Changes in mRNAs encoding steroidogenic acute regulatory protein, steroidogenic enzymes and receptors for gonadotropins during spermatogenesis in rainbow trout testes

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Abstract

In vertebrates, sperm development and maturation are directly regulated by gonadal steroid hormone secretion. The relationships among the expression of genes encoding steroidogenic proteins and receptors for gonadotropins, and testicular steroid production have not yet been comprehensively determined in male teleosts. In this study, the changes in levels of mRNAs encoding follicle-stimulating hormone (FSH) receptor, luteinizing hormone (LH) receptor, steroidogenic acute regulatory protein (StAR), cytochrome P450 cholesterol side-chain cleavage, 3 β -hydroxysteroid dehydrogenase/ Δ 5–4-isomerase, cytochrome P450 17 α -hydroxylase/17,20-lyase, cytochrome P450 11 β -hydroxylase, 11 β -hydroxysteroid dehydrogenase and 20 β -hydroxysteroid dehydrogenase were determined by real-time, quantitative PCR assays and related to changes in serum steroid levels throughout the reproductive cycle in male rainbow trout. Serum 11-ketotestosterone and 17 α ,20 β -dihydroxy-4-pregnen-

3-one levels were measured by RIA. Although the pattern of change in the mRNA levels for the enzymes was variable, the increases in steroidogenic enzyme mRNAs started prior to a significant increase of serum steroid levels. The patterns of transcript levels of FSH and LH receptors suggest that changes in StAR and steroidogenic enzyme transcripts are largely mediated by the FSH receptor during early and mid-spermatogenesis and by the LH receptor during late spermatogenesis and spermiation. Levels of StAR (10-fold) and P450 17 α -hydroxylase/17,20-lyase (sevenfold) transcripts changed with the greatest magnitude and were closely related to the changes in serum steroids, suggesting that changes in StAR and P450 17 α -hydroxylase/17,20-lyase abundance are likely to be the major influences on overall steroidogenic output during the reproductive cycle in male rainbow trout.

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Introduction

In vertebrates, two types of gonadotropins (GTHs), follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and gonadal steroid hormones have major roles in the regulation of spermatogenesis and sperm maturation. In mammals, FSH and LH primarily regulate steroid production through control of steroidogenic enzyme gene expression (Richards 1994). Binding of FSH or LH to their specific cell-surface receptors leads to the production of second messenger molecules which initiate changes in expression and activity of key steroidogenic enzymes for the synthesis of specific steroid hormones at specific times.

In teleosts, two distinct pituitary GTHs, GTH I and GTH II, were first isolated from chum salmon (Suzuki *et al.* 1988) and coho salmon (Swanson *et al.* 1991). Later studies demonstrated that GTH I and GTH II are structurally and functionally similar to FSH and LH, and therefore GTH I and GTH II are currently recognized as FSH and LH respectively (Swanson *et al.* 1989, Van der Kraak *et al.* 1992, Koide *et al.* 1993, Tanaka *et al.* 1993, Okada *et al.* 1994, García-Hernández *et al.* 1997, Weltzien *et al.* 2003). In male rainbow trout, FSH in plasma was elevated during mid-spermatogenesis, whereas LH showed a significant increase in plasma during the spermiating stage (Prat *et al.* 1996, Gomez *et al.* 1999). For salmon testis *in vitro*, the relative potencies of FSH and LH

in stimulating steroid synthesis did not change before the late spermatogenesis stage and spermiation (Planas & Swanson 1995). However, during the later stages of spermatogenesis and spermiation, the sensitivity of the testis to the steroidogenic effects of LH on progesterone production increased, whereas the sensitivity of the testis to the effects of FSH decreased (Planas & Swanson 1995). These results imply that the differences in the steroidogenic actions of FSH and LH that are associated with particular stages of gonadal development are probably due to changes in expression of genes encoding their receptors. Although cDNAs for the FSH receptor (FSH-R) and the LH receptor (LH-R) have been isolated from several salmonid FSH-R – amago salmon (Oba *et al.* 1999a), rainbow trout (GenBank accession no. AF439405) and Atlantic salmon (GenBank accession no. AJ567667) – and LH-R – amago salmon (Oba *et al.* 1999b), rainbow trout (GenBank accession no. AF439404) and Atlantic salmon (GenBank accession no. AJ579790) – the intracellular mechanisms of steroidogenesis that are regulated by FSH and LH through their receptors in teleosts are not understood to the same degree as in mammals.

For male salmonids, the potent androgen, 11-ketotestosterone (11-KT) and the progesterone, 17 α ,20 β dihydroxy-4-pregnen-3-one (17,20 β -P), have key roles in the regulation of spermatogenesis. A distinct shift in the steroidogenic pathway from 11-KT to 17,20 β -P synthesis occurs in the testes of salmonid species around the onset of spermiation (Nagahama 1994). Studies on male salmonids indicate that spermiation is associated with a decrease in plasma 11-KT and an increase in plasma 17,20 β -P (Baynes & Scott 1985, Lou *et al.* 1986, King & Young 2001). Using Japanese eel testis fragments *in vitro*, Miura *et al.* (1991) demonstrated that 11-KT can induce all stages of spermatogenesis, including spermatogonial proliferation, meiotic division and spermiogenesis. These results indicate that 11-KT has important roles during spermatogenesis in teleost species (Schulz & Miura 2002). Although the capacity of the testis to produce 17,20 β -P is low during spermatogenesis, 17,20 β -P production sharply increases during spermiation (Ueda *et al.* 1983, 1984, Sakai *et al.* 1989). Two successive injections of 17,20 β -P induced precocious spermiation in nonspermiating amago salmon and goldfish (Ueda *et al.* 1985). These results suggest that 17,20 β -P plays a role in the process of final sperm maturation and spermiation in teleosts.

Previous studies have shown that the long-term changes in steroid secretion in mammalian ovaries are regulated by changes in steroidogenic enzyme gene expression (Doody *et al.* 1990a, 1990b, Aspden *et al.* 1998). It is likely that steroid hormone synthesis in teleosts is similarly regulated by changes in the activity of individual steroidogenic enzymes through the action of GTHs and/or endogenous steroids. Salmonids are excellent study animals to examine the changes in steroidogenic enzyme gene expression in relation to steroid hormone synthesis,

since germ cell development progresses in a synchronous fashion and dynamic changes in steroid hormone secretion occur during gametogenesis. In rainbow trout, cDNAs for most of the steroidogenic enzymes responsible for the synthesis of sex steroids have already been isolated: cytochrome P450 cholesterol side-chain cleavage (P450_{scc}) (Takahashi *et al.* 1993), cytochrome P450 17 β -hydroxylase/17,20-lyase (P450C17) (Sakai *et al.* 1992), 3 β -hydroxysteroid dehydrogenase/ Δ 5–4-isomerase (3 β -HSD) (Sakai *et al.* 1994), cytochrome P450 aromatase (P450 arom) (Tanaka *et al.* 1992), cytochrome P450 11 β -hydroxylase (P45011 β) (Liu *et al.* 2000, Kusakabe *et al.* 2002a), 11 β -hydroxysteroid dehydrogenase (11 β -HSD) (Kusakabe *et al.* 2003) and 20 β -hydroxysteroid dehydrogenase (20 β -HSD) (Guan *et al.* 1999). Recent studies reported changes in mRNA levels for key ovarian steroidogenic enzymes during a reproductive cycle in channel catfish (Kumar *et al.* 2000), Arctic char (von Hofsten *et al.* 2002) and rainbow trout (Nakamura *et al.* 2005), and a few recent studies in male teleosts reported seasonal changes in transcript levels of a single steroidogenic enzyme (Liu *et al.* 2000, Kusakabe *et al.* 2002a, 2003). However, comprehensive studies on the expression of testicular steroidogenic enzyme genes during spermatogenesis in teleosts have not been reported.

The rate-limiting step of tropic hormone-induced steroidogenesis in mammals is the rate of the delivery of cholesterol to P450_{scc} by steroidogenic acute regulatory protein (StAR) (Stocco 2000). Studies on mammalian StARs have mainly concentrated on the short-term (acute) changes in steroidogenesis with little information available on the regulation of StAR during prolonged seasonal cycles. Recently, cDNAs encoding StAR in brook trout and rainbow trout were isolated (Kusakabe *et al.* 2002b), and an increase in ovarian StAR gene expression during the late vitellogenesis, maturation and post-ovulatory stages in rainbow trout was demonstrated (Bobe *et al.* 2004, Nakamura *et al.* 2005). However, the changes occurring in testicular StAR gene expression in teleosts during spermatogenesis remain to be determined.

The aim of this study was to determine the changes in expression of the gene encoding steroidogenic enzymes and StAR in rainbow trout testes during spermatogenesis in relation to androgens and 17,20 β -P production, and to changes in mRNAs encoding FSH-R and LH-R. In the course of the study, we also had to address the problem of normalization of mRNA levels by expression of housekeeping genes. The trout testis changes 20-fold in size during spermatogenesis and has proven to be problematic in this regard. Kusakabe *et al.* (2002a) reported the progressive reduction of mRNA levels for the commonly used housekeeping gene, β -actin, during mid-spermatogenesis. In this study, we conducted further investigations of the stability of two other housekeeping genes, acidic ribosomal phosphoprotein P0 (ARP) and 18S ribosomal RNA.

Materials and Methods

Study animals

Two-year-old, male rainbow trout were obtained monthly from Wanaka Trout Hatchery (Wanaka, New Zealand) from January to November in 2001. For each sampling, 3–5 fish were anesthetized with 300 mg/l MS222 (3-aminobenzoic acid ethyl ester; Sigma) buffered in sodium bicarbonate. Testes were isolated by dissection, and blood was sampled from the caudal vein with syringes. Blood was allowed to clot, and serum was obtained by centrifugation and stored at -20°C for steroid assay. Testis fragments were frozen with liquid nitrogen and stored at -80°C for RNA extraction. Testis fragments were also fixed with Bouin's fixative to determine the developmental stage. Animal care and use were approved by the University of Otago Committee on Care and Use of Animals.

Histologic analysis of rainbow trout testes

The fixed testis fragments were dehydrated and embedded in paraffin, sections (8 μm) were stained by hematoxylin and eosin, and the developmental stages in each sample were determined, according to Grier (1981), as follows:

1. Early spermatogenesis – spermatogonia and spermatocytes are dominant.
2. Mid-spermatogenesis – spermatogonia, spermatocytes, spermatids and sperm are present.
3. Late spermatogenesis – sperm become dominant and very few spermatogonia and/or spermatocytes are present.
4. After spawning – the testes size declines and no developing germ cells are present.

Serum sex steroids

Sex steroids were extracted from serum with ethyl ether, as previously described by Kagawa *et al.* (1981). Levels of 11-KT and $17\alpha,20\beta\text{-P}$ were measured by RIA, as previously described (Kagawa *et al.* 1981, Ueda *et al.* 1985, Young *et al.* 1986).

Expression of genes encoding steroidogenic proteins and gonadotropin receptors

Previous studies have shown that the sites of expression of steroidogenic enzymes and StAR in rainbow trout testes are restricted to Leydig cells (Kobayashi *et al.* 1998, Kusakabe *et al.* 2002a, 2002b, 2003). Furthermore, the localization of Leydig cells in testis during spermatogenesis changes with the maturational stage (Kusakabe *et al.* 2003). Therefore, in order to avoid biasing the number of Leydig cells in each sample, testes samples for RNA extraction were collected as 300 mg cross-sections taken from the widest point, approximately 3 cm from the

anterior end. Testis fragments from 3–5 males were collected for each month. Relative expression of transcripts was measured by real-time, quantitative RT-PCR as described below.

Total RNA was isolated from the testis fragments with Trizol reagent, as described by the manufacturer (Invitrogen). Concentrations of total RNA were carefully measured at least three times with NanoDrop ND-100 (NanoDrop Technologies, Wilmington, DE, USA), and equal amounts of RNA were subjected to reverse transcription to synthesize cDNAs.

An amount of 300 ng total RNA was incubated with 0.6 unit of DNase I, Amp Grade (Invitrogen) at room temperature for 15 min to eliminate genomic DNA contamination. DNase I was inactivated by heat denaturation at 65°C for 10 min. Subsequently, single-strand cDNAs were synthesized from the DNase I-treated RNA with the High-Capacity cDNA archive kit as described by the manufacturer (Applied Biosystems, Foster City, CA, USA).

For the relative quantitation of StAR, steroidogenic enzyme and GTH receptor gene expression, real-time, quantitative RT-PCR was performed with the ABI PRISM 7300 sequence detection system (Applied Biosystems). Primers and TaqMan probes for real-time PCR were designed with Primer Express, Version 1.5 (Applied Biosystems). The nucleotide sequences for the primers and probes are listed in Table 1. For normalization of data, an 18S ribosomal gene was measured with commercially available primers and probes purchased from Applied Biosystems.

Each real-time PCR reaction contained 25 μl PCR mixture made from 12.5 μl ABI Universal PCR Master Mix, 0.9 μM forward primer, 0.9 μM reverse primer, 0.2 μM fluorescent-labeled probe and 3.0 μl cDNA template. Real-time PCR cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. A standard curve was generated by serial dilutions of cDNA for each PCR reaction to determine the levels of transcripts. Standard curve dilutions were run in triplicate, and correlation coefficients of the standard curves ranged from 0.99 to 1.00. RNA samples not subject to reverse transcription were used to check for genomic DNA contamination during RNA preparation.

In order to present changes in steroidogenic enzyme and StAR mRNA levels on the basis of absolute amounts per testes, the total testes weight in each fish and the weight of frozen tissue used for total RNA extraction were measured. The yield of total RNA extracted from each testis sample was calculated by measuring the concentration of total RNA. Therefore, it was possible to estimate the total amount of total RNA contained within the testes. Given the result obtained from real-time PCR in the formula, the relative amount of target mRNA per animal normalized to relative gonad size was determined by the following formula:

Table 1 Nucleotide sequences for steroidogenic enzyme and StAR primers and probes

Target	Forward primer (5'–3')	Probe (5'–3')	Reverse primer (5'–3')
FSH-R	CAGTCACCTGACGATCTGCAA	ACTGGACTGAGGGTCTACCTAACTTCTCCCG	TGCAGGTCAGCAGAAACG
LH-R	CAACTGAATATAGTCAATGAACCTGT	TCTTGGTCCCAITAAAGGCATAGTCTTGTAATTTCTCTA	CGGTATTTCTTCAAAACCAATTTATTT
StAR	AAGAGGTCAAGATCCTCCAGAGAT	ACCACGAGGTGTGGGACCC	CGGCCCCACCAAGTT
P450 _{scc}	ACATGCTACAGATGCTGAAGATGAT	TCAGCGCTCTTTGACCCAGCGG	TGGATGAAGCCTCAGCGTTT
P450 _{c17}	CGCATCCGGCCTGTTG	CCCTCTACTCATCCCCCATGTAGCCC	GAACTTGCCAATACTGGTGTCTGT
P450 _{11β}	TGCTCAACAGGGAGGTGATG	TGGCTCTGCGGTACGTGCGTT	TCCCTCGCTACTTCTGTCTAGGA
3β-HSD	TCCACACTGCGTCCCTCAT	TGAAGTCACTGTATAACACCTTCCCGGTG	CTGGGTCTCTTTGACGTTGAC
11β-HSD	TGCGCCACGAAATGGAA	CAAAGTGTCCACCATACTGCCATCTCAT	GGTTACCGGACTGACCTGCTT
20β-HSD	CAGGAAGTTTAACTCATCTCACAGACT	AGGAAATTTGCTCAAAAGATTCTGCCAATTC	GTCAACAAACCTATGTTCGAATGC
ARP	GAAATCATCCAAATGCTGGATG	CTATCCCAATGTTTCATTTGTCGGCGC	CTCCACGCAAGGACAGA

$$\begin{aligned} \text{Target mRNA levels} &= \frac{\text{real-time PCR value}}{18\text{S}} \\ &\times \frac{\text{total RNA amount from RNA extraction}}{\text{tissue weight for RNA extraction}} \\ &\times \frac{\text{gonad weight}}{\text{body weight}} \end{aligned}$$

Data are presented as relative mRNA levels. Mean of initial samples in January 2001 was set as 1.

Statistical analysis

Serum steroid hormones, FSH-R, LH-R, StAR and steroidogenic enzyme mRNA levels were analyzed by one-way ANOVA followed by the Fisher PLSD test. The data were log transformed where appropriate before analysis to obtain approximately normal distributions. For the sake of clarity, not all significant differences are shown on graphs. Instead, only prominent increases or decreases are indicated. Linear regression analysis was used to examine relationships between serum steroid hormone levels and GTH receptors, StAR and steroidogenic enzyme mRNA levels, and the relationship between GTH receptor mRNA levels and StAR or steroidogenic enzyme mRNA levels.

Results

Changes in gonadosomatic index (GSI)

Changes in GSI (gonad weight/body weight × 100) from January to November 2001 are shown in Fig. 1. The GSI was maintained at low levels during the early spermatogenesis in January (0.2% ± 0.07) and February (0.4% ± 0.07). The GSI increased significantly (*P* < 0.0001) to a peak in March (3.5% ± 0.82). High GSI values (2.5–3.5%) were maintained during mid-spermatogenesis and gradually declined during the late spermatogenesis. The GSI in November (0.6% ± 0.12) decreased to the same levels as the early spermatogenesis.

Changes in size and structure of testes

Representative sections of testes in each month (Fig. 2)
1. *Early spermatogenesis (January–February).* Spermatogonia and spermatocytes were dominant in January. In February, more spermatocytes and spermatids appeared and a few sperm were first observed. The size of the cross-sections continuously increased from January to February.
2. *Mid-spermatogenesis (March–June).* In March, small clusters of spermatozoa were observed in the center of cysts, although spermatocytes and spermatids were still dominant. The incidence of spermatocytes and spermatids

Figure 1 Changes in gonadosomatic index (GSI; gonad weight/body weight $\times 100$) during spermatogenesis. Data are shown as the mean $\pm$ S.E. Number of samples per data point is shown in parentheses. One-way ANOVA was performed ($P < 0.0001$). Significant differences identified by ANOVA and Fisher PLSD test are indicated.

declined continuously from April to June, while numbers of spermatozoa increased. Cross-sectional area of testes appeared to become maximal during this stage when the mean GSI reached maximum levels.

3. *Late spermatogenesis (July–October)*. Sperm became dominant, and very few spermatocytes or spermatids were observed during late spermatogenesis stage (July–October). Spermiation occurred in August–October, as evidenced by breakdown of cyst walls and ability to collect sperm with gentle abdominal pressure. The area of cross-sections appeared to decrease at the end of the late spermatogenesis stage (October), when mean GSI also declined.

4. *Post-spawning (November)*. The size of testes dramatically declined because of sperm release in November. The amount of sperm decreased, and spermatogonia and connective tissue occupied the rest of the cross-sectional areas.

Seasonal changes in serum steroid levels

Serum 11-KT levels Serum 11-KT levels (Fig. 3A) were low during early and mid-spermatogenesis (5–7 ng/ml, January–April), increased significantly ($P = 0.0001$) in the mid-spermatogenesis stage from May (17.76 ± 1.63 ng/ml) to June (49.15 ± 8.61 ng/ml), reached a peak during late spermatogenesis (65.6 ± 14.3 ng/ml, September), and then decreased significantly to low levels in the post-spawning (3.0 ± 0.7 ng/ml, November) ($P = 0.0001$).

Serum 17,20 β -P levels 17,20 β -P levels (Fig. 3B), although low, progressively increased from 0.12 ± 0.02 ng/ml in January (early spermatogenesis) to 0.8 ± 0.4 ng/ml in June (mid-spermatogenesis). Levels then

increased significantly between June and August ($P = 0.003$) and peaked at the late spermatogenesis stage (16.7 ± 4.2 ng/ml, September) ($P = 0.0003$). The elevation in 17,20 β -P levels between July and September occurred when spermiation was evident (Fig. 2). When all testes reached the post-spawning stage in November, 17,20 β -P levels significantly decreased (0.46 ± 0.1 ng/ml) ($P = 0.0002$).

Changes in total RNA amount/testes

The total RNA amount was estimated from the yield of RNA from 300 mg tissue and total gonad weight (Fig. 4). The levels of total RNA/testes were low (2.5 – 7.0 mg/testis) during the early spermatogenesis in January and February, and increased significantly to the highest levels (32.6 – 35.5 mg/testes) ($P < 0.0001$) in March and April. The levels of total RNA/testes decreased significantly in May (16.8 ± 3.7 mg/testes) ($P = 0.004$) to reach low levels during late spermatogenesis (July–October, 1.7 – 3.3 mg/testis) and the post-spawning period (November, 10.6 ± 1.4 mg/testes).

Seasonal changes in ARP mRNA and 18S RNA levels

The seasonal changes in ARP mRNA and 18S RNA levels during spermatogenesis are presented in Fig. 5. The ARP mRNA levels changed significantly during the sampling period ($P < 0.0001$). ARP mRNA levels increased in February and then declined in March. From March to November, the ARP mRNA levels were relatively stable. Notably, the average ARP mRNA levels in February were 3–4 times higher than the levels in the latter half of the study. In contrast, 18S RNA levels were relatively stable through the experimental period and showed no significant differences between sampling points ($P = 0.105$).

Seasonal changes in FSH-R, LH-R, StAR, P450_{scc}, P450C17, P45011 β , 3 β -HSD, 11 β -HSD and 20 β -HSD mRNA levels

The transcript levels shown in Fig. 6 were normalized to relative abundance, accounting for total testis mass, RNA yield and body mass by the formula given in Materials and methods. The changes in FSH-R, LH-R, StAR, P450_{scc}, P450C17, P45011 β , 3 β -HSD, 11 β -HSD and 20 β -HSD mRNA levels during spermatogenesis are presented as a ratio of the levels in January. The mean of initial samples in January 2001 was set as 1.

FSH-R Transcript levels of FSH-R fluctuated during the study. Levels increased significantly (approximately threefold) as testes entered mid-spermatogenesis

Figure 2 Developmental stages of rainbow trout testes. Representative hematoxylin and eosin-stained sections for each month showing seasonal changes in cell components. SG, spermatogonia; SCY, spermatocyte; ST, spermatid; SP, spermatozoa.

Figure 3 Changes in the (A) serum 11-ketotestosterone (11-KT) and (B) serum 17 α ,20 β dihydroxy-4-pregnen-3-one (17,20 β -P) levels of male rainbow trout during spermatogenesis. Data are shown as the mean $\pm$ S.E. Number of samples per data point is shown in parentheses. One-way ANOVA was performed (11-KT, $P < 0.0001$; 17,20 β -P, $P < 0.0001$). Significant differences identified by ANOVA and Fisher PLSD test are indicated.

(February–March) ($P = 0.0002$). Although other statistical differences were observed, transcript levels varied by only twofold or less for the remainder of the study.

LH-R Levels of LH-R mRNA increased fourfold ($P < 0.0001$) between February (early spermatogenesis) and May (mid-spermatogenesis) and twofold ($P = 0.039$) between mid-spermatogenesis and the middle stages of late spermatogenesis (May–August). The second rise in LH-R mRNA occurred during the onset of spermiation (Fig. 2). Transcript levels then slightly decreased in October (late spermatogenesis) and November (post-spawning).

StAR Relative levels of StAR transcripts were lowest during early spermatogenesis (January–February). StAR mRNA levels increased significantly (threefold) in March (mid-spermatogenesis) ($P < 0.0001$) and continued to increase until late spermatogenesis (August–September), when serum steroid levels were maximal, and then significantly decreased in October ($P = 0.035$) to reach low levels by the post-spawning period (November).

Figure 4 Changes in total RNA amount per fish during spermatogenesis. Data are shown as the mean $\pm$ S.E. Number of samples per data point is shown in parentheses. One-way ANOVA was performed ($P < 0.0001$). Significant differences identified by ANOVA and Fisher PLSD test are indicated.

P450scc During early spermatogenesis (January–February), P450scc mRNA levels were low. Gradual, progressive and significant increases occurred between February and September ($P < 0.0001$). P450scc mRNA levels declined moderately, but not significantly during the late spermatogenesis (October) to post-spawning period (November).

P450C17 P450C17 mRNA levels were low during early spermatogenesis (January–February). From then on, P450C17 mRNA levels progressively and significantly increased ($P < 0.0001$) to a peak during late spermatogenesis (August–September). P450C17 mRNA levels quickly declined in October ($P = 0.0034$), and low levels were maintained in the post-spawning period (November).

P45011 β P45011 β mRNA levels were low during early spermatogenesis (January and February), and slightly but significantly increased in March (mid-spermatogenesis) ($P = 0.0009$). Levels were relatively stable until a significant increase ($P = 0.0044$) occurred between October (late spermatogenesis) and November (post-spawning).

3 β -HSD 3 β -HSD mRNA levels were low during early spermatogenesis (January–February) and significantly increased ninefold ($P < 0.0001$) to a peak in March and April (mid-spermatogenesis). The transcript levels then decreased in May ($P = 0.0042$) and were maintained at low levels until post-spawning (November), although levels decreased transiently ($P = 0.031$) in October.

11 β -HSD Transcript levels of 11 β -HSD were low during early spermatogenesis (January and February), and

Figure 5 (A) Changes in mRNA levels of acidic ribosomal phosphoprotein P0 (ARP) and (B) ribosomal 18S RNA during spermatogenesis. The data for ARP mRNA levels were log transformed before analysis to achieve approximately normal distributions. One-way ANOVA was performed (ARP, $P < 0.0001$; 18S, $P = 0.105$).

significantly increased in March (mid-spermatogenesis) ($P < 0.0001$). Transcript levels then significantly increased approximately threefold during late spermatogenesis from July to August ($P = 0.014$), with a significant decline between September and October ($P = 0.042$).

20 β -HSD 20 β -HSD mRNA levels were low during early spermatogenesis (January and February), but increased threefold in March ($P = 0.0033$). A slight but significant ($P < 0.0001$) decline occurred between March and June. Levels then declined ($P = 0.0004$) to lowest levels in July and were stable until October. Transcript levels in post-spawning testes (November) were significantly elevated ($P < 0.0001$).

Regression analysis

Linear regression analysis (Table 2) identified a moderate but significant relationship between changes in serum 11-KT levels and transcript levels of LH-R ($P < 0.0001$, $r^2 = 0.32$), StAR ($P < 0.0001$, $r^2 = 0.31$), or P450C17

($P < 0.0001$, $r^2 = 0.30$). StAR transcript levels were also significantly correlated with serum 17,20 β -P levels ($P < 0.0001$, $r^2 = 0.31$). A moderate but significant negative relationship ($P = 0.0002$, $r^2 = 0.29$) existed between serum 17,20 β -P and 20 β -HSD mRNA. FSH-R mRNA levels were moderately related to StAR, 3 β -HSD, P450C17 and P45011 β transcript levels ($P < 0.001$, $0.23 < r^2 < 0.45$), with a stronger relationship between FSH-R mRNA and P450scc ($P < 0.0001$, $r^2 = 0.53$) and 11 β -HSD ($P < 0.0001$, $r^2 = 0.72$) transcripts. LH-R transcript levels were strongly related to StAR, P450scc, P450C17 and 11 β -HSD transcripts ($P < 0.0001$, $0.61 < r^2 < 0.65$) and displayed a moderate relationship with P45011 β transcript levels ($P < 0.0001$, $r^2 = 0.36$).

Discussion

Recent studies in female teleosts have demonstrated seasonal changes in mRNA levels encoding key steroidogenic enzymes and StAR (Kumar *et al.* 2000, Nakamura *et al.* 2005). However, a comprehensive understanding of the pattern changes in expression of GTH receptors, StAR and steroidogenic enzymes is limited in male teleosts. In this study, we determined the seasonal changes in FSH-R, LH-R, StAR and six steroidogenic enzymes (P450scc, P450C17, P45011 β , 3 β -HSD, 11 β -HSD, 20 β -HSD) mRNA levels in male rainbow trout.

Before discussing these changes, the issue of the use of housekeeping genes for tissues that exhibit significant changes in size and cellular components, such as teleost gonads, needs to be addressed. Rainbow trout testes changed 20-fold in mass during the course of this study, for example. Normalization of RNA levels using housekeeping genes is commonly used to verify the amount of starting materials and efficiency in reverse transcription and PCR reactions. The usage of housekeeping genes is currently controversial and recently has been discussed by Bustin (2002). Previously, we reported significant changes in transcripts for β -actin, a classical housekeeping gene, during the reproductive cycle of male rainbow trout (Kusakabe *et al.* 2002a). In this study, we conducted further investigations of the stability of two other commonly used housekeeping genes, ARP mRNA and 18S ribosomal RNA. ARP mRNA levels changed significantly during spermatogenesis, similar to β -actin transcripts, with a greater than threefold difference between the highest (February) and lowest (June) levels, whereas 18S ribosomal RNA showed no significant changes through the reproductive cycle (Fig. 5). Therefore, we used 18S ribosomal RNA as an internal control gene.

In this study, the GSI of the male rainbow trout increased 17-fold from January to March, followed by a prolonged gradual decrease for the remainder of the study (Fig. 1). The cellular composition of testes also underwent dramatic changes (Fig. 2). These changes in size and

Figure 6 Changes in mRNA levels of FSH-R, LH-R, StAR, P450scc, P450C17, P45011β, 3β-HSD, 11β-HSD and 20β-HSD during spermatogenesis. Data are shown as the mean $\pm$ S.E., and expressed as relative abundance accounting for total testis mass, RNA recovery and body mass (see Materials and Methods). Number of samples per data point is shown in parentheses. All the data were log transformed before analysis to achieve approximately normal distributions. One-way ANOVA was performed (FSH-R, $P=0.0008$; LH-R, $P<0.0001$; StAR, $P<0.0001$; P450scc, $P<0.0001$; P450C17, $P<0.0001$; P45011β, $P<0.0001$; 3β-HSD, $P<0.0001$; 11β-HSD, $P<0.0001$; 20β-HSD, $P<0.0001$). Significant differences identified by ANOVA and Fisher PLSD test are indicated.

composition were accompanied by a significant pattern of change in the amount of total RNA per testes, which varied 30-fold (Fig. 4), with the highest yield during March to April. This variation in total RNA yield was probably due to extreme increases in certain transcripts related to development of germ cells (also see Liu *et al.* 2000,

Kusakabe *et al.* 2002a). For example, protamine is a nuclear protein that is synthesized only in the spermatid cells of the testis and substitutes for histones in sperm chromatin during the haploid phase of spermatogenesis (Gilmour & Dixon 1972, Iatrou & Dixon 1978). Protamine transcripts increased 300–400 times in rainbow

Table 2 Regression analysis among changes in serum steroid hormones, FSH-R, LH-R, StAR and steroidogenic enzyme transcript levels

	11-KT	17,20 β -P	FSH-R	LH-R
FSH-R	$P=0.2375$, —	$P=0.1684$, —		
LH-R	$P<0.0001$, $r^2=0.32$	$P<0.0007$, $r^2=0.24$		
StAR	$P<0.0001$, $r^2=0.31$	$P<0.0001$, $r^2=0.31$	$P<0.0001$, $r^2=0.38$	$P<0.0001$, $r^2=0.61$
P450 _{scc}	$P<0.0022$, $r^2=0.20$	$P<0.0117$, $r^2=0.14$	$P<0.0001$, $r^2=0.53$	$P<0.0001$, $r^2=0.65$
P450 _{c17}	$P<0.0001$, $r^2=0.30$	$P<0.0183$, $r^2=0.13$	$P<0.0001$, $r^2=0.49$	$P<0.0001$, $r^2=0.62$
P450 _{11β}	$P<0.9625$, —	$P<0.4500$, —	$P<0.0001$, $r^2=0.45$	$P<0.0001$, $r^2=0.36$
3 β -HSD	$P<0.1556$, —	$P<0.1474$, —	$P<0.0009$, $r^2=0.23$	$P<0.8778$, —
11 β -HSD	$P<0.0136$, $r^2=0.14$	$P<0.0005$, $r^2=0.25$	$P<0.0001$, $r^2=0.72$	$P<0.0001$, $r^2=0.62$
20 β -HSD	$P<0.0038$, $r^2=0.18$	$P<0.0002$, $r^2=0.29$	$P<0.1997$, —	$P<0.4356$, —

trout testes during the stage of spermatogenesis when spermatids predominated (Kusakabe 2002). In this study, when real-time, quantitative RT-PCR data were normalized only to 18S, there was an apparent average fourfold decrease in abundance of both FSH-R and LH-R transcripts and steroidogenic protein transcripts between January and March, when both GSI and total RNA yield per testes peaked (data not shown), an effect probably due to the dilution of Leydig cell target mRNAs by mRNA, such as protamine, that are expressed in the multiplying and differentiating germ cells (Liu *et al.* 2000, Kusakabe *et al.* 2002a). To assess the functional importance of changes in transcript abundance (i.e. potential gonadal steroidogenic capacity of an individual animal), knowing the weight of sample tissue used for RNA extraction and the amount of total RNA extracted, we were able to estimate target transcript abundance within individual testes. We then normalized data to GSI, in order to account for gonad weight varying with fish body weight, as in the work of Kusakabe *et al.* (2002a, 2003).

Steroid profiles in this study were similar to previous studies (Baynes & Scott 1985, Lou *et al.* 1986), with serum 11-KT increasing during mid-spermatogenesis to reach a peak a few months before serum 17,20 β -P increased. We hypothesized that expression of certain steroidogenic protein genes was pivotal in controlling steroid biosynthesis. Although there are some variations in the seasonal changes in expression of genes encoding the steroidogenic proteins, the patterns of steroid hormone secretion during the reproductive cycle appear to be largely regulated by changes in expression of particular steroidogenic protein genes that increased before prominent rises in plasma steroid hormones.

Previous work has shown that substantial *in vitro* 11-KT production by coho salmon testis fragments occurs in response to short-term incubation with GTHs at least 1 month prior to a prominent increase in 11-KT levels in the plasma (Planas & Swanson 1995). These results indicate that the steroidogenic machinery for 11-KT production is in place some time before plasma levels increase, consistent with the results of this study. The increase in

plasma 11-KT levels in male coho salmon coincided with an increase both in the sensitivity of the tissue to LH, and in 11-KT-synthesizing capacity (Planas & Swanson 1995). In the present study in trout, 11-KT levels increased during a period of significant increases in LH-R transcripts. Gomez *et al.* (1999) demonstrated that in trout, plasma FSH levels were higher than plasma LH levels, were moderately increased during mid-spermatogenesis, and reached maximal levels during spermiation of rainbow trout, whereas plasma LH levels were low but steadily increased through spermatogenesis to reach a peak during the spermiation period.

In fish, very little information is available on the specific roles of FSH and LH in regulating androgen and 17,20 β -P production by the testis. In salmon, the relative potency of FSH and LH in stimulating *in vitro* steroid production by testis fragments at different stages of spermatogenesis were compared by Planas & Swanson (1995). These authors demonstrated that the increase in plasma 11-KT levels coincided with an increase both in the sensitivity of the tissue to LH, and in 11-KT-synthesizing capacity. This may be due to increases in LH-R, because in the present study in trout, 11-KT levels increased during a period of significant increases in LH-R transcripts. Interestingly, the second increase in LH-R transcripts in trout occurred when plasma levels of 11-KT were maintained at maximum levels, plasma 17,20 β -P increased, and spermiation became evident. A similar pattern in changes of plasma steroids in coho salmon was associated with increased sensitivity of the testis to LH for both 11-KT and 17,20 β -P production, supporting the idea that increases in LH-R occur at that time. Although plasma levels of gonadotropins were not measured in the present study, Gomez *et al.* (1999) demonstrated that in rainbow trout, plasma LH levels were generally low (less than 1 ng/ml) compared with FSH (2–10 ng/ml), and first detectable when spermatocytes were the predominant germ cell stage (comparable to the March samples in the present study). Plasma LH increased 15-fold as numbers of spermatids and spermatozoa increased, although maximum levels were still less than 1 ng/ml at spermiation. Thus, increases in

plasma LH levels in trout during spermatogenesis appear to occur in parallel with increases in LH-R transcripts, and probably LH-R protein. Even though plasma FSH levels are substantially higher than LH during spermatogenesis in trout (Gomez *et al.* 1999), a number of studies in fish have shown that the LH receptor is specific for LH (reviewed by Bogerd *et al.* 2005), and autoradiography studies in salmon have localized an LH-specific receptor in Leydig cells (Miwa *et al.* 1991). Taken together, these data suggest that, in salmonids, a major regulatory point for steroid production by the testis could be increases in LH signaling, induction of LH receptor in Leydig cells of the testis, and increases in plasma LH.

In contrast to the pattern of LH-R gene expression, FSH-R transcripts fluctuated during spermatogenesis and were only 2–3-fold higher than initial levels toward the end of spermatogenesis. The initial rise in FSH-R transcripts (February–March) occurred prior to any significant change in LH-R gene expression and was associated with high meiotic activity in the testis (increase in spermatocytes and spermatids), but without a major change in plasma 11-KT levels. At this stage, Gomez *et al.* (1999) reported significant increases in plasma FSH levels in trout. Few data are available on specific functions of FSH in spermatogenesis in fish; however, studies suggest that FSH stimulates spermatogonial proliferation in trout (Loir 1999) and 11-KT production *in vitro* (Planas & Swanson 1995). Furthermore, autoradiography studies in salmon localized FSH-R to presumed Sertoli cells, but could not determine whether Leydig cells contained FSH-R (Miwa *et al.* 1994). More recently, Schulz *et al.* (2003) reported that FSH also stimulates proliferation of Sertoli cells in African catfish. Thus, as in mammals, a major target of fish FSH appears to be Sertoli cells, and FSH plays important roles in regulating spermatogenesis. But, because fish FSH also stimulates androgen production, it is possible that either FSH-R is present in Leydig cells or other FSH-induced Sertoli cell products affect steroid biosynthesis. Thus, increases in transcripts for StAR and several steroidogenic enzymes that occur early in spermatogenesis in trout may be due to increases in FSH signaling (see discussion below).

Abundance of P450_{scc}, P45011 β and 11 β -HSD transcripts changed significantly but only moderately during the reproductive cycle. These three transcripts increased significantly in March, 3 months before serum 11-KT increased significantly. von Hofsten *et al.* (2002) reported a significant rise of transcripts for P450_{scc} in Arctic char testes when plasma 11-KT levels were high *in vivo*. This difference may be due to the different ways of quantifying data, since the P450_{scc} mRNA levels of Arctic char were presented on a unit RNA base, whereas the P450_{scc} mRNA levels in this study were normalized by total testes RNA and GSI (Fig. 6). Although the magnitude of changes in transcript levels of P45011 β was lower (three-fold increase) than seen in the previous study, where

transcript levels were determined by Northern blotting (Kusakabe *et al.* 2002a), transcript levels were highest when serum 11-KT levels increased significantly. FSH-R and LH-R transcript levels exhibited moderately to strongly significant linear relationships with P450_{scc}, P45011 β and 11 β -HSD transcripts, suggesting that the expression of the P450_{scc}, P45011 β and 11 β -HSD genes is at least partially regulated by FSH and/or LH. It appears that there is a distinct gender difference with regard to the changes in 3 β -HSD and 20 β -HSD mRNA levels during gametogenesis in rainbow trout. In male rainbow trout, testicular 3 β -HSD mRNA levels showed a prominent 8–9-fold increase to peak levels in the early stage of mid-spermatogenesis (March and April), followed by a rapid decline to levels 2–3-fold higher than the initial sample for the remainder of the study. In contrast, ovarian 3 β -HSD mRNA levels in female rainbow trout increased during late vitellogenesis and post-ovulation (Nakamura *et al.* 2005). These observations imply that expression of the testicular 3 β -HSD gene may be regulated differently from those for other key steroidogenic enzymes, although the functional significance of the rapid increase of testicular 3 β -HSD transcripts during mid-spermatogenesis is still unclear. In female rainbow trout, 20 β -HSD mRNA levels significantly increased sevenfold during the late reproductive stages (Nakamura *et al.* 2005). Ovarian 20 β -HSD transcripts in ayu (Tanaka *et al.* 2002) and tilapia (Senthikumar *et al.* 2002) also showed a distinct increase during late oogenesis stages. In contrast, 20 β -HSD transcripts in rainbow trout testes appeared to have an inverse relationship with seasonal serum 17,20 β -P levels, 20 β -HSD mRNA levels decreasing during late spermatogenesis when serum 17,20 β -P levels peaked. One possible explanation for the weak relationship is that there may be more than one type of 20 β -HSD in some teleosts. In male amago salmon, an *in vitro* study demonstrated that sperm converted 17 α -hydroxyprogesterone to 17,20 β -P in rainbow trout (Ueda *et al.* 1984, Sakai *et al.* 1989), suggesting the existence of 20 β -HSD enzyme in the salmonid sperm. However, Vizziano *et al.* (1996) reported 20 β -HSD activity in nonflagellated germ cells obtained from early spermatogenic rainbow trout testes, although serum 17,20 β -P levels are very low at this stage. In this study, 20 β -HSD mRNA levels were high during the mid-spermatogenesis stages, whereas levels during late spermatogenesis were almost nondetectable. Histologic analysis showed that mid-spermatogenic testes are occupied with a considerable proportion of nonflagellated germ cells (Fig. 2). Therefore, the 20 β -HSD mRNAs detected in the rainbow trout testes may be derived from nonflagellated germ cells. Further investigation is required to clarify the regulation of 20 β -HSD associated with testicular 17,20 β -P production. All in all, it appears that the moderate changes in P450_{scc}, P45011 β , 11 β -HSD, 3 β -HSD and 20 β -HSD transcripts may have only minor effects on the changes in seasonal steroid production.

StAR mRNA correlated moderately with serum 11-KT levels ($r^2=0.31$, $P<0.0001$). Of all the transcripts measured, the 10-fold increase in StAR mRNA was the greatest change observed. During the later stages of spermatogenesis, StAR transcripts increased significantly, corresponding to the increases of serum sex-steroid levels. These results suggest that StAR abundance at least partially determines the levels of steroid production during spermatogenesis in rainbow trout. Previous mammalian studies have demonstrated an induction of StAR protein by LH treatment in rat Leydig cells (Luo *et al.* 1998). Interestingly, there was a significant linear relationship between LH-R and StAR mRNA levels ($r^2=0.61$, $P<0.0001$), which was higher than that of FSH-R and StAR mRNA ($r^2=0.38$, $P<0.0001$). These results, along with data from other studies on plasma FSH and LH levels, suggest that expression of the StAR gene may be under the control of FSH during early stages of spermatogenesis, and of LH during the later stages of spermatogenesis.

Like StAR transcripts, P450C17 transcripts had a moderately linear relationship with serum 11-KT levels ($r^2=0.30$, $P=0.0001$). Both StAR and P450C17 started increasing in March and April when spermatocyte abundance started to increase (Fig. 5). P450C17 is a single microsomal enzyme that catalyzes two distinct steroid biosynthesis activities, 17 α -hydroxylase and 17,20-lyase. Phosphorylation of P450C17 and the presence of cytochrome b5 are important factors determining whether the enzyme has 17,20-lyase activity (Katagiri *et al.* 1995, Zhang *et al.* 1995). These factors partly explain the tissue-specific differences in P450C17 activities between adrenal gland and gonads (Miller *et al.* 1997). As rainbow trout P450C17 also has two distinct steroid biosynthetic activities (Sakai *et al.* 1992), the relative enzymatic activity of 17 α -hydroxylase and 17,20-lyase probably regulates the biosynthesis of androgens and progestogen. The lack of 17,20 β -P production during the early and mid-spermatogenesis stages, despite the presence of 20 β -HSD mRNA (this study) and evidence of protein (Vizziano *et al.* 1996) in the testes, may be due to high C17-C20 lyase activity resulting in low availability of the immediate precursor for 17,20 β -P, 17 α -hydroxyprogesterone. Knowledge of the regulation of the enzymatic activities of P450C17 is important to understand the mechanisms of testicular 17,20 β -P production in teleosts.

This study provides an overall picture of changes in steroidogenic enzyme gene expression changes during spermatogenesis and their relationships to steroid production. The patterns of steroid hormone secretion during the reproductive cycle appear to be largely regulated by changes in expression of genes encoding StAR, P450C17, and gonadotropin receptors. Expression of genes encoding various steroidogenic enzymes increased before prominent rises in levels of plasma steroid hormones that were presumably initiated by FSH and/or LH signals.

Planas *et al.* (1997) reported that two intracellular signaling pathways, cAMP/protein kinase A and protein kinase C/ Ca^{2+} , mediate the effects of FSH and LH on ovarian steroidogenesis in brook trout. How these signaling pathways affect gene expression of steroidogenic enzymes and related factors has not yet been clarified. Experimental approaches will be required for further understanding of the regulation of steroidogenesis at the level of gene expression, such as analyzing the effects on expression of genes encoding these proteins through *in vitro* tissue culture with GTHs.

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