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The onset of reproductive bioindicators of young Amur common carp (*Cyprinus carpio haematopterus*) during different seasons in the Tarai region of Uttarakhand, India

N Soranganba and IJ Singh

Abstract
The onset of reproductive bioindicators differ with age, sex and species. The objective of the study was to study the level of reproductive biomarkers in young Amur common carp using some of the reproductive biomarkers during different seasons. Using RP-HPLC and analytical kit methods, steroidal hormones like testrosterone, estradiol, DHP and cortisol and biochemical parameters like total protein, cholesterol, triglycerides, HDL, LDL, VLDL and phospholipids were analysed in 0+ year age Amur common carp, *Cyprinus carpio haematopterus*. It was found that plasma testosterone and cortisol levels were detected in summer, which declined in autumn and continued to decline to the lowest level in winter season. Plasma E2 and DHP levels were detected only in winter season. Serum CHO, TG, HDL VLDL and PHO levels showed an increasing trend from summer season to autumn being at the lowest level in winter season. Thus, the onset of maturity seems to have been initiated during winter season after 5-6 months in Amur common carp.

Keywords: amur common carp, biomarkers, season

Introduction
Fishes undergo several changes from the time of hatching through larval stages until the attainment of adulthood and many physiological changes occurred during sexual maturation and their ability to reproduce may vary between the first timers (pubertal) and adult that has bred already. Age plays an important role in reproduction of fishes with intra- and inter-specific variations (Bagenal, 1969) [5]. Okuzawa (2002) [24] defined puberty as process by which an immature animal acquires the capacity to reproduce for the first time. Onset of maturity in fishes is important as some take several years before onset of puberty while precocious maturations are observed in species like common carp. The reproductive cycle of teleost fishes is controlled by the endocrine system under the influence of seasonal environmental stimuli (Munro, 1990) [23]. Seasonal especially temperature plays a greater role in the timing of reproductive cycles in common carp. Fish of different age groups and sexes show marked alteration in terms of their hormonal, biochemical and physiological responses.
The physiological processes involved in the gonadal development of fishes vary according to the taxonomic status and reproductive process of the species (Balon, 1975) [6]. Okuzawa (2002) [24] observed an increase in the production and release of gonadal sex steroids during spawning period and speculated that the onset of maturity could have involved the brain-pituitary-gonadal (BPG) axis and secretion of GnRH and FSH in responses to environmental conditions. Similarly, Taranger *et al.,* (2010) [34] also expressed about activation of BPG axis by factors such as growth, adiposity, feed intake, temperature etc. In teleosts, the initiation of puberty occur following gonadal sex differentiation (Strüssmann and Nakamura, 2002) [32] characterized by the onset of spermatogenesis in males (Schulz and Miura, 2002) [28] and vitellogenic oogenesis in females (Patiño and Sullivan, 2002) [25]. Sex steroids have been observed as the natural inducers for the onset of maturity in male African catfish (Schulz and Goos, 1999) [27]. Dufour *et al.,* (1999) [12] suggested that sex steroids might act an 'amplifier' but not as activator for the onset of maturity by positive feedback mechanism. Shearer and Swanson (2000) [30] observed that the maturation of Chinook salmon (*Oncorhynchus tshawytscha*) was initiated in males approximately a full time last year prior to the fish will spawn. Maturity may depend on exceeding thresholds of growth rate, body size, lipid stores or rate of lipid storage, stage of gonadal development or a combination of such factors

(Taranger *et al.*, 1999) [35]. Hulata, *et al.*, (1974) [19] described about the effect of age differences in two races of *Cyprinus carpio* (European and Chinese races). Eenennaam and Doroshov (1998) [13] observed that the gonadal development in females takes a longer time, compared with males in Atlantic sturgeon. Aliniya *et al.*, (2013) [1] observed age-dependent differences on the reproductive performance of brooders of two age groups male and female common carp. Apparently, no reports are available about physiological changes occurring prior or at the time of onset of first maturity in younger specimens of Amur common carp, *Cyprinus carpio haematopterus*. The objective of the study was to study the level of reproductive biomarkers in young Amur common carp using some of the reproductive biomarkers during different seasons.

Materials and Methods

Maintenance of experimental specimens

Amur common carp (*Cyprinus carpio haematopterus*) specimens of 0+ year age group obtained from Instructional Fish Farm (IFF) of the College of Fisheries, G. B. Pant University of Agriculture & Technology, Pantnagar (Uttarakhand) was held in earthen pond and later sampled for analysis. Fish were fed once daily @ 3% body weight with supplementary pellet floating feed containing 25% protein.

Experimental site and climatic conditions

The experimental site is situated at IFF inside the Experimental farm facility of College of Fisheries, G.B. Pant University of Agriculture & Technology, Pantnagar (Uttarakhand) located at the latitude of 29.01°N, longitude 79.3°E, 344 metres above mean sea level (MSL) at Tarai region, The Shivalik range of the Himalayas. The region has a sub-tropical type of climatic with very hot, humid and dry summer, monsoon and very cold winter.

Sample collection, schedule and anaesthesia

Samples for 0+ year's age group were collected only from pond B during summer, autumn and winter seasons. Clove oil @ 30 mg/l (Velisek *et al.*, 2005) [36] was used to anesthetize the fish prior to regular handling or experimental procedure of the specimens. After collection of specimens from experimental ponds, the fish were anesthetized using clove oil for collection of blood for hormonal and biochemical assay. Caudal amputation for 0+ year's age group was carried out and blood samples were collected and pooled within 5-10 mins (as far as possible). The blood drawn was dispensed into a lithium heparin coated plasma tubes for hormonal estimation and another into normal serum tubes for biochemical analysis. The heparinized blood was centrifuged at 10,000 rpm (11180 x g) for 12 mins at 4^0C and the supernatant was collected in 2 ml microcentrifuge tubes and analyzed immediately or stored at below -20^0C sealed with parafilm till analysis. Similarly, the whole blood sample for the biochemical study was allowed to clot for 15 - 30 mins at normal room temperature and centrifuged at 4000 rpm (1788 x g) for 10 mins at 4^0C and the supernatant was collected in 2 ml microcentrifuge tubes and analyzed immediately or stored at below -20^0C sealed with parafilm till analysis.

Observations on water quality parameters

Water quality parameters of the two experimental ponds which included water temperature, total dissolved solids (TDS), pH, dissolved oxygen (DO) and free carbon dioxide (free CO_2) were recorded during all the four sampling seasons. Temperature, TDS (accuracy ±2%) and pH (accuracy 0.01 pH) were measured using a digital meter whereas DO and CO_2 were analyzed using titrimetric method (APHA, 1992).

Hormonal and Biochemical Estimation

Estimation of the steroidal hormones – Testosterone (T), Estradiol (E2), 17α, 20β-dihydroxy-4-pregnen-3-one (DHP), cortisol in blood plasma were carried out using reversed phased high-performance liquid chromatography (RP-HPLC) of Dionex Ultimate 3000, operated by Chromeleon software (version 6.8). Chromatographic condition including validation, quantitation, linearity of the assay, accuracy, stability, repeatability and precision were carried according to Soranganba, N and Singh, I.J. (2018) [21]. Each aliquot was pre-treated with SPE (Solid Phase Extraction) LiChrolut RP-18 (40-63 µm) 100 mg 1 ml standard PP-tubes [119855] as per Budzinski *et al.*, (2006) [8] and Chen-Hao Zhai *et al.*, (2009) [9] with certain modifications. Biochemical analysis for serum total protein (TP), cholesterol (CHO), triglycerides (TG) and high density lipoprotein (HDL) were carried out using analytical kits from Erba, Germany. Further, the derivation of low density lipoprotein (LDL) and very low density lipoprotein (VLDL) were calculated using Friedewald *et al.*, (1972) [16] and phospholipids (PHO) by Covaci *et al.*, (2006) [11].

Statistical Analyses

Data were statistically analyzed by analysis of variance (ANOVA – one way). Differences were considered significant at $p < 0.05$. Data were expressed in mean ± SEM.

Result

Water quality parameters observed in different seasons were given in Table 1. Observations on plasma steroidal hormones (T, E2, DHP, cortisol) and serum biochemical

Table 1: Observations on water quality parameters of the experimental ponds in different seasons

Water Parameters	Sampling seasons		
	Summer	Autumn	Winter
Water Temp. (^{0}C)	28.90±0.61	25.78±0.55	14.73±0.61
TDS (ppm)	255.25±5.11	227.25±5.31	218.25±2.14
pH	7.25±0.05	7.31±0.05	7.91±0.03
DO (ppm)	7.40±0.11	7.57±0.05	7.75±0.03
Free CO_2 (ppm)	1.10±0.04	1.05±0.03	1.20±0.02

Parameters (TP, CHO, TG, HDL, LDL, VLDL, PHO) of 0+ year's age group during summer, autumn and winter seasons are given in Table 2. The plasma T and cortisol levels were detected in summer, which declined in autumn and continued to decline to the lowest level in winter season (Figures 1 and 2). Changes in plasma T and cortisol levels were found to be statistically significant ($p< 0.05$) during all three seasons. Plasma E2 and DHP were detected only during winter season (Table 2). Serum total protein (TP) level showed statistically non-significant differences in all three seasons (Figure 3). Serum CHO, TG, HDL VLDL and PHO levels showed an increasing trend from summer season to autumn being at the lowest level in winter season (Figures 4, 5, 6, 8, 9). Statistically significant differences ($p< 0.05$) were observed in all three seasons for serum CHO, TG, HDL, VLDL and PHO levels however, serum LDL level did not exhibit any variation during the study (Figure 7).

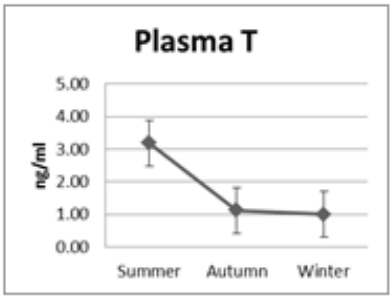

Fig 1

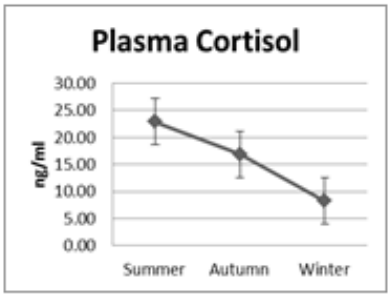

Fig 2

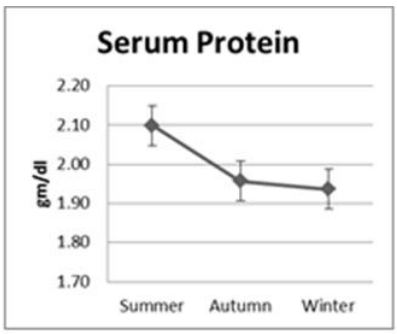

Fig 3

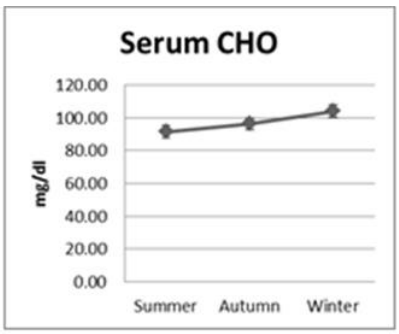

Fig 4

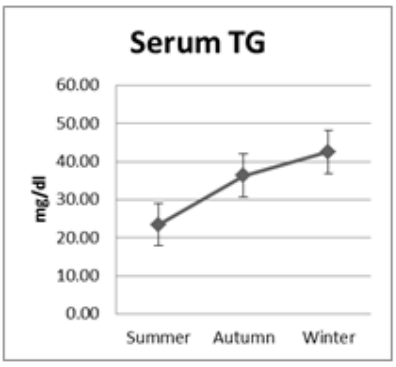

Fig 5

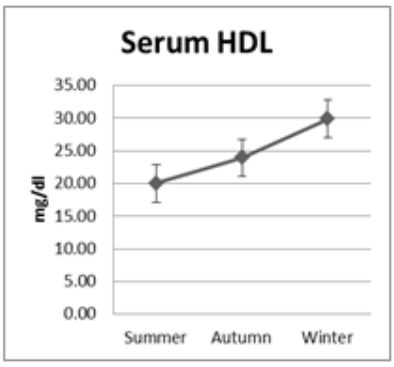

Fig 6

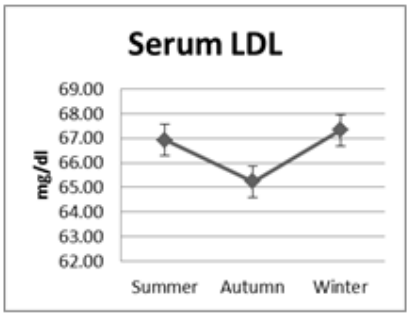

Fig 7

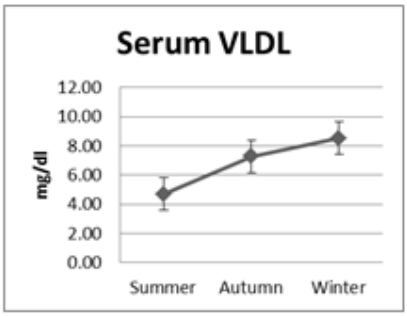

Fig 8

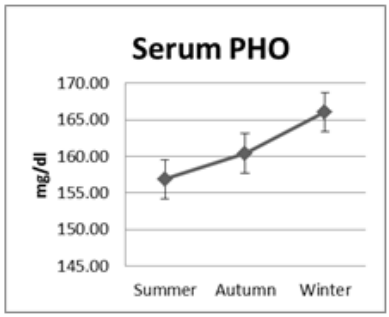

Fig 9

Fig 1-9: 1, 2 represents plasma testosterone and cortisol level during whereas figures from 3, 4, 5, 6, 7, 8 and 9 represents seasonal change in serum CHO, TG, HDL, LDL, VLDL and PHO in 0+ year's age Amur common carp during summer, autumn and winter seasons

Discussion

The significant seasonal variations with initial detection of plasma T and its highest level in 0+ year's age group of *Cyprinus carpio haematopterus* specimens might be indication of initiation of reproductive activity probably as preparatory activity. Detection of

Table 2: Plasma steroidal hormones and serum biochemical parameters of 0+ year's old amur common carp in different seasons

Observations		Summer	Autumn	Winter
Plasma Hormones	T (ng/ml)	3.15±0.03	1.12±0.02	1.01±0.01
	E2 (ng/ml)	-	-	0.67±0.03
	DHP (ng/ml)	-	-	0.37±0.04
	Cortisol (ng/ml)	22.90±0.32	16.84±0.14	8.17±0.11
Serum Biochemical parameters	TP (gm/dl)	2.08±0.05	1.97±0.03	1.91±0.03
	CHO (mg/dl)	91.62±0.54	96.24±0.47	104.45±0.82
	TG (mg/dl)	23.65±0.55	36.48±0.66	42.73±0.62
	HDL (mg/dl)	20.35±0.99	23.92±0.76	30.19±0.70
	LDL (mg/dl)	66.56±1.37	64.84±1.04	67.48±1.26
	VLDL (mg/dl)	4.73±0.11	7.29±0.13	8.54±0.12
	PHO (mg/dl)	156.88±0.40	160.26±0.34	166.25±0.60

[Data are given as mean ± SEM (n=5). T - testosterone; E2 - 17β-estradiol; DHP - 17α, 20β-Dihydroxy-4-pregnen-3-one; TP – total protein; CHO - cholesterol; TG - triglycerides; HDL - high density lipoprotein; LDL - low density lipoprotein; VLDL - very low density lipoprotein; PHO – phospholipids]

E2 and DHP only during winter season along with T and cortisol might be correlated with the onset of reproductive activities in Amur common carp and act as preparatory phase for further gonadal development. Cortisol level of young Amur common carp might be correlated with seasonal changes in temperature as its highest level coincides with the highest water temperature in summer season. Changes in cortisol level seems to be more correlated with the variations in environmental factors especially temperature which seem to indicate the role of this steroid, stress hormone in metabolic activity and growth. Ching-Fong and Mei-Ru (1990) [10] observed high level of plasma sex steroids (E2 and T) in 5 to 6 months old juveniles and young common carps during the non-spawning season and concluded about their differential role in physiological functions. Similar observations on high level of T during immature stages were detected by Heidari *et al.,* (2010) [18] in *Rutilus frisii kutum* and Assem *et al.,* (2016) [4] in *D. dentex*. The late maturing behaviour of Amur common carp could be related to detection of E2 only in winter season during young age. Felip *et al.,* (2008) observed that an unbalanced production of androgen led to early arrest of puberty in male sea bass. The detection of plasma E2 and DHP levels only during winter season might be an indication for onset of gonadal development in young Amur common carp. Sen *et al.,* (2002) [29] observed seasonal and cyclic variations in plasma T, E2 and DHP to be correlated with the presence of vittelogenenic or post-vittelogenic oocytes in *L. rohita*, which indicated intricate relationship between gonadal development and these hormones. An increase in cortisol concentrations with increase in water temperature was reported in *Cyprinus carpio* (Saha *et al*., 2002) [26].

Non-significant differences in all three seasons in serum TP level of 0+ year's age group in the present study indicated that these were not influenced by water temperature rather might be correlated with the feeding regime and metabolic activity. Ghoroghi *et al.,* (2009) [17] reported that fluctuations in serum TP concentrations were influenced in *Ctenopharyngodon idella* by seasonal environmental conditions like water temperature and feeding regime. Detection of all lipid classes (CHO, TG, HDL, VLDL, PHO) during summer season and their highest levels in the winter season might be correlated with the level of reproduction related activities and consequent events of gonadal development. These patterns in young Amur carp might be due to deposition of lipids after active feeding preceding warmer environment. Lipids are forms an integral part for cellular growth, energy reserves and later as substrate for steroid biosynthesis. Many investigators have reported higher serum lipids and lipoprotein during warm seasons as compared to cold seasons due to higher metabolic rate and feeding activity as reported in *Tinca tinca* (Svoboda *et al.,* 2001) [33], *Dicentrarchus labrax* (Kavadias *et al.,* 2003) and *Capoeta capoeta umbla* (Bayir, 2005) [7]. Cholesterol (CHO) is the precursor for steroidal hormones and increase in CHO level may be for initiating gonadal development and maturity in 0+ year's age group fish. Young *et al.,* (2004) reported the occurrence of high CHO levels in fish as a prerequisite for gonadal steroidogenesis and production of basal steroid throughout the year. Many authors have provided information on seasonal fluctuation in serum CHO during reproductive cycle in *Capoeta trutta* (Eroğlu and Şen, 2017) [29], HDL and VLDL in *Leuciscus cephalus* (Aras *et al*., 2008), PHO in *Clarias batrachus* (Lal and Singh, 1987) [21] and *Morone saxatilis* (Lund *et al*., 2000) [22]. Wallaert and Babin (1994) observed circannual variation in the LDL level irrespective of differences in age and sexual maturity in both sexes of trout (*Oncorhynchus mykiss*).

Observations on changes in steroidal hormones and biochemical parameters related to reproductive activity would be helpful in understand the age for onset of maturity in young Amur common carp. The changes in the levels of steroidal hormones and biochemical parameters with higher levels in spring and summer seasons seem to be correlated with higher level of gonadal development and possibility of spawning under favourable environmental conditions. This study might be helpful in formulating for future research programmes towards achieving off season breeding and seed production of Amur common carp.

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