Serum Carotenoid Concentration Changes during Sexual Maturation in Farmed Rainbow Trout (*Oncorhynchus mykiss*)†

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One hundred forty individually tagged rainbow trout (*Oncorhynchus mykiss*) were fed diets supplemented with astaxanthin (100 mg/kg) or canthaxanthin (80 mg/kg) during sexual maturity. Only 20 subjects were followed to the end of experience. Blood sampling was withdrawn each month to evaluate pigment concentrations. In females a decrease took place 1 month before spawning, whereas in males variations were not so marked. Females had a higher carotenoid concentration than males. Mean serum levels of astaxanthin ranged in female rainbow trout from 2.80 to 3.33 μg/mL and from 0.67 to 2.32 μg/mL for canthaxanthin. Serum astaxanthin levels in male trout ranged from 0.99 to 3.28 μg/mL and for canthaxanthin from 0.78 to 1.28 μg/mL. Between-subject (V_b) and within-subject (V_s) variations appeared. Serious error can occur when single measurement are used to categorize individuals. Carotenoid levels in the serum of trout during spawning period would not be an adequate indicator for muscle pigmentation.

Keywords: *Carotenoids; serum; sexual maturity; between-within-subject variability; rainbow trout*

INTRODUCTION

The red color of the flesh of salmonids is due to the ingested ketocarotenoid, astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) and canthaxanthin (β,β-carotene-4,4′-dione) (André, 1926). As salmonids cannot synthesize carotenoids *de novo*, they must obtain them from their diet. In the wild carotenoids are ingested with prey items, while in intensive rearing systems carotenoids are added to the diet to meet the animal's pigmentation.

The pigmentation is a complex phenomenon governed by several factors and one of the most important is the influence of sexual maturity. In salmonids, the sexual cycle is well known: gametogenesis in summer and spawn in autumn and in winter (Billard, 1979). Sexual maturation of salmonids involved significant changes in the metabolism of carotenoids (reviewed by Choubert, 1986). During salmonid sexual maturation there is generally a drastic reduction of muscular carotenoids in wild environments (Crozier, 1970; Kitahara, 1983; Ando, 1986) and in farmed rainbow trout (Torrisen and Torrisen, 1985; Choubert and Blanc, 1993). Spawned females progressively recovered muscle carotenoid concentrations equivalent to those observed before spawning in a few months. In contrast, during this time, males recovered only one third of their initial muscle carotenoid concentrations (Choubert and Blanc, 1993).

Biological variation can be divided into an intra-individual component, reflecting changes occurring in the same individual over time (within subject), and an interindividual component, representing the differences between individuals (between subjects). These variations are large enough to impose significant constraints on the design and interpretation of studies of serum carotenoids.

Within-subject and between-subject variation was not available for many micronutrients. Extreme variation in concentrations of carotenoids in human milk, both within and between individuals, has previously been quantitated (Giuliano *et al.*, 1994). Previous studies for Tangney *et al.* (1987) reported data on the within-subject and between-subject variances for β-carotene, retinol, and tocopherol in plasma and diet. Lux and Naidoo (1994) determined the magnitude of within- and between-subject variation of β-carotene in plasma in healthy individuals. Gobantes *et al.* (1997) found a high variability in serum carotenoid concentrations in immature rainbow trout. Data on the biological variations of carotenoids are important in salmonid aquaculture.

With this background the aim of this work was (a) to study the distribution of astaxanthin and canthaxanthin in the serum of rainbow trout (males and females) during the sexual maturation and (b) to investigate the biological variation of carotenoids in mature rainbow trout.

MATERIALS AND METHODS

Experimental Design. The experiment was conducted in the INRA experimental fish farm of Lées-Athas (southwestern France) provided with spring water (constant temperature = 8 ± 1 °C, pH = 7−8, oxygen = 10−12 mg of O_2/L). The experimental period lasted 7 months, beginning 3 months before spawning and ending 3 months after spawning.

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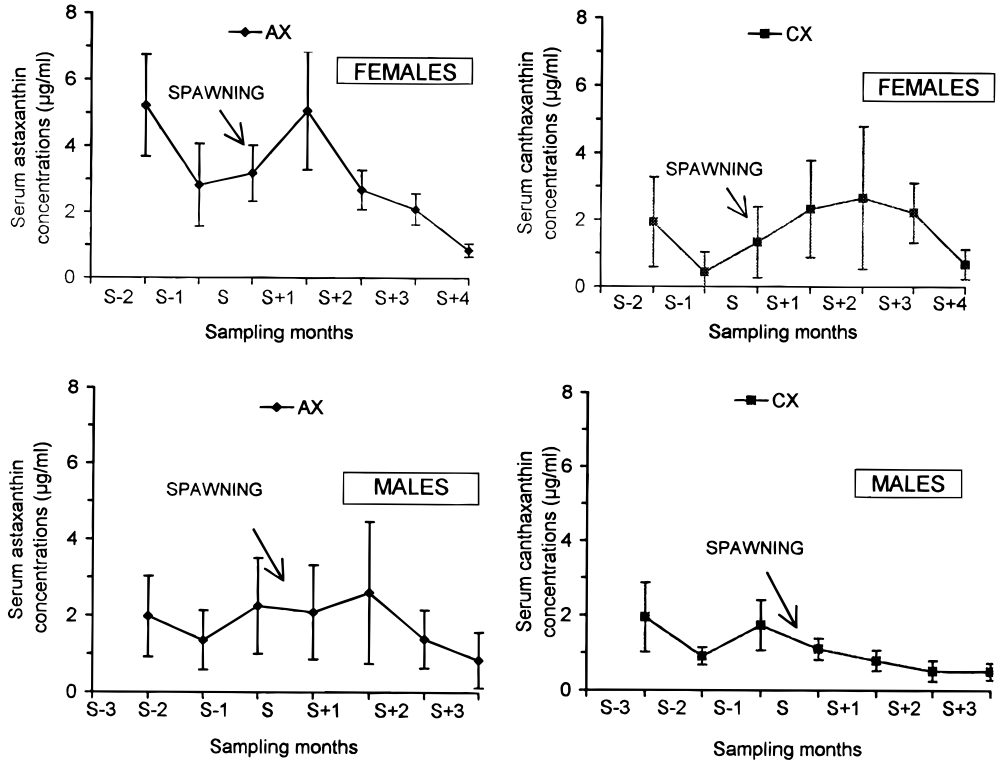

Figure 1. Serum carotenoid [astaxanthin (AX) and canthaxanthin (CX)] concentration (μg/mL of serum) patterns during sexual maturity in female and male rainbow trout.

A total of 140 rainbow trout *(Oncorhynchus mykiss),* 70 females and 70 males in their first sexual maturation, with a mean weight of 800 g, were distributed into six tanks of 1 m^3. Fish were individually tagged with an indelible dye (Alcian blue).

Feed and Feeding. The fish received a basal diet (fish meal 35%, soybean meal 30%, pregelatinized starch 25%, fish oil 6%, vitamin mix 1.5% (Labbé *et al.*, 1993), mineral mix 1.0% (Labbé *et al.*, 1993), sodium alginate 1.5%) supplemented with 100 mg of astaxanthin (F. Hoffmann-La Roche, Basel, Switzerland)/kg of feed or with 80 mg of canthaxanthin (F. Hoffmann-La Roche, Basel, Switzerland)/kg of feed. Carotenoid concentrations in feed were chosen according EC legislation. The carotenoids pigments were added as gelatin stabilized beadlets. The fish were hand-fed each day, once a day *ad libitum* during the experimental period.

Sampling and Analysis. Blood (approximately 2.5 mL/fish) was withdrawn every month after anesthesia (2-phenoxyethanol, Prolabo, Paris, France) in the caudal peduncle (Le Bail *et al.*, 1981). Blood samples were allowed to clot (overnight at +4 °C) and the sera were recovered after centrifugation (2000*g* for 5 min).

Carotenoid analyses of the serum were carried out following the precautions for isolation and handling (Fiasson *et al.*, 1969). Carotenoids were determined by high-performance liquid chromatography (HPLC) procedure using a 30 × 2.1 mm i.d. guard column and a 250 × 4.6 mm i.d. column, both packed with C_{18} reversed-phase material of 5 μm particle size (Lichrosorb, Merck, Darmstadt, Germany). Elution was performed with an isocratic solvent (acetonitrile, dichloromethane, methanol, water, propionic acid, 71:22:4:2:1, by volume) (Guillou *et al.*, 1993). The system was consisted of a pump (Model M2200, Bischoff, Leonberg, Germany) and a spectrophotometric detector (Model Lambda 1000, Bischoff, Leonberg, Germany). The detection of carotenoids was carried out at 480 nm for astaxanthin and at 472 nm for canthaxanthin (Guillou *et al.*, 1993). Data were evaluated in the Pic 3 software (ICS, Toulouse, France).

All system (column and mobile phase) was thermoregulated at 3 °C by a recirculating system to avoid variations in peak heights and retention times caused by change of ambient temperature. External standards of known concentrations were used for peak identification and quantification.

Statistical Analyses. Analysis of variance (ANOVA) with repeated measurements was applied. After each ANOVA a paired *t*-test was performed. Level of significance was fixed at 95%. The data were processed by using the IV-package of the BMDP program (BMDP Statistical Software, Inc., Los Angeles, CA).

RESULTS

From the 140 initial trout some blood sample could not be collected because tagged fish were not identified. Some others were discarded to keep the same number of individuals along the statistic calculation, and some died before the end of experiment. Therefore, only 20 subjects (10 males and 10 females) were really followed during maturity period.

For clarity in the text as well as in the figure, S means for the spawning time, S − 1 and S − 2 1 and 2 months before spawning, S + 1, S + 2, ... 1, 2, ... months after spawning. The pattern of variation in carotenoid serum levels in rainbow trout females and males is shown in Figure 1. In females the highest astaxanthin serum concentration was reached 2 months before spawning (S − 2). For both carotenoids, their serum levels decreased from S − 2 to S − 1, despite the fact that rainbow trout were fed. Then the serum levels increased with a maximum for astaxanthin 1 month after spawning (5.07 ± 1.78 μg/mL of serum) and for canthaxanthin 2 months after spawning (2.66 ± 2.13 μg/mL of serum).

In male trout, for convenience, the spawning months were reported to results obtained for females. Even if males were mature earlier and for a longer time than females, no significant differences in astaxanthin concentrations were found. In contrast significant differences were observed for canthaxanthin levels until spawning time.

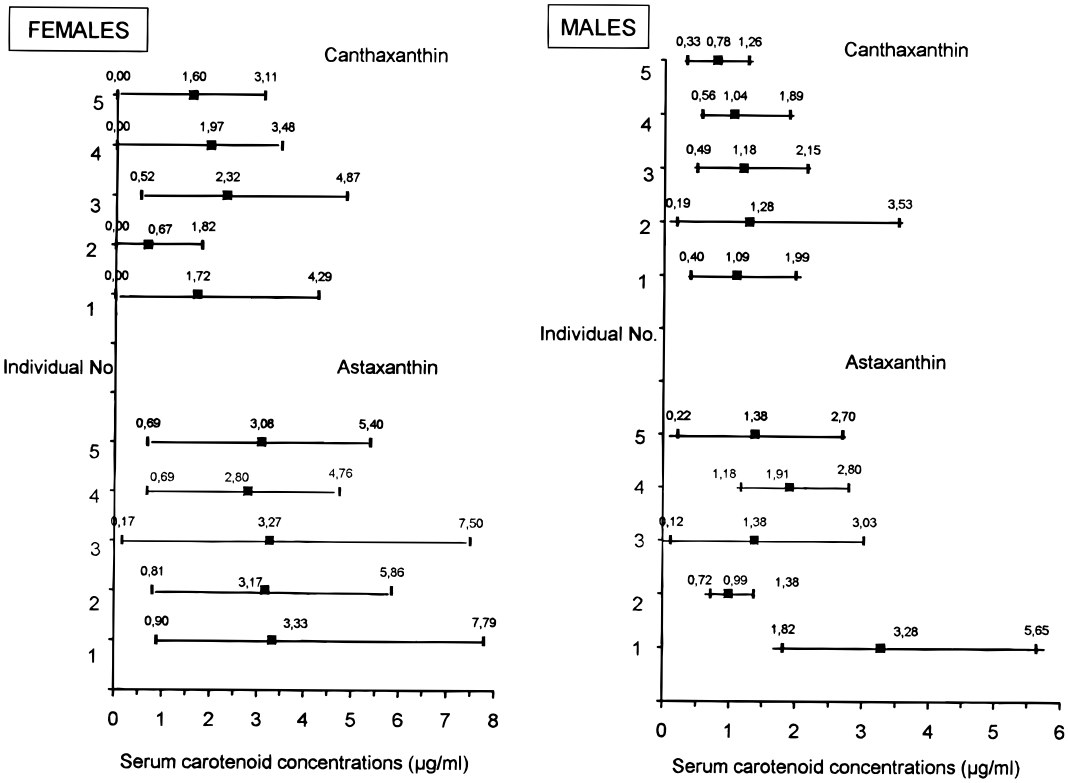

Figure 2. Parametric means and absolute ranges for serum carotenoid concentrations results from fed astaxanthin and canthaxanthin female and male rainbow trout. Each bar represents mean and standard deviation for individuals calculated from monthly measurements during 7 months.

Table 1. Mean Squares and Level of Significance ($P < 0.05$) for the Different Sources of Variation on Carotenoid Concentrations in Mature Rainbow Trout

source of variation	df	serum concn mean square	F	probability level of significance
sex	1	43.52	47.90	0.0001
pigment	1	30.94	34.05	0.0001
month	6	12.36	13.61	0.0001
individual (sex + pigment)	16	1.74	1.92	0.0275
error	96	0.91		

A total of 140 serum samples were obtained from measurement of ketocarotenoid levels once a month in 20 subjects over a period of 7 months. The results of the ANOVA with repeated measurements are shown in Table 1. Females had a higher carotenoid concentration than males. Ketocarotenoid levels in female serum were represented twofold higher than the value of ketocarotenoid levels in males serum. On the other hand a highly significant difference was observed between the two ketocarotenoids. Astaxanthin concentrations were always higher than those of canthaxanthin whatever the sex of the fish. Time of sampling had a significant effect on serum carotenoid concentration. Serum carotenoid concentration was variable from fish to fish at each sampling time. This effect was significant for carotenoid and sex as well.

The mean and range of values obtained for mature rainbow trout fed the two carotenoids are shown in Figure 2. Each bar represents the mean and standard variation for five individuals calculated from monthly measurements during 7 months. In female trout the mean astaxanthin concentration ranged from 2.80 to 3.33 μg/mL and the mean canthaxanthin from 0.67 to 2.32 μg/mL. In males the mean astaxanthin level ranged from 0.99 to 3.28 μg/mL and the mean canthaxanthin from 0.78 to 1.28 μg/mL.

Table 2. Calculated Average Components of Variance ($n = 20$)[a]

	components of variance				
	within subject		between subject		ratio of within to between variance
subjects	V_S	V_S/V_T	V_b	V_b/V_T	$(V_S/V_b)^{1/2}$
females AX[b]	3.53	74.5	1.21	25.4	1.71
females CX[c]	1.84	53.9	1.56	46.0	1.08
males AX[d]	0.92	40.6	1.34	59.4	0.82
males CX[e]	0.24	50.1	0.24	49.9	1.00

[a] V_S, within-subject variation; V_b, between-subject variation; V_T, percentage of each total variance. [b] Females fed astaxanthin. [c] Females fed canthaxanthin. [d] Males fed astaxanthin. [e] Males fed canthaxanthin.

Analyses of within- and between-subject variability for both carotenoids and for both sexes are represented in Table 2. Within-subject variability was highest for astaxanthin in female trout, while variability between individuals was highest for canthaxanthin in female trout. Within-subject variation was higher than between-subject variation for astaxanthin and canthaxanthin serum concentrations in female rainbow trout. Ratios of within-subject to between-subject variances were <1 for males rainbow trout fed astaxanthin but were >1 for the others.

DISCUSSION

The patterns of serum carotenoid levels associated with spawning were different in male and female rainbow trout. The decrease in serum carotenoid levels 1 month before spawning in females could be due to a mobilization of these pigments to the ovaries. Nassour and Léger (1989) reported that in the trout lipids are

mainly stored in the carcass and to a less extent in the gut and ovaries. The distribution ranged from 68 to 73%, 31 to 6%, and 0.1 to 20% respectively from the beginning to the end of sexual maturation.

The disposition of total lipids in these three deposition sites was closely related to the reproductive cycle. An increase in total lipids takes place in the ovary during ovulation. These results are in agreement with those previously reported (Choubert and Blanc, 1989), in which the canthaxanthin amounts expressed by lipid amounts are accumulated at a higher rate in the ovary at spawning time. On the other hand, pigmented eggs give less mortality compared to egg with low carotenoid content (Hartmann *et al.*, 1947; Deufel, 1965). In the same way, a mobilization of carotenoids from the muscle to ovaries during sexual maturation has been reported in wild environments (Steven, 1949; Crozier, 1970; Shnarevitch and Sakhnenko, 1971; Kitahara, 1983; Ando, 1986). The decrease in pigment levels observed during the last months may be due to a deposition of these pigments in muscle, since females recovered their muscle carotenoid concentrations 4 months after spawning (Choubert and Blanc, 1993).

The results observed in males are influenced by an earlier maturity than the females. This sexual maturity remained so over a longer period of time. This is in accordance with the results of Sano (1960) for rainbow trout and Aksness *et al.* (1986) for Atlantic salmon in sea water farming conditions.

Astaxanthin serum levels of either male or female trout were higher than those of canthaxanthin. This fact is not related to the different initial concentration of carotenoids. Higher serum accumulations of astaxanthin than those of canthaxanthin have been reported earlier either for immature (Choubert *et al.*, 1987, 1992, 1994; Gobantes *et al.*, 1997) or mature rainbow trout (Guillou *et al.*, 1992). In addition, a higher deposition of astaxanthin than that of canthaxanthin in salmonid muscle has been reported (Foss *et al.*, 1984; Torrisen, 1986, 1989; Choubert and Storebakken, 1989; Bjerkeng *et al.*, 1990; No and Storebakken, 1992).

In females, astaxanthin levels decreased down to 54% compared to 22% for canthaxanthin. These observations suggest that astaxanthin is more affected by the sexual maturity process than canthaxanthin. On the other hand, the conversion of astaxanthin into vitamin A is possible. Moreover, this conversion could increase during sexual maturity (Al-Khalifa and Simpson, 1988; Guillou *et al.*, 1989). However, no conversion of canthaxanthin into vitamin A has been reported.

Between-subject and within-subject variations in the amount serum carotenoid appeared. This variability has been reported in fish works (Choubert *et al.*, 1987; March *et al.*, 1990; Guillou *et al.*, 1992), although is not explained. Extreme variations in concentrations of carotenoids in trout serum or muscle could impose significant constraints on the design and interpretation of carotenoid studies. This problem was also observed in humans (Tangney *et al.*, 1987; Lux and Naidoo, 1994) and in human milk by Giuliano *et al.* (1994), reporting significant between-subject and within-subject variations in carotenoid concentrations.

Due to within-subject variation, repeated measurements from the same trout over time are required to accurately determine serum astaxanthin or canthaxanthin for female rainbow trout for an individual. In contrast, due to between-subject variation larger sample sizes are needed to accurately measure changes in astaxanthin or canthaxanthin for female rainbow trout and in astaxanthin for male rainbow trout in serum. These results suggest that serious error can occur when a single measurement is used to categorize individuals. Multiple independent measurements might be obtained for each individual, but this approach could pose substantial logistical problems and diminish subject number. For these reasons we think that carotenoid levels in the serum of trout during the spawning period would not be an adequate indicator for muscle pigmentation. This conclusion does not agree with the results found with Atlantic salmon (Storebakken and Goswami, 1996). Usually, the fish producer should avoid the pigmentation during sexual maturity as has been reported for rainbow trout (Choubert and Blanc, 1993) and for Atlantic salmon (Akness *et al.*, 1986).

However, the high astaxanthin levels found in females in an early stage of maturation could be related to hormonal changes during sexual maturation. Under our experimental conditions the results suggest that the best moment for slaughtering and processing female rainbow trout could be in this period. This hypothesis requires further investigation.

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