



SHBG Immunoreactivity in male *Labeo rohita*.

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ABSTRACT

SHBG acts as the main delivery protein for sex steroids within the blood of numerous mammals, different vertebrates, and fish varieties. Made chiefly by the liver, circulating SHBG manages how available its attached steroid hormones are and how quickly they are cleared metabolically. Nevertheless, in mammals, the SHBG gene is also active in various tissues outside the liver, suggesting SHBG might have functions separate from the bloodstream. Among aquatic animals, the particular way SHBG attaches to steroids is quite unlike that in mammals, since certain fish possess SHBG that binds androgens with greater affinity than oestrogens. This current research examined where the SHBG molecule is located in this specific vertebrate through immunohistochemistry. Precisely, we aimed to pinpoint the cellular location of SHBG within mature male Indian major carp (*Labeo rohita*). The results we have now suggest that the liver creates SHBG mainly when fish enter their reproductive period. This aligns with a possible function in assisting the release of steroids through bile. Since steroids are gradually used up and gone by the height of gonad development, the need for SHBG as a carrier protein might lessen, thus affecting how much steroid is present in the plasma or other tissue amounts.

Keywords: SHBG; androgens; immunoreactivity; testes; *Labeo rohita*

INTRODUCTION

SHBG serves as the primary transport protein for sex hormones found in the bloodstream of various mammals and other vertebrate classes, including fish (Westphal, 1986). The liver generates plasma SHBG, which manages the availability and metabolic clearance of its steroid ligands within the bloodstream (Siiteri, 1982), although SHBG genes in mammals are present in tissues aside from the liver, indicating that SHBG may fulfill distinct roles outside the circulatory system (Hammond, 1995). For example, the testis generates a homolog of SHBG, referred to as the testicular androgen-binding protein, which is released into the seminiferous tubular lumen and controls androgen availability in the male reproductive system (Joseph, 1994).

The SHBG gene in humans is also present in the germ cells of the testis, and an SHBG variant with an unclear function accumulates in the sperm acrosome (Selva et al., 2002). In mammalian fetal development, there is a temporary surge of SHBG gene activity in the liver that aligns with key phases of reproductive organ formation (Sullivan et al., 1991; Jänne et al., 1999), and there is some evidence suggesting its expression in the fetal gastrointestinal tract (Jänne et al., 1999). Therefore, alterations in tissue-specific SHBG expression are likely to significantly influence the effects of sex steroids throughout development, and our understanding of this vital aspect of sex steroid function in fish is limited.

While SHBG is detectable in the bloodstream of fish, detailed knowledge regarding the structure of fish SHBGs or the locations of SHBG expression within this class of vertebrates remains scarce. Research examining the steroid-binding properties of SHBG in several fish species (Ovrevik et al., 2001; Hobby et al., 2000; Van der Kraak and Biddiscombe, 1999; Laidley and Thomas, 1994; Pasmanik and Callard, 1986; Tollefsen, 2002; Callard and Mak, 1988; Mak and Callard, 1987) has indicated that the protein's affinity for natural sex steroids (like testosterone and estradiol) and xenobiotics (Tollefsen, 2002; Milligan et al., 1998; Kloas et al., 2000; Tollefsen et al., 2002; Pryce-Hobby et al., 2003) differs across species. There is also some indication that plasma SHBG concentrations fluctuate in fish throughout the reproductive cycle (Hobby et al., 2000; Laidley and Thomas, 1997; Foucher et al., 1992; Suresh and Bagade, 2022), yet the precise sites of SHBG production in fish or the regulatory mechanisms governing SHBG gene expression remain unclear. Research about SHBG gene activity during the early stages of fetal development in mammals is also limited, and zebrafish serve as an excellent model for investigating the timing and location of specific gene expression during developmental stages (Westerfield, 2000).

The current study focused on identifying the localization of the SHBG molecule within this nonmammalian vertebrate species using immunohistochemical methods. An effort was made to determine the cellular distribution of SHBG in adult male and female Indian major carp, *Labeo rohita*, through immunohistochemistry.

MATERIAL AND METHODS

Immunohistochemistry (IHC)

The sections are made dewaxed and wet by moving through three rinses using xylene that last five minutes for every rinse, after which there are two rinses lasting ten minutes each using one hundred percent ethanol and two more rinses using ninety-five percent ethanol for ten minutes apiece. The slides are subsequently rinsed using plain water and PBS to get them ready for antigen retrieval, heating them in a ten mM sodium citrate buffer at a pH of six point zero for sixty seconds. After the antigen retrieval step is complete, the slides get to cool down and are then treated using one percent hydrogen peroxide for a period lasting ten minutes, followed by covering every section with one hundred to four hundred µl of a blocking solution for sixty minutes at room temperature. Next, one hundred to four hundred µl of the primary antibody that has been diluted (anti-SHBG) is put onto every section (the antibody ought to be mixed into the blocking solution) and left overnight at four°C. After this specific stage is finished, the antibody mixture is thrown out, and sections are rinsed in PBS three times, with each rinse taking five minutes. Then, one hundred to four hundred µl of the secondary antibody, which is also mixed in blocking solution, is added at room temperature. Should the ABC avidin/biotin approach be employed, prepare the ABC solution and place one hundred to four hundred µl of it onto each section, then follow this by adding one hundred to four hundred µl of the DAB solution, making sure to keep a close eye on how the coloring develops. If it seems needed, slides are able to be restained with hematoxylin for ten seconds. In conclusion, the sections are rinsed in dH₂O, dried out, and treated with xylene before the usual procedure for mounting takes place (Suresh, 2007; 2023).

RESULTS AND DISCUSSION

Immunohistochemistry was utilized as the method to identify the presence of SHBG across various tissues in *Labeo rohita*. The tissues assessed for IHC reactions included the liver, which produces the globulin, the kidney, a key site for synthesizing most body steroids, the gonads, where sex hormones are carried by these blood globulins, and the brain, where SHBG localization may play a role in the regulation or

influence by neurotransmitters or neuromodulators. A reaction was observed against anti-SHBG (DSL, Texas, USA) in all tissues except for the brain, signifying the essential role of these carrier globulins in steroid processing within the gonads rather than being involved in modulation via brain neurotransmitters or

neuromodulators. The specificity of the antibody was analyzed using pre-incubated anti-SHBG with the globulin in serum, which revealed no reactions in the liver, kidney, and gonads, thereby confirming that the specific anti-SHBG used did not cross-react with any other peptides.

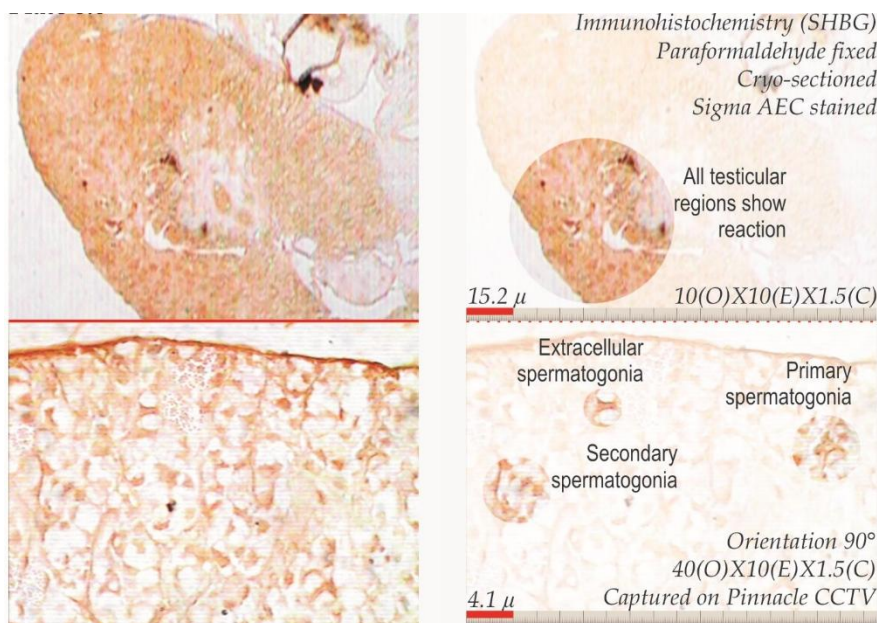


Plate 1. Cross section at low & transverse section at higher magnification of preparatory testis of *Labeo rohita* showing spermatogonia reacted to anti-SHBG. Right image is masked replica highlighting reacted details.

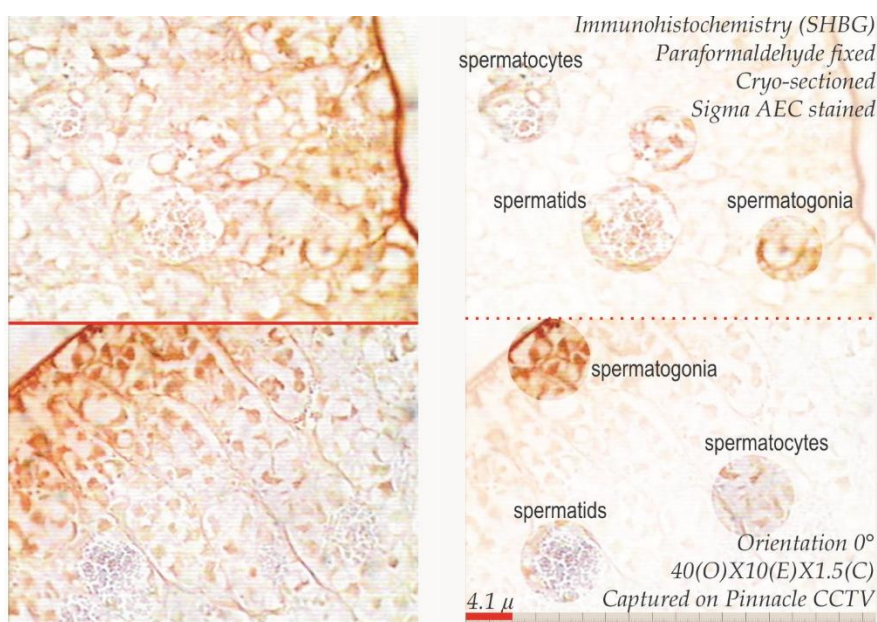


Plate 2. Transverse section of pre-spawning testis of *Labeo rohita* showing spermatogonia, spermatocytes and spermatids reacted to anti-SHBG. Right image is masked replica highlighting reacted details.

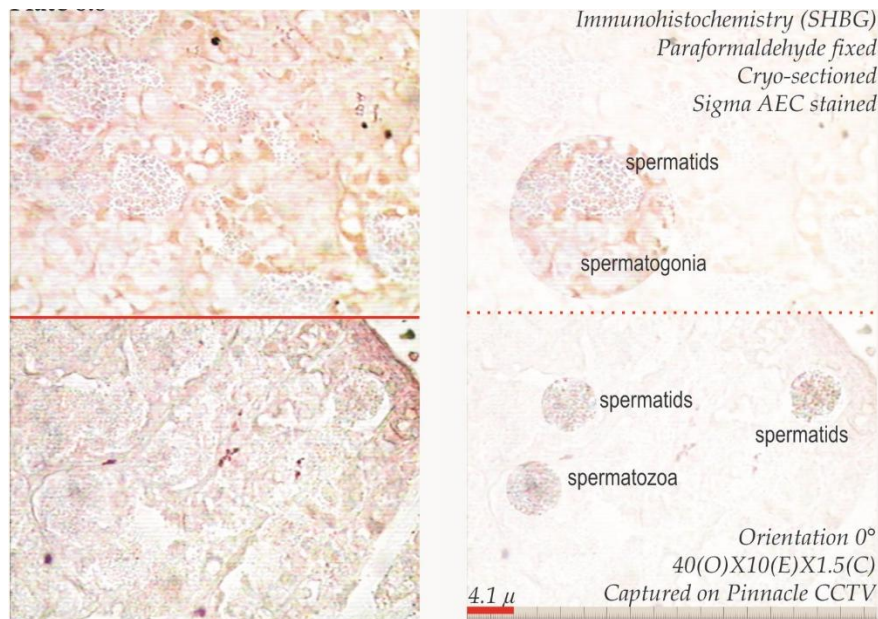


Plate 3. Transverse section of spawning testis of *Labeo rohita* showing spermatocytes and spermatids strongly- and spermatozoa feebly-reacted to anti-SHBG. Right image is masked replica highlighting reacted details.

Tissue specimens from the liver of both male and female *Labeo rohita* across all stages of reproduction were analysed using immunohistochemistry against anti-SHBG. The findings indicated a moderate presence of SHBG throughout the entire season, with a notably stronger presence during the late pre-spawning and spawning stages of the fish's reproductive cycle. A similar reaction trend was noted in males as well. The liver's hepatocytes exhibited a comparable level of reaction intensity throughout the season, though the liver arteries and sinusoidal spaces in various sections demonstrated stronger reactivity to anti-SHBG. The kidney cortex and medulla serve as the locations for the production of various steroids within the body, and they are mainly carried by distinct globulins. The localization indicates the essential role of SHBG, which is to manage the metabolic reduction of sex steroids in the fish's body. The reaction was consistently moderate across the seasons, but it intensified during the spawning period and was evident in numerous tubules of both genders (Suresh, 2023)

SHBG activity within the gonads was also evident across various reproductive stages of female *Labeo rohita*. All oocyte types across different phases display SHBG presence, intensifying until the fish enters the spawning period, at which point the activity decreased because the steroids in this phase are depleted and the ovarian tissue holds less SHBG.

The positioning of SHBG across three developmental stages of oocytes during the preparatory phase of the reproductive cycle. The developing oocytes stain quite intensely indicating the existence of sex steroids which are carried along by this SHBG protein. Likewise, the developmental stages III and V oocytes exhibit (plate 4) a powerful response in the pre-spawning period of the fish cycle when the usage of sex steroids reached its peak. Following the ample consumption of sex steroids by the oocytes in their matured state nearing the spawning period, the SHBG reaction lessened inside the oocytes (Suresh, 2023).

Plates 1-3 illustrate the distribution of SHBG within the testes of the fish species *Labeo rohita* during various reproductive stages. The entirety of the testes exhibited a response (plate 1), indicating that all components contributing to male reproductive success are present. These components can be seen in detail at increased magnification across different reproductive phases. The lower portion of plate 1 highlights both primary and secondary spermatogonia, alongside extracellular spermatogonial spaces that showcase a robust response indicative of the preparatory stage in the fish's reproductive cycle. The two images in plate 2 distinctly display the reaction in spermatogonia, spermatocytes, and spermatids within the testes during the pre-spawning phase of the cycle. The upper section of plate 3 similarly allows for the observation

of spermatids exhibiting a strong reaction to anti-SHBG during the spawning phase, while the lower section of the same plate indicates a weak response in spermatozoa, suggesting that sex steroids are depleted by the time of their formation. Throughout the season, both the liver and kidneys demonstrated a reaction, indicating that SHBG plasma levels did not drop to zero but maintained a relatively stable level throughout the cycle, except when approaching the spawning phase.

Similar to the SHBGs found in mammals, the SHBG present in fish is classified as a glycoprotein. It is commonly thought that N-glycosylation of plasma proteins affects their biological half-lives; however, it has also been proposed that a well-preserved consensus site for N-glycosylation within the carboxy-terminal laminine G (LG) domain of mammalian SHBGs may serve an additional functional purpose (Hammond and Bocchinfuso, 1995; Suresh et al., 2021). Therefore, it was unexpected to find that this site is not preserved in the SHBG sequences of fish, and none of the three consensus sites for N-glycosylation in fish SHBGs are found in the carboxy-terminal LG domain as they are in mammalian SHBG sequences. Furthermore, all three N-glycosylation sites identified in the zebrafish SHBG are retained within the amino-terminal LG domains of the SHBG sequences of Fugu fish and several other species of fish. These evolutionary variations in the positioning of N-linked carbohydrate chains within SHBG sequences might provide new insights into the specific functional roles of certain oligosaccharide chains associated with this protein.

The specificity of SHBG binding to steroids in fish species shows a broader variation compared to mammals. In some fish, SHBG displays a stronger binding affinity for androgens over estrogens (Hobby et al., 2000; Van Der Kraak and Biddiscombe, 1999; Pasmanik and Callard, 1986), while the opposite trend is observed in others (Ovrevik et al., 2001; Hobby et al., 2000; Tollefsen, 2002). In certain fish, two variants of the androgen receptor have been reported, which differ in their relative affinities for androgens (Takeo and Yamashita, 1999; Ikeuchi et al., 1999; Sperry and Thomas, 1999), and 11-ketotestosterone stands out as the quantitatively predominant androgen present in the blood of numerous fish species during their breeding period (Borg, 1994). It is noteworthy that,

based on current studies, fish SHBGs exhibit a reduced affinity for this androgen compared to testosterone.

The concentration of SHBG in the plasma of adult *Labeo rohita* is observed to be between 192 and 417 nM for females (Suresh et al., 2008), while for males, it ranges from 218 to 424 nM. This minor increment during the fish's reproductive phase is not statistically significant and has been similarly noted in a variety of other fish species, such as goldfish, flounder, and spotted seatrout. Notably, there appears to be no difference between sexes in SHBG levels, a finding that has been previously documented in goldfish. It is important to note that these levels represent some of the highest SHBG concentrations recorded in fish, with several species exhibiting plasma SHBG levels of under 50 nM. Reports indicate that plasma SHBG levels in certain fish increase by merely about 50% during breeding periods, and substantial rises in plasma 11-ketotestosterone could lead to the release of testosterone and androgen precursors like androstenedione from the binding sites on SHBG, thereby facilitating their transport to tissues sensitive to androgens. Additionally, in species where SHBG binds preferentially to estradiol, significant elevations in plasma 11-ketotestosterone among both females and males may also improve the availability of this potent estrogen. This situation emphasizes the necessity to explore how the variation in SHBG gene expression may influence the behaviours of both androgens and estrogens concerning their specific target tissues during reproductive cycles across various fish species.

Research into the localization in major carp, specifically *Labeo rohita*, during gametogenesis reveals that the liver, kidney, and gonads act as primary sites for SHBG expression (Suresh, 2023). This likely leads to SHBG being released into the developing circulatory system where it may affect the influence of sex steroids on gonadal development at this critical growth phase.

The adult female major carp displays significant SHBG reactivity within its reproductive organs, specifically in the ovaries, which highlights the participation of sex steroids in the maturation of oocytes (Suresh, 2023). Comparable findings have been noted in the testes, where SHBG immunoreactive protein is localized to the outer

edges of the seminiferous tubules. Although this layout aligns with the presence of Sertoli cells in fish testes, SHBG immunoreactivity is not restricted to specific cell types like spermatogonia, spermatocytes, or spermatids; rather, it is apparent across all cell types involved in maturation, and it likely serves a different role in regulating androgen access for developing sperm than it does in mammals, such as rats, according to Joseph in 1994. Additional research has highlighted the existence of a specific isoform of human SHBG that accumulates in the sperm acrosome, a possibility that can also be suggested in fish due to detectable yet minimal SHBG immunoreactivity in spermatozoa. This isn't surprising, given the significant differences in testis morphology and SHBG gene expression sites, even among various mammalian species, as noted by Selva et al. in 2002.

CONCLUSION

Fish SHBG is identified as a glycoprotein, widely believed that the N-glycosylation of plasma proteins has an effect on their biological half-lives. In fish SHBG sequences, the region responsible for N-glycosylation within the carboxy-terminal LG domain is not conserved. In aquatic species, the steroid-binding specificity of SHBG differs significantly compared to mammals, with some fish having SHBG that binds androgens more strongly than estrogens. A better understanding of SHBG gene expression could influence how androgens and estrogens act within their designated target tissues during the reproductive processes of various fish species. Findings indicate that the liver generates SHBG during the reproductive stage in fish, hinting at its potential involvement in the biliary excretion of steroids. As the steroids are utilized by the point of maximal gonadal maturation, SHBG levels might decline, with the bioavailability of these steroids being facilitated by the globulin carrier protein, SHBG. The activity in testes serves as a clear indication of how androgen access to developing sperm is regulated.

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