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Role of adipocytes in the muscle tissue of Atlantic salmon (*Salmo salar*) in the uptake, release and retention of water-soluble fraction of crude oil hydrocarbons

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Abstract The uptake and depuration of the water-soluble fraction (WSF) of hydrocarbons of crude petroleum by Atlantic salmon (*Salmo salar*) has previously been examined in terms of whole muscle. The hypothesis that the tainting WSF in the muscle was retained primarily by adipocytes has been investigated by the isolation of adipocytes and the subsequent analysis for hydrocarbons in adipocytes. After 96 h exposure of market-sized Atlantic salmon to 0.2 ppm WSF, adipocytes isolated from the belly flap region of the muscle tissue accumulated 14.3 times more WSF (59.4 ppm) than the dorsal white muscle (4.2 ppm), while 54% of the tainting WSF in the dorsal white muscle was found to be stored in associated adipocytes. When returned to clean seawater, WSF accumulated in the dorsal white muscle was released much faster than that in the adipocytes. These results indicated that the loose association of WSF with the nonlipid portion of white muscle, mainly muscle cells and intercellular fluid, is responsible for the rapid discharge of WSF from the dorsal muscle tissue in the early stages of depuration. After 4 d of depuration, the adipocytes became the principal storage site of residual WSF in white muscle and the depuration of WSF from muscle tissue then reflected the release of WSF from adipocytes in the muscle tissue. After 20 d of depuration, 10.7 ppm of tainting WSF in the form of high molecular weight aromatic hydrocarbons (mainly C_4-benzenes, naphthalene and alkylated naphthalenes) were still present in adipocytes, while in the dorsal white muscle only a trace of total WSF was detected. Increases in the number of aromatic rings and the alkylations on the rings enhanced the accumulation and retention of individual hydrocarbons in both adipocytes and white muscle. From these studies we conclude that it is the adipocytes in the muscle tissue which control the actual accumulation and release of hydrocarbons in the whole muscle tissue of Atlantic salmon.

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Introduction

Fish tainted with the water-soluble fraction (WSF) of petroleum hydrocarbons are unmarketable and must be held in clean water for a period of time until the objectional flavours disappear. Various investigations have been conducted to study the accumulation and release of hydrocarbons in aquatic organisms, but they have usually been based on the whole tissue as a unit (Stegeman and Teal 1973; Fossato and Canzonier 1976; Neff et al. 1976; Heras et al. 1992). Much of the work emphasized only the fate and metabolism of hydrocarbons, especially aromatic hydrocarbons, in aquatic organisms (Pedersen et al. 1974; Lee et al. 1976; Thomas and Rice 1981; Cravedi and Tulliez 1986). The mechanisms of hydrocarbon uptake and storage behaviour in individual cell compartments have rarely been studied and remain largely unknown.

Any species-specific difference in the accumulation and release of hydrocarbons is reported to be related to tissue lipids (Stegeman and Teal 1973; Neff et al. 1976; Boryslawskyj et al. 1988; Hebert and Keenleyside 1995). Lean fish and shellfish, such as cod and scallops, took up small amounts of WSF in their muscle and were freed of the tainting WSF very rapidly when returned to clean water (Ernst et al. 1987, 1989). Atlantic salmon, a fatty fish, accumulated much more WSF hydrocarbons under similar exposure conditions than lean fish, and the tainting aromatic hydrocarbons of high molecular weights could still be detected in muscle tissue after 1 month of depuration (Heras et al. 1993). It has been proposed that the accumulated hydrocarbons are associated with lipids and that this is probably a process of

partitioning between the exposure water and the tissue lipids (Stegeman and Teal 1973; Neff et al. 1976; Johnsen and Lloyd 1992). In fact, octanol–water partition coefficients have been used in defining the uptake of organic pollutants from water by aquatic organisms at equilibrium (Chiou et al. 1977; Boese 1984). Lipid normalization has been used as an approach to adjust the concentrations of organic pollutants for variations in tissue lipid contents (Hebert and Keenleyside 1995), and has been applied in modelling bioaccumulation of organic pollutants in aquatic organisms (Thomann and Connolly 1984; Connolly and Pederson 1988).

The muscle tissue of aquatic organisms is mainly composed of muscle cells and to a much lesser extent of adipocytes as well as other tissue components. It is important to understand the roles of different cell compartments in the uptake and release of hydrocarbons in order to see the actual role of lipid-containing compartments and to clarify the species-specific phenomenon of hydrocarbon behaviour observed in different aquatic organisms. The main objective of this work is to reveal the role of adipocytes in the muscle tissue of Atlantic salmon in relation to the uptake, release and retention of WSF hydrocarbons by the whole of the muscle tissue.

Materials and methods

Exposure of Atlantic salmon to WSF

WSF stock solution was prepared by stirring Flotta North Sea crude oil (courtesy of Esso Petroleum Canada) with cold seawater in a ratio of 1:99 for 24 h, followed by 48 h of settlement at room temperature. The preparation of WSF was conducted in a cylindrical tank with a water holding capacity of 800 litre. The prepared WSF stock solution was stored in plastic bags fitted into a 70 litre bucket before being admitted to the exposure tank (Heras et al. 1995). Exposure of Atlantic salmon was conducted in a fish tank 2 m in diameter with an overflow system (2500 litre water holding capacity). The tank was covered with clear plastic film sealed in place during the exposure period to maintain a stable WSF hydrocarbon profile in the exposure water. WSF stock solution and well-oxygenated seawater were continuously supplied to the exposure tank in an appropriate proportion to obtain the desired WSF concentration by dilution and to maintain the oxygen level in the water of the exposure tank (80 to 110% saturation). The concentration of total WSF in the tank was controlled at approximately 0.2 ppm for 96 h. Water samples in both plastic storage bags (stock solution) and exposure tank were taken twice daily for the analysis of WSF concentration. The fluctuations of WSF in the exposure tank were kept to a minimum by immediately adjusting the flow rate of WSF stock solution according to the WSF levels found in the water when analyzed by gas-liquid chromatography (GLC).

A total of 18 market-sized Atlantic salmon, *Salmo salar*, were used for the exposure experiment (average weight ± SD: 2434 ± 520 grams, length: 59 ± 3 cm); of these, 12 salmon were female and contained mature eggs (4 to 5 mm in diameter). The fish had been previously held in aerated sand-filtered seawater for 3 months at the Dalhousie University Aquatron and fed a diet of Fundy Choice feed (Corey Mills Ltd, Fredericton, New Brunswick). The fish were starved for 24 h before the exposure to reduce faeces in the exposure water. The temperature of the exposure water was 4.5 to 6.5 °C. The photoperiod was set for 12 h light:12 h dark. In addition, six control fish were kept in clean seawater under the same conditions. At the end of exposure, three experimental fish were killed for the immediate dissection of muscle tissue and for the isolation of adipocytes, and the remaining fish were transferred to a clean seawater tank for depuration. Salmon were depurated for 20 d in seawater at temperatures ranging from 6.5 to 8.0 °C and the fish (three each time) were taken on Days 1, 4, 10 and 20 for analyses of hydrocarbons in adipocytes and white muscle. Three of the six control fish were sacrificed on Days 0 and 10, respectively.

Sampling of white muscle and isolation of adipocytes

The live Atlantic salmon removed for study were immediately transported in chilled seawater to the Canadian Institute of Fisheries Technology at the Technical University of Nova Scotia. The fish were anaesthetized with 2-phenoxyethanol and blood was taken. The fish were then killed with a blow on the head and the gut cavities were cut open along the middle of the abdomen. The viscera were then removed and the fish were thoroughly washed with cold water. A cylinder-shaped portion of dorsal white muscle (about $2 \times 2 \times 10$ cm) was excised adjacent to the vertebrae, 2 cm from the head. The dorsal white muscle portions thus dissected from each of three fish were cut into several pieces, pooled, and separated into two portions. One portion was used for hydrocarbon analysis and the other portion for the determination of total lipids and lipid storage in the myosepta of the dorsal white muscle tissue. For maximum adipocyte recovery, strips of belly flaps, about 2 cm in width, were cut along one side of the half fillet from the pectoral fin to the pelvic fin. The skins of the belly flap strips and bundles of muscle fibers were then removed and the resulting enriched belly flap tissues from the three fish were pooled. All dissection work was performed in a cold room at 5 °C.

About 9 g of the dissected belly flaps were immediately placed in a petri dish containing Krebs–Ringer phosphate buffer with 1% albumin (bovine, Fraction V, Sigma Chemical Co., St. Louis, Mo.). The tissues were cut into small pieces in the petri dish and added to a 4 oz Nalgene plastic bottle containing 22 ml of 1% albumin in phosphate buffer plus 180 mg collagenase (Type II, Sigma). The digestion processes applied to tissue from the belly flaps and the technique for discrimination between isolated adipocytes and free fat have been described elsewhere by Zhou et al. (1996). The resulting dialysis tubing containing sections of the isolated adipocytes, the free fat layer and the buffer solution was immediately frozen at −35 °C. The adipocyte block was then cut off and stored at −35 °C until analysis. Isolation of adipocytes from belly flaps was performed in duplicate.

Hydrocarbon analysis

The hydrocarbons in the dorsal white muscle and the isolated adipocytes were recovered by modification of the procedures of Ackman and Noble (1973). The steam distillation apparatus consisted of a 250 ml (for dorsal white muscle) or 125 ml (for adipocytes) round-bottomed flask with a magnetic stirring bar, a 20 ml Barrett-type distilling receiver (Teflon stopcock) and a water-cooled condenser. The flask was filled with 80 ml (for dorsal white muscle) or 50 ml (for adipocytes) distilled water and heated to boiling using an electric heating mantle. The distillation was terminated when 20 ml of water condensate was collected. The flask was then cooled to room temperature and the water condensate discarded.

The frozen dorsal white muscle portions were thawed in a domestic refrigerator and immediately upon softening were minced in a Sorvall Omni-Mixer. The minced dorsal white muscle (20 g) was added to the remaining water in the 250 ml flask for steam distillation of hydrocarbons. The frozen adipocyte block was left at room temperature for only a few minutes until the frozen block could be slipped out of the dialysis tubing. The frozen adipocyte block was transferred intact to the predistilled water in a 125 ml

flask. Methylene chloride (1 ml) was added to both flasks. The distillation resumed until 15 ml of condensate had been collected. Dichloromethane (1 ml) was then immediately added through the top of the condenser. After distillation the condensate was drained into a 50 ml graduated centrifuge tube cooled in ice. Methylene chlo-ride (200 µl) containing *n*-heneicosane as internal standard was added to the centrifuge tube. The water and methylene chloride in the centrifuge tube were vortexed for 1 min and centrifuged. The methylene chloride layer was removed by syringe and used for GLC analysis. Distillation of the dorsal white muscle from the control fish and of the untainted adipocytes isolated from belly flaps was performed by exactly the same procedures as for the tainted samples. Both dorsal white muscle and adipocyte samples were distilled in duplicate and each hydrocarbon extract was analysed in duplicate.

The recovery efficiency for hydrocarbons by steam distillation was evaluated by spiking 21 hydrocarbon standards ranging from benzene to methylnaphthalenes into both the control adipocyte block and the control dorsal white muscle and by performing the same recovery procedures. The amounts of tainting hydrocarbons in the samples were calculated according to the internal standard added, the recovery efficiency and the GLC response factors of individual hydrocarbons reported by Ernst et al. (1989). Table 1 shows the recovery efficiency of some of the 21 hydrocarbon standards spiked to the dorsal white muscle and the adipocyte block by steam distillation. In both sample types all of the spiked hydrocarbons were recovered with percent recoveries higher than 50% of the spiked amounts, and with the recovery of methylnaphthalenes being the lowest. The procedures for the determination of WSF in seawater and the conditions of hydrocarbon analysis by GLC have been described elsewhere by Zhou et al. (1994).

Determination of lipid content

The lipid content of dorsal white muscle portions was determined in duplicate by following the method of Bligh and Dyer (1959). The analyses of lipid classes and of lipid stored in myosepta of dorsal white muscle were carried out as described by Zhou et al. (1995).

The total lipid in each collected adipocyte block was recovered immediately after the termination of the steam distillation. Upon removal of the distillation receiver, the flask was cooled and 5 ml of chloroform was added. The water and chloroform in the flask were then pipetted into a 50 ml centrifuge tube. The flask was again rinsed three times with 5 ml of chloroform and all portions of chloroform were combined in the centrifuge tube. Methanol (5 ml) and saturated sodium chloride solution (2 ml) were subsequently added to the centrifuge tube. The centrifuge tube was flushed with nitrogen, vortexed and centrifuged. The bottom chloroform layer was quantitatively transferred to a weighed round-bottom flask. The lipids in the flask were initially stripped of solvent on a rotary evaporator and finally dried with a mechanical vacuum pump. The effect of distillation on the recovery of lipids from the adipocyte block was evaluated by spiking 2 g of belly flap lipids into the flask and then performing the same distillation procedures. The amount of lipids recovered from the adipocyte block was considered as the total weight of adipocytes used for distillation since lipids are the predominant component of adipocytes (~90%). The percentages of WSF stored in adipocytes of dorsal white muscle were calculated under two assumptions: (1) adipocytes in the white muscle and adipocytes in the belly flap regions have the same characteristics with respect to the accumulation and release of WSF and (2) membrane lipids are composed of only phospholipids and sterols and all the other neutral lipid components are associated with adipocytes. Histological studies on the adipocyte and lipid distribution in dorsal white muscle showed that both adipocytes and lipid droplets (or sacs) were present in the intercellular connective tissue (Zhou et al. 1996). However, since those lipid droplets were present in low proportion compared with adipocytes they were classified as part of adipocytes in this study.

Table 1 *Salmo salar*. Recovery efficiency of spiked hydrocarbons from the untainted dorsal white muscle and adipocyte block by steam distillation. The concentration of each spiked hydrocarbon is ~0.7 ppm (wet tissue basis). Values are the average of triplicate (white muscle) or duplicate (adipocytes) determinations with standard deviations

Spiked hydrocarbons	Recovery efficiency (%)	
	Dorsal white muscle	Adipocyte block
Benzene	84.43 ± 4.31	90.49 ± 5.26
Methylcyclohexane	56.04 ± 6.66	60.28 ± 3.17
Toluene	78.88 ± 2.07	85.35 ± 2.95
Ethylbenzene	75.09 ± 4.85	77.35 ± 2.60
m+*p*-Xylenes	70.93 ± 1.27	64.32 ± 5.33
o-Xylene	70.32 ± 5.24	67.79 ± 2.01
Propylbenzene	74.38 ± 3.44	76.07 ± 2.17
1,3,5-Trimethylbenzene	67.22 ± 2.17	62.84 ± 4.36
1,2,4-Trimethylbenzene	83.76 ± 6.05	77.21 ± 3.61
1,2,4,5-Tetramethylbenzene	63.70 ± 2.54	59.87 ± 3.47
Naphthalene	60.19 ± 3.42	54.65 ± 1.75
2-Methylnaphthalene	55.49 ± 2.37	51.94 ± 3.46
1-Methylnaphthalene	57.71 ± 3.75	50.77 ± 4.87

Results

Adipocytes were successfully isolated from the belly flaps of the muscle tissue through enzymatic digestion and further purified through the flotation procedures. The distribution of adipocytes in the muscle tissue of Atlantic salmon and the characterization of isolated adipocytes have been discussed by Zhou et al. (1996). Steam distillation of the isolated adipocytes had no important effect on the gravimetric recovery of the amount of isolated adipocytes since 99% of the similar spiked belly flap lipid was recovered after stripping of hydrocarbons by steam distillation.

Figure 1 is a typical GLC chromatogram of the WSF stock solution sampled after 50 h of exposure. The WSF hydrocarbon profile of the stock solution displays a pattern similar to that characterized by Zhou et al. (1994) with respect to the total number of hydrocarbon components and their relative concentrations. Alkylated benzenes, ranging from benzene to C_4-benzenes, dominate the WSF in the exposure tank and only traces of *n*-alkanes are present. The concentrations of polynuclear aromatic hydrocarbons (PAH) in the water column are relatively low compared with those of alkylated benzenes (Fig. 1). The total hydrocarbon concentrations of the WSF stock solution used during the entire 96 h exposure period ranged from 3.87 to 6.06 ppm. However, the effect of this fluctuation on the actual WSF concentrations in the exposure tank was largely minimized by the immediate adjustment of flow rates according to the GLC analysis of WSF concentrations in the stock solution and the exposure water. The average concentration of WSF in the exposure water was 0.2 ppm.

Variations in lipid contents of dorsal white muscle ranging from 3.11 to 4.81% were observed among

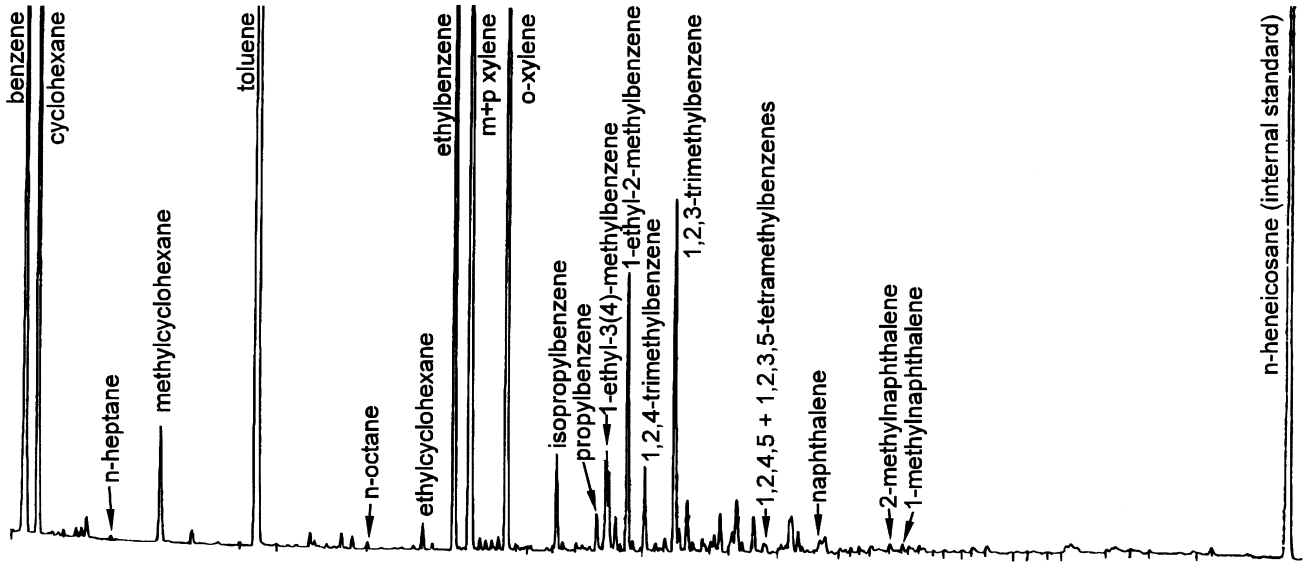

Fig. 1 A typical GLC chromatogram of water-soluble fraction (WSF) stock solution of Flotta North Sea crude oil sampled after 50 h of exposure period

Atlantic salmon sampled at different depuration stages (Table 2). Examination of the lipid distribution revealed that approximately 40% of the dorsal white muscle lipid (35.3 to 42.0%) was stored in the myosepta of the dorsal white muscle tissue (Table 2). The average membrane lipid content in dorsal white muscle was calculated to be approximately 0.6% (wet tissue) (Table 2), a figure close to that reported for the very lean muscle of Atlantic cod *Gadus morhua* (Bligh and Dyer 1959; Jangaard et al. 1967).

Both dorsal white muscle and adipocytes isolated from the belly flap region of the muscle tissue accumulated high levels of WSF hydrocarbons after long-term exposure (96 h), but the difference in the concentrations of tainting WSF between the dorsal muscle tissue and the adipocytes was substantial (Fig. 2; Table 3). The adipocytes bioaccumulated 59.4 ppm of WSF which is 300 times higher than that in the exposure water, while the dorsal white muscle took up only 4.2 ppm of WSF (Fig. 2). Moreover, the dorsal white muscle per se contained 4.48% (w/w) lipid, of which 86% was in the form of adipocytes or lipid sacs (considered adipocytes hereafter) in the muscle connective tissue and 42% (of the 4.48%) was specifically in the adipocytes associated with myosepta (Table 2). At the end of 96 h exposure, the total adipocyte lipids in dorsal white muscle accounted for the storage of 54% of the total tainting WSF in the dorsal white muscle (Fig. 3). After subtracting the WSF stored in adipocytes from the total tainting WSF accumulated in the dorsal white muscle, the nonadipocyte portion of the dorsal white muscle, predominantly composed of white muscle cells, was calculated to have taken up only 2.0 ppm of WSF, an amount only ten times higher than the WSF concentration in the exposure water.

The depuration rates of WSF from adipocytes and dorsal white muscle also showed large differences (Fig. 2). The characteristic depuration of WSF from dorsal white muscle was distinguished from that of adipocytes by showing a sharp release of WSF on the first day of depuration. The WSF in the dorsal white muscle dropped from 4.2 to 1.9 ppm, accounting for 55% of the total accumulated WSF, while only 19% of the accumulated WSF in adipocytes was released during

Table 2 *Salmo salar*. Total lipid and membrane lipid contents of pooled dorsal white muscle (wet tissue basis) from three fish, and percentages of lipid stored in the myosepta of dorsal white muscle, sampled during the 20-d period of depuration after 96 h exposure of Atlantic salmon to WSF. All values are the average of triplicate determinations with standard deviations, except for the total lipid contents which were only determined in duplicate. Values for membrane lipid content were calculated from lipid class analysis, assuming that lipids in the cell membranes are primarily composed of only phospholipids and sterols; percentage of the amount of lipids stored in the myosepta of dorsal white muscle were compared to the total lipid of the same muscle tissue

	Duration times (d):					
	0	1	4	10	20	Average
Total lipid content (%)	4.48 ± 0.11	3.11 ± 0.20	4.81 ± 0.08	4.55 ± 0.06	3.25 ± 0.14	4.04 ± 0.71
Membrane lipid content (%)	0.62 ± 0.04	0.57 ± 0.03	0.62 ± 0.05	0.58 ± 0.03	0.58 ± 0.02	0.60 ± 0.02
Lipids in myosepta (%)	42.0 ± 5.1	35.3 ± 3.5	41.8 ± 5.0	38.1 ± 4.8	37.1 ± 6.4	38.9 ± 2.64

the same first day of depuration. The release of WSF from dorsal white muscle then slowed down and showed a pattern similar to that from the isolated adipocytes. This characteristic is clearly demonstrated when the depuration rates are expressed as the amount of WSF released per day and per unit of initial WSF in the samples at different time intervals of depuration (Fig. 2). From Day 0 to 1, the adipocytes showed a depuration rate (Fig. 2) of only 0.21 (ppm d^{-1} ppm^{-1} initial hydrocarbons), while a depuration rate of 0.76 was observed for the dorsal white muscle. Afterwards, the depuration rate of WSF in dorsal white muscle from Day 1 to 4 decreased to 0.21, slightly higher than the 0.18 rate of the isolated adipocytes for the same period. A similar depuration rate of about 0.05 was then maintained to the end of the depuration study for both dorsal white muscle and adipocytes. After 20 d of depuration, the dorsal white muscle was almost free of the accumulated WSF and only 0.2 ppm of WSF was detected; however 10.7 ppm of WSF were still stored in the adipocytes.

Figure 3 further illustrates the role of adipocytes in the retention of tainting WSF hydrocarbons in dorsal white muscle at different stages of depuration. The percentages are expressed as the amount of WSF stored in the adipocytes of the dorsal white muscle as a percentage of the total amount of WSF in the same white muscle. At the end of the exposure period, 54% of the tainting

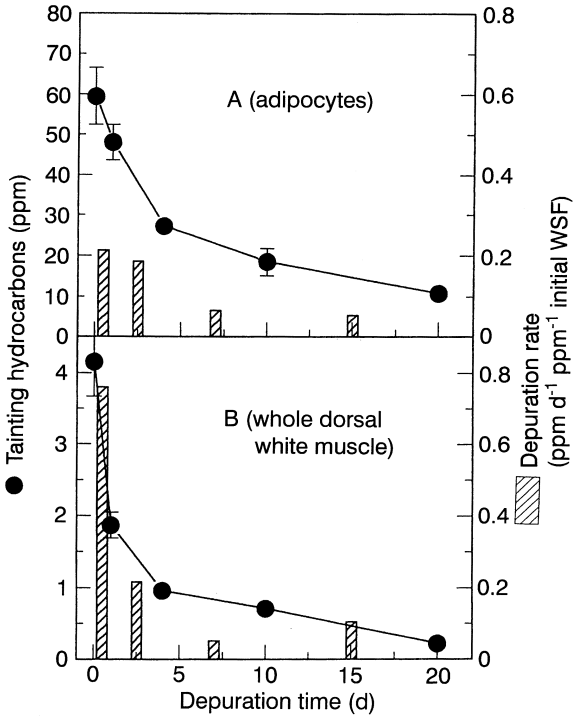

Fig. 2 *Salmo salar*. Release of the tainting WSF from **A** adipocytes and **B** dorsal white muscle during 20 d period of depuration. Depuration rate was calculated as the average concentration of WSF released per day and per unit of initial WSF remaining in dorsal white muscle or adipocytes during the time intervals of Day 0 to 1, Day 1 to 4, Day 4 to 10, and Day 10 to 20

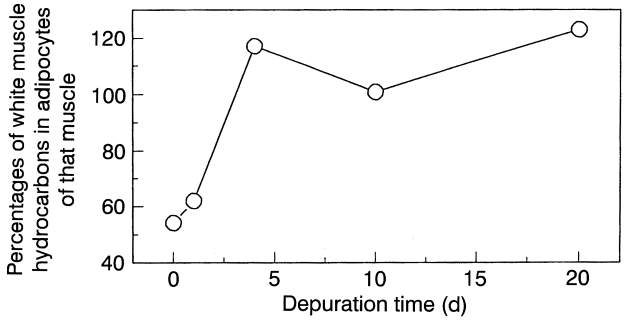

Fig. 3 *Salmo salar*. Changes in calculated percentages of tainting WSF in adipocytes of the dorsal white muscle accounting for the total tainting WSF in the same muscle tissue

WSF in the dorsal white muscle was found to be in the adipocytes. Tainting WSF in the dorsal white muscle cells was discharged almost completely and the adipocytes became the principal storage site of WSF in dorsal white muscle after 4 d of depuration. The release of tainting WSF from dorsal white muscle thereafter was exclusively the depuration of WSF from the adipocytes in the muscle tissue.

Bioaccumulation factors of individual hydrocarbons in the adipocytes were much higher than those in the dorsal white muscle (Table 3). It was found that the bioaccumulation of individual hydrocarbons increased along with the number of aromatic rings and the extent of alkylation on the aromatic ring. Immediately after exposure the bioaccumulation factor of methylnaphthalenes was almost 23 times higher than that of a single ring counterpart (toluene) in the dorsal white muscle and about 13 times higher in adipocytes. Methylnaphthalenes in the exposure water were concentrated by a factor of 2970 by adipocytes and were the most concentrated components of the tainting WSF hydrocarbons in specific cell compartments of the white muscle. That the bioaccumulation factors were also more-or-less related to the number of substituent alkyl-groups on the ring is noteworthy. Generally, the greater the number of alkyl substitutions, the higher the bioaccumulation factor. This is clearly illustrated for the alkylated benzenes (Table 3). An exceptional case in both dorsal white muscle and adipocytes was that ethylbenzene and xylenes were slightly more concentrated than isopropylbenzene and propylbenzene. The bioaccumulation factor of methylcyclohexane was observed to be unusually high in both dorsal white muscle and adipocytes (31 and 480, respectively) relative to that for aromatic hydrocarbons having similar molecular weights, e.g., the bioaccumulation factors of toluene were only 11 and 230 for dorsal white muscle and adipocytes, respectively.

The discrimination factors in Table 3 reflect the ratios of relative abundance of individual hydrocarbons between the dorsal white muscle or the adipocytes and the exposure water. The ratios of relative abundance of benzene were the lowest in both the dorsal white muscle

Table 3 *Salmo salar*. Bioaccumulation factor and discrimination factor of hydrocarbons in dorsal white muscle and adipocytes isolated from the belly flap region of muscle tissue of Atlantic salmon after 96 h exposure to 0.2 ppm WSF. Bioaccumulation factor was calculated as the ratio of hydrocarbon concentration in dorsal white muscle or adipocytes divided by hydrocarbon concentration in exposure water; discrimination factor was calculated as the ratio of individual hydrocarbon concentration to the total hydrocarbon concentration in the dorsal white muscle or adipocytes divided by the equivalent ratio in the exposure water

Hydrocarbons	Bioaccumulation factor		Discrimination factor	
	White muscle	Adipocytes	White muscle	Adipocytes
Total WSF hydrocarbons	21	300	–	–
Benzene	4	100	0.2	0.3
Methylcyclohexane	31	480	1.4	1.6
Toluene	11	230	0.5	0.8
Ethylbenzene	26	430	1.2	1.4
Xylenes	47	570	2.2	1.9
Isopropylbenzene	20	270	0.9	0.9
Propylbenzene	36	430	1.7	1.4
Ethyl-methylbenzenes	51	520	2.4	1.7
Trimethylbenzenes	74	740	3.4	2.4
Methylnaphthalenes	230	2970	10.7	9.7

and the adipocytes (0.2 and 0.3, respectively). The percentage of toluene in the accumulated WSF of adipocytes was 80% of that in the exposure water, while it was only 50% in the dorsal white muscle tissue. The discrimination factors of tainting aromatics in dorsal white muscle and adipocytes exceeded those in the exposure water when two carbons or more of alkylations were present on the ring, except for isopropylbenzene which had a discrimination factor of 0.9 for both the dorsal white muscle and the adipocytes. The relative abundance of methylnaphthalenes in the dorsal white muscle and the adipocytes was ten times higher than that present in the exposure water.

The depuration of individual hydrocarbons was dependent on their alkyl substitutions, number of aromatic rings, and to a large extent their storage sites in the muscle tissue. Benzene in dorsal white muscle was completely depurated within only 1 d, while it took 4 d to be depurated from the adipocytes. One methyl alkylation on the benzene ring (toluene) required 4 and 10 d for complete depuration from the dorsal white muscle and the adipocytes, respectively. The depuration rates of individual hydrocarbons sharply slowed down with increasing alkyl substitutions. The depuration of methylnaphthalenes was so slow that it would take months before they were completely released from the adipocytes. These characteristics are reflected through the expression of biological half-lives, meaning the time required for the depuration of half the accumulated individual hydrocarbon in dorsal white muscle and adipocytes (Table 4). An important observation was that all of the individual hydrocarbons, including those highly alkyl substituted, were rapidly discharged from the dorsal white muscle at the beginning of depuration with a biological half-life of less than 4 d, while in adipocytes only benzene and toluene showed this characteristic. The benzenes alkylated with three or more carbons were released so slowly that it took at least 15 d to depurate to the 50% level in adipocytes. The depuration of polynuclear aromatic hydrocarbons from adipocytes, e.g., methylnaphthalenes, was the slowest among all of the tainting aromatics and the biological half-life exceeded 20 d of depuration in clean water. It is interesting to note that the biological half-life of methylcyclohexane was unexpectedly much longer than that of toluene, and even of xylenes and ethylbenzene, in both dorsal white muscle and adipocytes.

Discussion

The muscle tissue of Atlantic salmon is mainly composed of muscle cells which are held together through connective tissues. Adipocytes in the white muscle tissue of Atlantic salmon are concentrated in part of this connective tissue, specifically in the myosepta (Zhou et al. 1996), and are cells specialized for the storage of triacylglycerols. It is the myosepta, the principal connective tissue of white muscle, that stores about 40% of the total white muscle lipids although the volumetric proportion of myosepta in dorsal white muscle is negligible. In farmed Atlantic salmon the muscle fat content ranges from 10 to 12% (Ackman 1989), or even higher

Table 4 *Salmo salar*. Biological half lives (d) of individual hydrocarbons accumulated in dorsal white muscle and adipocytes when depurated in clean seawater

Hydrocarbons	White muscle	Adipocytes
Benzene	0.5	0.6
Methylcyclohexane	2.5	12.0
Toluene	0.6	0.8
Ethylbenzene	0.8	3.9
Xylenes	0.9	6.8
Isopropylbenzene	3.3	17.0
Propylbenzene	3.8	18.0
Ethyl-methylbenzenes	2.5	15.0
Trimethylbenzenes	2.9	16.0
Methylnaphthalenes	3.5	> 20.0

depending on the size and nutritional status of the fish. We have observed, through both lipid class analysis (Table 2) and histological examination (Zhou et al. 1996), that except for the membrane lipid most of the lipid in white muscle is found in adipocytes or lipid droplets trapped in connective tissue. Histologically no lipid droplets were visible by light microscope within the section of each individual white muscle cell, whereas finely dispersed lipid droplets were a characteristic of dark muscle cells. Therefore, the accumulation and release of WSF in dark muscle are expected to be more complicated than in white muscle. The belly flap is well-known for a very high fat content which we have found to be almost solely due to adipocytes (Zhou et al. 1996).

The exposed salmon exhibited signs of physiological stress, e.g., reduction in daily diet intake, due to the long-term hydrocarbon exposure and to their transfer from tank to tank at the beginning and end of exposure. However, examination of the lipid content of dorsal white muscle at different depuration stages (Table 2) did not show an obvious trend of lipid depletion caused by the lower food intake. The variations in the lipid content of dorsal white muscle among Atlantic salmon sampled at different depuration stages can mainly be attributed to the physical and nutritional differences among individual fish. No distinction in lipid in muscle was apparent in samples, including the female fish containing eggs, but fish were not examined on an individual basis.

The large difference found in the accumulation and release of hydrocarbons between adipocytes and white muscle cells confirms that the species-dependent characteristics with respect to the accumulation and retention of hydrocarbons are actually dependent on both the tissue lipid content and the lipid storage format. Our present study on the separation of adipocytes from Atlantic salmon muscle tissue and the subsequent analysis of the tainting hydrocarbons in this specific compartment of the tissue have revealed that adipocytes in salmon muscle tissue do play an important role in controlling the uptake and release of hydrocarbons. The WSF accumulated in the tissue was primarily stored in the adipocytes and was also retained by the adipocytes during depuration in clean seawater.

Neely et al. (1974) suggested that the accumulation and release of hydrocarbons in the tissue is most probably a passive process of partitioning of the hydrocarbons between the exposure water and the tissue lipids. The actual accumulation and release processes of WSF into and from the muscle tissue are probably composed of several partitioning and diffusion steps before WSF finally reaches the lipid droplets in the adipocytes. Studies have shown that the primary route for the uptake of hydrocarbons from water and the excretion of the accumulated hydrocarbons was via the gills (Lee et al. 1972; Thomas and Rice 1981). The uptake of WSF from water probably proceeds in the following steps: WSF in the exposure water are first partitioned through gills and are subsequently associated with the lipoproteins and chylomicrons in the blood fluid, which carry the WSF through the circulatory system to the whole fish body including muscle tissue. The lipoproteins and chylomicrons then adhere to the luminal surface of the capillaries (Porter and Bonneville 1973). The WSF is released from lipoproteins or chylomicrons and diffuses to the endothelial cells of the capillaries. Due to the concentration gradient of WSF between the bulk lipid droplets of adipocytes and the endothelial cells, the WSF continuously diffuses and passes through the capillary endothelium into the connective tissue ground substance (Wheater et al. 1979). Tissue fluid is loosely bound to the ground substance, thereby forming the medium for passage of WSF throughout connective tissue. The WSF diffuses through the ground substance and reaches the membrane surrounding individual adipocytes. The WSF then dissolves and easily passes through the bi-phospholipid layer membrane, and finally enters the single large lipid droplet in the adipocyte. The accumulation of WSF continues until an equilibrium is reached. Our unpublished results show that high levels of tainting WSF are detected in the muscle tissue of Atlantic salmon after only 1 h of exposure. Rapid accumulations of hydrocarbons in the tissues of other fish or shellfish are also reported within hours or even minutes (Lee et al. 1972; Stegeman and Teal 1973; Johnsen and Lloyd 1992). Unfortunately these examinations only considered whole tissue and there is no report on the accumulation rates of hydrocarbons in specific tissue compartments. It is believed that most of the hydrocarbons detected initially are probably associated only with tissue fluids and it would take a longer exposure time to show their predominant accumulation in the adipocytes (Heras et al. 1993).

The nonadipocyte portion of white muscle is mainly composed of water, but the accumulated hydrocarbons are most probably associated with the cell membrane polar lipids or the hydrophobic groups of proteins and glycolipide, etc. Boryslawskyj et al. (1988) indicated that lipids in membranes are a key factor in the partitioning of hydrophobic xenobiotics in a freshwater mussel, but changes in the membrane lipid composition did not show a significant effect on the uptake of hydrocarbons. The association of WSF with these cell lipid components is expected to be looser than their actually dissolving into the bulk triacylglycerol lipid in the adipocytes. Some of the WSF in the nonadipocyte portions are presumably free to move around if they are merely associated with tissue fluids such as plasma. This WSF of hydrocarbons can be quickly transported to the gills or to the liver. Thus they are more readily released into the clean water column or metabolized by the liver (Thomas and Rice 1981). This loose association of WSF with the components in the muscle cells and its presence in tissue fluids are probably the causes of the sharp release of tainting hydrocarbons, mostly lower molecular weight aromatics, from white muscle at the early stage of depuration (Day 0 to 4).

Most of the lipids in the muscles of cod and scallop are membrane lipids (< 1%) (Jangaard et al. 1967;

Ackman and McLeod 1988) and the presence of adipocytes would obviously be negligible. Scallop adductor muscle exposed to 2.66 ppm WSF for 24 h took up only 15.59 ppm hydrocarbons (bioaccumulation factor of 5.9), of which 14.34 ppm was released during the first 24 h of depuration in clean seawater (Ernst et al. 1989). Similar hydrocarbon uptake and rapid depuration behaviours were also observed for Atlantic cod muscle (Ernst et al. 1987). Thus the low bioaccumulation characteristics of WSF hydrocarbons and their subsequently rapid depuration from lean cod and scallop muscles is further evidence of the fast release of WSF from the nonadipocyte portion of marine organisms. After the rapid discharge of WSF from the nonadipocyte portion of the white muscle, i.e. 4 d of depuration in clean seawater, the adipocytes in the muscle tissue became the principal site for hydrocarbon storage. This view is supported by the similarity in depuration rates of the tainting WSF from the dorsal white muscle and from the isolated adipocytes on Day 4 and thereafter (Fig. 2), and also by the changes in the calculated percentages of WSF stored in the adipocytes of the dorsal white muscle during the 20 d period of depuration (Fig. 3).

When the tainted fish return to hydrocarbon-free seawater, the lipid/water partition coefficients for hydrocarbons favour the release of WSF from tissue to water. Rapid depuration rates of WSF from adipocytes were also observed in the early depuration periods (Fig. 2), but these were not as fast as the depuration rates from dorsal white muscle and apparently can be attributed to the release from the adipocytes of the more water-soluble hydrocarbons such as benzene and toluene (Table 4). The highly alkylated benzenes and polynuclear aromatics such as methylnaphthalenes did not show any sign of rapid release at the early stage of depuration. Theoretically, depuration is the reverse of the accumulation process, but this does not necessarily mean identical rates for the accumulation and the depuration of WSF from adipocytes. Both the accumulation and the release of WSF are controlled by the hydrophobicity of components in the cell compartments and the degree of vascularization surrounding the compartments. It is the affinity of the triacylglycerols in adipocytes for the WSF hydrocarbons which leads to the slow release of the accumulated WSF from adipocytes. The persistent retention of polynuclear aromatic hydrocarbons in adipocytes of the exposed fish must be emphasized because of their carcinogenic properties and the potential health hazard they may pose to consumers of marine foods (Heidelberger 1964).

Individual hydrocarbons of WSF accumulated in the dorsal white muscle and adipocytes displayed differences in their abundances in the total tainting WSF when compared with those in the WSF of the exposure water. The selective accumulation of WSF is probably due to the combined effects of differences in their water solubility and in the metabolic processes of Atlantic salmon (Gerhart and Carlson 1978; Melancon and Lech 1978; Cravedi and Tulliez 1981). The livers of fish, including salmonids, contain aryl hydrocarbon hydroxylase (AHH), an enzyme system capable of metabolizing petroleum hydrocarbons (Pederson et al. 1974; Gruger et al. 1977; Statham et al. 1978). Kennish et al. (1988) investigated the metabolic conversion of toluene and ethylbenzene and found that both can be metabolized by microsomes from Pacific salmon liver. Coho salmon were also capable of biotransforming hydrocarbons, and the retention and accumulation of parent compounds and metabolites were found to increase along with the increases in aromatic rings (Roubal et al. 1977). Thomas and Rice (1981) compared the metabolism of ^{14}C-labelled toluene and naphthalene by Dolly Varden char (*Salvelinus malma*) and reported that toluene was more easily metabolized than naphthalene. The metabolic conversion of WSF in Atlantic salmon must have begun shortly after the fish were exposed to the WSF and the process continued for the 96 h of exposure. Concurrently, the reverse diffusion of WSF from tissue compartments to tissue fluids would have released relatively more of the hydrocarbons with higher water solubility.

The relatively slower depuration of the nonaromatic, cyclic methylcyclohexane from adipocytes and dorsal white muscle is probably caused by its lower water solubility and a greater difficulty in its being metabolized by the livers of fish than would be the case for their aromatic counterparts (Cravedi and Tulliez 1986).

In Fig. 3 the calculated percentage of tainting WSF stored in adipocytes was higher than 100% of the total tainting WSF in the dorsal white muscle (120%). This could probably be attributed to a number of error sources such as the hydrocarbon recoveries by steam distillation, the lipid content determination by $CHCl_3$/MeOH and the membrane lipid analysis by thin-layer chromatography–flame ionization detection (TLC-FID). Moreover, the assumption that all lipids were stored in adipocytes except for membrane lipids also contributed to the apparent excess of over 100% hydrocarbon storage in adipocytes since a small fraction of nonmembrane lipids would be expected to be present in the nonadipocyte cells as an energy reserve. However, in spite of the additive errors in the calculated values of percent hydrocarbon storage in adipocyte of the dorsal white muscle, it is obvious from the trend that adipocytes became the main storage site for the accumulated hydrocarbons after the first 4 d of depuration in clean seawater.

In conclusion, adipocytes in the muscle tissue of Atlantic salmon were found to be the principal cell compartments for the storage and retention of WSF hydrocarbons, particularly after the initial rapid discharge of the accumulated WSF from the muscle cells during the depuration periods. The role of adipocytes in the storage and retention of xenobiotics may be applicable not only to the WSF hydrocarbons but also to various other organic pollutants due to their similarity in hydrophobicity. This key role of adipocytes would also be expected to occur in various other aquatic organisms, particularly those with high lipid contents. The presence

of high lipid reserves in some tissues implies that adipocytes would be the major form for lipid storage and would be abundant in such tissues, while in lean tissues most of the lipids are present in the membranes. This would satisfactorily explain the species-specific phenomena of hydrocarbon behaviours observed in many aquatic organisms.

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