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Diversity and biological significance of sex hormone-binding globulin in fish, an evolutionary perspective

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Abtract

In fish, two different genes, *shbga* and *shbgb*, exist that encode for very different proteins. Shbga is the ortholog of mammalian Shbg and was found in all investigated teleosts. In contrast, Shbgb is highly divergent and appears to be a salmonid-specific protein. Here, we review existing data on fish Shbga and Shbgb that have been obtained in chondrichthyes and osteichtyes. Even though other significant expression sites exist, existing data indicate that Shbga is mainly expressed in liver and subsequently secreted into the blood as a homodimer. In contrast, Shbgb is mainly expressed in the ovary, probably secreted as a monomer, and could contribute to the regulation of local steroid action. Binding studies indicate a specialization of circulating Shbg during evolution towards the preferential binding of estradiol and testosterone in teleosts. In contrast, specific fish steroids such as 11-oxo-androgens and oocyte maturation-inducing steroids that are crucial for reproduction are poorly bound by either form of Shbg.

Introduction

Sex hormone-binding globulins (Shbg) are mainly known as carrier blood proteins involved in the transport of sex steroids in plasma and in the regulation of their bioavailability to target organs. The Shbg protein has initially been identified in the beta-globulin fraction of the human serum (Rosner et al., 1969). This field has subsequently benefited from many molecular studies of the genes and transcripts encoding Shbg in many mammalian species (Joseph et al., 1991;Kahn et al., 2002;Rosner, 2006;Nakhla et al., 2009) (see also reviews in the present issue). In fish, Shbg has been found in the plasma of an elasmobranch, the skate (*Raja radiata*) (Freeman and Idler, 1969), and of a teleost, the rainbow trout (*Oncorhynchus mykiss*) (Fostier and Breton, 1975) a few decades ago, and subsequently identified and studied in a wide variety of fish species as reviewed here. However, in contrast to mammals, the molecular analysis of teleost *shbg* genes was not carried out until recently (Miguel-Queralt et al., 2004;Miguel-Queralt et al., 2005;Bobe et al., 2008b) and available data remain scarce. Here, after providing a phylogenetic characterization of Shbg proteins in fish, we first aim at reviewing existing molecular and biochemical data that have been obtained not only in osteichtyes, but also in chondrichthyes. The recent molecular data on *shbg* genes will be reviewed and discussed in the light of existing biochemical data that have previously been gathered in a wide variety of species. In addition, we will take advantage of the basal evolutionary position of teleosts within the vertebrate lineage to discuss the evolution of binding characteristics of vertebrate Shbg proteins. Finally, although several alternatively spliced transcripts have been identified in mammals (Nakhla et al., 2009), it should be stressed that, in contrast to mammals, two highly divergent Shbg proteins exist in fish. Shbg alpha form (Shbga) is clearly the ortholog of the mammalian Shbg and has been found in all investigated teleost species. In contrast, the Shbg beta form (Shbgb) initially identified in

rainbow trout (Bobe *et al.*, 2008b) has subsequently been found in other salmonid species (Miguel-Queralt et al., 2009) but could not be identified in any other non-salmonid teleost species investigated to date. This offers a great opportunity to discuss the evolution of Shbgb binding characteristics and putative biological functions with regards to sequence divergence and conserved sequence features.

For clarity reasons, Shbga and Shbgb symbols will be used in the text when proteins with known sequences were specifically studied while Shbg symbol will be used for Shbg proteins in general or when binding or biochemical studies were carried out directly from plasma or tissues.

Shbg genes, structure and evolutionary history

Evolution of fish Shbg genes

To date, Shbg proteins have only been characterized molecularly in bony fish (osteichtyes class), more specifically in the teleost superorder that includes all modern ray-finned fish species (actinopterygians fishes). The phylogenetic reconstruction of the evolutionary history of Shbg proteins in these teleost fish (Figure. 1) indicates that most species have only one Shbg, designated as the Shbg alpha form (Shbga), that is closely related to mammalian Shbg proteins. However, in some fish species, such as the common carp (*Cyprinus carpio*), two duplicated genes are found (*shbga1* and *shbga2*) that are probably the result of the recent whole genome duplication reported in this species (Larhammar and Risinger, 1994). In contrast to these recent gene duplications that resulted in highly similar Shbg proteins, a highly-divergent Shbg beta form, designated as Shbgb, has been found in the salmonid lineage (Bobe *et al.*, 2008b;Miguel-Queralt *et al.*, 2009). The phylogenetic position of Shbgb proteins is intriguing as they all branched together with high bootstrap values at the root of the tetrapod Shbg branch (Figure. 1). This topology suggests an ancient duplication followed by a

subsequent lost in all non-salmonid vertebrate species (Miguel-Queralt *et al.*, 2009). However, it is also possible that *shbga* and *shbgb* genes are the result of a duplication of a common *shbg* ancestor gene that occurred after salmonid radiation. The additional whole genome duplication (3R) that occurred in salmonids would be consistent with this hypothesis (Allendorf and Thorgaard, 1984). The topology of the phylogenetic tree could thus be explained by a long-branch attraction artifact that is classically observed for highly divergent sequences (Delsuc et al., 2005). It should also be stressed that accelerated molecular evolution appears to be quite frequent following fish genome duplications (Steinke et al., 2006). The high divergence between Shbga and Shbgb would thus suggest a functional shift through either sub- or neo-functionalization that would be consistent with the very different expression patterns observed (Bobe *et al.*, 2008b;Miguel-Queralt *et al.*, 2009).

Structure of Shbg-alpha (Shbga) in teleosts

In all species studied to date the classical Shbg (Shbga) exists as a homodimeric glycoprotein characterized by a common structure composed by two laminin G (LG) like domains that contains two sets of conserved cysteines that form intramolecular disulphide bridges. As secreted proteins, a signal peptide sequence is cleaved from the precursor protein to give rise to the mature protein. Apart from its role as a steroid carrier protein, there is some evidence that this protein can mediate steroid signaling by binding to membrane associated proteins (Hryb et al., 2002). Accordingly, a putative N-terminal membrane receptor-binding domain that could bind to specific receptors on cell membranes has been characterized in humans (Khan et al., 1990). Plasma Shbg is normally glycosylated and this is thought to influence the biological half-life of the protein (Cousin et al., 1999). Classical Shbg (Shbga) sequences in fish have been characterized recently but only in a very few number of fish species including zebrafish (*Danio rerio*) (Miguel-Queralt *et al.*, 2004), Atlantic sea bass (*Dicentrarchus labrax*) (Miguel-Queralt *et al.*, 2005), rainbow trout (Bobe *et al.*, 2008b) and coho salmon

(Miguel-Queralt *et al.*, 2009). Other information on fish Shbg sequences can also be deduced from published cDNA, expressed sequence tags (ESTs) databases, and from the analysis of genomic sequences available in some fish species. The analysis of these sequences and their deduced proteins indicates that the fish classical Shbga proteins are typically 380-400 amino acid long. These proteins are poorly conserved at the amino-acid level as they only share 40 to 80 % identity with each other, depending on their evolutionary proximity, and around 25 % identity with the human sequence (Table 1). Despite this relatively poor sequence identity, all fish Shbga have classical Shbg features including, the two well conserved LG-domains (N and C-terminal) with the conserved cysteine residues that participate in the disulphide bridges, a signal peptide sequence that is cleaved from the precursor protein to produce the mature protein, and a putative N-terminal membrane receptor-binding domain (Figure 2). Fish Shbga also contain three N-glycolysation sites that are relatively well conserved among fish sequences (Figure 2) with the exception of the Japanese medaka (*Oryzias latipes*) that does not share the third site of N-glycosylation (Miguel-Queralt *et al.*, 2005). However, these N-glycosylation sites are unique to fish Shbga and are not conserved in mammalian SHBG sequences (Miguel-Queralt *et al.*, 2005).

A few biochemical determinations of fish Shbg molecular weight have been performed on native or recombinant proteins using gel filtration and/or polyacrylamide gel electrophoresis: 80 Kda in skate (Freeman and Idler, 1969), 150-180 Kda in spiny dogfish (*Squalus Acanthias*) (Ho et al., 1980), 64 Kda in Japanese eel (*Anguilla japonica*) (Chang et al., 1994), 194 Kda in common carp (Chang and Lee, 1992), 105±8.7 Kda in zebrafish (Miguel-Queralt *et al.*, 2004), 118.3±11.5 Kda in Atlantic sea bass (Miguel-Queralt *et al.*, 2005), and 153 Kda for the coho salmon Shbg alpha form (Miguel-Queralt *et al.*, 2009). With the exception of the Japanese eel these observations are in agreement with the presence of a circulating homodimer made from glycosylated subunits (Miguel-Queralt *et al.*, 2005). Besides species differences, variability in

molecular weights may also be due to the occurrence of Shbg aggregation / polymerization which is less pronounced when dithiothreitol or 2-mercaptoethanol was used as reducing agents in buffer preventing disulfide bonds (Freeman and Idler, 1969;Ho *et al.*, 1980). These differences could also be due to different numbers of oligosaccharides chains since, as indicated above, three N-glycosylation sites may be found in several species (Miguel-Queralt *et al.*, 2004). In mammals, observed isoforms have been considered to be the result of partial use of the two N-glycosylation sites on each Shbg subunit (Danzo et al., 1989;Bocchinfuso et al., 1992;Cousin *et al.*, 1999).

The structure of the *shbga* gene has been initially described in the zebrafish (Miguel-Queralt *et al.*, 2004) in which the transcription unit encoding the Shbga protein has been found to span close to 13 kb in length and to contain 8 exons. This gene organization with 8 exons is well conserved in teleost fish; at least within *shbga* genes deduced from species with an available genome sequence (Figure 3). Despite important differences in gene length (from 2.2 kb for the Japanese pufferfish (*Takifugu rubipres*) to more than 13 kb in the zebrafish) all intron-exon boundaries are conserved (data not shown). This highly conserved structure of the *shbga* gene among teleost species was not totally unexpected as the zebrafish gene organization was already found to be well conserved with the cognate human sequence, including a perfect conservation of the intron-exon boundaries (Miguel-Queralt *et al.*, 2004). In contrast, none of the regulatory regions, that are conserved in human and rat genes, have been identified in the 1 kb proximal promoter of zebrafish and Japanese pufferfish *shbga* genes (Miguel-Queralt *et al.*, 2004).

Structure of the salmonid-specific Shbg beta form (Shbgb)

The salmonid-specific Shbgb proteins are highly divergent Shbg proteins (Bobe *et al.*, 2008b) as they share less identity (around 20%) with the classical fish Shbg (Shbga) than the human SHBG with fish Shbga (around 25%). However, despite these important sequence

divergences (see Table 1 and Figure 2) with Shbga proteins, they still display a good structural conservation of the tandem repeats of laminin G-like domains that are crucial for steroid-binding and dimer formation, or of the putative N-terminal amino-acid stretch that could bind to a Shbg membrane receptor. This suggests some well conserved functionality even though major structural differences have been found between Shbga and Shbgb proteins. For instance, and in contrast to Shbga, the Chinook salmon Shbgb has an estimated molecular weight of 68 kda and is only found as a monomer in plasma or when expressed as a recombinant protein (Miguel-Queralt *et al.*, 2009). The fact that Shbgb would not homodimerize spontaneously has been correlated with the lack of the highly conserved Val89 and Leu122 (Miguel-Queralt *et al.*, 2009) that have been found to be crucial for dimerization in human (Avvakumov et al., 2001). Other hypotheses explaining this absence of dimerization include major differences in N-glycosylation sites, as none of these sites are conserved with the Shbga form (Figure 2), or the lack of essential cation biding sites required for this dimerization (Miguel-Queralt *et al.*, 2009). To date, no gene structure has been reported for these highly divergent salmonid-specific *shbgb* genes. The gene structure information would, however, provide important information as the comparison of the vicinity of *shbga* and *shbgb* genes in salmonids would probably help to conclude whether or not these genes arise from a duplication that took place following the salmonid-specific genome duplication or following a more ancient duplication. Comparison of regulatory sequences between the proximal promoter sequences of *shbga* and *shgbb* genes may also be of great interest with regards of the important tissues specific expression of these two *shbg* genes.

Tissue distribution and expression sites

Shbga

The tissue distribution of *shbga* transcript has been studied in a limited number of teleost species. In zebrafish, *shbga* mRNA was detected by *in situ* hybridization and RT-PCR in digestive tract and hepatopancreas (Miguel-Queralt *et al.*, 2004). In addition, a low expression was detected in testis using RT-PCR (Miguel-Queralt *et al.*, 2004). Using immunocytochemistry, zebrafish Shbga protein was immunodetected in hepatopancreas, intestine, and testis (Miguel-Queralt *et al.*, 2004). In Atlantic sea bass, northern blot analysis showed that *shbga* mRNA was strongly expressed in liver but could not be detected in brain, digestive tract, kidney, or gonads (Miguel-Queralt et al., 2007). Using immunocytochemistry, seabass Shbga protein was detected in liver, intestine, and testis. Shbga immunoreactivity was also detected in the connective tissue around the ovary and a positive signal was observed around the post-vitellogenic oocytes of a mature female (Miguel-Queralt *et al.*, 2007). This would be in agreement with the finding of a Shbg-type binding in ovarian interstitial fluids of another perciformes species, the spotted weakfish *Cynoscion nebulosus* (Laidley and Thomas, 1994). In salmonids, *shbga* is also strongly expressed in liver (Bobe *et al.*, 2008b;Miguel-Queralt *et al.*, 2009). In rainbow trout, a strong mRNA expression was also observed in spleen while low expression levels could be detected in gills, stomach, and pituitary using quantitative RT-PCR (Bobe *et al.*, 2008b). In coho salmon, a low expression could also be detected in gills, stomach, and brain (Miguel-Queralt *et al.*, 2009). Together, these data indicate, in agreement with mammalian literature, that liver is the main expression site of Shbga in teleost fish. It is thus likely that the hepatic expression of Shbga, followed by secretion into the blood stream, is resulting in plasma Shbga. This is fully supported by existing data demonstrating that Shbg present in the culture medium of rainbow trout

hepatocytes and in plasma had similar steroid binding characteristics and electrophoretic mobility (Foucher et al., 1991). However, the significant expression found in several other tissues (e.g. spleen) suggests that, at least in some species, circulating Shbga could also be from extra-hepatic origin. In mammals, a local expression of Shbg in several target organs has also been evidenced and associated with a modulation of the steroidogenic signal (Hryb *et al.*, 2002;Kahn et al., 2002). Similarly, non-hepatic expression sites of Shbga in teleosts could also be associated with local action in target organs. However, further investigation would be necessary in fish to identify potential transcripts variants given that the transcriptional regulation of vertebrate *shbg* genes could be somewhat complex. For instance, 19 unique transcripts exhibiting differential tissular expression and deriving from 3 different promoters have been recently identified in humans (Nakhla *et al.*, 2009).

In mammals, a protein initially named androgen-binding protein (Abp) due to its binding affinity for androgen was found in the male reproductive tract of several species (Joseph, 1994). Further investigations have however demonstrated, in several mammalian species, that both Shbg and the so-called Abp were in fact encoded by a single copy *Shbg* gene (Joseph et al., 1987;Hammond et al., 1989;Hammond and Bocchinfuso, 1996). Numerous mammalian studies have further confirmed that testis was an important expression site of Shbg (Selva and Hammond, 2006;Nakhla *et al.*, 2009). In fish, Shbg binding was found in cytosol originating from trout testis that had been previously perfused to avoid blood contamination, in seminal plasma, and in the incubation medium of testicular explants (Foucher and Le Gac, 1989). It was thus hypothesized by these authors that *shbg* expression could occur in the rainbow trout testis. The binding found in testis was very similar to the binding found in plasma with regards to electrophoretic mobility and relative binding affinity for testosterone (T) and E2 (Foucher and Le Gac, 1989). In contrast, the recombinant Shbgb protein corresponding to the expressed form in the trout ovary has a similar affinity for T and E2 (Bobe *et al.*, 2008b), thus

suggesting that the testicular Shbg corresponded to Shbga rather than to Shbgb. In agreement with this hypothesis made in trout was the Shbg immunoreactivity observed in zebrafish testis around the seminiferous tubules as well as the testicular expression of *shbga* mRNA (Miguel-Queralt *et al.*, 2004). Surprisingly, no testicular mRNA expression of *shbga* or *shbgb* was detected in the two salmonid species investigated so far (Bobe *et al.*, 2008b;Miguel-Queralt *et al.*, 2009). Similarly, no mRNA expression was seen in Atlantic sea bass testis by Northern blot analysis, even though Shbga immunoreactivity could be observed in the interstitial spaces between testicular lobules (Miguel-Queralt *et al.*, 2007). Together, data obtained in fish indicate that testicular expression of *shbga* mRNA is much lower than what has been evidenced in mammals. In addition, the discrepancy between the lack of testicular *shbga* expression and the presence of the corresponding Shbga protein observed at least in some species does not rule out uptake and accumulation of it from blood by testicular tissues (Foucher and Le Gac, 1989). Further investigations are required to clarify the expression of *shbga* gene and corresponding protein in the fish testis.

During development, zebrafish *shbga* mRNA was detected in larvae 5 and 6 days after fertilization (Miguel-Queralt *et al.*, 2004) while Atlantic sea bass *shbga* mRNA was detected at all assayed stages between 8 and 150 days post-fertilization (Miguel-Queralt *et al.*, 2007). In both species, it was assumed that the expression occurred in the hepatopancreas or the liver. In mammals, *Shbg* mRNA is detected as early as 11 days of gestation in rabbit fetal liver and its expression increases dramatically at day 30 to remain high until parturition (Ng et al., 2005) while the transcript is also detected in fetal rat liver (Sullivan et al., 1991). Together, these available data indicate that *shbga* expression occurs relatively early during fish development, in agreement to what is observed in some mammals.

Shbgb

To date, the tissue distribution of *shbgb*, the highly divergent paralog of *shbga* that has only been found in salmonids was only studied in rainbow trout (Bobe *et al.*, 2008b) and, more recently, coho salmon (Miguel-Queralt *et al.*, 2009), two species belonging to the *Oncorhynchus* genus. In rainbow trout females, a strong ovarian expression was observed while a lower expression was evidenced in muscle and stomach. In contrast, no expression could be detected in brain, pituitary, gills, heart, liver, spleen, intestine, kidney, and skin. Similarly, no expression was found in rainbow trout testis (Bobe *et al.*, 2008b). In coho salmon, the strong ovarian expression of *shbgb* mRNA was evidenced in pre-smolt females. A significant expression was also observed in gills, stomach, and muscle. It should however be stressed that differences were observed for these expression sites depending on the sex and the sexual maturity of the fish (Miguel-Queralt *et al.*, 2009). Within the ovary, Shbgb is expressed in the granulosa cells as shown by both *in situ* hybridization and immunohistochemistry (Bobe *et al.*, 2008b). Based on the binding characteristics of coho salmon Shbga and Shbgb recombinant proteins (Shbga binds androstenedione and ethinylestradiol with high affinity, whereas Shbgb binds E2 preferentially), it was concluded that Shbgb was present in the plasma in both immature and mature fish. However, the protein has never been immunodetected, to date, in salmon or trout plasma. In addition, the estimated amount of Shbgb was much lower than the quantity of Shbga present in the plasma of both pre-smolts and mature fish of both sexes (Miguel-Queralt *et al.*, 2009).

Binding characteristics of Fish Shbg proteins

The specificity of steroid binding has mostly been investigated in teleost fish. While the amount of information remains limited, available data are consistent with differences in binding characteristics that correspond to the species position in the phylogenetic tree. In

addition to studies of the proteins in plasma, the binding characteristics of some fish Shbg has been studied in hepatocyte cell culture medium (Foucher *et al.*, 1991), testis extracts (Foucher and Le Gac, 1989), and as recombinant proteins (Miguel-Queralt *et al.*, 2004;Miguel-Queralt *et al.*, 2005;Miguel-Queralt *et al.*, 2009). As discussed above, their characteristics should all correspond to Shbga and are listed in Table 2.

Circulating shbg

The existence of sex steroids and sex steroid receptors is clearly established in lampreys (Bryan et al., 2008) but to our knowledge, studies on circulating steroid binding proteins are very scarce in these species. Two steroid binding proteins, a α1 and a β globulin, have been reported in the sea lamprey (*Petromyzon marinus*). Both proteins bind progesterone (P4) and E2 but very poorly testosterone (T), the α1 globulin having the highest specificity for P4 and the β globulin for E2 (Boffa et al., 1972). They showed lower affinities for T and corticosterone. Further analyses in agnathan species would be valuable due to their evolutionary position in the vertebrate lineage. In gnathostoma, data are unfortunately missing in the primitive ray-finned bony fishes, and to our knowledge, only one preliminary study indicated that testosterone was bound in blood of a chondrostean fish, the Siberian sturgeon (*Acipenser baerii*, Acipenseriformes) (Bennetau-Pelissero et al., 1998).

Shbg has been, in contrast, characterized in the blood of both chondrichthyes (eslasmobranch: sharks and rays), and osteichtyes (mostly teleostei) (Table 2). Plasma Shbg shows a lower specificity in the first fish group than in the second one since it binds C18 (E2), C19 (T) and C21 (P4, corticosterone) steroids while C18 and C19 steroids are the main steroids able to bind Shbg in teleosts (Freeman and Idler, 1969;Freeman and Idler, 1971;Martin, 1975;Ho *et al.*, 1980). Thus, the dissociation constant of female spiny dogfish plasma for P4 (Kd=23.8 nM) was similar for T and a little lower than for E2. Furthermore, P4 showed also a similar competition to T against E2 binding for the spiny dogfish testicular Shbg (Mak and Callard,

1987), while it competed only a little against T binding for the trout testicular Shbg (Foucher and Le Gac, 1989). Furthermore, in almost all studies performed in teleosts, P4 has been shown to be a relatively poor competitor against E2 or T for the circulating Shbga. This is also true for the Shbgb paralog recently found in rainbow trout ovary (Bobe *et al.*, 2008b) and coho salmon blood (Miguel-Queralt *et al.*, 2009). Surprisingly, P4 has been claimed to be a high competitor against E2 in the common carp (Kloas et al., 2000), although the other two studied cyprinidae species, the goldfish, *Carassius auratus* (Van der Kraak and Biddiscombe, 1999) and the tench, *Tinca tinca* (Scott et al., 2005), lacked this characteristics.

Species differences in binding affinities are also apparent since osteichtyes show a 5 to 10 fold higher affinity for E2 and T than chondrichthyes, with the exception of *Scyliorhinus canicula* in which affinity for E2 was similar to teleosts (Martin, 1975). In most teleosts, the dissociation constant for E2 or T ranged from 1 to 10 nM, which is similar to human Kd for E2 (Petra et al., 2001). In addition, both association and dissociation rates are rapid in several teleosts, i.e. $t_{1/2} < 5$ min (Pasmanik and Callard, 1986;Laidley and Thomas, 1994;Hobby et al., 2000) while the dissociation rate was found to be slower in the spiny dogfish (i.e: $t_{1/2}= 100$ min). Finally, it should be stressed that a rapid dissociation rate is one of the characteristics classically used to distinguish Shbg proteins from steroid receptors (Table 3).

Differences between studies may be due to methodological discrepancies, especially for the separation of bound and free steroids (Fostier and Breton, 1975) or to the analysis of either complete or partially purified serum (Martin, 1975). Other binding systems, with lower affinities but higher capacities (Cortiscosteroid Binding Globulin like protein, albumin) may exist and interfere in non-purified or non-diluted plasma or serum (Laidley and Thomas, 1994). Thus, blood binding capacities can hardly be compared between species when different methods have been used to estimate bound and unbound steroid fractions (Table 4). For instance, several authors have stressed the possible underestimation of binding capacities due

to over exposure to dextran-coated charcoal as the separation reagent (DCC) (Foucher and Le Gac, 1989;Laidley and Thomas, 1994;Hobby *et al.*, 2000). Nevertheless, when comparing data obtained with similar methods, estimated Shbg binding capacities for T are close among studied species, including between elasmobranches and teleosts (Table 4). However, differences could also be explained by physiological species specificities depending on natural circulating steroid levels (Laidley and Thomas, 1997;Hobby *et al.*, 2000), or differences between sexual stages, although binding characteristics are generally similar between sexes (Tollefsen et al., 2004). Nutritional factors such as non-esterified fatty acids (Van der Kraak and Biddiscombe, 1999) or environmental estrogens (Tollefsen, 2002) may also modulate Shbg affinity properties.

The progestins 17,20β-dihydroxy-4-pregnen-3-one (17,20ß-P) and 17,20β,21-trihydroxy-4-pregnen-3-one have a major physiological role in fish as they are involved in teleost oocyte maturation(Bobe et al., 2008a). Interestingly, these progestins compete even less than P4 against E2 or T. However, a dimeric 110 kDa protein able to bind 17,20β-P has been purified from female rainbow trout plasma (Yoshikuni et al., 1994). Its overall amino acid composition was similar to that of human SHBG, but its binding characteristics were not classical for a Shbg-like protein. Although its affinity for 17,20β-P appears to be moderate (Kd=21 nM), T and E2 did not compete strongly against 17,20β-P, showing relative binding affinity values (RBA) of 79 and 31, respectively. Furthermore, 17-hydroxyprogesterone and progesterone had higher and similar RBA than E2; i.e. 65 and 32 respectively. Although salmon Shbga binds progesterone and 17,20β-P better than Shbgb, this is at a lower level than androgens and estrogens (Table 5). These features do not fit with the properties of other Shbgs and are closer to the binding characteristics of another binding protein found in trout plasma and able to bind C-18, C-19 and C-21 steroids, including some corticosteroids like cortisone (that has not been tested by Yoshikuni *et al.*) but not cortisol (Fostier and Breton,

1975). However, the amino acid composition and the dimeric structure of this purified 17,20ß-P binding protein would exclude it from the Cbg protein family. Finally, direct measurement of E2 and T affinities would have been helpful to better compare this protein to other Shbgs.

It is also noteworthy that another physiologically important steroid, 11-ketotestosterone, the fish-specific androgen (Borg, 1994), binds poorly to plasmatic Shbg. Finally, estriol and estrone, which have low biological activity in fish, have also a low affinity for Shbg. Altogether, existing data indicate a specialization of circulating Shbg during evolution towards the preferential binding of E2 and its precursor, T. Both steroids show biological activities in fish. P4 binding, which is still observed in the ovoviviparous elasmobranches, was lost in teleosts. Finally, specific fish steroids as 11-oxo-androgens and oocyte maturation-inducing steroids which are actively involved in reproduction are poorly bound by fish Shbg.

Testis Shbg binding

A counterpart of the mammalian Abp, which is, as indicated above, encoded by a single copy *Shbg* gene, has been first described in the spiny dogfish (Mak and Callard, 1987). The authors claimed that it was identical to the circulating Shbg in its broad specificity but differed by a longer rate of dissociation (T1/2 = 160 min vs <30 min) and a faster electrophoretic mobility on polyacrylamide gel. However, such differences were not found for the rainbow trout testicular Shbg which has been found to be secreted by testicular explants (Foucher and Le Gac, 1989). Several Shbg characteristics, especially for tissular Shbg, are classically used for discriminating Shbg binding from T or E2 receptor binding, e.g. higher association and dissociation rates, lower affinity (higher Kd), higher capacity, absence of binding to DNA (Table 3). However, except for the last one, such differences between Shbg and receptors are not always valid as previously stressed (Bryan et al., 2007).

Shbg recombinant proteins (RPs)

Binding studies using native purified or recombinant proteins (RP) give the opportunity to perform the characterization of binding features without possible interference due to other binding proteins present in the blood such as Corticosteroid Binding Globulin like protein (Cbg) (Fostier and Breton, 1975) or albumin (Baker, 1998), even though fish Cbg still remains to be further characterized (Mommsen et al., 1999;Fast et al., 2008).
Plasma and RP binding studies (Table 5) gave similar affinity constant estimations in Atlantic sea bass (Kd = 6.8 and 8.8 nM, respectively; (Miguel-Queralt *et al.*, 2005) and similar electrophoretic mobilities in Atlantic sea bass and zebrafish (Miguel-Queralt *et al.*, 2004). Interestingly, in salmonids, RPs have been used to specifically characterize the two Shbg forms (Bobe *et al.*, 2008b;Miguel-Queralt *et al.*, 2009). Altogether, data interpretation is made difficult by the diversity of tritiated steroids used to estimate affinity constants and relative binding affinities. E2 and T show high affinities for both Shbga and Shbgb with a possible higher affinity of Shbgb for E2. In contrast, estriol, cortisol and progestins have low or very low affinities. Androstenedione (A4), 5α-dihydrotestosterone (DHT), 17α-ethynyl-estradiol and, to a lesser extent, estrone and 11-ketotestosterone show a higher affinities for Shbga than for Shbgb. Finally, Shbgb binds 2-methoxy-estradiol with a higher affinity than Shbga.

Binding to xenobiotics

The potential binding of xenobiotics to Shbg is of particular interest because that could be a mechanism of disturbing action for these molecules. Concerning pharmaceutical steroids, 17α-ethynylestradiol and diethylstilbestrol showed low or high affinity for Shbg depending on the species, but other xenobiotics like phytotoxins, pesticides and industrial estrogenic compounds compete poorly for Shbg E2 binding (McPherson et al., 1988;Milligan et al., 1998;Tollefsen *et al.*, 2004;Miguel-Queralt *et al.*, 2005). However, these compounds could

interfere with native steroid binding to Shbg because they may be found at high concentrations (Milligan *et al.*, 1998). Estrogenic pollutants could thus modulate Shbg synthesis by the liver and change the blood binding capacities (Pryce-Hobby et al., 2003). Interestingly, a recent study explored the possibility to screen *in silico* the binding potentialities of 80,000 commercial chemicals listed by the European Chemical Bureau and Environment Canada by using a zebrafish Shbg model (Thorsteinson et al., 2009). Six non-steroidal substances of the top hits were shown to displace effectively ^{3}H-5α-DHT bound to a zebrafish recombinant Shbga, and three of them bound in the micromolar range.

Putative functional roles of Shbg proteins

As in mammals, fish Shbg proteins are considered to be involved in sex steroid transport, regulation, and action. However, to our knowledge, no study in fish is clearly showing a direct action of Shbg on specific biological mechanisms. In addition, circulating Shbg has received most of the attention so far, but it may be reasonably hypothesized that the function of this circulating form, or forms, could be very different from the functions of Shbg proteins expressed locally in target organs and tissues.

Protective and transport functions of Shbg

Shbg-type binding fluctuated in blood in parallel to sex steroids levels in spotted weakfish (Laidley and Thomas, 1997) and Indian major carp, *Labeo rohita* (Suresh et al., 2008) but not in common carp (Chang and Chen, 1990) and brown trout, *Salmo trutta* (Pottinger, 1988). A direct relationship may be due to the stimulation of liver Shbg synthesis by E2 (Foucher *et al.*, 1991). However, when studied in details in spotted weakfish, no significant correlation could be found between Shbg binding and E2 or T plasma levels (Laidley and Thomas, 1997). In contrast, the hypothesis of a protective role of Shbg for biologically active plasma steroids would be consistent with Shbg binding characteristics found in teleosts and elasmobranches.

As already known, E2 and T have a peripheral action in both Elasmobranches and Teleosts and are both able to bind Shbg. In contrast, ovarian progestins have mainly a local action in teleosts (Bobe et al., 2008a) while P4 has a peripheral action in Elasmobranches. Indeed, it can be noted that Elasmobranch Shgb can bind P4 (Jones and Baxter, 1991) while Shbg has very little affinity for progestins in teleosts species (Table 5). Thus, the associated loss of both Shbg binding and peripheral action of progestins during evolution would be in favor of a major protective and transport role of Shbg for plasma steroids.

As evidenced in Atlantic sea bass, circulating Shbga levels decrease in both males and females during reproductive season (Miguel-Queralt *et al.*, 2007). Similarly, circulating Shbg levels decrease in male rainbow trout towards the end of the reproductive cycle, e.g. during spermiation (Foucher et al., 1992) and the plasma-binding capacity of both mature and immature brown trout declined during the spawning period (Pottinger, 1988). Such a decrease in blood E2 at the end of the female cycle (Fostier et al., 1978;Goetz et al., 1987;Bobe et al., 2003) is necessary for the completion vitellogenesis but also to release an E2 inhibition on the maturation inducing steroid synthesis by granulosa, as shown in rainbow trout (Jalabert and Fostier, 1984a;Jalabert and Fostier, 1984b;Fostier and Baek, 1993). Such blood E2 decrease is related to the decrease of ovarian aromatase activity (Young et al., 1983) and gene expression (*cyp19a1a*) (Bobe *et al.*, 2008b), but is amplified also by a higher metabolic clearance of E2 (Baroiller et al., 1987). This higher clearance could be due to a decrease of Shbg-protected E2 levels. In conclusion, blood Shbg might play a major role in fish for the regulation of E2 and T catabolism since binding capacities reach levels (110-3500 nM; Table 4) sufficient to bind a large part, if not all, of the circulating sex steroids (roughly in the range of 3 to 300 nM, according to sexual stages and species). However, the protective function of Shbg against steroid catabolism is not completely accepted. After comparing radioactive steroids affinities for tench Shbg and their clearances, which have been estimated by the accumulation of their

radioactivity in the gale bladder, it was suggested that Shbg could enhance the ability of the steroid to be metabolized by the liver (Scott *et al.*, 2005). In fact, peripheral metabolization of steroids is a multi-compartments system in which metabolizing enzymes levels and affinities, with their own regulation, have to be taken into account. Shbg should be considered only as a part of this complex system.

Facilitation of liphophilic steroid release into the environment

In fish, the regulation of steroid metabolism and excretion in the aquatic environment has a particular biological significance because some free and conjugated steroids play an important pheromonal role in both males and females (Stacey et al., 2003). Hydrophilic conjugated steroid are not bound by Shbg (Hobby *et al.*, 2000;Tollefsen, 2002) and can be release freely into urine. Interestingly, no significant *shbga* or *shbgb* expression could be detected in the kidney, as indicated above. In contrast, liphophilic non-conjugated steroidal pheromones can be released by the gills (Vermeirssen and Scott, 1996) and Shbg may regulate their diffusion (or uptake) between water and blood through the gill membranes, depending on their affinities for the protein (Scott *et al.*, 2005). Considering a protective function of Shbg, the protein might also help in providing unmetabolized active steroids to the fish environment. These various functions need to be provided at the gills level and Shbg, which has been found to be expressed in gills, could have such local physiological functions, regardless of its potentiality to uptake xenobiotics from the aquatic environment (Miguel-Queralt and Hammond, 2008).

At the local ovarian level, salmonid *shbgb* and *cyp19a1a* are expressed in the same follicular compartment, the granulosa layer, as shown by *in situ* hybridization in trout (Bobe *et al.*, 2008b) and the exchange of T and E2 between Shbg and the aromatase enzyme is worth considering. As suggested by Pasmanik and Callard (1986), aromatase and Shbg affinities for T are close (aromatase Km usually in the range 5-50 nM according to Piferrer.and Blazquez, 2005), thus facilitating the transfer of T from Shbg to the enzyme. After T aromatization, E2

may be quickly bound by Shbg for a protected transportation to its targets. In that case, a cellular local couple Shbgb-Aromatase might be a fully functional system to maintain a proper E2 production. Together, these observations suggest a local participation of Shbgb in steroidogenesis and/or steroid action during late oogenesis in salmonids, possibly in follicle-enclosed maturational competence acquisition. Given the remarkably high levels of brain aromatase found in fish (Piferrer and Blazquez, 2005), the transfer of T to aromatase might also occur in pituitary and brain where *Shbga* transcripts have been found to be expressed in salmonids (Bobe et al., 2008b; Miguel-Queralt et al., 2009).

Shbga Shbgb and tissular receptors: 3 actors involved in E2/T availability

Finally, a finely tuned equilibrium may also occur, in teleosts, for E2 and T between circulating or local Shbg and their nuclear or membrane receptors. In fact, affinities of E2 and T for their receptors are close to their affinities for Shbg (Table 3) and range from 1 to 5 nM (Lazier et al., 1985;McPherson *et al.*, 1988;Pottinger and Pickering, 1990;Kloas *et al.*, 2000;Tollefsen et al., 2002;Gale et al., 2004;Thomas et al., 2006).

Conclusion

In fish, two different genes, *shbga* and *shbgb*, exist that encode for Shbga and Shbgb proteins respectively. Shbga is the ortholog of mammalian Shbg and has been found in all teleost species investigated thus far. In contrast, Shbgb has only been characterized from salmonid species and it remains unclear whether or not this highly divergent paralog results from salmonid-specific duplication or from a more ancient duplication followed by a subsequent loss in all non-salmonid vertebrate species. The genomic sequences obtained from species with an available genome sequence suggest that *shbga* genomic organization is well conserved in teleosts. In contrast, the genomic sequence of *shbgb* remains currently unknown.

Shbga is mainly expressed in the liver where it is subsequently secreted into the blood. Other important sites of Shbga expression exist that could also contribute to plasma Shbga levels and/or support a local function of this protein in target organs. In contrast, Shbgb is mainly expressed in the ovary where it could contribute to the regulation of local steroid action. In coho salmon, Shbgb is expressed in both males and females and is also detected in plasma, thus suggesting possible species-specific differences among salmonids.

In the blood of sea lamprey, an agnathan vertebrate that occupies a key phylogenetic position between cephalochordates and gnathostomes, two globulins are able to bind E2 and P4, but not T. Testosterone binding is established in elasmobranches and is maintained in teleosts while P4 binding, which is still observed in elasmobranches, was lost in teleosts. Altogether, existing binding studies indicate a specialization of circulating Shbg during evolution towards a preferential and more specific binding of E2 and its precursor, T. As previously stressed, specific fish steroids such as 11-oxo-androgens and oocyte maturation-inducing steroids which are actively involved in reproduction are poorly bound by Shbg. In addition, several bodies of evidence indicate that circulating Shbg might play a major role in the regulation of E2 and T transport and availability for metabolization or target organs, as binding capacities reach levels sufficient to bind a large part, if not all, of the circulating sex steroids in investigated fish species.

Further studies are needed to better understand the evolutionary history of Shbg proteins in vertebrates. This is especially true for the salmonid-specific Shbgb form. New insights in the local expression and potential action of both Shbga and Shbgb protein in target organs are also required to better understand the biological significance of each protein and the possible contribution of the non-hepatic expression sites to circulating Shbga, and possibly Shbgb, levels. In addition, the striking dynamic co-expression of *cyp19a1a* and *shbgb* in the rainbow trout ovarian follicle during late oogenesis raises the question of the participation of Shbgb in

some key steps of fish reproduction, in which E2 plays a key role. In the light of existing mammalian literature on the local role of Shbg in target organs, this suggests a possible participation of Shbg in the local modulation of reproductive functions by steroids that will require future investigations.

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Tables

Table 1. Cross-species amino acid sequence identities (in % of identity) among vertebrate Shbg proteins.

		Fish Shbga					SHBG	Shbgb
	Species	*D. rerio*	*C. carpio I*	*C. carpio II*	***O. mykiss***	*D. labrax*	*H. sapiens*	***O. mykiss***
Fish Shbga	*D. rerio*	100	86.3	80.2	**51.8**	42	25.9	**21.2**
	C. carpio I	86.3	100	88	**51.4**	42.5	26.5	**21.6**
	C. carpio II	80.2	88	100	**49.3**	39.9	24.6	**20.7**
	O. mykiss	**51.8**	**51.4**	**49.3**	**100**	**50.1**	**27.7**	**20.8**
	D. labrax	42	42.5	39.9	**50.1**	100	25.6	**22**
SHBG	*H. sapiens*	25.9	26.5	24.6	**27.7**	25.6	100	**19.8**
Shbgb	***O. mykiss***	**21.2**	**21.6**	**20.7**	**20.8**	**22**	**19.8**	**100**

Table 2. Shbg binding characteristics in gnathostoma fish. The sex and sexual stage (M: mature, IMM: immature) are indicated.

(1) Samples used for the binding studies might be serum, plasma, a partially purified fraction (P.P.F) or a recombinant protein (R.P.). Various methods might be used to separate bound and free steroids, i.e. equilibrium dialysis (Dial.), gel filtration (GF), DEAE (gel of filter), dextran-coated charcoal (DCC). Temperature at which incubation was performed separation is mentioned. The duration of incubations were usually 16h or 'overnight'.
(2) Except when indicated, Relative binding affinity values (RBA) are estimated by the ratio of the concentration of radioinert T, E2 or DHT to those of tested steroids resulting in a 50% reduction in the specific binding of tritiated T, E2 or DHT.
(3) Steroids are abreviated as follow: E2= estradiol-17β; E1= estrone; E3= estriol; T= testosterone; A4= androstenedione; DHT= 5αdihydrotestosterone; 11KT= 11-keto-testosterone; P4= progesterone; 17,20P= 17,20β-dihydroxy-4-pregnen-3-one; 20βS= 17,20β,21-trihydroxy-4-pregnen-3-one; F= cortisol
(4) These values are non competitive binding relative to testosterone = % bound of 0.1µM (Idler and Freeman, 1969) or 0.1µM (Freeman and Idler, 1969) of cold steroid / % bound of the same concentration of cold T.
(5) In that case, 100 ng of displacing steroid has been used in competition with 0.1 ng ^{3}H--testosterone.
(6) The relative ability of various steroid to displace ^{3}H-T, ^{3}H-E2 or ^{3}H-P4 has been estimated by the only competition of competing steroid at the concentration of 1 x 10^{-7}M against ^{3}H-steroid at the concentration of 1.2-1.7 x 10^{-10}M.
(7) These values have not been given by the authors but have been roughly estimated according to their graphical data in order to indicate the rank of competition.
(8) RBA was estimated from the estimated Kd of each competing steroid relatively to E2 Kd.
(9) The relative ability of various steroid to displace ^{3}H-T, ^{3}H-E2 or ^{3}H-P4 has been estimated by the only competition of competing steroid at the concentration of 2 x 10^{-7} M against ^{3}H-steroid at the concentration of 2 x 10^{-9}M.
(10) Media of primary culture of hepatocytes

CLASS Order *Species*	Sample and method[1]	Sex	Sexual stage	Kd (nM)		Relative Binding Affinities[2]											References
						Bound ^{3}H-St	Competitive steroids against ^{3}H-St binding[3]										
							C18 steroids		C19 steroids				C21 steroids				
				T	E2		E2	E1	T	A4	DHT	11KT	P4	17,20P	20βS	F	
CHONDRICHTHYES (elasmobranchs)																	
Rajiformes																	
Raja radiata	Serum, Dial. 3°C	M	M				101		100	46			130			63	Freeman and Idler, 1969 [4]
		M	IMM	17.4													Freeman and Idler, 1971
		F	IMM	12.3													
	P.P.F., Dial. 3°C	M	M			T	110	76	100	2			118			19	Freeman and Idler, 1969 [4,5]
Carcharhiniformes																	
Scyliorhinus canicula	Serum, Dial. 4°C	F	IMM	10.9	2.1												Martin, 1975 [6]
		F	M	13.3	2.9												
		M	M	13.3	2.8												
						T	275		100				413				
						E2	100		35				50				
						P4	133		64				100				
	P.P.F., Dial. 4°C					P4	104	76	51	8.5	98		100			*15*	
Squaliformes																	
Squalus acanthias	Plasma, DCC, 4°C	M	M		39												Ho et al., 1980 [7]
		F	M	25	36	E2	*100*	*45*	*1100*		*245*		*100*			20	
	Testis, G.F., 4°C	M	M	2.2	2.5	E2	*100*	*<10*	*80*		*15*		*55*				Mak and Callard, 1987 [7]
OSTEICHTHYES(teleosts)																	
Anguilliformes																	
Anguilla anguilla	Plasma, Dial.	F	IMM	1.9	5	T	66	37	100	90	100	47	36			0.6	Quérat et al., 1983
						E2	100	25	72	26	55	51	18			0	
Anguilla japonica	P.P.F, DCC,4°C	mixed	IMM			T	193	0.5	100	0.6		25	<0.1	<0.1		<0.1	Chang et al., 1994
						E2	100	<0.1	73.9	0.9		14.9	<0.1	<0.1		<0.1	
Cypriniformes																	
Carassius. auratus	Serum, DCC, 4°C	M&F	M	1.9	2.1	T	*20*	*1*	*100*		*5*	*<1*	*6*			*<0.1*	Pasmanik and Callard, 1986 [7]
						E2	100	*<0.1*	*75*		*20*	*2*	*3*			*<0.1*	
	Serum, DCC, 4°C	M&F	Various stages	2.1	1.9	E2	*100*	*20*	*100*	*60*			*5*	*0.5*			Van Der Kraak and Biddiscombe, 1999 [7]
Cyprinus carpio	Plasma, DCC, 4°C					T	17	7	100	39		4	8	3		0.2	Chang and Lee, 1992
	Plasma, DCC, 4°C	M&F	IMM			E2	100		104		111	51				0.9	Kloas et al., 2000
Tinca tinca	Plasma, DCC, 4°C	M&F	IMM	3.4	4.0	T	19		100	162		7		8		<0.1	Scott et al., 2005
						A4	10		45	100		4		3		<0.1	
Danio rerio	R.P. DCC, 4°C			1.8	2.2	T	37	10	100	106	39	11	3	2		<0.3	Miguel-Queralt et al., 2004
Catostomus commersoni	Plasma, DCC, 4°C			0.9	2.7	E2	100	15	313	87			15			0.3	Pryce-Hobby et al., 2003 8
Catostomus catostomus	Plasma, DCC, 4°C			1.9	3.1	E2	100	9	165	82			11			0.2	

Siluriformes																	
Ictalurus punctatus	Plasma, DCC, 4°C	F	M		1.9	E2	100	23									Gale et al., 2004 [9]
Salmoniformes																	
Oncorhynchus mykiss	Plasma, Dial. 4°C	F	M	2.6		T	23		100			15	19				Fostier and Breton, 1975
					5.5	E2	100	10	108				10	<1			
	Liver [(10)]DEAE, 4°			4.7		T	*30*		*100*					*10*		*<0.5*	Foucher et al., 1991 [7]
	Testis cytosol, DEAE, 4°	M	maturing	2.5		T	30		100	167	160	8	15	11		<1	Foucher and Le Gac; 1989
	Plasma, DCC, 4°C	F	M		20	E2	100		*8*			*12*					Pottinger and Pickering, 1990 [7]
	Plasma, DEAE, 4°C					E2	100		100		90						Milligan et al., 1998
						DHT	20		110	0.3	100						
	Plasma, DCC, 4°C	M&F	IMM/M		2.1	E2	100		87			13	0.2			$8x10^{-4}$	Tollefsen, 2002
	Plasma, DCC, 4°C	F			0.4	E2	100	0.07	29	0.3		4	<0.01	<0.01	<0.002	<0.002	Hobby et al., 2000 [8]
	Liver [(8)]DEAE, 4°			4.7		T	*32*		*100*					*9*		*<0.6*	Foucher et al., 1991 [7]
	Testis cytosol, DEAE, 4°	M	maturing	2.5		T	30		100	167	160	8	15	11		<1	Foucher and Le Gac; 1989
Salmo salar	Plasma, Dial., 3°C	M		11		100						12					Freeman and Idler, 1971 7
		F		7		100						8					
	Plasma, DCC, 2°C				13												Lazier et al., 1985
Salmo trutta	Plasma, DCC, 4°C	M&F	IMM/M	33													Pottinger, 1988
Salvelinus alpinus	Plasma, DCC, 4°C	M&F	IMM/M	0.6-4.4		*100*			*75*			*7*					Tollefsen et al 2004 [7]
	Plasma, DCC, 4°	M	M		1.9												Øvrevik et al., 2001 8
		F	M		3	E2	100	1.5	76			8	<0.01				
			IMM		1.9												
Salvelinus fontinalis	Plasma, DCC, 4°	F	M			E2	100				*<10*		*<10*				McPherson et al., 1998 [7]
Gadiformes																	
Gadus morhua	Plasma, Dial., 3°C	M		83												*0.2*	Freeman and Idler, 1971 [7,8]
		F		38												*0.1*	
Perciformes																	
Cynoscion nebulosus	Plasma, DCC, 4	F	M	4.9	3.1	T	167	118	100	49	143	14	13	3	0.8	0.7	Laidley and Thomas, 1994
	Ovarian fluid					T	115	100	100	51	115	12	14	3	0.8	0.7	
Acanthopagrus butcheri	Plasma, DCC, 4	F			3.4	E2	100	4	6	4		2	0.7	0.1	<0.1		Hobby et al.,2000 [8]
Pagrus auratus	Plasma, DCC, 4	F			11	E2	100	19	51	20		6	1	<0.002			
Dicentrarchus labrax	Serum, DCC				8.8												Miguel-Queralt et al., 2005
	R.P., DCC				6.8	E2	100	39	46	24	31	6	2	<1		<1	
Pleuronectiformes																	
Rhombosolea tapirina	Plasma, DCC, 4°				85	E2	100	35	215	21		20	5.6	10	2.6	1.4	Hobby et al.,2000 [8]

Table 3: Comparison of binding characteristics between fish Shbg and T or E2 nuclear receptors

Species	Tissue	Binding type	Tritiated ligand	Kd (nM)	Time of equilibrium	Half-life of Dissociation	DNA binding	References
Squalus acanthias	Plasma	Shbg	E2	35.7	<5min	100 min		Ho et al., 1980
						<30 min		Mak and Callard, 1987
	Testis			2.5	45 min	160 min	-	
		Receptor		1.5	30 min		+	(Callard and Mak, 1985)
			T	4.4	4 h	4h	+	Cuevas and Callard, 1992
Cyprinus carpio	Plasma	Shbg	E2		15 min			Kloas et al., 2000
	Liver (female)	Receptor		2.1	12h	*6 h* (1)		
Oncorhynchus mykiss	Plasma	Shbga	E2	0.4	15 min	<5 min		Hobby et al., 2000
	Testis		T	2.5	2 h	1-2 min		Foucher and Le Gac, 1989
	Recombinant protein	Shbgb	T	0.8	30 min	3 min		Bobe et al., 2008b
Oncorhynchus kisutch	Ovary	Receptor	Mb(2)	0.32	12 h		+	Fitzpatrick et al., 1994
Salvelinus alpinus	Plasma	Shbg	E2	3.0	*30 min*(1)	3 h		Øvrevik et al., 2001.
Salmo trutta	Plasma	Shbg	E2	48			+	Pottinger, 1986
	Liver	Receptor		2.6			-	
Cynoscion nebulosus	Ovary and plasma	Shbg	T	4.9	*5 h* (1)	<90 sec		Laidley and Thomas, 1994

(1)The value has been estimated according to graphical data
(2) Mb= Miborelone, or dimethylnortestosterone, is a potent synthetic androgen

Table 4 : Binding capacities of fish blood Shbg for testosterone.

Species [(1)]	Bmax (nM)	Bound/Unbound separation[(2)]	References
G. morhua	1400-3500	Dial	Freeman and Idler, 1971
O. mykiss	2400	Dial	Fostier and Breton, 1975
S. canicula	1600-1900	Dial	Martin, 1975
S. salar	900-1900	Dial	Freeman and Idler, 1971
A. anguilla	1700	Dial	Querat et al., 1983
R. radiata	700-1400	Dial	Freeman and Idler, 1971
O. mykiss	1300	Gel Filtration	Fostier and Breton, 1975
O. mykiss	350-750	DEAE Biogel	Foucher and Le Gac, 1989
O. mykiss	500	DEAE Biogel	Foucher *et al*., 1992
C. nebulosus	300-500	DCC; 30 sec; 0°C; 8 mg/ml	Laidley and Thomas, 1997
S trutta	130-360	DCC; 5 min; 0°C; 4 mg/ml	Pottinger, 1988
C. auratus	270	DCC ; 7 min ; 4°C ; 2.5 mg/ml	Pasmanik and Callard, 1986
T. tinca	200	DCC; 1 min; 0°C; 4 mg/ml	Scott *et al*., 2005
S. acanthias	110	DCC; 2 min, 2.5 mg/ml	Ho *et al*, 1980
S. trutta	24	DCC, 10min, 4°C, 7.5 mg/ml;	Pottinger, 1988

(1) Full names are given in table 2. Elasmobranches species are underlined.
(2) Dial = equilibrium dialysis; DCC = dextran coated charcoal. The duration of DCC action before centrifugation, treatment temperature and charcoal concentration are specified, respectively.

Table 5. Recombinant Shbga and Shbgb binding characteristics

		Species				
		Shbg/Shbga			**Shbgb**	
		D. rerio (1)	*D. labrax* (2)	*O.kisutch* (3)		*O. mykiss* (4)
Kd (nM)	Androstenedione	1.8		2.8		
	Testosterone					0.77
	17ß-Estradiol	2.2	6.8		0.8	
Competitive steroids		**Relative binding affinities to:**				
		Testosterone	**Estradiol**	**5α-dihydro-testosterone**	**Estradiol**	**Testosterone**
17ß-Estradiol		37.2	100	31	100	100
Estrone		9.9	38.6	21	<0.1	
Estriol		1.3	1.6	<1	<0.1	
2-methoxyestradiol		<0.3	<1.0	<1	15	26
17α ethynylestradiol			99.0	160	<1	
Testosterone		100	45.5	344	30	100
Androstenedione		105	24.0	365	<1	
11-ketotestosterone		10.9	5.5	57	2	9
5-androstene-3β,17β-diol		2.9	49.8	10	58	
5α-dihydro-testosterone		38.9	30.7	100		2
Progesterone		3.1	2.2	9	<0.1	
17-hydroxyprogesterone						0.1
17,20βdihydroxy 4-pregnen-3-one		2.4	1.4	12	<0.1	
cortisol		<0.2	<1.0	<1	<0.1	0.1

See table 2 for relative binding affinity definition

(1) Miguel-Queralt *et al*., 2004

(2) Miguel-Queralt *et al*., 2005

(3) Miguel-Queralt *et al*., 2009

(4) Bobe et al., 2008b.

Figure legends

Figure 1. Evolutionary relationships of teleost fishes sex hormone binding proteins (Shbg). This consensus n/p/l tree (fusion of neighbor joining (n), maximum parsimony (p), and maximum likelihood (l) trees) was computed using the FIGENIX platform (Gouret et al., 2005) with the *D. rerio* growth arrest-specific 6 (Gas6) protein (**AAH53117**) as an outgroup and the following Shbg proteins dataset: human and mouse Shbg (**CAA34398** and **AAI20788**); Shbg alpha (Shbga) from zebrafish (**AAU14174**); barfin flounder, *Verasper Moseri* (**BAG84605**); Atlantic sea bass, *Dicentrartchus labrax* (**AAW23033**); spotted green pufferfish, *Tetraodon nigroviridis* (**CAF93818**); three-spined stickleback, *Gasterosteus aculeatus* (ENSORLP00000008096); Japanese medaka, *Oryzias latipes* (ENSORLP00000008096); Japanese pufferfish, *Takifugu rubipres* (ENSTRUP00000031810); common carp, *Cyprinus carpio* ShbgaI and ShbgaII (**BAE48780**, **BAE48781**); salmonids Shbg alpha and Shbg beta (Shbgb) from rainbow trout, *Oncorhynchus mykiss* (**BAE48779**, **ABQ45411**); Chinook salmon, *Oncorhynchus tshawytscha* (**ACJ25981**, **ACJ25982**); coho salmon, *Oncorhynchus kisutch* (**ACJ25979**, **ACJ25980**). The Atlantic salmon, *Salmo salar* Shbg partial proteins were deduced from Expressed Sequence Tags matching Shbga (**DV106329**, **CK888851** and **CK888675**) and Shbgb (**DY735728**, **DY694623**, **DY719210**, **DW561629**, **DY699232**, **CK884863**, **CA056042**, **DY699233**, and **DY719211**). Significant bootstrap values are given in percentage at the main nodes of the consensus tree for each of the n_p_l method used (*: non significant).

Figure 2. Comparison of the structure of different teleost fishes *shbga* genes. Exons are boxed and lines indicate the introns. Out of scale introns are indicated by broken lines and their lengths are given in bp. Exon numbers are given above exon boxes. All intron-exon boundaries were predicted according to the Ensembl gene prediction for three-spined

stickleback, *Gasterosteus aculeatus* (ENSORLP00000008096); Japanese medaka, *Oryzias latipes* (ENSORLP00000008096) and Japanese pufferfish, *Takifugu rubipres* (ENSTRUP00000031810).

Figure 3. Alignment of Shbga sequences from zebrafish (**AAU14174**), rainbow trout (**BAE48779**), sea bass (**AAW23033**) with human SHBG (**CAA34398**) and rainbow trout Shbgb (ABQ45411). Amino-acids sequences conserved in at least 4 sequences are shaded. Amino acid residues specific of the rainbow trout Shbgb are shown below the alignment respectively with a hyphen (-). The conserved cysteine residues are identified below the alignment with a bold type C letter and the two intra-molecular disulfide bridges (IMDB1 and IMDB2) linking these cysteines are represented. Consensus sites for N-glycosylation are indicated in bold italics. Amino acids corresponding to the signal peptides are underlined and have been obtained from the following published sequences (Miguel-Queralt *et al.*, 2004;Miguel-Queralt *et al.*, 2005).