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Reproductive Phase-related Plasma Proteins in *Labeo rohita*

Suresh DVNS[1*], Bagade RS [1] and Baile VV [2]

[1]Department of Zoology, Dr Ambedkar College, Deekshabhoomi, Nagpur 440010, Maharashtra, India.
Email: sureshdvns@gmail.in, ramdasbagade63@gmail.com
[2]Ex-Professor, Post Graduate Teaching Department of Zoology, RTM Nagpur University, Nagpur 440033, Maharashtra, India.
*Corresponding author.

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ABSTRACT

A study of plasma content of proteins in *Labeo rohita* in the present project gives a base for the understanding of carrier proteins in the plasma specifically sex hormone-binding globulins, a type of carrier protein, which carry the sex hormones with different affinity and capacity associations. Their electrophoretic patterns in different phases of reproductive cycle of the fish will also give a preliminary idea at what molecular weight these carrier proteins might get separated in the gel for further isolation and identification. The trend of the plasma protein levels showed a significant increase in prespawning-phase of the fish breeding cycle. The values as recorded went up to 28.940 ± 0.404 mg/mL in females and 19.600 ± 0.115 mg/mL in males. The study has not only revealed the protein content of the plasma of both sexes but also kept in synchronous the records of electrophoregrams of plasma proteins of reproductive and non-reproductive phases of the cycle. Protein content in plasma increases as important estradiol-dependent proteins such as vitellogenins, eggshell proteins, which are known to be synthesized in liver, have higher or lower molecular weights and are produced in much lower amounts during the maturing phase of the fish reproduction.

Keywords: Carrier proteins, SDS-PAGE, Prespawning, Reproductive phase, *Labeo rohita*

INTRODUCTION

The disturbances in the gas exchange, nitrogenous waste excretion, acid-base and ionic balance are due to the change in water pH cause stress in fish affecting its body physiology and growth (Pickering, 1981; Jeney *et al.*, 1992). Though majority of the fish farmers in India take precautions to prevent abrupt changes in water pH in aquaculture operations, such situations may occur due to the excessive inputs of supplementary feed, manures and inorganic fertilisers to get higher

production per unit area. The three Indian major carps, catla, *Catla catla* (Ham.), rohu, *Labeo rohita* (Ham.) and mrigal, *Cirrhinus mrigala* (Ham.) account for more than 80% of the country's aquaculture production amounting to over 1.8 million ton (FAO, 2003). The optimum pH range for culture of these carps is 7.5-8.5 (Banerjea, 1967), and the majority of the Indian carp culture ponds have a pH in this range. Since, the change in water pH influences nitrogenous waste excretion, ion balance and respiratory homeostasis, we predicted there would also be altered haematology of carps (Das *et al.*, 2006). Accordingly, we studied the changes in size, shape and population of blood cells and concentrations of hemoglobin (Hb), blood sugar and serum protein which could be used as tools to indicate the stress level in these fishes during exposure to changed water pH.

A study of plasma content of proteins in *L rohita* in the present project gives a base for the study of carrier proteins in the plasma specifically sex hormone-binding globulins (a type of carrier protein) which carry the sex hormones with different affinity and capacity associations. Their electrophoretic patterns in the present study in different phases of reproductive cycle of the fish will also give a preliminary idea at what molecular weight these carrier proteins might get separated in the gel for further isolation and identification.

MATERIAL AND METHOD

Protein Assay (Hartree-Lowry Assay)

Under alkaline conditions the divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with Folin reagent to produce an unstable product that becomes reduced to molybdenum/tungsten blue. *Reagent A* consists of 2 gm sodium potassium tartrate x 4 H20, 100 gm sodium carbonate, 500 ml 1N NaOH, H20 to one liter (that is, 7mM Na-K tartrate, 0.81M sodium carbonate, 0.5N NaOH final concentration). Keeps 2 to 3 months. *Reagent B*t consists of 2 gm 2 gm sodium potassium tartrate x 4 H20, 1 gm copper sulfate (CuSO4 x 5H20), 90 ml H20, 10 ml 1N NaOH (final concentrations 70 mM Na-K tartrate, 40 mM copper sulfate). Keeps 2 to 3 months. *Reagent C* consists of 1 vol Folin-Ciocalteau reagent diluted with 15 vols water. Prepare a series of dilutions of 0.3 mg/ml bovine serum albumin in the same buffer containing the unknowns, to give concentrations of 30 to 150 micrograms/ml (0.03 to 0.15 mg/ml). Final assay volume is 5 ml. Measure absorbance at 650 nm in 1 cm cuvettes. Prepare a standard curve of absorbance versus micrograms protein (or *vice versa*), and determine amounts from the curve. Determine concentrations of original samples from the amount protein, volume/sample, and dilution factor, if any.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate (SDS) is an anionic detergent which denatures proteins by "wrapping around" the polypeptide backbone - and SDS binds to proteins fairly specifically in a mass ratio of 1.4:1. It is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size: this is done with 2-mercaptoethanol or dithiothreitol. In denaturing SDS-PAGE separations therefore, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight. Assemble two glass plates (one notched) with two side spacers, clamps, grease, etc. as shown by demonstrators. Stand assembly upright using clamps as supports, on glass plate. Pour some pre-heated 1% agarose onto glass plate, place assembly in pool of agarose this seals the bottom of the assembly. *Gel concentration of 12.5% in 0.25 M Tris-HCl pH 8.8 is resolving gel Gel concentration of 4.5% in 0.125 M Tris-HCl pH 6.8 is stacking gel.* Grind a little leaf material (eg. 2 grams) in a mortar, centrifuge for 3 min. Take supernatant and mix 100ul 1:1 (v:v) with SDS-PAGE disruption mix: this is 125mM Tris-HCl pH 6.8 / 10% 2-mercaptoethanol / 10% SDS / 10% glycerol, containing a little bromophenol blue. Layer samples under buffer on stacking gels. Connect up apparatus and electrophorese. Make up stain: 0.2% *Coomassie Brilliant Blue* (CBB) in 45:45:10 % methanol: water: acetic acid. Cover gel with staining solution, seal in plastic box and leave overnight on shaker (RT) or for 2 to 3 hours at 37 degree Celsius. Destain with 25% 65% 10% methanol water acetic acid mix. Rinse gel in distilled water and seal in a plastic.

RESULTS AND DISCUSSIONS

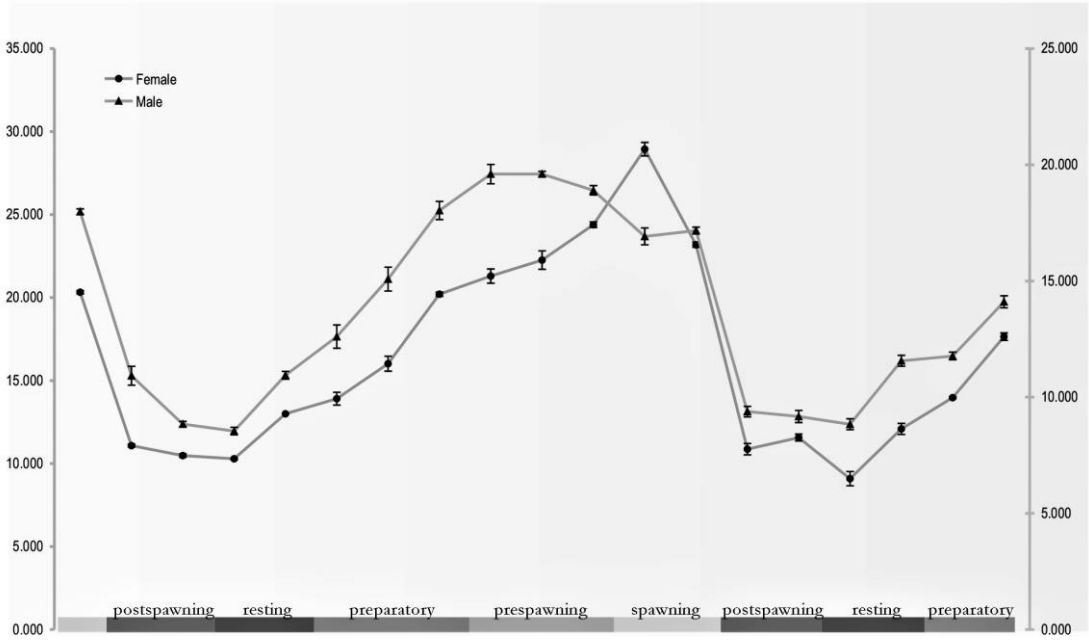
Female *Labeo rohita*

Mean readings collected of 6-8 female and male animals every month in two fortnightly batches for

eighteen months starting from August 2013 to February 2015. The plasma protein levels estimated are tabulated in Table 1 and comparative seasonal mean levels are graphically depicted in Graph 1.

Table 1: Monthly plasma protein in *Labeo rohita* during August 2013 & February '2015 (*Values in (mg/mL) ± SEM (Standard Error of Mean); *P>0.05; rest P<0.001*).

Month	(Female)		(Male)	
AUG '13	20.320	± 0.100	17.977	± 0.123
SEP '13	11.087	± 0.079 *	10.920	± 0.404 *
OCT '13	10.487	± 0.105 *	8.840	± 0.117 *
NOV '13	10.287	± 0.057	8.540	± 0.162
DEC '13	13.000	± 0.012	10.947	± 0.168
JAN '14	13.910	± 0.387	12.607	± 0.499
FEB '14	16.013	± 0.450	15.080	± 0.510
MAR '14	20.207	± 0.127	18.033	± 0.393
APR '14	21.293	± 0.431	19.600	± 0.416
MAY '14	22.260	± 0.553	19.600	± 0.115
JUN '14	24.387	± 0.173	18.900	± 0.200
JUL '14	28.940	± 0.404	16.917	± 0.363
AUG '14	23.183	± 0.156	17.167	± 0.145
SEP '14	10.867	± 0.343 *	9.380	± 0.223 *
OCT '14	11.567	± 0.208 *	9.167	± 0.260 *
NOV '14	9.093	± 0.428	8.833	± 0.233
DEC '14	12.087	± 0.330	11.567	± 0.233
JAN '15	13.973	± 0.057	11.773	± 0.165
FEB '15	17.647	± 0.226	14.100	± 0.260



Graph 1: Comparative graphical representation of seasonal mean plasma protein levels recorded in different reproductive phases of female and male *Labeo rohita.* (values are in mg/mL);

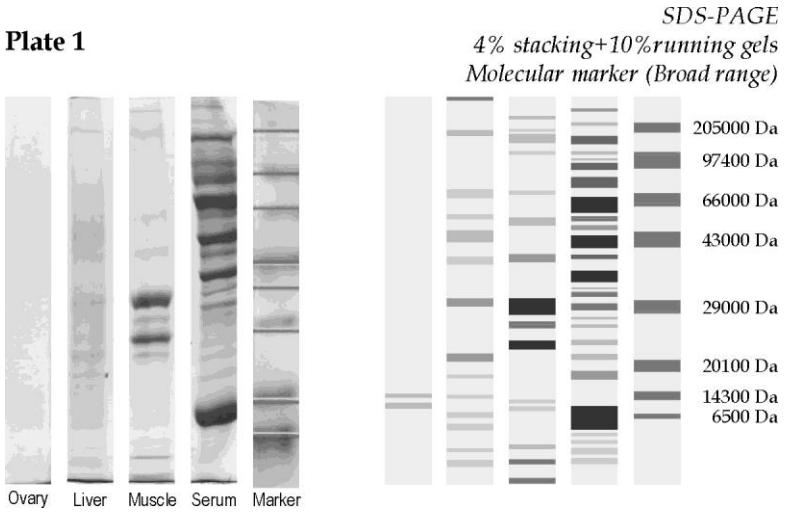


Plate 1: SDS-PAGElectrophoretic banding patterns of the samples of serum and the extract samples of ovary, liver & muscle of *L rohita* in non-reproductive phase (Nov. to Jan.). Image besides is the computer generated electrophoregram.

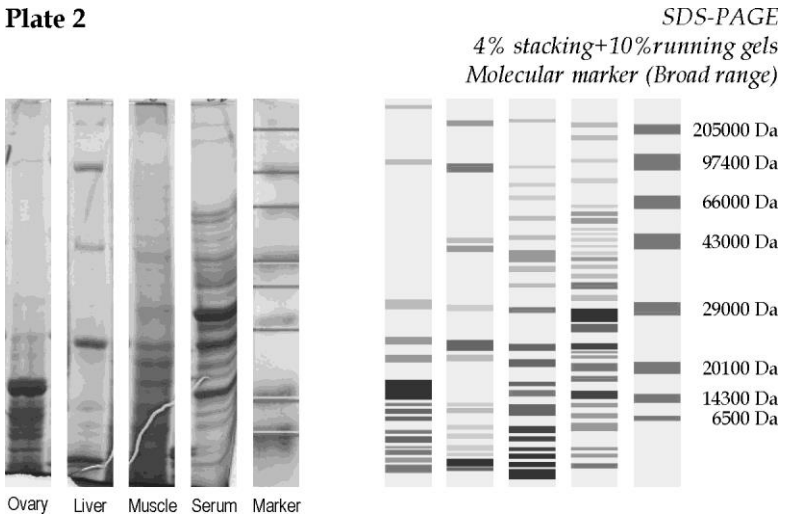


Plate 2 : SDS-PAGElectrophoretic banding patterns of the samples of serum and the extract samples of ovary, liver & muscle of *L rohita* in reproductive phase (Feb. to Aug.). Image besides is the computer generated electrophoregram.

The trend of the plasma protein levels showed a significant increase in prespawning-phase of the fish breeding cycle. The values went up to 28.940 ± 0.404 mg/mL in the month of July which subsequently reached lowest of the season in November with value 10.287 ± 0.057 mg/mL in year 2013 and 9.093 ± 0.208 mg/mL in year 2014.

Male *Labeo rohita*

Similarly, the plasma protein levels estimated in males are also tabulated in Table 1 and graphically depicted in Graph 1. The values went up to 19.600 ± 0.115 mg/mL in the month of May which subsequently reached lowest of the season in November with value 8.540 ± 0.162 mg/mL in year 2013 and 8.833 ± 0.233 mg/mL in year 2014.

Electrophoretic Patterns

The SDS-PAGE separation of plasma and few tissue levels such as ovary, liver and muscle proteins was also performed in non-reproductive (Plate 1) and reproductive (Plate 2) phases to have an idea of increased protein content during the breeding cycle of the fish that is correlated with carrier proteins and other steroid dependent proteins in the plasma.

DISCUSSION

Among the serum proteins, albumin and globulin are the major proteins, which play a significant role in the immune response. Globulins like gamma globulin are absolutely essential for maintaining a healthy immune system and contain all the immunoglobulin in the blood. A higher serum globulin level in the pre-challenge period is in agreement with Kumar *et al.* (2005) who reported a higher globulin level due to non-gelatinous carbohydrate feeding in *L. rohita* juveniles. The lowest globulin level at 2.0% n-3 PUFA supplemented groups both in the pre- and post-challenge period suggests an immunosuppressive action of high n-3 PUFA. Gelatinised carbohydrate fed groups registered lower serum protein levels, in agreement with Hemre *et al.* (1996) and Kumar *et al.* (2005). Hemre *et al.* reported a negative correlation of serum protein with dietary carbohydrate. After challenge study, reduction of serum protein may be due to vascular leaking of serum protein because of increased permeability (Quinn *et al.*, 1990; Rushmore *et al.*, 1988) along with impaired synthesis and non-specific proteolysis of serum protein (Salte *et al.*, 1993).

In view of the above discussion of the plasma proteins and other related factors like, the feed, lysozymal activity, environmental cues definitely affect the physiology of the fish more or less in a significant manner. Therefore, the plasma protein content of the both sexes was estimated in *L* rohita.

The experiments with juvenile *L. idus* presented in Allner *et al.*, (1999) study clearly show that compared to untreated fish the synthetic estrogen ethinyl estradiol causes the occurrence of an additional protein in blood. The molecular weight of this additional protein corresponds to the molecular weights of vitellogenins from other fish as reported in the literature (Tyler, 1991; Kishida *et al.*, 1992; Utarabhand and Bunlipatanon, 1996; Komatsu and Hayashi, 1997; Roubal *et al.*, 1997; Suresh *et al* 2008). This similarity justifies the assumption that the detected estrogen-dependent protein is vitellogenin. Further evidence is provided by the differences between male and mature female plasma protein spectra showing that male fish contain only very small amounts or no vitellogenin in blood. Exposure of adult male or juvenile fish to estrogens including the xenoestrogen 4-nonylphenol resulted in the same protein pattern as exhibited in plasma of mature females. This finding also supports the assumption that the estrogen-dependent protein is vitellogenin. Other estradiol-dependent proteins, for example eggshell proteins, which are also known to be synthesized in the liver, have higher or lower molecular weights and are produced in much lower amounts (Pelissero *et al.*, 1993).

According to Allner *et al.*, 1999, the advantage of the electrophoretic method is that only very low amounts of blood are required to show the induction of vitellogenin. The relative quantification of vitellogenin in relation to the entire blood protein does not depend on accurate blood volume determination which is a very time-consuming procedure when sampling a large number of fish in monitoring programs. Furthermore, the measurement of additional plasma proteins allows to distinguish specific vitellogenin induction from unspecific anabolic effects resulting in modified protein levels including vitellogenin.

CONCLUSION

Protein content in plasma increases, as important estradiol-dependent proteins, *e. g.* vitellogenins, eggshell proteins, that are known to be synthesized in the liver, have higher or lower molecular weights and are produced in much lower amounts during the maturing phase of the fish reproduction. The present study has not only revealed the protein content of the plasma of both sexes but also kept the records of the electrophoregrams of the plasma proteins of all different phases of the cycle to distinguish the sex hormone binding globulins from the electrophore-grams for further estimating/ separating/ isolating, the future objective of the study.

Conflicts of Interest: The authors declare no conflict of interest.

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