IJST (2015) 39A2: 141-146
Iranian Journal of Science & Technology

http://ijsts.shirazu.ac.ir

Changes in plasma vitellogenin, phosphorus and calcium in wild-caught female carp (*Cyprinus carpio*) as potential criteria for selecting brood-fish for restocking natural populations

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Abstract

Despite two decades of artificial spawning of wild carp (*Cyprinus carpio*) to rehabilitate natural populations in the southeastern Caspian Sea, there are still no appropriate physiological criteria for selecting competent brooders during induced spawning. To this end, the plasma concentrations of vitellogenin (Vtg), phosphorus (P) and calcium (Ca) were measured during ovarian development, as potential criteria for selecting ripe brooders. Vtg was measured by ELISA using pure carp Vtg and its specific antibody. P and Ca contents of plasma samples were also determined photometrically and colorimetrically, respectively, by Autoanalyzer using commercial specific kits. The plasma levels of Vtg showed no significant differences during sampling times, whereas its highest significant value (922±301 µg/ml) was recorded in late vitellogenesis ($P \leq 0.05$). The plasma concentrations of Ca also showed no significant differences in different stages of ovarian development except for late vitellogenesis (16.65±1.77mg/dl) and during sampling times except for June (17.24±1.76mg/dl) that rose to its peak. The plasma levels of P increased gradually during sampling times and reached maximum value in June (35.22±11.12mg/dl). Level of P also increased gradually during ovarian growth and peaked at late vitellogenesis (33.94±8.02mg/dl) and slowly decreased afterward. The regression analysis indicated that plasma P levels showed a high correlation with plasma Vtg concentrations. Therefore due to easier and lower cost of plasma P measurement in fish in comparison with Vtg, measurement of plasma P levels is recommended as criterion for selecting ripe females for artificial spawning.

Keywords*:* Wild carp; vitellogenin; phosphorus; calcium; brooder selection

1. Introduction

Wild common carp (*Cyprinus carpio*) (hereafter wild carp) is a native threatened species of the Caspian Sea. From long ago, wild carp has been counted as a species with high economic value in southeastern Caspian Sea and contributed to major employment and livelihood of the coastal residents (Vazirzadeh et al. 2011). Similar to other commercial fish species, the stocks of wild carp showed noticeable fluctuations during the last 5 decades (Fatemi et al. 2009). The landing of the species has declined in spite of increasing fisheries efforts (Vazirzadeh et al. 2011), mainly due to fishing pressure and destruction of the spawning ground (Fatemi et al. 2009).

The Iranian Fisheries Organization (IFO) started propagation of carp in 1997, using wild-caught brood-fish to rehabilitate natural stocks, and more than 150 million fingerlings had been released to Iranian parts of the Caspian Sea by 2011 (IFO, 2012, unpublished data). However, the program has experienced several difficulties, including low responsiveness of brood-fish to hormones administered to induce spawning, and poor performance of induced brood-fish which resulted in production of low numbers of fry by each spawning fish (Vazirzadeh et al. 2011).

Despite the importance of wild carp and other commercially valuable cyprinids in Asia and Europe, there are only a few researches describing the methods of artificial spawning, reproductive biology and hatchery techniques of wild cyprinids (Kouril et al. 2007; Krejszeff et al. 2010; Kucharczyk et al. 2005; Kujawa et al. 2011; Targońska et al. 2011). Also, development of controlled spawning techniques of wild cyprinids is still necessary as an essential component of wild

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Received: 9 May 2014 / Accepted: 5 July 2014

fish conservation efforts (Kucharczyk et al. 2005; Wildt et al. 1993).

Traditionally, brooders have been selected based on the external features mainly fullness and softness of belly, but fish response to hormonal induction of ovulation has shown that these are not accurate criteria for selection of ripe brooders. It is conceivable that some setbacks particularly lack of appropriate criteria in choosing qualified broodstocks for artificial propagation, are involved. It has been clearly shown that incompetent broodstocks, that did not accomplish vitellogenesis, would never favorably respond to hormonal inductions (Yaron 1995; Zohar and Mylonas 2001). Almost all fish show somehow reproductive dysfunctions when kept in captive conditions (reviewed by Zohar and Mylonas, 2001), but these problems are very serious in wild-caught species (Kucharczyk et al., 2005).

Vitellogenin (Vtg), a glycolipophosphoprotein, is synthesized in liver and in most finfish species yolk droplet accumulation in oocytes is accompanied by increase in circulating minerals, especially calcium and phosphorus (Specker and Sullivan 1994; Sun and Pankhurst 2004; Tyler et al. 1996). A positive correlation between plasma vitellogenin concentration and the level of those minerals in bloodstream has been shown in some cyprinids (Rinchard et al. 1997; Tyler and Sumpter 1990).

The aims of the present research were to study the plasma vitellogenin changes during different oocyte developmental stages of wild carp and to examine its correlation with phosphorus and calcium contents of plasma to achieve appropriate criteria for selecting suitably competent broodstocks for artificial propagation.

2. Materials and methods

a) Fish sampling and ovarian histology

Nine consecutive monthly samplings from Iranian waters of the southeastern Caspian Sea, Golestan province were carried out from October 2009 to June 2010. No samples were taken in summer because of banned commercial fisheries activity. A total of 65 wild carp females were randomly chosen from those caught by commercial beech seine. Blood samples were immediately taken from caudal vein using heparinized syringes after sedation with clove powder (100 ppm). Blood samples were placed on ice upon centrifugation at 6500 g for 10 minutes. Finally plasma were transferred to cryotubes and stored at –80 ˚C until analyses. Total length of fish measured using biometry board to nearest 1mm. Fish weight was recorded to nearest 0.1 g. They were immediately killed by a sharp blow to the head and ovaries and livers were dissected out and weighted to nearest 0.01 g. Slices about 1 cm^2 were prepared from right and left ovaries of each fish and were fixed in Bouin's solution for histological examinations (Hinton 1990). Histological preparations were done as described previously (Vazirzadeh et al., 2014). Defining ovaries developmental stages was according to an eight–stage method consisted of Balbiany bodies (stage 1), cortical alveoli (stage 2), previtellogenesis (stage 3), vitellogenesis (stage 4), late vitellogenesis (stage 5), final maturation or migratory nucleus (stage 6), ovulated (stage 7) and atretic oocytes (stage 8) (details are reported in Vazirzadeh et al., 2014).

b) Plasma vitellogenin, phosphorus and calcium measurement

Vitellogenin content of plasma samples was determined using pure vitellogenin of common carp and its specific antibody (common carp Vtg kit, Biosense, Norway) by ELISA according to protocol described by Mourot & Le Bail (1995). The procedure consists of competition between vitellogenin coated onto microplate wall and free vitellogenin from plasma samples to bind with the antibody. The method was briefly as follows:

Coating

150 µl of 100 ng/ml vitellogenin solution was incubated for 3 hrs at 37˚C in sodium carbonate buffer (pH 9.6 containing 5 mg/l gentamicin).

Saturation

To reduce the background effect, the free sites were blocked using 2% pig serum in TBS for 30 minutes at 37˚C.

Antigen incubation with specific antibody

Each well was pipetted with 75 µl of standard solution (pure vitellogenin with 3.9–4000 ng/ml concentrations) or plasma samples and 75 µl of diluted specific antibody (final dilution rate of 1.15). Incubation was extended to 16 hrs at 20˚C. To eliminate unspecific bands 4 wells were left without adding specific antibody solution. The data were duplicated.

Secondary antibody incubation

Subsequently complex of coated antibodies with antigens were incubated with 1.4000 dilution of secondary antibody (Sheep–anti–rabbit) in TBS for further 2 hrs at 37˚C.

Peroxidase–antiperoxydase incubation (PAP)

Wells were incubated for another hour at 37˚C through adding 150 µl solution of 1.5000 PAP in TBS.

Table 1. Number of samples and plasma concentrations of vitellogenin (Vtg), Calcium (Ca) and Phosphorus (P) of female wild carp collected at each sampling time. The gonadosomatic index (GSI) and hepatosomatic index (HSI) are also presented

Date	No. of samples	Vtg (µg/ml)	Ca (mg/dl)	P (mg/dl)	GSI (%)	HSI (%)
October 2009	8	684±241 [a]	12.89±1.22 [a]	16.15±1.21 [a]	9.56±2.47 [a]	1.78±0.24 [ac]
November	8	701±154 [a]	13.75±1.42 [a]	15.46±2.01 [a]	11.78±3.39 [a]	1.81±0.31 [a]
December	6	626±200 [a]	12.97±1.52 [a]	14.66±1.91 [a]	12.85±3.33 [ab]	1.61±0.27 [c]
January 2010	7	760±281 [a]	13.44±1.23 [a]	15.84±1.78 [a]	15.00±1.39 [b]	1.95±0.36 [a]
February	8	868±146 [a]	13.70±1.30 [a]	15.90±1.74 [a]	15.83±2.62 [b]	2.14±0.28 [ab]
March	8	718±372 [a]	13.56±0.54 [a]	15.18±3.32 [a]	11.95±5.44 [a]	2.11±0.53 [ab]
April	8	770±345 [a]	13.61±1.28 [a]	19.09±5.48 [ab]	16.91±3.54 [bc]	1.81±0.25 [a]
May	6	813±489 [a]	13.63±1.41 [a]	28.40±8.08 [bc]	16.81±6.04 [bc]	1.38±0.42 [cd]
June	6	746±465 [a]	17.24±1.76 [b]	35.22±11.12 [c]	21.91±2.47 [c]	0.99±0.16 [d]

Data are shown as mean ± S.D. Values with same letters in each column showed no significant differences ($P \geq 0.05$).

Revelation

Peroxidase activity was terminated using a solution composed of 200 µl of O–phenylendiamin (0.5 g/l) and 30% hydrogen peroxide (0.5 ml/l) in ammonium–acetate buffer (50 mM, pH 5.0) at darkness for another hour.

Washing

After each step wells were washed out 5 times with Tris–HCl buffer (10 mM, pH 7.5) containing 150 mM NaCL, 0.1% tween 20 and 5 mg/l gentamicin sulfate.

Measurements of optical density

ODs were read with microplate reader (Micro plate auto reader, Biotech Instrument) at 490 nm. Data were converted to vitellogenin concentrations by plotting vitellogenin standard curve.

Phosphorus and calcium contents of plasma samples were also determined photometrically and colorimetrically, respectively by autoanalyzer using commercial specific kits (Man Co, Italy).

c) Statistical analysis

The uniformity of the variance of the dependent variables was evaluated with the Levene's test of equality of error variances. Standard normality test of Kolmogorov–Smirnov was applied to determine normality of data sets. One–way ANOVA and Tukey's HSD tests were used to elucidate whether there were significant differences among plasma metabolites or not. Correlations between vitellogenin with calcium and phosphorus were tested by linear regression. All comparisons were performed using SPSS 15 statistical software package at the significance level of $P \leq 0.05$. All results were reported as Mean ± S.D.

3. Results

a) Vitellogenin

There were no substantial differences among different sampling times regarding circulating vitellogenin. The lowest (626±200 µg/ml) and highest (868±166 µg/ml) values were measured in December 2009 and February 2010, respectively (Table 1). Plasma levels of vitellogenin in the course of sampling time as well as at each sampling time showed relatively high variation.

Plasma levels of vitellogenin gradually increased, consistent with ovarian growth (Fig. 1) and reached its peak in stage 5 (922±301 µg/ml), which was significantly higher than other developmental stages ($P \leq 0.05$). Afterward the level of vitellogenin decreased but never reached to its level before vitellogenesis. The thing worth considering was that fish even with ovulated ovaries had higher plasma content of vitellogenin (442±264µg/ml), which was in good agreement with the presence of vitellogenic oocytes in their histological examinations.

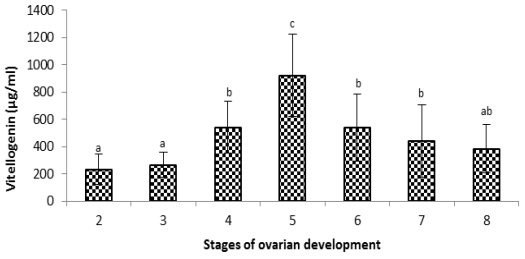

Fig. 1. Plasma concentrations of vitellogenin in different stages of ovarian development in wild carp. Data are shown as Mean ± S.D. Values with same letters showed no significant differences *($P \geq 0.05$)*

b) Phosphorus and calcium

Plasma levels of calcium and phosphorus of various samples are shown in Table 1. Calcium content of plasma gradually increased from 12.89±1.22 mg/dl in October 2009 to 17.24±1.76 mg/dl in June 2010. Except for June, there were no considerable differences among sampling times with regard to plasma calcium concentration. Circulating phosphorus concentration slowly

increased during spring season and culminated in 35.21±11.12 mg/dl in June from 14.66±1.91 mg/dl in December. Its plasma concentration in spring was also significantly higher than those of winter and autumn.

Samples at stages 5 showed higher plasma level of calcium but there were no noticeable differences among plasma calcium concentrations of fish at other ovarian developmental stages, whilst fish at their 4th and 5th sexual developmental stages showed significantly higher plasma phosphorus concentration (Fig. 2). Meanwhile, those ovulated fish also depicted higher plasma phosphorus content.

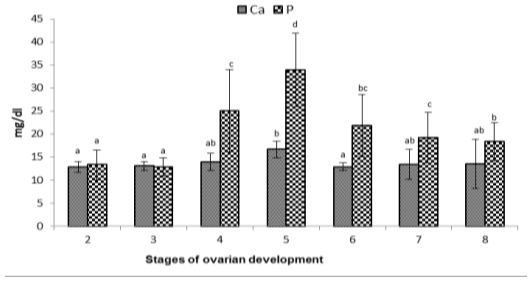

Fig. 2. Plasma levels of calcium (Ca) and phosphorous (P) in different stages of ovarian development in wild carp. Data are shown as Mean ± S.D. Values with the same letters showed no significant differences *(P ≥ 0.05)*

c) Plasma vitellogenin, calcium and phosphorus correlation patterns

Plasma contents of vitellogenin, calcium and phosphorus implied that there was significant correlation between vitellogenin and calcium and phosphorus concentrations in the present study (Figs. 3 and 4). However, results showed that there was higher correlation between phosphorus and vitellogenin than calcium and vitellogenin. The plasma vitellogenin and phosphorus levels between stage 2 and 5 differed by about 3 to 4 times, and the plasma calcium levels increased by more than 20% above basal level.

4. Discussion

Vitellogenin content of plasma did not significantly differ among various sampling times. However, inter-individual variation was noticeable, which might imply an asynchronous reproduction in the population. Vitellogenin synthesis, as a precursor for primary indigenous nutrient storage of next generation, was mediated via E2 on hepatocytes and exported to developing oocytes through bloodstream (Garcia–Lopez et al. 2007; Specker and Sullivan 1994). Plasma content of vitellogenin progressively increased along with oocyte development, implying its developmental stage before final oocyte maturation (Lv et al. 2009; Tyler and Sumpter 1990; Tyler et al. 1996). The plasma levels of vitellogenin of fish at their vitellogenic stage were in good agreement with the previous findings to the degree that it was statistically significant from those of remaining stages. Its plasma concentration also surged during vitellogenesis similar to domestic common carp (Tyler and Sumpter 1990; Tyler et al. 1996), Chinese loach (*Misgurnus anguillicaudatus*) (Lv et al. 2009) and other cyprinids (Rinchard et al. 1997; Tyler et al. 1996). Higher variation of plasma vitellogenin at each sampling time might be due to inter-individual variation or to different sexual maturity of fish, a well-documented phenomenon in common carp (Tyler and Sumpter 1990; Tyler et al. 1996;). In the case of the availability of plasma vitellogenin measurement facilities, according to the literature it is possible to use plasma vitellogenin moiety as a reliable index to choose competent broodstocks. Research obviously revealed that those fish that did not completely undergo vitellogenesis would be irresponsive to hormone injection (Mylonas et al. 1997; Mylonas et al. 2009; Tyler and Sumpter 1990). Consequently, results from the present study might be helpful to recognize competent wild broodstocks for hormonal spawning induction.

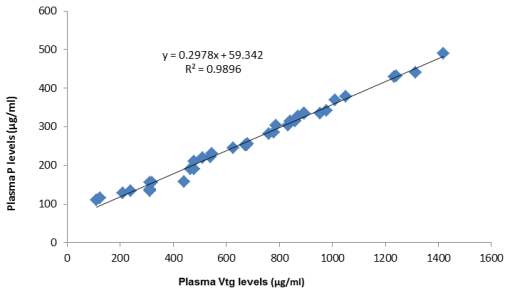

Fig. 3. The correlation between plasma phosphorus (P) and vitellogenin (Vtg) levels in female wild carp. (n=36; *P≤0.001*)

It may be important to note that most of the information regarding the vitellogenin content of plasma in common carp have documented the probable effects of endocrine disruptor chemicals in males (Petrovic et al. 2002; Tyler et al. 1996). Therefore, vitellogenin determination techniques and relevant results would be implemented as a benchmark to study the plausible outcomes of pollutants on cyprinids endocrine system of Iranian Caspian Sea stocks or other water bodies. Since there was remarkable consensus in chemical moiety of vitellogenin in studied cyprinids; it would be possible to successfully employ commercially available vitellogenin kits for common carp in studying closely related cyprinid fish (Petrovic et al. 2002; Tyler and Sumpter 1990; Tyler et al. 1996).

Plasma calcium content did not differ significantly at different ovarian maturity stages and different sampling times except for June. Nonetheless, plasma phosphorus concentration gradually increased in spring season and attained its upper limits during spawning season. Phosphorus content of ovaries was also significantly high at stages 4 and 5 of their development. Meanwhile, results from the present research brought out a strong correlation between plasma contents of vitellogenin and phosphorus, while it was weak in the case for plasma calcium concentration. Because of the involvement of the calcium and phosphorus in yolk synthesis in hepatocytes, their plasma concentrations have been implemented as desirable indices of examining ovarian maturation state in fish (Rinchard et al. 1997; Tyler and Sumpter 1990; Tyler et al. 1996). Contrary to previous findings implying a relevant correlation between plasma contents of vitellogenin and calcium in rainbow trout (*Oncorhynchus mykiss*), common carp did not indicate such a strong correlation owing to the lower rate of calcium increment in comparison to progressive increase of vitellogenin during oocyte development (Tyler and Sumpter 1990). For instance, in the present study plasma calcium content increased less than 1.2 times (3 mg/dl) from vitellogenic stage to late vitellogenesis, whilst plasma vitellogenin concentration simultaneously showed 4 times increment in value (600 µg/ml). Meanwhile, plasma phosphorus content was in good agreement with plasma vitellogenin content and showed even better synchronization in fluctuation with oocyte sexual maturity. Their plasma content culminated in vitellogenin accomplishment and decreased to some extent after spawning, but it never became less than previtellogenic values. This finding confirmed the presence of oocytes with different developmental stages even during the peak of spawning season. Former researchers reported the same pattern in other cyprinids and marine species with asynchronous spawning strategy (Guzman et al. 2008; Jensen et al. 2001; Rinchard et al. 1997; Sun and Pankhurst 2004; Tyler and Sumpter 1990).

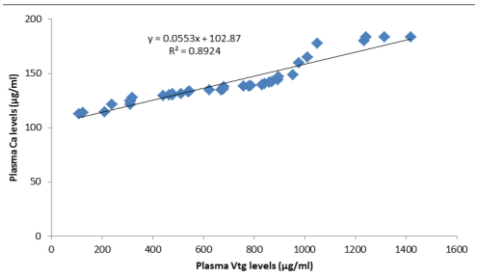

Fig. 4. The correlation between plasma calcium (Ca) and vitellogenin (Vtg) levels in female wild carp. (n=36; *P≤0.001*)

5. Conclusion

Taking all these finding into consideration, the presence of high correlation between plasma vitellogenin and phosphorus content fluctuations and also the concordance of all these changes with ovarian developmental stages and much lower cost and easier method of measuring phosphorus in comparison to vitellogenin assay, it would be reasonable to measure plasma phosphorus concentration for better evaluation of the sexual maturity state of broodstocks and better management of hatcheries for successful artificial propagation programs.

Acknowledgments

The authors would like to thank H. Nazari, E. Najafi, M. Shekiba and staff of Sijowal Center for Fish Propagation and Farming for their kind assistance during sampling period. This research was partially supported by ICRP, Ministry of Science, Research and Technology of Iran. We also appreciate Shiraz University Research Council for financial support of this work.

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