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Influence of Hypophysectomy, Castration, Fasting, and Spermiation on SBP Concentration in Male Rainbow Trout (*Oncorhynchus mykiss*)

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The influence of different experimental and physiological conditions on sex steroid binding protein (SBP) concentrations in the blood (and in hepatic and testicular cytosols) has been studied on male rainbow trout. SBP was measured with a specific binding assay. The aim was to further understanding of regulation of the SBP and in particular to determine the respective influences of reproductive and metabolic status. Twelve days after hypophysectomy, pituitary and steroid hormones were dramatically decreased and SBP concentrations were significantly lowered in blood (−32%) and in hepatic cytosol (−46%) while the binding protein concentration remained constant in testicular cytosol. Castration of maturing animals did not influence SBP concentration in blood and liver cytosol. Toward the end of the reproductive cycle, a dramatic decrease (−80%) of plasma SBP concentration occurred that appears independent of androgen changes that take place during this period, but is concomitant with a rapid increase of plasma growth hormone (GH) levels (and possibly secondary to a natural arrest of food intake). Long-term fasting that increases endogenous GH but decreases plasma IGFs (insulin-like growth factors) concentrations also induces a limited but significant decrease in SBP concentration. Treatment of intact control trout with recombinant bovine GH (1 μg/g wt, twice a week, for 6 weeks) increased plasma IGF concentrations but did not significantly increase SBP levels. These results suggest that in mature male trout, testicular androgens have little or no influence on SBP regulation. GH levels or liver GH responsiveness and IGF might be involved in SBP regulation. This would, in part, explain the large decrease in SBP around spermiation in trout.

Steroid binding protein (SBP or SHBG in human)[1] is a plasma glycoprotein present in vertebrates (Martin, 1980; Corvol and Bardin, 1973), including teleost (Freeman and Idler, 1971; Fostier and Breton, 1975) and elasmobranch fish (Idler and Freeman, 1969; Martin, 1975; Callard and Callard, 1987).

By transporting and protecting androgens and estrogens it is thought to regulate the balance between free and active fractions of these steroid hormones. However, several studies have shown that SBP and androgen binding protein (produced in the testis) could also play a role in the selective delivery of sex steroids to target tissues (Pardridge, 1988) or participate in sex-steroid action by entering target cells (Bordin and Petra, 1980; Pelliniemi *et al.*, 1981). SHBG binding to specific receptors on cell membranes might be a necessary step in

[1] Abbreviations used: T, testosterone (17β-hydroxy-4-androsten-3-one); 11KT, 11-ketotestosterone (17β-hydroxy-4-androsten-3,11-dione); E2, estradiol (1,3,5(10)-estratriene-3,17β-diol); 17α,20βDHP, 17β,20β-dihydroxy-4-pregnene-3,20-dione; RIA, radioimmunoassay; GtH, gonadotropin; PRL, prolactin; SBP, steroid binding protein; IGFs, insulin-like growth factors; GH, growth hormone; ^{125}I-rhIGF1, iodinated recombinant human IGF1.

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certain mechanisms of steroid action (see Rosner, 1990).

In vivo, sex, age, gestation, and endogenous sex-steroid levels influence SBP concentration in plasma.

In mammals, since Anderson's work (1974), there is much evidence that blood SBP concentrations are regulated by sex steroids and reflect androgen/estrogen balance (E2 increasing and testosterone (T) decreasing SBP levels). Hypophysial regulation of SBP has also been suggested and includes specific actions of ACTH, LH/HCG (Forest *et al.*, 1986), and prolactin (Plymate *et al.*, 1988). Finally, many physiological and clinical observations show metabolic status (insulin, corticosteroids, thyroid hormones, growth hormone (GH), somatomedins, nutritional status, obesity, and physical exercise) to affect serum SBP levels (see von Shoultz and Carlström, 1989; Vermeulen *et al.*, 1986). However, most *in vivo* situations do not distinguish between direct or indirect mechanisms of action of these factors.

In vitro, estrogens and sometimes androgens (in contrast to *in vivo*) stimulate SBP production by hepatoma cell lines (Mercier-Bodard *et al.*, 1987; Lee *et al.*, 1986); other hormones including thyroid hormones (Mercier-Bodard *et al.*, 1987), insulin (Plymate, 1988), and dexamethasone (Mercier-Bodard *et al.*, 1987) also influence SBP production.

In fish, Ng and Idler (1980) showed in flounders that plasma binding of 11-[^{3}H]ketotestosterone became undetectable after hypophysectomy and that normal values appeared after injection with pituitary extracts. In intact trout, gonadotropin treatment increased androgen concentration in males and females, but increased SBP only in females (Ng and Idler, 1980).

Ho *et al.* (1980) and Martin (1975) noted no variation of the plasma binding protein during the sexual cycle of two elasmobranch species. In mature male brown trout, the mean plasma concentration of SBP has been shown to decrease at the end of the reproductive cycle, a time of elevated blood plasma concentrations of testosterone and 11-ketotestosterone (11KT) (Pottinger, 1988), while Pasmanik and Callard (1986) did not find seasonal changes in SBP levels in goldfish.

In rainbow trout, an androgen binding protein present at high concentration in the testis and seminal fluid (Foucher and Le Gac, 1989) was similar to the plasma SBP and was apparently produced by testicular explants. Pituitary extracts and purified maturational GtH_2 added to the explants induced a strong production of androgens but had no influence on the binding protein production (Foucher and Le Gac, 1989). The role of gonadotropin hormone and sex steroids in SBP regulation in fish remains unclear.

In the present study, several *in vivo* experimental investigations were undertaken (including hypophysectomy, castration, and fasting) and the changes around spawning time were investigated in individual animals to differentiate the origins of the testicular and the plasma binding proteins and to tentatively determine which reproductive or metabolic factors (growth endocrine status and nutritional status) is/are major determinants of plasma SBP levels in male fish.

METHODS

Animals. Male rainbow trout were obtained from fish farms and acclimated to the laboratory in a recirculating water system at 12°–15°, under artificial lights mimicking natural photoperiod.

Chemicals. [1,2,6,7-^{3}H]testosterone (90 Ci/mmol; 3 TBq/mmol) was obtained from Amersham. Unlabeled steroids were purchased from Steraloids (Wilton, NH). Vitamin K1 was obtained from Laboratoires Delagrange (Chilly-Mazarin, France) and DEAE Biogel A from Bio-Rad (Richmond, CA).

Recombinant bovine growth hormone (rbGH) was obtained from Monsanto, and recombinant human IGF1 was a gift from CIBA–GEIGY (Switzerland).

Testosterone binding assay. Steroid binding protein was measured as previously described (Johnson *et al.*, 1985; Foucher and Le Gac, 1989). Briefly, aliquots

(100 μl) of diluted blood plasma (diluted 1:150 in buffer A: 10 m*M* Tris–HCl, 1.5 m*M* EDTA, 12 m*M* monothioglycerol, 10% glycerol, pH 7.8) or cytosols (diluted 1:2 in buffer A) were incubated for 0.5 hr in glass assay tubes with 300 μl buffer A and 300 μl DEAE-Bio-gel washed and diluted 1:2 with buffer A. After centrifugation, the supernatant was discarded and ^{3}H[T], plus or minus unlabeled T, was added to the DEAE-protein complex. Incubations were stopped after 2 hr by rapid filtration and DEAE binding protein T complexes were collected on Whatman GFA filters; the measurement of radioactivity on filters determined total and nonspecific binding of ^{3}H[T]. Intra- and interassay variations were respectively ≤10 and ≤15%.

Scatchard plots of saturation data were obtained using pooled plasmas from control, castrated, and hypophysectomized animals. Since the affinity constants were similar (K_a = 3–5 × 10^8 M^{-1}), capacities were subsequently calculated using the law of mass action and the appropriate K_a.

Hormone measurements. Steroids were assayed by radioimmunoassay (RIA). 11KT was measured following Fostier *et al.* (1982), and 17α,20βDHP was measured following Fostier and Jalabert (1986).

Gonadotropin (GtH), prolactin (PRL), and GH were measured by RIA following Breton *et al.* (1983), Prunet *et al.* (1985), and Le Bail *et al.* (1991), respectively.

IGF measurement. IGFs were measured in blood plasma using a protein binding assay similar to that described for human IGF measurement by Binoux (1984), and adapted for trout IGF by Niu (unpublished data). Briefly, IGFs are separated from their binding proteins after dissociation under acidic conditions (0.15 *M* NaCl, 1 *M* acetic acid, pH 3.2; yield, 90%). Neutralized trout IGF samples are then incubated with an aliquot of a preparation enriched in trout IGF binding proteins, in competition with a tracer concentration of iodinated recombinant human IGF1 (^{125}I-rhIGF1) in 200 m*M* Tris–HCl pH 9 and in the presence of 1% BSA (20 hr at 4°).

At the end of incubation, bound and free ^{125}I-rhIGF1 are separated by charcoal treatment. Specific binding of ^{125}I-rhIGF1 to the binding protein preparation measured for each sample is compared to a standard curve obtained with 0.1 to 25 ng of rhIGF1.

Serial dilutions of trout serum give a competition curve parallel to the standard curve. Human IGF2 also cross-reacts in this assay; thus the two growth factors cannot be differentiated. However, pig and fish insulin, and several other peptide hormones tested, do not cross-react in the IGF assay. The assay sensitivity is 0.4 ng/ml and the coefficients of variation are as follows: intraassay, 13%; interassay, 15%.

Preparation of cytosols. Freshly collected testes and livers were perfused with heparinized saline to eliminate blood contamination. Tissues were minced and homogenized in 4 vol homogenizing solution (10 m*M* Tris–HCl, 1.5 m*M* EDTA, 12 m*M* monothioglycerol, 10% vol glycerol; pH 7.8), with a tissue homogenizer (Polytron PC U2 Kinematica-Switzerland), in the presence of 1 m*M* phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 2000*g* (15 min) and 105,000*g* (1 hr) successively. Pellets were discarded. The resulting supernatants (cytosols) were kept at −30° until use.

Preparation of blood plasma. Blood samples were collected in heparinized tubes, from caudal vein of animals anesthetized with phenoxyethanol (3.5 ml/10 liters). After centrifugation (15 min, 4000*g*), plasma was stored at −30°.

Removal of steroids. One volume of Dextran-coated charcoal suspension (1% washed charcoal, 0.1% Dextran) was added to 9 vol of sample (45 min, 4°). Charcoal was removed by centrifugation (15 min, 4°).

Hypophysectomy. A transorbital technique was used (Nishioka *et al.*, 1987). Animals (300 to 500 g, just before spermiation) were pretreated 2 days beforehand with vitamin K1 (1 mg/kg) to avoid excess loss of blood during surgery. Anesthetized animals (phenoxyethanol 3.5 ml/10 liters water) were placed on a wet surface. Their gills were perfused with water containing the same amount of anesthetic. The right eye was removed and the orbital cavity was cleaned of any tissues. A hole in the orbitosphenoid bone was drilled with a biopsy punch (2 mm, Stieffel, France). The pituitary was removed by gentle suction. Sham operated animals underwent eye ablation and blood sampling.

A third group of intact fish was also prepared and kept (controls) while a fourth one was sacrificed to give initial values of hormones and SBP.

Fish from the first three groups were kept in the same tank. On Days 4 and 8 after surgery, 500-μl aliquots of blood were collected. On Day 12, all the remaining fish were sacrificed; testes, liver, and blood were collected and the absence of pituitary tissues was verified in hypophysectomized animals.

Castration. Animals (250–450 g at stage 3 to 4 of spermatogenesis–meiosis, following Billard and Escaffre, 1975) were anesthetized with phenoxyethanol and their gills were perfused as before during surgery.

A 15-cm incision was made between the genital papilla and pelvic fins. Testicular ducts were clamped and sectioned near the genital papilla. Testes were pulled away from the body of the fish until testicular veins and arteries ruptured. After removal, testes were examined to verify complete ablation. Hemostatic and antibiotic solutions were placed in the peritoneal cavity. The incision was then sutured. Sham operated animals were sutured after manipulation of the ducts. All fish were kept in the same tank. Every second week, 300-μl aliquots of blood were collected and animals were treated with antibiotics during wound healing. The fish were sacrificed on Day 119, and the absence of testicular tissues was verified in castrated animals. The sham operated animals were then at stage 7 to 8 of spermatogenesis.

Variations of SBP concentration at the end of the reproductive cycle. Blood was sampled over a 5.5-month period, including the spermiation phase, from three individual mature males of a spring spawning strain (2.5 kg body wt) and plasma was assayed for SBP, GH, and 11KT.

Effect of temperature. Two groups of 20 and 15 mature male rainbow trout were kept at 5° and 13°, respectively. After 12 weeks, blood samples were collected and assayed for SBP.

Long-term fasting and GH supplementation. Three groups of nine immature fish were kept in three different tanks for 6 weeks. Animals from the first group were used as controls and received the normal diet (1% body wt/day). Animals from the second group received the same diet and were given two injections of recombinant bovine growth hormone (1 μg/g body wt) per week. Animals from the third group were fasted throughout the experiment. After 6 weeks, blood plasma from all the animals was collected and assayed for SBP and IGF.

RESULTS

Hypophysectomy

Twelve days after hypophysectomy plasma, GH, PRL, and GtH concentrations were close to the lower detection limit of the assays (Fig. 1). 11KT fell toward its limit of detection and 17α,20βDHP concentrations dramatically decreased (Fig. 2). Large intragroup variations of steroid concentration observed in sham and intact animals resulted from rapid physiological changes related to the beginning of spermiation during the experiment. A small decrease of the gonadosomatic index was noted. Whitening of hypophysectomized animals occurred 3 or 4 days after surgery.

After hypophysectomy, binding of T remained constant in testicular cytosol, whereas it decreased in liver cytosol and blood plasma (Figs. 3 and 4). On Day 12, binding of T in liver cytosol from hypophysectomized animals was 54% that measured in sham-operated animals. Blood plasma SBP concentration decreased by 24% on Day 8 and 32% on Day 12 after hypophysectomy. An initial decrease of SBP concentration was seen between days 0 and 4 in blood of both sham operated and hypophysectomized animals.

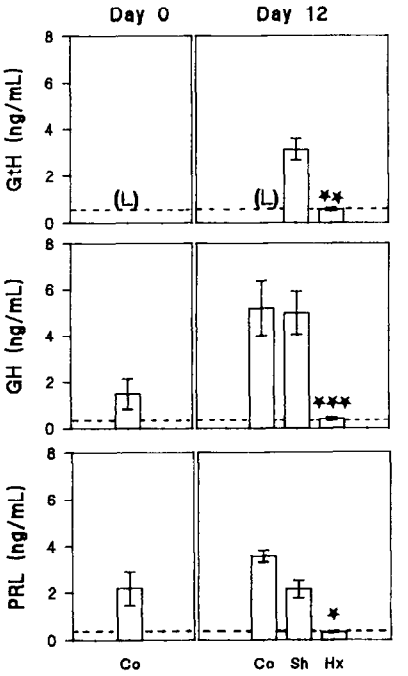

FIG. 1. Concentration of GtH, GH, and PRL in blood plasma from intact (Co), sham operated (SH), or hypophysectomized animals (Hx) on Day 0 or 12 Days after surgery. Results are means ± standard error of the mean (SEM; $n = 5$). L, lacking data. $^{*}P < 0.01$; $^{**}P < 0.005$; $^{***}P < 0.001$. Dashed lines, lower limit of assays.

Castration

After an initial 34% decrease of binding

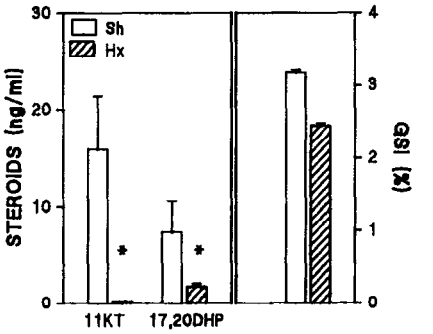

FIG. 2. Concentration of 11KT or 17α,20βDHP in blood plasma and gonado somatic index (GSI) values 12 days after surgery in sham operated (Sh) and hypophysectomized animals (Hx). Results are means ± SEM ($n = 5$). $^{*}P < 0.01$.

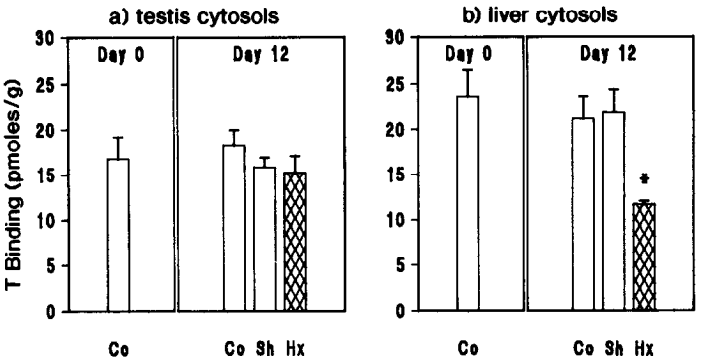

FIG. 3. Testosterone binding content (pmole/g tissue) in testis (a) or liver (b) cytosols from four groups of fish: intact (Co), at Days 0 and 12; sham operated (Sh) and hypophysectomized (Hx) sacrificed 12 days after surgery. Results are means ± SEM (n = 5). *$P < 0.01$.

in both control and castrated animals during the first 2 postoperative weeks, SBP concentrations slowly returned toward normal values during the following weeks (Fig. 5). At no time was there a difference in SBP blood concentration between castrated and sham operated animals. One month post-castration testosterone levels were 2010 ± 470 pg/ml (mean ± SE) in control animals and below the detection limit of the assay (50 pg/ml) in all castrated animals.

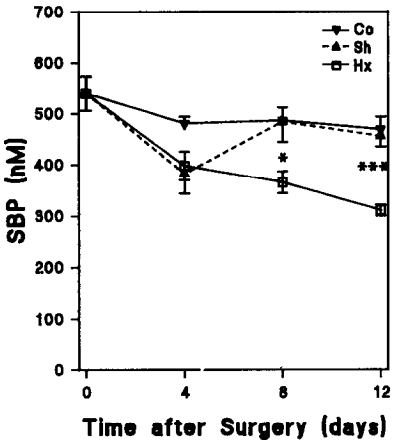

FIG. 4. SBP concentration in blood plasma from intact (Co), sham operated (Sh) and hypophysectomized animals (Hx) at different times after surgery. Values at Day 0 were obtained from five intact animals sacrificed at this time. Results are means ± SEM (n = 5). Statistical significance of the differences of SBP concentrations between groups were determined by Student t test., *$P < 0.01$; ***$P < 0.001$.

Variation in SBP and Hormones toward the End of the Reproductive Cycle

Large variations in SBP concentrations were recorded during the later phases of sexual maturation in three individual male trout (Fig. 6). Very low SBP concentrations were measured during spermiation, but no relation was found between individual androgen and SBP levels. The reduction in plasma SBP began when 11KT was high. Subsequently, SBP remained low when 11KT was decreasing and SBP increased again in two animals while 11KT (and testosterone, not shown) remained low.

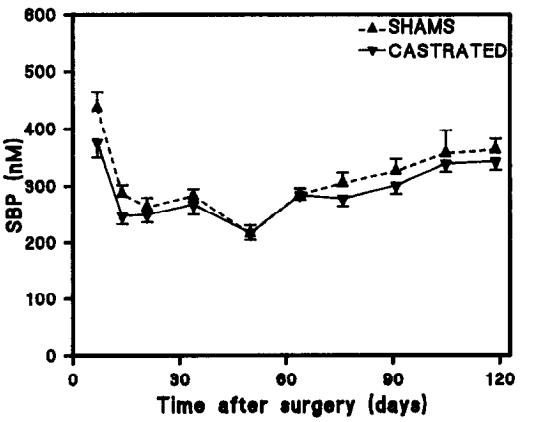

FIG. 5. SBP concentration in blood plasma from sham operated or castrated male rainbow trout at different times after surgery. Results are means ± SEM (n = 7 for the sham and 8 for the castrated animals).

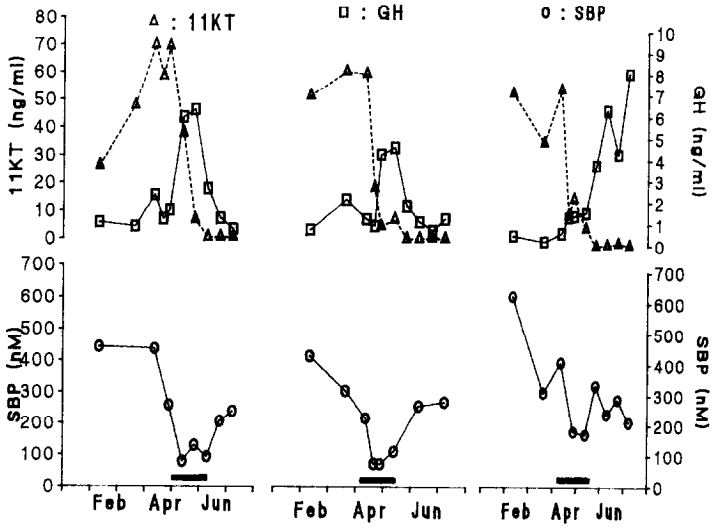

FIG. 6. SBP, 11KT, and GH concentration in blood plasma samples obtained from three individual fish over 5.5 months—before, during, and after the spermiation period. (▬) Indicates the spermiation period.

However, the decrease in SBP appeared to be related to a large elevation of growth hormone levels during spermiation: the SBP fall began slightly before the major GH peak (however, in two of the three fish it occurred during a preceding small GH peak); the lowest SBP levels coincided with the highest GH levels; and SBP then increased when GH decreased or, in one animal, SBP remained low while GH remained high.

Effect of Water Temperature

After 12 weeks of acclimation at 5° or 13°, plasma SBP concentrations were similar (Fig. 7), although at 5°, there was a larger individual variation.

Effect of Long-term Fasting and GH Supplementation

Long-term fasting significantly decreased SBP and IGF concentrations. On the other hand, GH treatment significantly increased circulating IGFs without affecting SBP level (Fig. 8).

To investigate further possible relationships between IGF and SBP concentrations, IGF was measured in blood samples from animals at the end of their reproductive cycle. Before the spawning period IGF concentration in blood plasma was high (225 ± 13 ng/ml, $n = 4$), decreased dramatically during spermiation (73.5 ± 10 ng/ml, $n = 6$), and increased again after this process (125 ± 6 ng/ml, $n = 3$).

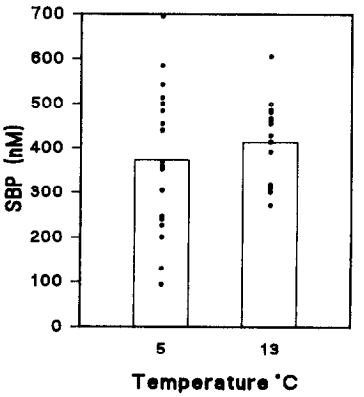

FIG. 7. SBP concentration in blood plasma from two groups of 11 and 15 fish kept for 3 months at 5° and 13°, respectively. Results are means and individual values.

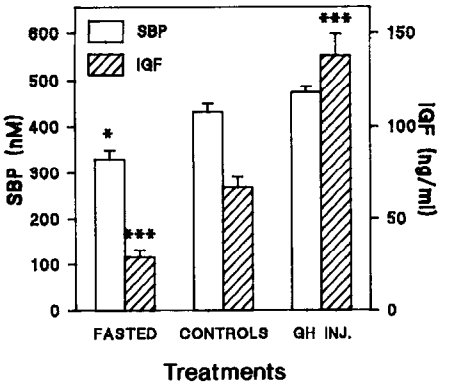

FIG. 8. SBP and IGF concentration in blood plasma from groups of control, fasted, or GH-injected fish (1 μg bovine GH/g wt, twice a week), after 6 weeks of treatment. $^{*}P < 0.01$; $^{***}P < 0.001$.

DISCUSSION

These studies reveal, in trout, experimental nonspecific inhibitory effects on plasma SBP concentrations: surgery has similar actions both in operated and sham operated animals; and similar effects were observed in infections or other treatment such as injections (data not shown). Stress, surgical shock, or general health (including reduced food intake) may be responsible. Such phenomena must be recognized when preparing protocols to study SBP levels *in vivo*.

There were no differences in blood SBP concentrations of castrated or sham operated male trout, neither in the short nor the long term. The plasma steroid binding protein that we measure is not considered to be to a noticeable degree of testicular origin. Our recent demonstration of SBP production by trout hepatocytes in culture (Foucher *et al.*, 1991) suggests the liver as the main source of circulating SBP in trout. The parallel decreases of T binding in liver cytosol and blood serum after hypophysectomy support this. Furthermore, the absence of T binding decrease in testicular cytosols suggests another site of production in the testis, as previously proposed (Foucher and Le Gac, 1989).

Castration is known to dramatically reduce the blood concentration of most androgens (Schulz and Blum, 1988) and the present study shows testosterone to be undetectable a few weeks after gonadectomy. The absence of difference in SBP concentration between the two groups suggests that variation of sex steroids of testicular origin (at least androgens) is not an important regulatory factor for SBP levels in mature male trout.

The present results are similar to most data obtained in human where SHBG is not affected by castration (von Shoultz and Carlström, 1989) (although, in macaca, a slight increase was reported, and normal values were obtained after subsequent T treatment (Kottler *et al.*, 1988)). Furthermore, in trout, castration at different stages induces a rapid and large increase in plasma gonadotropin levels (Billard and Breton, 1977); gonadotropins probably are not involved in SBP levels regulation therefore.

At the end of the spermatogenic cycle, the normally elevated SBP levels decrease during spermiation. Cyclic variations of SBP occur in mammals with discontinuous reproductive activity (Saboureau *et al.*, 1982; Audy *et al.*, 1982; Gustafson and Damassa, 1985). In the hedgehog (Saboureau *et al.*, 1982) SBP and T concentrations are inversely correlated, while in other species such correlations are not observed. There are cyclic variations of SBP (Pottinger, 1988) in brown trout in which mean binding of T was minimal when elevated mean levels of androgens were measured. However, the same variations exist in juveniles (where androgen concentrations are very low). In rainbow trout (this study), analysis of individual hormonal profiles show that the decreased SBP is not directly linked to androgen variations since it occurs before 11KT decreases and since SBP levels increase again while 11KT levels remain low. It should be noted that testosterone decreases before or simultaneously with 11KT and remains low during spermiation in salmonids (Baynes and

Scott, 1985; Le Gac *et al.*, unpublished data).

The hypothesis that, in winter, low water temperature could produce variations in SBP as described by Pottinger in a winter spawning (poikilotherm) species may be ruled out in view of our results at two different temperatures and in view of a SBP reduction during spermiation in a spring spawning strain kept at 13°.

The limited decrease in SBP of hypophysectomized trout is not comparable to the undetectable levels of [^{3}H]11KT binding found by Ng and Idler (1980) 1 month after hypophysectomy in flounder. It may be that in the present study 12 days were insufficient to allow the disappearance of SBP. However, the androgen (11KT) used by these authors in their binding studies had a poor affinity for the binding protein and was relatively insensitive for determinating plasma SBP.

The results of castration suggest that GtH (at least the maturational GtH_2, present toward the end of the reproductive cycle) and major male steroids are not directly involved in SBP regulation. To analyze the effect of hypophysectomy, direct or indirect effects of removing other hypophysial hormones must be considered. Concerning TSH, T3 (and T4) has been shown to be stimulatory in cultured human cell lines (Mercier-Bodard *et al.*, 1987). While the role of the thyrotropic axis remains to be clarified *in vivo*, a similar effect cannot be excluded in trout. ACTH may decrease SBP (Forest *et al.*, 1986) but this may have resulted from the increased cortisol levels. Prolactin is inhibitory (Vermeulen, 1986; Plymate *et al.*, 1988), so that its removal is probably not responsible for decreased SBP levels after hypophysectomy. While a direct action of GH on SBP levels has never been demonstrated, indirect evidence suggests a predominant role of the somatotropic axis on changes of SBP level in various physiological or clinical situations in human (von Shoultz and Carlström, 1989). A role for growth hormone is likely.

Around spermiation SBP decreases alongside an increase of GH concentration and at the same time there is a natural arrest of food intake for several weeks. In goldfish, elevated GH plasma levels have also been described toward the end of the annual cycle (Marchant *et al.*, 1989); no seasonal variation of SBP was found in this species (Pasmanick and Callard, 1986), which does not support the idea of a link between GH and SBP levels in fish. However, these authors did not indicate the reproductive stages studied.

The respective influences of GH and fasting were tested in trout. Since part of GH action in target tissues is relayed by IGF1, a potential role for this factor was also investigated.

Long-term fasting induced a limited but significant reduction in plasma SBP levels of trout, related to low circulating IGF levels. Fasting is known in trout to induce a large increase in endogenous GH (Sumpter *et al.*, 1991). It also provokes a remarkable decrease in hepatic GH receptors, which may explain the apparent contradiction between high GH and low IGF levels. (Yao *et al.*, in preparation). On the other hand experimentally increasing circulating GH by exogenous administration, at a dose that induced growth (data not shown) and increased circulating IGF, did not reduce SBP levels.

These results suggest that the considerable reduction of circulating SBP observed during spermiation in trout is not the direct consequence of elevated endogenous GH levels that occur at this time. It seems, in part, due to a diminished hepatic function, including decreased GH responsiveness and reduced IGF production, linked to the nutritional status of these animals.

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