

## Involvement of Four Hormones in Thyroxine Deiodination in Several Tissues of Immature Yearling Masu Salmon, *Oncorhynchus masou*

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**ABSTRACT**—Effects of estradiol-17 $\beta$ , testosterone, cortisol and ovine growth hormone on 3,5,3'-triiodothyronine (T<sub>3</sub>) and 3,3',5'-triiodothyronine (rT<sub>3</sub>) generation (5'-monodeiodinase and 5-monodeiodinase activity) from thyroxine (T<sub>4</sub>) in liver, gill, kidney and head kidney of immature yearling masu salmon were investigated. Reverse T<sub>3</sub> generation was observed in all tissues. Estradiol-17 $\beta$  significantly depressed both serum T<sub>4</sub> and T<sub>3</sub> levels and T<sub>3</sub> generation in gill and liver. Ovine growth hormone enhanced serum T<sub>3</sub> levels and T<sub>3</sub> generation in gill and liver. Although testosterone increased serum T<sub>4</sub> levels, it was not effective on either T<sub>3</sub> or rT<sub>3</sub> generation in each tissue. T<sub>3</sub> generation was found in the gill following cortisol treatment. However, cortisol showed no effects on serum thyroid hormones. Our results suggest two T<sub>4</sub> deiodination pathways in the masu salmon. The observed decrease in serum T<sub>3</sub> may be due to depressed 5'-monodeiodinase activity and stimulated 5-monodeiodinase activity following estradiol-17 $\beta$  treatment; and the observed increase in serum T<sub>3</sub> levels may be due to enhanced 5'-monodeiodinase activity following ovine growth hormone treatment.

### INTRODUCTION

Thyroid hormones are known to play an important role on various physiological phenomena in fishes. In salmonids, circulating thyroxine (T<sub>4</sub>) shows high levels and dramatic change, whereas 3,5,3'-triiodothyronine (T<sub>3</sub>) is low and stable [27]. T<sub>3</sub> seems to be produced by extrathyroidal monodeiodination of T<sub>4</sub> [16, 23] and active form of thyroid hormone in salmonids [2, 55]. T<sub>4</sub> 5'-monodeiodinase (5'D) was observed in various tissues (e.g. liver, kidney, ovary, brain) in mammals [21, 39]. 5'D activity also has been found in several tissues of fishes (liver [10, 30, 34, 38] and kidney [30]). Recent studies indicate that rainbow trout has 5'D and 5-monodeiodinase (5D) [50].

Recent studies [31, 34, 35, 54] have indicated

that several hormones (e.g. estradiol-17 $\beta$ , testosterone, growth hormone, corticosteroids) have a potential to affect deiodination in hepatocytes, however, the results have only demonstrated 5'D activity. In the present study, we investigated T<sub>4</sub> deiodination pathways and effects of estradiol-17 $\beta$ , testosterone, cortisol and ovine growth hormone on T<sub>4</sub> deiodination in liver, gill, kidney and head kidney in immature yearling masu salmon, *Oncorhynchus masou*.

### MATERIALS AND METHODS

#### *Fish*

Twenty immature yearling masu salmon (75–130 g body weight) obtained from Hokkaido Fish Hatchery were transferred to Hokkaido University in September, and were held in a 250-liter circulating tank at 12°C under a natural photoperiod. Fish were acclimated to rearing conditions for two days before hormone injections. Fish were divided into

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five groups ( $n=4$ ) (control, estradiol-17 $\beta$ , testosterone, cortisol, ovine growth hormone).

#### *Hormone treatment*

After anesthetizing with *p*-aminobenzoate (1.5 g/10 liter water), fish were injected intraperitoneally with estradiol-17 $\beta$ , testosterone, cortisol (Sigma Chemicals, St. Louis, MO) or ovine growth hormone (oGH, NIADKK-oGH-12, NIH), 1  $\mu$ g/g body weight. Injectate stock solutions of steroids were dissolved in 50% ethyl alcohol, and ovine growth hormone in masu salmon Ringer's solution (NaCl 8.6 g, KCl, 0.23 g, MgSO<sub>4</sub>7H<sub>2</sub>O 0.07 g, NaHPO<sub>4</sub>2H<sub>2</sub>O 0.149 g, MgCl<sub>2</sub>6H<sub>2</sub>O 0.2 g, CaCl<sub>2</sub>2H<sub>2</sub>O 0.5 g, glucose 1.0 g, N-(2-Hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid 2.38 g in one liter, adjust pH to 7.4 by 1 N NaOH). Final injection volume of ethyl alcohol and masu salmon Ringer's solution was equivalent (0.1 ml). Control group fish received an equivalent volume of both ethyl alcohol and masu salmon Ringer's solution. Steroid-treated groups and growth hormone-treated group received an equivalent volume of masu salmon Ringer's solution and 50% ethyl alcohol, respectively. Injections were given between 10:00 and 11:00 on day 0 and day 3, and sampled between 10:00 and 11:00 on day 7. The fish were not fed during the experimental period.

#### *Thyroxine deiodination*

Blood was collected immediately from the caudal blood vessels, followed by centrifugation at 12,000 rpm for 15 min. The obtained serum was stored at -40°C for later analysis of thyroid hormones. Liver, gill, kidney and head kidney were collected from each group. The following incubation conditions were determined by our previous experiments [55]. The tissues were rinsed in ice-cold masu salmon Ringer's solution (MSR), followed by weighing and homogenizing with five-fold volume of MSR containing 5 mM dithiothreitol (DMSR). The homogenates were diluted to the appropriate protein concentrations (2 mg protein/100  $\mu$ l in final assay volume, determined by the Lowry method) with ice-cold DMSR containing 5 mM (DTT). [3'- and 5'-<sup>125</sup>I]T<sub>4</sub> (specific activity >211 MBq/ $\mu$ g, carrier free, New England Nuclear) were chromatographed and purified us-

ing a high performance liquid chromatography system as described below, and purified [<sup>125</sup>I]T<sub>4</sub> were used to deiodination experiments. To determine non-enzymatic deiodination at 15°C for 2 hr, control samples were heated at 100°C for 5 min to block the enzyme activity, and compared with non-heat-treated control group. Moreover, 6-propyl-2-thiouracil (PTU, final concentration 3 mM) were added into the control sample as a blocker of enzymatic T<sub>4</sub> deiodination. Final assay sample of 100  $\mu$ l was pipetted into the 12×75 mm glass tubes, and then 400  $\mu$ l of [<sup>125</sup>I]T<sub>4</sub> substrate was added into the tubes to become final concentrations of 0.1 nM, and incubated for 2 hr at 15°C. The T<sub>4</sub> substrate was prepared by dilution of [<sup>125</sup>I]T<sub>4</sub> with DMSR. After incubation, radioactive compounds of interest were extracted from 500  $\mu$ l of medium with 500  $\mu$ l of *n*-butyl alcohol (*n*-BuOH), from which the extraction recovery of each sample was determined. This extraction resulted in recoveries of 70–80% of <sup>125</sup>I activity. In preliminary studies, we verified that there were no qualitative differences in chromatographic results of one extraction versus 3 extractions (90–96% recovery). A volume of 400  $\mu$ l of extract (*n*-BuOH layer) was transferred to a 12×75 mm glass tube, and evaporated by a centrifugal evaporator at 40°C. T<sub>4</sub> to T<sub>3</sub> and rT<sub>3</sub> conversion rates were evaluated by measuring 3,5,3'-<sup>125</sup>I-T<sub>3</sub> and 3,3',5'-<sup>125</sup>I-T<sub>3</sub> generation from <sup>125</sup>I-T<sub>4</sub>.

#### *High performance liquid chromatography*

Radioactive thyroid hormones were redissolved in 50  $\mu$ l methyl alcohol containing 10 nmole thyroid hormone standards, and a volume of 20  $\mu$ l was injected to high performance liquid chromatography (HPLC). Fractionation of extracted radioactive thyroid hormones was performed using HPLC (JASCO, model 800) with reverse-phase (Nucleosil 3C18, 4.6×100 mm, Chemco Co.). Thyroid hormone standards (T<sub>4</sub>, T<sub>3</sub> and rT<sub>3</sub>, Sigma Chemicals, St. Louis, MO) were employed for determination of the elution times. For T<sub>3</sub>, rT<sub>3</sub> and T<sub>4</sub>, these were 4.7, 6.3 and 8.8 min, respectively (Fig. 1a) using a mobile phase of acetonitrile-water (37:63, v/v) containing 0.1% trifluoroacetic acid at 40°C run at 1.0 ml/min. Twenty-five fractions (0.5 ml/fraction) were collected and the

radioactivity of each fraction was counted by  $\gamma$ -counter (Aloka, model 300) for calculation of  $T_4$  metabolite concentrations.  $5'D$  and  $5D$  activities were calculated from generated  $T_3$  and  $rT_3$  (fmol/mg protein/2 hr).

#### Radioimmunoassay

Serum concentrations of  $T_4$  and  $T_3$  were determined by radioimmunoassay according to the method of Suzuki and Suzuki [49].

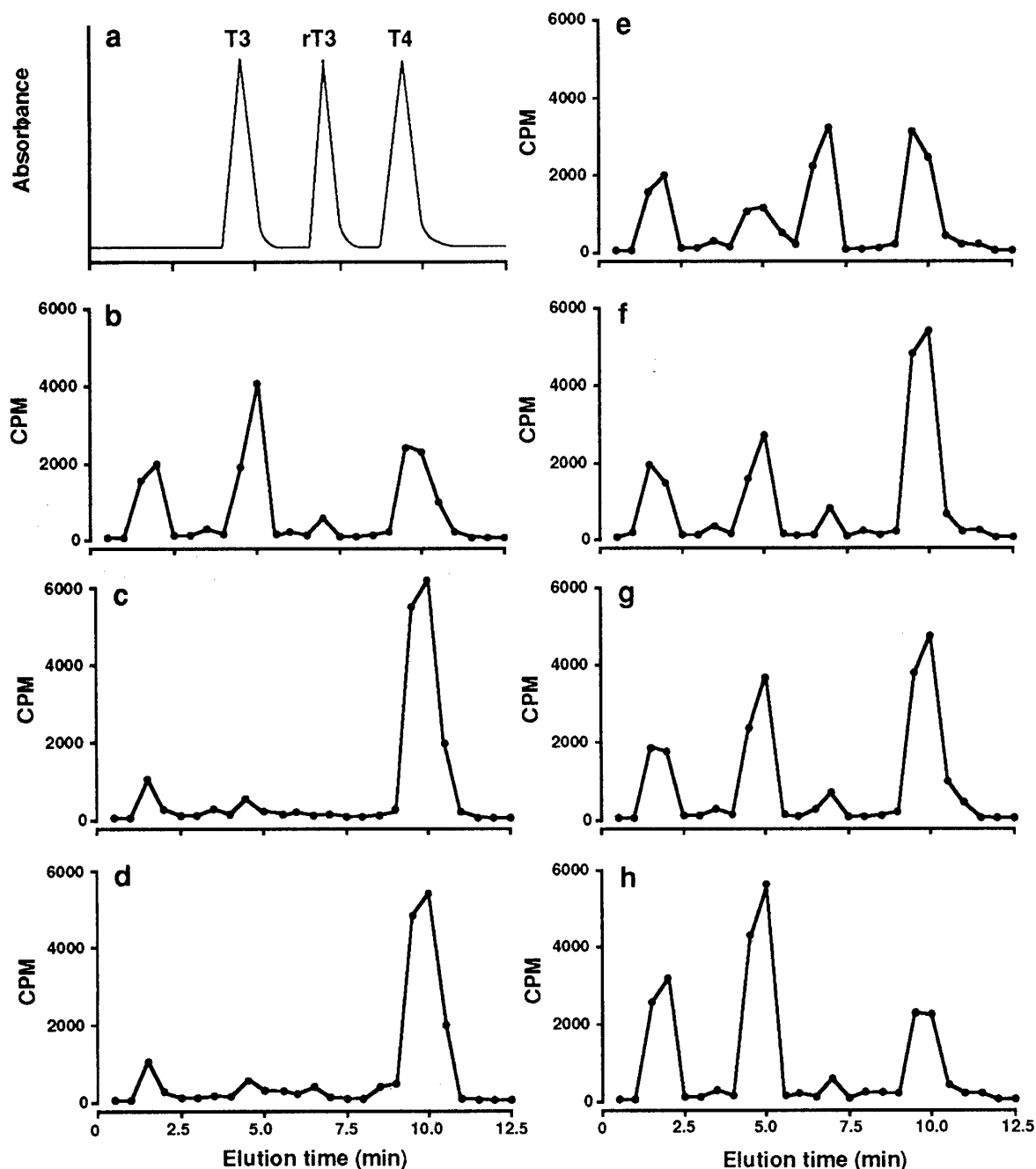


FIG. 1. HPLC chromatogram of unlabeled (a) and radiolabeled (b~h) 3,5,3'-triiodothyronine, 3,3',5'-triiodothyronine and thyroxine under various conditions in the liver. a) elution pattern of standards, b) control, c) heat-treated control, d) PTU-treated control, e) estradiol-17 $\beta$ -treated, f) testosterone-treated, g) cortisol-treated, h) growth hormone-treated. Standard iodothyronines (a) were measured by absorbance at 235 nm, and radiolabeled and unlabeled iodothyronines were separated by following HPLC conditions. Column: 100 $\times$ 4.6 mm I.D. packed with Nucleosil 3C18; mobile phase, water-acetonitrile (63:37) containing 0.1% (v/v) trifluoroacetic acid; flow rate, 1.0 ml/min; detection: UV at 235 nm.

TABLE 1. Serum thyroxine ( $T_4$ ) and 3,5,3'-triiodothyronine ( $T_3$ ) concentrations in the immature masu salmon treated with estradiol-17 $\beta$  ( $E_2$ ), testosterone (T), cortisol (F) and ovine growth hormone (oGH). (n=4)

Treatment	Dose ( $\mu$ g/g BW)	Mean fish wt (g)	Serum $T_4$ (ng/ml)		Serum $T_3$ (ng/ml)	
			Mean	SEM	Mean	SEM
C	1.0	124.1	5.27	0.42	0.79	0.04
$E_2$	1.0	115.0	2.79*	0.27	0.19*	0.05
T	1.0	105.0	7.97*	0.90	1.02	0.28
F	1.0	100.3	5.62	1.21	0.41	0.10
oGH	1.0	105.1	4.96	0.48	1.82**	0.26

\* Significantly different from control ( $P<0.05$ ).

\*\* Significantly different from control ( $P<0.01$ ).

### Statistics

Testing for differences in the amount of  $T_3$  and  $rT_3$  generation between control and experimental groups was accomplished using analysis of variance (ANOVA), followed by Duncan's Multiple Range Test.

## RESULTS

### Serum thyroid hormones

Table 1 shows serum  $T_4$  and  $T_3$  levels following administration of estradiol-17 $\beta$ , testosterone, cortisol and ovine growth hormone. Estradiol-17 $\beta$  treatment decreased both  $T_4$  and  $T_3$  levels significantly ( $P<0.05$ ) compared to controls. Testosterone treatment increased serum  $T_4$  levels ( $P<0.05$ ), but not serum  $T_3$  levels. Cortisol had no effect on serum  $T_4$  and  $T_3$  levels. Ovine growth hormone significantly enhanced serum  $T_3$  levels ( $P<0.01$ ), but not serum  $T_4$  levels.

### Elution patterns of labeled and unlabeled iodothyronines

Heat-treated control of liver homogenates (Fig. 1c) showed significantly lower  $^{125}$ I-generation than that of non-treated control (Fig. 1b).

In the PTU-treated liver homogenates,  $T_4$  to other iodothyronines conversion were significantly inhibited by recognized blockers of enzymatic conversion of thyroid hormones compared with non-heat-treated control group (Fig. 1d).

Typical elution patterns of labeled iodothy-

ronines in the liver homogenates were shown in Fig. 1 (e:  $E_2$  treated, f: cortisol treated, g: testosterone treated, h: ovine growth hormone treated). Other elution patterns of kidney, head kidney and gills were not shown. However, those patterns were almost similar to that of the liver, except for the amount of radioactivities of each iodothyronine.

### Head kidney

In head kidney, estradiol-17 $\beta$  treatment depressed  $T_3$  generation ( $P<0.05$ ), and increased  $rT_3$  generation ( $P<0.05$ , Fig. 2a). Ovine growth hormone, testosterone and cortisol treatment were not effective on modifying  $T_4$  deiodinations.

### Kidney

Estradiol-17 $\beta$ , testosterone, cortisol and ovine growth hormone treatment were not effective on  $T_3$  and  $rT_3$  generation in kidney (Fig. 2b).

### Gill

Estradiol-17 $\beta$  treatment depressed  $T_3$  generation ( $P<0.05$ ), and increased  $rT_3$  generation ( $P<0.05$ , Fig. c). Ovine growth hormone treatment enhanced  $T_3$  generation ( $P<0.05$ ), but not  $rT_3$  generation. Testosterone treatment was not effective on  $T_4$  deiodination, while an increase in  $T_3$  generation ( $P<0.05$ ) was observed for cortisol treatment.

### Liver

Estradiol-17 $\beta$  treatment decreased  $T_3$  generation ( $P<0.05$ ), and increased  $rT_3$  generation ( $P<$

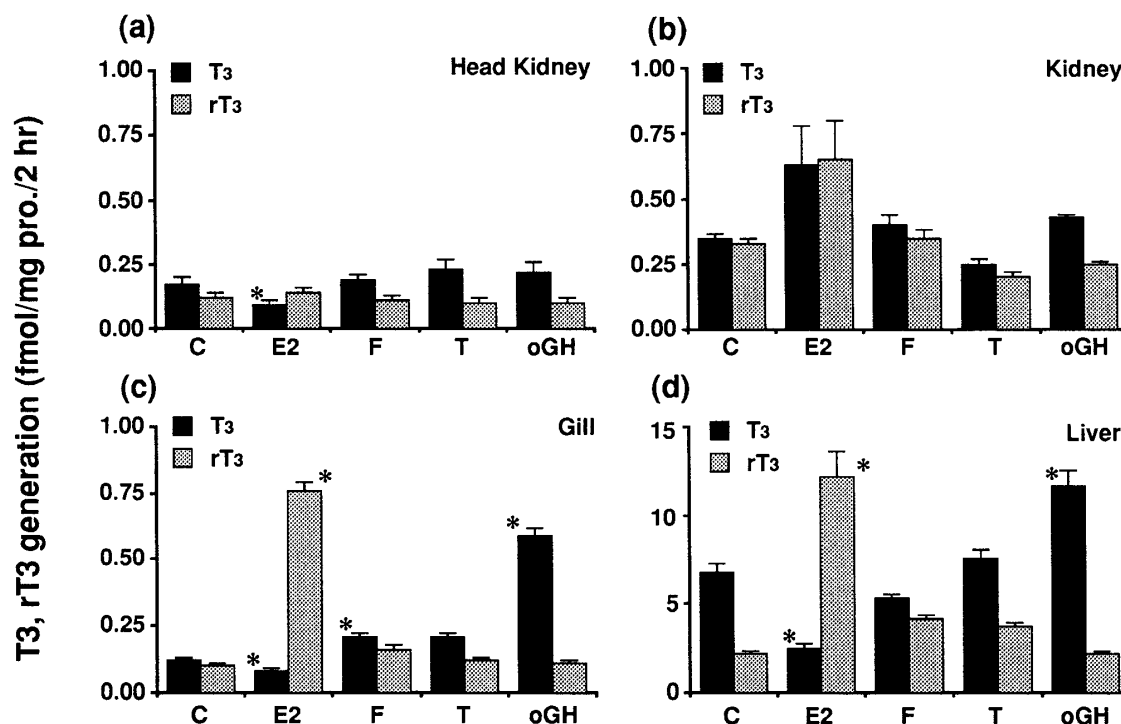


FIG. 2. Effect of estradiol-17 $\beta$  (E<sub>2</sub>), testosterone (T), cortisol (F) and ovine growth hormone (oGH) on T<sub>4</sub> to T<sub>3</sub> and rT<sub>3</sub> conversion in head kidney (a), kidney (b), gill (c) and liver (d) of masu salmon. The vertical bars represent  $\pm$  SEM. \*Significantly different from the value of controls (C) at  $P < 0.05$ .

0.05, Fig. 2d). Ovine growth hormone treatment increased T<sub>3</sub> generation ( $P < 0.05$ ). Testosterone and cortisol were not effective on either T<sub>3</sub> or rT<sub>3</sub> generation.

## DISCUSSION

The objectives of this study were to investigate T<sub>4</sub> deiodination pathways, and possible involvement of estradiol-17 $\beta$ , testosterone, cortisol and ovine growth hormone on these pathways, in the immature yearling masu salmon.

In the present study, radiolabeled T<sub>4</sub> and its metabolites eluted in the order T<sub>3</sub>, rT<sub>3</sub> and then T<sub>4</sub>, virtually coincident with peaks of unlabeled iodothyronines injected into the same HPLC system. Other HPLC systems used in similar studies resulted in the same order of elution of T<sub>3</sub>, rT<sub>3</sub> and T<sub>4</sub> [44, 45, 52]. T<sub>3</sub> and rT<sub>3</sub> also can be deiodinated to 3,3'-T<sub>2</sub>, and this product eluted before T<sub>3</sub> [45]. In the present study, a small peak, not significantly higher than the baseline, was observed before the peak of [<sup>125</sup>I]T<sub>3</sub>, at 4.3 min retention time, that was

likely radiolabeled 3,3'-T<sub>2</sub>. Recently, Sweeting and Eales [50] detected 3,3'-T<sub>2</sub> in the hepatic microsomes of thyroid hormone treated rainbow trout using similar HPLC system.

In the present study, T<sub>3</sub> and rT<sub>3</sub> were found in the head kidney, kidney, gills and liver. This means there are two T<sub>4</sub> deiodination pathways, 5'D and 5D, in the masu salmon. In the rainbow trout, rT<sub>3</sub> was not detected in the plasma after administration of [<sup>125</sup>I]T<sub>4</sub> [16, 23], and specific radioimmunoassay for rT<sub>3</sub> also showed negligible presence of rT<sub>3</sub> in the plasma [18]. From these results, Eales *et al.* [18] suggested that T<sub>4</sub> deiodination was restricted to T<sub>3</sub> formation. However, recent studies reported the presence of rT<sub>3</sub> in plasma of Tilapia [4] and in incubated hepatic microsomes of rainbow trout [50] using labeled T<sub>4</sub> and HPLC detection. The apparent differences between our findings and the previous literature are likely to be a result of the different methods used for determining T<sub>4</sub> deiodination (thin-layer chromatography: [23]; gel filtration: [16, 18]). The HPLC system we used in the present study is much

more sensitive for identification and separation of thyroid hormones in these types of samples. However, the production of  $T_3$  and  $rT_3$  was in fmole amounts in comparison with pmole amounts in previous reports. The reason for such low  $T_3$  and  $rT_3$  values can be explained by the differences in enzymatic reaction methods. Metabolized  $T_3$  and  $rT_3$  in incubated samples was assessed as both  $T_3$  and  $rT_3$  in one milligram protein in previous reports and in the present study. But, we used whole tissue homogenate for the deiodination reaction, and partially purified microsomes [10, 50] were used in previous reports. Reverse  $T_3$  was measurable in bile, urine and plasma following injection of [ $^{125}I$ ] $T_4$  in the plaice, *Pleuronectes platessa* [37], using thin layer chromatography. However, these investigators injected a high dose of [ $^{125}I$ ] $T_4$  into the plaice, in contrast with earlier reported studies. In the present study,  $rT_3$  generation was lower than that of  $T_3$  generation. Our findings suggest that, if low doses of [ $^{125}I$ ] $T_4$  are injected into experimental fish,  $rT_3$  may be detectable only if a sufficiently sensitive chromatography system is used. Therefore, differences in the results of Osborn and Simpson [37] and the previous literature can be explained by not only the size of the injected dose, but also the chromatography method, and lower 5D activity than 5'D activity described in the present study.

Reverse  $T_3$  is present in humans [7] and other mammals [8, 29, 51], and  $rT_3$  production may equal or exceed the production of  $T_3$  from  $T_4$  [21, 39]. Moreover,  $T_3$  and  $rT_3$  are eventually metabolized to  $T_0$  in mammals. Mammals have a limited iodine supply and, for this reason, extrathyroidal iodothyronine deiodination is necessary for iodine economy in mammals. Rainbow trout, which have high iodine availability, also have both 5'D and 5D pathways [50]. The presence of two  $T_4$  deiodination pathways observed in the masu salmon and rainbow trout suggest that  $T_4$  and  $rT_3$  can be further metabolized to 3,3'-diiodothyronine and 3'- $T_1$ , and these may be no differences in thyroxine metabolic pathways between mammals and salmonid fish, even if both animals are in different environment of iodine availability.

It is well known that  $T_4$  is synthesized in the thyroid gland, secreted into blood, taken up by its

target cells, and then deiodinated to  $T_3$  by 5'D. Some of this  $T_3$  is bound to specific receptors in cells, and some is released into blood again. This view supports the concept that almost all circulating  $T_3$  is derived from peripheral  $T_4$  deiodination [20]. Estradiol-17 $\beta$  treatment decreased serum  $T_4$  levels in the present study. Undoubtedly, estradiol-17 $\beta$  depresses thyroidal activity [29]. Furthermore, as in rainbow trout [19, 31], estradiol-17 $\beta$  depressed serum  $T_3$  levels and the  $T_4$  to  $T_3$  conversion rate in liver, gill and head kidney in the present study. High dose treatment of estradiol-17 $\beta$  depressed 5'D activity in rainbow trout [10]. Therefore, the observed decrease in serum  $T_3$  levels was probably caused by decreased  $T_4$  to  $T_3$  conversion rate in peripheral tissues following estradiol-17 $\beta$  treatment.

During maturation, plasma thyroid hormone levels decrease and estradiol-17 $\beta$  levels increase in female Atlantic salmon [14], rainbow trout [9] and masu salmon (unpublished results). We also observed an increase in serum estradiol-17 $\beta$  levels in 2-year-old masu salmon in May (unpublished results), followed by upstream maturational migration. Moreover, upstream migrating salmonids do not usually feed, but migration needs high energy. In such conditions, masu salmon must get energy from body lipids for upstream migration, and use limited iodine for thyroid economy. Thyroid hormone decreases body lipids (rainbow trout, [1]; catfish, [47]), and enhances lipid metabolism in fish [40]. Therefore, the limited iodine is generated not only from  $T_3$  deiodination but also from increased  $rT_3$  deiodination. Male as well as female fish have a response that induces vitellogenin following estrogen treatment, which suggests that the basic function of estrogen is not different between males and females. Therefore, it is likely that the male masu salmon, like the female, has the same response of  $T_4$  deiodination to estradiol-17 $\beta$ .

Testosterone appears to stimulate thyroidal activity, such as increased follicle cell height and radioiodide uptake [24, 53]. Testosterone treatment increased serum  $T_4$  levels, but not  $T_3$  levels in the present study, suggesting that testosterone stimulated thyroidal activity, as in previous studies. Similarly, testosterone propionate [28] and testosterone [34] stimulate conversion of  $T_4$  to  $T_3$

in rainbow trout. Moreover, a recent study indicated that testosterone enhanced  $T_3$  generation in liver of masu salmon during and after smoltification, but is not effective before smoltification (Yamada *et al.*, in preparation). However, testosterone treatment was not effective on  $T_4$  to  $T_3$  conversion in the present study. Judging from these findings, it seems that the stimulatory effect of testosterone on  $T_4$  to  $T_3$  conversion depends on the developmental stage.

Vijayan *et al.* [54] indicated that high doses and long-term treatment with cortisol increased *in vitro* hepatic 5'D activity. However, cortisol treatment was not effective on serum  $T_4$  and  $T_3$  levels and  $T_4$  deiodination in the liver, kidney and head kidney. Our injection dose and period were lower and shorter than in previous reports. High doses and long-term treatment may be necessary for the possible involvement of  $T_4$  deiodination in these tissues. In fish, *in vivo* cortisol treatment enhanced gill  $Na^+-K^+-ATPase$  activity [41, 42, 43]. Effects of cortisol on thyroid function and deiodination in trout were investigated [3]. In coho salmon, cortisol increased  $Na^+-K^+-ATPase$  activity *in vitro* [33], and it has been suggested that cortisol induces  $Na^+-K^+-ATPase$  abundance in Tilapia directly. In the present study, cortisol increased  $T_4$  to  $T_3$  conversion only in gills. It seems possible that cortisol supports  $T_4$  to  $T_3$  conversion, but the dose response on cortisol in gills was higher than that in other tissues examined in the masu salmon.

de Luze *et al.* [12, 13] observed an increase in serum  $T_3$  levels following growth hormone treatment in the European eel, and suggested that *in vivo* 5'D is stimulated by growth hormone. In addition to their reports, MacLatchy and Eales [35] have found same effect of growth hormone on *in vitro* 5'D activity in rainbow trout. Ovine growth hormone enhanced  $T_4$  to  $T_3$  conversion in gills and liver and also increased serum  $T_3$  levels in the present study. It is unclear that increased serum  $T_3$  levels were caused by enhanced  $T_4$  to  $T_3$  conversion in peripheral tissues, and it is important to consider whether growth hormone effects on  $T_4$  deiodination are direct or indirect. Growth hormone receptors are observed in the liver of salmon and eel [25, 36, 46] and also in the gills [46]. Therefore, it seems that increased  $T_4$  to  $T_3$  conver-

sion is a direct action of growth hormone mediated by growth hormone receptors. However, growth hormone induces insulin-like growth factors as a second messenger hormone in the liver of mammals [6, 11, 22] and also of fish [32, 15]. These findings suggest that liver is an important organ for growth hormone activity, by production of insulin-like growth factors as second messenger hormones. To clarify the relationship between growth hormone and thyroid hormone, *in vitro* studies on the effects of growth hormone action on  $T_4$  deiodination are required.

In conclusion, the present study has demonstrated two  $T_4$  deiodination pathways, and has also shown that decreases in serum  $T_3$  levels are followed by inhibition of 5'D and stimulation of 5D pathway by estradiol-17 $\beta$ . Ovine growth hormone stimulated the 5'D pathway, resulting in an increase in serum  $T_3$  levels. These results strongly suggest that these hormones are closely involved in  $T_4$  deiodination. The physiological role of  $rT_3$  production in the masu salmon remains to be determined.

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