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Biochemical composition and changes in biological indices associated with maturation of the ovary in the spiny cheek grouper *Epinephelus diacanthus* (Valenciennes, 1828)

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ABSTRACT

Changes in biological indices (gonadosomatic index GSI, hepatosomatic index HSI and condition factor) and biochemical composition of body tissues and serum, associated with ovarian development were examined in *Epinephelus diacanthus* sampled for two years. GSI, HSI and Condition factor values increased with the maturation of gonads. Mobilisation of biochemical constituents from somatic tissues to the gonadal tissues with the maturation of the ovary was noticed. Highest protein percentage was in stage III (24.81%) ovary. The total lipid percentage of the ovary showed a gradual increase from Stage I (6.56%) to Stage III (13.48%). Total cholesterol showed a steady declining trend and carotenoid levels of the ovary showed a positive relationship with ovarian maturation. Ash content of the ovary increased from stage I (0.82%) to stage III (1.20%).

Keywords: *Epinephelus diacanthus*, Gonadal maturation, Gonadosomatic index, Proximate composition, Spiny cheek grouper

Introduction

The success of any fish species is ultimately determined by the ability of its members to reproduce successfully in a fluctuating environment and thereby to maintain a viable population. Reproduction is a highly integrative function, which involves complex physiological changes at intracellular and intercellular level. The gonads show a series of developmental alterations with the onset of maturation which are closely accompanied by conspicuous cellular, biochemical, molecular and endocrinological changes (Nagahama, 1983; Guraya, 2000). These complex physiological changes control the spawning activity in natural conditions. As the gonads increase in size, somatic growth slows down and eventually stops. At this stage, proteins and lipids are mobilized from the somatic tissues and transferred to the gonads (Aksnes *et al*., 1986).

Groupers are popular food fish farmed in south-east Asia and have the potential to become an important aquaculture species, owing to their fast growth, efficient feed conversion, and high market price. However, availability of seed from wild resources is limited. The fluctuating and capricious nature of wild seed availability prompted investigations into the development of captive brood stock and hatchery seed production. Most of the reports from India are on induced sex reversal and spawning; not much information is available on changes in body composition associated with maturation of gonads in groupers, which is infact essential for the development of standardised hatchery technology.

The present investigation was taken up on the key biological indices and body composition changes associated with the maturation of ovary in the spinycheek grouper, *Epinephelus diacanthus.*

Materials and methods

Collection and preservation of samples

Live specimens of *E. diacanthus* were collected onboard *Matsya Varshini* during her cruises off Quilon region (latitude: 8º 55' N and longitude: 76º 30' E) and off Ratnagiri region (latitude: 15º 42' N and longitude: 73º 16' E) at a depth of 50 m. Fishes were also collected from trawlers operated off Quilon region. Live fishes collected onboard were transferred to buckets containing seawater mixed with 100 ppm anesthetic clove oil (Koya, 2003). The anesthetized fishes were measured accurately to the nearest millimeter (mm) for total length, standard length and total weight. Length and weight ranges of the specimens were 17.00 – 39.20 cm TL and 64 – 825 g respectively. Blood sample was collected by cardiac puncture from anesthetized fishes. Disposable syringes of 5 ml with heparinised needles were used for blood collection. Serum separation and preservation was carried out by following the method of Utarabhand and Bunlipatanon (1996). Serum samples were preserved at -30 ^{0}C until further studies were carried out and the samples wherein haemolysis was

noticed, were discarded. Immediately after collection of blood, each fish was dissected to remove the liver, ovary and body tissue for biochemical studies. The dissected tissues were weighed, labelled and packed in 4 x 5 cm polythene bags. These bags were preserved at -20 ^{0}C until the completion of biochemical analyses.

Ovaries of fishes collected onboard were carefully observed in the laboratory under stereoscopic dissection microscope to study the maturity stages. The gonads were assigned three maturity stages – stage I (Immature), II (Maturing) and III (Mature/Ripe) following the method of Moe (1969).

Biological indices

The gonadosomatic index (GSI) and hepatosomatic index (HSI) for each fish was calculated using the method of Yuen (1955) and Crupkin *et al*. (1988) respectively. The 'ponderal index' or condition factor' (K) for each fish was calculated using the method suggested by Clark (1934). The range and average values of GSI, HSI and 'K' were determined for each maturity stage.

Biochemical analysis

Moisture, total protein, total carbohydrates, total lipids, total cholesterol, total carotenoids and ash contents in the muscle, liver and gonad tissues of *E. diacanthus* were estimated at different stages of maturation. The serum was also analaysed for all the above biochemical parameters except for moisture and ash contents. The moisture content of ovary, liver and muscle tissues was determined by placing pre-weighed wet samples at 70 ºC in hot air oven till constant weights were obtained. The loss in weight was taken as the moisture content and expressed as percentage. Total proteins of the tissues were quantified by the method of Lowry *et al*. (1951). The concentration of total carbohydrates was determined by the phenol sulphuric acid method of Dubois *et al.* (1956). Total lipids were estimated as per Barnes and Blackstock (1973). Total cholesterol content of the tissues were estimated by Henly's method (1957) as given by Varley (1976). The method described by Olson (1979) was adopted to estimate the total amount of carotenoids in different tissues and blood serum. Ash content was estimated as per AOAC (1990). All the values were given on wet weight basis. The values obtained were estimated for 100 mg per 100 ml tissue except for carotenoids and expressed in percentage. Total carotenoids were expressed in µg carotenoid g^{-1} in the muscle, liver and ovary and in the case of serum µg ml^{-1}. All the biochemical analyses were carried out with five replications.

Statistical analysis

The data on biochemical analyses of liver, ovary and body tissue for different maturity stages were analysed by one way ANOVA using the SYSTAT software version 7.0.1 and the significance was tested at 1% level. The analysis of variance was worked 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. The variation in the composition of blood serum at different stages of maturity was investigated by one way ANOVA, applied separately for each biochemical component.

Results

Gonadosomatic index (GSI)

The GSI values of *E. diacanthus* ovary showed correlation with the maturation of gonads (Fig. 1). Immature ovaries showed a GSI value of 0.062%, for the maturing ovaries value was 0.234% and for the ripe stage ovaries, the value was 3.064%. Highest GSI value was observed in stage III of gonadal maturation.

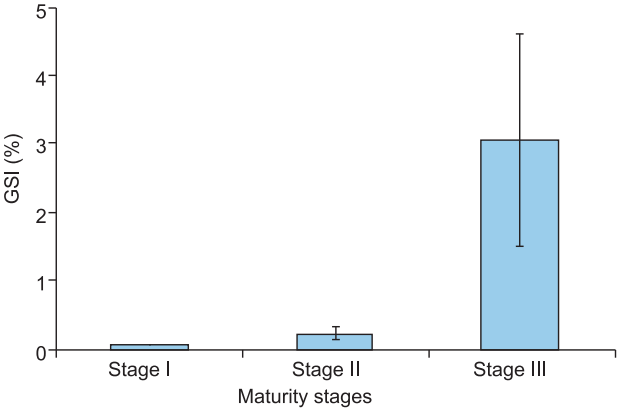

Fig. 1. Trends in the GSI values with the maturation of gonads (Mean ± SD)

Hepatosomatic index (HSI)

The hepatosomatic index was found to increase gradually from immature to ripe stages in female *E. diacanthus*. Highest hepatosomatic index was noticed in stage III (2.549%) of ovarian maturation (Fig. 2).

Condition factor (K)

In the present study, condition factor in *E. diacanthus* was in the range of 1.15 – 1.61. Highest condition factor (1.61) was observed in stage III of gonadal maturation. (Fig. 3).

Biochemical changes during maturation

Muscle

Highest moisture content was observed in the maturity Stage I (76.84%); moisture percentage gradually reduced in Stage II and III (Table 1), showing an inverse relation.

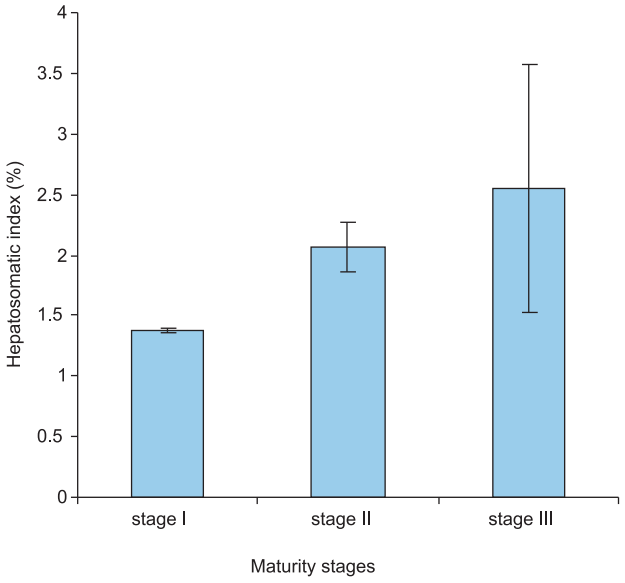

Fig. 2. Trends in the HSI values with the maturation of gonads (Mean ± SD)

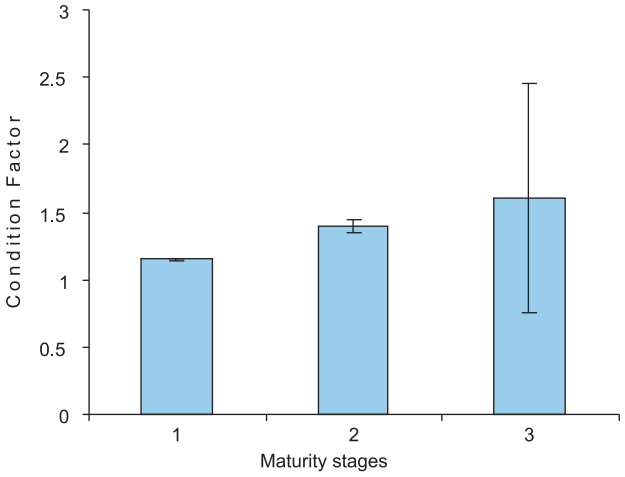

Fig. 3. Trends in Condition factor with the maturation of gonads (Mean ± SD)

Trend in total protein with ovarian maturation of muscle showed a positive relation. Protein content increased from stage I (18.8%) to Stage III (32.8%). Carbohydrate content decreased from stage I (3.09%) to stage III (1.3%). Lipid content in the muscle was 2.468 % in stage I and it increased slightly to 2.72 % in stage II. Eventhough the lipid content of muscle increased from stage I to II it declined to 1.648% in stage III. Cholesterol content of muscle decreased from stage I (1.13%) to stage III (0.762%), showing an inverse relationship with the maturation of gonad. Total carotenoids showed a positive relationship with gonad maturation with a value of 3.8 µg g^{-1} in stage I and 8.71 mg g^{-1} in stage III. Muscle ash percentage showed an inverse relationship with ovarian maturation. Ash content decreased from Stage I (1.81%) to Stage III (0.39%).

Liver

In the liver, moisture content showed an inverse relation with the gonad maturation. It decreased from 66.0% in stage I to 57.2% stage III (Table 2). However, protein percentage showed a positive relationship with gonad maturation. Highest protein percentage was observed in stage III (22.30%) and lowest in stage I (11.5%). Liver carbohydrate content in stage I showed 13.23%, which decreased to 10.96% in stage II and further increased to 14.27% in stage III. Lipid content of the liver showed an inverse relationship with ovarian maturation. In stage I, the value was 16.17% and decreased to 7.08 % in stage III. The total cholesterol percentage in liver tissue was higher than in any other body tissues. Cholesterol content showed an inverse relationship with maturation, decreasing from stage I (3.42%) to stage III (1.67%). The carotenoid content of liver showed a steady increase from stage I to stage III showing a positive relationship with gonad maturation. Highest carotenoid content was considered in stage III (11.3 µg g^{-1}) and lowest in stage I (3.6 µg g^{-1}). Highest ash percentage was observed in stage I (0.81%) while the lowest ash percentage was recorded in stage III (0.39%).

Blood serum

Total protein in the blood serum showed a positive trend in relation to gonad maturation with the highest value (10.98) recorded in stage III (Table 3). The carbohydrates level was 6.52% which increased to 8.29% in stage II and decreased further to 6.8% in Stage III. Blood serum lipids showed a positive relation with gonad maturation. A steady increase in values from stage I (1.86%) to stage III (2.61%) was noticed.

Table 1. Biochemical constituents in the muscle of the grouper *E. diacanthus* (Mean ± SD)

Stage	Moisture (%)	Total proteins (%)	Total carbohydrates (%)	Total lipids (%)	Total cholesterol (%)	Total carotenoids (µg g^{-1})	Ash (%)
I	76.84 ± 2.74	18.80 ± 0.049	3.096 ± 0.009	2.46 ± 0.012	1.13 ± 0.016	3.8 ± 0.037	1.81 ± 0.007
II	72.15 ± 2.03	24.64 ± 0.018	1.372 ± 0.013	2.72 ± 0.022	0.83 ± 0.023	5.19 ± 0.015	0.55 ± 0.002
III	68.22 ± 1.55	32.81 ± 2.107	1.723 ± 0.029	1.648±0.019	0.762 ± 0.015	8.71 ± 0.016	0.39 ± 0.001

* Wet matter basis

Table 2. Biochemical constituents in the liver of the female grouper, *E. diacanthus* (Mean ± SD)

Stage	Moisture (%)	Total proteins (%)	Total carbohydrates (%)	Total lipids (%)	Total cholesterol (%)	Total carotenoids (µg/g)	Ash (%)
I	66.02 ± 0.90	11.59 ± 0.255	13.23 ± 0.025	16.17 ± 0.09	3.42 ± 0.16	3.6 ± 0.07	0.81 ± 0.005
II	61.60 ± 0.78	15.029 ± 0.094	10.96 ± 0.102	11.66 ± 0.24	2.27 ± 0.14	7.14 ± 0.054	0.55 ± 0.002
III	57.20 ± 0.56	22.30 ± 0.468	14.27 ± 0.046	7.08 ± 0.17	1.67 ± 0.02	11.3 ± 0.16	0.39 ± 0.003

*Wet matter Basis

Table 3. Biochemical constituents in the blood serum of the female grouper, *E. diacanthus* (Mean ± SD)

Stage	Total protein (%)	Total carbohydrates (%)	Total lipids (%)	Total cholesterol (%)	Total carotenoids (µg ml^{-1})
I	6.61 ± 0.013	6.52 ± 0.02	1.86 ± 0.039	0.45 ± 0.019	0.17 ± 0.01
II	8.73 ± 0.001	8.27 ± 0.07	2.13 ± 0.016	0.37 ± 0.006	0.33 ± 0.02
III	10.98 ± 0.05	6.83 ± 0.11	2.61 ± 0.05	0.63 ± 0.018	0.95 ± 0.01

*Wet matter Basis

Table 4. Biochemical constituents in the ovary of the grouper, *E. diacanthus* (Mean ± SD)

Stage	Moisture (%)	Total Proteins (%)	Total Carbohydrates (%)	Total lipids (%)	Total Cholesterol (%)	Total Carotenoids (µg/g)	Ash (%)
I	71.66 ± 1.15	14.02 ± 0.059	1.46 ± 0.008	6.56 ± 0.01	2.06 ± 0.03	1.42 ± 0.03	0.820 ± 0.005
II	63.56 ± 0.25	17.93 ± 0.07	1.23 ± 0.001	7.89 ± 0.06	1.65 ± 0.019	2.69 ± 0.05	1.01 ± 0.008
III	60.55 ± 0.19	24.81 ± 0.28	1.38 ± 0.005	13.48 ± 0.04	1.27 ± 0.03	7.98 ± 0.021	1.20 ± 0.003

*Wet matter basis

Variations in serum cholesterol levels were observed with the maturation of gonads. In stage I, It was 0.45% in Stage I, which decreased to 0.37% in stage II and increased to 0.63% in stage III. Carotenoid content of 0.17 µg g^{-1} in stage I rose to 0.95 µg g^{-1} in stage III.

Ovary

The moisture content of the ovary showed a steady decrease from stage I (71.66 %) to stage III (60.58 %) (Table 4). Total protein exhibited a positive relation with the maturation of gonads. In stage I, it was 14.02, whereas in stage III, it was 24.81%. However, total carbohydrate content showed a fluctuating trend. High carbohydrate percentage observed in stage I (1.43), declined to 1.23 in stage II and increased to 2.38 in stage III. Total lipid percentage showed a positive relationship with a gradual increase from 6.56% in stage I to in 13.48% stage III. Total cholesterol levels of ovary showed an inverse relationship with maturation showing a steady decline from stage I (2.06%) to stage III (1.27%). In stage I, the carotenoid percentage which was 1.42 rose to 7.98 in stage III. Ovary tissue had more ash content than the liver. The values showed a positive relation with gonad maturation increasing from 0.82 %. In stage I, to in 1 stage III .

Discussion

The condition factor (K) and hepatosomatic index are a measure of fish energy reserves. Condition factor values follow inter annual variations and seasonal cycles (Lambert and Dutil, 1997). The results of the persent investigation are in accordance with that of Gopalakrishnan (1991) who reported the increase of condition factor with the advancement of maturation in *Mugil cephalus* and Hernandez *et al*. (2003) who observed similar trend in the *D. puntazzo*.

In *E. diacanthus,* moisture content decreased in the ovary lines and muscle with the imaturation of gonads. The low muscle moisture percentage in stage III may be due to its replacement with proteins. The results are in agreement with Sivakami *et al.* (1986) who recorded decrease in muscle moisture percentage in *Cyprinus carpio*. With the advancement of maturation, major energy reserves in the muscle are mobilized for the growth of gonads. Masurekar and Pai (1979) noticed fluctuating muscle water content during the maturation of gonads in *Cyprinus carpio.*

In *E. diacanthus,* muscle protein percentage showed increase with gonadal maturation. Muthukaruppan (1987) also observed increase of muscle protein from stage I to stage III of *Liza parsia* during gonadal maturation. Ando and Hatano (1986) found a positive correlation between gonadosomatic index and muscle protein content in chum salmon. Sivakami *et al*. (1986) found that muscle protein percentage increased from the second stage of gonadal maturation in *C. Carpio*.

Muscle carbohydrate content pattern in the present study showed variation from stage I to stage III. Muthukaruppan (1987) also observed the same trend in *L. parsia*. It is discerned that carbohydrate content decreased with the translocation of carbohydrates from depot site to where the energy prompt is required. However, Sivakami *et al*. (1986) observed gradual increase of muscle carbohydrate content with the maturation of gonads in the *C. carpio*.

In the present study, it has been observed that muscle lipid content of *E. diacanthus* got depleted with the maturation of gonads. The results agree with that of Masurekar and Pai (1979) who observed muscle fat content depletion with the maturation of gonads in *C. carpio* and Nelson and Mc Pherson (1987) who observed decrease in lipid content in muscle and viscera of brook char (*Salvelinus fontinalis*) with the progress of reproduction.

Earlier works in *Sparus auratus* (El-sayed *et al*., 1984), pelagic sculpins (Kozlova, 1997), chum salmon (Ando and Hatano, 1986) and *L. parsia* (Muthukaruppan, 1987) have also shown correlation of muscle fat depletion with the maturation of gonads. However, muscle lipid content increased with the maturation of gonads in *C. carpio* (Sivakami *et al.,* 1986) and in the common dentex (Chatzifotis *et al*., 2004).

Cholesterol plays the major role in the synthesis of steroids, which in turn influence the maturation phenomena. In the present study, it was observed that the cholesterol levels decreased in the muscle with the maturation of gonads. It can be inferred that muscle cholesterol has a role in steroid synthesis.

In *E. diacanthus,* muscle carotenoids increased with the maturation of gonads. A gradual increase of carotenoid content with the maturation of gonads was noticed. However, Kitahara (1983) observed that chum salmon muscle carotenoid content decreased with the maturation of gonads. He also noticed mobilization of muscle carotenoids to the skin. Crozier (1970) detected that the high carotenoid content of the Sockeye Salmon muscle decreased with the Groupers consuming large amount of crustaceans such as crabs (were found rich in carotenoids (Tessy, 1994).

In the present study, ash content decreased in the muscle from stage I to stage III, but increased in the ovary from stage I to stage III. This indicates that of mobilization of minerals from muscle to ovary takes place with the maturation of gonads. Sivakami *et al*. (1986) observed decline of muscle ash content from stage III to stage V in the female *C. carpio*.

The liver moisture content showed inverse relationship with the ovarian maturation. Similar results were observed in *Mugil cephalus* (Gopalakrishnan, 1991) and in *Diplodus puntazzo* (Hernandez *et al*. 2003). Moisture content of the liver of *E. diacanthus* decreased from immature stage (stage I) to ripe ovary stage (stage III) while protein content increased. It may be concluded that proteins have replaced the moisture content of liver. Liver protein percentage has increased from stage I to stage III. Aida *et al*. (1973) reported the synthesis of liver protein in Ayu fish with the maturation of gonads.

In the present study, *E. diacanthus* carbohydrate content in liver showed high fluctuations with the maturation of gonads. Lal (1991) observed similar trend in carbohydrate content with the ovarian maturation in *Lates calcarifer*.

Liver lipid percentage of *E. diacanthus* decreased with the development of gonads. El-sayed *et al*. (1984) observed minimum liver total lipid content with the progress of gonadal maturation in *Tilapia nilotica* and *Sparus auratus*. Kozlova (1997) reported utilization of lipids in the liver of pelagic sculpins for gonadal development and its subsequent depletion after maturation. The total lipid content of the liver was observed to have decreased with the maturation of gonads of steelhead trout (Sheridan *et al.,* 1983), in *L. parsia* and *M. cephalus* (Muthukaruppan, 1987 and Goplakrishnan, 1991) and red drum, *Sciaenops ocellatus* (Craig *et al*., 2000). In common dentex (*D. dentex*), variation of liver lipid levels with the maturation of gonads was noticed (Chatzifotis *et al*., 2004). Depletion of the liver total lipid content with the maturation of female germ cells was observed in the protandrous hermaphrodite *L. calcarifer* (Lal, 1991).

Liver cholesterol levels of *E. diacanthus* decreased with the maturation of gonads as also observed Lal (1991) by in *L. calcarifer.* Low levels of cholesterol in liver may be due to higher physiological needs of gonadal development. The liver carotenoid content increased from stage I to III of gonadal maturation in *E. diacanthus* and was high compared to ovary, muscle and serum. According to Leger (1985), liver is the site of synthesis of lipoprotein. Liver plays the major role in mobilization of carotenoids to the ovary with the maturation of gonads. Patnaik (2001) found that in *Priacanthus hamrur* carotenoid content of liver increased from stage I to stage II of gonadal maturation and decreased during subsequent stages upto stage VI.

Blood serum plays a major role in the maturation of gonads. In the present study, serum protein percentage showed a gradual increase with the progress of maturation. Chatzifotis *et al*. (2004) found that serum protein percentage increased with the maturation of gonads in the common dentex (*Dentex dentex*). However, serum carbohydrates showed a pattern opposite to the trends observed in muscle, ovary and liver. The results indicate that serum played the

role of a transporting agent of carbohydrates from body tissues to reproductive organs. Chatzifotis *et al*. (2004) observed similar trend in serum glucose concentration of common dentex, which decreased from stage I to stage II and increased during subsequent stages.

Highest cholesterol level was recorded in stage III. Lal (1991) observed the same trend in total lipid and serum cholesterol levels with the maturation of female germ cells in the *L. calcarifer*. Chatzifotis *et al*. (2004) also observed increase in total lipid and serum cholesterol from stage I to stage IV in female common dentex. Furrel and Muni (1983) also reported elevation of serum cholesterol level with spawning in Atlantic salmonids. Serum carotenoid content increase during onset of vitellogenesis. In the present study, carotenoid content was found to increase with the maturation of gonads and the highest serum carotenoids were noticed in the stage III of gonadal development. It indicates that the carotenoids are transported by serum to the developing eggs in the ovary. Plack and Woodhead (1966) also reported increase of carotenoid compounds in the serum of ripe female cod.

In the present study, protein content of the ovary increased with the maturation of gonads in *E. diacanthus*. Robards *et al*. (1999) found that protein percentage of gonad increased in the Pacific sand lance in relation to maturity. In *M. cephalus*, Gopalakrishnan (1991) observed that ovary protein percentage increased with the maturation of gonads. The same trend was noticed by Muthukaruppan (1987) in *L. parsia*. The study did not exhibit any subsequent decrease of body tissue proteins content with the increase of ovary protein. Hence it may be inferred that exogenous factors such as food intake was the major source for protein percentage increase of gonads. Intake of protein rich food increased from immature to ripe fishes in *E. diacanthus* (Tessy, 1994).

Ovary carbohydrate percentage of *E. diacanthus* increased from stage II to stage III. Similar results were observed earlier by Sivakami *et al*. (1986), Muthukaruppan (1987), Gopalakrishnan (1991) and Lal (1991) for *C. carpio*, *L. parsia, M. cephalus* and *L. calcarifer* respectively.

Much of the yolk material in the eggs of teleost fishes generally contained a lipophosphoprotein complex. Mobilization of lipids to the gonads takes place during the spawning period to the gonads. In the present study, it was observed that the lipid content of ovary increased drastically from stage I to stage III. El-sayed *et al*. (1984) recorded similar trend in lipid content change in *Tilapia nilotica* and *Sparus auratus*. Sivakami *et al*. (1986) observed that the lipid levels increased in the ovary of *C. carpio* with the maturation of gonads. Muthukaruppan (1987) in *L. parsia* and Gopalakrishnan (1991) in *M. cephalus* also observed similar trends in ovary lipid with the progress of gonadal development.

Lal (1991) observed correlation in the lipid content in ovary with the maturation of gonads in *L. calcarifer* and found that lipid content increased with the maturation of female from stage I to stage IV. However, Chatzifotis *et al*. (2004) noticed decrease of ovary lipid content from stage I to stage II and subsequent increase in the process of gonadal development in the common dentex.

The correlation in the lipid content in different body tissues of *E. diacanthus* varied with the maturation of gonads. It was observed that liver lipid content drastically decreased from stage I to stage III and that consequently ovary lipid content increased with maturation of gonads. This is suggestive of the mobilization of liver and muscle lipids to the ovary with the progress of vitellogenesis. In the present study, cholesterol content of the ovary was observed to decrease from stage I to stage III. Cholesterol level depletion has indicated the progress of steroid synthesis as the gonad becomes fully mature. Lal (1991) also observed similar trend in the ovary cholesterol content with the maturation of female gonads in *L. calcarifer*. Jayashree and Srinivasachar (1979) noticed the lowest level of cholesterol in the ripe male in *Clarias batrachus*.

However, Diwan and Krishnan (1986) observed fluctuation in cholesterol levels of the gonads with maturation in gonads in *Etroplus suratensis*. In immature female fish, gonadal cholesterol was high and as maturation progressed in subsequent stages cholesterol level showed variations. They concluded that steroid synthesis progressed with the gonadal development leading to fluctuations in gonadal cholesterol levels.

In the present study, *E. diacanthus* ovary carotenoids increased from stage I to stage III. During sexual maturation considerable amount of carotenoids are transferred to the yolk (No and Storebakken, 1992). According to Bjerking *et al*. (1992) the carotenoid content in the female gonad increases moderately with maturation. Patnaik (2001) noticed increase in carotenoid content of the ovary of *P. hamrur* with the maturation of gonads.

Fishes get most of the minerals required for germ cell production through osmosis, but less mineral mobilization for gonad development takes place. In the present study, mineral content decreased in the muscle but increased in the ovary from stage I to stage III. It indicates that mobilization of minerals from muscle and liver to ovary takes place with the maturation of gonads.

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