Cold Acclimation or Grapeseed Oil Feeding Affects Phospholipid Composition and Mitochondrial Function in Duckling Skeletal Muscle

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ABSTRACT: The phospholipid fatty acid (FA) composition and functional properties of skeletal muscle and liver mitochondria were examined in cold-acclimated (CA, 4°C) ducklings. Phospholipid FA of isolated muscle mitochondria from CA birds were longer and more unsaturated than those from thermoneutral (TN, 25°C) reared ducklings. The rise in long-chain and polyunsaturated FA (PUFA, mainly 20:4n-6) was associated with a higher State 4 respiration rate and a lower respiratory control ratio (RCR). Hepatic mitochondria, by contrast, were much less affected by cold acclimation. The cold-induced changes in phospholipid FA profile and functional properties of muscle mitochondria were reproduced by giving TN ducklings a diet enriched in grapeseed oil (GO, rich in n-6 FA), suggesting a causal relationship between the membrane structure and mitochondrial functional parameters. However, hepatic mitochondria from ducklings fed the GO diet also showed an enrichment in long-chain PUFA but opposite changes in their biochemical characteristics (lower State 4, higher RCR). It is suggested that the differential modulation of mitochondrial functional properties by membrane lipid composition between skeletal muscle and liver may depend on muscle-specific factors possibly interacting with long-chain PUFA and affecting the proton leakiness of mitochondrial membranes.

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When chronically exposed to cold, most endotherms are able to enhance their metabolic capacity to generate heat to compensate for the rise in heat loss and maintain homeothermy. Small rodents are thus well known to develop nonshivering thermogenic (NST) capacity in the cold (1). Some species of birds, including chickens, ducks, and penguins, can also develop NST in response to cold acclimation or acclimatization (2–5). This remarkable adaptation to cold exposure differs from the situation in mammals, as birds lack the specialized thermogenic brown adipose tissue (BAT) of newborn mammals, hibernators, and small rodents (6–8). In the absence of BAT, skeletal muscle appears as the main site of cold-induced NST (9).

Muscle NST may be based on a loose-coupling of mitochondrial oxidative phosphorylation controlled by fatty acids (FA) on the basis of results obtained with isolated mitochondria in cold-acclimated (CA) ducklings (3,10,11). These studies have contributed to clarify the earlier observation of loose-coupled mitochondria and possible involvement of FA in CA birds (12,13). The molecular mechanisms of such loose-coupling are still unclear, but involve an increase (+40%) in the mitochondrial membrane conductance to protons in CA ducklings (11). No uncoupling protein with protonophoric properties similar to those expressed in mammalian tissues has yet been described in CA birds (7,8). Nevertheless, because phospholipid membranes are inherently leaky to protons (14,15) and the degree of proton leak correlates with the composition in unsaturated FA (16,17), a change in the phospholipid composition of mitochondrial membrane may contribute to the increased leakiness of mitochondrial membranes in CA ducklings. Differences in nonphosphorylating respiratory rates of isolated mitochondria from various species have already been related to mitochondrial phospholipid composition (18). Consistent with this idea is the observation that in CA rats, the membranes of BAT mitochondria contain more polyunsaturated FA (PUFA), possibly contributing to the thermogenic activation of BAT mitochondria (19). Furthermore, an enrichment of membranes with PUFA obtained through changes in the lipid composition of the diet was associated with an increased skeletal muscle oxygen consumption (20).

The aim of this study was therefore to assess whether the development of skeletal muscle NST in CA ducklings was accompanied by changes in the FA composition of tissue membranes. In a first experiment with whole tissues, we found that there was an enrichment in PUFA in skeletal muscle phospholipids of CA ducklings. In a second experiment, we examined whether the cold-induced changes in phospholipid composi-

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Abbreviations: ANOVA, analysis of variance; BAT, brown adipose tissue; CA, cold acclimated; FA, fatty acid; FOG, fast-oxidative glycolytic; GO, grapeseed oil; MUFA, monounsaturated fatty acid; NST, nonshivering thermogenesis; PUFA, polyunsaturated fatty acid; RCR, respiratory control ratio; SFA, saturated fatty acid; TN, thermoneutral control ducklings; U.A.R., Usine d'Alimentation Rationnelle.

tion were causally related to the functional characteristics of isolated mitochondria. To this purpose, mitochondrial membrane FA composition was altered at thermoneutrality by changing the lipid composition of the diet (21). The consequences of a diet supplemented with grapeseed oil rich in n-6 FA on the phospholipid composition and functional properties of hepatic and muscle mitochondria were thus examined in ducklings kept at thermoneutrality and compared to those observed after cold acclimation.

MATERIALS AND METHODS

Animals. Animals were cared for under the French Code of Practice for the Care and Use of Animals for Scientific Purposes, and the experimental protocols were approved by the French Ministry of Agriculture Ethics Committee (section animals). Male Muscovy ducklings (*Cairina moschata* L., pedigree R51, Institut National de la Recherche Agronomique, France) were obtained from a commercial stockbreeder (Ets Grimaud, France). They were fed *ad libitum* with a commercial mash (Genthon 5A; Genthon, Cheyssieu, France, see Table 1) and had free access to water. From 1 wk of age, ducklings were assigned to experimental groups.

A first batch of ducklings was divided into two groups; one was kept at 25°C and constituted the thermoneutral (TN) control group, while the other one was reared in the cold (4°C) and constituted the CA group. These birds were kept on the Genthon 5A commercial mash. This cold-acclimation schedule was shown to stimulate the development of skeletal muscle NST by 5 wk of age (6,9). Ducklings were killed by decapitation and tissues (gastrocnemius muscle and liver) were sampled and frozen in liquid nitrogen and kept at −70°C until FA analysis of total tissue phospholipids.

Following the results of the first experiment, a second batch of ducklings was divided into three experimental groups. Two groups of TN or CA ducklings were fed *ad libitum* with a commercial mash [UAR 115; Usine d'Alimentation Rationnelle (U.A.R.), Villemoisson, France]. A third group of ducklings kept at thermoneutrality was fed with the UAR 115 mash supplemented with 6.5% (w/w) grapeseed oil (GO; U.A.R.). Composition and energy content of diets are presented in Table 1. By 5 wk of age, ducklings were killed by decapitation. As the CA group grew a little bit more slowly (Fig. 1), they were killed a few days later at the same body weight as the TN and GO groups. Red internal gastrocnemius, rich in slow-oxidative and fast-oxidative glycolytic (FOG) fibers, white external gastrocnemius, rich in fast glycolytic and FOG fibers (22), and liver samples were taken and used for the analysis of mitochondrial function and FA composition of mitochondrial phospholipids. Photoperiod was 8 h/16 h (light/dark). Food consumption and body weight of ducklings were measured daily.

Isolation of skeletal muscle mitochondria. Internal and external gastrocnemius muscle and liver samples were rapidly taken out, freed of fat and connective tissues, and mixed in a cold isolation medium with a Teflon glass homogenizer. Mus-

TABLE 1
Fatty Acid Composition (in %) of Diets

	Genthon 5A	UAR 115	GO diet
14:0	3.49	0.31	0.15
14:1n-7	0.24	0.06	0.03
16:0	15.68	11.39	8.46
16:1n-9	0.26	0.07	0.04
16:1n-7	1.13	1.00	0.42
18:0	5.76	2.78	3.37
18:1n-9	27.98	22.02	20.28
18:1n-7	1.30	1.41	1.00
18:2n-6	37.98	49.61	60.52
20:0	0.41	0.41	0.30
20:1n-9	2.28	5.10	2.21
20:2n-6	0.15	0.19	0.12
20:3n-6	0.19	0.05	0.07
22:0	—	0.04	0.05
20:4n-6	0.05	0.17	0.07
20:5n-3	—	0.67	0.26
24:0	0.10	0.28	0.17
24:1n-9	—	0.20	0.06
22:5n-3	—	0.12	—
22:6n-3	0.20	1.38	0.54
Total SFA	25.24	15.21	12.50
Total MUFA	33.78	29.86	24.04
Total PUFA	39.25	52.19	61.58
Total n-6	38.64	50.02	60.78
Total n-3	0.92	2.17	0.80
n-3/n-6	0.02	0.04	0.01
Unsaturation index[a]	1.14	1.42	1.50
Mean chain length[b]	17.08	17.47	17.57
Lipid content (%)	4.5	3.5	9.5
Energetic value[c] (kcal/g)	4.20	3.38	3.74

[a]Unsaturation index = $(\sum m_i \cdot n_i)/100$, where m_i is the mole percentage and n_i is the number of C–C double bonds of the fatty acid *i*.
[b]Mean chain length index = $(\sum m_i \cdot n_i)/100$, where m_i is the mole percentage and n_i is the number of C of the fatty acid *i*.
[c]Energetic value has been calculated according to 4 kcal/g for digestible proteins and carbohydrates and 9 kcal/g for digestible lipids. GO, grapeseed oil; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

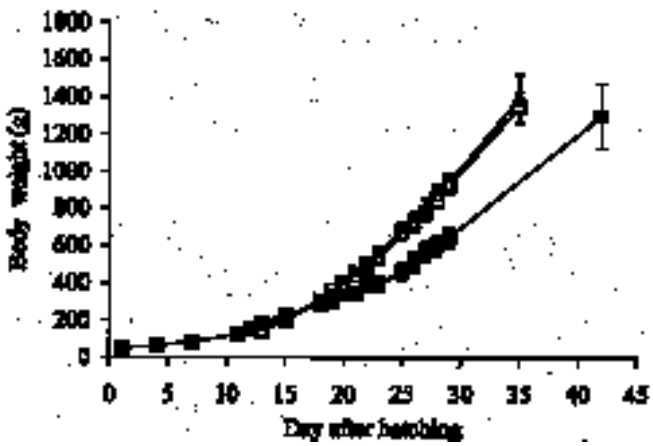

FIG. 1. Effects of grapeseed oil (GO) feeding or cold acclimation on growth rate. GO ducklings reared at thermoneutrality (△) and CA ducklings (■) are compared with thermoneutral controls (□). Values are means ± SE (n = 7).

cle intermyofibrillar mitochondria and liver mitochondria were isolated as described previously (10,11).

Lipid analysis. Tissue and mitochondrial total lipids were extracted according to Folch *et al*. (23). Residual water of the extracts was removed by oil-vacuum. The lipid extracts were weighed, diluted in chloroform, and stored at –20°C until use.

Phospholipids and triglycerides from tissue extracts were separated by thin-layer chromatography on silica gel (G60 plates) using diisopropyl ether as migration solvent. Phospholipids were recovered by scratching the gel and then methylating according to Slover and Lanza (24) before FA analysis.

The FA analysis of phospholipids from mitochondrial extracts was performed with total lipids because the presence of tri- and diglycerides was found negligible, and only traces of cholesterol were detected. The FA of total lipids were therefore assumed to be those of phospholipids. Methylation was performed according to Lepage and Roy (25) using acetylchloride.

FA methyl esters were stored in hexane (high-performance liquid chromatography grade). Gas–liquid chromatography of FA methyl esters was performed using a Chrompack CP9001 chromatograph (Chrompack, Middelburg, The Netherlands) equipped with a 50-m capillary column (CP-Sil 88, 0.25 mm internal diameter) and Chrompack Maestro2 integrator software. The split injector was at 270°C and the flame-ionization detector at 260°C. Temperature program was 150°C for 8 min, rising to 185°C by 10°C/min, 185°C for 10 min, rising to 200°C by 10°C/min, and then 200°C for 20 min. Nitrogen was used as carrier gas (100 kPa). FA were identified by comparison with commercially available standards (Supelco, Bellefonte, PA).

Mitochondrial respiration and enzymatic activities. The respiration of isolated mitochondria (0.5 mg mitochondrial protein/mL) was determined polarographically with a Clark oxygen electrode (oxygraph Gilson 5/6 H), in a glass cell of 1.5 mL volume, thermostated at 25°C as described previously (10,11). The ATP synthesis of mitochondria was determined by the bioluminescence procedure of Wibom *et al*. (26) at 25°C with some modifications as described previously (11). The cytochrome oxidase activity of isolated mitochondria was determined polarographically as described elsewhere (10). As the protein/lipid ratio remained unchanged in all the experiments (data not shown), the specific activity of the enzymes was expressed per mg of protein.

Statistics and chemicals. Data are presented as means ± SE. One-way or two-way analysis of variance (ANOVA) and post-ANOVA Fisher PLSD tests and Student's *t* tests were used to determine significant differences between groups. Statistical difference was accepted at $P < 0.05$.

Solvent of analytical grade for lipids extraction and analysis was from SDS (Peypin, France); the other chemicals were purchased from Sigma.

RESULTS

Experiment 1: Effect of cold acclimation on the FA composition of tissue phospholipids. At the time of killing, TN ducklings were slightly heavier than CA birds (1.43 ± 0.03 vs. 1.20

TABLE 2
Fatty Acid Composition (in %) of Total Phospholipids from Gastrocnemius Muscle and Liver in Thermoneutral (TN) or Cold-Acclimated (CA) Ducklings[a]

	Gastrocnemius muscle		Liver	
	TN	CA	TN	CA
16:0	12.6 ± 0.3	11.9 ± 0.2*	23.6 ± 0.4	21.2 ± 1.3*
16:1n-7	—	—	0.4 ± 0.1	0.7 ± 0.1*
18:0	17.5 ± 0.4	19.3 ± 0.1*	18.4 ± 0.3	19.3 ± 0.4
18:1n-9	19.8 ± 0.7	16.6 ± 0.4*	11.3 ± 0.4	13.6 ± 1.0*
18:1n-7	0.7 ± 0.1	0.5 ± 0.1	1.0 ± 0.1	1.2 ± 0.1
18:2n-6	16.0 ± 0.4	15.5 ± 0.3	8.4 ± 0.6	9.1 ± 1.2
20:3n-6	1.5 ± 0.1	1.1 ± 0.1*	2.4 ± 0.2	2.7 ± 0.5
20:4n-6	11.2 ± 0.4	14.2 ± 0.3*	21.1 ± 0.6	19.3 ± 2.9
22:4n-6	3.9 ± 0.2	4.1 ± 0.1	1.8 ± 0.1	1.3 ± 0.1*
22:5n-6	2.9 ± 0.1	3.1 ± 0.1	3.6 ± 0.2	2.7 ± 0.2*
22:5n-3	1.4 ± 0.1	1.5 ± 0.2	0.5 ± 0.1	0.8 ± 0.1*
22:6n-3	1.5 ± 0.1	2.4 ± 0.2*	1.9 ± 0.1	1.9 ± 0.3
Total SFA	31.9 ± 0.6	33.2 ± 0.2*	42.4 ± 0.3	40.9 ± 0.9
Total MUFA	20.7 ± 0.7	17.3 ± 0.4*	13.9 ± 0.5	16.7 ± 0.9*
Total PUFA	39.4 ± 0.9	43.0 ± 0.3*	42.2 ± 0.4	41.0 ± 1.7
Total n-6	36.0 ± 0.7	38.7 ± 0.2*	37.3 ± 0.6	35.5 ± 1.4
Total n-3	3.3 ± 0.2	4.4 ± 0.1*	3.1 ± 0.3	3.5 ± 0.3
n-3/n-6	0.092 ± 0.004	0.113 ± 0.004*	0.082 ± 0.006	0.097 ± 0.007
Unsaturation index	1.51 ± 0.04	1.66 ± 0.01*	1.70 ± 0.02	1.65 ± 0.09

[a]Values are mean ± SE from six (gastrocnemius) or five (liver) animals kept on the Genthon 5A diet. *$P < 0.05$ vs. TN. Fatty acids representing a small proportion of the total are not shown but values are included when possible in the totals presented. See Table 1 for abbreviations.

± 0.03 kg, $P < 0.05$) despite a lower food intake by 5 wk of age (106 ± 3 vs. 139 ± 4 kcal·kg$^{-0.75}$·d^{-1}). The FA profile of tissue phospholipids (Table 2) differed between the two tissues, the gastrocnemius muscle being poorer in saturated fatty acids (SFA) but richer in monounsaturated fatty acids (MUFA) than the liver. In the gastrocnemius muscle, cold acclimation led to a marked increase in the proportion of total PUFA and to a lesser extent of SFA at the expense of total MUFA. The cold-induced rise in total PUFA was mainly accounted for by 20:4n-6, and to a lesser extent by 22:4n-6 and 22:6n-3. The unsaturation index of total phospholipids was thus higher in skeletal muscle from CA than from TN ducklings. In liver, by contrast, the cold-induced rise in long-chain PUFA was not observed, and there was only a slight increase in the proportion of total MUFA mainly accounted for by 18:1n-9 FA in CA ducklings. The unsaturation index of liver phospholipids was similar between TN and CA ducklings.

Experiment 2: Effect of GO diet or cold on food intake and growth rate. Despite differences in the energy content of the diet, TN and GO ducklings had a similar energy intake by 5 wk of age (441 ± 3 and 439 ± 12 kcal·kg$^{-0.75}$·d^{-1}, respectively) and grew at similar rates (Fig. 1). Again, the energy intake of CA ducklings was higher (496 ± 5 kcal·kg$^{-0.75}$·d^{-1}) and their growth rate lower than those of the ducklings kept at thermoneutrality.

Experiment 2: Effect of GO diet or cold on the FA profile of mitochondrial phospholipids. Whatever the diet or cold-acclimation status, the FA composition of mitochondrial phospholipids differed between tissues (Table 3). As seen in the composition of tissue phospholipids, there were more SFA and less PUFA in the phospholipids of mitochondria isolated from liver than from gastrocnemius muscles. Mitochondrial phospholipids from the red internal part of the gastrocnemius muscle were richer in PUFA than those from the white part. Despite these differences, the unsaturation indexes were similar in the three tissues studied, mainly because of the differences in the pattern of n-6 FA. The content in 20:4n-6 was higher in hepatic than in muscle mitochondria, while the 18:2n-6 was the most abundant FA in muscle mitochondria. The n-3/n-6 ratio was similar in the three tissues.

The mitochondrial FA profile was differentially affected by cold acclimation in liver and skeletal muscles. In both parts of the gastrocnemius muscle, a higher unsaturation index and a longer mean chain length were observed in CA ducklings. These parameters reflected the increased proportion of PUFA (especially 20:4n-6, and to a lesser extent

TABLE 3
Fatty Acid Composition (in %) of Total Phospholipids from Mitochondria of Internal, External Gastrocnemius Muscle, and Liver in TN, CA, and GO-Supplemented Ducklings[a]

	Internal gastrocnemius			External gastrocnemius			Liver		
	TN	CA	GO	TN	CA	GO	TN	CA	GO
14:1n-7	1.2 ± 0.1	1.5 ± 0.3	1.1 ± 0.2	2.6 ± 0.4	2.5 ± 0.4	2.0 ± 0.3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
16:0	10.5 ± 0.3^{a}	8.2 ± 0.2^{b}	8.8 ± 0.2^{b}	12.2 ± 0.4^{a}	9.5 ± 0.3^{b}	10.9 ± 0.4^{c}	19.0 ± 0.8	19.3 ± 0.6	17.9 ± 0.5
16:1n-9	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.03
16:1n-7	0.4 ± 0.0^{a}	0.3 ± 0.0^{b}	0.2 ± 0.0^{c}	0.6 ± 0.1^{a}	0.4 ± 0.0^{b}	0.2 ± 0.0^{c}	0.9 ± 0.1^{a}	0.9 ± 0.1^{a}	0.3 ± 0.0^{b}
18:0	19.1 ± 0.4^{a}	21.7 ± 0.2^{b}	21.5 ± 0.3^{b}	15.1 ± 0.3^{a}	18.4 ± 0.2^{b}	17.2 ± 0.3^{c}	16.2 ± 0.3^{a}	17.0 ± 0.2^{a}	18.9 ± 0.3^{b}
18:1n-9	13.1 ± 0.5^{a}	10.8 ± 0.3^{b}	8.2 ± 0.3^{c}	16.5 ± 0.6^{a}	13.8 ± 0.4^{b}	10.7 ± 0.3^{c}	15.2 ± 0.4^{a}	16.9 ± 0.6^{b}	8.7 ± 0.5^{c}
18:1n-7	3.5 ± 0.1^{a}	3.7 ± 0.1^{a}	2.6 ± 0.1^{b}	3.7 ± 0.1^{a}	4.2 ± 0.1^{b}	2.6 ± 0.1^{c}	1.8 ± 0.1^{a}	1.6 ± 0.1^{a}	1.1 ± 0.1^{b}
18:2n-6	20.2 ± 0.2^{a}	19.4 ± 0.7^{a}	23.9 ± 0.8^{b}	20.7 ± 0.3^{a}	20.1 ± 0.9^{a}	25.7 ± 1.0^{b}	10.1 ± 0.3^{a}	9.0 ± 0.3^{b}	15.2 ± 0.4^{c}
20:0	0.3 ± 0.0^{a}	0.2 ± 0.0^{b}	0.3 ± 0.0^{c}	0.2 ± 0.0^{a,b}	0.2 ± 0.0^{a}	0.3 ± 0.0^{b}	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:1 + 18:3n-3	0.6 ± 0.0^{a}	0.6 ± 0.0^{a}	0.4 ± 0.0^{b}	0.6 ± 0.0^{a}	0.6 ± 0.0^{a}	0.4 ± 0.0^{b}	0.4 ± 0.0^{a}	0.4 ± 0.0^{a}	0.3 ± 0.0^{b}
20:2n-6	0.5 ± 0.0^{a}	0.4 ± 0.0^{b}	1.0 ± 0.0^{c}	0.5 ± 0.0^{a}	0.4 ± 0.0^{a}	1.1 ± 0.1^{b}	0.4 ± 0.0^{a}	0.3 ± 0.0^{a}	1.0 ± 0.1^{b}
22:0	0.8 ± 0.0^{a}	0.6 ± 0.1^{b}	0.5 ± 0.0^{b}	0.9 ± 0.1^{a}	0.7 ± 0.1^{b}	0.6 ± 0.1^{b}	2.1 ± 0.2	1.8 ± 0.2	1.4 ± 0.3
20:4n-6	12.4 ± 0.5^{a}	16.3 ± 0.7^{b}	15.7 ± 0.6^{b}	9.4 ± 0.4^{a}	12.2 ± 0.4^{b}	12.1 ± 0.6^{b}	18.1 ± 0.4^{a}	17.9 ± 0.7^{a}	20.8 ± 0.6^{b}
20:5n-3	1.0 ± 0.1^{a}	0.6 ± 0.0^{b}	0.3 ± 0.0^{c}	1.0 ± 0.0^{a}	0.7 ± 0.0^{b}	0.4 ± 0.0^{c}	0.7 ± 0.1^{a}	0.6 ± 0.1^{a}	0.2 ± 0.0^{b}
22:4n-6	1.9 ± 0.1^{a}	2.5 ± 0.1^{b}	2.8 ± 0.1^{c}	1.8 ± 0.1^{a}	2.5 ± 0.1^{b}	2.9 ± 0.2^{b}	1.4 ± 0.1^{a}	1.5 ± 0.1^{a}	2.2 ± 0.1^{b}
22:5n-6	1.2 ± 0.1^{a}	1.2 ± 0.1^{a}	2.1 ± 0.1^{b}	1.2 ± 0.1^{a}	1.3 ± 0.1^{a}	2.3 ± 0.2^{b}	1.6 ± 0.1^{a}	1.3 ± 0.2^{a}	3.0 ± 0.1^{b}
22:5n-3	1.3 ± 0.1^{a}	1.2 ± 0.1^{a}	1.0 ± 0.1^{b}	1.4 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	0.6 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
22:6n-3	6.0 ± 0.3^{a}	6.5 ± 0.2^{a}	4.7 ± 0.3^{b}	6.3 ± 0.4^{a}	7.0 ± 0.3^{a}	4.9 ± 0.4^{b}	6.4 ± 0.4	6.4 ± 0.4	5.6 ± 0.4
Total SFA	31.0 ± 0.4	31.0 ± 0.2	31.4 ± 0.3	28.6 ± 0.3	29.0 ± 0.2	29.2 ± 0.2	37.8 ± 0.5	38.5 ± 0.4	38.5 ± 0.4
Total MUFA	18.6 ± 0.7^{a}	16.6 ± 0.4^{b}	12.4 ± 0.3^{c}	23.9 ± 0.9^{a}	21.3 ± 0.4^{b}	15.8 ± 0.3^{c}	18.6 ± 0.6^{a}	20.0 ± 0.6^{a}	10.5 ± 0.6^{b}
Total PUFA	44.7 ± 0.6^{a}	48.2 ± 0.5^{b}	51.7 ± 0.3^{c}	42.4 ± 0.8^{a}	45.9 ± 0.4^{b}	50.8 ± 0.8^{c}	39.3 ± 0.7^{a}	37.8 ± 0.9^{a}	48.6 ± 0.8^{b}
Total n-6	34.4 ± 0.5^{a}	37.5 ± 0.5^{b}	42.9 ± 0.3^{c}	31.9 ± 0.4^{a}	34.3 ± 0.5^{b}	41.4 ± 0.5^{c}	30.3 ± 0.4^{a}	28.8 ± 0.6^{a}	40.1 ± 0.6^{b}
Total n-3	8.3 ± 0.4^{a}	8.3 ± 0.2^{a}	6.0 ± 0.3^{b}	8.7 ± 0.5^{a}	9.1 ± 0.4^{a}	6.5 ± 0.5^{b}	7.7 ± 0.4^{a}	7.5 ± 0.3^{a}	6.3 ± 0.4^{b}
n-3/n-6	0.24 ± 0.01^{a}	0.22 ± 0.01^{a}	0.14± 0.01^{b}	0.27 ± 0.02^{a}	0.27 ± 0.01^{a}	0.16 ± 0.01^{b}	0.25 ± 0.0^{a}	0.26 ± 0.01^{a}	0.16 ± 0.01^{b}
Unsaturation index	1.71 ± 0.03^{a}	1.86 ± 0.02^{b}	1.82 ± 0.03^{b}	1.67 ± 0.04^{a}	1.81 ± 0.02^{b}	1.79 ± 0.05^{b}	1.70 ± 0.03^{a}	1.67 ± 0.04^{a}	1.87 ± 0.03^{b}
Mean chain length	18.4 ± 0.0^{a}	18.5 ± 0.0^{b}	18.5 ± 0.0^{b}	18.2 ± 0.1^{a}	18.4 ± 0.0^{b}	18.4 ± 0.1^{b}	18.4 ± 0.0^{a}	18.4 ± 0.0^{a}	18.5 ± 0.1^{b}

[a]Values are means ± SE ($n = 7$). FA representing less than 0.4% are not shown, but are taken into account for the calculation of the totals. Each tissue value with a different roman superscript is significantly different ($P < 0.05$), two-way analysis of variance (ANOVA) and Fisher post-ANOVA test. See Tables 1 and 2 for abbreviations.

22:4n-6). The proportion of 22:6n-3 was not significantly increased in mitochondrial phospholipids. The rise in long-chain PUFA was somehow compensated for by a decrease in the proportion of MUFA (especially 18:1n-9) and an increase in the mean chain length of SFA (rise in 18:0 and drop in 16:0). In liver, by contrast, the rise in long-chain PUFA was not observed, and cold acclimation induced only minor changes in mitochondrial FA profile, i.e., a slight increase in 18:1, compensated for by a decrease in 18:2n-6. The n-3/n-6 ratio was not affected by cold acclimation in the three tissues.

Contrary to cold acclimation, the GO diet at thermoneutrality affected the three tissues studied similarly and induced a nonspecific increase in the proportion of all n-6 phospholipid FA. In muscle mitochondria, these increases were at least up to the levels observed in CA ducklings. Consequently, in muscle mitochondria of GO ducklings, the mean chain length and the unsaturation index reached the values observed in CA ducklings, while in hepatic mitochondria, these parameters were higher than in both TN and CA ducklings. In the three tissues of GO ducklings, the increased proportion of n-6 FA was compensated for by a decreased proportion of MUFA and the n-3/n-6 ratio was markedly decreased.

Experiment 2: Effect of cold acclimation or GO diet on the biochemical characteristics of isolated mitochondria. Cold acclimation affected the functional characteristics of skeletal muscle mitochondria (Table 4), especially in the red part of gastrocnemius, but not those of liver mitochondria (Table 5). In both parts of the gastrocnemius muscle, there was a higher State 4 respiratory rate, and consequently a lower respiratory control ratio (RCR) in CA than in TN ducklings. The ADP/O ratio was, however, not affected by cold acclimation. The specific (per mg mitochondrial protein) ATP synthesis was higher in muscle mitochondria from CA ducklings, but the specific cytochrome oxidase activity was unaffected. Because of a higher amount of mitochondrial proteins in skeletal muscles of CA ducklings, the total oxidative capacity and ATP production capacity per organ were increased by cold acclimation in both skeletal muscles.

The GO diet affected the functional characteristics of hepatic and skeletal muscle mitochondria differently. In liver, the GO diet led to a higher ADP/O ratio and a higher RCR due to a lower respiratory State 4 as compared with ducklings fed the standard diet. The other parameters remained unchanged. In both parts of the gastrocnemius muscle, the GO diet induced a lower RCR due to a higher respiratory State 4 than in control TN ducklings. The specific ATP synthesis activity was intermediate between that of TN and CA ducklings, but because of the increased mitochondrial amount, the total ATP production per gram of muscle reached the level observed after cold acclimation. Taken together, present results

TABLE 4
Respiratory and Functional Parameters of Mitochondria from Internal and External Gastrocnemius Muscle in TN, CA, and GO-Supplemented Ducklings[a]

	Internal gastrocnemius[b]			External gastrocnemius[b]			P^c		
	TN	CA	GO	TN	CA	GO	Effect of treatment	Effect of muscle type	Interaction treatment × muscle type
State 4	23.0 ± 1.8	26.8 ± 1.0	26.7 ± 1.9	21.8 ± 1.0	26.4 ± 1.6	25.0 ± 1.0	<0.05$^{\$,£}$	NS	NS
State 3	116.5 ± 10.0	116.9 ± 3.1	122.3 ± 4.9	121.8 ± 6.2	133.6 ± 6.2	128.1 ± 5.2	ns	NS	NS
RCR	5.05 ± 0.14	4.38 ± 0.15	4.63 ± 0.17	5.62 ± 0.31	5.12 ± 0.27	5.14 ± 0.17	<0.05$^{\$,£}$	<0.01	NS
ADP/O	1.19 ± 0.04	1.15 ± 0.04	1.26 ± 0.02	1.24 ± 0.03	1.30 ± 0.05	1.31 ± 0.02	NS	<0.01	NS
Cytochrome-oxidase specific activity	0.88 ± 0.02	0.88 ± 0.03	0.85 ± 0.02	0.80 ± 0.03	0.80 ± 0.03	0.81 ± 0.03	NS	<0.05	NS
Total cytochrome oxidase activity	53.2 ± 2.5	64.8 ± 6.6	63.9 ± 4.3	34.5 ± 2.5	42.5 ± 3.2	38.3 ± 2.6	<0.05$^{\$,£}$	<0.0001	NS
Mitochondrial proteins	60.8 ± 2.9	74.06 ± 7.1	75.5 ± 4.5	43.2 ± 3.1	53.1 ± 3.4	48.0 ± 4.4	<0.05$^{\$,£}$	<0.0001	NS
Specific ATP-synthesis	66.7 ± 6.5	85.2 ± 2.4	78.9 ± 5.6	84.1 ± 7.5	100.9 ± 8.6	92.2 ± 4.5	<0.05$^{\$}$	<0.01	NS
Total ATP production	4.16 ± 0.56	6.58 ± 0.86	6.00 ± 0.60	3.57 ± 0.34	5.42 ± 0.50	4.47 ± 0.51	<0.01$^{\$,£}$	<0.05	NS

[a]The respiratory reaction medium contained 200 mM sucrose, 5 mM KH_2PO_4, and 20 mM Tris-HCl, pH 7.4 with a final fatty-acid-free bovine serum albumin concentration of 2 mg/mL (0.2% wt/vol). The controlled state of respiration (State 4) was initiated by the addition of 5 mM succinate (sodium salt) in the presence of rotenone (5 μM) and the active state of respiration (state 3) was initiated by the addition of 100 μM ADP. The method of Estabrook (44) was used for the calculation of State 4 and state 3 respiration and the respiratory control ratio (RCR). The latter respiratory parameter is a measure of the degree of control imposed on oxidation by phosphorylation. ADP/O ratio was calculated by using the total oxygen consumed during phosphorylation of a pulse of ADP added to initiate state 3 respiration.

[b]Values are means ± SE. State 4 and State 3 in nmol O/min/mg protein, ADP/O in nmol ADP/nmol O, cytochrome-oxidase specific activity in μmol O/min/mg protein, total cytochrome-oxidase activity in μmol O/min/g muscle, mitochondrial proteins in mg protein/g muscle, specific ATP-synthesis in pmol ATP/min/μg protein, and total ATP production in μmol ATP/min/g muscle.

[c]Values of two-way ANOVA. [$]Significant difference between CA and TN; [£]significant difference between GO and TN; NS, nonsignificant ($P > 0.05$). See Tables 1, 2, and 3 for other abbreviations.

TABLE 5
Respiratory and Functional Parameters of Mitochondria Liver in TN, CA, and GO-Supplemented Ducklings[a]

	Liver		
	TN	CA	GO
State 4	11.4 ± 1.0[a,b]	12.3 ± 0.7[a]	9.0 ± 0.9[b]
State 3	51.3 ± 4.9	53.5 ± 2.5	48.4 ± 3.4
RCR	4.49 ± 0.28[a]	4.37 ± 0.09[a]	5.47 ± 0.33[b]
ADP/O	1.39 ± 0.02[a]	1.44 ± 0.02[a]	1.52 ± 0.02[b]
Cytochrome-oxidase specific activity	0.42 ± 0.04	0.39 ± 0.02	0.31 ± 0.04
Total cytochrome-oxidase activity	81.1 ± 8.3	85.5 ± 4.9	66.9 ± 5.3
Mitochondrial proteins	195.2 ± 19.2	217.4 ± 6.4	222.3 ± 14.3
Specific ATP-synthesis	29.4 ± 3.0	31.6 ± 4.0	27.5 ± 3.0
Total ATP production	5.67 ± 0.69	6.84 ± 0.82	5.31 ± 0.61

[a]Values are means ± SE (n = 7). State 4 and State 3 in nmol O/min/mg protein, ADP/O in nmol ADP/nmol O, cytochrome-oxidase specific activity in µmol O/min/mg protein, total cytochrome-oxidase activity in µmol O/min/g muscle, mitochondrial proteins in mg protein/g muscle, specific ATP-synthesis in pmol ATP/min/µg protein and total ATP production in µmol ATP/min/g muscle. Each tissue means with a different roman superscript is significantly different ($P < 0.05$), one-way ANOVA and Fisher post-ANOVA test. See Tables 1, 2, and 3 for abbreviations.

showed that in skeletal muscles, but not in liver, the GO diet at thermoneutrality globally mimicked the changes in the mitochondrial lipid composition and biochemical parameters induced by cold acclimation.

DISCUSSION

Two main results emerged from the present study. First, CA ducklings showed alterations in the lipid composition of skeletal muscle membranes leading to an increased proportion of long-chain PUFA. Second, the enrichment of mitochondrial phospholipids with n-6 PUFA, created by giving ducklings kept at thermoneutrality a diet rich in GO, partly reproduced the alterations in the functional properties of skeletal muscle mitochondria observed in CA ducklings.

Present results clearly show that cold acclimation altered the FA composition of tissue and mitochondrial phospholipids in the gastrocnemius muscle. Indeed, there was a higher proportion of the longer and more unsaturated FA (mainly 20:4n-6) in tissue and mitochondrial phospholipids from gastrocnemius muscle of CA than in those of TN ducklings resulting in increased (+10%) unsaturation index and mean chain length of membrane FA. By contrast, these changes in FA profile were not observed in liver mitochondria. We do not favor the possibility that the difference between liver and skeletal muscle is related to the fact that these tissues experience different temperatures during cold acclimation. Although living at low temperature indeed results in increased unsaturation in ectotherms (see Ref. 27 for a recent review), the difference between liver and skeletal muscle temperature is likely to be small in endothermic ducklings and would not exceed a few degrees. This is far less than the thermal challenge used to induce phospholipid changes in ectotherms. A similar enrichment in PUFA resulting in an increase in the total FA unsaturation index of mitochondrial phospholipids has been described in the thermogenic BAT of rodents (19). By contrast, there was a decrease in the proportion of total unsaturated FA in heart mitochondria from CA rats (28), mainly resulting from a rise in the proportion of 18:0 and a decrease in that of 18:1. No marked changes in the unsaturation index of phospholipid FA have been reported in hepatic mitochondria of CA rodents (29). These results therefore indicate a tissue-specific modulation of the FA composition of membrane phospholipids toward an enrichment in long-chain PUFA in the thermogenic organs (BAT or skeletal muscles) of endotherms. Such enrichment in long-chain PUFA in mitochondrial membranes of CA ducklings suggests specific roles of these FA in the biophysical properties of these membranes. It is indeed generally accepted that changes in FA profile of biological membranes alter the fluidity, leakiness to protons, and may contribute to the modulation of the activity of membrane-bound enzymes (30–33).

The FA composition of mitochondrial phospholipids could be altered by giving ducklings kept at thermoneutrality a diet rich in GO, providing more n-6 PUFA. This diet composition, rich in 18:2n-6, was chosen in order to provide ducklings with the FA that were the most affected by cold acclimation as shown in the first experiment with total tissue phospholipids (Table 2). Results of experiment 2 indicate that in birds, as in mammals (21), the mitochondrial membrane FA composition can be manipulated by the FA composition of the diet. However, the enrichment in membrane PUFA is lower than what could be expected from the diet composition, indicating some homeostatic control of the membrane lipid composition already noted by others (34). Because the diet similarly affected the FA profile of mitochondrial phospholipids of the three tissues investigated, it follows that the tissue-specific changes observed after cold acclimation are precisely regulated and, for instance, are not dependent on the higher food intake or alterations in the supply of blood lipids (35). These changes may therefore reflect active processes controlling the incorporation of specific long-chain highly unsaturated FA into

membranes for particular metabolic and thermogenic purposes. Thyroid hormones are good candidates for the modulation of phospholipid composition in CA ducklings. Indeed, they are known to increase 20:4n-6 and decrease 18:2n-6 (36,37) and concomitantly increase the unsaturation index (38). Furthermore, they are suspected to play a role in the development of NST in birds (4,5).

Cold acclimation as well as GO feeding, both leading to an enrichment of mitochondrial phospholipids in n-6 FA, similarly altered the functional properties of isolated skeletal muscle mitochondria. In both cases, there was an increase in State 4 respiration rates and a decrease in respiratory control, indicating a lower degree of control imposed on oxidation by the phosphorylation. Similar loose-coupling of muscle mitochondria in CA ducklings has already been reported (11,22) and has been related to an increase in mitochondrial membrane conductance to protons (11). The present results support the hypothesis that the FA composition of ducklings' mitochondrial membranes may contribute, at least in part, to an increased proton leak across mitochondrial inner membrane. In mammals as well, the degree of proton leak is correlated with the composition in unsaturated FA (16,17) and differences in nonphosphorylating respiratory rates of isolated mitochondria from various species are related to mitochondrial phospholipid composition (18). However, the PUFA composition of mitochondrial membranes may not be the only factor responsible for the altered coupling of muscle mitochondria as similar changes in FA composition induced by the GO diet were observed in liver mitochondria, while there were opposite changes in mitochondrial characteristics (decreased State 4, increased RCR). This difference is difficult to interpret, but may possibly be related to the muscle-specific expression of mitochondrial membrane proteins interacting with membrane phospholipids and modulating membrane proton leakiness. This hypothesis is based on the recent description of an uncoupling protein 3 expressed at high levels in mammalian skeletal muscles and BAT, but not in hepatocytes (39,40) although the presence of a similar protein in duckling muscle is still not demonstrated. It is also based on the observation that reconstituted liposomes of differing fatty acid composition do not exhibit differences in proton leak (41), while in intact mitochondria, differences in FA composition affect membrane proton leakiness (17). It is therefore tentatively postulated that a muscle-specific avian uncoupling protein, the protonophoric activity of which may be potentiated by the FA composition of the mitochondrial membrane, contributes to the functional properties of muscle mitochondria induced by either cold acclimation or the GO diet. More studies are required to confirm the existence and clarify the regulatory activity of such protein.

On an other hand, the lipid composition of mitochondrial membranes may be important in modulating the activity of membrane-bound enzymes, and the enrichment in PUFA induced by cold acclimation was indeed related to an increase of the ATP synthesis in skeletal muscles. The ATPase complex activity was already shown to be regulated by the chain length and the degree of unsaturation of membrane FA (42). The GO diet had less clear-cut effects on the ATP synthesis, despite marked changes in membrane PUFA, possibly because the proportion of the 18:2n-6 was also markedly increased. In rats, the ATP-synthase activity was indeed shown to be inversely related to the content in 18:2n-6 (43). A specific role of the 20:4n-6 or longer n-6 FA in modulating these activities remains to be clarified.

In conclusion, this study showed that in CA young birds, there is a muscle-specific enrichment of mitochondrial phospholipids in long and unsaturated FA. Similar changes do not occur in liver mitochondria. Feeding a GO diet rich in n-6 PUFA induced similar changes in lipid composition and mitochondrial functional activity in skeletal muscles as did cold acclimation. It is suggested that the FA composition of mitochondrial membranes may contribute to the increased proton leakiness of muscle mitochondria observed in CA ducklings, but that muscle-specific factors control this effect.

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