HOKKAIDO UNIVERSITY

Title	STUDIES ON THE FOOD BIOCHEMICAL ASPECTS OF CHANGES IN CHUM SALMON, ONCORHYNCHUS KETA, DURING SPAWNING MIGRATION : MECHANISMS OF MUSCLE DETERIORATION AND NUPTIAL COLORATION
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STUDIES ON THE FOOD BIOCHEMICAL ASPECTS OF CHANGES IN CHUM SALMON, *ONCORHYNCHUS KETA*, DURING SPAWNING MIGRATION: MECHANISMS OF MUSCLE DETERIORATION AND NUPTIAL COLORATION*

By

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Chapter I. General introduction

Pacific salmon (*Oncorhynchus* spp.) are typical anadromous fish that grow in the Pacific Ocean and breed in fresh water. The salmon therefore appear at certain times of the year in many Far Eastern coastal regions, enter their natal rivers for spawning, and then die.

It has been ascertained that all species of Pacific salmon do not feed during the entire spawning migration period. The amazing ability of all these fish to extensively deplete lipid and protein reserves has been established. The increase in snout length (particularly for males), the increase in skin thickness, the uptake of water accompanying losses in lipid and protein, the increased weight of the gonads (particularly for females), the loss of muscle pigmentation, the decreased enzymic activity of the stomach and other changes have been qualitatively or semi-quantitatively established for some species (Greene, 1926; Pentegoff *et al.*, 1928; Davidson and Shostrom, 1936). These changes in migrating salmon are considered to be closely related to their physiological state, although detailed studies have mainly been published on sockeye salmon (*Oncorhynchus nerka*) (Idler and Clemens, 1959).

Idler *et al.* have reported on the biochemical changes in sockeye salmon during spawning migration. They have thoroughly examined a large number of sockeye salmon (216 fish) returning to spawn and have attempted to establish the physiological state of the standard salmon (Idler and Tsuyuki, 1958). The muscle lipid content markedly decreased during the early stage of spawning migration, while a decrease in muscle protein (water soluble and salt soluble proteins) was found during the final stage of spawning migration. The muscle moisture content gradually increased, while no marked changes in cholesterol levels of muscle were found during spawning migration (Idler and Bitners, 1958, 1959; Duncan and Tarr, 1958). Wood (1958) has investigated the non-protein compounds in muscle and revealed a marked decrease in histidine level during the early stage of spawning migration. In addition, Idler *et al.* have analyzed the components of organs such as liver, gonads and plasma, as well as muscle. Idler and Bitners (1960) have revealed that the ovaries consumed 8% of the energy expended from the body of the female while the testes accounted for only 0.5% of the energy expended from the body of the male during spawning migration. Such components as glucose, protein and non-protein compounds varied in plasma during spawning migration (Jonas and MacLeod, 1960). Chang *et al.* (1960) have detailed the distribution of phosphorus compounds, creatine and inositol in the muscle, gonads and liver of migrating salmon.

Furthermore, Idler *et al.* (1959) and Idler and Tsuyuki (1959) have reported the levels of steroid hormones for sockeye salmon with regard to their physiological state during spawning migration. A new blood steroid, 11-ketotestosterone, has been isolated from the post-spawned male sockeye salmon (Idler *et al.*, 1960a). 11-Ketotestosterone has been shown to have androgenetic activity for this species and influenced skin thickness and coloration, muscle pigmentation, and spermatogenesis in the male (Idler *et al.*, 1961; van Overbeeke and McBride, 1971). Schmidt and Idler (1962) have suggested the possibility of a "hormone trigger theory" for spawn-

ing migration on the basis of the changes in plasma steroid hormone levels of sockeye salmon. Idler and Truscott (1963) have investigated the *in vivo* metabolism of steroid hormones in sockeye salmon and found that testosterone and 17α-hydroxyprogesterone were precursors of 11-ketotestosterone. In addition, pregn-4-ene-17α, 20β-diol-3-one was isolated from the plasma of spawning sockeye salmon (Idler *et al.*, 1960b). Idler and his collaborators have thus investigated in detail the biochemical and physiological changes in sockeye salmon during spawning migration.

There have also been several reports describing the changes of muscle proximate composition during spawning migration of chum salmon (*Oncorhynchus keta*) (Pentegoff *et al.*, 1928 ; Igarashi and Zama, 1953 ; Uno *et al.*, 1956 ; Takahashi *et al.*, 1975, 1976 ; Hatano *et al.*, 1983 ; Konagaya, 1983). They have revealed that as chum salmon move from the estuary to the spawning grounds, the moisture content in the muscle increases and the lipid and protein contents decrease, as well as sockeye salmon. They have not discussed in detail, however, the relation between the changes of proximate composition and the physiological state of chum salmon during spawning migration. There are very few reports regarding the steroid hormones in chum salmon, although a detailed study has been reported on the peptide hormones (Yoneda and Yamazaki, 1976 ; Kawauchi, 1984). Yamazaki (1972) has revealed that 17α -methyltestosterone is capable of inducing hypertrophic changes in the skin and gonads of chum salmon. Ueda *et al.* (1984) have recently investigated the changes in serum concentrations of steroid hormones such as estradiol-17β, androgens, and 17α, 20β -dihydroxy-4-pregnen-3-one during spawning migration of chum salmon and found that the final maturation is caused by 17α, 20β -dihydroxy-4-pregnen-3-one. To date, the biochemical and physiological studies on chum salmon during spawning migration are very limited, despite the fact that chum salmon are very important fisheries resources in Japan.

About thirty-one million chum salmon returned to their natal rivers in Hokkaido, and the number of fish returning to Japan was more than forty-eight million in 1985. Chum salmon returning to Japan to spawn have become stable fisheries resources within the last ten years, although there remain several problems to be resolved in their utilization and processing (Kamimura, 1984 ; Zama and Takahashi, 1985). Further, biochemical and physiological studies will be required for the resolution of these problems. It is assumed that chemical changes in chum salmon during spawning migration are closely related to their physiological state, however, no attempt has been made to verify this hypothesis. Therefore, both biochemical and physiological approaches have been attempted to clarify various changes in chum salmon during spawning migration. The objective of the present study has been to clarify the causes of muscle deterioration and nuptial coloration which, from the food chemistry, as well as biochemical and physiological points of view, are the most important and interested phenomena. Furthermore, the relationship between sex steroid hormones, muscle deterioration and nuptial coloration will be discussed.

Chapter II. Muscle deterioration occurring in chum salmon during spawning migration

Muscle deterioration, *i.e.*, the changes in proximate composition (decrease in protein and lipid) and the activation of protease, which occurred during chum salmon spawning migration has been a great problem in the utilization and processing. However, little information is available concerning the causes of muscle deterioration. This chapter discusses the relationship between muscle deterioration and the physiological state of the fish.

Section 1. Changes in muscle proximate composition

Pacific salmon (*Oncorhynchus* spp.) are typical anadromous fish that grow in the ocean and breed in fresh water. Pacific salmon appear in the North Pacific Ocean at certain times of the year, enter their natal rivers for spawning, and then die. It is considered that this biological feature governs patterns of changes in their chemical composition.

There have been a number of detailed reports describing the biochemical changes of sockeye salmon (*Oncorhynchus nerka*) during spawning migration (Idler and Tsuyuki, 1958; Idler and Bitners, 1958, 1959, 1960; Duncan and Tarr, 1958). However, there have been very few reports on the changes of proximate composition during spawning migration of chum salmon (*Oncorhynchus keta*). Taking the Amur chum salmon as an example, Pentegoff *et al.* (1928) have established that as the fish move from the estuary to the spawning grounds, the moisture content in the muscle increases and the lipid and protein contents decrease. They have not discussed in detail, however, the relation between the changes of proximate composition and the physiological state of chum salmon during spawning migration.

The aim of the investigations reported in the present section is therefore to clarify the characteristic changes in chemical composition during spawning migration of chum salmon with regard to their physiological state.

Materials and Methods

Materials

Chum salmon specimens (*Oncorhynchus keta*) in four different physiological states were used as the materials (Table 1). Three males and 3 females were analyzed for each sample group. These samples were kept at −20°C until use. Only dorsal muscle was analyzed for the proximate composition.

Blood was collected from the caudal vasculature of live salmon and left at room temperature for several hours. The clotted blood was centrifuged to obtain the serum. Sera thus obtained were stored at −20°C until use.

Proximate composition

The dorsal muscle was analyzed in duplicate for moisture, protein and ash according to the usual methods (Horwitz, 1965). Lipid extraction from the dorsal muscle was carried out by the method of Bligh and Dyer (1959).

Table 1. Characteristics of chum salmon used in the study.

Stage	Sex	Collecting date and locality	Fork length (cm)	Body weight (g)	Gonadosomatic index*	Age	Remarks
Feeding migration	Male	Aug. 1-6, 1983. Lat. 45°59′N-49°29′N Long. 167°07′E-175°30′E	46±1	1370± 180	0.1±0.0	02-04	Sexually immature adult
	Female		50±3	1590± 410	1.3±0.3	03	No breeding color
Spawing migration	Male	Oct. 21, 1983. Yakumo coast of Hokkaido	66±2	3270± 270	6.3±0.5	03	Mature adult
	Female		68±1	3570± 270	17.2±0.3	03	Very faint signs of breeding color
Upstream migration	Male	Nov. 25, 1983. Lower reaches (1.8 km) of Yurappu River, Hokkaido	82±3	6540± 790	3.0±0.8	04	Mature adult
	Female		76±4	5130±1020	18.7±5.5	03-04	Clearly visible signs of breeding color
Spent	Male	Nov. 24, 1983. Lower reaches (1.8 km) of Yurappu River, Hokkaido	79±1	5140± 250	1.1±0.2	03	Mature adult
	Female		77±4	4270± 280	1.0±0.1	04	Very pronounced signs of breeding color

*(Gonad weight/Body weight)×100.
Values represent the mean±standard deviation of three samples.

The total lipid was analyzed quantitatively by thin-layer chromatography (TLC). The TLC plates (Kieselgel 60, a ready-made plate from Merck) were developed using *n*-hexane-diethyl ether-acetic acid (85 : 15 : 1, v/v/v) for non-phospholipid. The TLC plate was sprayed with a 3% copper acetate-8% phosphoric acid solution, heated on a hot plate, and quantitated using an Ozumor 82 densitometer. The amount of phospholipid was calculated from lipid-phosphorus assayed by the method of Fiske and Subbarow (1925).

The preparation of extracts from the dorsal muscle was made with 5% trichloroacetic acid. Total and extractive nitrogens were determined by the micro-Kjeldahl method. Ninhydrin positive substances of extracts were measured using the procedure of Yemm and Cocking (1955).

The dorsal muscle pigments, expressed as astaxanthin, were determined by the method of Kanemitsu and Aoe (1958). The surface color of dorsal muscle was measured with a color difference meter (Color-Ace-TCA-1 ; Tokyo Denshoku Co. Ltd.,).

Radioimmunoassay (RIA)

Serum levels of testosterone and estradiol-17β were measured by RIA methods (Nakamura *et al.*, 1974).

Results

Changes in gonadosomatic index (GSI) values

Changes in the GSI are shown in Fig. 1. The GSI values for females were markedly elevated during their spawning migration stage and high values were maintained during their upstream migration. In contrast, the GSI values for males slightly increased during the spawning migration phase.

Changes in serum concentration of steroid hormones

High levels of serum testosterone were shown during the spawning migration

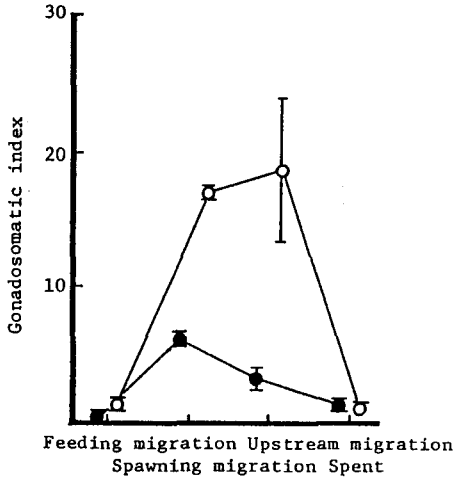

Fig. 1. Changes in the gonadosomatic index values during the spawning migration of male (●—●) and female (○—○) chum salmon. The vertical bars represent the mean±standard deviation of three samples.

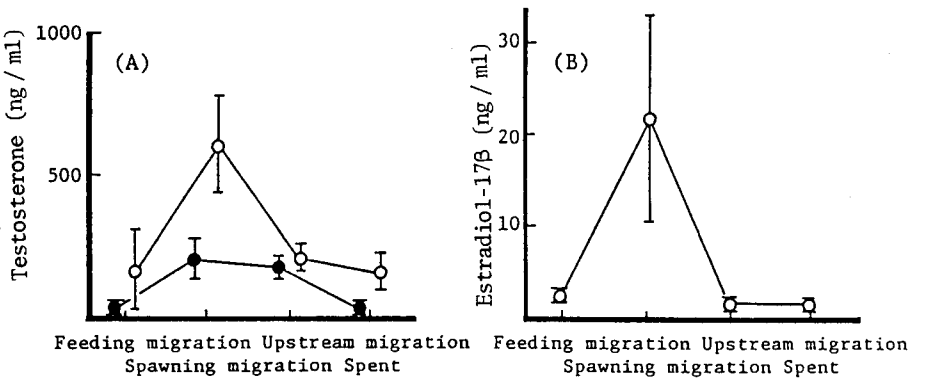

Fig. 2. Changes in serum testosterone (A) and estradiol-17β (B) levels during the spawning migration of male (●—●) and female (○—○) chum salmon. The vertical bars represent the mean±standard deviation of three samples.

Table 2. Changes in proximate composition of dorsal muscle during the spawning migration of chum salmon.

	Sex	Moisture	Protein*	Lipid	Ash	Extractive-N
		g/100 g muscle				
Feeding migration	Male	75.21 ± 0.99	18.62 ± 0.04	2.87 ± 0.98	1.22 ± 0.09	0.401 ± 0.02
	Female	75.11 ± 0.73	18.80 ± 0.27	3.57 ± 1.85	1.23 ± 0.07	0.407 ± 0.03
Spawning migration	Male	74.83 ± 0.37	21.36 ± 0.27	1.07 ± 0.31	1.30 ± 0.02	0.387 ± 0.02
	Female	76.45 ± 0.86	20.21 ± 0.12	1.01 ± 0.16	1.31 ± 0.06	0.424 ± 0.01
Upstream migration	Male	78.91 ± 0.18	17.37 ± 1.39	1.18 ± 0.20	1.17 ± 0.06	0.393 ± 0.02
	Female	80.19 ± 0.95	17.12 ± 0.92	1.34 ± 0.06	1.16 ± 0.05	0.442 ± 0.01
Spent	Male	82.97 ± 0.86	14.69 ± 0.12	0.80 ± 0.03	0.94 ± 0.06	0.402 ± 0.02
	Female	82.61 ± 2.35	16.29 ± 1.63	0.81 ± 0.06	0.98 ± 0.11	0.417 ± 0.02

*(Total N—Extractive-N) × 6.25.
Values represent the mean±standard deviation of three samples.

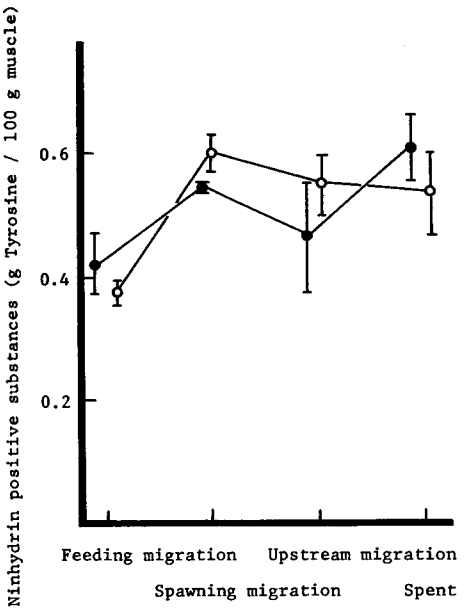

Fig. 3. Changes in ninhydrin positive substances of the muscle extracts during the spawning migration of male (●—●) and female (○—○) chum salmon. The vertical bars represent the mean ± standard deviation of three samples.

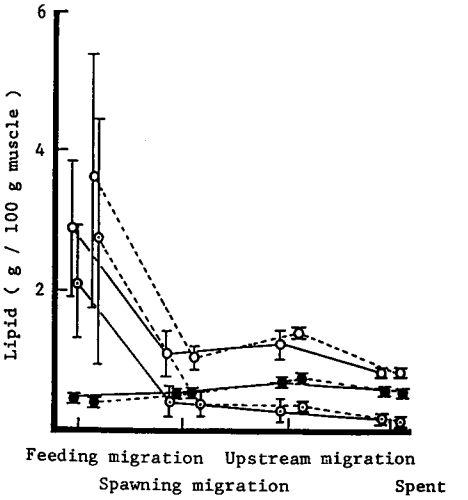

Fig. 4. Changes in total lipid (○), triglyceride (⊙), and phospholipid (●) contents of dorsal muscle during the spawning migration of male (——) and female (------) chum salmon. The vertical bars represent the mean ± standard deviation of three samples.

phase, and this tendency was preponderant in females (Fig. 2A). Testosterone levels markedly decreased during the upstream migration phase.

Serum levels of estradiol-17β were high during the spawning migration phase, and greatly decreased during the upstream migration phase (Fig. 2B).

Proximate composition of dorsal muscle at different migration stages

Table 2 shows the proximate composition of the dorsal muscle of chum salmon caught at different migration stages. The moisture content gradually increased, while the ash content gradually decreased in both males and females during upstream migration.

Protein content in both males and females was maintained during the spawning migration stage, but this markedly decreased during the upstream migration stage, but this markedly decreased during the upstream migration stage. It was also found that the changes in protein content were related to those in ninhydrin positive substances, which might correspond to free amino acids and low molecular peptides, of the extracts (Fig. 3). The ninhydrin positive substances gradually increased during spawning migration.

Figure 4 shows the changes in lipid composition during spawning migration. The lipid content markedly decreased during the spawning migration stage and this

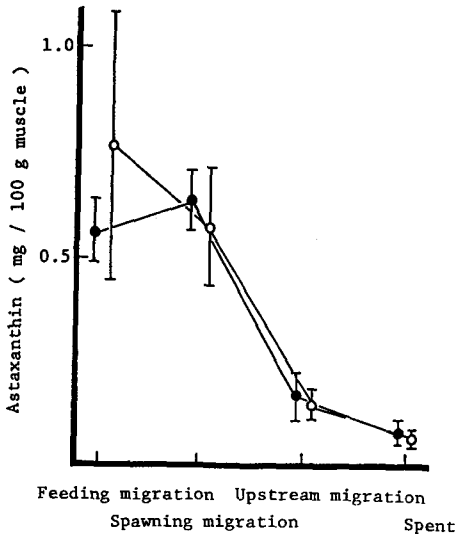

Fig. 5. Changes in astaxanthin content of dorsal muscle during the spawning migration of male (●—●) and female (○—○) chum salmon. The vertical bars represent the mean±standard deviation of three samples.

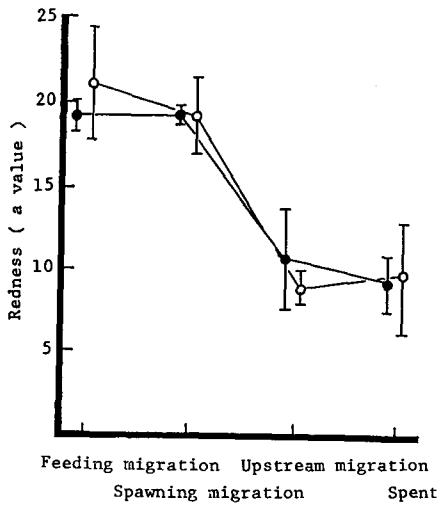

Fig. 6. Changes in redness of dorsal muscle during the spawning migration of male (●—●) and female (○—○) chum salmon. The vertical bars represent the mean±standard deviation of three samples.

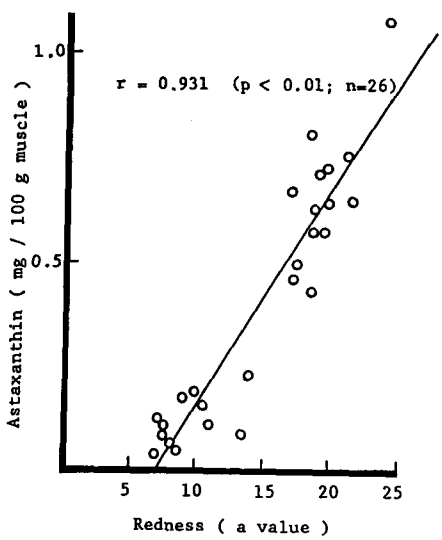

Fig. 7. Relationship between astaxanthin and redness of dorsal muscle during the spawning migration of chum salmon.

tendency was preponderant in females. As phospholipid was maintained at a constant level during spawning migration, triglyceride appears to have been responsible for the decrease in lipid content.

Changes in color of dorsal muscle at different migration stages

Figure 5 shows the changes in carotenoid content, which are represented as astaxanthin, of the dorsal muscle of chum salmon caught at different migration stages. Astaxanthin content in both males and females was maintained during the spawning migration stage, and this markedly decreased during the upstream migration stage.

Figure 6 shows the "a" value of dorsal muscle, which expresses the red color of muscle. The changes in "a" value were parallel with those of the astaxanthin content, as indicated by a coefficient of correla-

tion of 0.931 (as in Fig. 7)

Discussion

The results of this section have demonstrated that the proximate composition of dorsal muscle markedly changed in chum salmon while ascending the river to spawn.

It was shown that the changes in GSI values were related to those of testosterone and estradiol-17β levels. GSI values markedly increased during the spawning migration stage. The lipid content markedly decreased during the spawning migration stage, and this tendency was preponderantly recognized in females. The decrease in lipid content was caused by triglyceride but not by phospholipid. Therefore, the maturation of gonads in chum salmon during spawning migration was considered to be closely related to the changes in triglyceride.

Why did the protein content decrease during the upstream migration stage but not during the spawning migration ? It has been found that the chum salmon were feeding during the spawning migration stage but not during upstream migration. Since the chum salmon during the upstream migration stage were not feeding and deficient in lipid, it was assumed that the protein finally decreased at this stage. It was thought that the lipid of chum salmon muscle was utilized in order to mature the gonads and protein was used as an energy source to ascend the river. Thus, the decrease in lipid and protein content in chum salmon muscle was considered to be related to their physiological state. Considering that the ninhydrin positive substances in the extracts gradually increased during spawning migration, the decrease in protein content while ascending the river might be caused by the protease activity of muscle (Konagaya, 1982, 1983, 1985a, b ; Nomata *et al.*, 1985a).

As shown in Fig. 8, no close correlation was found between lipid content and astaxanthin (fatty pigment) as indicated by the low coefficients of determination ($r^2 = 0.377$). Consequently, the chum salmon muscle during spawning migration was characterized by protein, lipid and astaxanthin. These relationships are shown in Fig. 9. The chum salmon caught during the feeding migration stage showed high levels of protein, lipid and astaxanthin. However, during the spawning migration stage, a decrease in only the lipid content was found. The chum salmon after spawning showed lower levels of protein, lipid and astaxanthin than the pre-spawning salmon.

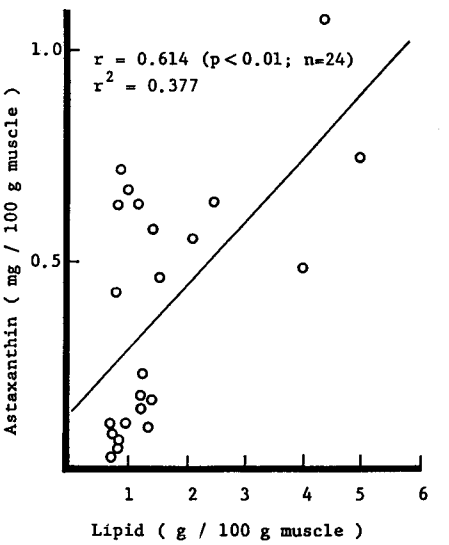

Fig. 8. Relationship between astaxanthin and lipid of dorsal muscle during the spawning migration of chum salmon.

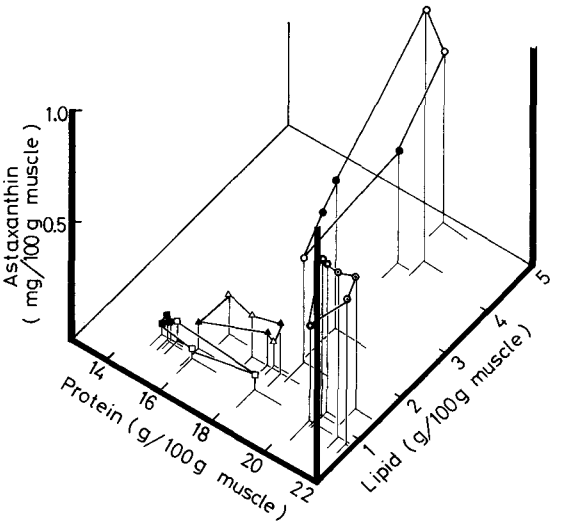

Fig. 9. Characteristics of dorsal muscle by protein, lipid and astaxanthin during the spawning migration of chum salmon. Feeding migration stage, (● male; ○ female); spawning migration stage, (⊙ male; ◎ female); upstream migration stage, (▲ male; △ female); spent, (■ male; □ female).

Section 2. Consumption of muscle lipid

In Section 1, gonadosomatic index values markedly increased, while a decrease in muscle lipid (particularly in triglyceride) content was found during spawning migration. This suggested that the gonadal maturation in chum salmon during spawning migration was closely related to the changes in triglyceride.

It is widely known that the lipid in muscle decreases during spawning migration. The decrease in muscle lipid may be due to gonadal maturation and the energy required for upstream migration. There are several reports stating that progressive spawning migration induces changes in lipid composition and fatty acid composition of muscle lipid, as well as a decrease in lipid content (Lovern, 1934; Zama and Igarashi, 1954; Ōta and Yamada, 1971, 1974a, b). It has been considered that selective consumption of some specific fatty acids occurs during progressive spawning starvation. It is, however, still unresolved as to whether or not the enzyme systems hydrolyzing some specific fatty acids are present in the chum salmon muscle during spawning migration.

The present study, using the analytical data of fatty acid composition and lipid composition, was therefore conducted to ascertain whether or not the selective consumption of fatty acids in muscle lipid occurred during spawning migration of chum salmon.

Materials and Methods

Materials

The same chum salmon muscles as shown in Table 1 were used as materials.

Lipid analyses

Procedures used for lipid extraction and lipid composition analysis were as previously described (Section 1).

Fatty acid composition was analyzed as follows. A portion of lipid was applied to preparative TLC (Wakogel B-0). The plate was sprayed with a Rhodamine 6G reagent, and non-phospholipid and phospholipid were detected under ultraviolet light. Fatty acid compositions of non-phospholipid and phospholipid were analyzed by gas-liquid chromatography (GLC) after methylation with methanol containing anhydrous hydrogen chloride (Prevot and Mordret, 1976). GLC analysis was performed with a Hitachi 063 gas chromatograph equipped with a hydrogen flame ionization detector, using a glass column (3 mm i.d. and 3 m length) packed with Unisol 3000 on a Uniport C (80-100 mesh).

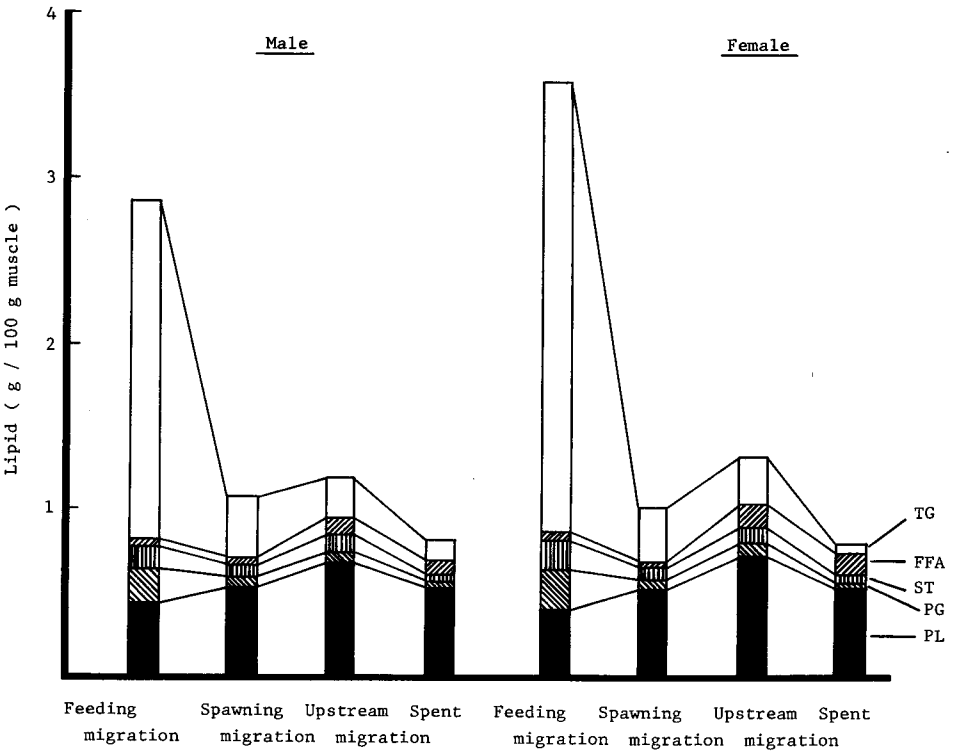

Fig. 10. Lipid composition of dorsal muscle. Abbreviations: PL, phospholipid; PG, partial glyceride; ST, sterol; FFA, free fatty acids; TG, triglyceride.

Results

Changes in lipid composition of dorsal muscle at different migration stages

Figure 10 shows the changes in lipid composition during spawning migration. Total lipid in muscle markedly decreased during spawning migration, in particular, the triglyceride. Free fatty acid gradually increased, while phospholipid was maintained at a constant level during spawning migration.

Changes in fatty acid composition of dorsal muscle at different migration stages

The relative ratio of monoenoic fatty acids to total lipid gradually decreased, while that of polyenoic fatty acids gradually increased during spawning migration. No significant difference in the relative ratio of saturated fatty acids to total lipid was found (Table 3). These results might suggest that monoenoic fatty acids in total lipid were selectively consumed during spawning migration.

However, the relative ratio of phospholipid to muscle lipid markedly increased (Fig. 10). It was proved that phospholipid was mainly composed of polyenoic fatty acids (Table 5). This might be the reason for the relative increase of polyenoic fatty acids during spawning migration of chum salmon. The changes in fatty acid compositions of non-phospholipid (Table 4) and phospholipid (Table 5) during spawning migration were smaller than those of total lipid (Table 3).

Discussion

Selective consumption of some specific fatty acids during progressive spawning migration starvation has been suggested for many years (Lovern, 1934 ; Zama and Igarashi, 1954 ; Ōta and Yamada, 1971, 1974a, b). As described above, however, it was proved that the changes in fatty acid composition of total lipid during spawning migration depended upon lipid composition. Therefore, in order to ascertain the possibility of selective consumption of fatty acids, the fatty acid contents of muscle lipid were estimated on the basis of the following assumptions :

1) Total lipid in muscle is composed of non-phospholipid and phospholipid.
2) Non-phospholipid is composed of partial glyceride (monoglyceride and diglyceride), sterol, free fatty acid, and triglyceride.
3) Phospholipid is composed of phosphatidylcholine.
4) Fatty acid compositions of partial glyceride, free fatty acid, and triglyceride are the same of non-phospholipid.
5) Fatty acid composition of phosphatidylcholine is the same of phospholipid.
6) Mean molecular weight of fatty acid in non-phospholipid is calculated from the fatty acid composition of non-phospholipid.
7) Mean molecular weight of fatty acid in phosphatidylcholine is calculated from the fatty acid composition of phospholipid.

Based on the result of Fig. 10 and above assumptions, fatty acid contents of total lipid during spawning migration of chum salmon were estimated as shown in Table 6. It was apparent that consumed fatty acid contents, which were induced from the result of Table 6, were higher in females than in males (Fig. 11). This

Table 3. Fatty acid composition of total lipid in dorsal muscle (weight %).

Fatty acids	Feeding migration		Spawning migration		Upstream migration		Spent	
	Male	Female	Male	Female	Male	Female	Male	Female
14 : 0	7.5	8.3	5.9	4.5	3.3	2.8	3.8	4.6
15 : 0	0.5	1.6	—	1.3	0.8	1.0	0.6	—
16 : 0	17.0	15.7	15.7	12.3	21.5	16.2	17.9	18.9
17 : 0	1.1	1.6	1.3	2.9	1.1	0.7	1.4	2.2
18 : 0	3.8	3.3	3.4	4.8	6.9	5.9	4.9	5.0
20 : 0	1.9	2.7	1.2	1.4	1.0	1.0	2.5	1.1
22 : 0	1.2	1.3	0.9	0.6	0.5	—	0.6	—
Saturated	33.0	34.5	28.4	27.8	35.1	27.6	31.7	31.8
16 : 1	6.2	6.4	8.3	5.5	1.6	5.5	3.8	2.3
17 : 1	0.5	1.0	0.9	—	1.0	0.7	0.1	—
18 : 1	25.4	21.0	27.5	24.3	20.9	20.0	12.7	17.4
19 : 1	1.3	1.5	1.0	1.3	1.4	1.1	2.3	1.1
20 : 1	6.3	8.1	7.1	8.1	3.3	2.9	7.5	6.0
22 : 1	6.5	7.7	2.2	4.5	1.7	1.4	2.3	3.8
Monoenoic	46.2	45.7	47.0	43.7	29.9	31.6	28.7	30.6
18 : 2	2.2	2.3	2.4	2.7	1.9	2.0	5.9	2.2
20 : 4	0.1	0.4	—	0.7	—	1.0	1.3	1.1
20 : 5	7.1	5.3	5.8	3.2	5.9	10.2	6.9	9.5
22 : 5	1.0	0.7	1.3	2.0	1.2	2.1	1.4	1.5
22 : 6	10.4	11.1	15.1	19.9	26.0	25.5	24.1	23.3
Polyenoic	20.8	19.8	24.6	28.5	35.0	40.8	39.6	37.6

Table 4. Fatty acid composition of non-phospholipid in dorsal muscle (weight %).

Fatty acids	Feeding migration		Spawning migration		Upstream migration		Spent	
	Male	Female	Male	Female	Male	Female	Male	Female
14 : 0	8.7	8.0	5.1	4.0	3.1	3.3	3.3	6.5
15 : 0	—	0.5	0.3	0.3	—	—	—	0.2
16 : 0	17.2	13.6	11.4	8.9	8.9	7.5	6.7	7.7
17 : 0	1.2	1.2	1.3	0.9	0.7	0.8	0.8	0.7
18 : 0	3.6	2.8	3.1	2.8	4.2	1.9	1.8	2.3
20 : 0	2.0	2.9	1.6	1.4	0.5	1.2	1.0	0.8
22 : 0	1.3	1.4	1.2	0.8	0.4	0.5	0.3	0.5
Saturated	34.0	30.4	24.0	19.1	17.8	15.2	13.9	18.7
16 : 1	7.0	7.1	8.1	7.4	5.5	10.7	3.3	6.1
17 : 1	—	0.6	1.1	0.6	1.0	0.6	0.7	0.6
18 : 1	25.2	23.1	25.4	27.0	34.4	33.7	14.0	20.1
19 : 1	1.4	1.5	1.0	0.9	0.7	1.1	1.2	0.5
20 : 1	6.2	10.4	9.3	14.5	10.5	6.0	29.8	18.5
22 : 1	6.5	11.0	7.6	8.4	6.6	2.7	17.8	13.3
Monoenoic	46.3	53.7	52.5	58.8	58.7	54.8	66.8	59.1
18 : 2	1.8	2.0	1.7	1.8	1.1	1.2	2.2	1.3
20 : 4	—	0.5	0.6	0.5	0.7	0.5	0.4	0.6
20 : 5	6.8	4.6	6.7	3.3	3.6	5.0	3.1	6.1
22 : 5	1.2	1.1	2.2	2.5	2.0	2.9	1.2	2.7
22 : 6	9.9	7.7	12.3	14.0	16.1	20.4	12.4	11.5
Polyenoic	19.7	15.9	23.5	22.1	23.5	30.0	19.3	22.2

Table 5. Fatty acid composition of phospholipid in dorsal muscle (weight %).

Fatty acids	Feeding migration		Spawning migration		Upstream migration		Spent	
	Male	Female	Male	Female	Male	Female	Male	Female
14 : 0	3.2	2.6	1.0	1.0	0.9	1.3	2.0	1.9
15 : 0	0.5	0.6	0.2	0.2	0.2	0.1	0.3	0.2
16 : 0	22.1	22.1	16.9	19.0	22.7	25.3	21.2	19.0
17 : 0	1.0	0.9	0.6	0.8	0.6	0.7	0.6	0.6
18 : 0	2.9	2.4	4.2	7.0	6.9	6.5	3.9	4.5
20 : 0	0.5	0.7	0.3	0.3	0.2	0.3	0.9	0.6
22 : 0	0.8	0.9	0.5	0.6	0.4	0.5	1.0	0.6
Saturated	31.0	30.2	23.7	28.9	31.9	34.7	29.9	27.4
16 : 1	1.2	1.1	0.7	1.2	1.0	1.5	0.9	1.6
17 : 1	0.2	0.3	0.1	0.2	0.2	0.1	0.2	0.2
18 : 1	9.7	8.7	8.1	11.7	11.3	13.8	8.0	10.2
19 : 1	0.7	0.7	0.4	0.4	0.4	0.5	0.9	0.5
20 : 1	1.5	1.9	1.1	2.3	1.4	1.4	4.5	2.4
Monoenoic	13.3	12.7	10.4	15.8	14.3	17.3	14.5	14.9
18 : 2	0.8	0.9	0.5	0.7	0.6	0.6	1.9	0.5
20 : 4	0.8	0.8	1.1	1.4	1.3	1.2	1.4	1.0
20 : 5	10.7	8.7	9.3	9.3	10.4	14.0	11.9	13.8
22 : 5	1.9	1.6	2.3	2.3	1.8	1.7	1.7	3.3
22 : 6	41.5	45.1	52.7	41.6	39.7	30.5	38.7	39.1
Polyenoic	55.7	57.1	65.9	55.3	53.8	48.0	55.6	57.7

Table 6. Fatty acid contents of non-phospholipid, phospholipid, and total lipid in dorsal muscle.

	Feeding migration		Spawning migration		Upstream migration		Spent	
	Male	Female	Male	Female	Male	Female	Male	Female
	g/100 g muscle							
Partial glyceride	0.20	0.23	0.06	0.06	0.05	0.08	0.02	0.02
Partial glyceride-fatty acid	0.17	0.20	0.05	0.05	0.04	0.07	0.02	0.02
Sterol	0.15	0.20	0.08	0.07	0.11	0.10	0.07	0.06
Sterol-fatty acid	—	—	—	—	—	—	—	—
Free fatty acid	0.05	0.05	0.03	0.03	0.11	0.14	0.08	0.15
Free fatty acid-fatty acid	0.05	0.05	0.03	0.03	0.11	0.14	0.08	0.15
Triglyceride	2.06	2.70	0.38	0.34	0.23	0.30	0.10	0.07
Triglyceride-fatty acid	1.97	2.59	0.36	0.33	0.22	0.29	0.10	0.07
Non-phospholipid	2.46	3.18	0.55	0.50	0.50	0.62	0.27	0.30
Non-phospholipid-fatty acid	2.20	2.84	0.45	0.41	0.37	0.50	0.19	0.23
Phospholipid	0.41	0.39	0.52	0.51	0.68	0.72	0.53	0.51
Phospholipid-fatty acid	0.29	0.28	0.37	0.36	0.48	0.51	0.38	0.36
Total lipid	2.87	3.57	1.07	1.01	1.18	1.34	0.80	0.81
Total lipid-fatty acid	2.49	3.12	0.82	0.77	0.86	1.01	0.57	0.60

Table 7. Individual fatty acid contents of total lipid in dorsal muscle.

Fatty acids	Feeding migration		Spawning migration		Upstream migration		Spent	
	Male	Female	Male	Female	Male	Female	Male	Female
14 : 0	0.186	0.259	0.048	0.035	0.029	0.028	0.022	0.028
15 : 0	0.014	0.051	—	0.010	0.007	0.011	0.004	—
16 : 0	0.423	0.490	0.129	0.095	0.184	0.163	0.102	0.113
17 : 0	0.027	0.051	0.011	0.022	0.009	0.007	0.008	0.013
18 : 0	0.093	0.103	0.028	0.037	0.059	0.059	0.028	0.030
20 : 0	0.046	0.084	0.010	0.011	0.009	0.010	0.014	0.006
22 : 0	0.031	0.039	0.007	0.005	0.004	—	0.004	—
Saturated	0.820	1.077	0.233	0.215	0.301	0.278	0.182	0.190
16 : 1	0.155	0.200	0.068	0.042	0.014	0.055	0.022	0.014
17 : 1	0.013	0.030	0.007	—	0.008	0.006	—	—
18 : 1	0.633	0.653	0.226	0.188	0.180	0.202	0.073	0.104
19 : 1	0.032	0.046	0.008	0.010	0.012	0.012	0.013	0.007
20 : 1	0.158	0.253	0.058	0.063	0.028	0.030	0.043	0.036
22 : 1	0.162	0.240	0.018	0.035	0.015	0.014	0.013	0.023
Monoenoic	1.153	1.422	0.385	0.338	0.257	0.319	0.164	0.184
18 : 2	0.056	0.073	0.020	0.021	0.016	0.021	0.034	0.013
20 : 4	—	0.011	—	0.005	—	0.010	0.008	0.007
20 : 5	0.176	0.166	0.047	0.025	0.051	0.103	0.039	0.057
22 : 5	0.025	0.021	0.011	0.015	0.011	0.021	0.008	0.009
22 : 6	0.259	0.345	0.124	0.154	0.223	0.257	0.138	0.139
Polyenoic	0.516	0.616	0.202	0.220	0.301	0.412	0.227	0.225
Total	2.489	3.115	0.820	0.773	0.859	1.009	0.573	0.599

Table 8. Individual consumed fatty acid contents of total lipid in dorsal muscle during spawning migration.

	Spawning migration		Upstream migration		Spent	
	Male	Female	Male	Female	Male	Female
Fatty acids	g/100 g muscle					
14 : 0	0.138 (8.27)	0.224 (9.56)	0.157 (9.63)	0.231 (10.97)	0.164 (8.56)	0.231 (9.18)
15 : 0	0.014 (0.84)	0.041 (1.75)	0.007 (0.43)	0.040 (1.90)	0.010 (0.52)	0.051 (2.03)
16 : 0	0.294 (17.61)	0.395 (16.86)	0.239 (14.66)	0.327 (15.53)	0.321 (16.76)	0.377 (14.98)
17 : 0	0.016 (0.96)	0.029 (1.24)	0.018 (1.10)	0.044 (2.09)	0.019 (0.99)	0.038 (1.51)
18 : 0	0.065 (3.89)	0.066 (2.82)	0.034 (2.09)	0.044 (2.09)	0.065 (3.39)	0.073 (2.90)
20 : 0	0.036 (2.16)	0.073 (3.12)	0.037 (2.27)	0.074 (3.51)	0.032 (1.67)	0.078 (3.10)
22 : 0	0.024 (1.44)	0.034 (1.45)	0.027 (1.66)	0.039 (1.85)	0.027 (1.41)	0.039 (1.55)
Saturated	0.587 (35.17)	0.862 (36.80)	0.519 (31.84)	0.799 (37.94)	0.638 (33.30)	0.887 (35.25)
16 : 1	0.087 (5.21)	0.158 (6.75)	0.141 (8.65)	0.145 (6.88)	0.133 (6.94)	0.186 (7.39)
17 : 1	0.006 (0.36)	0.030 (1.28)	0.005 (0.31)	0.024 (1.14)	0.013 (0.68)	0.030 (1.19)
18 : 1	0.407 (24.39)	0.465 (19.86)	0.453 (27.79)	0.451 (21.42)	0.560 (29.23)	0.549 (21.82)
19 : 1	0.024 (1.44)	0.036 (1.54)	0.020 (1.23)	0.034 (1.61)	0.019 (0.99)	0.039 (1.55)
20 : 1	0.100 (5.99)	0.190 (8.11)	0.130 (7.97)	0.223 (10.59)	0.115 (6.00)	0.217 (8.63)
22 : 1	0.144 (8.63)	0.205 (8.75)	0.147 (9.02)	0.226 (10.73)	0.149 (7.78)	0.217 (8.63)
Monoenoic	0.768 (46.02)	1.084 (46.29)	0.896 (54.97)	1.103 (52.37)	0.989 (51.62)	1.238 (49.21)
18 : 2	0.036 (2.15)	0.052 (2.22)	0.040 (2.45)	0.052 (2.47)	0.022 (1.15)	0.060 (2.38)
20 : 4	— (—)	0.006 (0.26)	— (—)	0.001 (0.05)	−0.008 (−0.42)	0.004 (0.16)
20 : 5	0.129 (7.73)	0.141 (6.02)	0.125 (7.67)	0.063 (2.99)	0.137 (7.15)	0.109 (4.33)
22 : 5	0.014 (0.84)	0.006 (0.26)	0.014 (0.86)	— (—)	0.017 (0.89)	0.012 (0.48)
22 : 6	0.135 (8.09)	0.191 (8.15)	0.036 (2.21)	0.088 (4.18)	0.121 (6.31)	0.206 (8.19)
Polyenoic	0.314 (18.81)	0.396 (16.91)	0.215 (13.19)	0.204 (9.69)	0.289 (15.08)	0.391 (15.54)
Total	1.669 (100)	2.342 (100)	1.630 (100)	2.106 (100)	1.916 (100)	2.516 (100)

Values in parenthesis are percentage of total consumed fatty acid contents.

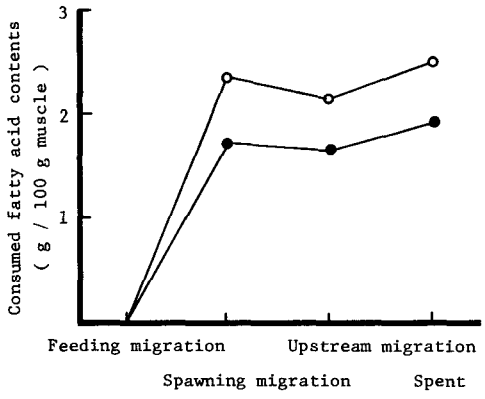

Fig. 11. Consumed fatty acid contents of total lipid in dorsal muscle during spawning migration. Male, (●); female, (○).

might suggest that gonadal maturation was responsible for the consumption of fatty acids in muscle lipid. It was also shown that the consumed fatty acid contents of muscle lipid gradually increased during spawning migration. This might suggest that the fatty acids of muscle lipid were used as an energy source of spawning migration, as well as, the gonadal maturation.

Based on the results of Tables 3 and 6, the individual fatty acid contents of muscle lipid were estimated (Table 7). It was indicated that the relative ratio of individual consumed fatty acids, which was induced based on the result as shown in Table 7, was almost the same among chum salmon of different physiological state, although the consumed fatty acids gradually increased during spawning migration (Table 8).

As described above, it was consequently concluded that no selective consumption of fatty acids in muscle lipid occurred during spawning migration of chum salmon.

Section 3. Muscle protein degradation and protease activity

In Section 1 of the present chapter, it has been shown that the chemical composition of chum salmon muscle during spawning migration is closely related to their physiological state. Furthermore, the decrease in muscle protein content while ascending the river has been considered to be caused by the protease activity of muscle, because the ninhydrin positive substances of non-protein nitrogenous compound gradually increased during spawning migration.

Konagaya (1982, 1983, 1985a, b) and Nomata *et al.* (1985a) have reported that protease activity of muscle is enhanced during spawning migration of chum salmon and high level of protease activity is found in very mushy muscle. Mommsen *et al.* (1980) have reported that cathepsin D activity in muscle increases during spawning migration of sockeye salmon. It is suggested that the protease activity of muscle varies with physiological state of chum salmon.

The aim of the present study is therefore to clarify the relation between protein degradation and protease activity of chum salmon muscle during spawning migration with regard to their physiological state.

Materials and Methods

Materials

The same chum salmon muscles as shown in Table 1 were used as materials.

Determination of protein composition and SDS-polyacrylamide gel electrophoresis

Muscle protein was fractionated using a procedure shown in Fig. 12. The protein and non-protein fractions were determined using the biuret (Gornall *et al.*, 1949) and micro-Kjeldahl methods, respectively, and protein composition was calculated. Portions of the protein fractions were used for electrophoretic analysis.

SDS-polyacrylamide gel electrophoresis was carried out in a 10% polyacrylamide gel using a slab gel apparatus according to the method of Laemmli (1970).

Measurement of autolytic activity

Muscle autolytic activity was determined according to the method of Konagaya (1982). Muscle tissue was homogenized with 3 vol. of distilled water. The reaction mixture, consisting of 1 m*l* of muscle homogenate and 1 m*l* of McIlvaine's buffer (pH 2.2–8.0) was incubated at 37°C for 4 hr. After this time, 2 m*l* of 5% (w/v) trichloroacetic acid (TCA) was added. The mixture was allowed to remain for 1 hr at room temperature and then filtered through a Toyo No. 3 paper. Blank mixtures were not incubated but the muscle homogenate, TCA and the buffer were mixed in this order. The TCA-soluble products in the filtrate were determined by Cu-Folin (Lowry *et al.*, 1951) and ninhydrin methods (Yemm and Cocking, 1955). Specific activity was expressed as μmoles tyrosine equivalent released from the reaction mixture in 4 hr in the presence of 1 mg of protein from muscle homogenate, and tissue activity as that released per g of muscle.

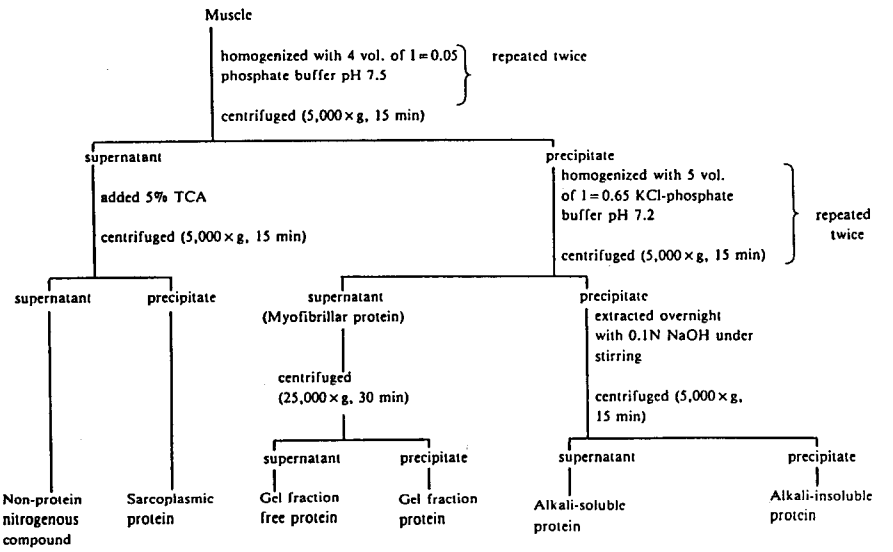

Fig. 12. Fractionation procedure of dorsal muscle proteins of chum salmon. I = ionic strength.

Measurements of acid and neutral proteinase activities

Muscle proteinase activity was determined according to the method of Makinodan *et al.* (1983). Muscle tissue was homogenized in 2 vol. of cold 33 mM phosphate buffer (pH 7.2). After standing for 3 hr at 4°C, the homogenate was centrifuged at 13,000×*g* for 30 min. The crude enzyme solution was prepared by dialyzing the supernatant overnight at 4°C against 33 mM phosphate buffer (pH 7.2).

A 5% (w/v) solution of hemoglobin was made by dissolution in 0.06 N hydrochloric acid and overnight dialysis against distilled water. The pH of the hemoglobin solution was adjusted to 3.0 with 1 N hydrochloric acid for acid proteinase assay, and to 7.0 with 1 N sodium hydroxide for neutral proteinase.

The reaction mixture for acid proteinase activity, consisting of 1.5 m*l* of McIlvaine's buffer (pH 2.2-5.0), 0.5 m*l* of hemoglobin solution, and 0.5 m*l* of crude enzyme solution was incubated at 37°C for 1 hr. Then, 2.5 m*l* of 5% (w/v) TCA was added. The mixture was allowed to stand for 1 hr at room temperature, and then filtered through a Toyo No. 3 paper. Blank mixtures were incubated separately from the other components of the reaction mixture and then combined after the addition of TCA. Cu-Folin value was measured by the method of Lowry *et al.* (1951). Specific activity, and tissue activity were expressed as described for measurement of autolytic activity.

The same procedure described above was used for neutral proteinase activity except that it was incubated using Kolthoff's buffer (pH 6.2-8.0) for 2 hr. The TCA-soluble components were determined by ninhydrin method (Yemm and Cocking, 1955).

Preparation of acid proteinase with Triton X-100

The same procedure described above was used except that it was homogenized in 2 vol. of cold 33 mM phosphate buffer (pH 7.2) containing 0.2% (v/v) Triton X-100.

Results

Changes in protein composition

Table 9 shows the changes in protein composition of dorsal muscle during spawning migration. It was noticeable that sarcoplasmic protein markedly decreased during spawning migration. In contrast, no change in myofibrillar content was found during spawning migration. Gel fraction protein which was fractionated from myofibrillar protein, however, gradually increased from samples ascending the river. Alkali-soluble and insoluble protein contents did not greatly change during spawning migration.

Changes in protein composition were detected by SDS-polyacrylamide gel electrophoresis (Fig. 13). The degradation of myosin heavy chain was apparently observed, while sarcoplasmic protein electrophoretograms were not changed, during spawning migration.

Table 9. Changes in protein composition of dorsal muscle during the spawning migration of chum salmon.

		Total protein	Sarcoplasmic protein	Myofibrillar protein	Gel fraction free protein	Gel fraction protein	Alkali-soluble protein	Alkali-insoluble protein
		g/100 g muscle						
Feeding migration	Male	18.26±0.04	7.15±0.99 (38.41±5.38)*	9.35±0.61 (50.24±3.16)*	3.73±0.79 (20.02± 4.23)	5.62±0.82 (30.20± 4.36)*	1.82±0.40 (9.79±2.16)*	0.29±0.13 (1.58±0.69)*
	Female	18.80±0.27	6.30±0.63 (33.49±2.93)	10.75±0.86 (57.24±5.32)	4.60±1.02 (24.46± 5.46)	6.16±1.52 (32.80± 8.38)	1.45±0.36 (7.68±1.78)	0.30±0.17 (1.57±0.86)
Spawning migration	Male	21.36±0.27	7.25±0.21 (33.93±0.78)	11.82±0.06 (55.36±0.63)	5.68±0.84 (26.58± 3.74)	6.15±0.79 (28.81± 4.00)	2.11±0.17 (9.88±0.75)	0.17±0.08 (0.81±0.37)
	Female	20.21±0.11	6.26±0.32 (30.97±1.43)	12.30±0.54 (60.88±2.76)	6.74±2.69 (33.40±13.49)	5.56±2.23 (27.50±10.96)	1.50±0.26 (7.44±1.29)	0.14±0.08 (0.71±0.38)
Upstream migration	Male	17.37±1.39	4.45±0.16 (25.76±2.71)	10.56±1.13 (60.71±1.99)	3.10±0.28 (17.86± 1.16)	7.46±0.92 (42.84± 1.95)	1.84±0.25 (10.61±1.06)	0.52±0.30 (2.93±1.52)
	Female	17.12±0.92	4.57±0.38 (26.70±1.84)	11.25±0.79 (65.69±2.19)	4.16±0.34 (24.34± 2.32)	7.09±0.97 (41.35± 4.49)	1.06±0.07 (6.19±0.66)	0.24±0.10 (1.39±0.57)
Spent	Male	15.09±0.67	3.62±0.33 (23.95±1.68)	9.32±0.42 (61.75±0.92)	2.07±0.42 (13.76± 3.10)	7.25±0.60 (47.99± 2.29)	1.73±0.35 (11.48±2.31)	0.42±0.07 (2.80±0.55)
	Female	15.89±1.90	3.52±0.94 (21.91±3.08)	9.92±1.95 (62.24±8.55)	2.61±1.03 (16.09± 4.31)	7.31±1.24 (46.16± 7.97)	1.96±0.95 (12.65±7.01)	0.49±0.31 (3.15±2.23)

* % of total protein.

Values represent the mean±standard deviation of three samples.

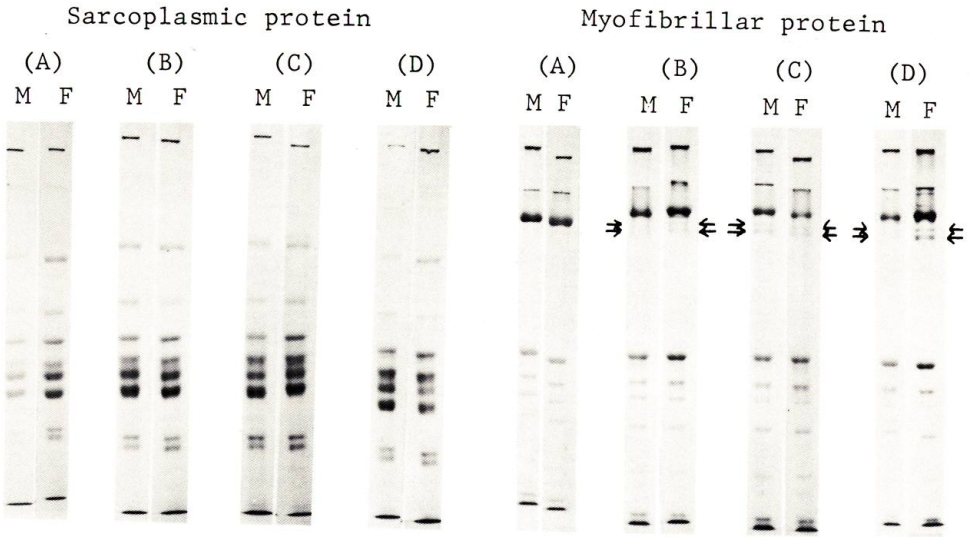

Fig. 13. SDS-slab-polyacrylamide gel (10%) electrophoretic patterns of muscle protein fractions during the spawning migration of male (M) and female (F) chum salmon. (A), feeding migration stage; (B), spawning migration stage; (C), upstream migration stage; (D), spent.

Changes in autolytic activity

Figure 14 shows the changes in autolytic activity of dorsal muscle during spawning migration. The autolytic activity which was expressed as Cu-Folin and ninhydrin positive substances increased when chum salmon were ascending the river to spawn. High levels of autolytic activity were found at around pH 3. It was noticeable that differences in autolytic activity were found between males and females.

Figure 15 shows the changes in autolytic activities at pH 3 and pH 6 during spawning migration. Autolytic activity at pH 3 markedly increased during the upstream migration stage and slightly decreased after spawning. It was also observed that autolytic activity at pH 6, corresponding to physiological state, gradually increased during spawning migration.

Changes in acid and neutral proteinase activities

High levels of acid and neutral proteinase activities were observed at pH 3 and pH 6.2. Changes in acid and neutral proteinase activities during spawning migration were almost the same as those in autolytic activity (Fig. 16).

Effect of Triton X-100 on acid proteinase activity

Table 10 shows the effect of Triton X-100 on acid proteinase activity during spawning migration. No marked activation in acid proteinase activity was found when this enzyme was extracted from muscle tissue in the presence of Triton X-100.

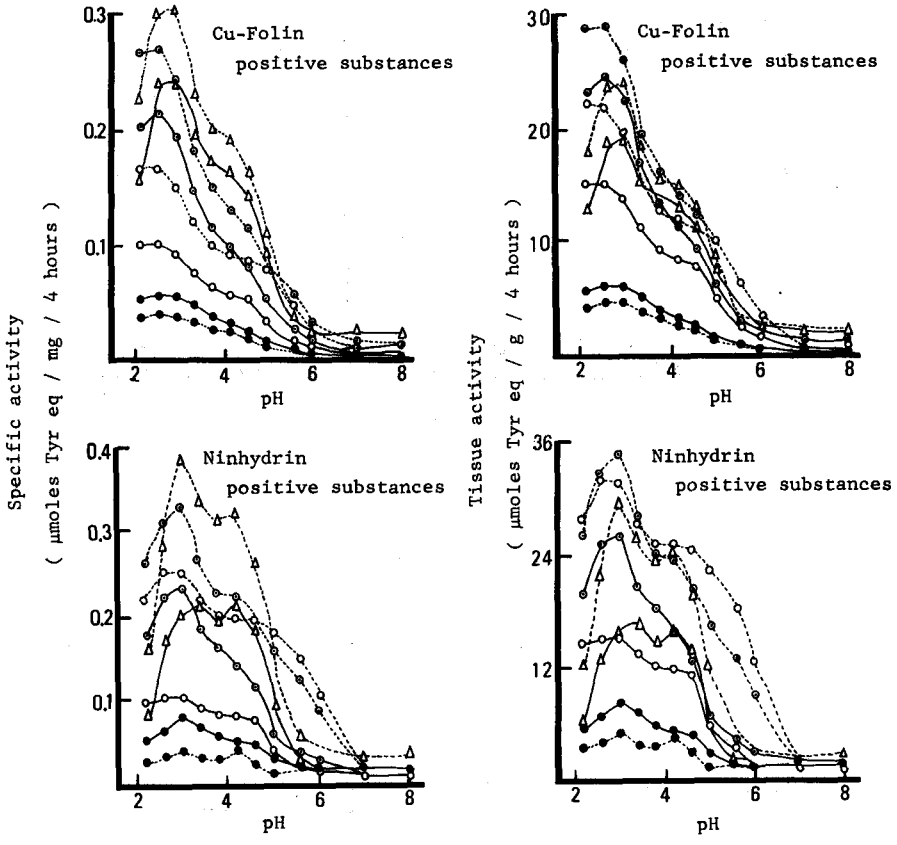

Fig. 14. Autolytic activity of dorsal muscle homogenate during the spawning migration of male (——) and female (------) chum salmon as a function of pH. Feeding migration, (●); spawning migration, (○); upstream migration, (⊙); spent, (△).

Discussion

In Section 1 of the present chapter, it has been considered that the decrease in protein during spawning migration may be related to the protease activity of muscle. The relation between protein degradation and protease activity will be discussed below with regard to the physiological state of fish.

Sarcoplasmic protein markedly decreased and ninhydrin positive substances of non-protein nitrogenous compound gradually increased during spawning migration (Fig. 3). This suggested that ninhydrin positive substances might have been caused by the degradation of sarcoplasmic protein. As shown in Fig. 17, a high coefficient of correlation was shown only between sarcoplasmic protein and ninhydrin positive substances ($r = -0.588$, $P < 0.01$). As acid and neutral proteinase activities markedly increased during spawning migration, it was considered that these proteinase activities were closely related to the degradation of sarcoplasmic protein. Judging

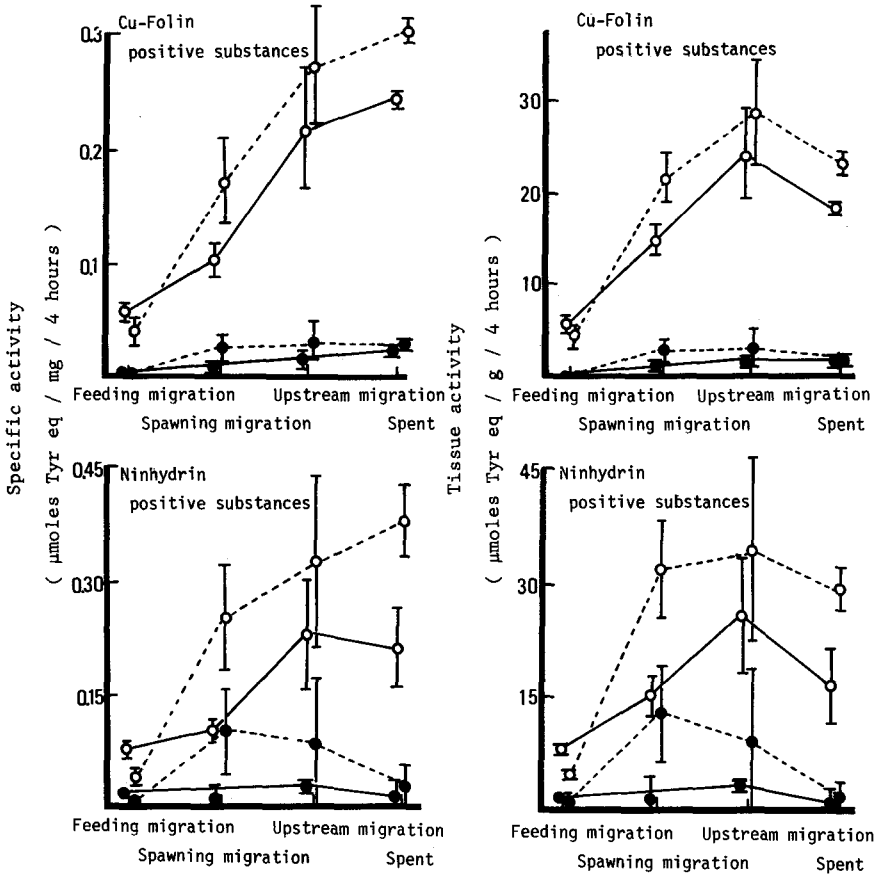

Fig. 15. Changes in autolytic activity of dorsal muscle homogenate at pH 3 (○) and pH 6 (●) during the spawning migration of male (——) and female (------) chum salmon. The vertical bars represent the mean±standard deviation of three samples.

from the fact that myosin heavy chain was gradually degraded during spawning migration, these proteinase activities might influence not only sarcoplasmic protein but also myofibrillar protein.

Why did the protease activity increase during spawning migration? High levels of protease activity were found at acidic region (around pH 3) and this protease activity markedly increased during spawning migration. It has been known that acid proteinases are contained in lysosomes (Reddi *et al.*, 1972; Dutson and Lawrie, 1974; Whiting *et al.*, 1975; Karvinen *et al.*, 1982; Makinodan *et al.*, 1982). Consequently, high levels of acid proteinase activity at the upstream migration stage might be caused by the injury of lysosomal membranes. If this is true, a marked activation of acid proteinase by Triton X-100 should be observed at the feeding migration stage. This assumption, however, was not confirmed based on the result as shown in Table 10.

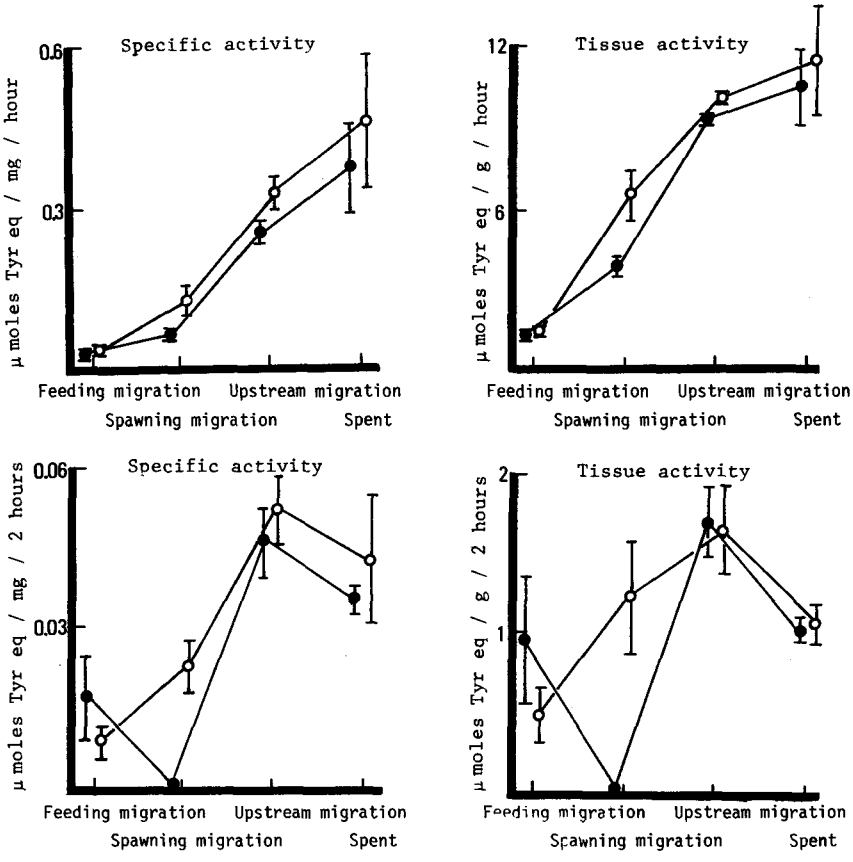

Fig. 16. Changes in acid (pH 3.0, upper column) and neutral (pH 6.2, lower column) proteinase activities of dorsal muscle during the spawning migration of male (●—●) and female (○—○) chum salmon. The vertical bars represent the mean ± standard deviation of three samples.

The changes in serum steroid hormones (Fig. 2) were similar to those in the autolytic activity, and acid and neutral proteinase activities during spawning migration. Serum levels of testosterone were markedly higher in females than in males and the same tendency was shown in acid and neutral proteinases. These results, therefore, strongly suggested that the activation of protease was closely related to the physiological state of fish. It was also shown that serum testosterone levels have greater influence in neutral protease than in acid protease (Fig. 18). High levels of protease activity, especially neutral proteinase, during spawning migration might be related to the utilization of protein as an energy source in order to ascend the river.

Table 10. Effect of Triton X-100 on acid proteinase activity of dorsal muscle during the spawning migration of chum salmon.

Stage	Sex	Activation of acid proteinase by Triton X-100 (pH)					
		2.2	2.6	3.0	3.4	4.0	5.0
Feeding migration	Male	123.2 ±37.5	115.7 ± 4.6	140.0 ±47.2	106.2 ± 3.2	98.5 ±14.5	108.7 ±29.0
	Female	108.8 ± 3.1	120.3 ± 8.8	120.1 ±23.2	110.0 ±21.3	104.8 ±15.2	76.9 ± 2.0
Spawning migration	Male	113.7 ± 4.2	150.9 ±37.4	131.1 ±36.9	93.0 ±35.9	120.4 ±12.3	87.5 ±24.6
	Female	106.5 ± 4.6	107.4 ± 5.4	106.7 ± 7.8	110.8 ± 5.1	115.4 ± 7.3	108.5 ± 3.8
Upstream migration	Male	101.1 ± 9.5	107.0 ± 3.9	110.2 ±12.2	106.2 ± 2.8	115.9 ± 8.4	97.6 ± 9.1
	Female	108.6 ±17.5	95.0 ± 7.0	106.3 ± 3.1	106.7 ± 2.5	115.3 ± 1.3	108.2 ±11.2
Spent	Male	95.3 ± 3.9	107.9 ± 6.8	106.2 ±10.7	98.3 ±13.1	104.2 ±11.9	106.0 ± 9.2
	Female	98.4 ± 6.2	98.6 ± 2.4	101.3 ±6.3	105.5 ± 4.4	105.2 ± 8.6	101.2 ±10.1

Values (mean±standard deviation) are percentage of acid proteinase activity with Triton X-100 to that without.

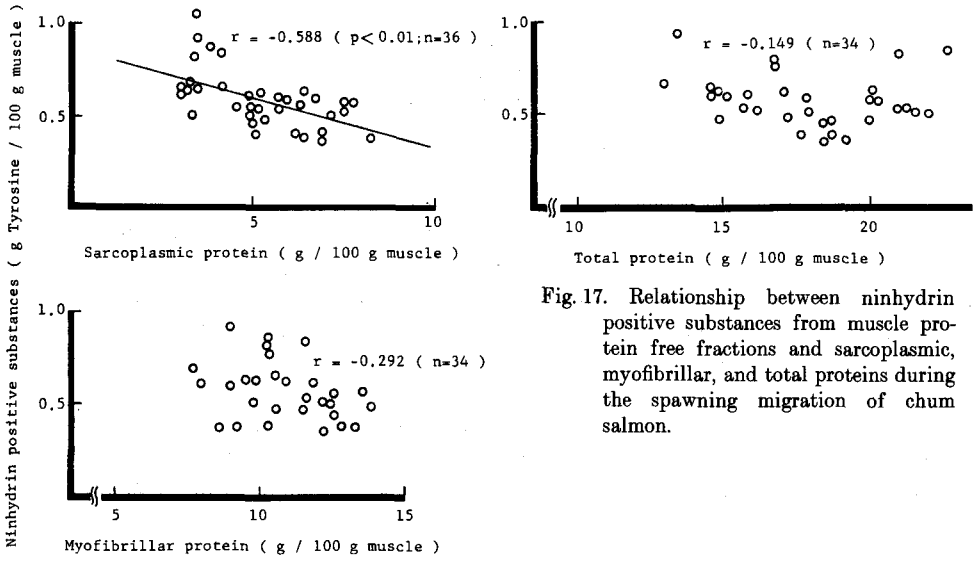

Fig. 17. Relationship between ninhydrin positive substances from muscle protein free fractions and sarcoplasmic, myofibrillar, and total proteins during the spawning migration of chum salmon.

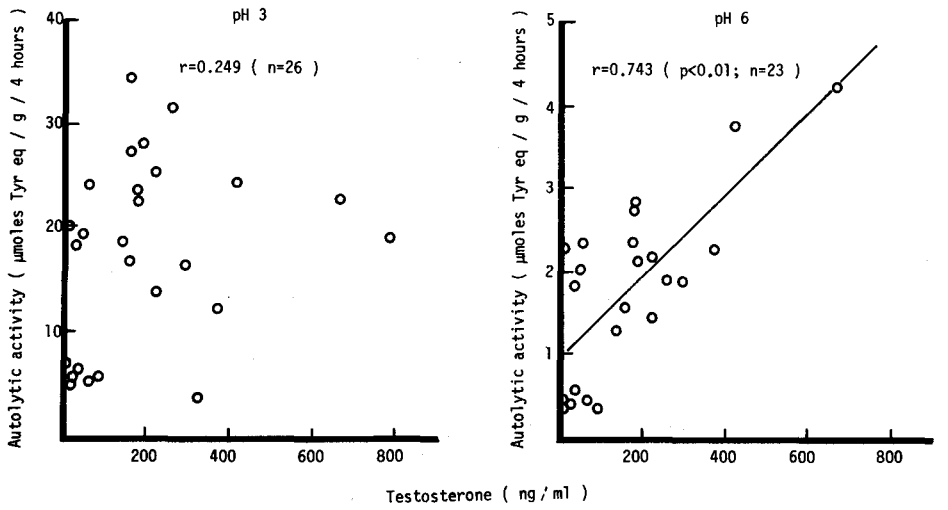

Fig. 18. Relationship between autolytic activity of dorsal muscle homogenate and serum testosterone levels during the spawning migration of chum salmon.

Section 4. Myofibrillar protein degradation, as evaluated by extractive N^{τ}-methylhistidine level

In Sections 1 and 3 of the present chapter, it has been shown that muscle proteins, sarcoplasmic and myofibrillar proteins, decrease during spawning migration of chum salmon. Furthermore, ninhydrin positive substances from muscle protein free fractions increased during spawning migration. However, no direct relation was demonstrated between the decrease of muscle proteins and the increase of ninhydrin positive substances.

N^{τ}-Methylhistidine (MeHis) has been used as an index of myofibrillar protein degradation *in vivo*. This amino acid is contained only in actin and myosin (Asatoor and Armstrong, 1967; Johnson *et al.*, 1967) and is formed by the post-translational methylation of specific histidine residue in the peptide chain of actin and myosin (Young *et al.*, 1970, 1972). MeHis is neither reutilized for protein synthesis nor metabolized in mammals, and is excreted quantitatively into urine (Young *et al.*, 1972; Long *et al.*, 1975). The presence of MeHis has also been reported in trout actin (Johnson *et al.*, 1967; Bridgen, 1971). Therefore, its level in muscle extracts may reflect the degree of myofibrillar protein degradation during spawning migration of chum salmon.

In the present section, the degree of myofibrillar protein degradation during spawning migration was estimated by MeHis amount in the extracts of chum salmon muscle. By measuring MeHis amount in the extracts, it was proved that myofibrillar protein was degraded during the spawning migration.

Materials and Methods

Materials

A list of chum salmon specimens collected at three different migratory stages is given in Table 11. These samples were kept at −20°C until use. Only dorsal muscle was analyzed for these experiments.

Preparation of extracts

Muscle extracts were prepared according to the method of Konosu *et al.* (1983). The minced muscle (30 g) was homogenized with 100 m*l* of absolute ethanol under cooling with ice-water. The homogenate was centrifuged at 5,000 rpm for 15 min and the precipitate was extracted twice with 100 m*l* of 80% ethanol in the same manner. The combined supernatants were evaporated under reduced pressure below 30°C to remove ethanol. The aqueous solution obtained was defatted several times with an equal volume of diethyl ether. The aqueous layer was evaporated under reduced pressure and made up to 25 m*l* with water.

Isolation of MeHis by ion-exchange column chromatography

Ion-exchange chromatography was performed using 100-200 mesh Dowex 50W-X8 (pyridine form) (Nishizawa *et al.*, 1978). The resin was packed by gravity into a column (2.0×30 cm) to give a bed height of 5.5 cm and a column volume of 17 m*l*. The column had previously been equilibrated with 0.2 M pyridine. The extracts, corresponding to 2–3 g of muscle, were evaporated to dryness and the residues were dissolved in 5 m*l* of 0.2 M pyridine and applied to the column. The acidic and neutral amino acids were eluted with 150 m*l* of 0.2 M pyridine. MeHis was then eluted with 60 m*l* of 1.0 M pyridine. The flow-rate of the eluent was *ca.* 1 m*l*/min. The MeHis fraction thus obtained was evaporated to dryness.

Table 11. Characteristics of chum salmon specimens.

Stage	Sex	Age	Date and locality of collection	Fork length (cm)	Body weight (g)	Gonadosomatic index*	Hepatosomatic index**
Feeding migration	Male	03	July 2, 1984. Lat. 44°00′N Long. 155°01′E	65	3900	0.64	1.44
	Female	03		59	2560	1.91	1.41
Upstream migration	Male	04	Nov. 16,1984. Lower reaches (1.8 km) of Yurappu River, Hokkaido	74	4290	4.31	2.26
	Female	04		76	4600	20.30	1.09
Spent	Male	04	Nov. 21, 1984. Lower reaches (1.8 km) of Yurappu River, Hokkaido	75	3920	0.74	1.22
	Female	05		74	3600	0.00	1.47

*(Gonad weight/Body weight)×100.
**(Liver weight/Body weight)×100.

Preparation of myofibril and actin

Myofibril and actin were prepared from the chum salmon muscle at the feeding migration stage according to the methods of Katoh *et al.* (1977) and Seki *et al.* (1973), respectively. Myofibril and actin, dialyzed thoroughly against cold water, were successively washed with 10% (w/v) trichloroacetic acid (three times), acetone, and diethyl ether (three times). The resulting residue collected by centrifugation was dried, weighed, and then subjected to hydrolysis with 4 N methanesulfonic acid at 115°C for 24 hr.

Amino acid analysis

Amino acids of the muscle extracts and the hydrolysate of myofibrillar protein were measured with a Hitachi 835 automatic amino acid analyzer.

Results

Amino acid composition of muscle extracts

The amounts of total extractive nitrogen in the muscles are shown in Table 12. The total extractive nitrogen tended to increase during spawning migration.

Profiles of free amino acids are given in Table 13. The level of histidine displayed a unique changes: The level at feeding migration stage was characteristically high, whereas it fell one tenth the initial level at upstream migration stage. At this stage, high contents of serine, glutamic acid, glycine, and alanine were detected instead. So was roughly the case with spent salmon. No MeHis was detected in the muscle extracts during spawning migration.

Detection of MeHis in muscle extracts

No MeHis was detected in the muscle extracts by the usual method, as shown in Table 13. Therefore, ion-exchange column chromatography was applied to detection of MeHis in the muscle extracts. After elution of acidic and neutral amino acids with 0.2 M pyridine, the fraction containing MeHis, if any, was eluted with 60 m*l* of 1.0 M pyridine (Table 14).

Table 12. Total extractive nitrogen in the muscle of chum salmon.

Stage	Sex	Total extractive nitrogen (mg/100 g muscle)
Feeding migration	Male	330
	Female	323
Upstream migration	Male	328
	Female	378
Spent	Male	370
	Female	355

Table 13. Free amino acid composition of chum salmon muscle extracts during spawning migration (mg/100g muscle).

	Feeding migration stage		Upstream migration stage		Spent	
	Male	Female	Male	Female	Male	Female
Asp	0.18	0.27	0.33	0.44	2.33	0.61
Thr	3.24	3.71	7.05	6.90	7.65	2.39
Ser	2.88	1.25	7.92	17.33	13.10	9.64
Glu	8.38	3.79	21.67	11.89	21.45	14.48
Gly	9.60	3.77	18.96	43.64	28.15	19.06
Ala	13.06	11.44	42.51	28.35	17.48	8.86
Cys/2	0.28	0.25	0.37	0.62	0.40	0.32
Val	3.02	3.93	7.12	7.49	17.74	4.86
Met	1.06	1.62	2.51	4.67	10.44	9.99
Ile	1.31	1.81	4.47	5.06	13.33	2.99
Leu	1.90	2.90	6.71	8.24	20.20	5.15
Tyr	1.49	1.80	2.09	3.80	10.24	3.05
Phe	1.34	0.86	2.36	3.68	12.32	2.97
Lys	3.34	0.92	12.88	4.04	8.61	0.94
His	86.17	57.50	7.03	1.02	8.97	6.67
Trp	0.88	0.00	0.71	0.24	4.04	0.00
Arg	0.97	0.60	1.49	1.39	1.13	0.00
Pro	5.27	1.74	6.94	5.07	8.86	1.75

Table 14. Retention volumes of amino acids with a Dowex 50W-X8 (pyridine form) column.

Eluate	Retention volume (m*l*)	Amino acids
0.2M pyridine	150	Acidic and neutral amino acids
1.0M pyridine	0-60	Trp > MeHis* > Ser > His > Gly
	60-110	His > Ser > Cys/2
	110-170	His
	170-240	Ser > His > Gly > Cys/2

* N^{τ}-Methylhistidine.

Figure 19 shows the concentration of MeHis in the muscle extracts. MeHis levels markedly increased during spawning migration of chum salmon, reflecting directly in the degradation of myofibrillar protein. MeHis levels of female fish generally were higher than those of males.

Amino acid composition of myofibril and actin

A trace of MeHis was detected in myofibril, while this amino acid was clearly

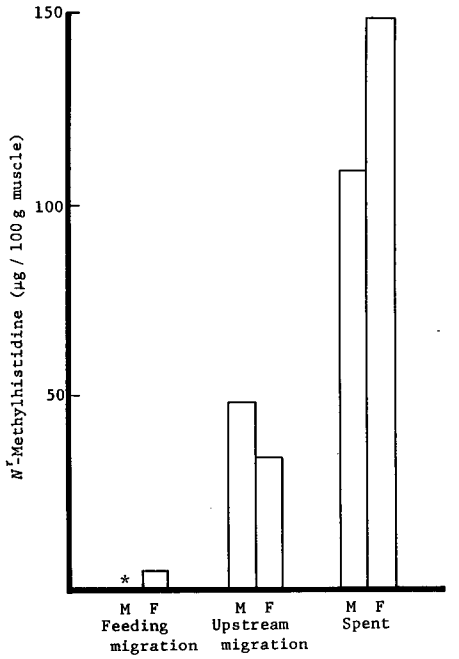

Fig. 19. Changes in N^{τ}-methylhistidine levels in the muscle extracts of chum salmon during spawning migration. Asterisk indicates no detection of N^{τ}-methylhistidine. M, male; F, female.

Table 15. Amino acid content of myofibril and actin prepared from chum salmon muscle at feeding migration stage (mol/100,000 g)

	Myofibril	Actin
Asp	81.22	81.44
Thr	40.02	47.56
Ser	32.63	41.06
Glu	123.23	104.28
Gly	50.92	56.53
Ala	70.26	75.95
Cys/2	0.84	0.79
Val	48.95	42.85
Met	23.22	28.40
Ile	41.18	53.08
Leu	68.82	64.91
Tyr	23.45	30.18
Phe	26.24	25.31
Lys	72.33	57.48
His	18.62	17.34
Trp	1.46	5.50
Arg	40.39	40.48
Pro	28.50	37.30
MeHis*	Tr	1.01

*N^{τ}-Methylhistidine.

detected in actin (Table 15). These results showed that the degradation of myofibrillar protein was responsible for MeHis in the muscle extracts.

Discussion

MeHis in skeletal muscle tissue is known to be formed by post-translational methylation of specific histidine residues in peptide chain of actin and myosin (Young *et al.*, 1970, 1972). When myofibrillar protein is degraded, MeHis is firstly released into the muscle extracts and then excreted quantitatively into urine through the blood without reutilization for protein synthesis (Nishizawa, 1983). Therefore, urinary excretion of MeHis is often used as an index of the degradation of myofibrillar protein in mammals (Nagasawa *et al.*, 1982, 1984a, b; Kadowaki and Noguchi, 1984).

There are very few reports dealing with the occurrence of MeHis in fish muscle extracts (Suyama and Suzuki, 1975; 1975; Konosu *et al.*, 1983; Shirai *et al.*, 1983), although a number of reports on fish muscle extracts have been published from the

viewpoint of food chemistry (Sakaguchi, 1981). No studies have so far been performed on the relation between the extractive MeHis and the degradation of myofibrillar protein.

For the first time the degree of myofibrillar protein degradation during spawning migration of chum salmon could be clearly elucidated, taking the extractive MeHis level as a parameter. To attain this, it was necessary to concentrate the muscle extracts by ion-exchange column chromatography.

The summary of the previous and present sections on the deterioration of chum salmon muscle during spawning migration was as follows:

1) Sarcoplasmic protein content markedly decreased and myosin heavy chain was gradually degraded during spawning migration (Section 3).
2) Muscle protease activity markedly increased during spawning migration. The protease activity was higher in females than in males (Section 3).
3) Ninhydrin positive substances in the muscle extracts slightly increased during spawning migration (Sections 1 and 3).
4) MeHis was detected in myofibril and actin at the feeding migration stage.
5) MeHis in the muscle extracts markedly increased during spawning migration. MeHis level of female fish generally was higher than that of males.

The above results indicate that myosin and actin, as well as sarcoplasmic protein, are degraded by muscle proteases activated during spawning migration (Fig. 20). Amino acids except for MeHis released from muscle protein are reutilized for protein synthesis, and hence do not accumulate in the muscle extracts during spawning migration. This implies that a certain size of amino acid pool might be maintained in the muscle extracts. Therefore, the degree of deterioration of chum salmon muscle during spawning migration could not be estimated only from extractive amino acid levels. However, since only MeHis in the muscle extracts is not reutilized, the degree of muscle deterioration can be estimated by the level of this amino acid. In this connection, MeHis was detected in a heated salmon muscle extracts (Konosu *et al.*, 1983; Shirai *et al.*, 1983).

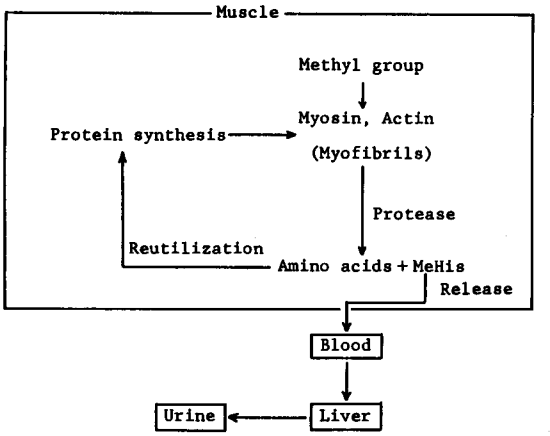

Fig. 20. A scheme of N^{τ}-methylhistidine synthesis and release in chum salmon muscle.

Extractive MeHis can thus be regarded as a useful index of the degradation of muscle protein in fish as well as in mammals.

Section 5. Changes in serum protease inhibitory activity

It has been reported that protease activity of muscle is enhanced during spawning migration of chum salmon (Konagaya, 1982, 1983, 1985a, b ; Nomata *et al.*, 1985a) and sockeye salmon (Mommsen *et al.*, 1980). In Sections 1 and 3 of the present chapter, the same phenomena as described above have been confirmed and the close relation between protease activity and physiological state of chum salmon has been pointed out. High levels of protease activity in salmon muscle have been found at acidic region (around pH 3). However, high levels of acid protease activity were not caused by the injury of lysosomal membranes (Table 10). Then, why is the protease of chum salmon muscle activated during spawning migration ?

It has been well known that protease inhibitors in serum are to control proteolysis of cells and tissues (Heimburger, 1975 ; Travis and Salvesen, 1983). α_1-Proteinase inhibitor in serum has been isolated and characterized not only from man (Pannell *et al.*, 1974) but also from several mammals, such as rabbit (Koj *et al.*, 1981), mouse (Takahara and Sinohara, 1982) and rat (Kuehn *et al.*, 1984). There are very few reports about proteinase inhibitors of fish serum, except for the latest study of Hjelmeland (1983).

A relation between protease inhibitory activity and physiological states of silkworm has been suggested (Eguchi and Kanbe, 1982). It may be conceivable that muscle protease activity of chum salmon during spawning migration is controlled by protease inhibitors in serum. It was, therefore, of interest to clarify the relation between muscle protease activity and protease inhibitory activity in serum of chum salmon during spawning migration with regard to their physiological state.

Materials and Methods

Materials

The same chum salmon muscles and sera as shown in Table 1 were used as materials.

Assay of trypsin inhibitory activity

The reaction mixture for trypsin inhibitory activity of serum, containing 2.0 m*l* of Kolthoff's buffer (pH 7.6), 0.5 m*l* of 0.02% trypsin (Sigma Chemical Co.) solution, and 0.5 m*l* of serum was preincubated at 37°C for 10 min. Then, 1.0 m*l* of 5% casein (Wako Pure Chem. Ind. Ltd.) solution was added to the reaction mixture. After incubation at 37°C for 30 min, the reaction was stopped with 2.0 m*l* of 10% trichloroacetic acid (TCA) and the absorbance at 278 nm of the supernatant fraction was measured. Blanks were incubated separately from the other components of the reaction mixture at 37°C for 40 min and then combined after the addition of TCA. Trypsin inhibitory activity was expressed as the percent of inhibition against the protease activity with no serum as 100%.

Measurement of autolytic activity

Procedures used for the measurement of autolytic activity were as previously described (Section 3).

Autolytic activity of chum salmon muscle was also measured in the presence of serum from the different migration stages.

Results

Changes in trypsin inhibitory activity of serum

Figure 21 shows the trypsin inhibitory activity of serum from chum salmon during spawning migration. The absorption at 278 nm of TCA-soluble products decreased proportionally with increasing blood serum protein. The slope was steepest at the feeding migration stage, while that was gentlest at the upstream migration stage. It was noticeable that trypsin inhibitory activity at the spawning migration stage was higher in males than in females.

Figure 22 shows the relation between muscle protease activity and trypsin inhibitory activity of serum during spawning migration of chum salmon. A reverse relation was found between muscle protease activity and trypsin inhibitory activity of serum.

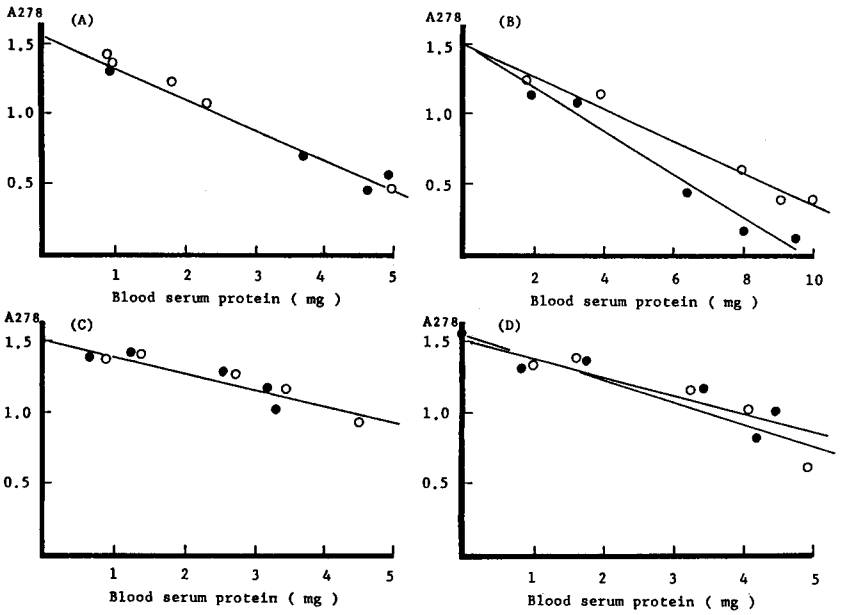

Fig. 21. Changes in serum trypsin inhibitory activity during spawning migration of male (●) and female (○) chum salmon. (A), feeding migration stage; (B), spawning migration stage; (C), upstream migration stage; (D), spent.

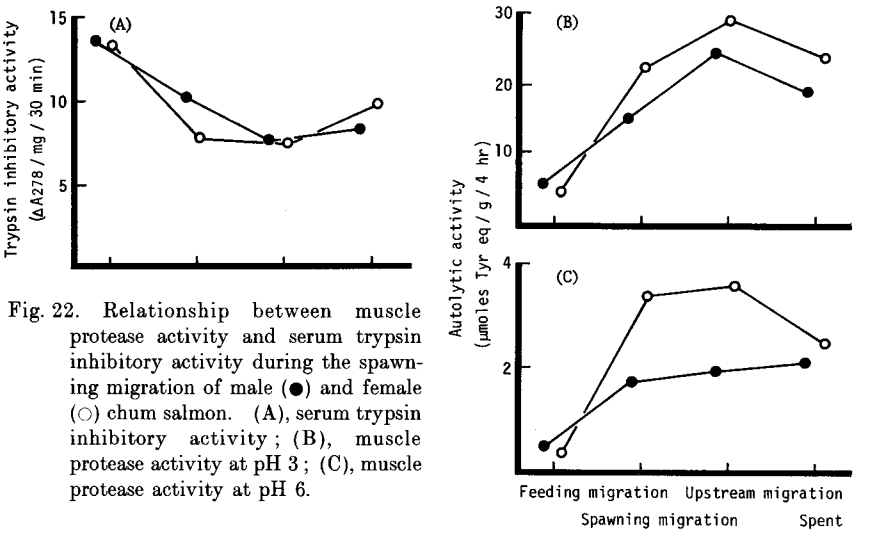

Fig. 22. Relationship between muscle protease activity and serum trypsin inhibitory activity during the spawning migration of male (●) and female (○) chum salmon. (A), serum trypsin inhibitory activity; (B), muscle protease activity at pH 3; (C), muscle protease activity at pH 6.

Effect of serum on autolytic activity of chum salmon muscle at the upstream migration stage

The autolytic activity of muscle homogenate from the upstream migration stage decreased following the addition of serum (Fig. 23). The inhibition of autolytic activity in muscle homogenate by serum was appreciably found at acidic region.

Discussion

In Section 3 of the present chapter, it has been shown that autolytic activity of muscle homogenate markedly increased during spawning migration of chum salmon. No relation was found between high levels of muscle protease activity and the injury of lysosomal membranes, while a close relation between protease activity and serum sex steroid hormones was suggested. A control of proteolysis of cells and tissues by protease inhibitors in serum has been suggested in mammals (Travis and Salvesen, 1983) and in insects (Eguchi and Kanbe, 1982). However, no possibility on the control of proteolysis by serum protease inhibitors has been obtained in fish so far. The relationship between muscle protease activity and serum protease inhibitor will be discussed below with regard to the physiological state of chum salmon.

The results of the present section strongly suggest the relationship between muscle protease activity and protease inhibitory activity in serum. The autolytic activity of chum salmon muscle markedly increased, while the trypsin inhibitory activity in serum markedly decreased during spawning migration (Fig. 22). This might suggest that the decrease of protease inhibitory activity in serum was the reason for the high levels of muscle protease activity during spawning migration. The possibility on the control of muscle protease by serum protease inhibitor was also shown in Fig. 23. It has been suggested that the protease inhibitor in muscle may derived from the serum (Hjelmeland, 1983). It was therefore considered that

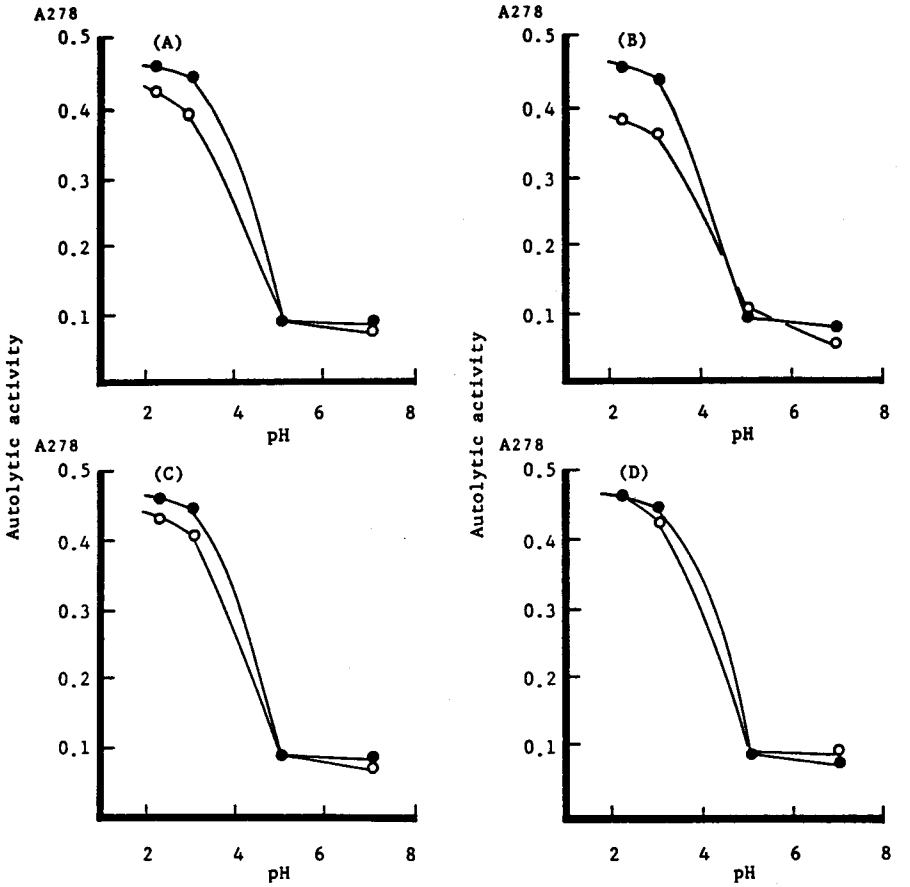

Fig. 23. Effect of serum on autolytic activity of chum salmon muscle. (A), feeding migration stage; (B), spawning migration stage; (C), upstream migration stage; (D), spent; (○), with serum; (●), without serum.

muscle protease activity was controlled by the protease inhibitor not only of serum but also of muscle. In this connection, Nomata *et al.* (1985b) have recently reported the existence of calpastatin and trypsin inhibitor in chum salmon muscle, and Hara *et al.* (1985) have isolated serine protease inhibitor from white croaker (*Argyrosomus argentatus*) ordinary muscle.

Taking into account the close relation between muscle protease activity and testosterone levels, it might be suggested that protease inhibitory activity of serum was controlled by androgens. This possibility will be discussed in the later chapter.

Chapter III. Nuptial coloration occurring in chum salmon during spawning migration

It has been well known that the muscle discolors in migrating chum salmon, and

the integument becomes dark yellow or red after entering a river. These changes in muscle or integument color may be closely associated with the physiological state, probably controlled by sex steroid hormones, of the fish (Idler *et al.*, 1961; Fagerlund and Donaldson, 1969; van Overbeeke and McBride, 1971; Yamazaki, 1972).

The present chapter describes the nuptial coloration occurring in chum salmon during spawning migration.

Section 1. Serum carotenoid-carrying lipoproteins

Kitahara (1983) has reported that carotenoids, mainly astaxanthin, in chum salmon muscle are transported into integument and gonads *via* the blood serum. However, no further information is available as to what kind of serum proteins transport astaxanthin, except for preliminary reports by Nakamura *et al.* (1985a, b). The presence of lipophorin has recently been demonstrated in insects, which transports such lipids as diacylglycerol, cholesterol, hydrocarbons, and carotenoids (Chino and Downer, 1982). Lipophorin is high density lipoprotein and the color is pale or deep yellow due to the presence of carotenoids (Chino *et al.*, 1969).

The present section describes the isolation and characterization of such carotenoid-carrying lipoproteins in the serum of chum salmon at various migration stages.

Materials and Methods

Materials

Blood was collected from the caudal vasculature of live salmon at each migration stage (Table 16) and left at room temperature for several hours. The clotted blood was centrifuged to obtain the serum. Sera thus obtained were stored at −20°C until use.

Table 16. Characteristics of chum salmon specimens[a].

Stage	Sex	Age	Date and locality of collection	Fork length (cm)	Body weight (g)	Gonadosomatic index*	Hepatosomatic index**
Feeding migration	Male	03-04	July 2, 1984. Lat. 44°00′N Long. 155°01′E	59	2860	1.5	1.3
	Female	03		59	2630	2.0	2.0
Spawning migration	Male	03-04	Oct. 16, 1984. Yakumo coast of Hokkaido	69	3510	5.4	1.7
	Female	03-04		68	3630	15.3	2.8
Upstream migration	Male	03-04	Nov. 16, 1984. Lower reaches (1.8 km) of Yurappu River, Hokkaido	75	4410	3.9	2.0
	Female	03-05		77	4770	18.8	1.3
Spent	Male	03-04	Nov. 21, 1984. Lower reaches (1.8 km) of Yurappu River, Hokkaido	69	3250	1.1	1.5
	Female	03-05		67	2820	2.3	1.4

[a] Values represent the mean of three samples.

* (Gonad weight/Body weight) × 100.

** (Liver weight/Body weight) × 100.

The dorsal muscle from the fish used as samples were also kept at −20°C until use.

Measurement of the absorption spectra of the sera

The absorption spectra of the sera of the chum salmon at each migration stage were measured with a Hitachi 556 double wavelength spectrophotometer.

Lipid extraction and assay of carotenoid levels

Lipid extraction from serum and muscle was carried out by the method of Bligh and Dyer (1959). Lipid classes were separated by thin-layer chromatography as previously described (Chapter II). Carotenoid content was calculated, assuming the $E^{1\%}_{1cm}$ value in acetone at 477 nm to be 2,200. Absorption spectra of the carotenoid solution were measured with a Hitachi 556 double wavelength spectrophotometer.

Thin-layer chromatography (TLC) of carotenoids

The concentrated carotenoids were studied by TLC. The TLC plate (Kieselgel 60, a ready-made plate from Merck) was developed by dichloromethane/ethyl acetate (4 : 1, v/v).

Preparation of lipoproteins

The bright orange serum from the upstream migrating chum salmon was analyzed by density gradients (Fig. 24), to confirm whether the carotenoids were associated with lipoproteins. An equal amount of 0.9% NaCl was gently layered over 5.5 m*l* serum containing 2.2 g of KBr, and centrifuged at 139,500×*g* at 10°C for 6 hr (Chung *et al.*, 1980). The protein content and absorbance at 480 nm of top, middle, and bottom fractions were measured.

Sequential ultracentrifugation (Havel *et al.*, 1955) was used on the serum from chum salmon of different physiological state, since carotenoids were found to be associated with lipoproteins. The density of the serum was brought to 1.063 g/m*l* by addition of a suitable amount of KBr solution (density 1.346 g/m*l*), and centrifuged at 139,500×*g* for 22 hr. After removal of low density lipoprotein (LDL, density 1.006−1.063 g/m*l*) by aspiration, the density of the infranatant was raised to 1.210 g/m*l*. The infranatant was then centrifuged at 139,500×*g* for 48 hr to fractionate high density lipoprotein (HDL, density 1.063−1.210 g/m*l*) and very high density lipoproteins ($VHDL_1$, density>1.210 g/m*l*; $VHDL_2$, density≫1.210 g/m*l*). All operations in the sequential isolation of lipoproteins were done at 10°C. All fractions contained 0.005% EDTA and 0.02% NaN_3. Proteins, lipids, and carotenoids of each fraction were measured.

Isolation of a carotenoid-carrying lipoprotein from HDL fraction

The HDL fraction from the chum salmon serum was dialyzed against 0.05 M phosphate buffer (pH 6.0) overnight and put on a column (1.5×9 cm) of DEAE-cellulose equilibrated with the above buffer solution. The carotenoid-carrying lipoprotein was strongly adsorbed at the top of the column. It was eluted with 0.3 M NaCl in 0.05 M phosphate buffer (pH 6.0). The carotenoid-carrying lipoprotein

thus eluted was put on a Toyopearl HW-55 column (1.5×81 cm) equilibrated with 0.5 M NaCl in 0.05 M phosphate buffer (pH 6.0) containing 0.005% EDTA and 0.02% NaN_3 to find the molecular weight.

Binding of astaxanthin to carotenoid-carrying lipoproteins

Serum was obtained from the male salmon at the feeding migration stage. A small amount of astaxanthin-containing acetone was added to 4.75 m*l* serum containing 1.9 g of KBr and mixed for 30 min at 4°C. An equal amount of 0.9% NaCl was gently layered over 4.75 m*l* serum and centrifuged at 218,000×*g* at 10°C for 5 hr. At the end of a run, the tubes were removed from the rotor and serum fractions were collected from each tube by pipette. The levels of proteins and carotenoids in each fraction were measured. Control serum without astaxanthin was worked up in a similar manner as above.

Electrophoresis

Electrophoresis in 5% polyacrylamide gel was done at pH 8.3 and 2 mA per tube, as described by Davis (1964). SDS-slab-15% polyacrylamide gel electrophoresis (PAGE) was done in the presence of 0.1% SDS, by the method of Laemmli (1970). Polyacrylamide gel isoelectric separation was done in the presence of Ampholine (pH 3.5-10) as carrier (Wrigley, 1971). After electrophoresis, gels were stained with Coomassie Brilliant Blue for protein and Sudan Black B for lipid. The electrophoretogram was measured by a Cosmo F-808 densitometer at 625 nm, if necessary.

Amino acid analysis

The amino acid composition of the delipidated carotenoid-carrying lipoprotein was found on a Hitachi model 835 automatic amino acid analyzer after hydrolysis with 4 N methanesulfonic acid at 115°C for 24 hr.

Measurement of proteins

Protein was measured by the biuret method (Gornall *et al.*, 1949), using bovine serum albumin as the standard.

Results

Coloration and absorption spectra of the serum at each migration stage

The serum became bright orange during spawning migration, suggesting the presence of carotenoids (Fig. 24). Each spectrum has two absorption maxima, one at 410-415 nm and the other at 470-480 nm. The latter absorption gradually increased during spawning migration, while the former was highest during the early stage of migration. The absorption at 410-415 nm might be derived from transferrin (Hara, 1984) or hemolysis (Nakamura *et al.*, 1985a, b), since this absorption was hardly affected by treatment of the serum with 25 vol. of cold ethanol-diethyl ether (3 : 1, v/v). The absorption at 470-480 nm disappeared upon the same treatment, indicating that carotenoids are associated non-covalently with serum proteins.

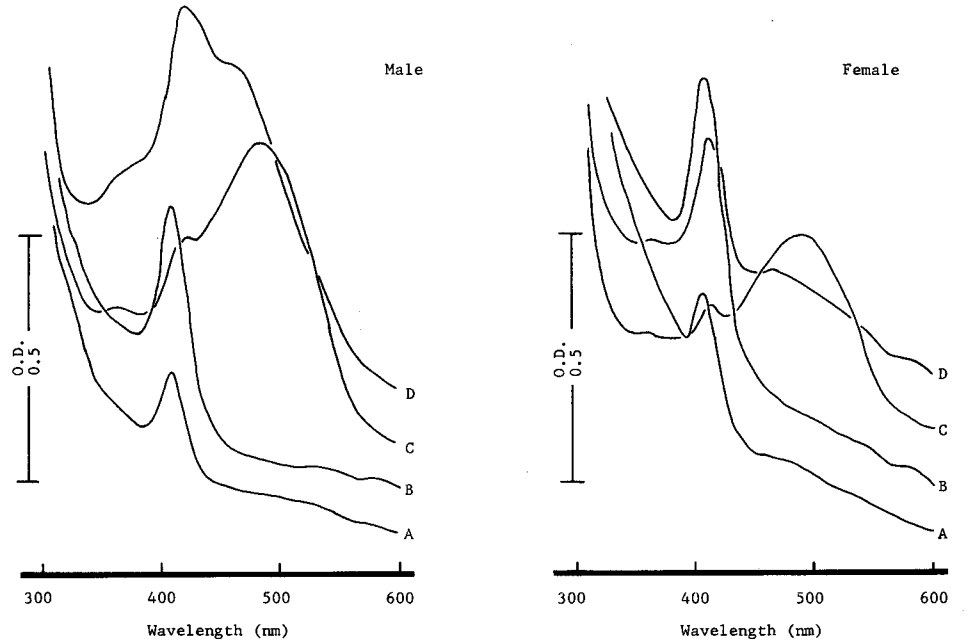

Fig. 24. Absorption spectra of serum from chum salmon of different physiological states. A, feeding migration stage ;B, spawning migration stage ; C, upstream migration stage ; D, spent.

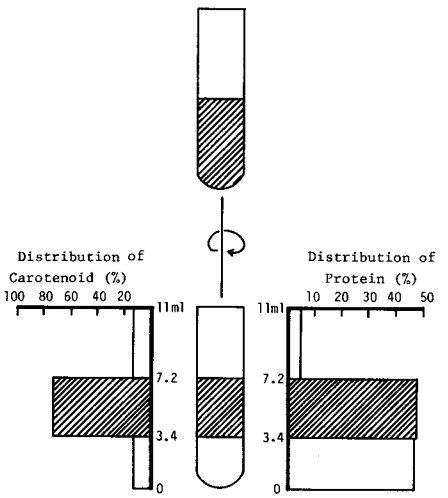

Fig. 25. Schematic representation of the distribution of carotenoid-carrying lipoproteins after density gradient ultracentrifugation of serum proteins from upstream migration male chum salmon. Diagonal areas show the orange coloration due to the presence of carotenoid-carrying lipoproteins.

Absorption spectra and TLC of the carotenoids from muscle and serum during spawning migration

The absorption spectra of carotenoids extracted from the muscle and serum at

each migration stage resembled each other, all having absorption maximum at 480 nm. Astaxanthin was considered to be the main carotenoids of serum from the result of TLC (Figs. 28 and 38).

Presence of carotenoid-carrying lipoprotein in serum

Density gradient ultracentrifugal analysis on the serum from an upstream migrating male chum salmon confirmed that the carotenoids were associated with lipoproteins. Three fractions (top, middle and bottom) were separated. Proteins were mainly distributed in the middle and bottom fractions, while carotenoids were

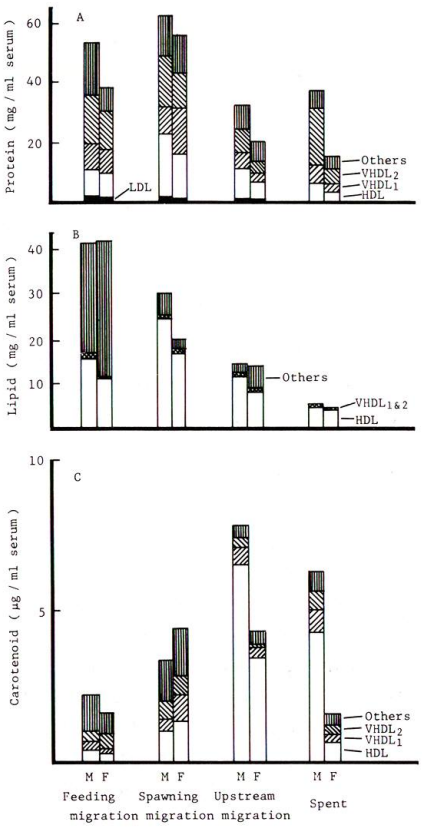

Fig. 26. Distributions of protein (A), lipid (B), and carotenoids (C) in serum lipoproteins from chum salmon of different physiological states. LDL, low density lipoprotein (d=1.006-1.063 g/m*l*); HDL, high density lipoprotein (d=1.063-1.210 g/m*l*); $VHDL_1$, very high density lipoprotein (d>1.210 g/m*l*); $VHDL_2$, very high density lipoprotein (d≫1.210 g/m*l*); M, male; F, female.

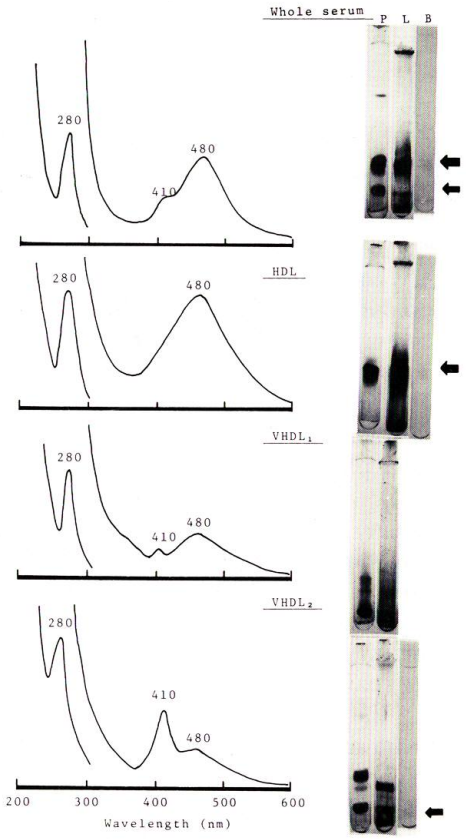

Fig. 27. Absorption spectra and polyacrylamide gel electrophoresis of serum lipoproteins from the upstream migrating male chum salmon. Arrows indicate the bands with orange coloration. P, protein staining; L, lipid staining; B, before staining.

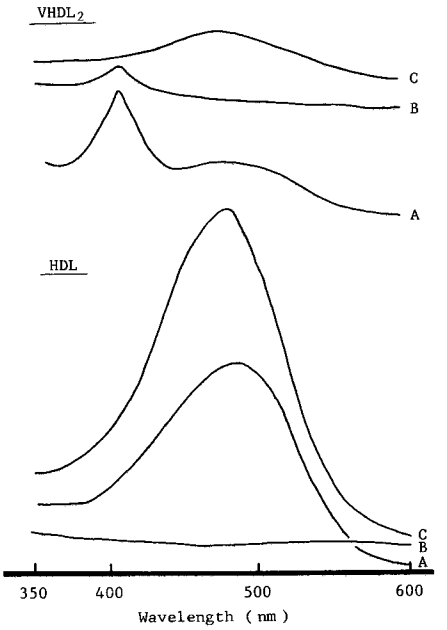

Fig. 28. Effects of delipidation treatment on absorption spectra of HDL and $VHDL_2$ fractions from the serum of upstream migrating male chum salmon. A, before treatment; B and C, apoprotein and lipid fraction, respectively, obtained from each fraction by treatment with cold ethanol-diethyl ether.

mainly contained in the middle fraction (Fig. 25), suggesting the presence of carotenoid-carrying lipoprotein in the male serum. Similar results were obtained for the serum of female salmon at the upstream migration stage.

Distributions of protein, lipid, and carotenoids in serum lipoproteins

Sequential ultracentrifugation was done on the serum from chum salmon of different physiological state, since the carotenoids were associated with lipoproteins (Fig. 25). The protein content of serum slightly increased at the spawning migration stage, and then greatly decreased during the upstream migration. The serum protein was mainly distributed in HDL, $VHDL_1$, and $VHDL_2$ fractions. LDL was detected as a minor fraction, and greatly decreased during spawning migration (Fig. 26A). The lipid content of the serum, particularly of LDL fraction, greatly decreased during spawning migration. The serum lipid was mainly in the HDL fraction during spawning migration (Fig. 26B). Carotenoids of the serum greatly increased during the upstream migration, and over 80% was in the HDL fraction at the upstream migration stage (Fig. 26C).

Figure 27 shows the absorption spectra and PAGE and male serum lipoproteins at the upstream migration stage. It was noticeable that serum carotenoids were present not only in the HDL but also in the $VHDL_2$ fraction. This result was supported by the data in Fig. 28, which showed the effects of delipidation with ethanol-diethyl ether on the compositions of HDL and $VHDL_2$ fractions. The absorption maximum of HDL fraction appeared at 480 nm, while $VHDL_2$ had two absorption maxima, one at 410 nm and the other at 480 nm. The absorption of both fractions at 480 nm disappeared upon the delipidation treatment, indicating that carotenoids were associated non-covalently with HDL and $VHDL_2$. PAGE of

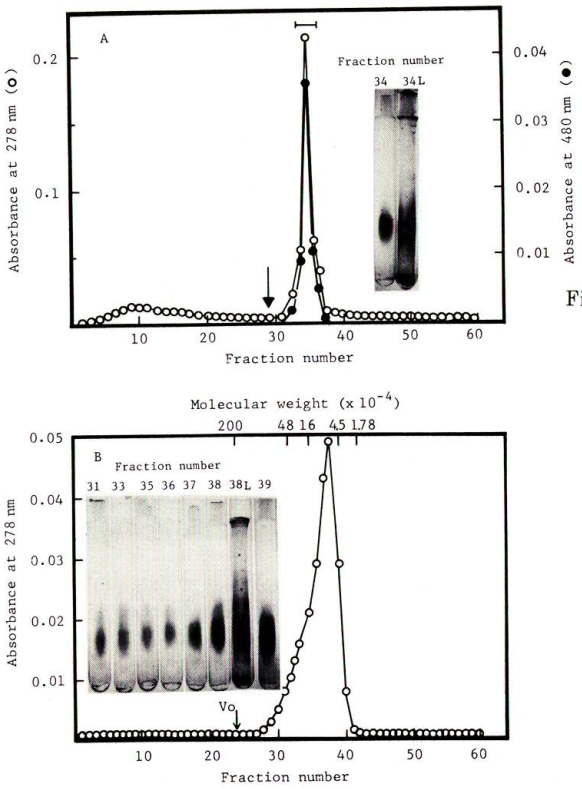

Fig. 29. DEAE-cellulose column (A) and Toyopearl HW-55 gel (B) chromatograms of HDL from the serum of upstream migrating male chum salmon.
A. Fractions eluted in the position indicated by a horizontal bar were collected and put on Toyopearl HW-55 gel column. Arrow indicates the beginning of elution with 0.3 M NaCl in 0.05 M phosphate buffer (pH 6.0). Each 3.8 m*l* fraction was collected at 50 m*l*/hr. Inset shows polyacrylamide gel electrophoresis of the main peak. L, lipid staining.
B. Each 2.3 m*l* fraction was collected at 10 m*l*/hr. Inset shows polyacrylamide gel electrophoresis of selected fractions. Vo, void volume; L, lipid staining.

serum lipoproteins was identical regardless of the physiological state of chum salmon, except that vitellogenin was detected in the female serum at the spawning migration stage.

Isolation of carotenoid-carrying lipoprotein from HDL fraction

Attempts were made to isolate the main carotenoid-carrying lipoprotein from the serum of upstream migrating male chum salmon.

The HDL fraction was mostly adsorbed when chromatographed on a DEAE-cellulose column with 0.05 M phosphate buffer (pH 6.0), but was eluted with 0.3 M NaCl in 0.05 M phosphate buffer (pH 6.0) (Fig. 29A). The fractions indicated by a bar were combined, concentrated, and then put on a Toyopearl HW-55 column. The elution pattern is shown in Fig. 29B. One broad peak ranging in molecular weight from 30,000 to 500,000 appeared, with a peak at 70,000 dalton. Some selected fractions exhibited almost the same PAGE patterns.

Similar results were obtained irrespective of the physiological state.

Characterization of carotenoid-carrying lipoprotein

The carotenoid-carrying lipoprotein thus isolated gave two subunits whose molecular weights were 24,000 and 12,000 when used for SDS-slab-PAGE in the presence of 2-mercaptoethanol (Fig. 30A). Assuming that the color intensity with

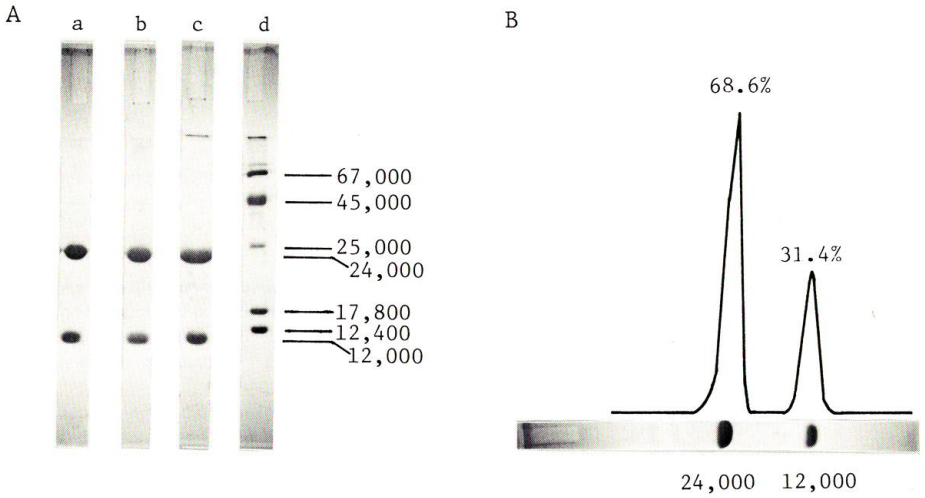

Fig. 30. SDS-slab-polyacrylamide gel electrophoresis of the carotenoid-carrying lipoprotein.
A. a, HDL; b, main peak on DEAE-cellulose column chromatogram; c, main peak on Toyopearl HW-55 gel column chromatogram; d, standard protein mixtures.
B. Densitometric pattern of subunits of the carotenoid-carrying lipoprotein from HDL. Figures represent percentages in area.

Coomassie Brilliant Blue was the same for the two subunits, the ratio of 24,000 dalton to 12,000 dalton subunits was calculated to be 2.18 : 1, or a molar ratio near to 1 : 1 (Fig. 30B). The carotenoid-carrying lipoprotein showed a pI of 5.1 (Fig. 31).

Table 17 shows the amino acid composition of carotenoid-carrying lipoprotein, along with that of the HDL fraction from pink salmon (Nelson and Shore, 1974). They were similar, being rich in glutamic acid, alanine, leucine, and lysine.

Carotenoid-carrying lipoproteins were then isolated from HDL fractions of different physiological state, and compared with regrad to subunit composition, pI, and amino acid composition, resulting in essentially the same results as described above.

Table 18 shows the lipid composition of carotenoid-carrying lipoprotein and that of the HDL fraction from pink salmon (Nelson and Shore, 1974), for comparison. In each of them, cholesterol ester, cholesterol, and triglyceride accounted for most of the neutral lipid of the carotenoid-carrying lipoprotein. The major components of phospholipid were phosphatidylcholine and sphingomyelin.

Astaxanthin binding by carotenoid-carrying lipoproteins

The binding of astaxanthin by carotenoid-carrying lipoproteins was confirmed by density gradient ultracentrifugal analysis (Figs. 32 and 33). Astaxanthin was bound to the lipoproteins with the density of 1.14 g/m*l*.

Table 17. Amino acid composition of the carotenoid-carrying lipoprotein from the upstream migrating male chum salmon.

Amino acids[a]	Carotenoid-carrying lipoprotein	HDL of pink salmon[b]
Asp	62.6	68.5
Thr	40.1	47.3
Ser	33.5	44.3
Glu	182.6	180.6
Pro	45.7	43.2
Gly	50.2	46.3
Ala	118.4	114.0
Cys/2	Tr.	Tr.
Val	76.4	74.8
Met	34.3	33.5
Ile	36.4	35.9
Leu	104.2	103.5
Tyr	48.0	45.4
Phe	16.5	17.1
His	13.6	16.6
Lys	93.0	90.7
Arg	44.3	39.0
Trp	0.0	0.0
Total	999.8	1000.7

[a] Residues per 1,000 amino acid residues.
[b] Nelson and Shore (1974).

Discussion

There are very few reports as to carotenoid-carrying lipoproteins in the serum. Chino and Downer (1982) have demonstrated the presence of lipophorin in insects, which transports diacylglycerol, cholesterol, hydrocarbons, and carotenoids. Lipophorin is a HDL and the color is pale or deep yellow due to the presence of carotenoids. Ashes *et al.* (1984) have reported the presence of a HDL component as a carrier of β-carotene in bovine plasma. Nakamura *et al.* (1985a, b) have recently found that astaxanthin, which is the main carotenoid in chum salmon muscle, is exclusively transported by the HDL fraction in the serum of the upstream migrating chum salmon. The results of the present section clearly showed that the serum astaxanthin of chum salmon was present not only in the HDL but also in the $VHDL_2$ fraction, although isolation and characterization of the carotenoid-carrying lipoprotein from the latter remain to be done (Figs. 27 and 28).

As shown in Fig. 26B, the serum lipid content greatly decreased during spawn-

Table 18. Lipid composition of the whole serum and the carotenoid-carrying lipoprotein from the upstream migrating male chum salmon.

Component	% Weight[a]			
	Chum salmon		Pink salmon[c]	
	Whole serum	Carotenoid-carrying lipoprotein	Whole serum	HDL
Protein	58.6	46.0	—[b]	39.9
Total lipid	41.4	54.0	—	60.1
Cholesteryl ester	30.5	26.4	28.6	30.5
Triglyceride	5.0	15.0	10.7	11.4
Free fatty acid	2.7	6.2	8.2	2.8
Cholesterol	15.0	9.2	5.0	5.3
Partial glyceride	1.1	0.2	—	—
Phospholipids	45.8	42.8	46.9	49.9
Phosphatidyl-choline	(82.8)	(85.5)	(82.5)	(83.0)
Phosphatidyl-ethanolamine	(4.5)	(2.8)	(2.2)	(2.3)
Lysophosphatidyl-choline	(3.2)	(4.3)	(4.2)	(3.1)
Sphingomyelin	(7.7)	(7.5)	(6.6)	(6.7)

[a]Lipid fraction are expressed as percentages of the total lipid. Each phospholipid is expressed as percentage of total phospholipids in parentheses.
[b] Not determined.
[c] Nelson and Shore (1974).

ing migration and the LDL fraction disappeared after spawning. Patton *et al.* (1970) have found the decrease of serum lipid in pink salmon during spawning migration. Nelson and Shore (1974) have reported that serum LDL disappeared at the pre-spawning migration stage and lipid was transported through serum HDL at the upstream migration stage of pink salmon. It was noticeable that no direct relation was found between the serum lipid content and serum carotenoid content during spawning migration (Fig. 26B and C). This result might suggest that the transport of carotenoids in salmon serum is not simple.

The molecular weight of carotenoid-carrying lipoprotein isolated from chum salmon was estimated to be from 30,000 to 500,000 by gel filtration, with a peak at 70,000. The carotenoid-carrying lipoprotein was split into two subunits (molecular weight 24,000 and 12,000, the molar ratio of 1 : 1) after reduction with 2-mercaptoethanol. However, fractions obtained by Toyopearl HW-55 gel chromatography gave rise to essentially the same PAGE patterns. These results suggested that carotenoid-carrying lipoprotein had a basic unit consisting of one mole each of 24,000 dalton and 12,000 dalton subunits. The molecular species of 70,000 dalton could have two basic units and that of 500,000 dalton 14 basic units. Serum HDL

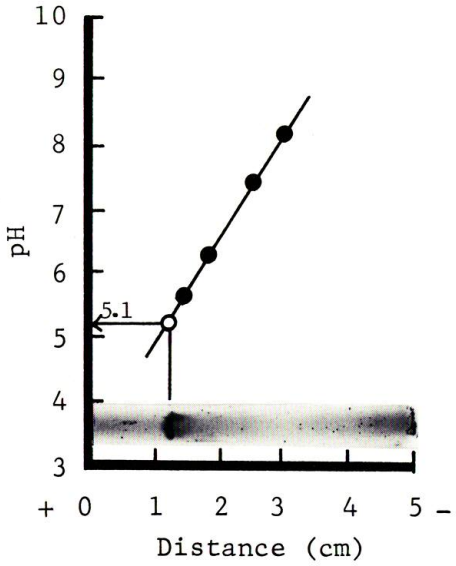

Fig. 31. Polyacrylamide gel isoelectric separation of the carotenoid-carrying lipoprotein.

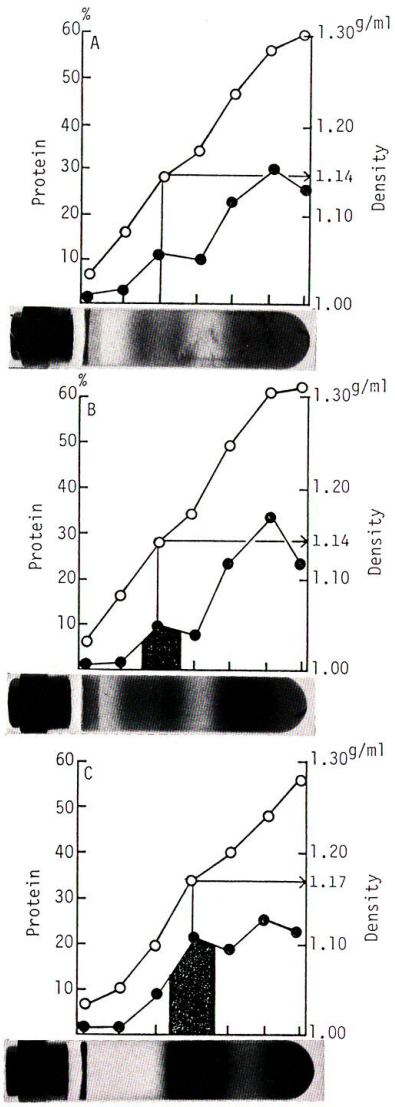

Fig. 32. Distribution of density and proteins in the fractions from the feeding-migrating male serum (A, control ; B, astaxanthin added) and the upstream migrating male serum (C) after ultracentrifugation at 218,000×*g* for 5 hr. Shadowed areas show the presence of carotenoid.

fraction contains species of various particle sizes (Chapman, 1980 ; Ashes *et al.*, 1984). Thus the carotenoid-carrying lipoprotein isolated here was considered to be a cluster of lipoproteins of various particle sizes.

Subunits of the carotenoid-carrying lipoprotein treated with only SDS were identical with those treated with SDS and 2-mercaptoethanol, indicating that there was no disulfide bond between the two subunits. It was considered that the HDL fraction at the feeding migration stage had the same carotenoid transport ability as the counterpart at the upstream migration stage, because both of them showed the

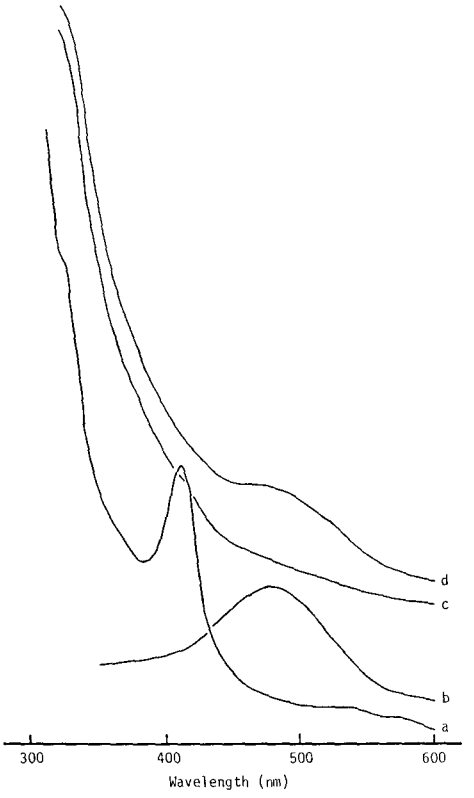

Fig. 33. Absorption spectra of serum fractions with the density of 1.14 g/m*l* (c and d) from the feeding migration stage. a, whole serum ; b, astaxanthin ; c, control ; d, astaxanthin added.

same subunit composition.

The carotenoid-carrying lipoprotein as described above was considered to transport astaxanthin, which is the main carotenoids in the muscle, into the integument and ovaries. However, carotenoid composition in chum salmon integument are very complex : *e.g.*, salmoxanthin, antheraxanthin, zeaxanthin, and doradexanthin in addition to astaxanthin have so far been detected in the integument (Kitahara, 1983). Ashes *et al.* (1984) have demonstrated the presence of HDL as a carrier of β-carotene in bovine plasma. The present results, along with these findings, might suggest that the carotenoid-carrying lipoprotein had the ability to transport not only astaxanthin but also other carotenoids. In the present section, it was proved that carotenoid-carrying lipoproteins were present in chum salmon serum, HDL and $VHDL_2$.

Section 2. Carotenoid transport associated with serum vitellogenin

Vitellogenin has been known as a sex-specific serum protein in amphibia, and is the immediate precursor of egg yolk proteins (Wallace, 1970 ; Redshaw and Follett, 1971). Vitellogenin is also present in fish, and is related to the gonadal maturation of eggs (Aida *et al.*, 1973 ; Hara, 1975 ; Hara *et al.*, 1983). Hara (1976, 1978) and Hara and Hirai (1978) have reported that fish vitellogenin is related to

iron transport as well. Vitellogenin of insects is a very high density lipoprotein, and it is yellow because of the presence of carotenoids (Chino *et al.*, 1976). It has been well known that the eggs of chum salmon have orange coloration. Thus, vitellogenin may be closely related to carotenoid transport in chum salmon from the muscle into the ovaries. These situations aroused to initiate the present study, and this section deals with the results obtained.

Materials and Methods

Materials

The dorsal muscle, serum and ovaries from the chum salmon specimens as shown in Table 16 were used as materials.

Preparation of vitellogenin

To the serum was added 9 volumes of cold water. The precipitate thus formed was collected by centrifugation and dissolved in 0.5 M NaCl (pH 6.0). This fraction, which showed a single clear band in a polyacrylamide gel (Fig. 37), is called the "vitellogenin solution" here.

Binding of carotenoids to vitellogenin

Carotenoids were extracted from muscle at the feeding migration stage of the fish. Various amounts of the carotenoids were added to 1 m*l* each of vitellogenin

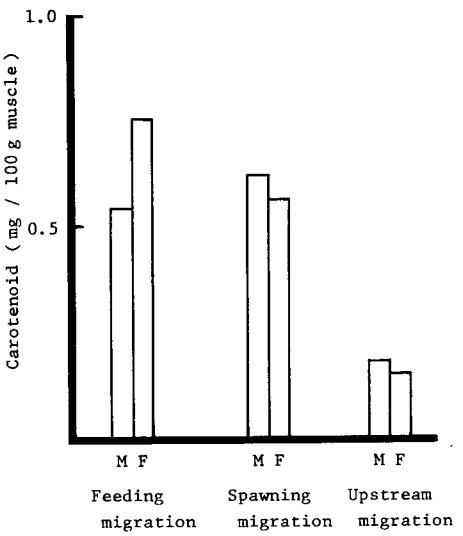

Fig. 34. Carotenoid levels of chum salmon muscle at various stages of migration. M, male; F, female.

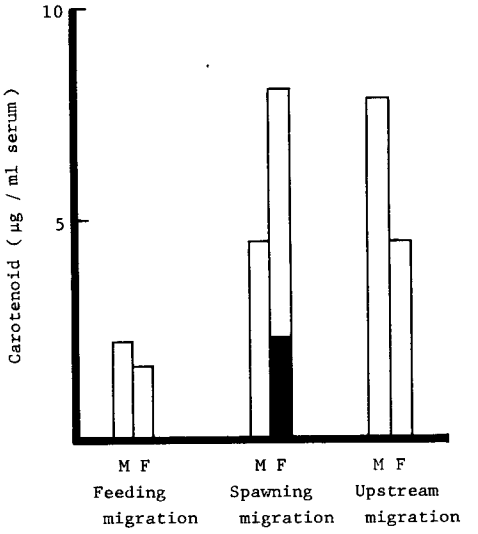

Fig. 35. Carotenoid levels of chum salmon serum at various stages of migration. The solid area represents carotenoids bound to the vitellogenin, that was obtained from 1 m*l* of serum. M, male; F, female.

solution and mixed in an automixer for 1 min. The mixture was then incubated with shaking at 37°C for 1 hr. After incubation, 0.5 m*l* of *n*-hexane was added to each reaction mixture, the whole mixed in an automixer for 1 min, and centrifuged at 3,000 rpm for 5 min to remove the upper layer containing untreated carotenoids. To the lower layer was added 10 m*l* of acetone to extract bound carotenoids, and then the sample was studied by spectrophotometry. A portion of the lower layer was also examined by polyacrylamide gel electrophoresis (PAGE).

Other analytical methods

Procedures used for carotenoid analysis, PAGE, and serum protein concentration were as previously described (Section 1 in the present chapter).

Results

Changes in carotenoid levels of muscle and serum during spawning migration

Figure 34 shows the changes in the level of muscle carotenoids of chum salmon caught at different stages of migration. Carotenoid content markedly decreased at the upstream migration stage.

Serum carotenoids markedly increased during spawning migration (Fig. 35). Vitellogenin was to a large extent responsible for the high carotenoid level in serum of females at the spawning migration stage.

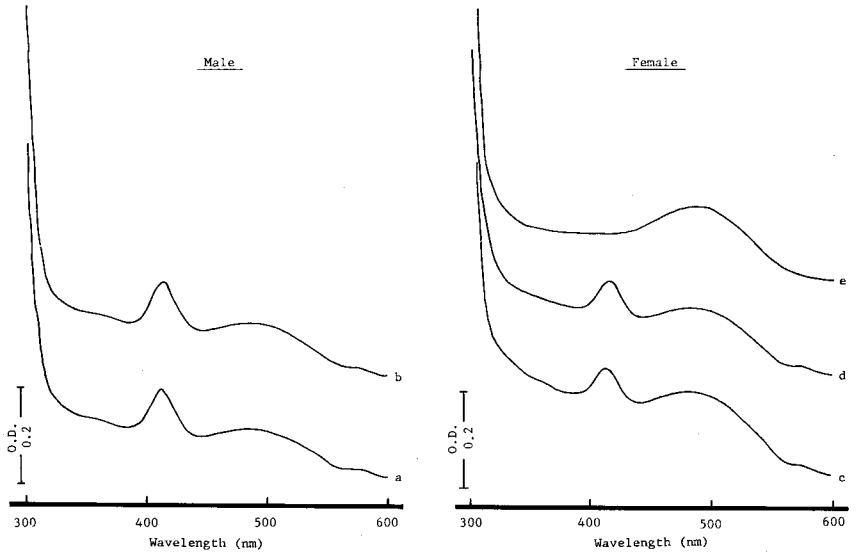

Fig. 36. Absorption spectra of serum and serum fractions at the spawning migration stage. Protein concentrations were: whole serum from male (a), 5.0 mg/m*l*; the water-soluble fraction obtained from a (b), 5.1 mg/m*l*; whole serum from female (c), 7.9 mg/m*l*; the water-soluble fraction obtained from c (d), 4.3 mg/m*l*; the water-insoluble fraction obtained from c (e), 13.7 mg/m*l*.

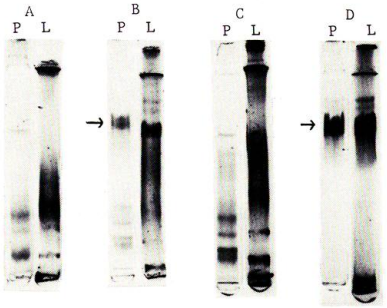

Fig. 37. Polyacrylamide gel electrophoresis of chum salmon serum at the spawning migration stage. Arrows indicate vitellogenin. A, whole serum from male; B, whole serum from female; C, the water-soluble fraction obtained from B; D, the water-insoluble fraction obtained from B; P, protein staining; L, lipid staining.

Table 19. Protein and carotenoid distribution in chum salmon serum at the spawning migration stage[a].

	Male		Female	
	Protein (mg)	O.D.$_{480}$	Protein (mg)	O.D.$_{480}$
Whole serum	99 (100)[b]	2.2 (100)[b]	158 (100)[b]	3.8 (100)[b]
Supernatant	99 (100)	2.2 (100)	84 (53)	2.9 (76)
Precipitate	—[c](—)	—[c](—)	74 (47)	0.9 (24)

[a] To 2 m*l* of male and female serum (protein content, 99 mg and 158 mg, respectively) was added 18 m*l* of cold water. The diluted serum was allowed to stand at 4°C for 4 hr, and centrifuged at 25,000 x *g* for 30 min to separate the supernatant (water soluble fraction) and the precipitate (water insoluble fraction). The precipitate regarded as vitellogenin was dissolved with 0.5 M NaCl (pH 6.0). The serum and each fraction were measured for carotenoid content by O.D. at 480 nm, and for protein content by the biuret method.
[b] Values in parenthesis represent percentages, taking that of the whole serum as 100%.
[c] Not detected.

Characteristics of serum vitellogenin at the spawning migration stage

Figure 36 shows the absorption spectra of whole serum, of the water-soluble fraction and of the water-insoluble fraction (containing vitellogenin) of these salmon at the spawning migration stage. Only serum of females produced a precipitate upon addition of cold water. Sera from both males and females had two absorption maxima, at 410 and at 480 nm. The first might be from transferrin (Hara, 1984) or hemolysis (Nakamura *et al.*, 1985a, b), and the second from carotenoids. The spectrum of vitellogenin, which was checked by PAGE (Fig. 37), had only one absorption maximum, at 480 nm, suggesting that vitellogenin can bind with carotenoids.

Table 19 shows the protein and carotenoid distribution in serum at the spawning migration stage. Vitellogenin made up was 47% of female whole serum protein,

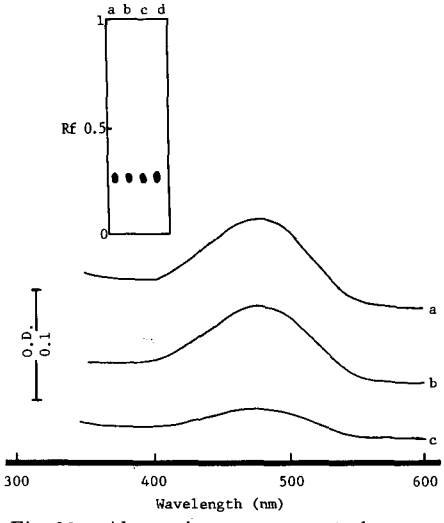

Fig. 38. Absorption spectra (solvent; acetone) and TLC of carotenoids from chum salmon serum at the spawning migration stage. Carotenoid concentrations were: whole serum from male (a), 0.39 μg/ml; whole serum from female (b), 0.34 μg/ml; vitellogenin (c), 0.14 μg/ml. Lane d of the TLC has astaxanthin extracted from chum salmon muscle at the feeding migration stage.

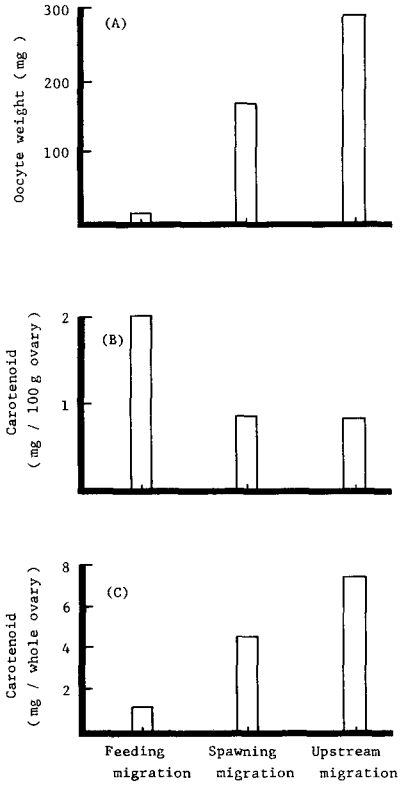

Fig. 39. Changes in oocyte weight (A) and carotenoid level (B, C) of chum salmon ovaries at various stages of migration.

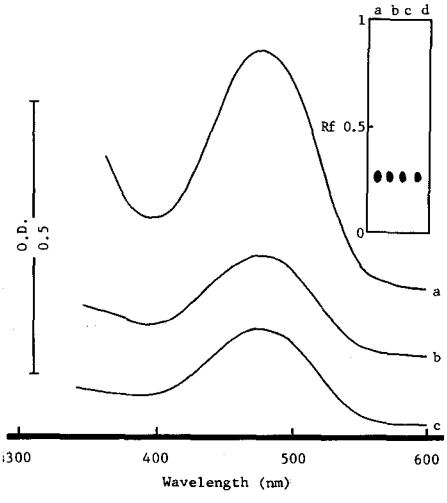

Fig. 40. Absorption spectra (solvent; acetone) and TLC of carotenoids from chum salmon ovaries during spawning migration. Carotenoid levels were as follows: in the feeding migration stage (a), 2.01 μg/ml; spawning migration stage (b), 0.84 μg/ml; upstream migration stage (c), 0.81 μg/ml. Lane d of the TLC has astaxanthin extracted from chum salmon muscle at the feeding migration stage.

and 24% of the carotenoids in serum of females was bound to vitellogenin. There was no water-insoluble protein in whole serum from males. The carotenoid level (24%) calculated from the absorption of vitellogenin solution at 480 nm was near that (27%) found by extraction from vitellogenin (Fig. 35). This finding suggests that the absorbance of vitellogenin solution at 480 nm reflected the carotenoid level of vitellogenin. Absorption spectra of carotenoids extracted from whole serum and vitellogenin had almost the same patterns, with absorption maxima at 480 nm. Astaxanthin was the main carotenoids bound to these proteins from the result of TLC (Fig. 38).

Changes in carotenoid level of the ovaries during spawning migration

The ovary of chum salmon matured rapidly during spawning migration (Fig. 39A). The carotenoid level per 100 g of ovaries was highest at the feeding migration stage, and markedly decreased during spawning migration (Fig. 39B). However, the amount of carotenoids in each whole ovaries markedly increased during spawning migration, indicating that much carotenoids accumulated there in this stage (Fig. 39C).

The absorption spectra of carotenoids extracted from the ovaries at different physiological state resembled each other, with absorption maxima all at 480 nm. TLC showed that astaxanthin was the main carotenoids of the ovaries (Fig. 40).

Carotenoids binding by vitellogenin

The binding of carotenoids by vitellogenin markedly increased with the amount of carotenoids added (Fig. 41). This was also shown by PAGE: the mobility of vitellogenin tended to change as carotenoids were added.

Discussion

Vitellogenin, a precursor of yolk proteins, that is synthesized by hepatocytes, accumulates in serum of non-mammalian vertebrate females during oocyte growth, and is therefore specific to females (Wallace, 1970; Redshaw and Follett, 1971). Vitellogenin has also been found in fish with regard to the gonadal maturation (Aida *et al.*, 1973; Hara, 1975; Hara *et al.*, 1978). Hara (1976, 1978) and Hara and Hirai (1978) have found that the vitellogenin in fish also binds iron. Kitahara (1983) has reported that the amount of carotenoids in chum salmon ovaries increases during spawning migration. It was interested in whether this compound is involved in transporting carotenoids to the ovaries.

The present section suggested the possibility that some of the muscle carotenoids is transported into the ovaries by the vitellogenin. The carotenoid level in muscle markedly decreased during spawning migration, so muscle carotenoids might be transported elsewhere, such as into the blood serum, integument, and ovaries (Fig. 34). Carotenoid level in blood serum markedly increased during spawning migration (Fig. 35). This suggests that muscle carotenoids were in fact transported into blood serum. Only the serum obtained from female salmon at the spawning migration stage gave rise to a precipitate when diluted with cold water (Fig. 36, Table 19). In addition, the carotenoids, which seemed to be astaxanthin,

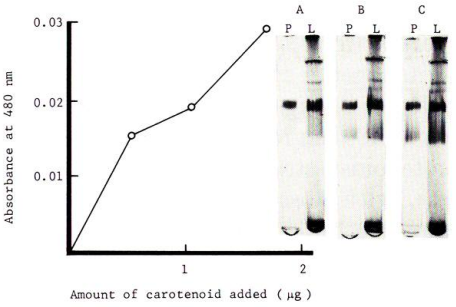

Fig. 41. Carotenoid binding by vitellogenin, as seen by spectrophotometry (solvent; acetone) and polyacrylamide gel electrophoresis. A, vitellogenin (4.5 mg) without carotenoids; B, vitellogenin (4.5 mg) plus 0.52 μg of carotenoids; C, vitellogenin (4.5 mg) plus 1.69 μg of carotenoids. P, protein staining; L, lipid staining.

were found in the precipitate (vitellogenin). The carotenoid level of vitellogenin-free serum protein from females at the spawning migration stage was almost the same as that of serum protein at the upstream migration stage (Fig. 35), indicating that some of the muscle carotenoids was transported by vitellogenin.

The amount of carotenoids per whole ovaries markedly increased during spawning migration, although that per 100 g of ovaries decreased. This suggests that much carotenoids accumulated in the ovaries during the spawning migration of these salmon. It was considered that some other lipoproteins in serum are related to carotenoid accumulation into the ovaries at this time; vitellogenin and some lipoproteins are related to this accumulation during the upstream migration, because vitellogenin was detected in the serum during the spawning migration but not during the upstream migration. Vitellogenin can bind carotenoids (Fig. 41).

Section 3. Egg yolk proteins associated with carotenoid-carrying lipoproteins

The results presented in Sections 1 and 2 on carotenoid-carrying lipoproteins in the serum of chum salmon, demonstrated that: (1) the muscle carotenoids are transported into the integument and gonads through the serum; (2) the main carotenoid-carrying lipoprotein in the serum is isolated from HDL fraction; (3) the other carotenoid-carrying lipoprotein is present in $VHDL_2$ fraction; and (4) vitellogenin in the serum of the spawning migration stage is related to the transport of carotenoids from muscle into ovaries.

There have been a number of detailed reports describing the characteristics of egg yolk proteins in fish (Ito *et al.*, 1963; Markert and Vanstone, 1968, 1971; Mano, 1970; Hara and Hirai, 1978). The carotenoid composition of fish eggs has also been examined and astaxanthin is the main carotenoid of eggs of chum salmon (Miki *et al.*, 1982; Kitahara, 1983). However, no further information is available as to which kinds of egg yolk proteins, lipovitellin and/or phosvitin, are associated with astaxanthin, except for the reports by Nakagawa and Tsuchiya (1969, 1971). They have investigated the natures of protein, lipid, and carbohydrate in the immature egg of rainbow trout (*Salmo gairdneri irideus*). The present section describes the presence of carotenoids bound to an egg yolk protein with regard to the characteristics of egg yolk proteins in chum salmon.

Materials and Methods

Materials

Ovulated eggs were collected from chum salmon specimens which were captured from the Yurappu River, Hokkaido, in November 1984 (Table 16), and kept frozen at −20°C until use.

Preparation and isolation of egg yolk proteins

To isolate egg yolk proteins, the eggs were washed with 0.75% NaCl and broken by a mixer. The mixture was strained through a Buchner funnel to remove egg membranes, and centrifuged at 25,000×*g* for 60 min at 4°C. A clear middle layer with an orange color was collected, and was dialyzed against cold distilled water overnight at 4°C. The precipitate formed was separated by centrifugation at 25,000×*g* for 30 min. The pellet with an orange color was dissolved in 0.5 M NaCl-0.02 M Tris-HCl buffer (pH 7.5) containing 0.005% EDTA and 0.02% NaN_3, and

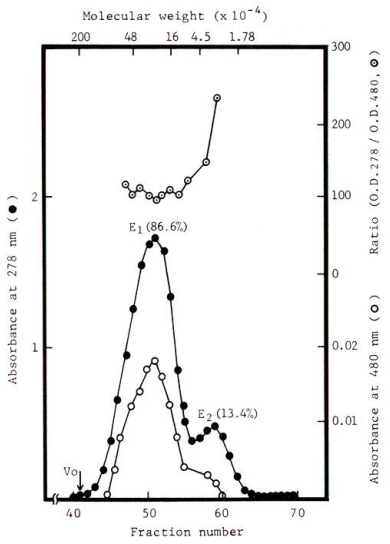

Fig. 42. Gel filtration of egg yolk proteins of chum salmon. Each 3.8 m*l* fraction was collected at 15 m*l*/hr. Materials: water-insoluble proteins isolated from chum salmon egg yolk. Column: Toyopearl HW-55 (2.2×94 cm). Elution buffer: 0.5 M NaCl-0.02 M Tris-HCl (pH 7.5) containing 0.005% EDTA and 0.02% NaN_3. Vo: void volume. Two major components were designated as E_1 and E_2. Each value in parenthesis represents the percentage of area.

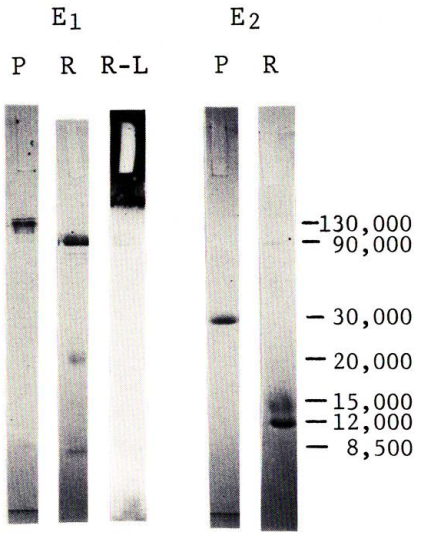

Fig. 43. SDS-slab-polyacrylamide gel (15%) electrophoresis of E_1 and E_2 from egg yolk proteins of chum salmon. "R" indicates the reduction with 2-mercaptoethanol. P, protein staining; L, lipid staining.

filtered on a Toyopearl HW-55 gel column (2.2×94 cm), with the same buffer.

Analytical methods

Procedures used for SDS-polyacrylamide gel electrophoresis and amino acid composition were as previously described (Section 1 of the present chapter).

Results and Discussion

Two peaks, E_1 and E_2, appeared as shown in Fig. 42. The molecular weights, estimated by gel filtration, were 250,000 for E_1 and 36,000 for E_2. The ratio of E_1 to E_2 (as protein) was estimated to be 6.5 to 1. This, along with their molecular weights, allowed us to calculate their molar ratio, 1 to 1. Absorbance at 480 nm due to the presence of carotenoids was mainly found in E_1 but not in E_2, thus suggesting

Table 20. Amino acid composition of two chum salmon egg yolk proteins, E_1 and E_2[a].

Amino acids[b]	E_1		E_2	
	Chum salmon	Rainbow trout[e]	Chum salmon	Rainbow trout[e]
Asp	7.34	7.66	13.88	15.69
Thr	5.16	5.33	4.19	3.99
Ser	4.29	4.80	9.30	10.86
Glu	10.06	11.60	10.55	9.97
Pro	5.65	5.36	3.38	3.17
Gly	4.13	4.05	6.60	5.72
Ala	13.32	14.24	4.25	3.51
Cys/2	0.38	—[c]	0.96	2.74
Val	8.25	7.69	8.70	7.76
Met	2.59	2.55	4.27	3.03
Ile	6.38	5.91	5.12	5.00
Leu	10.30	10.41	7.44	6.99
Tyr	3.10	2.89	3.66	3.89
Phe	4.59	4.63	2.17	1.72
His	2.53	2.20	3.22	2.25
Lys	7.06	6.27	9.42	10.59
Arg	4.45	4.41	2.07	3.15
Trp	0.40	—[d]	0.78	—[d]
Total	99.98	100.00	99.96	100.03

[a] Conditions: hydrolysed with 4 N methanesulfonic acid at 115℃ for 24 hr; determined with a Hitachi 835 automatic amino acid analyzer.

[b] The results are expressed as mol/100 mol of amino acid.

[c] Not detected.

[d] Not determined.

[e] Hara and Hirai (1978).

that carotenoids (mainly astaxanthin) were bound to E_1. This was confirmed by the ratio of absorbance at 278 nm to that at 480 nm.

Hara and Hirai (1978) have demonstrated that two egg yolk proteins, E_1 and E_2, were present in rainbow trout. The molecular weights were 300,000 for E_1 consisting of two subunits whose molecular weights were 90,000 and 15,000, and was 35,000 for E_2 consisting of a subunit whose molecular weight was 15,000. E_1 and E_2 from chum salmon were similar to the corresponding components of rainbow trout in molecular weight, but not in subunit compositions. As shown in Fig. 43, E_1 consisted of three subunits whose molecular weights were 90,000, 20,000, and 8,500, whereas E_2 consisted of two subunits whose molecular weights were 15,000 and 12,000. The 90,000 dalton subunit was positive to lipid staining, suggesting that it bound carotenoids.

Table 20 shows the amino acid composition of E_1 and E_2 from egg yolk proteins of chum salmon with the corresponding data from rainbow trout for comparison (Hara and Hirai, 1978). E_1 and E_2 from chum salmon and rainbow trout appeared similar to each other, as far as the amino acid profiles are concerned. In rainbow trout E_1 has been considered to be lipovitellin, but E_2 is not phosvitin due to a low level of serine. This seems to be true for both components of chum salmon.

It was reported that the egg yolk proteins prepared from rainbow trout had a clear yellowish color (Hara and Hirai, 1978), indicating the possible binding of carotenoids to lipovitellin. This could also be true for chum salmon.

Section 4. Metabolic pathways of carotenoids

In the previous sections, it was proved that various serum lipoproteins such as high density lipoprotein and very high density lipoproteins including vitellogenin transport the muscle astaxanthin into the integument and ovaries during spawning migration. Furthermore, it was suggested that the carotenoid-carrying lipoproteins

Table 21. Characteristics of chum salmon specimens.

Stage	Sex	Age	Date and locality of collection	Fork length (cm)	Body weight (g)	Gonadosomatic index*	Hepatosomatic index**
Feeding migration	Male	03	June 27,1985. Shizunai coast of Hokkido	68	4070	0.20	1.99
	Female	03		62	3170	4.59	2.49
Spawning migration	Male	03	Sept. 22, 1985. Shibetsu coast of Hokkido	67	3410	5.88	1.42
	Female	03		69	3800	13.06	2.73
Upstream migration	Male	03	Oct. 6, 1985. Lower reaches (2.4km) of Shibetsu River, Hokkido	68	3010	3.54	2.59
	Female	03		66	3060	20.27	1.43
Spent	Male	03	Nov. 19, 1985. Lower reaches (1.8km) of Yurappu River, Hokkido	82	5000	1.98	1.38
	Female	04		76	3840	1.25	1.67

*(Gonad weight/Body weight)×100.
**(Liver weight/Body weight)×100.

have the ability to transport not only astaxanthin but also other carotenoids. This might indicate that carotenoids have already been metabolized in the muscle. The present section describes the possibility that astaxanthin is reductively converted to zeaxanthin in the muscle of chum salmon during spawning migration.

Materials and Methods

Materials

Specimens of chum salmon in different physiological state were used as the materials (Table 21). Blood was collected from the caudal vasculature of live salmon at each migration stage and left at room temperature for several hours. The clotted blood was centrifuged to obtain the serum.

Extraction of carotenoids

Carotenoids were extracted from muscle, serum and ovaries with acetone. The

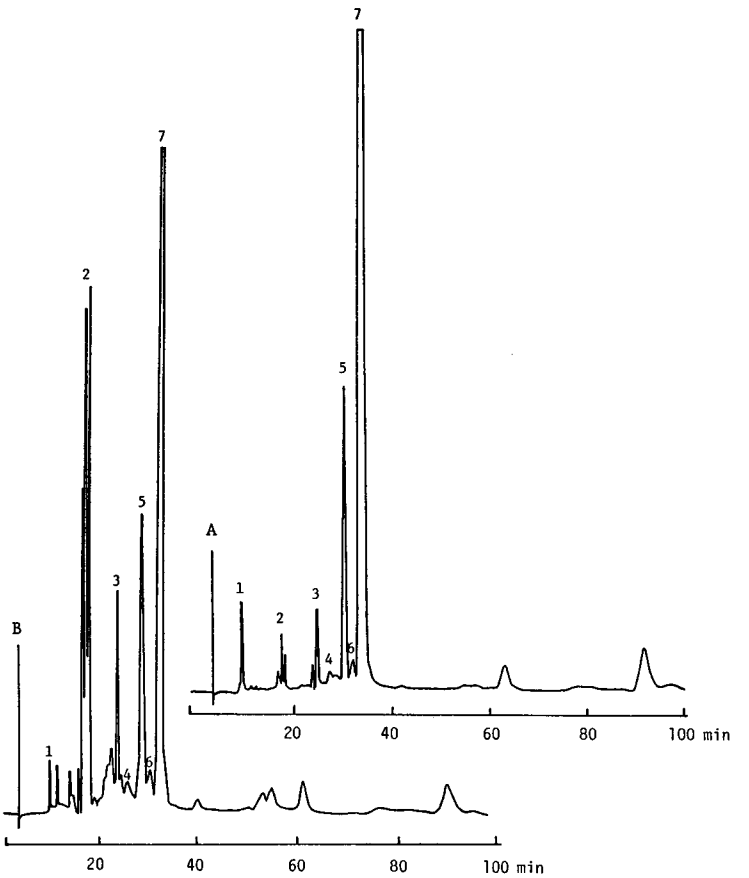

Fig. 44. HPLC separation of carotenoids extracted from the male chum salmon muscles at the feeding migration stage (A) and after spawning (B).

Table 22. Content and percentage composition of carotenoids in the chum salmon muscle during spawning migration.

	Feeding migration stage		Spawning migration stage		Upstream migration stage		Spent	
	Male	Female	Male	Female	Male	Female	Male	Female
Total carotenoids (mg/whole muscle)	16.18 (100)[b]	12.46 (100)	12.33 (100)	11.24 (100)	0.57 (100)	0.48 (100)	1.05 (100)	1.44 (100)
Canthaxanthin (1)[a]	0.12 (0.73)	0.04 (0.32)	0.04 (0.32)	0.03 (0.27)	tr (0.35)	tr (0.54)	tr (0.47)	tr (0.24)
Zeaxanthin (2)	0.19 (1.15)	0.10 (0.77)	0.32 (2.61)	0.31 (2.80)	0.07 (11.85)	0.04 (8.44)	0.26 (23.75)	0.29 (20.04)
4-keto-zeaxanthin (3)	0.34 (2.08)	0.20 (1.61)	0.45 (3.69)	0.48 (4.30)	0.03 (4.99)	0.02 (4.27)	0.05 (4.99)	0.05 (3.42)
Diatoxanthin (4) + Cynthiaxanthin (4)	0.06 (0.35)	0.05 (0.37)	0.07 (0.55)	0.12 (1.06)	0.01 (1.53)	tr (0.43)	0.01 (0.85)	tr (0.36)
Astaxanthin fraction	14.52 (89.75)	11.42 (91.70)	10.68 (86.71)	9.44 (84.00)	0.42 (73.43)	0.37 (78.56)	0.70 (64.63)	1.02 (70.69)
(3R, 3′R)-Astaxanthin (5)	1.28 (7.90)	1.65 (13.23)	1.28 (10.42)	1.00 (8.90)	0.04 (6.63)	0.04 (8.94)	0.11 (10.46)	0.19 (12.96)
(3R, 3′S; *meso*)-Astaxanthin (6)	0.07 (0.43)	0.08 (0.67)	0.06 (0.52)	0.07 (0.64)	tr (tr)	tr (tr)	0.01 (0.58)	0.01 (0.52)
(3S, 3′S)-Astaxanthin (7)	13.17 (81.42)	9.69 (77.80)	9.34 (75.77)	8.37 (74.46)	0.38 (66.80)	0.33 (69.62)	0.58 (53.59)	0.82 (57.21)
Unidentified	0.95 (5.94)	0.65 (5.23)	0.77 (6.12)	0.86 (7.57)	0.04 (7.85)	0.05 (7.76)	0.03 (5.31)	0.08 (5.25)

[a] Peak numbers on chromatogram.

[b] Values in parentheses represent percentages, with total carotenoids being 100%.

total carotenoid content was calculated, assuming the $E^{1\%}_{1cm}$ value in acetone at 477 nm to be 2,200.

After concentration under reduced pressure, the carotenoids were transferred to diethyl ether by the addition of distilled water. The aqueous phase was extracted with ether several times. The combined ethereal layer was concentrated under reduced pressure. The residue was submitted to high performance liquid chromatography (HPLC).

HPLC analysis of carotenoids

HPLC was carried out on a Shimadzu LC-6A instrument with a Shimadzu SPD-2A VIS spectrophotometer set at 470 nm. The column used was a 250×4 mm I.D. stainless steel column packed with 5 μm Sumipax OA-2000 (Sumitomo Chemical Co., Ltd.). Separation was achieved with a mobile phase of *n*-hexane-CH_2Cl_2-EtOH (50 : 20 : 0.5), flow-rate of 0.8 m*l*/min. Identification of each carotenoid was accomplished by co-thin-layer chromatography and co-HPLC with authentic specimens. The authentic carotenoids used were as follows : Astaxanthin diester, astaxanthin monoester and astaxanthin were extracted and purified from the Antarctic krill *Euphausia superba* (Yamaguchi *et al.*, 1983 ; Maoka *et al.*, 1985). Zeaxanthin, diatoxanthin and cynthiaxanthin were extracted and isolated from *Spirulina maxima* (Miki *et al.*, 1986).

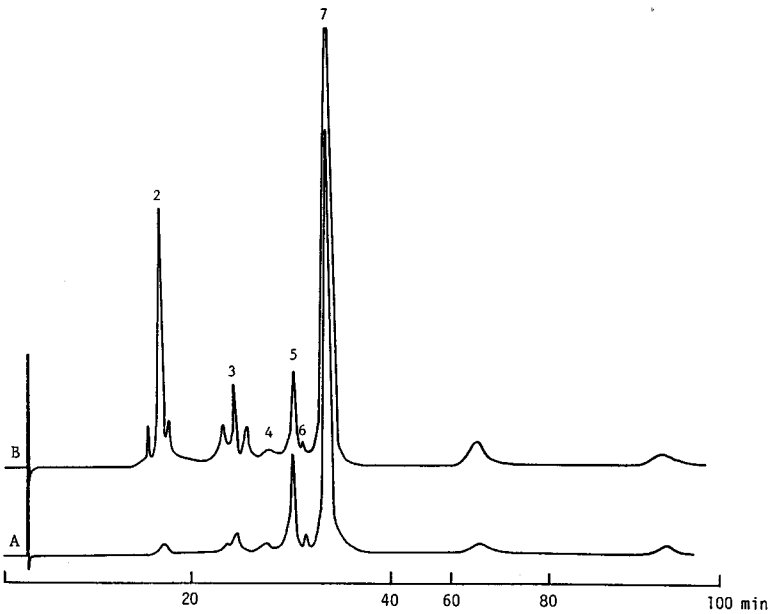

Fig. 45. HPLC separation of carotenoids extracted from the male chum salmon sera at the spawning migration stage (A) and after spawning (B).

Table 23. Content and percentage composition of carotenoids in the chum salmon serum during spawning migration.

	Spawning migration stage		Upstream migration stage		Spent	
	Male	Female	Male	Female	Male	Female
Total carotenoids (ng/m*l* serum)	3298 (100)[b]	4298 (100)	9449 (100)	8259 (100)	6250 (100)	8139 (100)
Zeaxanthin (2)[a]	18 (0.55)	47 (1.10)	816 (8.64)	370 (4.48)	1241 (19.86)	159 (1.95)
4-keto-zeaxanthin (3)	85 (2.57)	49 (1.15)	734 (7.77)	446 (5.40)	506 (8.10)	418 (5.13)
Diatoxanthin (4) + Cynthiaxanthin (4)	36 (1.10)	9 (0.22)	106 (1.12)	12 (0.14)	61 (0.97)	38 (0.47)
Astaxanthin fraction	3000 (90.92)	3856 (89.68)	6954 (73.59)	6762 (81.87)	3800 (60.80)	7173 (88.13)
(3R, 3′R)-Astaxanthin (5)	469 (14.22)	346 (8.04)	879 (9.30)	1514 (18.33)	459 (7.34)	1403 (17.24)
(3R, 3′S; *meso*)-Astaxanthin (6)	29 (0.88)	14 (0.33)	52 (0.55)	38 (0.46)	29 (0.46)	88 (1.08)
(3S, 3′S)-Astaxanthin (7)	2502 (75.82)	3496 (81.31)	6023 (63.74)	5210 (63.08)	3312 (53.00)	5682 (69.81)
Unidentified	159 (4.86)	337 (7.85)	839 (8.88)	669 (8.11)	642 (10.27)	351 (4.32)

[a] Peak numbers on chromatogram.

[b] Values in parentheses represent percentages, with total carotenoids being 100%.

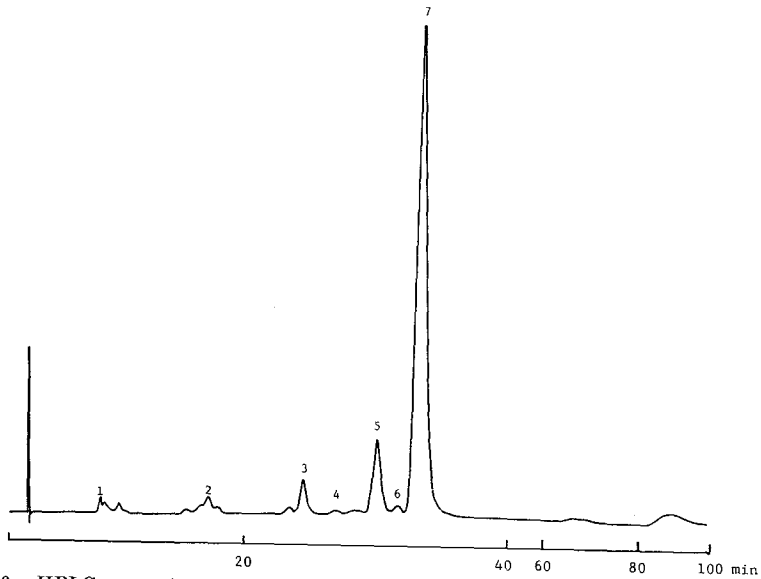

Fig. 46. HPLC separation of carotenoids extracted from the chum salmon ovaries at the upstream migration stage.

Table 24. Content and percentage composition of carotenoids in the chum salmon ovaries during spawning migration.

	Feeding migration stage	Spawning migration stage	Upstream migration stage
Total carotenoids (mg/whole ovaries)	2.510 (100)[b]	2.580 (100)	4.863 (100)
Canthaxanthin(1)[a]	0.043 (1.73)	0.016 (0.61)	0.020 (0.41)
Zeaxanthin(2)	0.056 (2.24)	0.137 (5.31)	0.070 (1.44)
4-keto-zeaxanthin(3)	0.049 (1.94)	0.164 (6.34)	0.193 (3.97)
Diatoxanthin(4) + Cynthiaxanthin(4)	0.011 (0.43)	0.034 (1.30)	0.046 (0.95)
Astaxanthin fraction	2.181 (86.90)	2.109 (81.75)	4.276 (87.92)
(3R,3'R)-Astaxanthin(5)	0.292 (11.64)	0.251 (9.73)	0.473 (9.72)
(3R,3'S; *meso*)-Astaxanthin(6)	0.014 (0.54)	0.014 (0.53)	0.032 (0.66)
(3S,3'S)-Astaxanthin(7)	1.875 (74.72)	1.844 (71.49)	3.771 (77.54)
Unidentified	0.170 (6.76)	0.121 (4.69)	0.258 (5.31)

[a] Peak numbers on chromatogram.
[b] Values in parentheses represent percentages, with total carotenoids being 100%.

Results

Typical chromatograms of muscle carotenoids are shown in Fig. 44. (3S, 3'S)-Astaxanthin was a dominant carotenoid at the feeding migration stage, while the percentages of zeaxanthin and 4-keto-zeaxanthin increased during spawning migration. However, the amounts of all carotenoids including astaxanthin, zeaxanthin and 4-keto-zeaxanthin decreased during spawning migration (Table 22). The similar behavior of carotenoid composition was observed in both males and females.

The serum carotenoid level markedly increased during spawning migration. Typical chromatograms of serum carotenoids are shown in Fig. 45. (3S, 3'S)-Astaxanthin was a dominant carotenoid at the spawning migration stage, while zeaxanthin and 4-keto-zeaxanthin as well as astaxanthin markedly increased at the upstream migration stage and after spawning. The increase of zeaxanthin and 4-keto-zeaxanthin was particularly found in male serum (Table 23).

The ovary carotenoids level markedly increased during spawning migration. A typical chromatogram of ovary carotenoids is shown in Fig. 46. (3S, 3'S)-Astaxanthin was a dominant carotenoid during spawning migration. The similar behavior of carotenoid composition was observed during spawning migration (Table 24).

Discussion

Several metabolic pathways, based on the contents and individual composition of carotenoids and on their stereochemical grounds, have been proposed. The reductive metabolic pathways of astaxanthin have been suggested in the integuments of yellowtail *Seriola quinqueradiata* (Fujita *et al.*, 1983a ; Miki *et al.*, 1985) and red sea bream *Pagrus major* (Fujita *et al.*, 1983b). Matsuno *et al.* (1985) have reported the reductive metabolic pathways of (3S, 3'S)-astaxanthin in the eggs of marine fish from the stereochemical point of view. Kitahara (1983) has suggested the following reductive metabolism of astaxanthin in the integument of chum salmon, astaxanthin→4-keto-zeaxanthin→β-carotene-triol→zeaxanthin. Thus, the reductive metabolic pathways of carotenoids are limited to the integuments and eggs of fish.

In this study, however, the reductive metabolic pathways of astaxanthin in the muscle of chum salmon were presumed from the following reasons :

1) The muscle carotenoid level greatly decreased during spawning migration. If the carotenoids are not metabolized in the muscle, the similar behavior of carotenoid composition is observed during spawning migration. As shown in Fig. 44 and Table 22, however, the percentages of zeaxanthin and 4-keto-zeaxanthin in the muscle carotenoids increased and those of astaxanthin decreased during spawning migration. This suggested that astaxanthin was reductively converted to zeaxanthin *via* 4-keto-zeaxanthin in the muscle. The similar metabolism of muscle carotenoids was observed in both males and females.

2) The reductive metabolic pathways of astaxanthin in the muscle were also

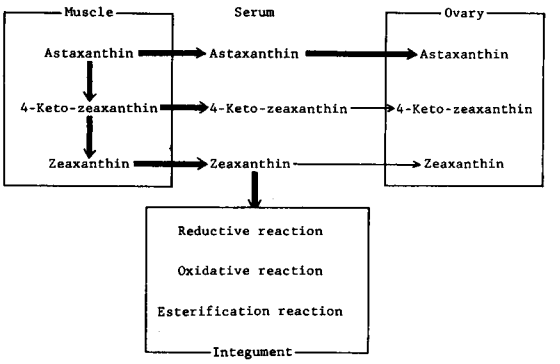

Fig. 47. A presumed metabolic pathway of carotenoids in the chum salmon during spawning migration. ⟹, main pathways; ⟶, supplemental pathways.

confirmed by the results of the carotenoid composition in the serum (Fig. 45 and Table 23). Appreciable amounts of zeaxanthin and 4-keto-zeaxanthin as well as astaxanthin were detected in male serum during spawning migration. The carotenoid metabolism in the muscle was considered to be responsible for the increase of carotenoids in the serum. The reductive metabolites of astaxanthin in female serum were much less than those in male serum, although astaxanthin was reductively metabolized in the muscle of both males and females. This indicated that zeaxanthin and 4-keto-zeaxanthin as well as astaxanthin in female serum were transported rapidly into the integument, since astaxanthin was a dominant carotenoid in the ovaries during spawning migration (Fig. 46 and Table 24). The sexual differences in the transport rate of carotenoids from the muscle to the integument *via* the serum might influence the carotenoid composition in the serum.

A presumed metabolic pathway, based on the contents and individual composition of carotenoids in the muscle, serum and ovaries of chum salmon during spawning migration, is given in Fig. 47. This pathway represents a new presumption, the reductive metabolism of astaxanthin in the muscle. This indicates that the reductive enzymes system of carotenoids are present not only in the integument but also in the muscle.

Section 5. Carotenoid decomposition

The composition of carotenoids in various tissues of salmon has been investigated in detail (Matsuno *et al.*, 1980a, b, c). Kitahara (1983) has reported on the behavior of carotenoids in chum salmon during their anadromous migration and found that the carotenoid content markedly decreased in muscle and increased in integument and ovaries as maturation advanced. These results, however, were explained on the basis of the relative concentrations of carotenoid in the tissues. To compare the carotenoid metabolism in various tissues of salmon, it is necessary to measure the levels of carotenoids. No such reports have been published except for the studies by Crozier (1970). He found that more than 60% of the carotenoids in sockeye salmon disappeared during spawning migration. This might suggest that carotenoids in fish are decomposed by some tissue, possibly the liver, during spawn-

ing migration.

In the present section, the actual amounts of carotenoids were measured in various tissues of spawning-migrating chum salmon and it was found that more than 60% of the carotenoids may be decomposed by heme proteins of the liver.

Materials and Methods

Materials

Chum salmon specimens as shown in Table 16 were used as materials.

Extraction of carotenoids

The tissues such as ordinary muscle, integument, gonads, and the others were repeatedly homogenized in acetone until the acetone layer became colorless. The carotenoid level was calculated, assuming the $E^{1\%}_{1cm}$ value in acetone at 477 nm to be 2,200.

Preparation of liver extracts

The liver was exhaustively washed with 0.75% NaCl, cut into small pieces with scissors, and homogenized with 4 vol. of 0.5 M KCl with cooling by ice-water. The homogenate was centrifuged at $10{,}000 \times g$ for 20 min at 4°C and the supernatant was assayed for astaxanthin bleaching activity.

Assay of astaxanthin bleaching activity

Astaxanthin bleaching activity was measured in the presence of 0.2 m*l* of 0.005% free astaxanthin-containing acetone, 0.2 m*l* of 2.5% linoleic acid-containing acetone, 0.2 m*l* of liver extracts, and 4.4 m*l* of 0.02 M phosphate buffer (pH 7.0). After incubation at 25°C for various periods in the dark, the reaction was stopped by

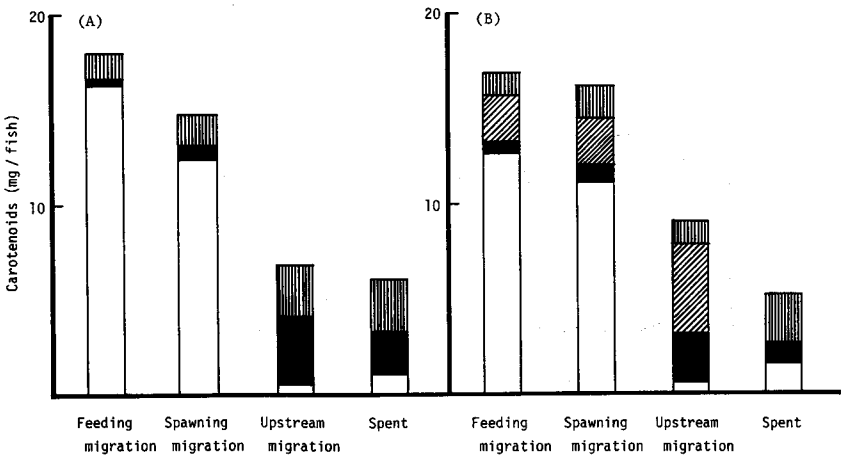

Fig. 48. Changes in carotenoid level of chum salmon during spawning migration. A, male; B, female; □, ordinary muscle; ■, integument; ▨, ovaries; ▥, others.

mixing with 3.0 m*l* of ethanol and 5.0 m*l* of diethyl ether, and the absorbance at 480 nm of the ether layer was measured. Astaxanthin bleaching activity was also measured in the presence of oleic or stearic acid. In addition, the astaxanthin bleaching activity of liver extracts heated at 100°C for 10 min was measured in the presence of linoleic acid.

Assay of thiobarbituric acid value

The thiobarbituric acid value of liver extracts with or without heat treatment (100°C, 10 min) was measured by the method of Vyncke (1972).

Results

Changes in carotenoid levels of chum salmon during spawning migration

As shown in Fig. 48, muscle carotenoid level in male fish greatly decreased during spawning migration, while the integument carotenoid level greatly increased. A similar behavior of carotenoids was observed in female fish, except for the gonads. The carotenoid content of ovaries greatly increased during spawning migration. The carotenoid level after spawning, however, was almost the same as that of spent male fish. More noticeable was a marked decrease of carotenoid levels at the upstream migration stage.

Decomposition of astaxanthin by chum salmon liver tissue

The marked decreae of carotenoid levels at the upstream migration stage was considered to imply the decomposition of fish carotenoids by some tissue. Preliminary experiments revealed that no carotenoid decomposition occurred in the integument. Therefore, the possibility of carotenoid decomposition by liver tissue was examined.

Only when fatty acids were present in the reaction medium, was astaxanthin bleaching activity recognized (Fig. 49A). The highest activity was detected in the

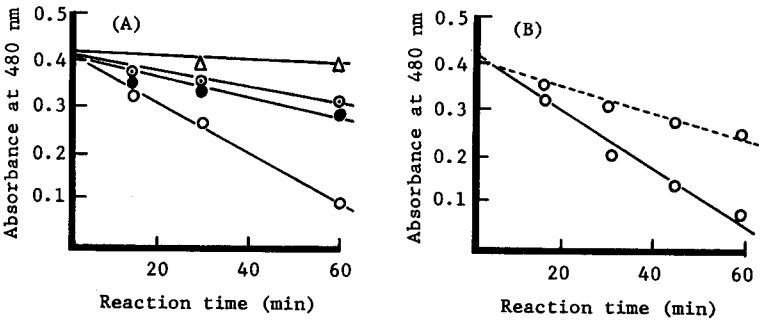

Fig. 49. Astaxanthin bleaching by liver extracts.
A. Effects of fatty acid : △, no fatty acid (control) ; ⊙, stearic acid ; ●, oleic acid ; ○, linoleic acid.
B. Effects of heat treatment of liver extracts. Astaxanthin bleaching activity was measured in the presence of linoleic acid. ——, native liver extracts ; ------, liver extracts heated at 100°C for 10 min.

Table 25. Thiobarbituric acid values of native and heated liver extracts*.

	Thiobarbituric acid value ($O.D._{538}$/g liver tissue)
Native liver extracts	0.144
Heated liver extracts	0.216

* The heated liver extracts were prepared from the native liver extracts, which were heated at 100°C for 10 min. The liver extracts were homogenized with 2 vol. of 7.5% trichloroacetic acid solution containing 0.1% EDTA and 0.1% propyl gallate. The mixture was filtered through a Toyo No. 3 paper. Five m*l* of filtrate was incubated with 5 m*l* of 0.02 M thiobarbituric acid solution at 100°C for 40 min and the absorbance at 538 nm was measured.

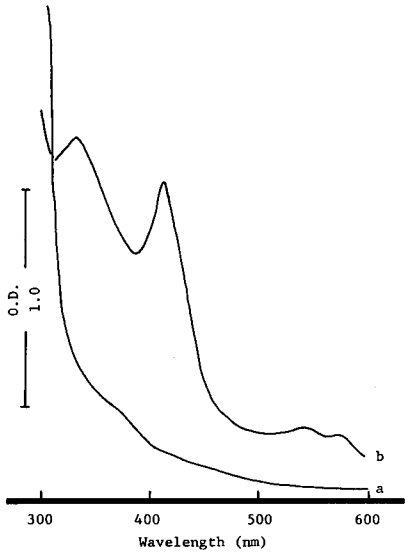

Fig. 50. Absorption spectra of native (b) and heated (a) liver extracts. The liver extracts heated at 100°C for 10 min was centrifuged at 10,000 × *g* for 20 min and the supernatant was used for absorption spectrum.

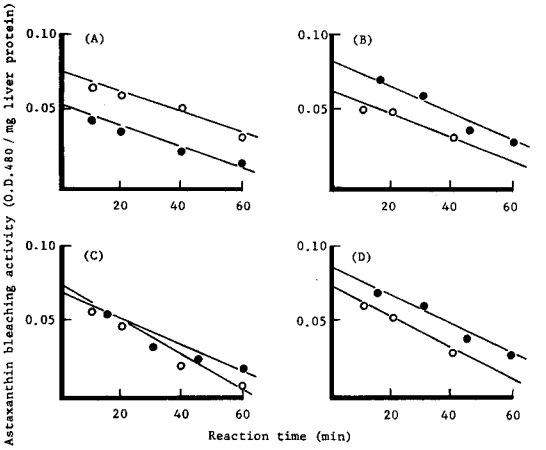

Fig. 51. Astaxanthin bleaching activities at four different migratory stages. A, feeding migration stage; B, spawning migration stage; C, upstream migration stage; D, spent; ●, male; ○, female.

presence of linoleic acid. Thin-layer chromatography of astaxanthin decomposition products showed an immobile spot, when developed with *n*-hexane/acetone (4 : 1, v/v). This activity was not completely inhibited by the heat treatment of liver extracts (Fig. 49B). After being heated at 100°C for 10 min, the liver extracts retained about 50% of the original activity.

The thiobarbituric acid value of liver extracts was measured to examine why

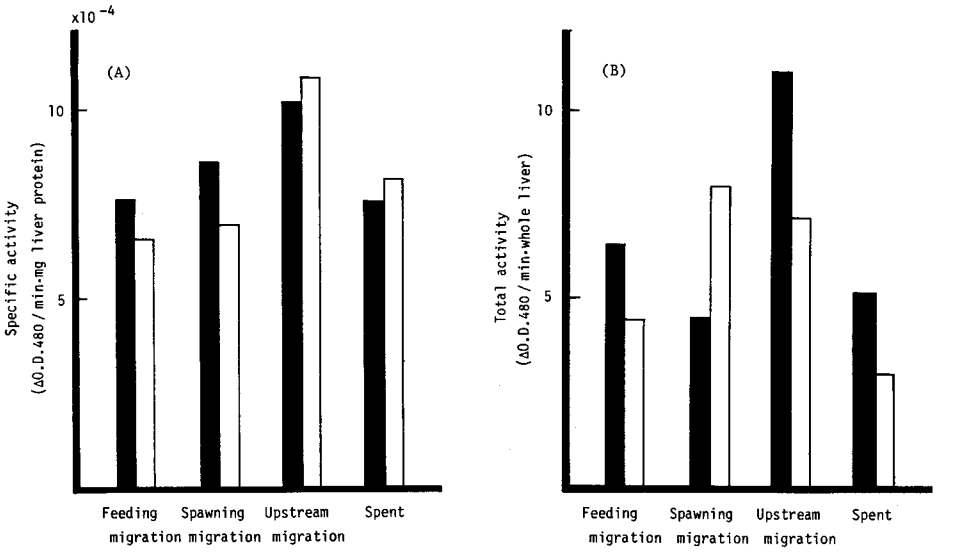

Fig. 52. Changes in the specific (A) and total (B) astaxanthin bleaching activity during spawning migration. ■, male; □, female.

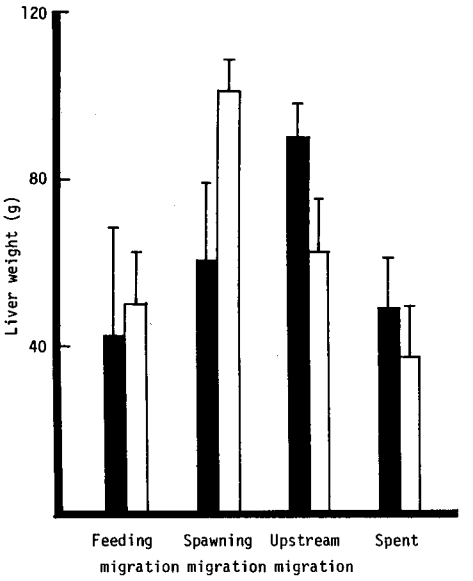

Fig. 53. Changes in liver tissue weight during spawning migration. The vertical bars represent mean±standard deviation of three samples. ■, male; □, female.

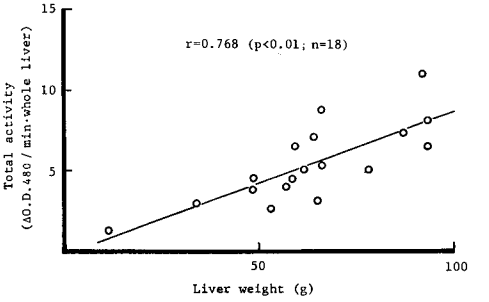

Fig. 54. Relationship between the total astaxanthin bleaching activity and the weight of liver.

astaxanthin bleaching activity was retained in the heated liver extracts. The heat treatmemt elicited 50% augmentation in the thiobarbituric acid value of liver extracts, suggesting the oxidation of fatty acids in the liver extracts (Table 25). This might be responsible for the high level of astaxanthin bleaching activity in the heated liver extracts, although the liver proteins are denatured by the

heat treatment.

The liver extracts showed an absorption maximum at 410-415 nm and two broad ones at 540-545 and 575-580 nm, reminding us of the spectrum specific to heme proteins. This spectrum, however, completely disappeared after heating under the above conditions (Fig. 50).

Changes in astaxanthin bleaching activity during spawning migration

Astaxanthin was bleached by the liver tissue irrespective of the stage of migration (Fig. 51). The specific activity, as evaluated by the slope of the straight line, tended to increase slightly during spawning migration (Fig. 52A).

Changes in the weight of chum salmon liver have been pointed out as a physiological characteristic during the spawning migration (Yamazaki, 1985). Liver weight in male fish increased at the upstream migration stage and then decreased greatly, while that in females increased at the spawning migration stage and decreased greatly at the upstream migration stage (Fig. 53). The total astaxanthin bleaching activity greatly increased at the upstream migration stage, indicating a marked decomposition of carotenoids (Fig. 52B). A parallel between the total activity and the liver weight can be seen in Fig. 54.

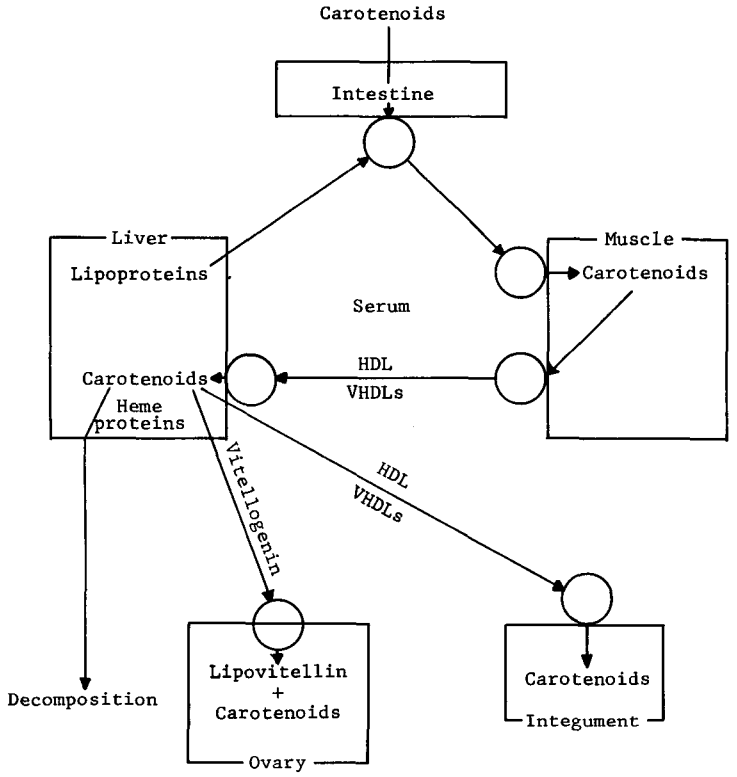

Fig. 55. A scheme proposed for carotenoid metabolism in chum salmon. Circles, carotenoid-carrying lipoproteins.

Discussion

Heavy metals and heme compounds catalyze the peroxidation of unsaturated lipids. The resulting hydroperoxides have an oxidizing potential and easily oxidize and discolor carotenoids and polyphenols (Tappel, 1962). Lipoxygenase was initially found in soybeans and later in a wide variety of plants. The presence of lipoxygenase-like activity in microorganisms (Satoh *et al.*, 1976; Matsuda *et al.*, 1976) and fish integument tissue (Tsukuda, 1972; Yamaguchi and Toyomizu, 1984; German and Kinsella, 1985). Tsukuda (1972) found that carotenoids were decomposed by such an enzyme in the integument of red fish.

The present study suggested that appreciable amounts of carotenoids in chum salmon were decomposed during spawning migration, some of the carotenoids being accumulated in the integument and ovaries. Carotenoids were considered to be decomposed by heme proteins in the liver because the liver extracts had a heme-protein-specific spectrum featuring absorption maxima at 410–415, 540–545, and 575–580 nm; because fatty acids were necessary for astaxanthin bleaching activity in the liver extracts. The activity was high in the presence of highly unsaturated fatty acids such as linoleic acid, and low in the presence of oleic or stearic acid. Also because astaxanthin bleaching activity did not completely vanish upon heating liver extracts at 100°C for 10 min, and because high astaxanthin bleaching activity occurred at the upstream migration stage when the carotenoid level decreased sharply. The specific astaxanthin bleaching activity in the liver remained at essentially the same level regardless of migratory stage, although the liver weight varied widely during spawning migration. Therefore, the changes in liver size were supposed to be responsible for the changes in carotenoid decomposition during spawning migratiom. Kusaka *et al.* (1985) have reported the high contents of polyunsaturated fatty acids in chum salmon liver. The *in vitro* carotenoid decomposition by the liver extracts shown here was considered to occur in the liver tissue *in vivo*.

In the previous sections, it has been demonstrated that various serum lipoproteins, such as HDL and the VHDL fractions including vitellogenin, transport

Table 26. Protein composition of hormone-

Treatment	Total protein	Sarcoplasmic protein	Myofibrillar protein	Gel fraction free protein
	g/100 g muscle			
Control	15.20	5.27 (34.67)*	8.93 (58.75)*	5.20 (34.21)*
Estradiol-17β	15.86	4.92 (31.02)	9.76 (61.54)	4.29 (27.05)
17α-Methyl-testosterone	13.21	3.48 (26.34)	8.92 (67.52)	4.78 (36.18)

* % of total protein.

astaxanthin, which is the main carotenoid in chum salmon muscle, into the integument and ovaries during spawning migration. The results of this study showed the possibility that carotenoid transported from muscle tissue by serum lipoproteins is decomposed by heme proteins of liver tissue. A scheme of the whole carotenoid metabolism in chum salmon is shown in Fig. 55. It was suggested that appreciable amounts of carotenoid in chum salmon are decomposed by heme proteins of liver during spawning migration, although the physiological roles of this carotenoid decomposition are obscure.

Chapter IV. Effect of sex steroid hormones on muscle composition of chum salmon

It is well known that sex steroid hormones in fish are concerned in secondary sex characters, spawning behavior, and thickness of the integument (Pickford and Atz, 1957). Fostier *et al.* (1983) have reviewed the physiological role of gonadal steroid hormones in reproduction.

In the previous chapters II and III, it has been suggested that serum sex steroid hormones such as testosterone and estradiol-17β were closely related to various phenomena occurring in chum salmon during spawning migration.

The present chapter describes the effect of sex steroid hormones on muscle composition of chum salmon.

Section 1. Changes in protein composition and protease activity of juvenile chum salmon muscle upon treatment with sex steroid hormones

In the previous chapters, it has been suggested that serum sex steroid hormones such as testosterone and estradiol-17β were closely related to the protein degradation and to high levels of protease activity of chum salmon muscle during spawning migration.

The aim of the present section is to ascertain whether 17α-methyltestosterone (MT), a synthetic androgen, and estradiol-17β are capable of inducing the protein degradation and the activation of protease.

treated juvenile chum salmon.

Gel fraction protein	Alkali-soluble protein	Alkali-insoluble protein	Ninhydrin positive substances (as Tyrosine)
g/100 g muscle			
3.73 (24.54)*	0.76 (5.00)*	0.24 (1.58)*	0.601
5.47 (34.49)	0.98 (6.18)	0.20 (1.26)	0.611
4.14 (31.34)	0.65 (4.92)	0.16 (1.21)	0.917

Materials and Methods

Materials

Juvenile chum salmon of about 4 g in average body weight and 8 cm in average fork length were used in the experiments.

The two groups were fed on dried pellets containing 30 μg MT and 30 μg estradiol-17β per gram diet, respectively, for 3 or 4 weeks. Control fish were fed on normal dried pellets for the same periods as the hormone-treated groups. The dried pellets were contained carotenoids (canthaxanthin) at the concentration of 2 mg per gram diet.

Determination of protein composition and SDS-polyacrylamide gel electrophoresis

Fish fed for 4 weeks were used for the determination of protein composition. The procedures were previously described (Chapter II).

Measurement of autolytic activity

Fish fed for 3 weeks were used for the measurement of autolytic activity. The same procedure reported in Chapter II was used.

Results

Changes in integument coloration

It was noticeable that only the samples treated with MT had nuptial coloration. Estradiol-17β induced no change in appearance and the fish retained the silvery body coloration (Ando *et al.*, 1986a).

Changes in protein composition

Table 26 shows the changes in protein composition upon treatment with the sex steroid hormones. Total protein and sarcoplasmic protein markedly decreased upon treatment with MT. No changes were found in myofibrillar protein upon treatment

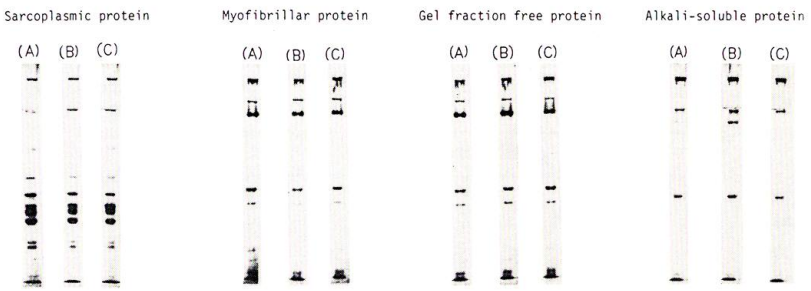

Fig. 56. SDS-slab-polyacrylamide gel (10%) electrophoretic patterns of muscle protein fractions of hormone-treated juvenile chum salmon. (A), control; (B), estradiol-17β; (C), 17α-methyltestosterone.

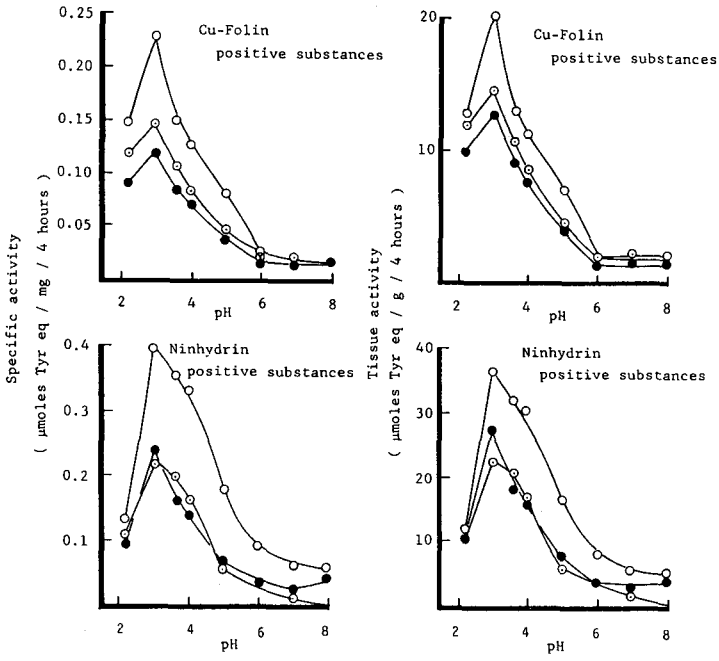

Fig. 57. Autolytic activity of hormone-treated juvenile chum salmon muscle homogenate as a function of pH. Control, (●—●); estradiol-17β, (⊙—⊙); 17α-methyltestosterone, (○—○).

with the sex steroid hormones. It was noticeable that ninhydrin positive substances from protein free fractions markedly increased upon treatment with MT. In contrast, estradiol-17β did not affect the protein composition.

The SDS-polyacrylamide gel electrophoretic patterns of the protein fraction did not significantly change upon treatment with these sex steroid hormones (Fig. 56).

Changes in autolytic activity

Figure 57 shows the changes in autolytic activity of muscle upon treatment with the sex steroid hormones. The autolytic activity which was expressed as Cu-Folin and ninhydrin positive substances markedly increased upon treatment with MT. High levels of autolytic activity were found at around pH 3. Autolytic activity at pH 6, corresponding to physiological state, slightly increased upon treatment with MT. In contrast, estradiol-17β did not significantly influence the autolytic activity.

Discussion

Konagaya (1982, 1983, 1985a, b) and Nomata *et al.* (1985a) have reported that the protease activity of muscle markedly increased during spawning migration and that high levels of protease activity were found in very mushy muscle. In Chapter II, the above phenomena have been confirmed and the close relation between

protease activity and hormonal conditions of chum salmon has been pointed out.

The results of the present section clearly suggested that androgens were responsible for various changes of chum salmon muscle during spawning migration. Upon treatment with MT a slight nuptial coloration was observed on the integument of juvenile chum salmon. Sarcoplasmic protein markedly decreased, while ninhydrin positive substances from protein free fractions markedly increased upon treatment with MT. Autolytic activity of the fish treated with MT markedly increased. It was therefore considered that the changes found in MT-treated juvenile chum salmon might correspond to those at the upstream migration stage.

In contrast, fish treated estradiol-17β retained the silvery body color of juvenile chum salmon. No changes were found in protein composition and autolytic activity of muscle upon treatment with estradiol-17β. It has been shown that estrogen is related to the maturation of the ovaries (Nagahama, 1982). This might be responsible for the lack of effect of estradiol-17β on muscle protein.

Section 2. Effect of 17α-methyltestosterone on muscle composition of chum salmon

In the previous section of the present chapter, it has been found that 17α-methyltestosterone, a synthetic androgen, but not estradiol-17β is involved in the deterioration of juvenile chum salmon muscle. The present study was conducted to ascertain whether 17α-methyltestosterone induced deterioration of sexually immature chum salmon muscle.

Materials and Methods

Materials

Injection of 17α-methyltestosterone (MT) into chum salmon was accomplished as follows. Immediately after capture in the North Pacific Ocean, the fish was injected intraperitoneally with MT (1.5 mg) dissolved in olive oil. The fish was then transferred to a 700 liter aquarium supplied with running seawater and retained unfed for 5 days on the board of the training ship Hokusei Maru, belonging to Faculty of Fisheries, Hokkaido University. Control fish received an injection of olive oil alone. These samples were kept at -20°C until use. Dorsal muscle was

Table 27. Characteristics of chum salmon specimens.

Treament (Sex)	Collection date and locality	Fork length (cm)	Body weight (g)	Gonadosomatic index*	Age	Remarks
Control (Female)	July 17, 1983. Lat.47°30'N Long.170°01'E	51	1480	1.2	03	Sexually immature adult No breeding color
17α-Methyltestosterone (Female)		54	1460	10.3	03	Intraperitoneal injection (1.5 mg/3 ml olive oil) Mature adult Very pronounced signs of breeding color

* (Gonad weight/Body weight) $\times$ 100.

collected for the following analyses. Also, blood samples were collected from the caudal vasculature.

Analytical methods

Procedures used for proximate, fatty acid and protein composition, SDS-polyacrylamide gel electrophoresis, autolytic activity, acid and neutral proteinase activities, and serum protease inhibitory activity, as well as, radioimmunoassay for serum measurements of testosterone and estradiol-17β were as previously described (Chapter II).

Results

Serum concentrations of sex steroid hormones

The location where the fish were captured, gonadosomatic index (GSI) value, age of fish, and responses in integument coloration to treatment are given in Table 27 (Ando *et al.*, 1986b).

Table 28 shows the changes in serum concentrations of sex steroid hormones following treatment with MT. It was noticeable that serum levels of testosterone significantly increased upon treatment with MT. No change in estradiol-17β concentration was found.

Proximate composition and fatty acid composition

In the hormone treated fish the lipid content showed a marked decreased while a modest increase was evident in the protein level of the muscle (Table 29).

Figure 58 shows a thin-layer chromatogram of lipid composition. MT induced

Table 28. Serum testosterone and estradiol-17β levels in chum salmon administered 17α-methyltestosterone (MT).

Treatment (Sex)		Testosterone (ng/ml)	Estradiol-17β (ng/ml)
Control (Female)		8.51	5.86
MT (Female)	0 day	82.30	2.81
	5 days	369.27	1.56

Table 29. Proximate composition of chum salmon dorsal muscle following treatment with 17α-methyltestosterone(MT).

Treatment (Sex)	Moisture	Protein*	Lipid	Ash	Extractive-N
	g/100 g muscle				
Control (Female)	74.64	19.11	4.30	1.30	0.382
MT (Female)	71.52	23.48	0.94	1.41	0.567

* (Total N − Extractive-N) × 6.25.

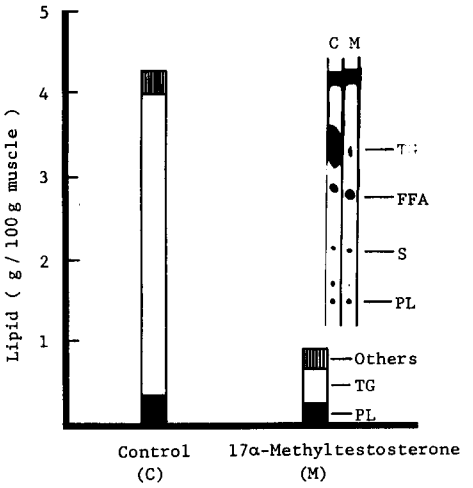

Fig. 58. Changes in total, triglyceride, and phospholipid contents of chum salmon dorsal muscle following treatment with 17α-methyltestosterone. Thin-layer chromatography plate was developed using n-hexane- diethyl ether-acetic acid (85 : 15 : 1, v/v/v). C, control samples ; M, 17α-methyltestosterone-treated sample ; TG, triglyceride ; FFA, free fatty acid ; S, sterol ; PL, phospholipid.

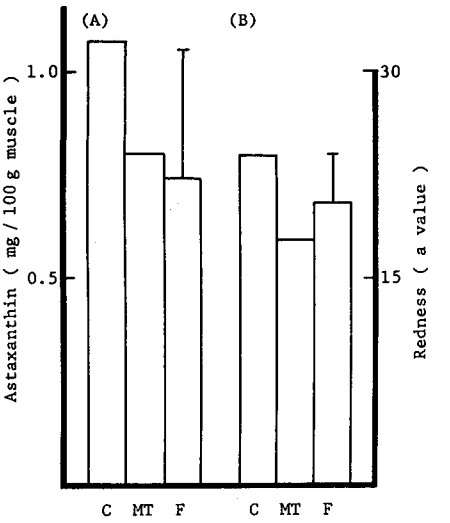

Fig. 59. Changes in astaxanthin content (A) and redness (B) of chum salmon dorsal muscle following treatment with 17α-methyltestosterone. The vertical bars represent the mean$\pm$standard deviation of astaxanthin content and redness of chum salmon at the feeding migration stage, respectively. C, control ; MT, 17α-methyltestosterone ; F, feeding migration stage.

a decrease in triglyceride. Phospholipid content did not change upon treatment (Table 30).

The relative ratio of saturated fatty acids to total lipid markedly decreased, while that of polyenoic fatty acids markedly increased following treatment with MT (Table 31). Similar changes in fatty acid composition were observed in phospholipid. However, no significant differences were found in the fatty acid composition of non-phospholipid following treatment with MT. It was noticeable that MT induced a relative increase in polyenoic fatty acids to total lipid.

Figure 59 shows the changes in astaxanthin content and "a" value (an index of red color in muscle), respectively. Neither of these components was significantly altered following treatment with MT.

Changes in serum color

Serum color of MT-treated fish was reddish. This might be related to the transport of muscle astaxanthin into the blood (Ando *et al.*, 1986b).

Protein composition

Treatment with MT resulted in marked increases in gel fraction protein, alkali-soluble protein, and ninhydrin positive substances (Table 32). Sarcoplasmic protein, however, was not affected.

Table 30. Effect of 17α-methyltestosterone (MT) on lipid content of chum salmon muscle.

Lipid class	Lipid contents (g/100 g muscle)	
	Control	MT
Phospholipid	0.35 (8.2)	0.26 (27.6)
Partial glyceride	0.31 (7.2)	0.02 (2.1)
Sterol	0.25 (5.8)	0.04 (4.2)
Free fatty acid	0.07 (1.6)	0.17 (18.1)
Triglyceride	3.31 (77.2)	0.45 (47.9)
Total lipid	4.29 (100)	0.94 (100)

Values in parentheses represent percentages, taking the total lipid as 100%.

Table 31. Fatty acid composition of total lipid, non-phospholipid, and phospholipid in chum salmon muscle (weight %).

Fatty acids	Total lipid		Non-phospholipid		Phospholipid	
	Control	MT	Control	MT	Control	MT
14 : 0	8.2	6.7	7.0	9.7	2.3	1.7
15 : 0	1.4	1.6	0.5	Tr	0.4	0.4
16 : 0	19.4	11.6	17.5	12.2	26.0	21.6
17 : 0	1.5	2.2	1.2	1.6	1.1	1.0
18 : 0	3.7	4.8	3.4	3.1	4.7	5.7
20 : 0	1.3	2.5	1.2	2.9	0.4	0.2
22 : 0	0.7	1.0	0.8	1.9	0.5	0.4
Saturated	36.2	30.4	31.6	31.4	35.4	31.0
16 : 1	7.5	5.3	7.4	5.8	1.2	0.5
17 : 1	0.8	2.8	0.8	Tr	0.1	0.2
18 : 1	23.8	22.2	25.5	23.9	10.5	9.0
19 : 1	1.0	2.1	1.0	1.8	0.3	0.3
20 : 1	5.2	7.9	6.1	11.1	2.0	2.0
22 : 1	5.2	5.1	7.3	5.1	Tr	Tr
Monoenoic	43.5	45.4	48.1	47.7	14.1	12.0
18 : 2	2.0	2.5	1.2	2.4	0.4	0.3
20 : 4	0.4	0.3	0.5	Tr	0.9	0.9
20 : 5	6.8	4.4	7.1	5.3	7.8	6.2
22 : 5	0.8	1.0	1.0	1.9	1.9	2.1
22 : 6	10.3	16.0	10.5	11.3	39.5	47.5
Polyenoic	20.3	24.2	20.3	20.9	50.5	57.0

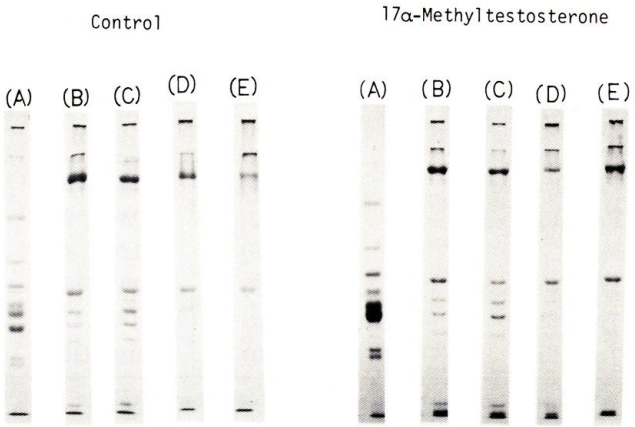

Fig. 60. SDS-slab-polyacrylamide gel (10%) electrophoretic patterns of muscle protein fractions of control and 17α-methyltestosterone-treated chum salmon. (A), sarcoplasmic protein; (B), myofibrillar protein; (C), gel fraction free protein; (D), gel fraction protein; (E), alkali-soluble protein.

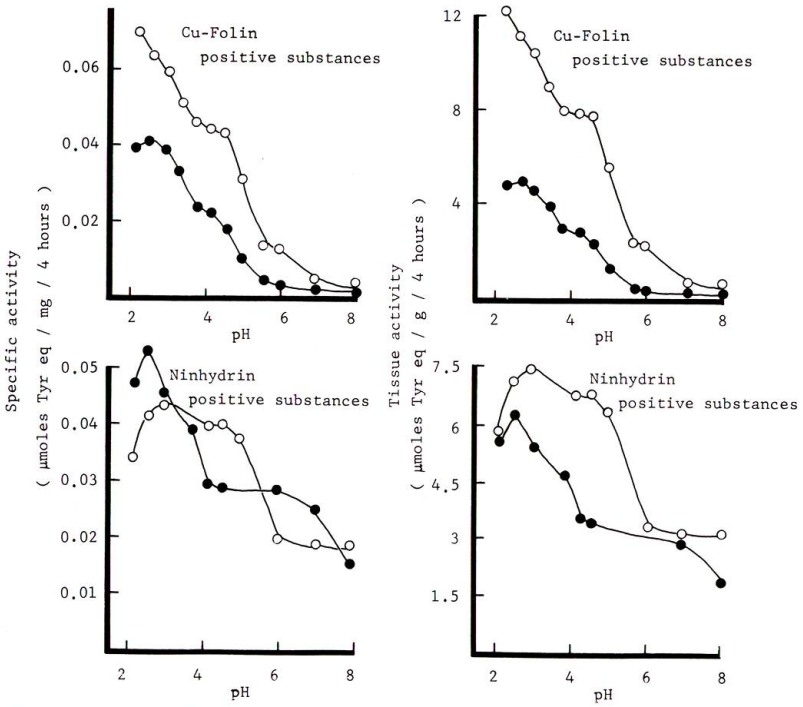

Fig. 61. Autolytic activity of 17α-methyltestosterone-treated chum salmon muscle homogenate as a function of pH. Control, (●—●); 17α-methyltestosterone, (○—○).

Changes in protein composition were detected by SDS-polyacrylamide gel electrophoresis (Fig. 60). The degradation of myosin heavy chain was slightly observed in MT-treated fish.

Changes in autolytic activity

The autolytic activity which was expressed as Cu-Folin and ninhydrin positive substances markedly increased in the MT-treated fish (Fig. 61). High levels of autolytic activity were found at around pH 3. Protease activity at pH 6, corresponding to physiological state, slightly increased upon treatment with MT.

Changes in acid and neutral proteinase activities

As shown in Fig. 62, MT induced an increase in acid and neutral proteinase activities. These changes were similar to those of the autolytic activity.

Effect of Triton X-100 on acid proteinase activity

Marked activation of acid proteinase was not noted in either control or MT-treated fish following treatment with Triton X-100 (Table 33).

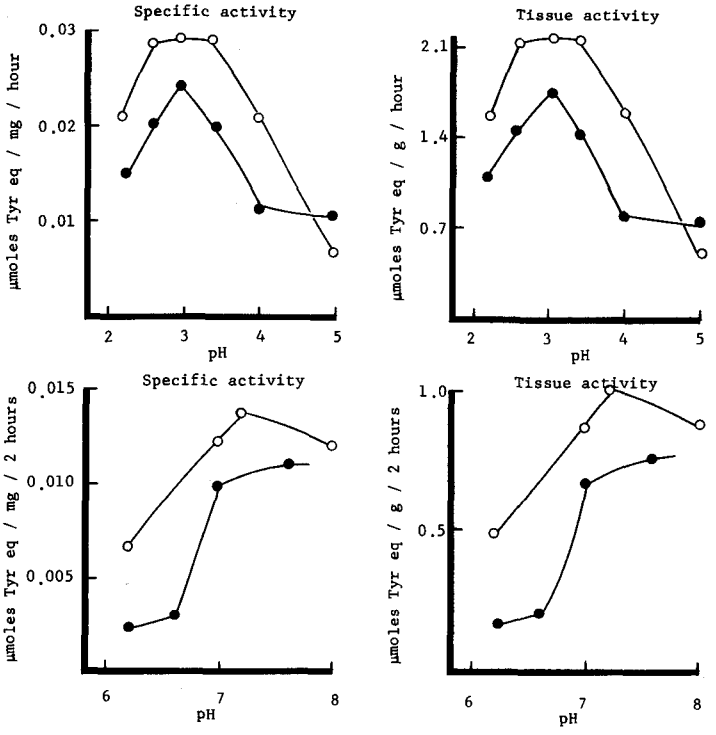

Fig. 62. Acid (upper column) and neutral (lower column) proteinase activities of dorsal muscle of 17α-methyltestosterone-treated chum salmon as a function of pH. Symbols are the same as in Fig. 61.

Table 32. Protein composition of chum salmon dorsal muscle

Treatment (Sex)	Total protein	Sarcoplasmic protein	Myofibrillar protein	Gel fraction free protein
	g/100 g muscle			
Control (Female)	19.11	6.95 (36.37)*	9.80 (51.28)*	4.64 (24.28)*
MT (Female)	23.48	7.96 (33.90)	12.15 (51.75)	5.01 (21.34)

* % of total protein.

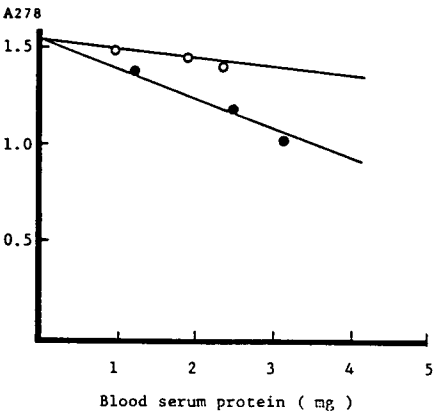

Fig. 63. Changes in serum trypsin inhibitory activity following treatment with 17α-methyltestosterone. (●), control; (○), 17α-methyltestosterone.

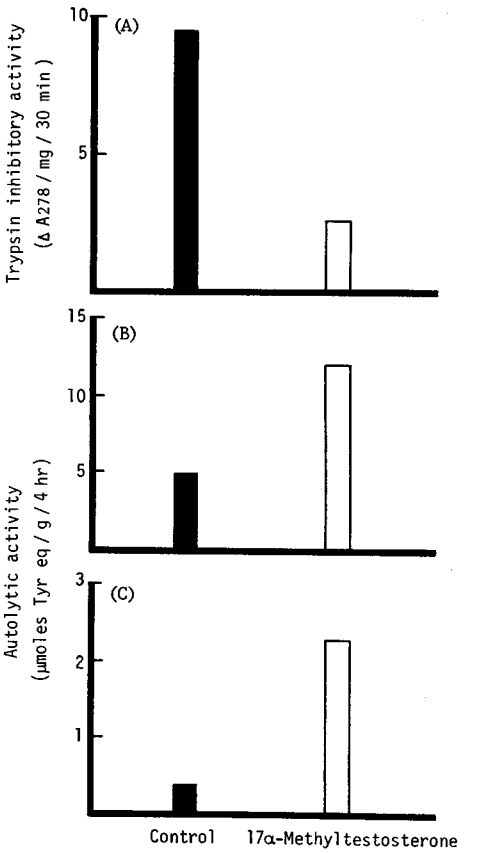

Fig. 64. Relationship between muscle protease activity and serum trypsin inhibitory activity following treatment with 17α-methyltestosterone. (A), serum trypsin inhibitory activity; (B), muscle protease activity at pH 3; (C), muscle protease activity at pH 6.

Changes in trypsin inhibitory activity of serum

Figure 63 shows the changes in trypsin inhibitory activity of serum following treatment with MT. A marked inactivation of trypsin inhibitor of serum was found following treatment with MT. A reverse relation was found between muscle protease activity and trypsin inhibitory activity of serum (Fig. 64).

following treatment with 17α-methyltestosterone (MT).

Gel fraction protein	Alkali-soluble protein	Alkali-insoluble protein	Ninhydrin positive substances (as Tyrosine)
g/100 g muscle			
5.16 (27.00)*	1.86 (9.73)*	0.49 (2.56)*	0.374
7.14 (30.41)	3.12 (13.29)	0.24 (1.02)	0.700

Table 33. Effect of Triton X-100 on acid proteinase activity of dorsal muscle in 17α-methyltestosterone-treated chum salmon (MT).

Treatment (Sex)	Activation of acid proteinase by Triton X-100					
	pH					
	2.2	2.6	3.0	3.4	4.0	5.0
Control (Female)	98.7	109.8	95.5	75.9	138.2	113.6
MT (Female)	105.6	130.2	134.1	121.1	128.6	—

Values are percentage of acid proteinase activity with Triton X-100 to that without.

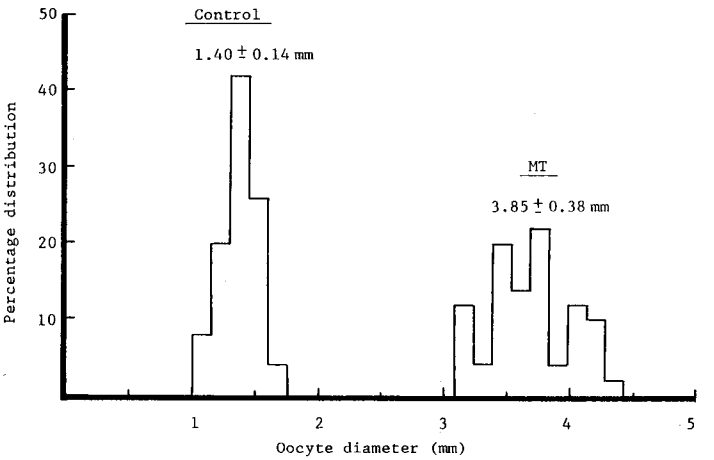

Fig. 65. Effect of 17α-methyltestosterone (MT) on oocyte diameter of chum salmon ovaries.

Discussion

In Section 1 of the present chapter, it has been found that MT induces a deterioration in the muscle of juvenile chum salmon. Also, marked changes in integument coloration were noted. The latter corresponds to the nuptial coloration. Furthermore, the GSI of MT treated fish was large (Fig. 65). The mean GSI value of 25 fish, which were captured at the same date and location as the present study, was less than 1 (Data Rec. Oceanogr. Obs. Expl. Fish., 1984). This suggested that the gonadal growth was caused by MT directly or indirectly but not by individual differences, although the only one fish was injected intraperitoneally with MT and retained unfed for 5 days. It was clear that androgen was intimately involved in various changes of chum salmon muscle during spawning migration.

Upon treatment with MT the lipid content (mainly triglyceride) of muscle markedly decreased and GSI value markedly increased. These changes in lipid and GSI value corresponded to those in chum salmon at the spawning migration stage. The biosynthesis of estradiol-17β and 11-ketotestosterone from testosterone has been demonstrated in teleost gonads (Nagahama, 1982). The role of MT in muscle lipid mobilization and gonadal maturation was suggested by the results of the present section, although it is unknown whether MT itself or its metabolites affected the muscle lipid and gonadal growth of chum salmon. The possibility of the transport of muscle lipid to the gonads was suggested in sockeye salmon (Idler and Bitners, 1960) and rainbow trout (Takashima *et al.*, 1971). The changes in the fatty acid composition, furthermore, as well as lipid composition were found following treatment with MT. These results might suggest that muscle lipid (particularly in triglyceride) was utilized as an energy source in the development of the gonads of chum salmon.

Protease activity of muscle markedly increased following treatment with MT. This suggested that the changes in hormonal conditions were responsible for the high levels of protease activity during spawning migration. It was also observed that MT did not result in a decrease in protein. This result differed from the previous section which showed a decrease in total protein and sarcoplasmic protein of juvenile chum salmon muscle.

Why did the effect of MT on muscle protein differ between chum salmon at the juvenile stage and at the feeding migration stage ? This difference might be due to : (a) different duration of hormone treatment in the two experiments (5 days and 3–4 weeks), (b) anabolic effect of testosterone on the muscle (Hibiya, 1976) and (c) difference in lipid content of muscle between juvenile in fresh water and adults in seawater. The lipid content of juvenile chum salmon muscle was low. In contrast, high levels of lipid were contained in the muscle of chum salmon at the feeding migration stage. Low levels of lipid content might be responsible for the decrease in protein following treatment with MT reported in the previous section with juvenile chum salmon. Possibly, where the lipid content is low, a greater use of protein is required to satisfy daily energy demands. Consequently, the results of the present study strongly suggest that androgens facilitate first lipid as an energy source in the development of the gonads and second protein as an energy source for

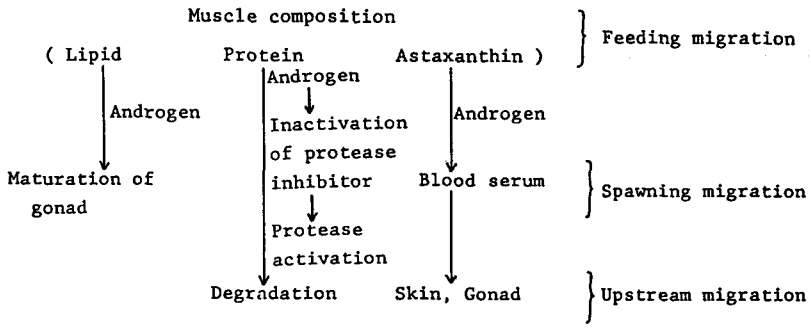

Fig. 66. A scheme of relationship between changes in muscle composition and hormone.

upstream migration.

The high level of muscle acid proteinase activity noted in the treated fish of the present study is consistent with the results obtained with similarly treated juveniles. It was repeatedly shown that injury of lysosomal membranes was not responsible for the activation of acid proteinase in MT-treated fish. A marked inactivation of serum protease inhibitor was found following treatment with methyltestosterone (Fig. 63). This suggests that protease inhibitory activity of serum was controlled by androgens.

It has been reported that the carotenoids in muscle are transported into the integument and the gonads through the blood (Kitahara, 1983). van Overbeeke and McBride (1971) found that androgen treatment in sockeye salmon induced the red skin color typical of sexually matured fish. Treatment with MT resulted in a marked decrease in lipid, but the coloration of the muscle and blood serum were reddish. It was also observed that the color of muscle and blood serum at the spawning migration stage were reddish and that these fish had low levels of lipid. Therefore, the colored blood serum in the treated fish might suggest that the carotenoids in muscle are transported into the blood.

The relationship between changes in muscle composition and hormonal conditions are shown schematically (Fig. 66). It is suggested that androgens were trigger to the inactivation of serum protease inhibitor, resulting in high levels of muscle protease activity during spawning migration. The decrease in muscle protein content while ascending the river might be caused by the high levels of muscle protease released from the protease inhibitor.

The present study proved that high levels of androgens were trigger to the deteriorations of chum salmon muscle including the changes in muscle composition and protease activity during spawning migration.

Chapter V. General discussion and conclusions

Biochemical and physiological studies on chum salmon during spawning migration are very limited, although chum salmon are very important resources in Japan. The results presented in Chapter II to IV demonstrate that the muscle deterioration and nuptial coloration occurring in chum salmon during spawning

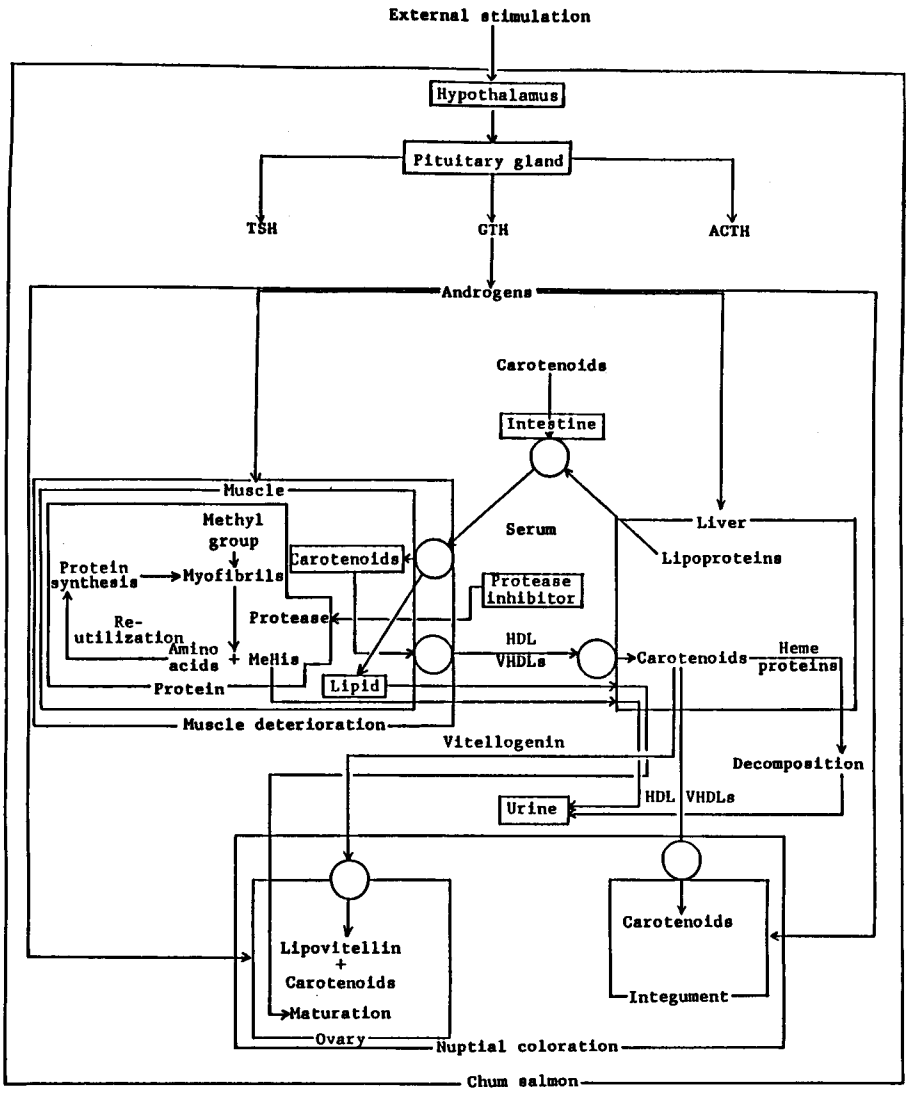

Fig. 67. A scheme of various phenomena occurring in chum salmon during spawning migration. Circles, carotenoid-carrying lipoproteins; HDL, high density lipoprotein; VHDLs, very high density lipoproteins; ACTH, adrenocorticotropic hormone; GTH, gonadotropic hormone; TSH, thyroid-stimulating hormone.

migration, which are the most important and interested phenomena from the standpoint of food chemistry as well as biochemistry and physiology, are closely related to their hormonal conditions, particularly androgen level.

In the present study, muscle deterioration and nuptial coloration were defined as the exclusive consumption of muscle composition during spawning migration and as carotenoid transport from the muscle to the integument and ovaries, respectively.

The relationship between muscle deterioration, nuptial coloration and androgen level is schematically shown in Fig. 67.

Androgens were trigger to the inactivation of serum protease inhibitor, resulting in high levels of muscle protease, acidic and neutral proteinase activities during spawning migration. Myofibrillar and sarcoplasmic proteins were degraded by the muscle protease activated during spawning migration. Amino acids were released into the muscle extracts as decomposition products of these muscle proteins, although amino acids except for N^{τ}-methylhistidine were reutilized for protein synthesis. High levels of protease activity might be necessary to the utilization of muscle protein as an energy source to ascend the river.

The role of androgens as trigger to muscle lipid consumption was confirmed by the administration of 17α-methyltestosterone to sexually immature salmon. The relative ratio of individual consumed fatty acids was almost the same among salmon of different physiological state, although consumed fatty acids in muscle lipid gradually increased during spawning migration. Muscle lipid (particularly in triglyceride) was utilized as an energy source in the development of the gonads.

17α-Methyltestosterone treatment induced the nuptial coloration and serum coloration of sexually immature salmon. This meant the transport of muscle carotenoids to the integument *via* serum. Various serum lipoproteins, such as high density lipoprotein and very high density lipoprotein fractions including vitellogenin, transported astaxanthin into the integument and ovaries during spawning migration. Appreciable amounts of carotenoids in salmon were decomposed during spawning migration, although muscle carotenoids were accumulated into the integument and ovaries. This decomposition of carotenoids was considered to occur in the liver tissue. Carotenoids transported from muscle to liver by serum lipoproteins were decomposed by heme proteins of liver tissue.

On the basis of the present works, the following conclusions can be drawn.

(1) The gonadosomatic index (GSI) values were markedly elevated at the spawning migration stage, and its high values were maintained during the upstream migration. The changes in GSI values were related to the hormone levels of testosterone and estradiol-17β.

(2) Muscle lipid (triglyceride) markedly decreased at the spawning migration stage, while phospholipid level of muscle was constant during spawning migration.

(3) The relative ratio of individual consumed fatty acids was almost the same among salmon of different physiological state, although consumed fatty acids in muscle lipid gradually increased during spawning migration. No selective consumption of fatty acids in muscle lipid occurred during spawning migration.

(4) Muscle lipid was utilized as an energy source in the development of the gonads.

(5) Sarcoplasmic protein markedly decreased and myofibrillar protein was gradually degraded during spawning migration. Muscle protein was utilized as an energy source to ascend the river.

(6) Acid and neutral proteinase activities markedly increased during spawning migration. These proteinase activities were higher in females than in males. High levels of acid proteinase were not caused by the injury of lysosomal membranes.

(7) The changes in serum sex steroid hormones were similar to those in the

protease activities during spawning migration. High levels of protease activity were considered to be closely related to the serum levels of androgens.

(8) N^{τ}-Methylhistidine (MeHiS) levels in the muscle extracts markedly increased during spawning migration and tended to be higher in female fish. MeHis could be detected in myofibrillar protein. These results indicated that muscle proteins were degraded by high levels of protease and the amino acids were released into the muscle extracts.

(9) The autolytic activity of salmon muscle markedly increased, while the trypsin inhibitory activity in serum markedly decreased during spawning migration. The protease inhibitory activity of serum was controlled by androgens. Androgens were trigger to the inactivation of serum protease inhibitor, resulting in high levels of muscle protease activity during spawning migration.

(10) Carotenoid-carrying lipoproteins (CCLs) were found in the high density lipoprotein (HDL, $d=1.063-1.210\,g/ml$) and very high density lipoprotein ($VHDL_2$, $d \gg 1.210\,g/ml$) fractions of the serum of salmon.

(11) The molecular weight of the CCL in the HDL fraction ranged from 30,000 to 500,000, with a peak at 70,000 dalton. The CCL gave rise to two subunits whose molecular weights were 24,000 and 12,000 by SDS-PAGE. The CCL had a pI 5.1, and was rich in glutamic acid, alanine, leucine, and lysine. The CCL showed a high content of lipid (54%), which consisted mainly of cholesterol ester, cholesterol, triglyceride, phosphatidylcholine, and sphingomyelin.

(12) Vitellogenin appeared in the surum at the start of spawning migration and disappeared during upstream migration. Appreciable amounts of carotenoids, probably astaxanthin, were bound to the vitellogenin. Vitellogenin might be closely related to the transport of carotenoids from muscle into the ovaries.

(13) The egg yolk proteins, E_1 and E_2, were present in salmon. The molecular weight was 250,000 for E_1 consisting of three subunits whose molecular weights were 90,000, 20,000, and 8,500, and was 36,000 for E_2 consisting of two subunits whose molecular weights were 15,000 and 12,000. Only E_1 (lipovitellin) had the absorbance at 480 nm due to the presence of carotenoids.

(14) Based on the contents and individual composition of carotenoids in the muscle, serum and ovaries of chum salmon during spawning migration, the reductive metabolism of astaxanthin to zeaxanthin was presumed in the muscle of both males and females. The metabolic rates of zeaxanthin and 4-keto-zeaxanthin in female serum were much faster than those in male serum.

(15) The liver extracts made astaxanthin bleaching in the presence of linoleic acid *in vitro*. The level of astaxanthin bleaching activity was closely related to the size of liver tissue. The liver extracts had an absorption maximum at 410–415 nm and two broad ones at 540–545 and 575–580 nm. These results suggested that carotenoids transported from muscle to liver by serum lipoproteins were decomposed in the presence of heme proteins of liver tissue.

(16) Injection of 17α-methyltestosterone, a synthetic androgen, into sexually immature salmon induced the muscle deterioration and nuptial coloration occurring in salmon during spawning migration.

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