically normal and not associated with coronary heart disease or "atherosclerosis" (Larsson & Fange, 1977). The present cholesterol data may however have some value in future atherosclerosis research.

Carotenoids

The bright yellowish-orange colour of the eggs of M. cephalus is due to the presence of carotenoids in the yolk. Carotenoids are not "just playful diversion of nature as is often assumed",but they fulfil important function(s) in reproduction (Craik, 1985). They are attributed with functions of maintaining egg quality, impairing colouration, controlling viability of eggs, protection from sunlight, as a fertilization hormone and as a precursor of vitamin A (Craik, 1985). They are dependant on diet variation and are found to be stored in muscle, liver and skin, and are mobilised from muscle and liver through the blood serum as carotenoglycolipoprotein complex and laid down in unesterified form in mature eggs (Kitahara, 1985; Ando, 1986a).

In the present study, the carotenoid level in the muscle increased from stage I to stage III, thereafter declined in the subsequent stages. Correspondingly, the gonad showed an increase in carotenoid content from stage I to stage IV, followed by a sharp decline in the spent condition. In blood serum, the carotenoid concentration increased upto the third stage, but in the subsequent stages, it showed a decrease. The preseent study thus substantiates the view of Crozier (1970), Kitahara (1983) and Ando (1986a).

Carotenoid composition of fish varies according to different environmental conditions. Matsuno et al. (1975) isolated lutein and zeaxanthin from the fin and skin of freshwater cultured M. cephalus, while the same species from seawater had zeaxanthin and diatoxanthin as major carotenoids

in the fin and skin. Though it has been found that carotenoids are deposited, in the liver also in stage II, no clear pattern of their mobilisation from this organ was evident to the developing gonads.

Ash

Fishes obtain minerals from dietary resources as well as from the water. Since mullets do not feed during the spawning migration, it is postulated that the minerals are being removed from the tissues such as liver and muscle through blood plasma by the developing gonads. In the present observation, ash content in ovary showed a steady increase upto stage IV, followed by a decline in the spent condition. In muscle, the ash content showed an elevation from stage I to II and in liver from stage I to stage III thereafter the level decreased in subsequent stages in both the tissues; thus showing depletion of minerals in muscle and liver and their redistribution in the developing ovary. The rise in ash content in the muscle and liver may be attributed to accumulation of minerals due to the voracious feeding. Vitellogenin contains phosphorous and shows affinity for calcium, iron and magnesium. The hike in ash content in stage III liver as observed in the present study, can be due to the peak synthesis of Vtg by the hepatocytes. Ando (1986a) in chum salmon and Muthukaruppan (1987) in Liza parsia have presented evidence suggesting the translocation of minerals from the liver and muscle to the developing ovary. The ash value of the ripe eggs in the present case (2.46%) fall in close agreement with that of Rhinomugil corsula (2.03%), reported by Hasan & Jafri (1964). Watanabe & Kuo (1986) have observed a small increase in the dry matter

of eggs of M. cephalus during hydration as a result of the intake of potassium, chloride, magnesium and sodium ions. However, the influence of gonadal maturation on the mineral content of migratory fishes has to be investigated further, as migration also causes an overall change ion concentrations.

Total Calcium and Iron

Calcium and iron are the two most important metals associated directly with vitellogenesis. An increase in the total plasma and ovarian calcium and iron levels has been observed during the sexual maturation of female teleosts (Oguri & Takada, 1967; Peterson & Shehadeh, 1971; Varghese, 1976; Yamane & Yamada, 1977; Tinsley, 1985; Wallace, 1985; Bjornsson et al., 1986; Hara, 1987). In the present case, a rise in calcium and iron contents was noticed in the blood serum upto stage III of maturity and in ovary upto stage IV, followed by a decrease in the subsequent stages. The pronounced rise in the concentration of these metals in stage III, could be associated with the presence of appreciable quantities of vitellogenin at this stage. Teleost vitellogenin and yolk proteins exhibit calcium and iron-binding property (Hara, 1987). The vitellogenin and the yolk protein bands in the present investigation showed a strong reaction for calcium. Total plasma calcium has frequently been used as an indirect index of exogenous vitellogenesis (Tinsley, 1985). Bjornsson et al. (1986) have shown that calcium and magnesium bind to vitellogenin at a ratio of 9:1 in Salmo gairdneri. These authors have also noticed a small, but significant drop in the free plasma levels of both calcium and magnesium, during the period

when the liver synthesis of vitellogenin is probably maximal. This drop could be due to the large demand for these metal ions,needed for vitellogenin binding. High calcium content of vitellogenin keeps this protein in solution and much of the calcium, iron and magnesium, bound to vitellogenin may be incorporated into the oocytes to serve as a vital reservoir of these metals for the embryogeny (Bjornsson et al., 1986).

As liver being the site of yolk synthesis, the estimation of calcium and iron in this gland might give a more clear picture of incorporation of these metals into Vtg in teleosts. But interpretation of such results will be complicated due to the contamination of the liver extract by blood, especially as the liver is highly vascularized.

EXECUTIVE SUMMARY

EXECUTIVE SUMMARY

1. The thesis presents a comprehensive account of the oogenesis in Mugil cephalus.

2. Following a review of literature on the biology and reproductive physiology of mullets, the material and methods employed in specimen collection, reproductive biology, histology, histochemistry, electrophoresis and biochemical analyses are detailed.

3. The female reproductive system consists of paired cylindrical compact ovaries, which are cystovarian type. Posteriorly, both the lobes of ovary are fused to form a common, short, muscular oviduct opening to the exterior through a common urinogenital aperture. Initially the ovaries make their appearance as two ribbon like, pinkish, translucent strands attached to the body wall at the dorsal side by means of a mesovarium. As the development proceeds, the ovaries enlarge, become yellowish and occupy almost the entire body cavity. Developing oocytes lie in the ovigerous lamellae in the ovocoel. On the basis of the morphological and histological changes, which the ovaries undergo during maturation, five stages of maturity are identified such as immature; maturing and recovering spent; mature; ripe, and spent. The characteristic features of each stage are discussed in detail.

4. The gonadosomatic index (GSI) and the condition factor (K) showed an increase as the development proceeded and the highest mean values of these parameters were recorded in the ripe stage. The hepatosomatic index (HSI) was highest in stage III. The maximum values of GSI, highest incidence of ripe specimens and the changes in environmental parameters (such as surface water temperature, salinity and dissolved oxygen) indicate that M. cephalus spawn in Cochin waters during the months of June, July and early August.

5. The oocyte size-frequency profiles indicate that only one distinct batch of ripe eggs is clearly demarked from a small clutch of immature eggs in a season and this single batch of ripe eggs spawn in its entirety; thus confirming the fact that M. cephalus has a "group synchronous type" ovary. The largest oocyte diameter of ripe eggs was 890 /um in the present study.

6. The fecundity of M. cephalus in Cochin area was found to vary between 5.27-35.6 lakhs in fish ranging in total lengths from 430 to 680 mm, whole body weight between 900 to 3200 g and total ovary weight between 170 to 590 g. A curvilinear relationship was obtained between fecundity and body length and linear relationships between fecundity and total body weight; and between fecundity and ovary weight.

7. From the histological study of oogenesis, a number of distinct developmental stages were delineated in M. cephalus, viz. the oogonia; chromatin nucleolus stage; early and late perinucleolus stages; early and late

lipid droplet stages; primary, secondary and tertiary yolk globule stages; migratory nucleus stage and ripe oocyte. The oogonia had a regular nuclear envelope and their less electron dense cytoplasm contained germ cell markers the "nuage" and "cement". The primary oocytes are distinguished from the oogonia by their highly electron-dense and basophilic cytoplasm and the undulating nuclear envelope. The basophilic yolk nucleus was composed of mitochondria, ribosomes and tubules of endoplasmic reticulum. During the secondary growth phase, three types of inclusions viz. the lipid droplets, protein yolk globules and cortical alveloli are formed in the oocytes. The lipid droplets (triglycerides) accumulated first, which was followed by the protein yolk. The exogenous, acidophilic, membrane-bordered fluid-filled protein yolk globules appeared first in the cortical cytoplasm and later they filled the entire ooplasm. These structures did not show any zonation internally. The third and quantitatively minor type of inclusion - the cortical alveoli - was less pronounced in the oocytes of M. cephalus. This inclusion appeared as a narrow zone immediately below the zona radiata, after both lipid yolk and protein yolk formation have started. The bipartite nature of zona radiata (ZR) was evident in electronmicrographs. The zona radiata externa had a maximum thickness of 1.5 μm and the zona radiata interna, 12.5 μm. The oocyte and follicular microvilli traversed the pore canals of ZR. The fine structure of the granulosa of follicle layer showed many sudanophilic lipid droplets, mitochondria with tubular cristae and smooth endoplasmic reticulum, during the secondary growth phase; thus pointing out the possible steroidogenic role of these cells. The thecal cells did not show any such structural modification and special thecal cells were not observed. Following GVBD, in the ripe oocytes,

the protein yolk coalesced to form an uniform mass and the lipid droplets into a large single oil drop.

8. Four oocyte atretic stages were identified in the present study, namely α, β, γ and δ stages. Detailed histological investigations revealed that, these residual yolky oocytes undergoing resorption did not have any steroidogenic capacity. The postovulatory follicles belonging to the A, B, C and D stages also did not show any endocrine potency.

9. Histological pictures of hypertrophied hepatocytes of mature fish showed the presence of numerous rough endoplasmic reticulum and ribsosomes and few lipid droplets. This indicate the possible role of liver in the synthesis of protein yolk.

10. The distribution of different biochemical components in the oocytes were studied using the histochemical tests. The ooplasm of primary oocytes, nucleoli and yolk nucleus stained strongly for proteins and RNA, while the yolk globules showed the presence of basic proteins, phospholipids, glycoproteins and calcium. Lipid droplets in all stages contained only neutral lipids (triglycerides), while those in the follicle layer for cholesterol. Rich amounts of polysaccharides and aminoacids like tyrosine and lysine were present in the zona radiata. Strong alkaline phosphatase and succinate dehydrogenase activity was detected in the yolk nucleus, while the follicle epithelium of vitellogenic oocytes showed the activity

of 3 /B HSD. Acid phosphatase activity was detected only in the corpora atretica and postovulatory follicles. In hepatocytes a steady increase of proteins, RNA, DNA glycogen and alkaline phosphatase activity was detected upto stage III and a decline of these parameters in the subsequent stages.

11. Electrophoresis of blood serum showed the presence of a strong vitellogenin band (R_f value 0.118) in the stages II and III. This band which stained intensely for proteins, lipids, glycogen and calcium, was absent in mature males. Two slow-moving egg protein bands were detected in the oocytes belonging to stages III and IV (R_f values 0.100 of 0.175). These bands also revealed their glycolipoprotein nature and affinity for calcium. Unlike many teleosts, the sera of M. cephalus showed the presence of a thick albumin and three narrow prealbumin bands.

12. An estimation of protein, carbohydrates, lipids, cholesterol, carotenoids ash calcium and iron in the muscle liver, ovary and blood serum during different stages revealed that there is a distinct depletion of these body resources from the somatic tissues during maturation. This depletion is found to be mainly due to the translocation of these substances to the ovary for the synthetic activities during oogenesis, and partly for meeting the energy requirements of fish during the final stages of maturation when the fish abstains from feeding.

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