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Gonadal development and steroid hormone profile of wild caught grey mullet (*Mugil cephalus*)

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The grey mullet *Mugil cephalus* is one of the popular and fast growing fishes being cultured in tropical and subtropical regions. In the present study, histological observation of gonadal development and corresponding changes in sex steroid levels from different maturity stages of wild caught male and female were studied. In female, testosterone and 17β-estradiol increased with the advancement of maturation and reached peak (17β-estradiol, 323 ± 13 pg/ml; testosterone, 938 ± 7.87 pg/ml) in mature stage, whereas the level of progesterone was maximum (488 ± 4.9 pg/ml) during ripe stage. Vitellogenin level in serum showed a similar trend as 17β-estradiol. In case of male, the testosterone level in serum increased gradually with advancement of maturation and was maximum (1820 ± 40.25 pg/ml) during ripe stage, whereas significant decrease in 17β-estradiol and progesterone was noticed with advancement of maturation. The fundamental information from this investigation would be useful for developing protocol for accelerating maturation and spawning under captive condition.

Keywords: mullet; gonad; maturation; sex steroid; vitellogenin; histology

1. Introduction

The grey mullet *Mugil cephalus* L. is considered as one of the popular and fast growing candidate species for culture in tropical and subtropical region of brackish and freshwater semi-intensive fish ponds (Thomson 1963). However, the culture of the fish is mostly dependent on the availability of wild fry as breeding in captive condition is not standardized. In teleost, it is well known that vitellogenesis and final oocyte maturation (FOM) are regulated by gonadotropic hormones that mediate their actions via steroids secreted by the follicular cells surrounding the oocyte (Nagahama et al. 1994). Spawning season of this species varies in different geographical location, for example, in Hawaii it spawns in January and February (Kuo & Nash 1975), in South Carolina in November and December (McDonough et al. 2003), in Tanshni estuary of North West Taiwan in November to January (Chang et al. 1999) and in South Indian estuary it breeds from October to January (Mohanraj et al. 1994). Correlation between changes in plasma levels of steroids and oocyte development have been documented in grey mullet by Das et al. 2014, and the reproductive periodicity of gonadal steroid hormone level has been studied in wild caught mullet by Yelghi et al. 2012. However, there is no

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report available on the level of different gonadal steroid and oocyte development of wild caught grey mullet during the breeding season. Therefore, the present study aims to describe the changes in the gonadal steroid and vitellogenin level in the serum, and their correlation with oocyte development in *M. cephalus* during its spawning season from east coast of India.

2. Materials and methods

2.1. Fish collection

Grey mullet were collected from east cost of India (Kovalam, Chennai; latitude: 12°49′N and longitude: 80°15′E), using bag nets locally called *Siruvalai*. Thirty specimens were collected during the mid of every month from October 2011 to January 2012.

2.2. Blood collection

Fishes were anesthetised with phenoxyethanol (0.3 ml L^{-1}). Concurrent with monitoring gonad maturation and sex, blood sample was collected by puncturing heart and allowed to clot for 1 h at 5°C, and centrifuged for 10 min at 5000 rpm. The serum was separated with pipette and stored at –20°C until further analysis. Minimum six fishes were sampled for each maturity stages ($n = 6$).

2.3. Gonadosomatic index and condition factor

Each fish in different maturity stages was weighed (wet weight, $W \pm 0.1$ g) and total length (total length, $L \pm 0.1$ cm) was measured, and thereafter the fish were dissected to observe the maturity stages (Mc Bride et al. 2002). The maturity stages used in the present study were corresponding to maturity scale of ICES (Mohanraj et al. 1994) for comparison. The gonad was dissected out to measure total length and weight. Gonadosomatic index (GSI) for each maturity stage was determined using the formula of Yuen (1955). GSI (%) = (Weight of gonad) (g)/(weight of fish) (g) × 100. The Pondreal index or condition factor (K) for each maturity stages was calculated using the formula suggested by Clark (1934). Condition factor (K) = (final mean body weight (g)/mean total length (cm) ^{3}) × 100.

2.4. Ovarian follicles

Ovary was dissected out and 5 mg of ovary from both left and right ovary was weighed and suspended in physiological saline (0.65% NaCl), and oocytes were separated from follicle with the help of fine needle. The oocyte diameter (OD) (oocyte sample = 30; ± 1.0 μm) was measured from ovary of different maturity stages using ocular micrometer

2.5. Histology of gonad

After dissecting the fish, the gonad samples were collected from the middle portion of gonad and fixed in 10% neutral buffer formalin. Gonad section was later embedded in paraffin wax, cut at 5 mm and stained with haematoxylin and eosin (H & E) as described by Roberts (1989).

2.6. Sex steroid and vitellogenin assay

Sex steroid hormones such as testosterone (T), 17β-estradiol (E2) and progesterone (P) in serum were estimated with Cyman's hormone enzyme immunoassay kits item No. 582701, 582251 and 582601, respectively, following manufacturer's protocol. Vitellogenin (Vtg) was estimated by Biosense rainbow trout vitellogenin ELISA kit (Prod. No. V01004402) following manufacturer's protocol.

2.7. Statistical analysis

Mean values of all the parameters were subjected to one-way ANOVA and *post hoc* test in all the cases were carried out using Duncan's multiple range tests to determine the significant differences between mean values, if any. Comparisons were made at 5% probability level. All the statistical analyses were performed with SPSS 17.0 for windows.

3. Results

3.1. Macroscopic observation of gonad

The ovary is paired and lobular of unequal length and is of cystovarian type in which matured eggs is released into ovarian cavity during the ovulation. In the matured female, the left ovary is longer than the right one. Testis is bilobed composed of many lobules which are separated from others by thin layer of connective tissue. As maturation progresses colour of ovary changes from light pink (immature) to yellow (maturing I and maturing II) and finally to red (mature and ripe), whereas the testis colour changes from light reddish pink (immature) to creamy white (maturing I and maturing II) and finally to white (mature and oozing). Immature, maturing I and II, mature and ripe gonad occupy 1/3rd, 2/3rd, 3/4th and full of gonadal cavity, respectively.

3.2. Gonadosomatic index%, condition factor and oocyte diameter

The mean values of GSI, *K* and OD are presented in Table 1. The mean value of GSI and *K* was increased significantly ($p < 0.05$) with the advancement of maturity in both sexes and was maximum in ripe stage. The correlation between GSI and *K* was higher in male ($r^2 = 0.803$) than the female. ($r^2 = 0.478$). Increase in OD was observed from immature (120 ± 5.20 μm) to ripe stage (635 ± 7.30 μm) of gonadal development.

3.3. Microscopic observation of gonad, sex steroids and vitellogenin

Histological observation of ovary showed that immature ovary is rich in pre-vitellogenic oocytes (mean diameter = 120 ± 5.20 μm). Pre-vitellogenic oocytes include chromatin nucleolar oocytes which were characterized by scant cytoplasm and centrally located nucleus, and the perinucleolar oocytes were recognized by increased cytoplasmic volumes, larger nuclei (germinal vesicles) and multiple nucleoli in the periphery of the nucleus (Figure 1(A)). Maturing I and II stage of ovary were rich with early- and mid-vitellogenic oocytes, respectively (mean diameter = 230 ± 3.80 μm and 523 ± 3.56 μm, respectively). These vitellogenic oocytes were characterized by prominent lipid droplets/oil droplets and appearance of yolk granules with a distinct vitelline membrane beneath the follicular epithelium (Figure 1(B)). Mature ovary oocytes (mean oocytes diameter = 620 ± 3.80 μm)

Table 1. Value of GSI %, Condition factor (*K*) of different maturity stages of male and female *M. cephalus* and OD (μm) in female.

Parameters	Maturity stages				
	Immature	Maturing I	Maturing II	Mature	Ripe
Female					
GSI	$1.60^{c} \pm 0.11$	$10.53^{b} \pm 0.61$	$10.6^{b} \pm 3.51$	$11.17^{b} \pm 0.34$	$17.08^{a} \pm 2.31$
K	$0.88^{b} \pm 0.04$	$0.88^{b} \pm 0.02$	$0.90^{b} \pm 0.23$	$0.89^{b} \pm 0.12$	$0.92^{a} \pm 0.78$
OD	$120^{e} \pm 5.20$	$230^{d} \pm 3.80$	$523^{c} \pm 3.56$	$620^{b} \pm 3.80$	$635^{a} \pm 7.30$
Male					
GSI	$3.20^{c} \pm 1.61$	$3.54^{c} \pm 0.09$	$5.26^{b} \pm 2.46$	$6.48^{b} \pm 1.20$	$8.45^{a} \pm 5.9$
K	$0.763^{c} \pm 0.34$	$0.669^{c} \pm 0.50$	$0.814^{b} \pm 0.45$	$0.976^{a} \pm 0.23$	$0.972^{a} \pm 1.7$

Notes: Different superscripts (a, b, c and d) in the same row indicate significant difference ($p < 0.05$) amongst different maturity stages (Duncan's multiple range test, $\alpha = 0.05$). Values are expressed as mean ± SE ($n = 6$).

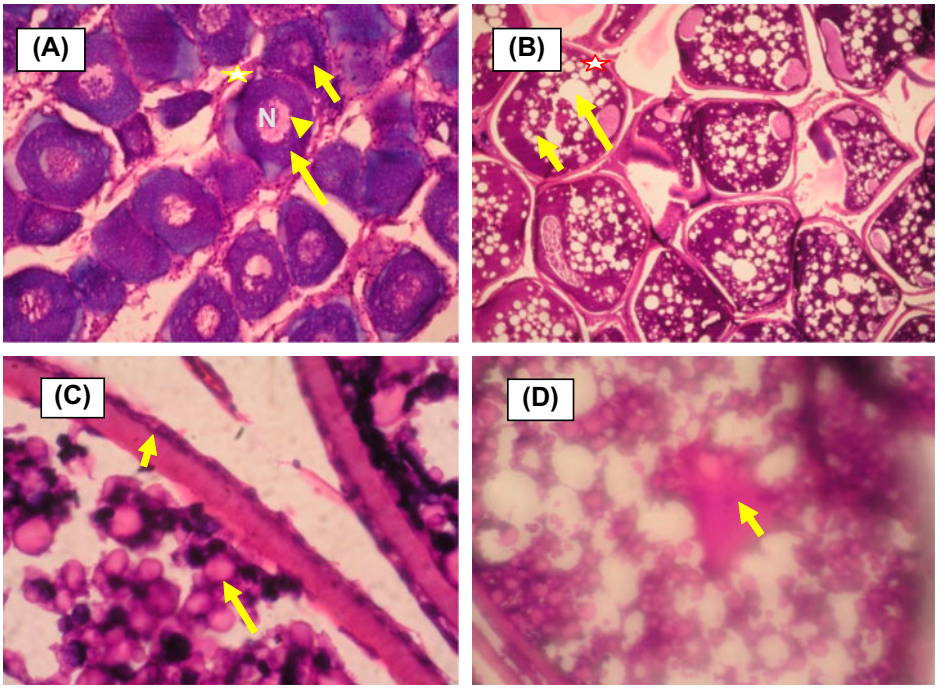

Figure 1. (Colour online) Light microscopic photograph of *M. Cephalus* ovary at different maturity stages. (A) Immature ovary rich in chromatin nucleolar oocytes, (small arrow); perinucleolar oocytes (large arrow) (arrow head showing nucleoli) and star showing follicle cells; 40×. (B) Vitellogenic oocytes showing lipid droplets (large arrow) and yolk granules (small arrow), star showing distinct chorion (vitelline membrane); 40×. (C) Mature ovary oocytes showingfusions of yolk granules (large arrow) and thickening of vitelline membrane (small arrow), 100×. (D) Ripe ovary oocyte showed clear nucleus (arrow) towards one side of the oocyte, 100× (N: Nucleus).

were characterized by fusion of yolk granules and thickening of vitelline membrane (Figure 1(C)). Ripe ovary oocytes (mean diameter = 635 ± 7.30 µm) have clear nucleus pushes to one side of the oocyte, and fusion of yolk granules and lipid droplets appears to varying degrees; however, no single large complete yolk mass can be observed (Figure 1(D)). Histological observation of testis showed that immature testis was in pre-spermatogenic stage and having only spermatogonia and spermatocytes (Figure 2(A)). Maturing testis (I and II) is in early-spermatogenic and mid-spermatogenic stage, respectively. Early-spermatogenic stage is characterized by the presence of predominantly spermatocytes and spermatids with few spermatozoa (Figure 2(B)), and mid-spermatogenic stage contained approximately equal numbers of spermatocytes, spermatids and spermatozoa (Figure 2(C)). Matured and ripe testis is in late-spermatogenic stage, which contains more spermatozoa (Figure 2(D)). Changes in serum levels of T, P and E2 in different stages of maturity of male and female are shown in Table 2. Serum T level was maximum (938 ± 7.87 pg/ml) during mature stage and minimum (312 ± 12.12 pg/ml) during ripe stage. E2 level increases significantly ($p < 0.05$) with advancement of maturation and reaches its peak during mature stage (323 ± 13 pg/ml) of ovarian development. Serum P level increased significantly ($p < 0.05$) as maturation advanced and was found maximum (488 ± 4.9 pg/ml) during the ripe stage of ovarian development. Serum Vtg protein level

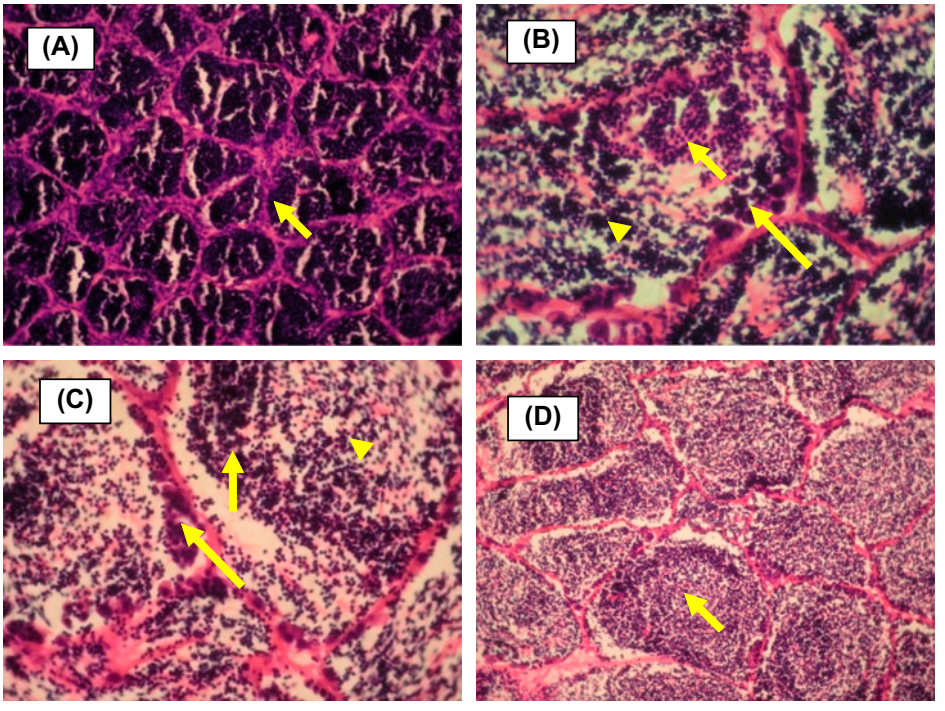

Figure 2. (Colour online) Light microscopic photograph of *M. cephalus* testis at various stages of spermatogenesis. (A) pre-spermatogenic testes contain spermatogonia and spermatocytes (arrow) (40×). (B) Early-spermatogenic testes containing predominantly spermatocytes (long arrow) and spermatids (short arrow) with few spermatozoa (arrow head) (100×). (C) Mid-spermatogenic stage contains approximately equal numbers of spermatocytes (long arrow), spermatids (short arrow) and spermatozoa (arrow head) (100×). (D) Mature and ripe testis has late-spermatogenic stage which contains more spermatozoa (arrow) (40×).

in different maturity stages of female mullet is shown in Table 2. Significantly highest value (367 ± 5.13 ng/ml) of vitellogenin was observed in mature fishes compared to other stages of development. Changes in serum levels of T, E2 and P in different maturity stages of male are shown in Table 2. The result revealed that the concentration of serum T in male increased gradually as maturity progresses and reaches its peak (1820 ± 40.25 pg/ml) at ripe stage; however, the trend of P and E2 was reversed.

4. Discussion

Reproductive system of *M. cephalus* includes a pair of gonads (ovary and testes), continued into a genital duct and ends in genital pore immediately behind the anus. The wall of the gonad is covered externally with a peritoneal layer. As maturation progress colour of ovary changes from light pink to red and colour of testis changes from light reddish pink to white. In case of female, left ovary was longer than the right ovary, which is similar to the observation made by Das et al. 2014. GSI is used as an indicator of the stage of ovary maturation and degree of ripeness (Htun-Han 1978). The GSI value of female was highly correlated with the OD ($GSI = 0.018 \times OD + 2.218$; $r^2 = 0.638$), which is similar to the finding of Kumar et al. (2014) in *Monodactylus argenteus* and

Table 2. Value of serum testosterone (T), progesterone (P), 17β-estradiol (E2) in different maturity stages of male and female *M. cephalus* and serum vitellogenin (Vtg) in different maturity stages of femle.

Parameters	Maturity stages				
	Immature	Maturing I	Maturing II	Mature	Ripe
Female					
T	$510^{d} \pm 2.03$	$628^{b} \pm 30.37$	$600^{b} \pm 23.76$	$938^{a} \pm 7.87$	$312^{c} \pm 12.12$
P	$78^{c} \pm 10.97$	$309^{b} \pm 4.93$	$367^{b} \pm 10.23$	$483^{a} \pm 6.36$	$488^{a} \pm 4.90$
E2	$25.34^{d} \pm 2.28$	$74.98^{c} \pm 4.8$	$186^{b} \pm 4.17$	$323^{a} \pm 13$	$210^{b} \pm 0.60$
Vtg	$40.02^{d} \pm 9.34$	$278^{b} \pm 13$	$286^{b} \pm 3.87$	$367^{a} \pm 5.13$	$181.67^{c} \pm 0.08$
Male					
T	$516^{d} \pm 6.56$	$734^{c} \pm 2.80$	$750^{c} \pm 70$	$914^{b} \pm 13.20$	$1820^{a} \pm 40.25$
P	$414^{a} \pm 6.69$	$130^{b} \pm 4.67$	$8.5^{c} \pm 1.33$	$2.7^{d} \pm 0.25$	$1.8^{e} \pm 0.14$
E2	$22.24^{a} \pm 40$	$14.16^{b} \pm 1.53$	$2.24^{c} \pm 1.2$	$1.2^{d} \pm 0.85$	$0.9^{d} \pm 1.40$

Notes: Different superscripts (a, b, c, d and e) in the same row indicate significant difference ($p < 0.05$) amongst different maturity stages (Duncan's multiple range test, $\alpha = 0.05$). Values are expressed as mean ± SE ($n = 6$). Units: pg ml^{-1} serum (T, P, E2); ng ml^{-1} serum (Vtg).

Das et al. (2014) in *M. cephalus.* We also observed that the mean value of GSI in female was higher than that of male and this may be due to the allocation of higher proportion of body reserve towards the development of ovary than testis, which is in accordance to the earlier findings in *Lethrinus nebulosus* (Mahmoud 2009). The condition factor (*K*) is a measure of energy reserves in fish and it changes with inter-annual variations and seasonal cycles (Lambert & Dutil 1997). In the present study, *K* values in both the sexes increased significantly ($p < 0.05$) with the advancement of sexual maturity, which is similar to the observation made by Gopalakrishnan (1991) in *M. cephalus*. Pre-vitellogenic oocytes include chromatin nucleolar oocytes and perinucleolar oocytes. Maturing I and II stage of ovary were rich with vitellogenic oocytes, which take up the hepatically derived vitellogenin under the influence of E2 (Patiño & Sullivan 2002). Mature ovary oocytes are characterized by fusion of yolk granules and thickening of vitelline membrane, which indicate the end of vitellogenesis. This observation is in accordance to the observation made by Shabanipour and Heidari 2004 in *Liza aurata*. Ripe ovary oocytes have amoeboid-shaped nucleus pushes to one side of the oocyte that indicates the germinal vesicle breakdown (GVBD) and FOM. Overall histological observation of ovary showed that ovarian development in *M. cephalus* is of group synchronous type where one clutch of oocyte matures annually with single annual-spawning episode which is in accordance to the finding of Wallace and Selman (1981). From the positive correlation between serum E2 and GSI ($r^2 = 0.362$), it could be inferred that during the phase of ovarian growth a larger ovary secretes more E2 than a smaller one, thereby confirming a role of E2 in vitellogenesis as reported in other teleost (Shafiei Sabet et al. 2009). In the present study, trend of Vtg concentration in serum was found to be similar to that of E2 and both are correlated to each other ($r^2 = 0.533$). These results suggest that the hormonal stimulation of vitellogenesis in *M. cephalus* is similar to that of other teleost. A sudden drop in the plasma E2 level from vitellogenic (mature) to post-vitellogenic (ripe) stage may be explained in terms of switching off the aromatase (CYP19) activity as oocytes progressed to final maturation that is parallel to the observation made by Das et al. 2014 in *M. cephalus*. A similar trend of T suggested that T was the precursor for E2 in *M. cephalus,* akin to the situation for another teleost such as medaka *Oryzias latipes* (Kobayashi et al. 1996) and Persian sturgeon *Acipenser persicus* (Nazari 2010). It is known that P is precursor of C21 steroid (Scott et al. 1983) and it includes 17α, 20β-dihydroxy-4-pregnen-3-one(17α, 20β-DP), 17α, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S), 20β dihydroprogesterone and 11-deoxycorticosterone (DOC) which are maturation-inducing steroid and induces GVBD FOM (Nagahama & Yamashita 2008). In the present study, we found that P increased progressively as maturation progresses in female and was found to be the maximum during ripe stage, which is indicated in histological slide of ripe ovary as shifting of nucleus towards animal pole similarly. The synthesis of male hormones occurs in Leydig cell and the major androgens produced by testicular tissue are T, 11-Ketotestosterone (11-KT) and androstenedione (Mylonas et al. 1998). The T has an effect on spermatogenesis such as spermatogonial multiplication and spermatocyte formation (Billard et al. 1982). The result revealed that the concentration of serum T in male increased gradually as maturity progresses and reaches its peak at ripe stage, which is in accordance to the observation of Zaki et al. 1995 in *M. cephalus*. The elevated T level may be responsible for the release of mature spermatozoa from their cysts at ripe stage, which is same as observation made by Barry et al. 1990. GSI follow the similar trend and is highly correlated to serum T level ($r^2 = 0.823$) and is similar to the finding of EL-Halfawy et al. 2007 in *Liza ramada*.

The present study from wild caught grey mullet could provide clue to accelerate/ regulate the maturation process in grey mullet through administration of exogenous hormone in captivity.

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Disclosure statement

There is no conflict between the authors regarding the research data presented in the paper.

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