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Effect of salinity on serum steroid levels and gonad development in common carp (*Cyprinus carpio* Linnaeus, 1758) reared in inland saline water

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Abstract

The effect of salinity on serum steroid levels and gonadal development were investigated in the common carp *Cyprinus carpio* Linnaeus, 1758. Fishes were stocked in eight rectangular earthen ponds at four different salinities of 0, 5, 10 and 15 ppt. Changes in serum concentration of cortisol, 17 β-Estradiol, progesterone; 17α, 20β dihydroxyprogesterone and androgens were investigated. Gonadal histology in male and female *C. carpio* was studied at different stages of gonadal maturity till ovulation or spermiation. Serum steroid analysis showed significantly high levels ($p<0.05$) in fish groups reared at 0 and 5 ppt compared to 10 and 15 ppt. Significantly higher cortisol levels were found during ovulation or spermiation at 10 and 15 ppt. In the present study, *C. carpio* was found to mature faster at 5 ppt as compared to higher salinities of 10 and 15 ppt, in inland saline water.

Introduction

Salinisation of land and groundwater is a common problem in India and an area of 8.62 million ha has been reported to be affected by soil salinisation (Lakra *et al.*, 2014). Salinisation is caused by both natural phenomena as well as by anthropogenic activities (Williams, 2001; Bennetts *et al.*, 2006; Bal *et al.*, 2021), poses a serious challenge for agriculture, especially aquaculture (Paital and Chainy, 2012). Conversely, these saline water reserves could be potentially used for the development of inland saline aquaculture. Techniques have been developed for the culture of commercial marine and brackishwater species, such as *Penaeus vannamei* (Jahan *et al.*, 2018) as well as a few freshwater species, such as *Heteropneustes fossilis* in inland saline waters (Bal *et al.*, 2021). Fish reproduction is generally influenced by environmental factors such as salinity, photoperiod, temperature and rainfall (Zohar *et al.*, 2009; Malik *et al.*, 2018; Iffat *et al.*, 2020). Salinity may significantly regulate reproductive activity and act as a limiting factor during gametogenesis stages (Haddy and Pankhurst, 1998; Pham, 2010). Common carp (*Cyprinus carpio* Linnaeus, 1758) is the second most important species in European freshwater aquaculture, with a production level of 164,755 t (FAO, 2020) and the fifth most cultured species in the world (FAO, 2020), contributing to 8% of total aquaculture production. Market value of the species is in the range ₹130-180/-regionally; ₹150-200/-nationally and US$ 3.13-3.24 internationally (FAO, 2020). It belongs to Cyprinidae family and the testicular and ovarian reproductive structures matures 2 times annually in tropical and subtropical areas of India, with peaks in January-March and July-August (FAO, 2013). However, there is no information on the effects of salinity on reproductive hormonal profile of *C. carpio* reared in inland saline water (ISW).

Teleost reproduction is regulated by the endocrine system through hormonal secretion from the hypothalamus-pituitary-gonad axis and controlled

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by environmental factors such as salinity, photoperiod and temperature (Nagahama *et al.*, 1997; Modesto and Canario, 2003). The key factors which regulate the reproductive mechanism are the secretion of gonadal steroids and gonadotropins (Barannikova *et al.*, 2004). Steroid hormones exert their stimulatory or inhibitory effects on the gonadotropins either directly or *via* the hypothalamus (Levavi-Sivan, 2005). Gonadal steroids such as 17 β-Estradiol (E_2); Progesterone; 17α, 20β Dihydroxyprogesterone (17α, 20β DHP), 11-ketotestosterone (11-KT) and Testosterone (T) regulates the reproductive activities (Kime, 1993). E_2 is a female-specific steroid hormone that regulates the vitellogenesis. After vitellogenesis, progestins such as Progesterone and 17α, 20β DHP promotes final oocyte maturation and ovulation, as the production of E_2 decreases (Nagahama, 1997). Testosterone may regulate gametogenesis in both males and females, but 11-KT specifically helps in the initiation of spermiation (Kime and Manning, 1982). During spermiation, the levels of androgens fall due to reduced conversion of 17b-hydroxyprogesterone into androgens and a shift in the steroidogenic pathway to the formation of the progesterone and 17α, 20β DHP (Yaron, 1995). Based on the above information, it could be assumed that salinity may affect the reproductive development of *C. carpio* in inland saline areas. Therefore, in the present study, the effects of salinity on reproductive activity of *C. carpio* were assessed in terms of serum 17 β-estradiol, progesterone; 17α, 20β-dihydroxyprogesterone, 11-ketotestosterone and testosterone levels at regular intervals.

Materials and methods

Experimental animal

Common carp (*C. carpio*) fingerlings (n= 840) having an average weight of 32 ± 2.5 g (2.5 months old) were procured from Sampla, Haryana, India. Following standard protocols, experimental fish were transported to the farm of ICAR-Central Institute of Fisheries Education (ICAR-CIFE), Rohtak, Haryana, India. Inland saline water of four different salinities (0, 5, 10 and 15 ppt) was prepared following the protocol of Raizada *et al.* (2015). Inland saline water of 15 ppt was pumped directly from a bore well. The water was then diluted with freshwater to achieve a salinity of 5 and 10 ppt. Freshwater was directly pumped into the pond (control groups) from a bore well. At the experimental site, fishes were acclimatised to different salinities (*viz*. 5, 10 and 15 ppt) in 1200 l capacity fibre reinforced plastic (FRP) tanks for 2-3 weeks by shifting the salinity at the rate of 2.5 ppt per 3 days with sufficient aeration. The experimental fish were then maintained in the respective salinities for the subsequent 7 days. During acclimatisation and the experimental period, no mortality was observed in any of the treatments.

Experimental design and maintenance

The experiment was conducted in eight uniform non-drainable rectangular earthen ponds (n=8) with a dimension of 21x10x1.50 m which comprised four treatment groups, *viz*. 0 (C), 5 (T1), 10 (T2) and 15 ppt (T3) in duplicate for 90 days. Inland saline water having salinity of 15ppt was mixed with freshwater to get the desired salinitiesof 5 and 10 ppt which were then held constant and were checked daily with a refractometer (ATAGO-S/Mill-E $0\text{-}100^0/_{00}$, Japan). Throughout the period of the experiment, the water quality parameters such as temperature (29-31°C), dissolved oxygen (6.58-6.75 mg l^1), pH (7.46-7.85), hardness (505-2451 mg l^{-1}), magnesium (86.66-490.88 mg l^{-1}), potassium (3.45-9.08 mg l^{-1}), sodium (396.53-2595 mg l^{-1}), calcium (56-820 mg l^{-1}), alkalinity (206.66-318.66 mg l^{-1}) and total ammonia-nitrogen (0.068-0.1 mg l^{-1}) levels were maintained at optimal levels. Fish were stocked at a density of 5000 nos. ha^{-1} in each pond and fed with commercial fish feed (CP AQUA, India) having crude protein content of 32%, at a rate of 2% of the body weight twice a day.

Sampling

Sampling of the experimental fishes were carried out using a drag net at regular intervals of 15 days. Maturing/mature fish (n =6) were sexed as males when white milt oozes out from genital opening and as females based on presence of swollen belly. All fish handling and treatment procedures were approved by the ethics and animal care committee of ICAR-Central Institute of Fisheries Education (ICAR-CIFE), Mumbai, India. Blood samples were collected from randomly selected male and female fish, from each treatment group, on 15, 30, 45, 60, 75 and 90 days of experimental period. Fish were anaesthetised and blood was collected from the caudal peduncle region using a sterile disposable syringe and needle (no. 23). The blood samples collected were kept for clotting at room temperature, centrifuged at 3000 *g* for 10 min at 4°C, serum was collected and stored at -20°C in Eppendorf vials unttil analysis for steroid hormones. Tissue samples of ovary/testis were also collected from the sampled fishes and fixed in 10% formalin, for histological studies. The histological sections of ovary and testis were prepared according to Bancroft and Stevens (1990). The tissues were dehydrated in alcohol, cleared in xylene, embedded in paraffin wax at 56°C, sectioned at 5-7 µm thickness and the sections were stained by Hematoxylin and Eosin (H & E). The DPX-mounted slides were observed under a light microscope (FSX 100, Olympus, Japan).

Hormonal assays

Cortisol was assayed in 50 µl of fish serum employing a competitive enzyme-linked immunosorbant assay kit (My BioSource Company, USA) following the manufacturer's instructions. The lower limit of detection was 10.65 ng ml^{-1}. 17 β-Estradiol (E_2) level in the serum samples was estimated using estradiol Elisa Kit (Cayman Chemical Company, USA), having lower detection limit of 0.89 ng ml^{-1}. For estimation of progesterone level in serum samples, Elisa Kit which works on AChE competitive binding enzyme linked immunoassay format

(Cayman Chemical Company, USA) with a lower detection limit of 0.94 ng ml^{-1} was used. 17α, 20β-dihydroxyprogesterone (17α, 20β DHP) level in serum samples was estimated using 17α, 20β DHP Elisa Kit (Cayman Chemical Company, USA) having lower detection limit of 0.09 ng ml^{-1}.

Testosterone (T) and 11-Keto testosterone (11-KT) levels were estimated using Elisa Kit (Cayman Chemical Company, USA) with a lower detection limit of 0.26 ng ml^{-1} and 0.23 pg ml^{-1} for T and 11-KT, respectively.

Statistical analysis

The data were analysed using two-way ANOVA in Microsoft Excel 2021, to determine the effect of varying water salinity on serum steroid hormone levels at different time intervals. The probability of establishing statistical significance was $p<0.05$.

Results

Effect of salinity on serum steroid levels

Female *C. carpio* underwent oocyte maturation after 30 days (June) of the experimental period and ovulation occurred 3 months later (August) during the trial (Fig. 1).

Mean serum 17α, 20β DHP level gradually increased as the maturation stage advanced, however, in fish held at 0 (4.5±0.1) and 5 ppt (5.35±0.45 ppt), high levels were

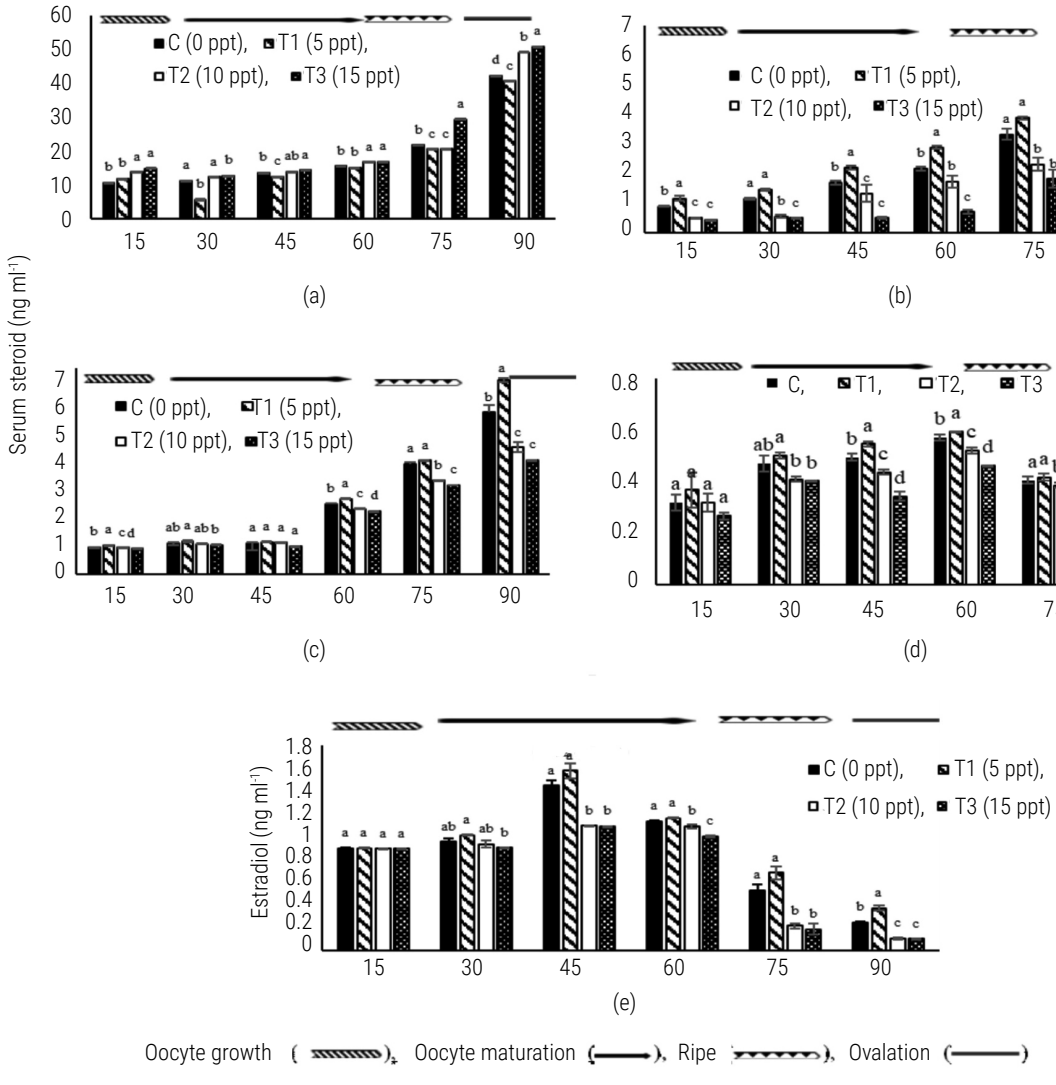

Fig. 1. Effect of salinity on serum steroid levels in maturing female *C. carpio* during the 90 days experimental period. (a) Cortisol, (b) 17α, 20β DHP, (c) progesterone, (d) Testosterone, (e) 17 β-Estradiol. Treatments bearing different alphabets differed significantly

found compared to other treatments (Fig.1b). Similarly, progesterone levels were found to be highly elevated during ovulation time after 90 days of the experimental period in treatment groups (Fig. 1c).

Serum testosterone levels were higher during the oocyte maturation stage and lowest during the ovulation stage (90 days) (Fig.1d) 17 β-Estradiol (E_2) concentration in serum increased during oocyte maturation (Fig.1e). Serum levels of all hormones analysed in the maturing females of *C. carpio* were found to by significantly influenced ($p<0.05$) by varying salinity.

Testosterone (T) levels were significantly high in maturing males reared in treatment C (0.61± 0.01) and T1 (0.66 ±0.001) (Fig. 2d).Serum 11-KT levels were low before spawning during the final maturation stage (Fig. 2e). Similarly, 11-KT levels were significantly high in 0 ppt (0.95±0.01) and 5 ppt (1.40±0.61) salinity levels.

Cortisol level was significantly high (59.3±2.88) for fish in T3 group, as compared to other treatment groups (Fig. 2a). Serum 17α, 20β DHP (0.009-0.225 ng ml^{-1}) and progesterone (0.94-1.07 ng ml^{-1}) levels were very low in males during developing stage (Fig. 2b, c). Still, a significant ($p< 0.05$) increase in the levels of serum 17α, 20β DHP levels in 0 and 5 ppt occurred during the spawning period. A similar result was also found for progesterone level. Serum hormone levels analysed were found to be significantly different ($p<0.05$) at varying salinities tested, in maturing male *C. carpio.*

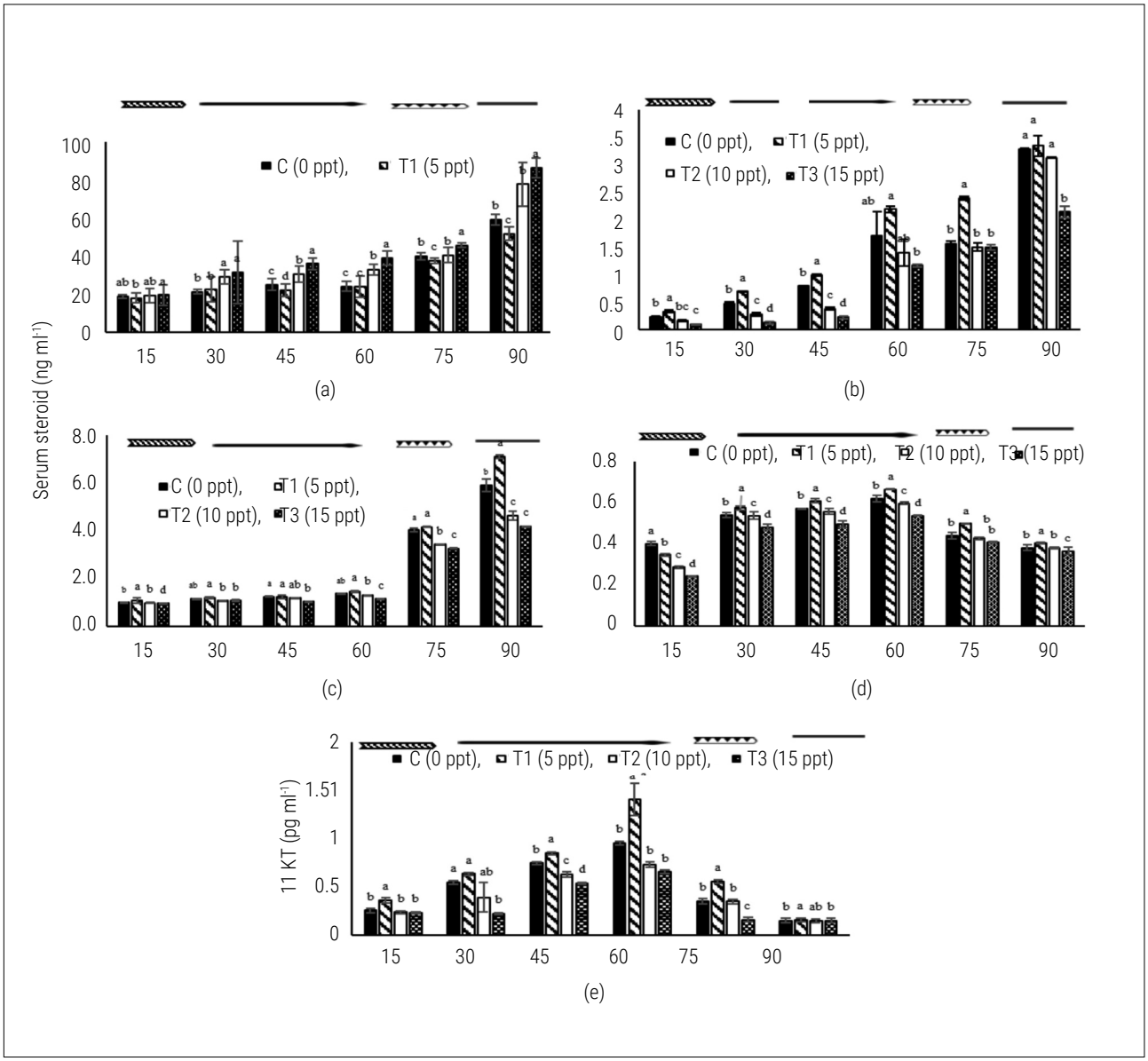

Fig. 2. Effect of salinity on serum steroid levels in maturing male *C. carpio* during the 90 days experimental period. (a) Cortisol, (b) 17α, 20β DHP, (c) progesterone, (d)Testosterone, (e) 11-ketotestosterone. Treatments bearing different alphabets differed significantly ($p<0.05$)

Histological architecture of female gonads

Six maturation stages of female gonads were observed based on histological structure

Stage 1 (Immature): Ovaries were small in size and tightly packed within the ovarian matrix. Oocytes were not visible to the naked eye.

Stage II (Developing): Oocytes with large central and irregular nucleus (n) with basophilic cytoplasm, which is the pre-nucleolar stage (PO). Started to develop after 30 days of experimental period in all treatment groups (Fig. 3d). Oocytes were found to be surrounded by inner granulosa and outer thecal cells formed by the follicular layer. This is known to be returning stage, because, after spawning, the ovaries return to this stage to start oogenesis, however; it occured after 90 days of experiment during ovulation (Fig. 3b).

Stage III (Maturing): Initial period (30 days) of the trial in all treatments, ovary size increased with formation of cortical alveoli (ca) in the periphery of the cytoplasm. The appearance of zona radiata (Zr) surrounded by follicular and thecal layers (Fig. 3a, 3c)

Stage IV (Mature or Ripe): After 45 days of the experiment, majority of the oocytes reared in varied salinities reached the yolked stage (vitellogenesis), where gonad size reached its maximum and the cytoplasm was densely filled with yolk granules (yg) and cortical alveoli. A decrease in the size of nucleus was observed (Fig. 3c).

Stage V (Spawning): Initiation of ovulation (75 days), which is indicated by germinal vesicle migration towards the animal pole or germinal vesicle breakdown (GVBD), which were mainly observed in 0, 5 and 10 ppt and characterised by dense yolk granules and blood vessels in the cytoplasm. The zona radiata became thin and separated from the follicular layers (Fig. 3a).

Stage V1 (Spent): At the end of the trial (90 days) where oocytes had an abundance of peri-nucleolar oocytes and suddenly dispersed among abundant post-ovulatory follicles (POF). Atretic oocytes were present throughout the ovarian matrix (Fig. 3b).

Effect of salinity on ovary development

Most of the fish held at 0 and 5 ppt, matured early as compared to those held at 10 and 15ppt. At the end of the experimental trial of 90 days, post-ovulatory follicles (POF) and atretic oocytes were observed in the ovaries (Fig. 3a and 3d)

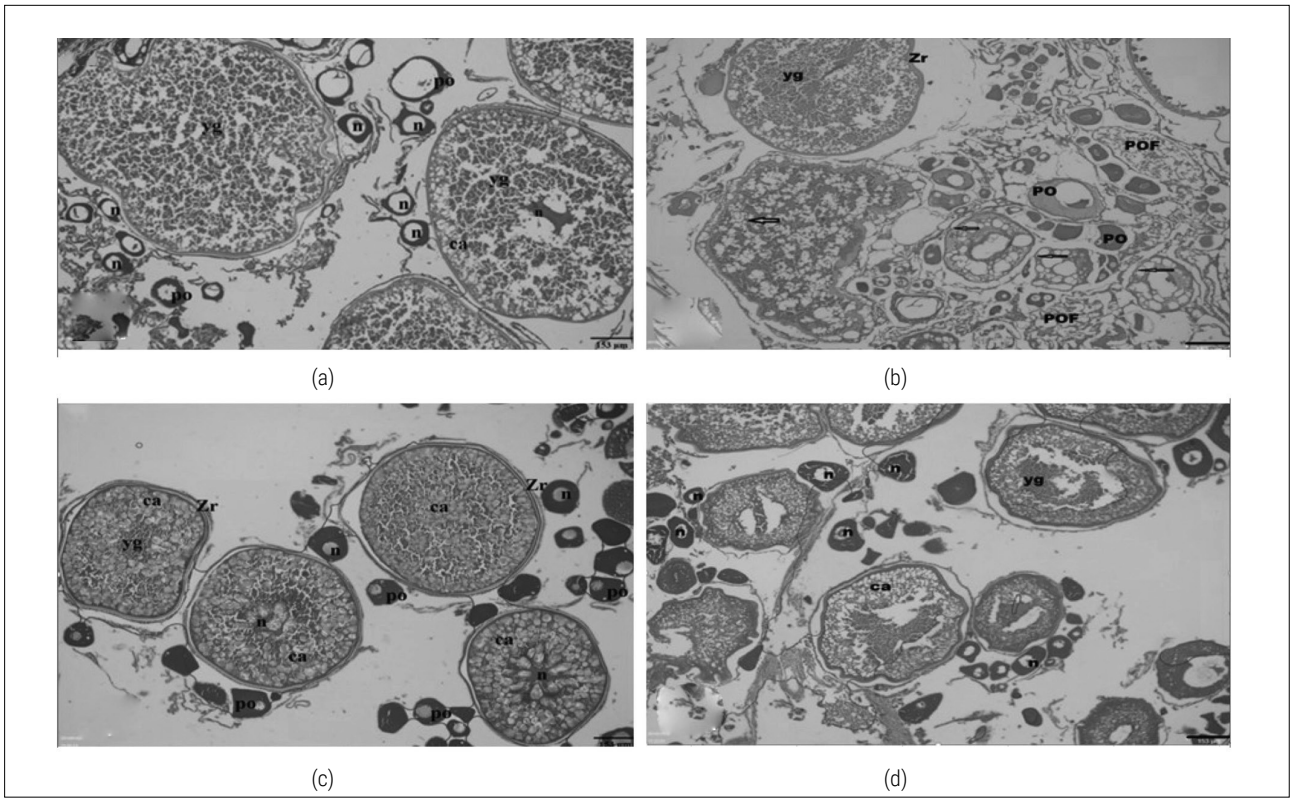

Fig. 3. Histological sections showing the maturation stages of common carp oocytes of different groups, (a) Control, (b) Treatment T1, (c) Treatment T2 and (d) Treatment T3. Perinucleolar stage shows perinucleolar oocytes (po) with large central nucleus (n), formation of cortical alveoli (ca) and pink stained zona radiata (zr) are evident. During final maturation stage, cytoplasm is densely filled with yolk granules (yg), cortical alveoli, nuclear migration stage, ovulated oocytes, abundance of peri-nucleolar oocytes and post-ovulatory follicles (POF). Arrows show yolk plates. Scale bar =153 µm.

Histological architecture of male gonads at different maturation stages

Testes were histologically classified in to five maturation stages.

Immature (Stage I): After 30 days of the rearing period, testis of fishes from all treatment appeared as thread-like, thin and whitish cream in colour. Spermatogonia and primary spermatocytes were found to be dominant (Fig. 4c).

Developing (Stage II): Spermatogonial cells were dominant and cysts of spermatocytes were also visible in the tubules (Fig. 4d).

Developed (Stage III): With the progress of maturation (45-60 days) the testes became elongated with the appearance of dark cream colour. Seminiferous tubules were found to be large and separated by interstitial tissue. Higher numbers of secondary and primary spermatocytes were present with very little spermatids and sperms (Fig. 4b).

Repining (Stage 1V): After 75 days of the rearing period, testis became large in size with plenty of sperm and spermatids but very few secondary spermatocytes were observed with well-developed interstitial cells. Milt was easily stripped by applying gentle pressure (Fig. 4a).

Spent (Stage V): After 90 days of the rearing period, size of the testis got reduced. There were very few sperm seen in the tubules as the fishes spawned. Milt was running more easily as in the former stage by applying gentle pressure at the abdominal region of the fish. Testis appeared flaccid because of the expulsion of sperm during the spawning period (Fig. 4b).

Effect of salinity on development of testis

Results of the study clearly indicated that male gonads of *C. carpio* started developing after 30 days of rearing in all the treatment groups. However, majority of the fish held at 0 and 5 ppt matured significantly faster as compared to fishes maintained at 10 and 15 ppt.

Discussion

Plasma cortisol is a steroid hormone that indicates stress in fishes, resulting from changes in season, reproductive stages and other factors (Bayunova *et al.*, 2002). In the present study, significantly elevated levels of plasma cortisol in post-ovulated and spermiated mature females and males respectively were noticed. A similar result was reported by Westring *et al.* (2008) who observed cortisol as the leading physiological event in post-spawning Pacific salmon.

17α, 20β-DHP and progesterone are maturation-inducing hormones, produced from follicle cells in females and Leydig

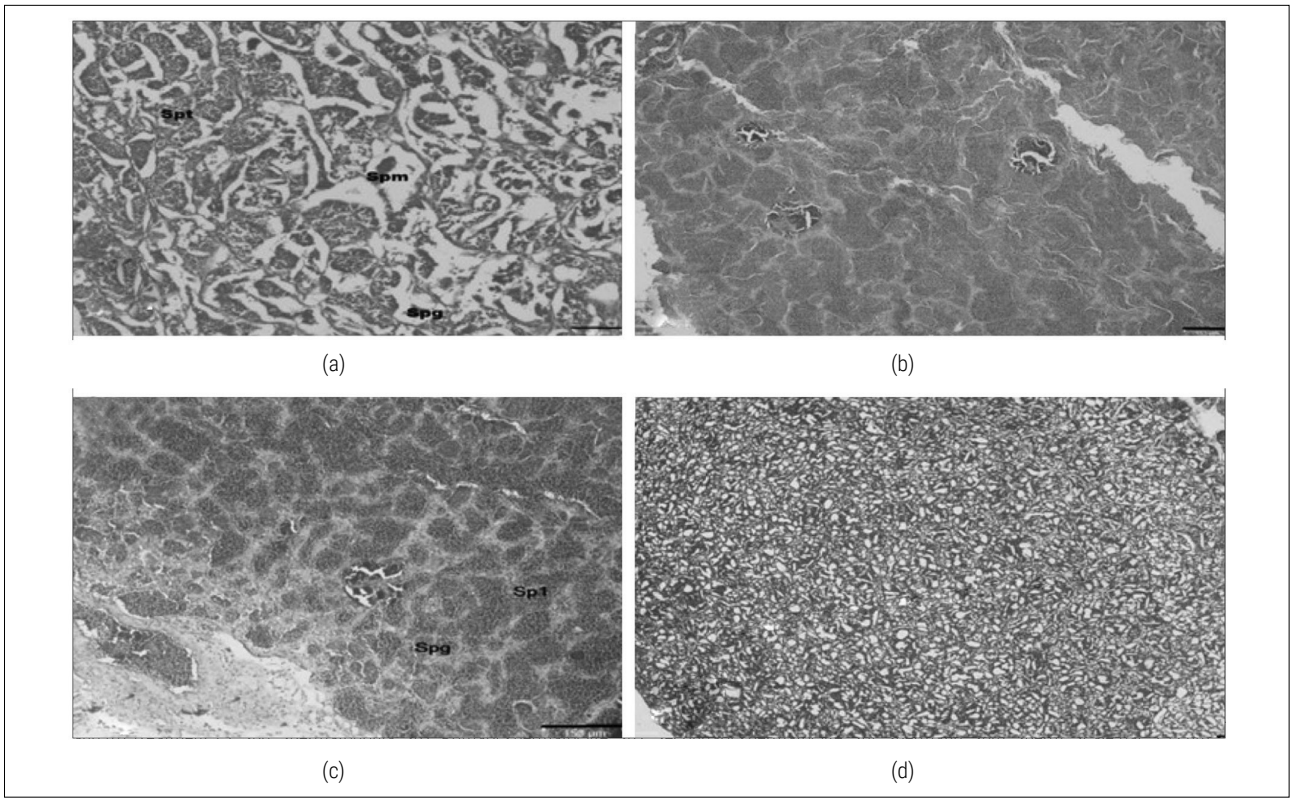

Fig. 4. Histological sections showing the maturation stages of common carp testes of different groups, (a) Control, (b) Treatment T1, (c) Treatment T2 and (d) Treatment T3. Spg: Spermatogonia, Sp1: Primary spermatocyte, Sp2: Secondary spermatocyte, Spt: Spermatid, Spm: Sperm. Scale bar=153 µm

cells in males under gonadotrophic releasing hormone (GtH) stimulation, which plays an integral role in final oocyte or testicular maturation that subsequently accelerates ovulation and spermiation during spawning season (Nagahama, 1997). The peak levels of these hormones were observed during final maturation and spawning in both the sexes at 0 and 5 ppt salinity when compared with 10 and 15 ppt. The physiological relevance of this finding is currently unclear because no study has so far been conducted in *C. carpio* reared in inland saline water. Miura *et al.* (2007) reported that 17α, 20β-DHP induces meiosis during spermatogenesis and oogenesis in *Hucho perryi* and *C. carpio*.

Plasma testosterone levels change with variation in salinity and progress of reproductive stages in both sex (McCormick and Saunders, 1990) Results of the present study suggests that testosterone is involved in gonad maturation which occurred prior to the production of maturation-inducing steroids. These findings agree with previous observations in teleost fishes (Webb *et al.*, 2002).

11-KT is an active male-specific androgen in teleost fishes that is associated with the development of testis and sexual characteristics (Patino and Sullivan, 2002) and it is a precursor of testosterone (Borg, 1994; Yaron, 1995). The level of 11-KT was low in developing and post-spawning males. It significantly increased in maturing individuals reared at 5 ppt salinity which is in accordance with the fact that 11-KT is critically responsible for the initiation of spermiation but falls sharply just after spawning (Kime, 1993; Barannikova *et al.*, 2004; Pham *et al.*, 2010).

E_2 is a universal female reproductive steroid hormone , the levels of which rise during vitellogenesis and decrease during the final oocyte maturation stage due to the production of progestins such as 17α, 20β DHP and progesterone in many teleost species (Barannikova *et al.*, 2004). It helps maintain the optimum osmoregulation mechanism in fish (Carrera *et al.*, 2007). During the experimental period, E2 levels in plasma showed significant differences among the various treatment groups and reproductive stages. The levels of E2 was closely similar to 11-KT; however, fishes (4 months old) reared at 0 and 5 ppt recorded higher levels during maturing stage (45 days old), which decreased during ovulation. E2 levels increased during vitellogenesis and decreased in stage III and IV and exhibited variation due to salinity changes (Tyler and Sumpter, 1996; Haddy and Pankhurst, 2000; Barannikova *et al.*, 2004).

The present study demonstrated significant effect of salinity in the developmental stages of the gonads, evidenced by the histo-architecture of gonads. In the current experiment, gonadal development was found to be significantly faster in the lower salinity levels (freshwater and 5 ppt) compared to higher salinity levels of 10 and 15 ppt. Similar results on the effect of salinity variation on sexual maturity have been reported in *Acanthopagrus butcheri* (Gray *et al.,* 2012), *Crassostrea gasar* (Paixao *et al.,* 2013) and *C. mandica* (Esmaeili, 2017).

This study has shown that *C. carpio* adapts well to inland saline water up to 15 ppt salinity. Under these circumstances, reproductive development usually continues and was found to be unaffected over a salinity range from 0 to 5 ppt Results of the study suggests that 0 and 5 ppt salinity had the best reproductive performance compared to salinity levels of 10 and 15 ppt. It is concluded that the optimum salinity for *C. carpio* culture in inland saline water is 5 ppt Further study is needed to prove that *C. carpio* is a potential candidate for expanding inland saline aquaculture.

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