Validation of Radioimmunoassays for Two Salmon Gonadotropins (GTH I and GTH II) and Their Plasma Concentrations Throughout the Reproductive Cycle in Male and Female Rainbow Trout (Oncorhynchus mykiss)¹

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ABSTRACT

RIAs were developed for the two salmon gonadotropins (GTH I and GTH II) and used to measure plasma GTH throughout the life of the rainbow trout, Oncorhynchus mykiss. The RIA for GTH II was specific and sensitive (< 0.001% cross-reaction with GTH I, mean sensitivity = 0.26 ± 0.02 ng/ml). The RIA for GTH I was less specific and less sensitive than the GTH II RIA (9.7% cross-reaction with GTH II, mean sensitivity = 2.34 ± 0.23 ng/ml).

In both males and females, the levels of GTH II remained undetectable (< 0.3 ng/ml) throughout most of the reproductive cycle, until shortly preceding spermatiation/ovulation, when they began to rise. Concentrations of plasma GTH II were maximal at spermatiation/ovulation. In both sexes, plasma profiles of GTH I differed from those of GTH II. The plasma GTH I concentration in females was elevated during the period of gonadal growth and could detect it only shortly before ovulation [4, 5].

In the late 1970s, Idler and Ng suggested that in fish, as in mammals, there were two GTHs [6]. Two putative GTHs were subsequently isolated (on the basis of their differing contents of carbohydrate), but it proved very difficult to separate the two GTHs on the basis of their function (both were able to stimulate steroidogenesis and ovarian growth), and data on concentrations in the plasma was forthcoming for only one of the two (maturational GTH).

In the late 1980s, advances in chemistry led to the identification of two distinct salmon GTHs in chum salmon (Onchorhynchus keta [7, 8]). They were called GTH I and GTH II and, like tetrapod FSH and LH, were shown to be glycoproteins with two subunits, α and β. Moreover, GTH I was shown to be chemically related to FSH, and GTH II to LH [9]. GTH I and GTH II have now been identified in other salmonids (coho salmon, O. kisutch [10]; rainbow trout, O. mykiss [11]) and in nonsalmonid fish, including carp (Cyprinus carpio) [12], red seabream (Pagrus major) [13], and Atlantic croaker (Micropogonias undulatus) [14]. Attempts to isolate GTH I in some fish, however, have proven unsuccessful (African catfish, Clarias gariepinus [15]).

In salmonids, both GTH I and GTH II have similar steroidogenic potencies in vitro for inducing the synthesis of 17β-estradiol (in vitellogenic follicles) and 17α-hydroxy-2003-dihydroprogesterone (in mature, postvitellogenic follicles) [16]. However, the available evidence on pituitary and plasma concentrations of GTH I and GTH II indicates that they are produced at different times of the reproductive cycle and therefore presumably have different physiological roles. Antibodies raised against GTH I and GTH II stain two distinctively different gonadotroph cell types in the pars distalis of salmonids [17], and GTH I-producing cells are present before, and increase in numbers during, vitellogenesis, whereas GTH II-producing cells do not appear until after the onset of spermatogenesis/vitellogenesis [17]. At ovulation, GTH II is the predominant GTH in the pituitary [18]. Swanson et al. [19] found that in juvenile (pre-vitellogenic and pre-spermatogenic) coho salmon, GTH I was the only GTH detectable in the plasma. Furthermore, at physiological concentrations, coho salmon GTH I stimulates vitellogenin uptake into rainbow trout oocytes, whereas GTH II does not [20].

Very few RIAs specific for GTH I have been developed [10, 18], and the data on cross-reaction between GTH I and GTH II in these assays are extremely limited. Despite this, the assays developed for coho salmon GTHs have been

INTRODUCTION

In tetrapods, two gonadotropins (GTHs) are involved in the control of gonadal development: FSH, regulating gonadal growth, and LH, regulating final maturation and ovulation. In contrast, gonadal development in fish was originally thought to be controlled by only one GTH [1]. The concept of a unique GTH in fish persisted for some time, as it appeared to induce all gonadotropic-ovarian functions [2, 3]. However, most researchers could not detect GTH in the blood during the period of gonadal growth and could detect it only shortly before ovulation [4, 5].

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Very few RIAs specific for GTH I have been developed [10, 18], and the data on cross-reaction between GTH I and GTH II in these assays are extremely limited. Despite this, the assays developed for coho salmon GTHs have been
used to measure GTH I in another species of Pacific salmon [21], and coho salmon GTH I has been used in physiological studies in both Pacific and Atlantic salmon [20, 22]. Furthermore, studies on the plasma concentrations of GTH in fish are limited to only parts of the reproductive cycle [9, 18, 21].

The aim of this study was to develop, and fully validate, GTH I and GTH II RIAs to measure plasma concentrations of GTH throughout gonadal development in an annually spawning salmonid, the rainbow trout (O. mykiss).

**MATERIAL AND METHODS**

Purified GTH I, GTH II, and β-subunits of GTH I and GTH II from rainbow trout were not available for this work; therefore GTH preparations from a closely allied Oncorhynchus species (coho salmon) were used. In competitive binding studies, however, rainbow trout GTH I and GTH II have been shown to be immunologically equipotent with coho salmon GTH I and GTH II [11].

**Standards**

Coho salmon (O. kisutch) GTH I and GTH II were used as standards, and antibodies were raised against their β-subunits. Intact hormones and β-subunits were kindly provided by Dr. Penny Swanson (see [10]).

**Production of Antiserum**

Antibodies against β-subunits of GTH I (βGTH I) and GTH II (βGTH II) from coho salmon were raised in rabbits. The β-subunits were dissolved in PBS (at a concentration of 50 μg/ml) and emulsified with an equal volume of Freund’s complete adjuvant. A suspension containing 25 μg of either GTH I or GTH II was injected into rabbits (2 rabbits were injected for each hormone). Each rabbit received five subsequent booster injections of 25 μg of GTH emulsified with Freund’s incomplete adjuvant. Rabbits were bled one month after the last injection. To determine the specificity of each assay, the ability of the heterologous GTH to compete with labeled homologous GTH for antibody binding was examined. If the heterologous GTH could compete, cross-reaction was calculated from the 50% inhibition level.

**Iodination of Gonadotropins**

Intact GTH I and GTH II were iodinated with 125I using the Iodogen method [23] according to Sumpter [24]. The labeled hormones were separated from free 125I by gel filtration on Sephadex G-25 columns (PD 10 columns; Pharmacia and Upjohn Inc., Kalamazoo, MI). Specific activities of GTH I and GTH II were between 80 and 100 μCi/μg. Radiolabeled hormones were stored at 4°C.

**Assay Validation**

Validation of the coho salmon GTH assays for use in measuring GTH in rainbow trout plasma was carried out as follows: 1) Parallelism tests: serial dilutions of rainbow trout plasma containing high titers of GTH were compared to serial dilutions of GTH standard. 2) Recovery tests: known amounts of GTH were added to plasma from immature rainbow trout (plasma containing little, if any, GTH I or GTH II). GTH I was “spiked” at 10 concentrations between 0 and 60 ng/ml. GTH II was “spiked” at 10 concentrations between 0 and 10 ng/ml. Assays were carried out according to the protocol described below.

**Assay Procedure**

Standards (50 μl/tube) were serially diluted in protein assay buffer (0.05 M phosphate buffer, containing 0.15 M NaCl, 0.01 M EDTA, 0.5% egg albumin, and 0.1% NaN3, pH 7.5). The concentrations of standards ranged from 0.2 ng/ml to 100 ng/ml for GTH I, and from 0.02 ng/ml to 10 ng/ml for GTH II. Antiserum against βGTH I was added (50 μl/tube) at a dilution of 1:3000 and antiserum against βGTH II at a dilution of 1:200 000, in 1:400 normal rabbit serum in protein assay buffer. After a 24-h incubation at 4°C, 50 μl of radiolabeled GTH was added to each tube (5000 cpm), and the tubes were incubated for a further 24 h at 4°C. On the third day, 50 μl donkey-anti-rabbit-γ-globulin, diluted to 1:40 in protein assay buffer, was added to each tube, and tubes were then incubated for 24 h at 4°C. On the fourth day, 200 μl of protein assay buffer was added to each tube, and the tubes were centrifuged at 3500 × g at 4°C for 1 h. The supernatant was aspirated, and radioactivity was counted in the precipitate for 5 min in an LKB Ultragamma (Pharmacia) scintillation counter.

**Chromatography of Plasma**

A pool of plasma from ovulated females was collected and gel-filtered on a 96 × 1.6-cm column of Sephadex G-75 superfine (Pharmacia) to determine whether the immunologically active material was present as intact hormone or β-subunit. The column was equilibrated and run with PBS, pH 7.3, at a flow rate of 10 ml/h, at 4°C. Fractions (1.3 ml) were collected and stored at −20°C until assayed.

**Annual Cycle Study**

Six hundred rainbow trout (South African strain) of mixed sex were placed into 3 outdoor 1500-L glass fiber tanks. Each tank was supplied with a constant flow of lake water at ambient temperature. The fish were fed once daily, five times per week, with a commercial feed (BP Mainstream; BP Nutrition UK Ltd., Cheshire, UK) at the rate recommended by the manufacturer.

Collection of plasma samples for measurements of GTH was started when the fish were 9-mo-old juveniles (January 1992), with a mean weight of 24 ± 1.2 g and mean length of 13 ± 0.2 cm (n = 22). Earlier in the trout's life history, the small size of the fish prevented sufficient volumes of plasma from being obtained for GTH assays. Every month
over a 2-yr period, 24 fish were netted (8 from each tank) and anesthetized (in 2-phenoxyethanol; 1:2000), and blood samples were collected from the caudal sinus into heparinized syringes. The blood was spun at 3000 × g for 10 min, and the plasma was collected and deep frozen at −20°C until required. The fish were killed, and weight and fork length were recorded. The body cavity was opened, and the gonads were removed and weighed to determine the sex and the gonadosomatic index (GSI = [gonad weight/body weight] × 100). The last samples were collected in April 1994, at which time the mean weight and length were 520 ± 39.3 g and 32.6 ± 0.68 cm (n = 20), respectively.

Female rainbow trout of the South African strain mature as 3- or 4-yr-olds, whereas males of this strain may mature a year earlier as 2-yr-olds (in this study, all males matured for the first time as 3-yr-olds). During 1993 (the third year of life), most, but not all, of the males and females started to mature. From July 1993 onwards, it was possible to distinguish between fish that were maturing that season and those that were not, on the basis of the size of their gonads; males and females with a GSI of 0.4 and higher were considered to be maturing, and fish with a GSI of less than 0.4 were considered to be immature; they were grouped accordingly for analysis.

Statistical Analysis

GSI and plasma concentrations of GTH I and GTH II were analyzed by one-way analysis of variance (ANOVA). When necessary, data were log-transformed. After ANOVA, a multiple-comparison Scheffé test was performed. In the final analysis, plasma levels of GTH I and GTH II during the third year of the fishes' lives were grouped and analyzed (ANOVA and a multiple-comparison Scheffé test) in relation to the GSI.

RESULTS

Specificity of the RIAs

The GTH I RIA had a 9.7% cross-reaction with GTH II (Fig. 1A). The GTH II RIA was highly specific, with less than a 0.001% cross-reactivity with GTH I (Fig. 1B). The assays appeared to measure both intact hormone and β-subunits in the plasma (Fig. 2).

Sensitivity of the RIAs

The midpoint of the GTH I standard curve was 21.1 ± 0.9 ng/ml (n = 12), and the practical detection limit (90% binding) was around 2 ng/ml. The GTH II RIA was considerably more sensitive than the GTH I assay; the midpoint of the standard curve was 1.29 ± 0.06 ng/ml (n = 10), and the practical detection limit was 0.25 ng/ml (Fig. 3). For the purpose of statistical analysis, any plasma sample that contained a concentration of GTH less than the amount that could be measured in the assays was ascribed a concentration corresponding to the assay detection limit.
Changes in GSI and Plasma Levels of Gonadotropins

Immature Fish

During the 2-yr study, fish that did not mature as 3-yr-olds increased in weight from 24 ± 1.2 g at the start of the experiment to 665 ± 58 g at its completion 2 yr later, in April 1994. GSIs in males and females that did not mature were less than 0.4 throughout. Concentrations of plasma GTH I and GTH II in immature fish showed no seasonal changes, and they were close to the detection limit of the assays throughout the study.

Maturing Females

In females that matured as 3-yr-olds, the maximum body weight was attained in January, shortly before ovulation (623 ± 37 g). At this time the GSI was 15.53 ± 0.85 (n = 6).

There were indications of a seasonal elevation in plasma GTH I in females during their second year (centered around July), but this rise was not statistically significant (Fig. 4). A significant increase in plasma GTH I, reaching 17 ng/ml, was seen in the following year (1993) and was centered around the period between July and August (p < 0.05 and p < 0.01, respectively). The first significant increase in the GSI detected in females was associated with the increase in the concentration of plasma GTH I. GTH I levels in the plasma of females increased from around 3 ng/ml in females with a GSI of less than 0.25, up to 10 ng/ml in females with a GSI of 0.25–0.38 (p < 0.01; Fig. 5A). Peak concentrations of GTH I during this initial growth of the ovary (17 ng/ml) were seen in females with a GSI of between 0.4 to 1 (p < 0.001). Subsequently, plasma concentrations of GTH I returned to basal levels, even though the GSI was increasing rapidly, until they rose again shortly before ovulation, in March–April (1994). At ovulation, in April, plasma concentrations of GTH I reached 34 ng/ml (p < 0.001). Thus, increases in the GSI above 1 were associated with decreasing concentrations of GTH I, until shortly before ovulation.

Plasma concentrations of GTH II remained low (between 0.2 and 0.5 ng/ml) throughout most of the female’s life. Not until January 1994 was there a significant increase in the plasma GTH II concentration, which increased to around 2 ng/ml (p < 0.001; Fig. 4). Thereafter, the plasma GTH II concentration increased very rapidly, peaking at around 70 ng/ml (p < 0.001) in March when the fish were ovulating.

Maturing Males

Males that matured as 3-yr-olds reached a maximum body size in December 1993 (556 ± 19 g). The GSI in maturing males was low (between 0.04 to 0.2) until June 1993, when the testes began to rapidly increase in size (p < 0.001; Fig. 6). The maximum GSI, which was around 7, was
reached in October 1993, two months before running males were observed in December, when the GSI was 4.34 ± 0.26 (n = 12). Overall, plasma levels of GTH I were lower in males than in females. Nevertheless, fluctuations in plasma GTH I in males appeared to occur at times similar to those seen in females. Statistical analysis of the data using ANOVA demonstrated significant increases (p < 0.05) in the concentration of plasma GTH I in males during May and July 1992 (concentrations at this time were around 6 ng/ml) and in September 1993 (5 ng/ml; Fig. 6). The highest concentrations of GTH I in males (around 6 ng/ml) were associated with a GSI of less than 0.4 (in May/July 1992) and with a GSI between 5 and 7 (5 ng/ml; p < 0.05, Fig. 5B).

Concentrations of plasma GTH II in males were low (between 0.3 and 0.5 ng/ml) until December 1993, when some of the maturing males began producing milt (Fig. 6). In February 1994, there was a significant increase in plasma GTH II (to 0.9 ng/ml; p < 0.001). The maximum GTH II concentrations in males (around 3 ng/ml) were seen in April (p < 0.001), when all of the maturing males were spermiating.

**DISCUSSION**

**RIAs for GTH I and GTH II**

The GTH I RIA developed in this study, using an antiserum raised against the βGTH I, was reasonably, but not completely, specific for GTH I. The degree of cross-reaction of GTH II in the GTH I assays (9.7%) was slightly less than that reported for GTH II in the chum salmon GTH I RIA (12%, [18]). In contrast, GTH I had essentially no cross-reaction in the GTH II assay (< 0.001%), and the GTH II RIA presented here is a considerable improvement on previous GTH II RIAs, compared both with those that have used antiserum raised against intact GTH (10%; [5]) and with others.
that have used antiserum raised against βGTH II (10%; [18]). However, cross-reaction of GTH II in the GTH I RIA does not pose any problems for interpreting plasma concentrations because the two GTHs are not secreted simultaneously, except at ovulation. Until more specific GTH I assays are developed, it will not be possible to say what the ratio of GTH I to GTH II is at ovulation, but the data strongly suggest that both GTHs are secreted at this time.

The greater sensitivity of the GTH II RIA compared to the GTH I assay (about 10-fold) is similar to that seen in mammals, where LH RIAs are usually more sensitive than FSH RIAs [25, 26]. The reason why the titer of antibodies raised against the two GTHs differed widely is not known, but it might relate to the differing contents of carbohydrate. In chum salmon, GTH I has a higher content of N-acetylated neuraminic (sialic) acid than does GTH II [7], as does FSH and chorionic gonadotropin (CG) compared with LH, in tetrapods [27]. This difference might account for why chum salmon GTHs and mammalian FSH are far less immunogenic than GTH II or LH, respectively. Moreover, it has been demonstrated that removing sialic acid in eCG, which has also a high sialic acid content, increases its immunoreactivity [28].

Rainbow trout plasma diluted parallel to both coho salmon GTH I and GTH II standards, and there were very accurate recoveries of known amounts of coho salmon GTH I or GTH II added to rainbow trout plasma. These data, together with the findings that rainbow trout GTHs and coho salmon GTHs are immunologically equipotent [11], validated the use of the coho salmon GTH assays for measuring GTH I and GTH II in rainbow trout plasma.

The GTH assays that were developed measured both intact hormone and free β-subunits. Surprisingly, free β-subunits appeared to be present in the plasma of ovulated females (free β-subunits were also present in the plasma of females before ovulation [data not shown]). The free β-subunits may have been released as such by the pituitary or they may have resulted from the degradation of intact hormones in the plasma. Recently, Naito et al. [29] observed the accumulation of free βGTH II in large globules in the pituitary GTH II-cells in rainbow trout, whereas intact hormone was found in small granules, but βGTH II is released into the plasma in much lower quantities than intact GTH II (Amano et al., manuscript in preparation; cited in [29]). In mammals, free α-subunits may be secreted from both gonadotrophs [30]. The functional significance of free GTH subunits in the plasma remains to be established.

Changes in the Concentrations of GTH I and GTH II in Plasma

This is the first study to look at the seasonal changes in plasma GTH I and GTH II during the reproductive cycle of a sequentially spawning fish. The data presented demonstrate that GTH I, but not GTH II, is associated with gonadal growth. In females, there was a rise in the plasma GTH I concentration during the initial phase of vitellogenesis, 8 mo or so prior to ovulation, whereas GTH II was undetectable in the plasma at this time. GTH I has been shown to stimulate synthesis of 17β-estradiol by vitellogenic oocytes in vitro [16] and also to increase the rate of vitellogenin uptake by the oocytes both in vivo and in vitro [20]. Uptake of vitellogenin into oocytes in the rainbow trout starts when the GSI is around 0.25 [31], and this coincides with the increase in GTH I observed in this study. Furthermore, the highest density of vitellogenin receptors (effecting vitellogenin uptake and therefore growth of the oocyte) are found in small vitellogenic follicles that have a diameter of 0.6 mm [32], when the GSI is about 0.5 and plasma levels of GTH I are high. Later in the cycle when GSI dramatically increases, the plasma GTH I levels decrease, suggesting that the primary function of GTH I is to initiate vitellogenic growth. In mammals, after recruitment of a group of follicles into the maturing pool, one follicle eventually outgrows the others (which then become atretic), and FSH levels in the plasma then fall to concentrations that are able to maintain maturation of the dominant follicle but are insufficient to stimulate further recruitment [33]. Although the dynamics of oocyte growth differ in fish (considered in detail in [34]) from those in mammals, the pattern of plasma concentrations of GTH I in female rainbow trout during a reproductive cycle appears similar to that of FSH in the estrous cycle of mammals. In mammals, FSH induces growth of a new batch of follicles, effects their initial growth rate [35], and influences fecundity [36]; in trout, GTH I effects recruitment of oocytes into vitellogenesis and/or their subsequent growth during the first part of vitellogenic development. Taken together, the evidence suggests that GTH I in fish probably plays a similar role to that of FSH in mammals.

GTH I concentrations in the plasma of females decreased to basal levels after the start of vitellogenesis until ovulation, when they were elevated again. The annual cycle of GTH I in female rainbow trout seen here differs from that reported in other Oncorhyncus species. Swanson [9] reported that the plasma GTH I concentration in coho salmon increased steadily during much of vitellogenesis, with the highest levels occurring during mid- to late-vitellogenesis, after which they decreased as ovulation approached. The reproductive strategy of the coho salmon differs, however, from that of the rainbow trout: coho salmon spawn only once and then die, whereas rainbow trout may spawn annually over a number of years, such that at the time of ovulation the ovary contains not only fully mature oocytes but also primary oocytes that have already begun growth [31]. It is possible that the surge in plasma GTH I at ovulation potentiates the ovulatory action of GTH II (as indeed occurs in mammals, where FSH up-regulates the number of LH receptors, [37]).
A further possibility is that the ovulatory surge of GTH I in the rainbow trout may have a function in initiating the next cycle of ovary growth. If this were the case, it would explain why concentrations of GTH I at ovulation are high in rainbow trout but not in coho salmon.

Assessing of the function of GTH I in males is more difficult since the plasma concentrations of GTH I were markedly lower in males than in females. The seasonal profile of plasma GTH I in male rainbow trout differed significantly from that reported in male coho salmon, where concentrations of up to 50 ng/ml were measured during the mid-stages of testicular growth (similar concentrations to those recorded in the plasma of female coho salmon [9]). In this study, maximum levels of plasma GTH I in male rainbow trout during their first reproductive season were around 5 ng/ml. This increase in plasma GTH I in male trout during mid- to late testicular growth was significant according to ANOVA, but not in a more stringent multiple-comparison Scheffé test. At this time of year (September), males with the larger GSIs had the highest concentrations of GTH I. In male trout, GTH I probably has a role similar to that of FSH in mammals, in which FSH affects the initiation and maintenance of testicular growth and spermatogenesis [38, 39].

In males, during late spring to early summer more than a year before the first reproductive cycle, there was an increase in the concentration of GTH I in the plasma. At this time, there also appeared to be an increase in the concentration of plasma GTH I in males (in females, however, it was not significant statistically). Previous studies on the rainbow trout have shown that gonadotrophs containing GTH I are present in the pituitary 60 days after fertilization, while gonadotrophs containing GTH II are not seen until the fish are 6 mo old [40]. This suggests that GTH I may have a function in gonadal growth a year before the first reproductive cycle. If we are to gain a further understanding of the possible role(s) of GTH I in males, and in early gonadal growth in both male and female rainbow trout, an improvement in the sensitivity of the GTH I RIA is required (we were not able to sensitize the GTH I assay either by adjusting the concentration of antibody added, by altering the amount of label added, and/or by increasing incubation times).

In female rainbow trout, GTH II concentrations were increasing rapidly one month before ovulation, when plasma levels of GTH I were still low, and were at their highest during ovulation. GTH II is more potent than GTH I in stimulating 17α-hydroxy-20β-dihydroprogesterone production in vitro [16], and there is little doubt that the primary function of GTH II in female fish is to effect ovulation. Plasma levels of GTH II measured here in males were considerably lower than in females. Maximal concentrations of GTH II were seen in males that were spermatiating, suggesting that GTH II is involved with controlling the later stages of spermatogenesis [5].

In summary, in fish there are two distinct gonadotropins, GTH I and GTH II, that are chemically related to FSH and LH, respectively, in mammals [7–9]. Studies to date on the physiological function(s) of GTH I and GTH II have shown that they have roles respectively similar to those of FSH and LH—GTH I in the initiation and maintenance of gonadal growth and GTH II in the regulation of the final stages of maturation and ovulation/spermiation. The seasonal patterns of plasma GTH I and GTH II, presented here for the rainbow trout, mimic the ovarian cycles of FSH and LH in mammals. On the basis of the evidence presented here, we suggest that GTH I and GTH II should be called fish FSH and fish LH, respectively.

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