The influence of both rearing temperature and dietary lipid origin on fatty acid composition of spermatozoan polar lipids in rainbow trout (Oncorhynchus mykiss). Effect on sperm cryopreservation tolerance.

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THE INFLUENCE OF BOTH REARING TEMPERATURE AND DIETARY LIPID ORIGIN ON FATTY ACID COMPOSITION OF SPERMATOZOAN POLAR LIPIDS IN RAINBOW TROUT (*Oncorhynchus mykiss*). EFFECT ON SPERM CRYOPRESERVATION TOLERANCE.

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ABSTRACT

A factorial experiment was designed to investigate the effect of dietary lipid (corn oil and fish oil) and water temperature (8°C and 18°C) on lipid composition of rainbow trout milt and the ability of frozen-thawed sperm to fertilize eggs.

One year old rainbow trout, 100 g in weight, were reared at two temperatures and two diets. After one year, semen of these now 1 Kg trout was collected regularly during a two month period. A part was frozen while cholesterol and fatty acids of the polar lipid fraction were assessed on the remaining sample.

Neither cholesterol nor total phospholipids contents were modified by the diet. The n-3/n-6 ratio was largely decreased in sperm phospholipids after corn oil supplemented feeding, reflecting the feature of the diet even when the 22:6n-3 levels are less influenced. A 8°C acclimation temperature seemed to decrease the cholesterol contents when compared to 18°C but this was not clearly repeatable. Sperm freeze-thaw fertility was two fold improved by a 18°C acclimation while no influence of the diet was observed.

INTRODUCTION

Cryopreservation of salmonid sperm has almost been mastered with the development of efficient freeze-thawing methods (LEGENDRE and BILLARD 1980, STOSS and HOLZ 1983). However, considerable individual variation was observed when comparing post-thaw fertility of semen from different males (STOSS and HOLZ 1983, MAISSE *et al.* 1988) and for a single male during its breeding season (MAISSE *et al.* 1988).

Trout, as each poikilotherms, respond by physiological and biochemical adjustments to temperature disturbance (WODTKE and COSSINS 1991) leading to modifications of the membrane lipid composition (HAZEL 1988). Moreover, quality of feed affects the reproduction of rainbow trout, showing the sensitiveness of sperm and eggs to dietary manipulations, mainly through modifications of the lipid composition (WATANABE *et al.* 1984, LERAY AND PELLETIER 1985, LERAY *et al.* 1985).

The purpose of our work was to investigate the influence of low rearing temperature (8°C) and high rearing temperature (18°C) with a linoleic acid rich diet (corn oil) and a n-3 fatty acid rich diet (cod liver oil), on spermatozoan phospholipid fatty acids and the associated influence on fertilizing ability of freeze-thaw trout sperm.

MATERIALS AND METHODS

ANIMALS

Four groups of one year old rainbow trout (*Oncorhynchus mykiss*) having an individual initial weight of 100 g were acclimated to 8°C or 18°C and fed two isoenergetic experimental pelleted diets one coated with corn oil (6 %) (CO) and the other with cod liver oil (6 %) (FO) (Table 1). The duration of the experiment was one year during which the animals went through gonadal development and reached sexual maturity.

Table 1 : Composition of the experimental diets

	Experimental diets	
Ingredients	**Corn oil (wt%)**	**Cod liver oil (wt%)**
Fish meal	45	45
Wheat starch (native)	20	20
Soybean LTI [1]	18	18
Pre-gelatinized starch	5	5
Mineral mix (INRA) [2]	2	2
Vitamin mix (INRA) [3]	2	2
Sodium alginate	2	2
Corn oil	6	0
Cod liver oil	0	6
Antioxidant (ppm)	100	100
Protein, %	43	43
Lipid, %	11	11
Digestible energy (kj/g)	15,5	15,5

1) Low trypsin inhibitor extruded full fat soybean.
2) Mineral mix contained the following ingredients (g/kg mix) : calcium carbonate, 215; magnesium hydroxyde, 124; KCl, 90; ferric citrate, 20; KI, 0.4; NaCl, 40; calcium hydrogen phosphate (CaHPO4), 500; copper sulfate, 3; zinc sulfate, 4; cobalt sulfate, 0.2; manganese sulfate, 3.
3) Vitamin mix contained the following diluted in cellulose (g/kg mix) : vit A (500 000 IU/g), 1.5; vit D3 (100 000 IU/g), 1.5; vit E (500 IU/g), 6; vit K, 0.25; thiamin, 0.75; riboflavin, 1.5; pyridoxine, 0.75; nicotinic acid, 8.75; vit C, 25; folic acid, 0.25; vit B12 (1000mg/kg), 2.5; inositol, 50; biotin (2%), 6.25; calcium pantothenate, 2.5; choline (50%), 200.

Milt from these rainbow trout weighing about 1 kg was collected at the beginning, the middle and towards the end of spawning season. Care was taken to avoid any contamination by blood and feces (MAISSE *et al.* 1988). A small volume of milt was frozen. Whole sperm cells analysis was performed on the remaining semen sample.

SPERM FREEZING

After males were stripped, milt from individual were stored on ice for 2-3 h. Motility was assessed for each sample. The extender solution (MOUNIB 1978 modified as follows :saccharose 125 mM, reduced gluthatione 6.5 mM, $KHCO_3$ 100 mM, egg yolk 10 %, DMSO 8 %) was added to the milt at a ratio of three parts extender to one part of milt. The diluted semen was immediately pelleted on dry ice (-79°C). The volume of a pellet was 0.1 cm^3. After 3 minute of freezing, the pellets were placed into small vials that were submerged in liquid nitrogen (-196°C) and stored for 3-4 months.

SPERM THAWING AND FERTILIZATION

Frozen pellets (2) were placed on 10 g freshly collected eggs pooled from several females (fed on a commercial diet and kept at 13°C) and 10 ml "DIA 532" (BILLARD 1977) kept at 25°C was simultaneously added. After gentle stirring from one bowl to another, inseminated eggs were incubated in recycled water thermoregulated at 10°C. Controls for fertilization to assess the quality of the eggs were carried out in the same way with the exception that fresh milt from same males was used in excess (i.e. 1 ml of a fresh milt pool was added to 10 g eggs and 10 ml DIA). Twenty days later, eyed eggs were recorded as fertilized and their number as a percentage of the control fertilized eggs was calculated.

LIPID ANALYSES

Spermatozoa were separated from seminal plasma by centrifugation (500g; 20 min). Lipids from total spermatozoan were extracted according to the method of FOLCH *et al.* (1957). The chloroform/methanol mixture (2:1 V/V) contained 0.02 % butyl hydroxy toluene.

Total cholesterol was determined by an enzymatic method (Biochemica test combination, Boehringer), adapted to carry out assays in 96-well plates, on the chloroform phase after evaporating (under nitrogen flux) and dissolving the extract in 2-propanol . Polar lipid phosphorus was determined after hydrolysing the chloroform extract with sulfuric acid according to the method of BARTLETT (1959). "Total phospholipid" was calculated from the inorganic phosphate and expressed as mole phosphate / mg protein.

Polar lipid was separated from neutral lipid according to the method of JUANEDA and ROCQUELIN (1985) : total lipid extract was dissolved in 0.5 ml chloroform then fractionated on sep-pak silica columns (Waters Chromatography Division, Millipore Corporation, MA-USA).

Methyl esters for fatty acid analysis were prepared as following : 4 ml of HCl / methanol / Dimetoxypropane / BHT (9.87/86.25/3.95/0.045 : v/v/v/w) reagent was added to 500 µg phospholipids. The tube was closed under nitrogen with a screw cap and heated in a 65°C water bath for 3 h. After cooling, the fatty acid methyl esters were extracted by adding 4 ml of hexane and rinsed three times with distilled water then dried and dissolved in 15 µl isooctane.

Quantitative analysis of fatty acids was carried out by gas chromatography (GC) using a fused silica column 50 m x 0.25 mm internal diameter coated with the liquid phase CP WAX 52 CB (Chrompack) under nitrogen carrier gas. The splitter injector temperature was 250°C, that of flame ionisation detector was 270°C. The oven temperature was programmed to rise from the injection temperature of 180°C to 225°C at 4°C/minute. The final temperature was maintained for 20 minutes. After a 1 µl injection, separated components were identified by reference to standards and quantitated (weight %) by a recording integrator (Spectra-Physics).

PROTEIN ASSAY

Protein content of milt was determined according to the method of SMITH *et al.* (1985) using Pierce "BCA Protein Assay Reagent" kit. Bovine serum albumin was used as a standard.

STATISTICAL ANALYSIS

Statistical analyses were performed by using the multivariates principal component analysis (ACP) (FOUCARD 1982).

RESULTS

The fatty acid composition of the experimental diet is similar to those used by CORRAZE *et al.* (1991) (Table 2). The total lipid content of corn oil (CO) and fish

Table 2. Fatty acid composition of the experimental diets (% of total fatty acids) *

	Experimental diets	
Fatty acids	Corn oil	Cod liver oil
14:0	2.3	6.7
16:0	18.5	24.7
16:1	2.3	8.2
18:0	2.8	3.7
18:1	22.8	20.5
18:2n-6	43.3	14.0
18:4n-3	0.3	1.2
20:1	0.2	2.0
20:4n-6	0.2	0.4
20:4n-3	-	0.2
20:5n-3	1.8	4.7
22:1	-	1.2
22:5n-3	0.2	0.4
22:6n-3	1.7	4.6
Total n-3 PUFA	5.8	13.3
Total n-6 PUFA	44.1	16.4
n-3/n-6	0.1	0.8

* From CORRAZE, 1991

oil (FO) diets was 11 to 12 % (wt) . The amounts of n-3 fatty acids (about 0.5 % of the corn oil diet and 1.1 % of the cod liver oil diet) and n-6 fatty acids (3.7 % and 1.36 %) in these diets met the essential fatty acids (EFA) requirements of broodstock fish (WATANABE 1985). Corn oil diet was 3 fold richer in linoleic acid than cod liver oil while the latter was 2.3 fold richer in n-3 fatty acid. The other feature of the two diets is that the HPUFA (highly polyunsaturated fatty acid)/SFA ratio is higher in cod liver oil compared to corn oil pellets (0.37 versus 0.24 respectively).

The fertilizing ability of sperm related to animals farming conditions are shown in Figure 1. For each value, 13 to 15 pools of 5 to 8 individual milts were tested. No significant difference was found between the two diets while the 18°C acclimated trout sperm demonstrated a greater fertilizing ability than the 8°C (two fold greater)($p < 0.01$; Student's test on arc sinus$\sqrt{x}$, x being the % of fertilized eggs). Motility assessed before cryopreservation did not reveal differences between the four groups (data not shown).

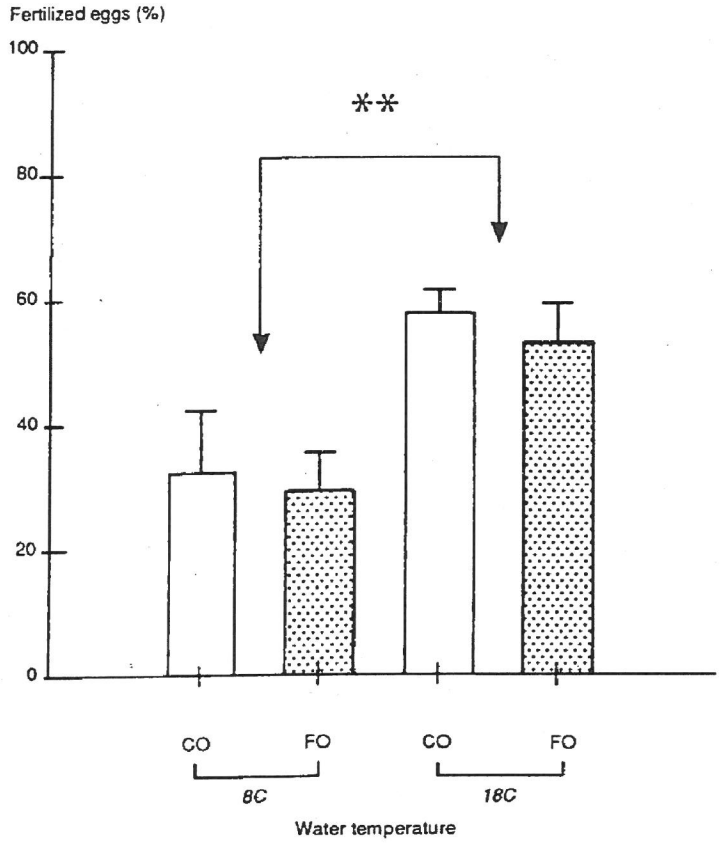

Figure 1. Effect of dietary lipid(1) on post-thaw fertility(2) of pellet-frozen milt from rainbow trout reared at 8°C and 18°C

(1) CO Corn oil diet, FO Fish oil diet (i.e. cod liver oil diet)
(2) *Values are determined as % of the control fertilized eggs (means ± STD from 12 to 15 milt pools, one determination for each pool)*
** $p < 0.01$, *Student's t test on "arc sin* $\sqrt{x}$"

Table 3. Effect of dietary lipid on cholesterol and polar lipid content of milt from rainbow trout reared at 8°C and 18°C

	Corn oil diet		Fish oil diet	
	8°C-reared *	18°C-reared **	8°C-reared *	18°C-reared *
Cholesterol/protein (nmoles/mg)	0.12 ± 0.02	0.14 ± 0.03	0.14 ± 0.04	0.13 ± 0.04
Polar lipid/protein (nmoles/mg)	0.42 ± 0.12	0.44 ± 0.11	0.47 ± 0.11	0.38 ± 0.09
Cholesterol/polar lipid (molar ratio)	0.30 ± 0.07	0.33 ± 0.07	0.32 ± 0.06	0.33 ± 0.08

* values are mean ± STD, n=8
** values are mean ± STD, n=7

Table 4. Effect of dietary lipid on fatty acid composition of milt phospholipids from rainbow trout reared at 8°C and 18°C

Fatty acids	Corn oil diet		Cod liver oil diet	
	8°C-reared *	18°C-reared **	8°C-reared *	18°C-reared *
14:0	1.0 ± 0.1	0.8 ± 0.2	1.4 ± 0.2	1.2 ± 0.2
16:0	25.9 ± 1.2	24.8 ± 1.2	25.5 ± 1.2	25.9 ± 1.5
DMA 16:1	0.7 ± 0.4	0.6 ± 0.4	0.7 ± 0.7	0.9 ± 1.1
16:1	1.0 ± 0.2	0.8 ± 0.1	1.6 ± 0.2	1.1 ± 0.3
18:0	4.2 ± 0.4	5.2 ± 0.3	4.0 ± 0.2	5.5 ± 0.4
DMA18:1	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.1
18:1n-9	9.3 ± 0.4	8.4 ± 0.3	9.6 ± 0.4	8.4 ± 0.5
18:1n-7	3.4 ± 0.3	3.2 ± 0.3	4.8 ± 0.4	5.1 ± 0.5
18:2n-6	9.0 ± 1.0	10.4 ± 0.6	4.6 ± 0.4	4.7 ± 0.7
20:1n-9	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.0	0.3 ± 0.1
20:2n-6	1.2 ± 0.2	1.7 ± 0.2	0.6 ± 0.1	0.7 ± 0.2
20:3n-6	1.8 ± 0.2	2.2 ± 0.2	0.4 ± 0.1	0.5 ± 0.3
20:4n-6	9.8 ± 1.0	10.0 ± 0.5	4.9 ± 0.3	5.3 ± 0.8
20:5n-3	8.1 ± 0.7	6.2 ± 0.9	14.5 ± 0.7	13.0 ± 1.5
22:5n-6	0.8 ± 0.0	1.2 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
22:5n-3	0.6 ± 0.1	0.6 ± 0.1	1.1 ± 0.1	1.1 ± 0.2
22:6n-3	21.0 ± 1.9	22.1 ± 0.9	23.0 ± 1.2	23.5 ± 2.1
Total n-3 PUFA	30.0 ± 2.7	29.2 ± 1.9	39.2 ± 2.2	38.1 ± 3.9
Total n-6 PUFA	22.6 ± 2.4	25.5 ± 1.6	10.9 ± 0.9	11.7 ± 2.1
n-3/n-6	1.3 ± 0.2	1.1 ± 0.1	3.6 ± 0.3	3.3 ± 0.5

* Values are means ± STD, expressed in weight % of total fatty acids identified, n=8
** Values are means ± STD, expressed in weight % of total fatty acids identified, n=7

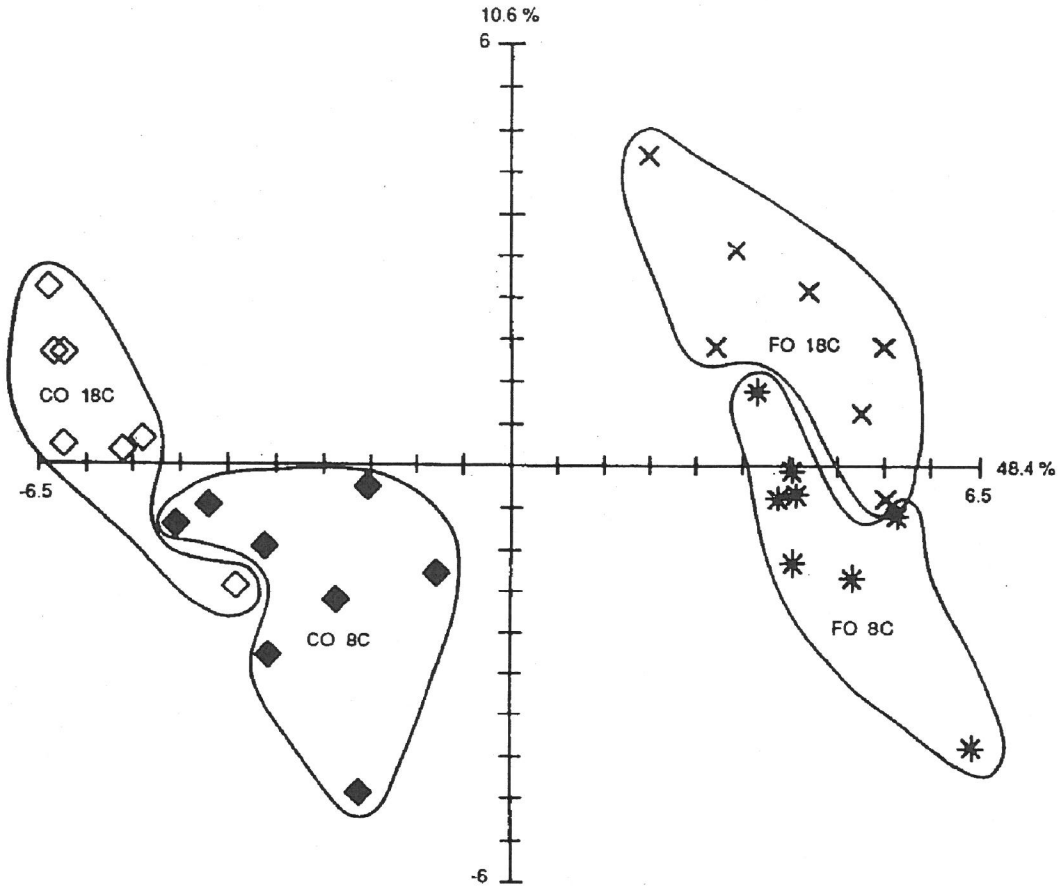

Figure 2. Effect of dietary lipid on the ACP distribution of milt from rainbow trout reared at 8°C and 18°C

X-axis axis 1 of the ACP reflecting the diet effect
Y-axis axis 3 of the ACP reflecting the rearing temperature effect
FO 18°C milt from 18°C-reared trout fed with fish oil (i.e. cod liver oil), FO 8°C milt from 8°C-reared trout fed with fish oil, CO 18°C milt from 18°C-reared trout fed with corn oil, CO 8°C milt from 8°C-reared trout fed with corn oil

Polar lipids and cholesterol contents of whole sperm regardless of the spawning sampled period are shown in table 3 ; Fatty acid composition of whole sperm polar lipids are shown in table 4. The multivariates principal component analysis (ACP) from these parameters related to the rearing conditions is presented in the Figures 2 and 3. The effects of both the diet and the acclimation temperature are expressed in the factorial plan formed with the axis 1 (X-axis) and the axis 3 (Y-axis) of the ACP. The distribution of the semen samples (Figure 2) along the axis one (X-axis) reveals the high contrast existing between the two diets : all the corn oil milts are localized on the left side of the axis while all the fish oil milts are localized on the right side of the axis. In the same way, the axis 3 (Y-axis) reveals the distribution of the semens related to temperature acclimation. Milt from the 18°C acclimated trout are on the upper side of the axis while those from the 8°C ones are on the lower side of the axis. Fourty eight point four % of the samples are significantly expressed on the axis 1 while 10.6 % are expressed on the axis 3. This points out that the effect of the diet on sperm lipid composition is much higher than that of the temperature acclimation of the trout.

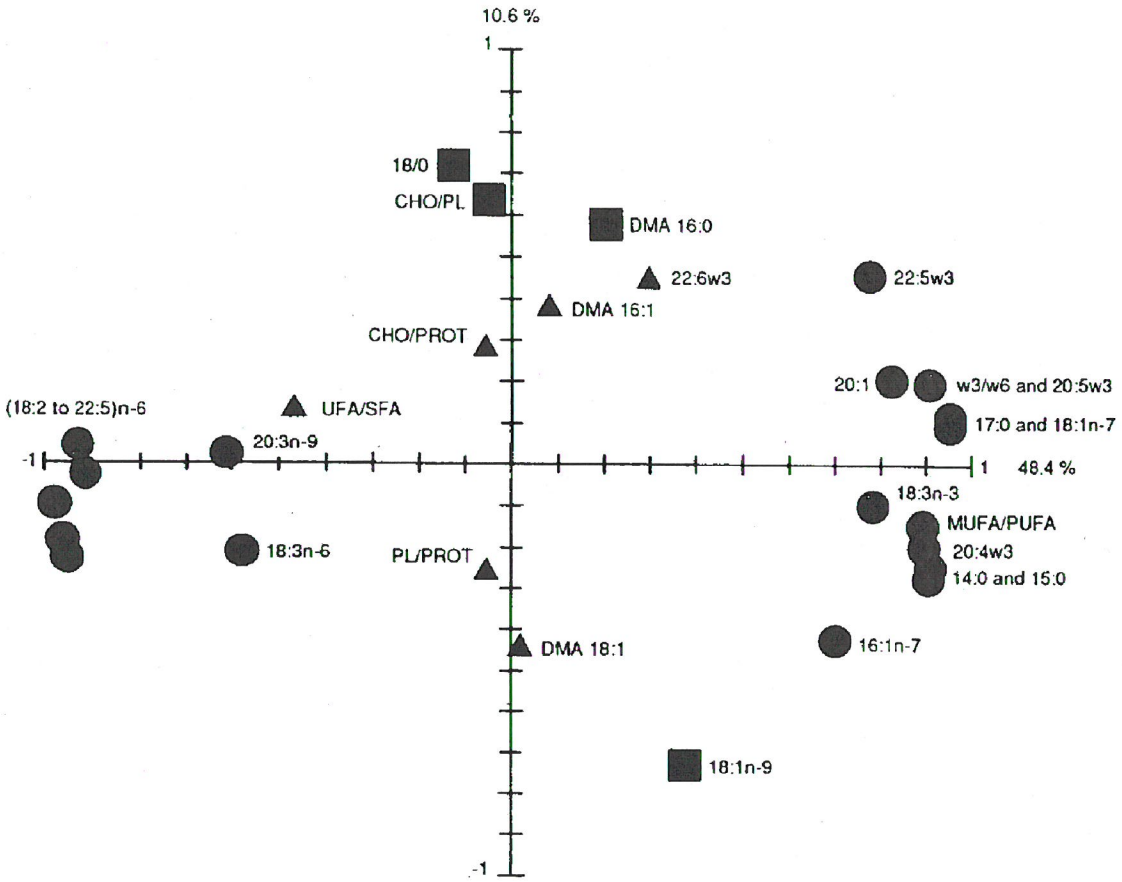

Figure 3. Effect of dietary lipid and rearing temperature on the correlation circle of the ACP (displaying milt parameters)

X-axis axis 1 of the ACP reflecting the diet effect
Y-axis axis 3 of the ACP reflecting the rearing temperature effect
● *parameters significantly expressed on the X-axis,* ■ *parameters significantly expressed on the Y-axis,* ▲ *parameters non-significantly expressed in this plane*

The distribution of the semen samples as revealed in the Figure 2 leads to the interpretation of the correspondance circle on the Figure 3 (displaying the distribution of the parameters assessed on sperm i.e. cholesterol, polar lipids and fatty acids contents). Parameters influenced by the diet spread along the axis 1 (X-axis). Those which are in high amounts in cod liver oil fed trout spermatozoa are on the positive side of the axis, those with high amounts in corn oil fed trout being on the negative side. In the same way, the parameters influenced by temperature spread along the axis 3 (Y-axis), the 18°C increased ones being on the positive side (up), the 8°C increased ones being on the negative side (down).

This correspondance circle reveals then that the phospholipids of sperm from corn oil fed trout are enriched in n-6 fatty acids while those from cod liver oil fed trout are enriched in n-3 fatty acids, reflecting the features of the pelleted diets. The MUFA/PUFA and n-3/n-6 ratios, higher in sperm from cod liver oil fed trout, also reflect the diet characteristics.

Concerning the influence of temperature acclimation, the cholesterol contents, stearic and palmitoleic dimethylacetal (DMA) fatty acid contents are higher in sperm from 18°C reared trout while oleic contents are higher in 8°C ones. However, further calculations with a higher number of sample made the cholesterol level variations unclear (data not shown), leading to the observation that this parameter is too heterogenous for any conclusion about the influence of temperature on the cholesterol/phospholipid ratio. Some parameters are not influenced by any of these rearing conditions, including the UFA/SFA, CHO/PROT and PL/PROT ratios, the docosahexaenoic fatty acid contents and the monounsaturated dimethylacetals (DMA 16:1 and DMA 18:1).

DISCUSSION

In contrast with previous papers where the effect of essential fatty acid (EFA) deficiency on trout reproductive process is described (LERAY *et al.* 1985, LERAY and PELLETIER 1985, WATANABE *et al.* 1984, WATANABE *et al.* 1983), none of the two fed groups were EFA deficient. No deficiency symptoms appeared with respect to the the growth rate or to the physiological integrity of the broodstock and they are now beginning their third year without any increase in mortality compared to commercial fed groups. We then confirmed that the distribution of fatty acids in trout spermatozoan phospholipids is greatly affected by dietary fatty acids of the broodstock even if the fish do not have to counteract any EFA deficiency. It appears that the most available lipid source is incorporated in sperm cell without alteration of its functionnal integrity as shown by the post-thaw fertility of pellet-frozen sperm from the two fed groups. However, an effect of diet is perhaps overcome by the influence of extender composition as described by BAYNES and SCOTT (1987).They pointed out that milt from sprat-fed rainbow trout gave significantly higher percentage fertilizations than that from the commercial pellet-fed fish when frozen in the absence of egg yolk while the mean percentage fertilization did not differ between the two groups when egg yok was added to the extender. The maintenance of the 22:6n-3 (docosahexaenoic fatty acid) levels in our experiment was also reported by CASTELDINE and BUCKLEY (1982) and they hypothesized that this could be due to the presence of an efficient recycling system. This would enable us to suggest that 22:6n-3 also plays a role in the conservation of the functional integrity of the cell.

In contrast with no influence of diet, the temperature acclimation of the broodstocks plays a major role in post thaw fertility of rainbow trout sperm (two fold better in 18°C acclimated trout than in 8°C one). The distribution of the cholesterol content remains to be clarified because of the great heterogeneity of the levels assessed in each groups. The fatty acid distribution in relation with temperature is also unclear with stearic fatty acid and palmitoleic DMA contents being higher at 18°C while oleic fatty acids contents are lower. In addition to a paper (in preparation) concerning the same experimental factors on spermatozoan plasma membrane and dealing with the phospholipid distribution and fatty acid pattern inside each class, further experiment about the biophysical properties of the membrane would allow to better elucidate these observations.

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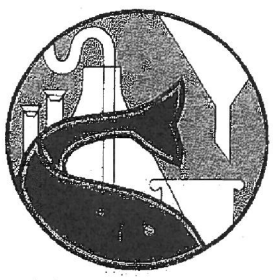

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