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***In vivo and in vitro effects of *Rhizopus* extract (RU) on  
body growth and steroid production in masu salmon,  
*Oncorhynchus masou* Brevoort***

Running title: RU effects in masu salmon

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## Abstract

This study investigated *in vivo* and *in vitro* effects of *Rhizopus* (filamentous fungus) extract (RU) in masu salmon *Oncorhynchus masou* Brevoort. Underyearling fish were fed with RU for 16 months. Monthly changes in body growth, gonadal maturation and serum levels of sex steroids were monitored. Gonads were also incubated at 0, 1, 10, 100 and 1000  $\mu\text{g RU mL}^{-1}$  Leibovitz's L-15 medium for 18 h. Levels of steroids in serum and cultured medium were measured. It was determined that RU-fed immature and mature males, when compared to control groups, showed significantly higher body growth during spring, summer, and the spawning period. Similarly, immature RU-fed females showed significantly higher fork length and body weight in autumn, spring and summer. Furthermore, RU-fed males showed significantly higher levels of serum testosterone (T) and 11-ketotestosterone (11-KT) levels in pre-spawning season, and  $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (DHP) in spawning season. *In vitro* RU incubation of gonads showed a dose-dependent and significant increase in T, 11-KT, estradiol- $17\beta$  and DHP release in the medium. It appears that the causes of enhanced body growth and increased steroid production herein observed in salmonids are the physiologically active substances that may be contained in the mycelium of the fungus.

**Key words:** *Rhizopus*, incubation, growth, steroids, masu salmon

## Introduction

Enhancement of body growth and acceleration of gonadal maturation are the main principles in modern aquaculture and are manipulated using peptides, amino acids and alteration of environmental factors. Obviously, the neuroendocrine functions in the somatotropic and gonadotropic axes play respective roles in the regulation of growth and maturation. Owing to increased knowledge of these functions, particularly the hormone cascades, great achievements have been made in aquaculture (Donaldson 1996).

In recent years, however, with development of successive filtration and powerful chromatography methods, purified forms of physiologically active substances have been investigated from microorganisms such as fungi and algae (Holland & Lakshmaiah 1999), proving unfounded the assumption that hormones are exclusively the product of vertebrate endocrine organs. It has been suggested that the vertebrate hormonal signal transduction mechanisms might have their origins in early eukaryotes (Janssens 1987; Pertseva 1991). Consecutively, the presence of mammalian-like peptide and steroid hormones, steroid enzymes and protein receptors in the mycelium of several fungus species has been reported (Kastelic-Suhadolc, Plemenitaš & Žigon 1994; Plementiaš, Kastelic-Suhadolc, Žigon & Žakelj-Mavrič 1999; Lanišnik & Žakelj-Mavrič 2000). These findings, mainly endogenous steroidogenesis and characterization of the bioactive substances in the cytosols of the fungi tested so far, may indicate the possibility of other fungal species possessing similar properties in their mycelium (Pogačar, Zorko & Žakelj-Mavrič 1998; Kristan, Lanišnik, Stojan, Gerber, Kremmer & Adamski 2003).

*Rhizopus* extract (RU) is a filamentous fungus and belongs to the family mucoraceae of group thallophyta. It is commonly used for the production of pharmacologically active

steroids through steroid hydroxylation (Charney & Herzong 1967). Previous studies indicate that the products of *Rhizopus* increase the rates of laying eggs, fertilization and hatching in hen (Ushikoshi 1963), pregnancy rates in cows (Sato 1976), ovarian steroidogenesis in rats (Horiuchi, Tabe, Ushikoshi & Seto 1985) and somatic growth enhancement in fish (Bhandari, Ushikoshi, Fukuoka, Koide, Yamauchi & Ueda 2002). These findings suggest that the physiologically active substances that might be contained in the mycelium of *Rhizopus* may play active roles in somatic growth and gonadal maturation, but their actual endocrine mechanisms remain unknown.

In this study, we examined *in vivo* and *in vitro* effects of RU in masu salmon. Long-term RU feeding was conducted in order to examine its effects on body growth and gonadal maturation. In addition, *in vitro* incubation of mature testicular fragments and ovarian follicles was carried out at different doses of RU to investigate its direct effects on the hormonal releases of the incubated gonadal tissues.

## **Materials and methods**

### **Fish and *in vivo* experiment**

Underyearling masu salmon of Mori strain, established at the Mori branch of Hokkaido Fish Hatchery, were transported to Toya Lake Station, Hokkaido University, Japan and were reared in 1400 L circular tanks under natural photoperiod with continuous flow of spring water (8.7 °C to 10.6 °C). Fish (males,  $21.41 \pm 1.29$  g; females,  $20.30 \pm 0.52$  g) were divided into two groups and fed commercial pellets (Oriental Feed Industry, Yokohama, Japan) with added feed oil ( $40 \text{ ml kg}^{-1}$  feed). Only individuals in the

experimental group also received refined RU ( $200 \text{ mg kg}^{-1}$  commercial pellets) mixed with the feed oil. The initial stocking densities in both RU-fed group and the control group were 400 fish per tank. They were fed daily at the rate of 2.5% (1% during winter) of body weight. Feeding rates were adjusted each month according to the body weight measured at monthly samplings. It was conducted from June 2002 to October 2003.

At each sampling, 15–20 fish from both RU-fed and control group were randomly selected and anaesthetized with 0.01% tricaine methanesulfonate (MS-222, Nakalai Tesque, Kyoto, Japan) buffered with an equal amount of sodium bicarbonate. Gentle pressure was applied on the abdomen to check whether spermiation or ovulation has occurred or not. Fork length and body weight were then measured. Blood samples were collected from caudal vasculature, kept on ice until and centrifuged at  $1000 \text{ g}$  for 10 minutes to obtain serum samples, which were stored at  $-30 \text{ }^{\circ}\text{C}$  until assayed. Finally, gonads were taken out and weighed. Gonadosomatic index (GSI) was computed as gonad weight  $\times 100 (\text{body weight})^{-1}$  for an estimate of gonadal maturity.

### **Preparation of gonads for incubation**

Fish that were reared by feeding commercial pellets only (different from the *in vivo* experiment) were used. They were anaesthetized with MS-222, and then body weight (male, 377.7 g; female, 491.6 g, one fish in each) were measured. Immediately, gonads (testes and ovaries) were carefully removed and transferred into large glass Petri dishes containing ice-cold Leibovitz's L-15 medium. Mature ovarian follicles (oocytes) and testes in late August (just a week before the anticipated final ovulation and spermiation) were used for this experiment. Testes were minced with razors into small fragments about

40–60 mg and ovarian follicles were isolated. Incubations of the mature testicular fragments and ovarian follicles were made at 0, 1, 10, 100 and 1000  $\mu\text{g RU mL}^{-1}$  L-15 medium (Gibco, New York, USA) for 18 h at 15 °C in a humidified incubator. Triplicate incubations were carried out in a 24 well microplates (Iwaki, Funabashi, Japan) for each treatment. After termination of the incubation period, the incubated gonadal tissues and media were collected and stored at  $-80$  °C until assayed.

### **Time-resolved fluoroimmunoassay**

Levels of sex steroid hormones [testosterone (T), estradiol-17 $\beta$  ( $E_2$ ), 11-ketotestosterone (11-KT), and 17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one (DHP)] in serum and their release in the medium of gonadal incubate were measured by time-resolved fluoroimmunoassays (TR-FIA). The protocols for T,  $E_2$ , 11-KT, and DHP assays followed those developed by Yamada, Satoh, Yamashita, Kambegawa & Iwata (1997). In brief, ether-extracted steroid hormones in the serum and media samples were evaporated in a 45 °C water bath with a continuous flow of nitrogen gas, reconstituted into 600  $\mu\text{L}$  of assay buffer [0.05 M Tris, 0.9% NaCl, 0.5% bovine serum albumin (BSA), 0.05%  $\text{NaN}_3$ , 0.01% Tween 40, 20  $\mu\text{M}$  diethylenetriamine-N, N, N', N'', N''-pentaacetic acid, pH 7.75]. Samples were applied to 96-well microtiter plates (Wallac Oy, Turku, Finland) in which BSA-conjugated antigen was immobilized by physical adsorption. Following incubation with BSA-conjugated antigen in dark at 4 °C overnight, europium (Eu)-labeled IgG was added to each well, incubated at room temperature for 2 h and was then stringently washed to remove unbound Eu-labeled IgG. Afterward, enhancement solution (0.1 M acetate-phthalate buffer, pH 3.2, containing 0.1% Triton X-100, 15  $\mu\text{M}$  2-naph-thoyltrifluoroacetone, 50

$\mu\text{M}$  tri-*n*-octylphosphine oxide; Perkin-Elmer) was pipetted and the intensity of fluorescence from dissociated Eu was measured with a time-resolved fluorometer (1234 DELFIA fluorometer; Wallac Oy, Turku, Finland) using DOS-based Multicalc software (Wallac Oy, Turku, Finland). In each assay, standard samples prepared from the pooled masu salmon were used as sub-controls. The intra- and inter-assay variabilities were 8.64 and 11.14% for T, 8.11 and 10.90% for 11-KT, 9.67 and 14.48% for E<sub>2</sub>, 8.88 and 13.70% for DHP, respectively.

### **Statistical analysis**

Data obtained were expressed as means  $\pm$  SEM (standard error of mean). To assess significant differences between RU-treated and control groups, data were subjected to one-way ANOVA analysis followed by Fisher's PLSD or Dunnett's multiple comparison tests. Differences were considered statistically significant when  $P < 0.05$ .

## **Results**

### **Body growth**

Number of fish sampled during the experimental period is shown in Table 1. RU-fed immature males showed a significantly higher body size increase in 0<sup>+</sup> September and October; and from 1<sup>+</sup> March to June (Fig. 1). RU-fed immature females showed a significantly higher body growth increase in 0<sup>+</sup> November and December; and 1<sup>+</sup> January and from May to August. From July 2003 onwards, the number of immature males was too low for meaningful statistical tests (Table 1).

Significant increases in fork length and body weight were observed in RU-fed maturing males in 1<sup>+</sup> August and October (Fig. 2). In 1<sup>+</sup> maturing females, there were no significant differences in body sizes between the RU-administered and control groups, except for the occurrence of a significant difference in body weight in 1<sup>+</sup> September.

### **Gonadal maturation**

GSI of immature and maturing fish in both RU-fed and control groups showed a similar pattern of changes, but the absolute values were low in the controls (Fig. 3). RU-fed fish did not show any significant difference in GSI as compared to controls. In June 2003 sampling, half of the sampled RU-fed females (none in the control group) were found in maturing. From June to October 2003 sampling, out of the total 1<sup>+</sup> sampled females, 36% and 17% of the RU-fed and control females, respectively, were in maturing or matured. Within the same period, 81% and 70% of the RU-fed and control males, respectively, were in maturing or matured. GSI peaked in 1<sup>+</sup> August and October in maturing males and females, respectively. In 1<sup>+</sup> October sampling, it was observed that all the RU-fed males were in spent condition while the controls were in fresh running milt condition.

### **Serum levels of steroid hormones in males**

There were gradual increases in serum T levels from 1<sup>+</sup> March and 11-KT levels from 1<sup>+</sup> April in immature males in both groups (Fig. 4). In RU-fed groups, serum T levels were significantly increased ( $P < 0.05$ ) in 1<sup>+</sup> March, April and June for immature males; and 1<sup>+</sup> July, September and October for maturing males. Serum levels of 11-KT were increased significantly in 1<sup>+</sup> April and May among RU-fed immature males and in 1<sup>+</sup> July among

maturing males, as compared to the controls. Serum DHP levels were low until July, but showed a surge increase in 1<sup>+</sup> September (Fig. 4). In RU-fed groups, serum DHP levels were significantly increased in 1<sup>+</sup> September and 1<sup>+</sup> October.

### **Serum levels of steroid hormones in females**

Serum T, E<sub>2</sub> and DHP levels in immature females were low throughout the sampling period (Fig. 5). In RU-fed maturing females, serum T levels started to rise in 1<sup>+</sup> June and peaked in 1<sup>+</sup> September, whereas in control maturing females, they increased in 1<sup>+</sup> August and September. Serum E<sub>2</sub> levels started to increase in 1<sup>+</sup> June in the RU-fed groups and in 1<sup>+</sup> July in controls. In RU-fed immature females, serum E<sub>2</sub> levels were significantly higher than control in 1<sup>+</sup> June and September (Fig. 5). In RU-fed and control groups, serum DHP levels of mature females showed a surge increase in 1<sup>+</sup> September and decreased in October (Fig. 5). In females, serum T and DHP levels did not show any significant differences between RU-fed and control groups.

### **Steroid release in the RU incubated gonads**

Concentrations of T, 11-KT and DHP in the culture medium of mature testicular fragments increased with RU dose increase (Fig. 6). Release of T was significantly increased at all dose levels of RU. The production of 11-KT and DHP was significantly increased at 100 and 1000 µg RU ml<sup>-1</sup> L-15 medium doses ( $P < 0.05$ ). The ability of RU to stimulate steroidal production has also been demonstrated in mature ovarian follicles (Fig. 7). At higher doses of RU incubation, increases in release of E<sub>2</sub> in the culture medium were significant ( $P < 0.05$ ), but this was not the case for T and DHP.

## Discussion

In this study, it has been shown that body growth of RU-fed individuals of both sexes was significantly higher than controls in 1<sup>+</sup> spring, summer and spawning time. No such differences were observed during the winter, which might be related to the generally low feed intake due to cold temperature. There was a 2 °C difference between the hot summer and cold winter. Similar growth promotion effects of RU in sockeye salmon *O. nerka* were reported by Bhandari *et al.* (2002). They suggested that acceleration of somatic growth might be brought by the essential amino acids (leucine and isoleucine) that might be contained in RU, which probably act via amino acid metabolism. Amino acids are essential nutrients whose infusion is known to lead to muscle growth in fish (Wilson 1989; Brown & Cameron 1991). Some of the key amino acids may also play active roles as metabolic substrates, oxidative fuels and precursors of protein. For example, arginine, being a secretagogue of insulin in pancreas, acts through growth hormone-insulin-like growth factor (GH-IGF) axis and is regarded a very powerful insulinotropin in fish (Mommsen 2001). Intraperitoneal injection of arginine also leads to long and sustained increases in the plasma levels of insulin (Carneiro, Navarro, Gutierrez & Plisetskaya 1993) and IGF-I (Banos, Planas, Gutierrez & Navarro 1999). In mammals, depletion of arginine results in induction of IGF-binding protein-I mRNA and protein (Bruhat, Jousse & Fafournoux 1999). Other amino acids as well, glutamine, glutamate and proline for example, act concurrently in the biosynthesis of protein and consequent muscle growth (Mommsen 2001). On the other hand, growth-enhancing effects of ingested steroids have been known in teleosts (Sower & Iwamoto 1985; Gannam & Lovell 1991). In this study, there is also the potential for growth enhancement in RU-fed groups via the anabolic

effects of steroids that might be contained in the RU-commercial pellets mix. Thus, the higher growth rates in RU-fed individuals, as documented in this study, might be explained by the direct involvement of amino acids contained in RU in protein synthesis or through activation of GH-IGF axis.

The work presented here demonstrated for the first time, *in vivo* and *in vitro*, that RU significantly stimulated the content and release of sex steroids in masu salmon. RU-fed males showed acceleration of spermiation as well as significant elevation in serum levels of androgens and DHP. RU-fed females showed significant elevation of serum E<sub>2</sub>, but not T and DHP levels. Besides, in 1<sup>+</sup> July sampling, maturing females were found in RU-fed groups, but none in controls. *In vitro* RU incubation also brought a significant increase in the release of T, 11-KT and DHP in testicular fragments and E<sub>2</sub> in ovarian follicles. Both *in vivo* and *in vitro*, the effects of RU on gonadal maturation were less pronounced in females. These results are consistent with the previous findings in sockeye salmon, where RU feeding significantly elevated plasma levels of androgens and accelerated spermiation in males but had no effects in females (Bhandari *et al.* 2002). The reasons behind the less pronounced effects of RU in T and DHP levels or productions in females both *in vivo* and *in vitro* are not clear. The administered or incubated RU may be too low to bring major physiological effects in females. On the other hand, *in vivo* and *in vitro* RU treatment more or less increased E<sub>2</sub> levels in serum and E<sub>2</sub> releases in the medium of mature ovarian follicles, which may indicate the involvement of bioactive substances present in RU in E<sub>2</sub> synthesis. There might be aromatase-inducing activities by RU but evidences, which show such activities, could not be found.

Furthermore, *Rhizopus* incubation of rat gonads accelerated ovarian and testicular steroidogenesis upon pretreatment with steroids or gonadotropins (Unpublished data). Thus, the significant increase in T, 11-KT, DHP and E<sub>2</sub> release in gonadal incubates might be due to the direct stimulation of the steroids in the gonad incubates by the physiologically active substances contained in the mycelium of RU. Those physiologically active substances could be T, androstenedione, progesterone, androgen-binding proteins, or other steroids and enzymes as investigated in other fungi species.

In RU-fed males, serum DHP was significantly elevated in 1<sup>+</sup> September and October as compared to the controls. That time was the peak period of spermiation and was marked by a 5-fold increase of serum DHP levels in RU-fed mature males. At the same time, *in vitro* RU incubation of mature testicular fragments brought a significant increase in DHP production in the medium. The fish used for *in vivo* and *in vitro* studies were of same origin (stock) but different batches and a minor stage difference. As mentioned in the Materials and methods, the gonadal explants used were sampled in late August, which was two weeks before 1<sup>+</sup> September sampling in the *in vivo* study. Nevertheless, the *in vivo* and *in vitro* effects of RU in males were correlated to each other because the gonadal explants used were mature and were more or less similar to the maturity stages of fish sampled in the *in vivo* study at 1<sup>+</sup> September. DHP typically increases in serum before spermatozoa and oocyte maturation (Nagahama 1987). In this study, the consistent increase in serum or culture medium DHP during the final maturation, *in vivo* or *in vitro*, most probably indicates that the physiologically active substances in RU could have stimulated the biosynthesis of DHP. This is quite likely, as it has long been known that RU contains certain physiologically active enzymes (Pazur & Okada 1967). For instance,

progesterone, membrane-bound progesterone receptors and guanosine triphosphatase (GTHase) activity have been investigated from *R. nigricans* (Bavec, Slajpah, Lenasi, Yorke & Breskvar 2000; Lenasi, Bavec & Zorko 2002). Prior to the onset of spermiation and ovulation, the shift from progesterone to  $17\alpha$ -hydroxyprogesterone and subsequently to DHP occurs in the presence of the enzymes  $17\alpha$ -hydroxylase (CYP17) and  $20\beta$ -hydroxysteroid dehydrogenase ( $20\beta$ -HSD), respectively (Young, Adachi & Nagahama 1986; Nagahama 1987). *In vitro* experiments have shown that administration of DHP, a final maturation-inducing hormone in salmonids (Nagahama 1987), and  $17\alpha$ -hydroxyprogesterone, a precursor of DHP, into the incubation medium induced final maturation of oocytes (Yamauchi & Yamamoto 1982; Ohta, Kagawa, Tanaka, Okuzawa, Iinuma & Hirose 1997). Hence, it is highly possible that the bioactive substances present in RU could have played active roles in the biosynthetic pathway of DHP.

Taken together, the promotion of somatic growth as well as the increase of hormonal production and release demonstrated *in vivo* and *in vitro* by this study could be explained by direct or indirect involvement of physiologically active substances apparently contained in RU, through potentiation of hormone and protein biosynthesis. These physiologically active substances seem to play active roles in body metabolism by providing substrate to the pathways, accelerating the turnover rate or direct involvement. In any case, it has been demonstrated that RU possessed growth promoting ability in masu salmon of both sexes, and markedly accelerated gonadal maturation, especially in males. These findings offer insights towards our understanding of direct or indirect effects of RU on somatic growth and steroid production, and are a potential basis for future use of RU as a tool for the enhancement of growth and reproduction in aquaculture.

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## Figure legends

Fig. 1 Changes in fork length (FL) and body weight (BW) of immature male and female masu salmon by long-term feeding of RU. The vertical bars represent the mean  $\pm$  SEM. Asterisks indicate significant differences between RU-fed and control groups (ANOVA,  $P < 0.05$ ).

Fig. 2 Changes in fork length (FL) and body weight (BW) of mature male and female masu salmon by long-term feeding of RU. The vertical bars represent the mean  $\pm$  SEM. Asterisks indicate significance differences between RU-fed and control groups (ANOVA,  $P < 0.05$ ).

Fig. 3 Changes in gonadosomatic index (GSI) of male and female masu salmon by long-term feeding of RU. The vertical bars represent the mean  $\pm$  SEM. IM: Immature; M: Mature.

Fig. 4 Changes in serum testosterone (T), 11-ketotestosterone (11-KT) and  $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (DHP) levels of male masu salmon by long-term feeding of RU. The vertical bars represent the mean  $\pm$  SEM. Asterisks indicate significant differences between RU-fed and control groups (ANOVA,  $P < 0.05$ ). IM: Immature; M: Mature.

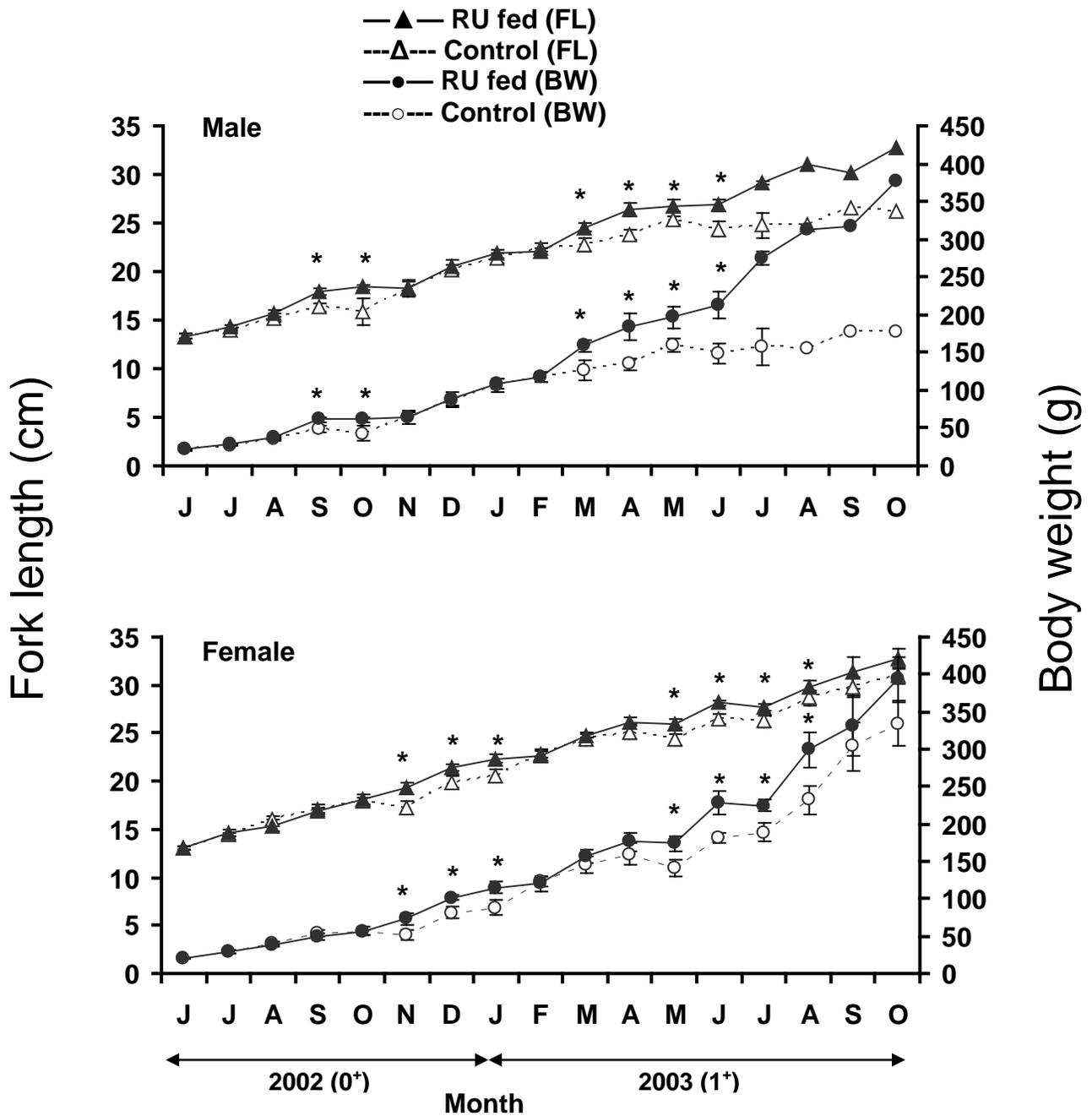
Fig. 5 Changes in serum testosterone (T), estradiol- $17\beta$  ( $E_2$ ) and  $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (DHP) levels of female masu salmon by long-term feeding of RU. The vertical bars represent the mean  $\pm$  SEM. Asterisks indicate significant differences between RU-fed and control groups (ANOVA,  $P < 0.05$ ). IM: Immature; M: Mature.

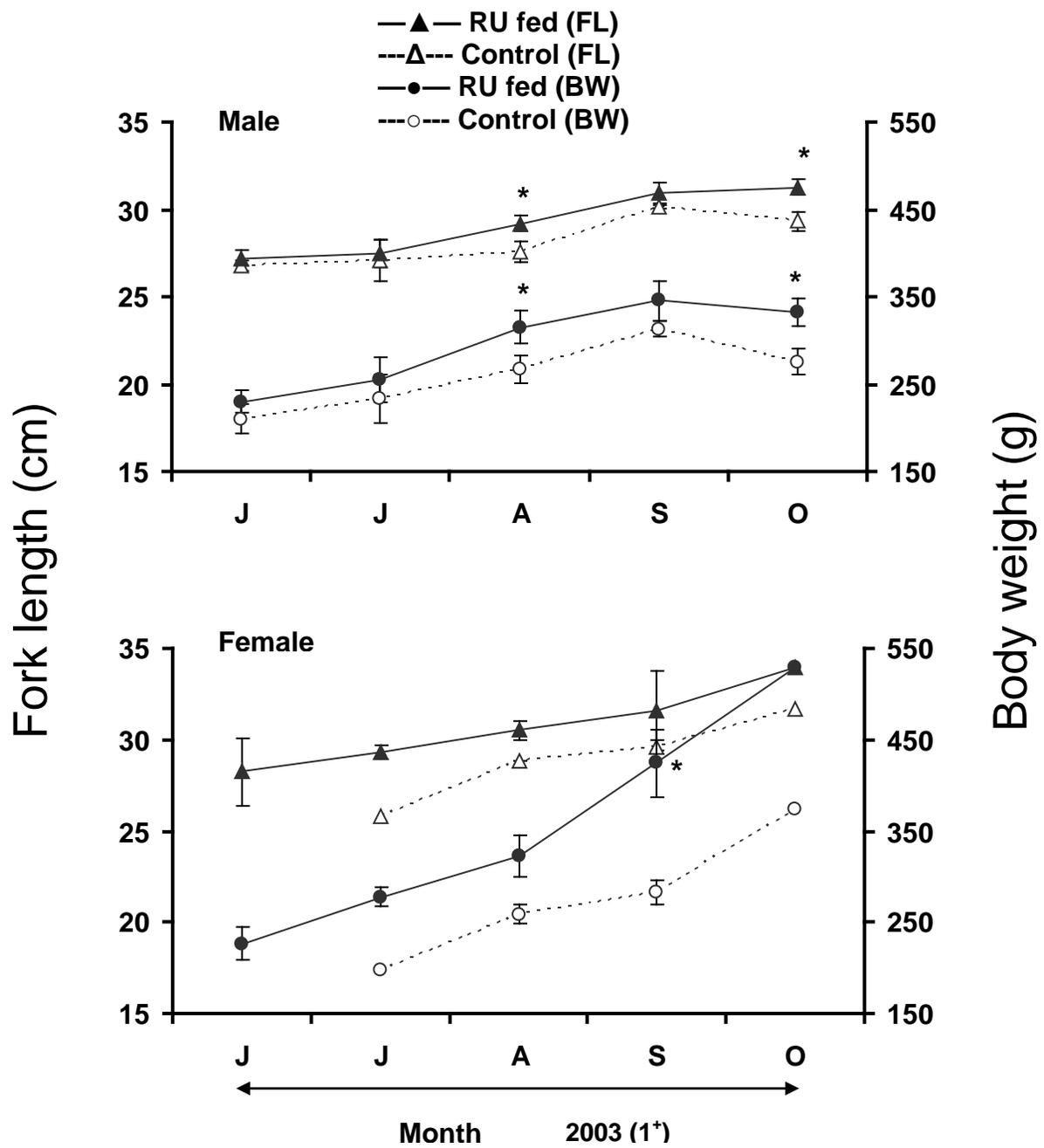
Fig. 6 Testosterone (T), 11-ketotestosterone (11-KT) and  $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (DHP) concentrations in the culture medium of mature testicular fragments from male masu salmon at different doses of RU incubation for 18 h ( $n = 3$ ). Asterisks indicate significant differences between RU incubates and controls (ANOVA,  $P < 0.05$ ).

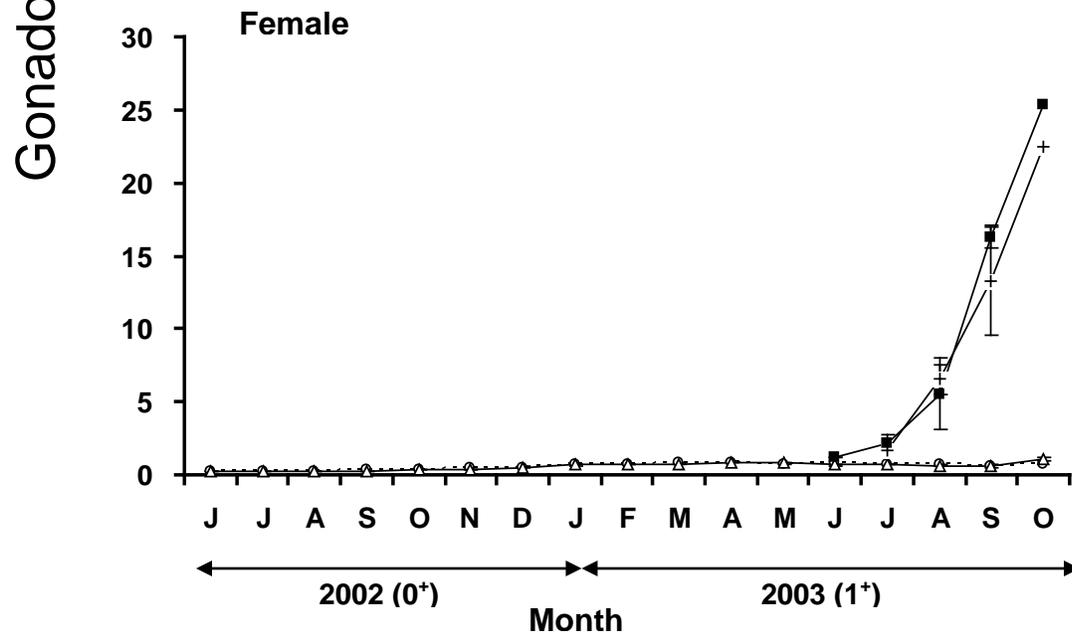
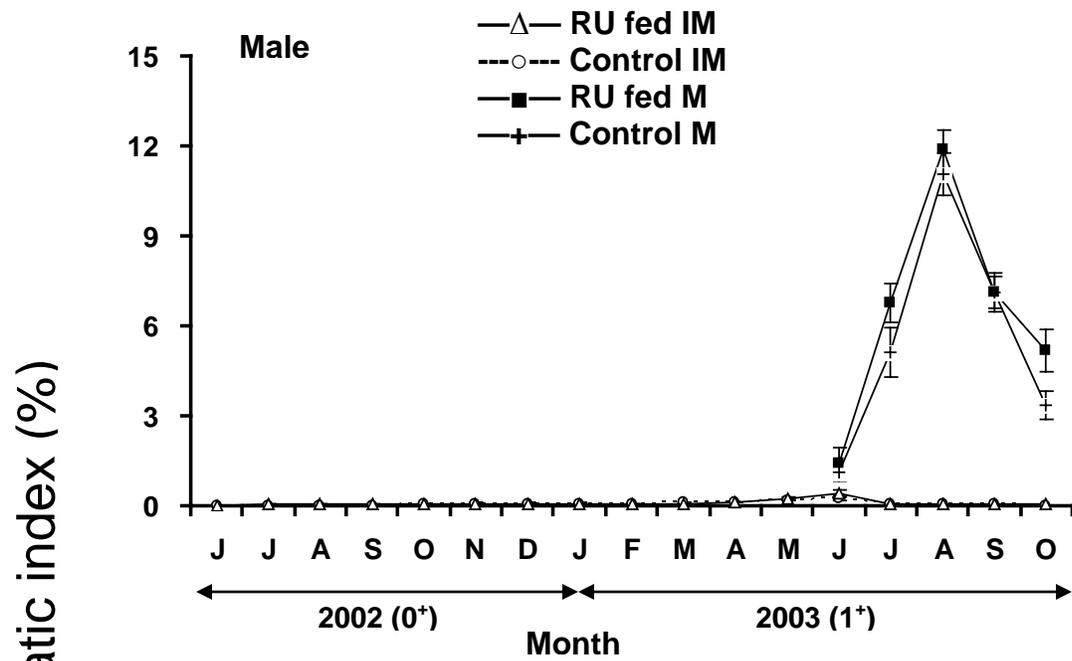
Fig. 7 Testosterone (T), estradiol- $17\beta$  ( $E_2$ ) and  $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (DHP) concentrations in the culture medium of mature ovarian follicles from female masu salmon at different doses of RU incubation for 18 h ( $n = 3$ ). Asterisks indicate significant differences between RU incubates and controls (ANOVA,  $P < 0.05$ ).

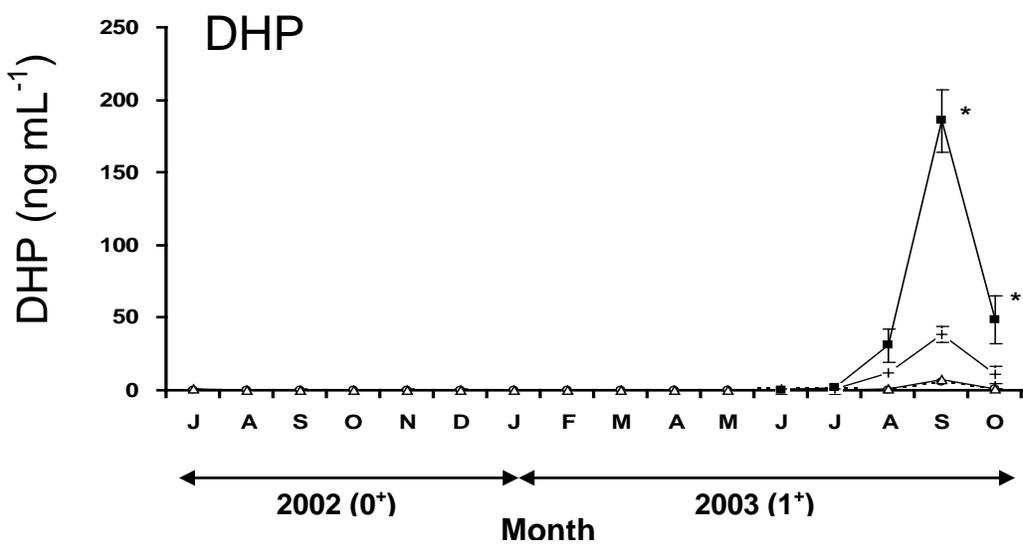
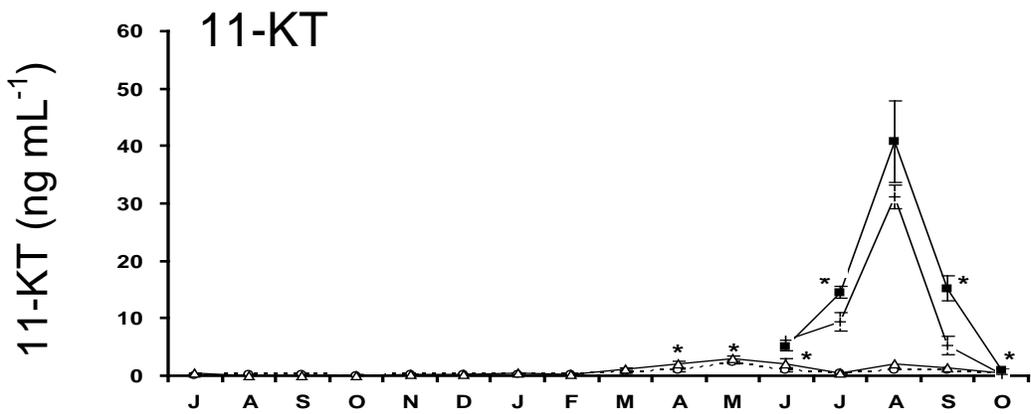
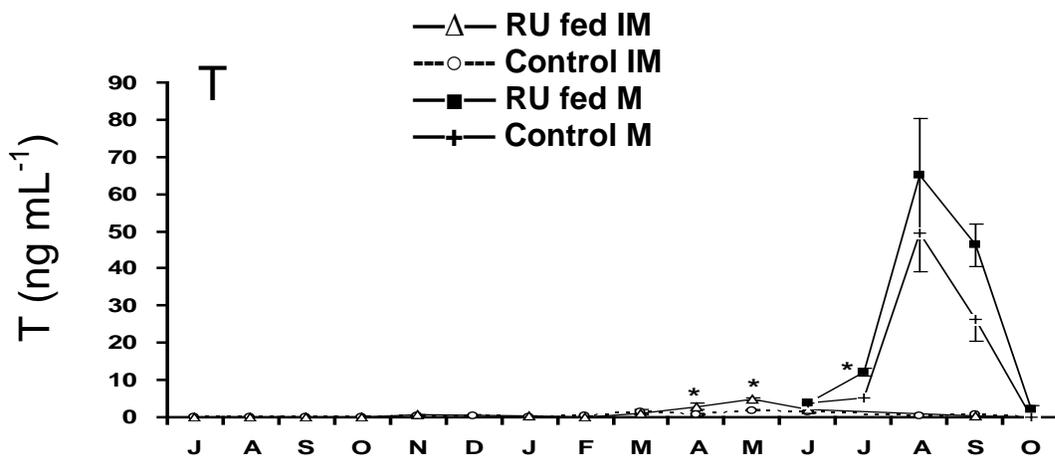
**Table 1** Number individuals sampled in each group of fish from June 2002 to October 2003

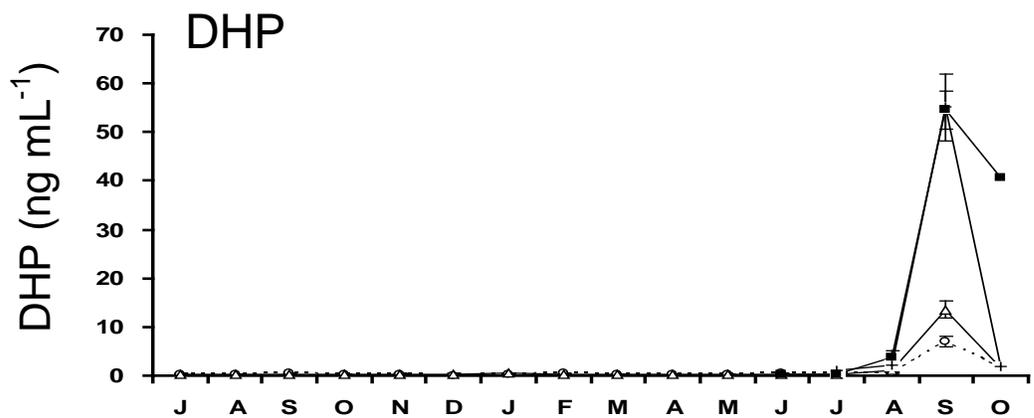
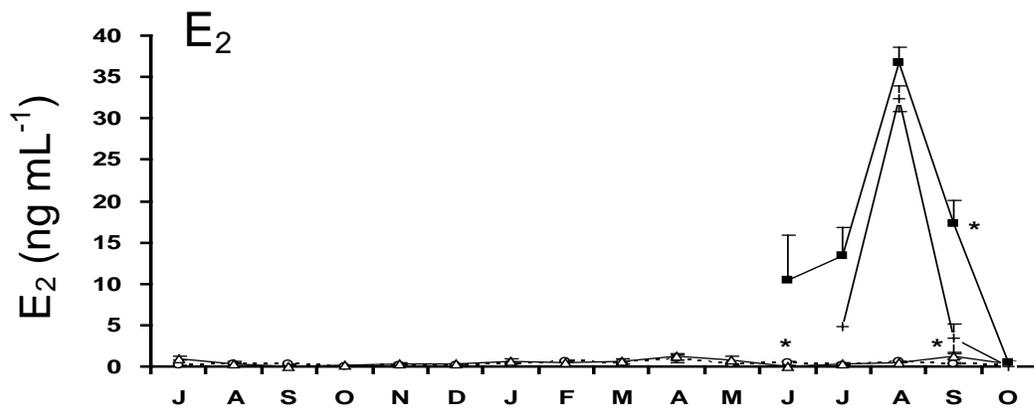
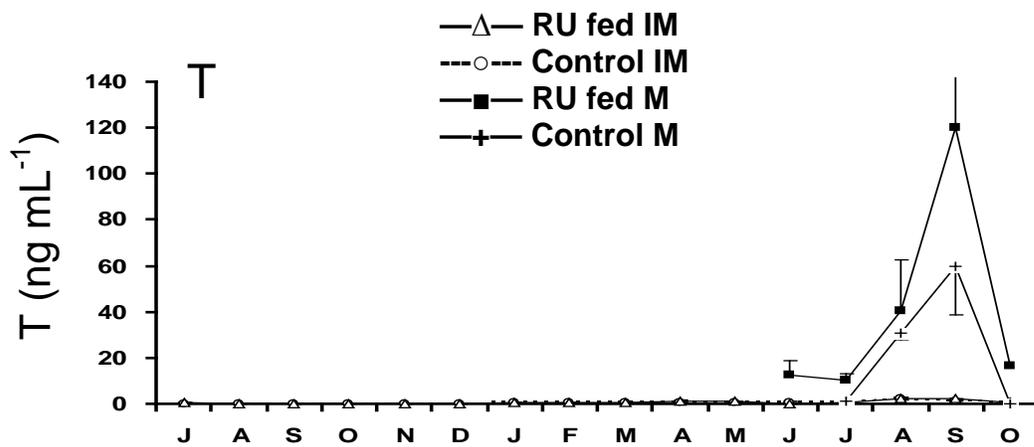
Months	RU fed				Control			
	Immature	Mature	Immature	Mature	Immature	Mature	Immature	Mature
	Males	Males	Females	Females	Males	Males	Females	Females
Jun	8		7		8		7	
Jul	11		8		12		7	
Aug	12		8		8		12	
Sep	7		10		8		10	
Oct	6		13		11		7	
Nov	9		10		10		7	
Dec	13		7		9		11	
Jan	11		8		12		7	
Feb	11		7		10		8	
Mar	10		8		10		8	
Apr	7		12		6		14	
May	10		9		6		12	
Jun	6	6	4	2	9	2	7	
Jul	2	6	5	4	2	6	9	1
Aug	1	10	5	2	1	8	6	2
Sept	1	13	3	3	1	7	7	3
Oct	1	14	4	1	1	11	6	1











← 2002 (0<sup>+</sup>)      2003 (1<sup>+</sup>) →

Month

