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Effect of salmon gonadotropic hormone on sex steroids in male rainbow trout: plasma levels and testicular secretion in vitro

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Summary. Male rainbow trout were treated with salmon gonadotropic hormone (GTH) at different stages of the circannual reproductive cycle; spawning fish were also treated with an antiserum against salmon GTH. Injection of GTH led to a several-fold increase of plasma sex steroid levels during spermatogenesis and in the spawning season but was without effect at early stages of testicular development. GTH neutralization during the spawning season was followed by a several-fold decrease of plasma sex steroid levels. During spermatogenesis and in the spawning season, both treatment regimes resulted in an increased sensitivity of testicular explants in response to a subsequent stimulation of steroid secretion in vitro. This up-regulatory response may facilitate and maintain the high sex steroid plasma levels observed during the spawning season. It may also be necessary to allow for concomitant peak values of plasma GTH and sex steroids in the spawning season, a situation difficult to understand within the negative feedback concept. The adaptive capacities of the testicular steroidogenic system indicate that it is not only an effector site for GTH but also an active part of the endocrine system controling reproduction.

Key words: Gonadotropic hormone – Sex steroids – Reproductive cycle – Testis – Rainbow trout, *Oncorhynchus mykiss*

Introduction

Male rainbow trout show important circannual changes of the sex steroid concentrations in blood plasma which are associated with the reproductive cycle. Low levels are found in immature fish, elevated levels during testicular growth, and peak levels in the spawning season (Scott and Sumpter 1989). Steroid secretion from trout testicular explants also differed in the course of a reproductive cycle in response to a PE from *Oncorhynchus* sp. (Schulz and Blüm 1990). Maximum sensitivity and steroid secretion capacity were recorded in the spawning season, a period during which LeGac and Loir (1988) found the circannual maximum of the testicular GTH receptor number. In mammals, the number of testicular LH receptors is subject to endocrine regulation. LH itself is a prominent regulatory factor (Wang et al. 1991) and changes in the receptor number are associated with changes in the LH responsiveness of androgen secretion (Dufau 1988). With respect to trout testicular steroid secretion, it may likewise be hypothesized that the PE sensitivity is modulated in response to pituitary stimuli. This paper addresses the question of whether a change of circulating GTH levels in male rainbow trout in vivo affects the testicular response to a subsequent stimulation of steroid secretion in vitro.

Abbreviations: BSA, bovine serum albumin; bw, body weight; E_2, 17β-estradiol; GnRH, gonadotropin releasing-hormone; GTH, gonadotropic hormone; LH, luteinizing hormone; OHT, 11β-hydroxytestosterone; OT, 11-ketotestosterone; 17–20P, 17α-hydroxy, 20β-dihydroprogesterone; PE, pituitary extract; raGTH, rabbit anti-GTH antiserum; rPS, rabbit preimmune serum; T, testosterone

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Materials and methods

Animals. Male rainbow trout *(Oncorhynchus mykiss)* were obtained from open-air ponds at a commercial hatchery (Forellenzucht Niggemann, Hattingen-Elfringhausen, FRG). Two weeks before the first injection, the fish were transferred to open-air ponds at Bochum University. Experiments were carried out with fish at four stages of testicular development: at the beginning of spermatogenesis (first cycle); at the end of spermatogenesis; in the spawning season; and at the beginning of the second cycle. At the beginning of the study (September 2) the fish were 18 months old. Their body weight (bw) ranged between 224 and 442 g; further details about the fish are given in Table 1.

BSA/GTH injection. Fish were injected with either bovine serum albumin (BSA) or with a partially purified GTH preparation from *Oncorhynchus* sp. (SGA–GTH, Syndel, Vancouver, Canada). A total dose of 30 $\mu g \cdot kg\ bw^{-1}$ was administered i.p. in two injections (10% and 90% of the total dose, respectively) 3 days apart. This treatment induced precocious ovulation in coho salmon (Jalabert et al. 1978). The fish were sampled 24 h after the second injection.

Table 1. Mean gonado-somatic index (GSI) and testicular histology of male rainbow trout treated with BSA or SGA-GTH or treated with rPS or raGTH

	Date	Treatment	(*n*)	GSI ± SE	Germ cell stages
Beginning of the first cycle	Sep 2	BSA	5	0.06 ± 0.01	Spermatogonia, spermatocytes, occasionally spermatids, no spermatozoa
		GTH	3	0.07 ± 0.02	
Full spermatogenesis	Nov 3	BSA	8	4.89 ± 0.32	All germ cell stages, spermatozoa in most lobuli
		GTH	9	4.63 ± 0.47	
Spawning	Jan 31	rPS	5	3.97 ± 0.39	See below
		raGTH	5	3.61 ± 0.61	
Spawning	Feb 2	BSA	8	3.87 ± 0.31	Lobuli filled with spermatozoa, occasionally cysts with spermatocytes and spermatids
		GTH	7	*5.88 ± 0.91	
Beginning of the second cycle	Aug 7	BSA	3	1.16 ± 0.37	Large lobuli with residual spermatozoa, small lobuli with spermatogonia and spermatocytes
		GTH	3	0.78 ± 0.15	

* Significantly different ($2P < 0.05$; Student's *t*-test) from BSA injected group

Blood plasma samples were stored at −20 °C until steroid quantification. Body and testis weight was determined and the gonado-somatic index (testis weight · 100 · bw^{-1}) was calculated. Testicular tissue from BSA- and SGA–GTH-treated fish was collected and processed for in vitro incubation. Because external sexing of immature fish was difficult and because of individual differences in gonad development, a selection of the fish was made during sampling: testis tissue and plasma samples were only included in the study when the gonado-somatic index and macroscopical examination indicated a similar stage of testicular development. This led to variable numbers of experimental animals (Table 1).

Passive immunization. The SGA–GTH treatment effects were clearest in mature fish. For the passive immunization experiment males in their first spawning season (22 months old and readily releasing milt) were selected. The fish were assigned to two groups of 20 fish. They received four intraperitoneal injections every 3rd day with rabbit preimmune serum (rPS) or with a rabbit antiserum (raGTH) raised against the GTH preparation of Donaldson et al. (1972). 20 µl of the sera were diluted with 180 µl of trout balanced salt solution (Jalabert et al. 1973) so that the fish received a total injection volume of 200 µl. Blood samples were taken 24 h after the last injection. Five fish were selected at random from each treatment group, and testicular tissue from rPS- and raGTH-treated fish was processed for in vitro incubation.

Histological techniques. Testicular tissue samples from the fish used for in vitro incubation were fixed and paraffin-embedded according to conventional techniques, after which 7-µm sections were stained with haematoxylin and the stage of spermatogenesis was recorded (Table 1).

To check for the presence of raGTH antibodies, plasma aliquots from the fish receiving the rPS and raGTH treatment were diluted 1:5 and were used to incubate Bouin-fixed and paraffin-embedded 7-µm sections of pituitaries from mature male trout [peroxidase anti-peroxidase technique; Sternberger and Joseph (1979)]. Incubations with plasma from raGTH-injected fish resulted in a labelling of cells which were also labelled after incubation with raGTH diluted 1:8000 [not shown; see for comparison Schäfer et al. (1989)]. Labelling was considered to indicate an excess of circulating antibodies. Incubations with plasma from rPS injected fish did not result in any labelling.

Testicular tissue incubation. Testicular tissue was processed for in vitro incubation as described previously (Schulz 1986). In brief, testis tissue was cut into small blocks which were aliquotted in 24-well Costar plates (about five blocks, weighing 25–35 mg per well). The tissue was incubated for 18 h at 10–12 °C in a carbogen atmosphere in 1.2 ml trout balanced salt solution. Groups of four wells were spiked with 0.1–10 µg of a PE from mature salmon (*Oncorhynchus* sp.; PE contained all water-soluble compounds and showed a GTH content of 10.4% w/w, according to Syndel). At the end of incubation 1 ml of medium was removed and heated for 1 h at 80 °C. After centrifugation the supernatants were stored at −20 °C until steroid quantification.

Steroid radioimmunoassays. The assay procedure followed for the androgens OT, T and OHT was that given by Schulz (1985). In addition, 17–20P (Mayer et al. 1989) and E, (Schulz 1984) were also quantified by radioimmunoassay. On analysis of incubation media there were no differences in the steroid levels measured when the samples were subjected to either ether extraction/thin-layer chromatography or heat treatment (Schulz and Blüm 1990). The same was true for blood plasma, except for OHT, which was overestimated 1.6-fold after heat treatment ($n = 24$; Wilcoxen test for paired observations). It was decided to adopt the assay procedure without ether extraction, i.e. heat-treated incubation medium was directly used in the assays. 400 µl blood plasma was mixed with 800 µl water containing 0.05% Na-azide, heat treated and centrifuged; the supernatants were used in the assays. Under these conditions, the detection limits were 0.24 ng · ml blood $plasma^{-1}$ and 0.1 ng · ml $incubation\ medium^{-1}$.

Statistics. In vitro data are given as picograms of steroid secreted per milligram of tissue incubated. Plasma levels are given as nanograms per millilitre. All data are expressed as means and standard error. The effect of increasing PE concentrations on steroid levels in the incubation media within the treatment groups was analysed by Duncan's multiple range test (significance set at $\alpha = 0.05$) after logarithmic transformation. Differences between hormone levels at the same PE dose but after different treatments in vivo (BSA or SGA–GTH and rPS or raGTH, respectively), and differences in steroid plasma levels were analysed by Student's *t*-test (linear data). A 2*P*-value of 0.05 was chosen as the limit of statistical significance.

Results

BSA/SGA–GTH injections

Plasma steroid levels in BSA-injected fish were below 1 ng · ml^{-1} at the beginning of the first and second reproductive cycles (Fig. 1). Higher levels were measured at the end of spermatogenesis and maximum values were recorded in spawning males. SGA–GTH injection had no statistically significant effect on steroid plasma levels at the beginning of the first and second cycles. At the end of spermatogenesis and in the spawning season the treatment significantly increased androgen levels 3- to 4-fold, except for T in spawning males; 17–20P levels were increased 8- to 9-fold. E_2 plasma levels were always below the detection limit (0.24 ng · ml $plasma^{-1}$; not shown).

The effect of PE on steroid secretion in vitro depended on the stage of testicular development and on the SGA–GTH pretreatment. (Fig. 2). Incubation with increasing amounts of PE mostly stimulated steroid secretion in a dose-dependent manner. Tissue of males at the end of spermatogenesis and in the spawning season was more reactive than at the beginning of the second

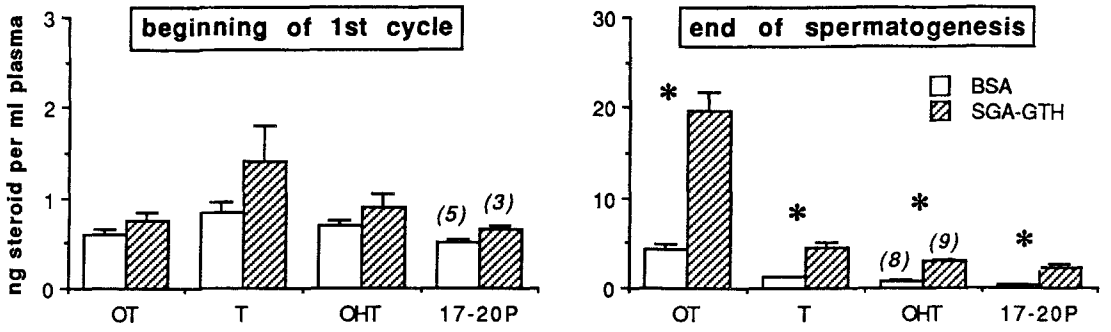


Fig. 1. Plasma steroid levels (ng · ml^{-1}) of male rainbow trout at four different stages of testicular development 24 h after the second of two BSA *(open columns)* or SGA–GTH *(hatched columns)* injections. The number of animals per group is given in parentheses. *Vertical bars* indicate standard error, an *asterisk* indicates a significant difference between BSA and SGA–GTH injected groups ($2P < 0.05$, Student's *t*-test). Note change in ordinate scaling

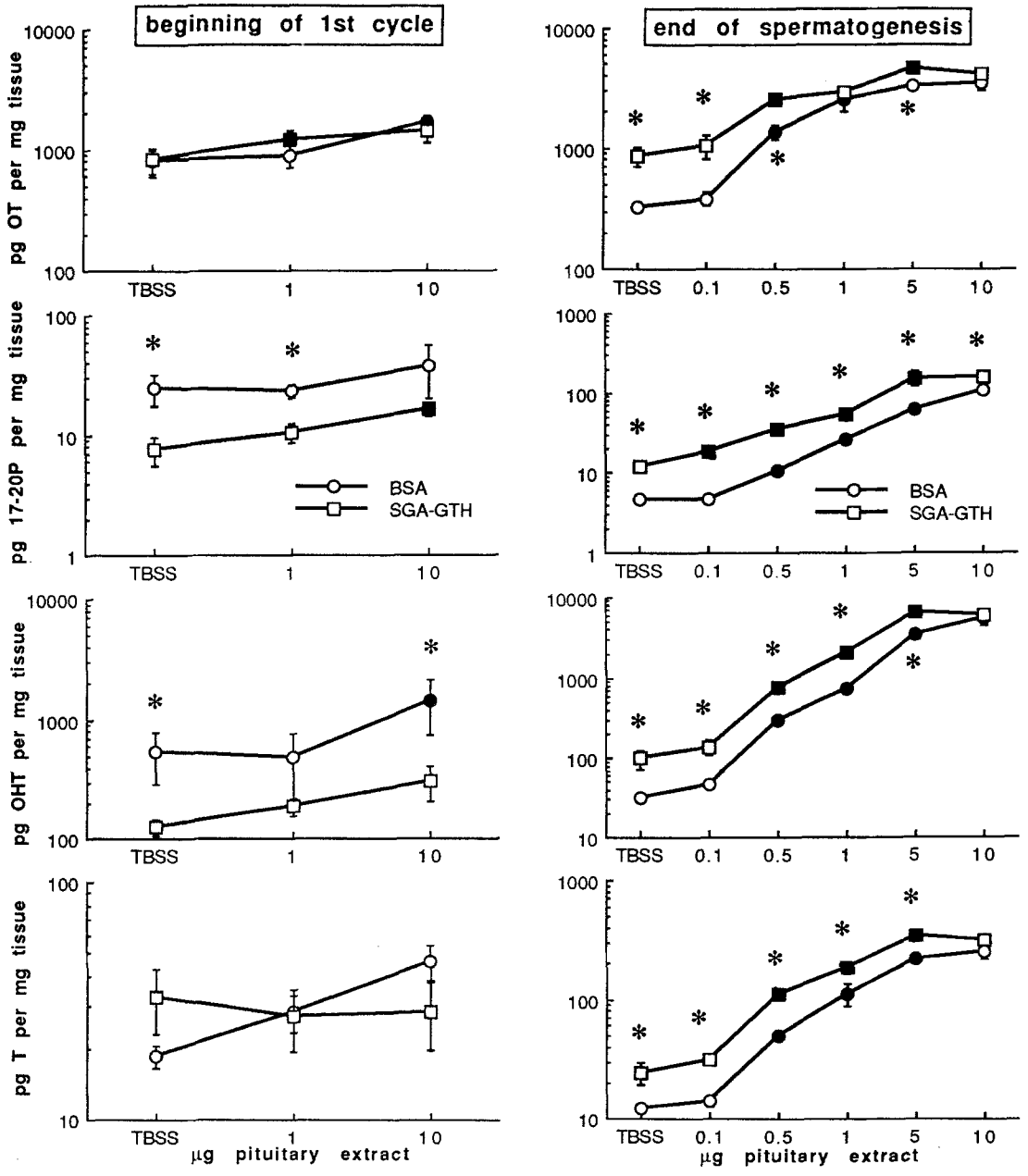


Fig. 2. Steroid levels in media (pg steroid · mg tissue^{-1}) from trout testis incubated in the absence (trout balanced salt solution – TBSS) or presence of 0.1–10 µg pituitary extract of mature salmon. Tissue was prepared at four different stages of development (Table 1) from fish after BSA *(circles)* and SGA–GTH injection *(squares)*. Data *points* show the mean of quadruplicate incubations. *Vertical bars*

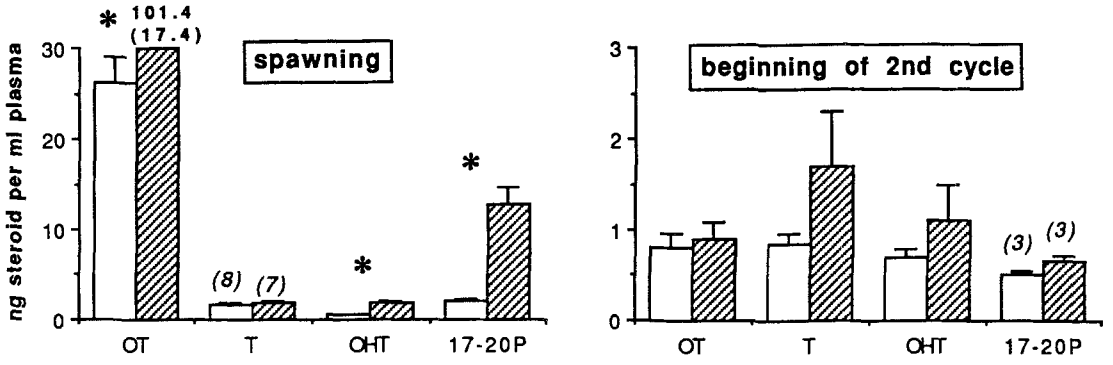
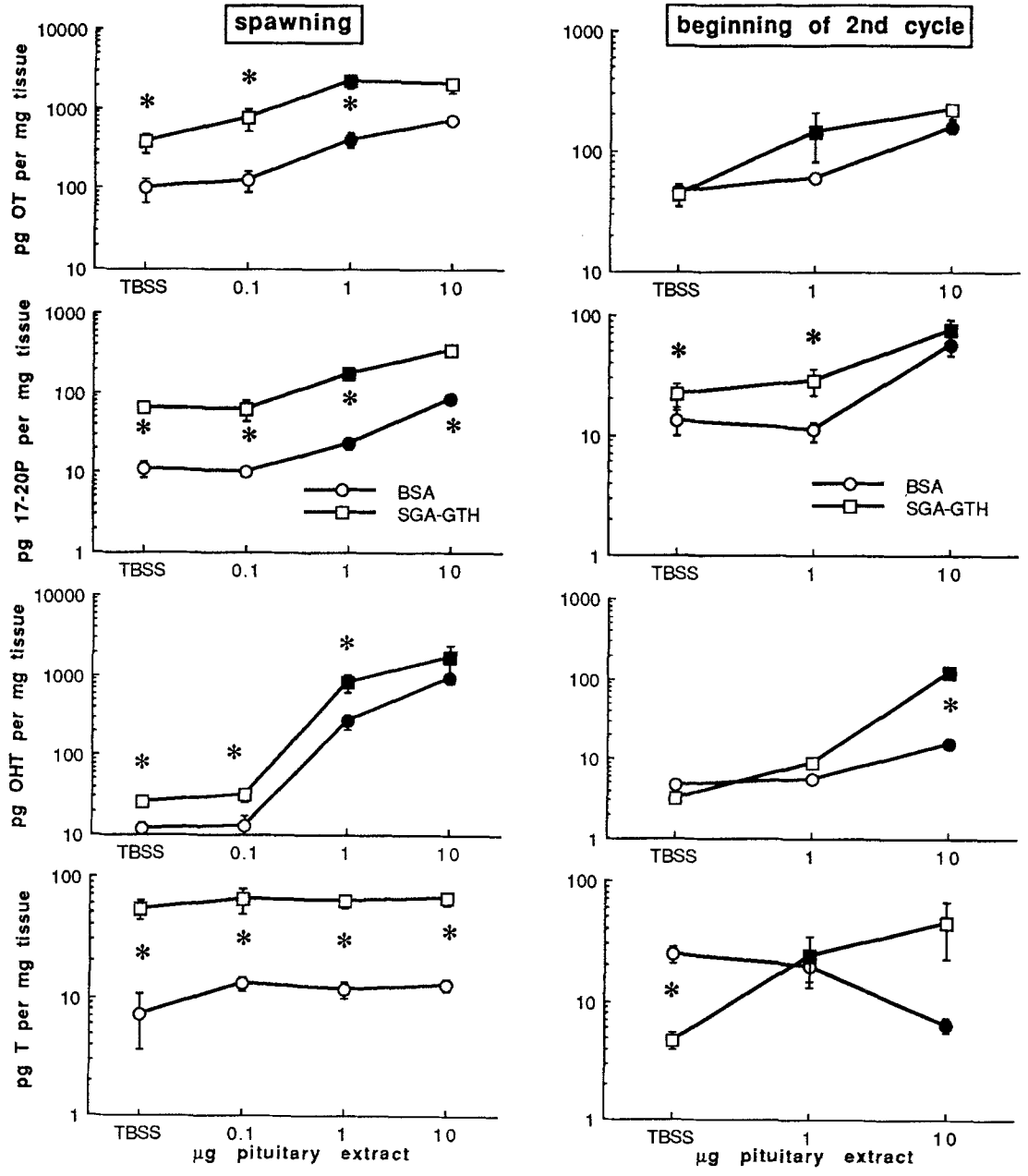


Fig. 1 (continued)



indicate standard error if not too small for the scale. Significant differences ($2P < 0.05$, Student's t-test) between the steroid amounts secreted at the same PE dose from tissue of BSA or SGA–GTH-injected fish are indicated by an *asterisk*. *Black circles* or *squares* indicate a significant difference (Duncan's multiple range test; $\alpha = 0.05$) as regards steroid secretion in response to the next smaller amount of pituitary extract. Note change in ordinate scaling

cycle, and more so than at the beginning of the first cycle. At the beginning of the first cycle, SGA–GTH treatment had either no statistically significant effect (Fig. 2: OT, T), or resulted in lower steroid levels than after BSA treatment (Fig. 2: OHT, 17–20P). At the end of spermatogenesis and in the spawning season, steroid levels in testis incubation media sampled from tissue of SGA–GTH-treated fish were significantly higher than in media sampled from tissue of BSA-treated fish, comparing the steroid amounts secreted at the same PE dose. Only at the highest PE dose did the stimulatory effect of the SGA–GTH pretreatment in vivo become non-significant for the androgens. At the beginning of the second cycle, slightly higher levels of 17–20P and of OHT were found after SGA–GTH injection. No changes were recorded with respect to OT, whereas T levels in the absence of PE were lower after SGA–GTH treatment.

E_2 levels in the incubation media (not shown) displayed a different pattern from the other steroids investigated. E_2 concentrations did not change significantly in response to PE in vitro, and was undetectable at the end of spermatogenesis. Treatment with SGA–GTH resulted in significantly lower (beginning of the first and second reproductive cycle) or significantly higher (spawning season) E_2 levels in the medium. In general, the levels were low, with a maximum of 60 pg · mg tissue^{-1} at the beginning of the first cycle.

Passive immunization

Treatment with raGTH led to a 2- to 3-fold decrease of plasma androgen concentrations (Fig. 3). The treatment effect was more pronounced in the case of 17–20P: 12 out of 20 fish showed plasma levels below the detection limit (0.24 ng · ml^{-1}), and the mean of the remaining 8 samples was more than 10-fold lower than in the rPS-injected group.

Steroid levels in the incubation media increased in a dose-dependent manner in response to increasing PE concentrations (Fig. 4). In the absence of PE, the levels of 17–20P, OHT and T were significantly higher after raGTH treatment than after rPS treatment, and significant increases of OT and OHT levels were recorded even at the lowest PE dose. Furthermore, at one or more of the PE doses used greater quantities of steroids were found in the incubation media after raGTH treatment of the tissue donors.

Discussion

The sensitivity of trout testis to pituitary stimuli and its capacity for steroid secretion increased during spermatogenesis and was highest in the spawning season (Schulz and Blüm 1990). The present study has shown that both injection and immunoneutralization of GTH led to a further up-regulation of the testicular steroidogenic response in advanced stages of the reproductive cycle. An up-regulation following GTH immunoneutralization may be understood as a compensatory reaction of a similar nature to that seen in mammals: low LH levels maintain Leydig cell LH receptors and steroidogenic enzymes in an up-regulated state (Dufau 1988). However, high LH levels down-regulated the LH receptor number and impaired LH-stimulated androgen secretion in mammals (Dufau 1988; Wang et al. 1991). It is likely that SGA–GTH injection led to GTH plasma levels similar to, or somewhat higher than, the circannual maximum in the spawning season. The latter was found to be between 2 and 12 ng · ml^{-1} (Fostier et al. 1982; Lou et al. 1986; Sumpter and Scott 1989), and Crim and Evans (1976) reported levels of 10–20 ng · ml plasma^{-1} 24 h after injection of 20 μg GTH · kg bw^{-1}. Nevertheless, the results of the present study indicate that the responsiveness of the steroidogenic system was also up-regulated after SGA–GTH treatment. This type of response therefore appears to be characteristic of advanced trout testis. Leydig cells may be of particular importance for this response; tissue PE sensitivity in terms of steroid secretion (Schulz and Blüm 1990), and GTH receptor and Leydig cell number (LeGac and Loir 1988; Loir 1990) were all high or maximal close to and during the spawning season.

Two gonadotropic hormones, GTH I and II, have been isolated from chum and coho salmon pituitaries (Suzuki et al. 1988; Swanson et al. 1991) and similar GTHs may be present in rainbow trout (Nozaki et al. 1990). SGA–GTH may have contained both GTH I and II, and the raGTH antiserum appears to recognize both GTHs (van Putten et al. 1983). It is therefore not possible to assign the up-regulatory response to a specific GTH.

Salmon growth hormone and prolactin also enhance trout gonadal steroid secretion (Singh et al. 1988). With the aim of a broad reflection of the total testicular capacity to react to pituitary stimuli, PE was used in this study to stimulate steroid secretion in vitro. The possibility that SGA–GTH or raGTH treatment might affect testicular sensitivity to growth hormone or prolactin therefore cannot be excluded.

SGA–GTH treatment had a limited effect on plasma steroid levels at the beginning of a reproductive cycle, but a clear effect at advanced stages of the cycle. This correlates well with the changes of testicular PE sensitivity (Schulz and Blüm 1990). 17–20P plasma levels were more sensitive than androgens to both GTH injection and immunoneutralization. This indicates of a differential control of progestin and androgen production. Sakai et al. (1989) proposed a shift in amago salmon testicular steroidogenesis from OT to 17–20P immediately prior to or during the spawning season. Barry et al. (1989) concluded from in vitro experiments with carp testis that 17–20P impaired 11β-hydroxylase and/or 11β-hydroxysteroid dehydrogenase activity. In the present and a previous study (Schulz and Blüm 1990) OHT was produced in large amounts in vitro in response to PE, suggesting that in the rainbow trout 11β-hydroxysteroid dehydrogenase, but not 11β-hydroxylase activity may be rate limiting for OT synthesis and may be affected by increasing 17–20P concentrations. However, OT levels in plasma and incubation media were several-fold higher than those of 17–20P and sensitive to SGA–GTH injection and PE. Therefore, a 17–20P-mediated inhibition of OT production and/or a shift in steroidogenesis at the expense of OT may be less strict in spawning trout. 20β-Hydroxysteroid dehydrogenase is the key enzyme for 17–20P production. The apparently high GTH dependency of 17–20P plasma levels may alternatively be

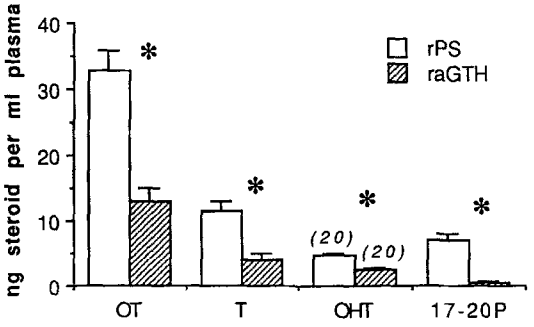


Fig. 3. Plasma steroid levels (ng · ml^{-1}) in mature male rainbow trout after rPS *(open columns)* or raGTH *(hatched columns)* treatment. The number of animals per group is given in parentheses. *Vertical bars* indicate standard error, an *asterisk* indicates a significant difference between rPS and raGTH injected groups ($2P < 0.05$, Student's *t*-test). 17–20P levels in the raGTH-injected group were below detection limit (0.24 ng · ml^{-1}) in 12 samples, so that the mean of the remaining 8 samples is given

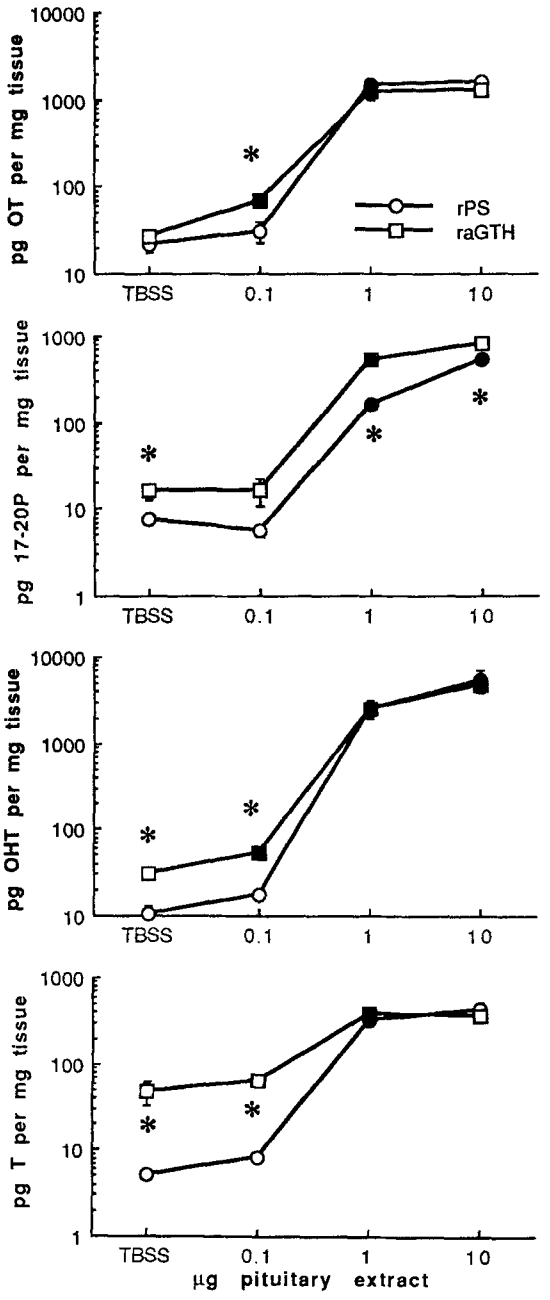


Fig. 4. Steroid levels in media (pg steroid · mg tissue^{-1}) from mature trout testis. Tissue donors were treated with rPS *(circles)* or raGTH *(squares)*. For further details see legend to Fig. 2

explained assuming a relatively low substrate (17α-hydroxyprogesterone) affinity of the enzyme. Under these circumstances only a strong pituitary stimulation would generate precursor concentrations high enough to allow for a noticeable 17–20P production. Conversely, GTH neutralization would inhibit 17–20P production at lower precursor concentrations. The latter, however, could still be sufficient for androgen synthesis, resulting in reduced but measureable plasma androgen levels after raGTH treatment.

What is the physiological significance of the up-regulatory response and of differential reactions to SGA–GTH during the cycle? Plasma GTH II and sex steroids reach the circannual peak levels during the spawning season (Scott and Sumpter 1989; Sumpter and Scott 1989). An up-regulatory mechanism may facilitate the comparatively much stronger increase in sex steroid levels, and it may permit sex steroids to attain high plasma levels despite the negative feedback they exert on the pituitary. Evidence for negative feedback effects of OT, T or E_2 on plasma GTH levels was provided by castration/steroid replacement experiments in male rainbow trout (Billard 1978). Trudeau et al. (1991) showed that treatment of intact male goldfish with T or E_2, but not with OT, up-regulated the GTH secretion response to GnRH injections, while basal GTH secretion remained unaffected. Whether the apparently different steroid feedback effects are related to species differences or to the experimental model (basal vs GnRH-stimulated GTH secretion, castrated vs intact fish) remains to be clarified. However, a potentiation of GnRH effects, in combination with an increasing sensitivity of the testicular steroidogenic system, would up-regulate the activity of the brain-pituitary-gonad axis towards a peak in the spawning season. At the end of the spawning season, trout testicular tissue became insensitive to pituitary hormones (Schulz and Blüm 1990), which may be a mechanism to end the up-regulated state of the brain-pituitary-gonad axis. As regards fish at the beginning of a cycle, it may be speculated that a restricted steroidogenic response prevents detrimental effects on spermatogenesis, considering that androgen treatment impaired the onset of spermatogenesis in trout (Billard et al. 1981). The strict GTH dependency of 17–20P plasma levels could limit gestagen production to periods when its physiological effects, for example on milt volume or seminal plasma composition (Ueda et al. 1985; Baynes and Scott 1985), are beneficial at the time of spawning. In this context, the data of Liley et al. (1986) is interesting in that the presence of ovulated and digging female trout triggered increases of plasma GTH, 17–20P and, to a much lesser extent, of OT levels in mature males.

In summary, mature trout testis adapts to increasing, as well as decreasing, levels of GTH bioactivity with increased sensitivity of the steroidogenic system to subsequent re-stimulation. The mechanisms resulting in up-regulation are not known. Several possibilities exist: for

example, changes in the GTH receptor number, changes in the sensitivity/activity of post-receptor mechanisms and changes in the half-life of messenger RNAs coding for steroidogenic enzymes; the mechanisms are not necessarily the same after increasing and decreasing GTH (and steroid) plasma levels. Furthermore, the treatment effects may be direct effects of pituitary hormones, but may also be indirect effects mediated by steroid hormones. Irrespective of the nature of the mechanisms involved, these adaptive capacities indicate that the testicular steroidogenic system is not only an effector site for gonadotropic regulation but is also an active part of the regulatory system controlling reproduction.

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