

Possible Immunolocalization of Sex-Hormone Binding Globulin (SHBG) in female *Labeo rohita*

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ABSTRACT

Sex hormone-binding globulin (SHBG) serves as the primary transport protein for sex steroids in the blood of many mammals, other vertebrates, and fish species (Westphal, 1986). Produced mainly in the liver, plasma SHBG controls the bioavailability and metabolic clearance of its bound steroid hormones (Siiteri, 1982). However, in mammals, SHBG gene expression also occurs in various extrahepatic tissues, suggesting potential roles for SHBG beyond the bloodstream (Hammond, 1995). A better understanding of SHBG gene expression patterns is important for clarifying how estrogens act in target tissues during reproduction across different fish species. The present findings indicate that the liver produces SHBG primarily during the reproductive phase in fish. The present study investigated the localization of cellular distribution of SHBG in adult female Indian major carp (*L. rohita*). This work provides insights into the tissue-specific presence of SHBG in a key aquaculture species, building on prior research into its plasma profiles and seasonal variations in this fish. As steroids are progressively utilized and depleted toward the peak of gonadal maturation, the demand for SHBG as a carrier protein may decrease, reducing its levels at that stage and thereby influencing steroid bioavailability.

Keywords: SHBG; estrogen; immunolocalization; bioavailability; *Labeo rohita*

INTRODUCTION

SHBG is the major transport protein for sex steroids in the blood of a wide variety of mammals and other vertebrate species, including fish (Westphal, 1986). Plasma SHBG is produced in the liver and regulates the bioavailability and metabolic clearance of its steroid ligands in the blood (Siiteri, 1982), but mammalian SHBG genes are expressed in tissues other than the liver, and this suggests that SHBG may act differently outside the blood system (Hammond, 1995). During fetal development in mammals, there is a transient burst of SHBG gene expression in the liver that coincides with critical stages of reproductive

organogenesis (Jänne *et al.*, 1999) and there is some indication that it is expressed in the fetal gut (Jänne *et al.*, 1999). Changes in the tissue-specific expression of SHBG are therefore likely to have a significant impact on the actions of sex steroids during development, and our knowledge of this critical component of sex steroid action in fish is lacking.

Although SHBG can be detected in the blood of fish, little is known about the structure of fish SHBGs or the site(s) of SHBG expression in this vertebrate class. Studies of the SHBG steroid-binding characteristics in several fish species (Ovrevik *et al.*, 2001; Hobby *et al.*, 2000; Van der Kraak and Biddiscombe, 1999; Laidley and Thomas, 1994; Tollefsen, 2002; Callard and Mak, 1988; Mak and Callard, 1987) have shown that its affinity for endogenous sex steroids (testosterone and estradiol), and xenobiotics (Tollefsen, 2002; Milligan *et al.*, 1998; Kloas *et al.*, 2000; Tollefsen *et al.*, 2002; Pryce-Hobby *et al.*, 2003) varies between species. There is also some evidence that plasma SHBG levels fluctuate in fish during the reproductive cycle (Hobby *et al.*, 2000; Laidley and Thomas, 1997; Suresh and Bagade, 2022) it is not exactly known where SHBG is produced in fish or how expression of the SHBG gene is regulated. Information about SHBG gene expression during early fetal development in mammals is also limited and zebrafish represent a powerful model system for studying the temporal and spatial expression of specific genes during development (Westerfield, 2000). Present study hereby involved the localization of SHBG molecule in this nonmammalian vertebrate species at both the immunohistochemical level. It has been attempted to localize SHBG cellular distribution in adult female Indian major carp, *Labeo rohita* by immunohistochemistry.

MATERIAL AND METHODS

Immunohistochemistry (IHC)

Deparaffinize/hydrate sections are incubated in three washes of xylene for 5 minutes each, then two washes of 100% ethanol for 10 minutes each and two washes of 95% ethanol for 10 minutes each. Washed sections in distilled water and PBS are prepared for the antigen unmasking (heat sections in 10 mM sodium citrate buffer (pH 6.0) for 1 minute. Cool slides after antigen unmasking and incubate them in 1% hydrogen peroxide for 10 minutes followed by blocking each section with 100–400 µl blocking solution for 1 hour at room temperature. Now add 100–400 µl diluted

primary antibody (anti-SHBG) to each section. (Dilute antibody in blocking solution.) Incubate overnight at 4°C. Remove antibody solution and wash sections in PBS three times for 5 minutes each and add 100–400 µl secondary antibody, diluted in blocking solution at room temperature. If using ABC avidin/biotin method, make ABC reagent add 100–400 µl ABC reagent to each section and finally add add 100–400 µl DAB reagent to each section and monitor staining closely. If desired, counterstain sections in hematoxylin for 10 seconds. Wash sections in dH₂O dehydrate sections and clear in xylene before normal mounting process (Suresh, 2007).

RESULTS AND DISCUSSION

Immunohistochemistry was adopted as the procedure to localize the SHBG in different tissues in *Labeo rohita*. The tissue levels checked for the reaction via IHC were liver, the manufacturing site of the globulin, kidney where most of the steroids of the body are synthesized, the gonads where the sex hormones are transported through these carrier globulins of the blood, and the brain too where the localization of SHBG might participate in the regulation or the modulation by any neurotransmitters or neuromodulators of the brain. The reaction occurred against anti-SHBG (DSL, Texas, USA) in all tissue levels except for the brain tissue which indicates the direct involvement of these carrier globulins in steroid metabolism at gonads and not any sort of modulation through neurotransmitter or neuromodulator of the brain.

The antibody specificity was also tested with pre-incubated anti-SHBG with the globulin in serum and no reaction was observed in the tissues like liver, kidney and gonads thus affirming no cross-reactivity with any of the other peptides by the used specific anti-SHBG.

Liver Tissue: Tissue samples of liver of female *Labeo rohita* in all the reproductive phases were subjected to IHC against anti-SHBG. The reaction showed a moderate occurrence throughout the season in the liver and more strong reaction during the late pre-spawning and spawning phases of the reproductive cycle of the fish. Rest of the period, the fish showed a non-significant change of SHBG in the plasma too in females which could come up with reaction of similar nature in the liver tissues of different seasons.

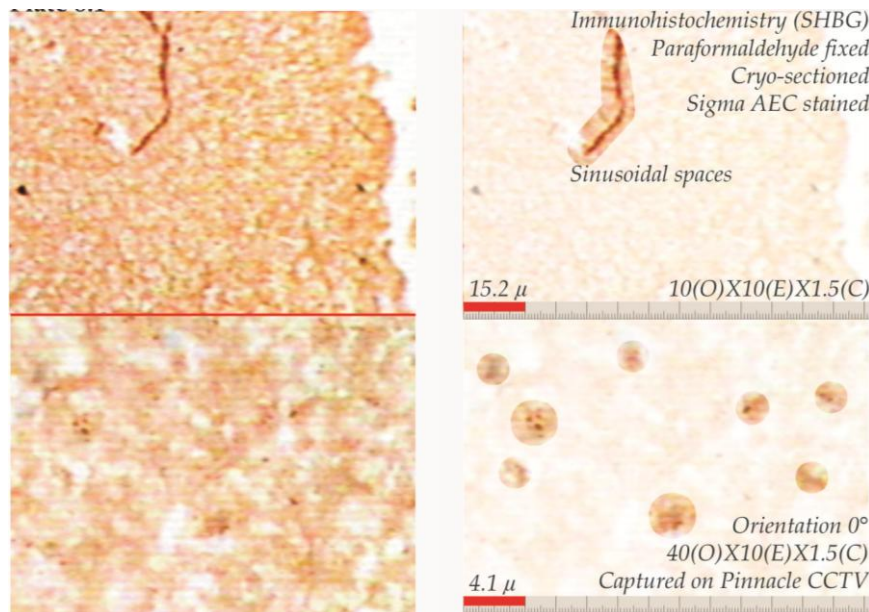


Plate 1. Transverse section of liver of *Labeo rohita* at lower and higher magnification showing hepatocytes & sinusoidal spaces reacted to anti-SHBG strongly. Right image is masked replica showing all reacted details.

The hepatocytes of liver showed a similar type of reaction strength throughout the season, but only the liver arteries and the sinusoidal spaces in different sections a showed the strong reactivity against anti-SHBG. Even extra-cellular areas of hepatocytes with glycogen stores were also found reacted which could help in the glycosylation in some or more way/amount of this carrier protein.

In plate 1, two immunohistochemically reacted sections of the fish liver are included to give an idea of localization of SHBG in the liver tissue. Sectioned used for the reaction were the *para*-formaldehyde fixed and cryo-sectioned liver tissue which support a positive, specific and significant reaction of the carrier protein SHBG in the fish, *Labeo rohita*.

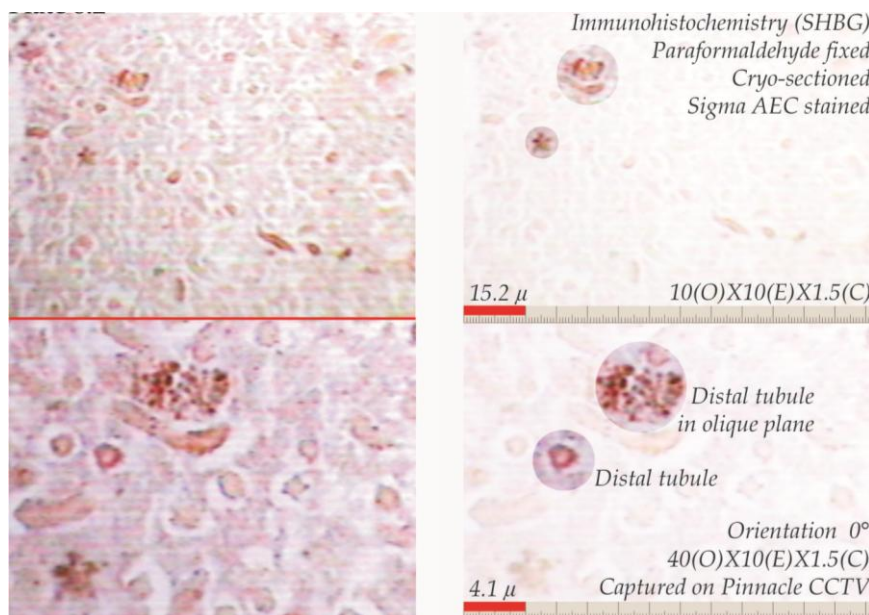


Plate 2. Transverse section of kidney tissue of *Labeo rohita* at low and higher magnification showing different collecting tubules reacted to anti-SHBG. Right image is masked replica highlighting all the reacted details.

Kidney Tissue: Kidney cortex and medulla are the sites for different steroid manufacture in the body and are primarily transported by different carrier globulins. There was a wide possibility of localization of this globulin in the kidney. Plate 2 shows two different photomicrographs at low and higher magnifications of kidney displaying reaction of anti-SHBG in different tubules. This localization signifies the prime function of SHBG that to regulate the metabolic loss of sex steroids in the body of the fish. The reaction was moderate throughout season, but

during the spawning phase the reaction appeared stronger and occurred in many tubule of both sexes.

Ovary: SHBG reaction at gonadal level was also demonstrated at different stages of reproductive cycle of female *Labeo rohita*. All the oocyte stages of different stages are shown to localize SHBG in an increasing order till the fish reaches the spawning phase where the reaction was diminished as the steroids in this phase are exhausted and the availability of SHBG diminishes in the ovarian tissue.

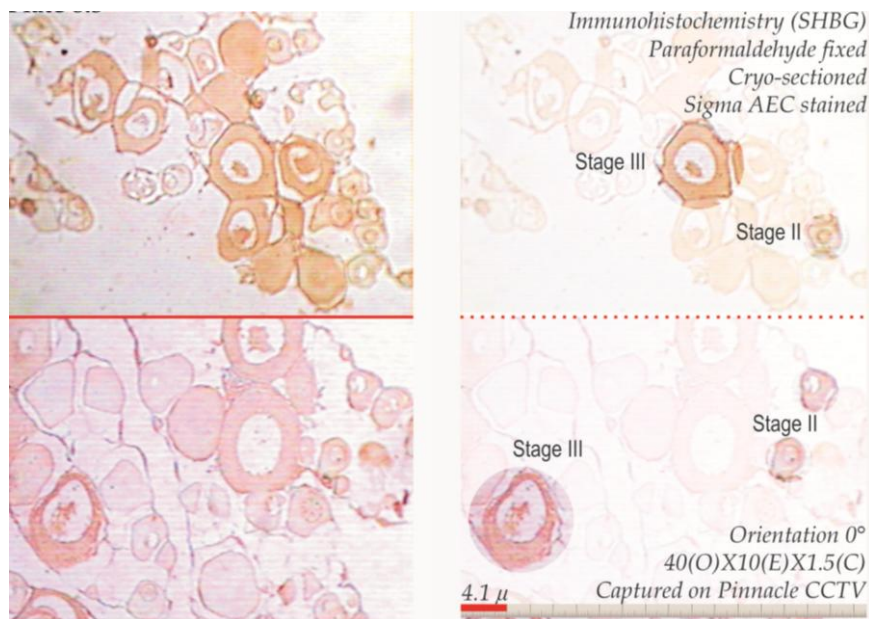


Plate 3. Transverse section of ovary of *Labeo rohita* at preparatory phase showing maturing oocytes of stage II & III reacted to anti-SHBG. Right image is masked replica showing all reacted details.

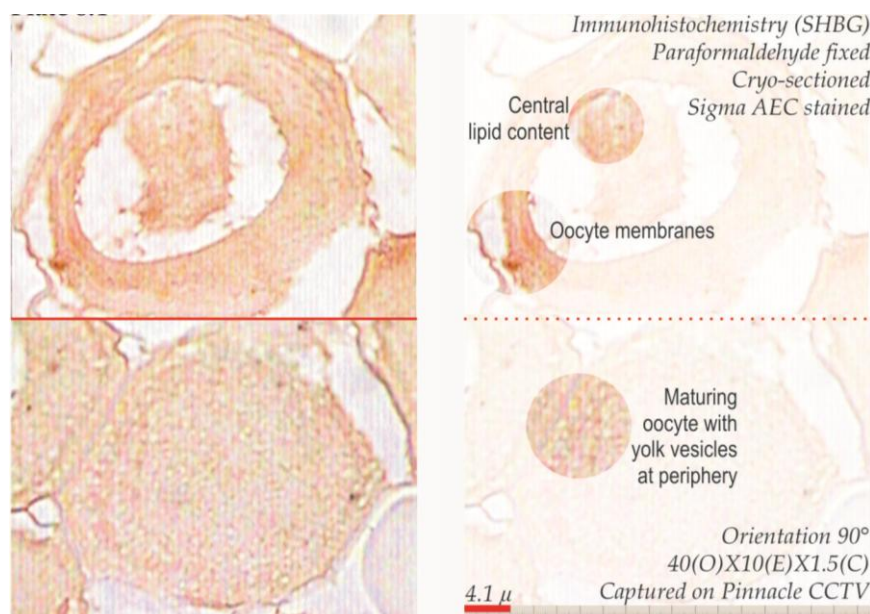


Plate 4. Transverse section of ovary of *Labeo rohita* at pre-spawning phase showing maturing oocytes of stage III & V reacted to anti-SHBG. Right image is masked replica showing all reacted details.

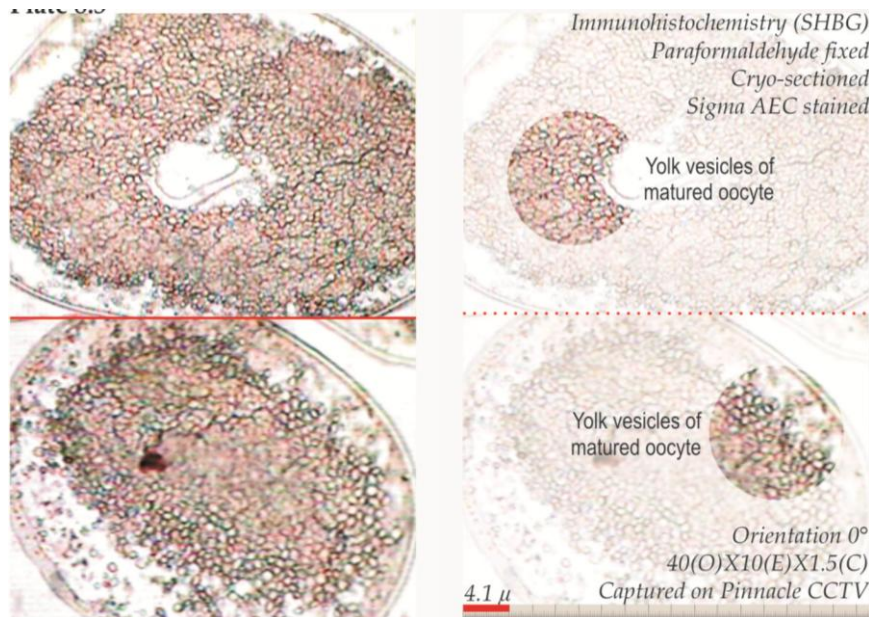


Plate 5. Transverse section of ovary of *Labeo rohita* at spawning phase showing maturing oocytes of stage VI reacted to anti-SHBG. Right image is masked replica showing all reacted details.

Plate 3 shows the localization of SHBG in three stages of oocytes of preparatory phase of the reproductive cycle. The maturing oocytes get stained strongly enough depicting the presence of sex steroids which are escorted with the help of this carrier protein SHBG. Similarly, the stage III and V maturing oocytes show (plate 4) strong reaction in the pre-spawning phase of the cycle of the fish where the utilization of sex steroids were at the maximum. As a consequence of sufficient utilization of sex steroids by the oocytes of matured state approaching spawning phase, the reaction of SHBG diminished within the oocytes. (Plate 5)

Like SHBGs in mammalian species, fish SHBG is a glycoprotein. The *N*-glycosylation of plasma proteins is generally assumed to influence their biological half-lives, but it has also been suggested that a highly conserved consensus site for *N*-glycosylation in the carboxy-terminal laminine G (LG) domain of mammalian SHBGs has some other functional role

(Hammond and Bocchinfuso, 1995). It was therefore surprising that this site is not conserved in fish SHBG sequences and that none of the three consensus sites for *N*-glycosylation sites in fish SHBGs are located within the carboxy-terminal LG domain as they are in mammalian SHBG sequences. Moreover, all three *N*-glycosylation sites in zebrafish SHBG are conserved within the amino-terminal LG domains of SHBG sequences of fugu fish and several other fish species.

These phylogenetic differences in the location of *N*-linked carbohydrate chains within SHBG sequences may provide new clues as to the functional roles of specific oligosaccharide chains associated with this protein.

Measurements of SHBG concentrations in the plasma of adult *Labeo rohita* are within range of 192- 417 nM in females increase in the reproductive period of the fish reproduction as reported in several other fish species including goldfish (Van Der Kraak and Biddiscombe, 1999), flounder (Hobby *et al.*, 2000), and spotted seatrout (Laidley and Thomas, 1994), with no sexual dimorphism in levels, as previously noted in goldfish. It should be noted, however, that these are among the highest plasma SHBG levels reported in fish species, with some species having plasma SHBG levels lower than 50 nM because plasma SHBG levels have been reported to increase by only about 50% in some fish during the breeding season (Hobby *et al.*, 2000; Laidley and Thomas, 1997; Foucher *et al.*, 1992). Furthermore, in those fish species in which estradiol is the preferred ligand of SHBG, large increases in plasma 11-ketotestosterone in females as well as males would also enhance the bioavailability of this active estrogen. This highlights the need for a greater understanding of how changes in *SHBG* gene expression may impact on the actions of both androgens and estrogens in the context of their specific target tissues during reproduction in different fish species.

Localization studies of major carp, *Labeo rohita*, during gametogenesis indicate that the liver, kidney and gonads are major sites of SHBG expression. As in mammalian fetuses (Jänne *et al.*, 1999) liver appears to produce SHBG during the reproductive phase of the fish as per the results obtained. The reaction of SHBG during spawning phase increases in the luminal regions of sinusoidal spaces and the duct areas but the hepatocytes show a consistent reaction throughout. The evidence of liver being the manufacturing site could also be affirmed by the results of Solange Miguel-Queralt *et al.*, (2004) where results prove that liver appears to produce SHBG during development of fish soon after its formation in zebrafish larvae may be in day 5 and day 6 larval fish with the help of in situ hybridization of mRNA of SHBG, and this most likely results in the secretion of SHBG into the developing blood circulation in which it presumably influences the way sex steroids influence gonadal development at this stage of development (Callard *et al.*, 2001; Kishida and Callard, 2001).

The amount of immunoreactive SHBG in *Labeo rohita* hepatocytes is average despite the fact that liver is clearly the major organ responsible for the production of plasma SHBG. This can be attributed to the rapid secretion of protein from hepatocytes, which would be in accordance with similar observations in mammals (Jänne *et al.*, 1998). Observations, however, were made of more intense SHBG immunoreactivity within the luminal aspect of cells lining intrahepatic bile ducts in adult fish and the sinusoidal spaces as captured in plate 8.1, suggesting that SHBG in this location may influence its manufacturing and release into the blood stream or to some extent in the biliary excretion of steroids in fish (Pryce-Hobby, 2003).

Like liver, kidney tissue also appears to show SHBG immunoreactivity in their tubular regions suggesting the involvement of SHBG in the sex steroid conservation from being metabolically lost.

The adult female major carp as it gives a concrete reactivity of SHBG in its gonads *i. e.* ovary, explains the involvement of sex steroids in the oocyte maturation and SHBG is the main globulin protein to carry them to this target organ. As the oocyte maturation is accomplished by the spawning period, SHBG reactivity in the ovaries diminishes giving clear indication that the need of SHBG might get reduced as the steroids are exhausted by this time of gonadal maturational climax

whose bioavailability is made possible by this carrier protein globulin, SHBG. Further studies have revealed that there is an isoform of human SHBG which is accumulated in the acrosome of the sperm and this could be suggested in fish too as SHBG immunoreactivity in spermatozoa is sufficiently visible, though very feeble. This was not unexpected because there is considerable variation in the morphology of the testis and the site of *SHBG* gene expression even in mammalian species (Selva *et al.*, 2002).

CONCLUSION

Like SHBGs in mammalian species, fish SHBG is a glycoprotein. The *N*-glycosylation of plasma proteins is generally assumed to influence their biological half-lives. The site for *N*-glycosylation in the carboxy-terminal LG domain is not conserved in fish SHBG sequences. The phylogenetic differences in the location of *N*-linked carbohydrate chains within SHBG sequences may provide new clues as to the functional roles of specific oligosaccharide chains associated with this protein. The need for a clearer understanding of *SHBG* gene expression may impact on the actions of estrogens in the context of their specific target tissues during reproduction in different fish species. The liver appears to produce SHBG during the reproductive phase of fish as per the results obtained, suggesting to some extent its role in the biliary excretion of steroids in fish SHBG might get reduced as the steroids are exhausted by the time of gonadal maturational climax whose bioavailability is made possible by this carrier protein globulin, SHBG.

Conflict of Interest: None of the authors have any conflicts of interest to disclose.

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