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HISTOCHEMICAL ARGUMENTS FOR MUSCULAR NON-SHIVERING THERMOGENESIS IN MUSCOVY DUCKLINGS

BY CLAUDE DUCHAMP, FRÉDÉRIQUE COHEN-ADAD, JEAN-LOUIS ROUANET AND HERVÉ BARRÉ

From the Laboratoire de Thermorégulation et Energétique de l'Exercice, URA 1341 CNRS, 8 avenue Rockefeller, F-69373 Lyon cédex 08, and the Laboratoire de Physiologie Générale et Comparée, Université Claude Bernard Lyon I, 43 Boulevard du 11 Novembre 1918, F-69622, Villeurbanne, France

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SUMMARY

1. The histochemical characteristics of gastrocnemius muscle were investigated in 6-week-old cold-acclimated (5 weeks, 4 °C) and glucagon-treated (5 weeks, 25 °C, 103 nmol/kg I.P. twice daily) muscovy ducklings, two groups able to develop non-shivering thermogenesis *in vivo*. A comparison was made with thermoneutral controls (25 °C) of the same age. All animals were fed *ad libitum*. Fibre type, fibre area and capillary supply have been studied. Further, a quantitative histochemical method for mitochondrial Mg^{2+}-ATPase activity was developed to characterize the mitochondrial coupling state *in situ*.

2. White gastrocnemius was composed of fast glycolytic (FG) and fast oxidative glycolytic (FOG) fibres, while red gastrocnemius contained FOG and slow oxidative (SO) fibres. In white gastrocnemius, the proportion of FG fibres was higher in glucagon-treated than in control or cold-acclimated ducklings. In red gastrocnemius, the proportion of SO fibres was higher in both cold-acclimated and glucagon-treated ducklings than in controls. The area of all fibres was generally lower in glucagon-treated than in other ducklings.

3. The capillary density was higher in both red and white components of the gastrocnemius muscle in cold-acclimated and glucagon-treated than in control ducklings, as a result of an increased number of capillaries around each fibre.

4. In all fibres, except the FG type in cold-acclimated ducklings, the staining intensity of the Mg^{2+}-ATPase reaction was higher in cold-acclimated and glucagon-treated than in control ducklings whereas the staining intensity with maximal decoupling of oxidative phosphorylation by dinitrophenol was unchanged. This indicated a more loose-coupled state of mitochondria *in situ* in all fibres of cold-acclimated ducklings, and in FOG fibres of white gastrocnemius and SO fibres of red gastrocnemius in glucagon-treated ducklings.

Address for reprint requests: Dr C. Duchamp, Laboratoire de Thermorégulation et Energétique de l'Exercice, URA 1341 CNRS, Faculté de Médecine, 8 avenue Rockefeller, F-69373, Lyon cedex 08, France.

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5. These results indicated a higher oxidative metabolism of skeletal muscle in both cold-acclimated and glucagon-treated than in control ducklings, and for most of the parameters studied, a similarity between cold acclimation and glucagon treatment. Because of the higher loose-coupled state of muscle mitochondria in cold-acclimated and glucagon-treated than in control ducklings, the higher oxidative capacity of skeletal muscle in these ducklings could be used for heat production rather than ATP synthesis and account for muscular non-shivering thermogenesis.

INTRODUCTION

Although it was long believed that non-shivering thermogenesis (NST) was absent in birds, this mechanism has been reported in cold-acclimated ducklings (Barré, Cohen-Adad, Duchamp & Rouanet, 1986*a*). Non-shivering thermogenesis can also be induced by a glucagon injection in chronic glucagon-treated ducklings reared at thermoneutrality (Barré, Cohen-Adad & Rouanet, 1987*a*), or by cold acclimatization in king penguin chicks (Duchamp, Barré, Delage, Rouanet, Cohen-Adad & Minaire, 1989). In each of these studies, skeletal muscle appeared to be the main site of NST in these birds devoid of thermogenic brown adipose tissue (Barré *et al.* 1986*a*). Skeletal muscle has additional oxidative capacity after acclimation to cold (Barré, Bailly & Rouanet, 1987*b*) or chronic glucagon treatment (Barré, Berne, Brebion, Cohen-Adad & Rouanet, 1989). However, it is not known whether this oxidative adaptation of muscle in birds is based on changes in fibre size, fibre-type distribution, or enzymatic content. Further, to be effective this higher oxidative capacity should require an increase in the oxygen supply capacity to muscle (Conley, Kayar, Rösler, Hoppeler, Weibel & Taylor, 1987), but this point has not been investigated in cold-acclimated birds.

In mammals, NST in brown adipose tissue is based on an increased respiration of uncoupled mitochondria because of the presence of the uncoupling protein (Nicholls & Locke, 1984). In birds, loose-coupled muscle mitochondria have also been implicated in NST (Barré, Nedergaard & Cannon, 1986*c*; Barré *et al.* 1989). However, in the absence of the uncoupling protein in muscles (Ricquier, Barlet, Garel, Combes-George & Dubois, 1983), it is questionable whether the loose coupling of isolated muscle mitochondria is functionally relevant *in situ* or if it results from the mitochondrial isolation procedures.

In order to answer these questions, we investigated in ducklings whether muscle adaptation in response to cold acclimation or chronic glucagon treatment involved a change in fibre-type distribution and muscle capillarization. In addition, we developed a quantitative histochemical characterization of the coupling state of skeletal muscle mitochondria *in situ* derived from that of Meijer & Vloedman (1980), enabling us to avoid the artifacts inherent in mitochondrial isolation procedures.

The present results showed, in cold-acclimated and glucagon-treated ducklings, an increase in muscle oxidative metabolism underlined by a change both in fibre-type distribution and capillary supply. This increased oxidative metabolism could be related to NST since muscle mitochondria *in situ* were found to be more loosely coupled in cold-acclimated and glucagon-treated than in thermoneutral control ducklings.

METHODS

Animals

Thirty male muscovy ducklings (*Cairina moschata* L, pedigree R31, Institut National de la Recherche Agronomique, France) obtained from a commercial stockbreeder (Ets Grimaud, France) were used. They were fed a commercial mash (Sanders 5061) *ad libitum* and had free access to water. The cold-acclimation schedule described by Barré *et al.* (1986*a*) was used: from the age of 1 week, ducklings were caged in groups of ten for a period of 5 weeks at either 4 or 25 °C ambient temperature. The glucagon treatment schedule described by Barré *et al.* (1987*a*) was used. From the age of 1 week, ducklings were caged in groups of ten for a period of 5 weeks at 25 °C ambient temperature and received twice daily an intraperitoneal injection of glucagon (103 nmol/kg). Ducklings were kept in a constant photoperiod (light/dark: 8/16 h). At the age of 6 weeks, ducklings were killed by decapitation and the morphological and functional properties of muscle were then examined histochemically. To assess whether the changes observed resulted from the experimental treatments or from growth, a fibre-type determination was also performed on 1-week-old ducklings.

Procedures

Immediately after the ducklings were killed, the gastrocnemius muscle was removed and weighed. The medial superficial part (just below the skin, white gastrocnemius) and the medial deep part (tibialis side, red gastrocnemius) of the gastrocnemius muscle were sampled at the same point of the muscle, and quickly frozen in liquid nitrogen. Muscle samples were kept frozen at −80 °C until histochemical analysis, except for the mitochondrial Mg^{2+}-ATPase study which was performed immediately after the nitrogen freezing. Serial 10 μm thin sections were cut at −30 °C in a cryostat. The plane of sections was at right angles to the longitudinal axis of the fibres. Muscle sections were stained for myofibrillar ATPase (mATPase) after acid (pH 4·4) or alkaline (pH 9·4) pre-incubation using the method of Guth & Samaha (1970), for ATPase to visualize capillaries using the method of Rosenblatt, Kuzon, Plyley, Pynn & McKee (1987), or for NADH-tetrazolium reductase (NADH-TR) according to Novikoff, Skin & Drucker (1961) to determine the oxidative state of the fibre (an activity closely related to the NADH dehydrogenase of the mitochondrial respiratory chain). Muscle fibres were characterized as slow oxidative (SO), fast oxidative glycolytic (FOG) and fast glycolytic (FG) fibres.

Fibre-type distribution

Distribution of fibre type was expressed as the number of fibres of each type relative to the total number of fibres per unit field. Measurements were made on at least 250 fibres in each section.

Cross-sectional areas

The areas were calculated by use of a computerized planimetry system coupled to a digitizer as described previously by Desplanches, Mayet, Sempore & Flandrois (1987). Mean areas were measured in thirty fibres of each type in each section.

Capillarization

Capillarization was expressed according to Andersen & Henrikson (1977) as capillary density (capillaries/mm^2 = number of capillaries in A divided by area of A). The number of capillaries in area A included half the number of capillaries on the borderline of A. Capillarization was determined on at least 300 capillaries in each section.

Capillarization was also expressed as capillaries per fibre (number of capillaries in A divided by number of fibres in A) and as capillaries in contact (number of capillaries in contact with each fibre type in A), and was determined on at least thirty fibres of each fibre type per section.

Histochemical detection of coupling state

The histochemical detection of mitochondrial coupling state in skeletal muscle is based on the *in situ* experimental demonstration of mitochondrial Mg^{2+}-stimulated ATPase described by Meijer & Vloedman (1980). The chemiosmotic-coupling hypothesis of Mitchell (1975) is the most widely accepted hypothesis to explain the mechanism by which energy produced by the oxida-

tion–reduction reactions of the electron transport chain is coupled to ATP synthesis. According to this hypothesis, the mitochondrial Mg^{2+}-ATPase is the terminal enzyme in oxidative phosphorylation and catalyses the reversible reaction: $ATP^{4-} + H_2O \leftrightarrow ADP^{3-} + HPO_4^{2-} + H^+$.

In our experimental conditions *in vitro*, this reaction was utilized in this sense of ATP degradation, but in mitochondria *in vivo*, this reaction functions in the sense of ATP synthesis in the presence of ADP, inorganic phosphate and a proton gradient. From the Mitchell hypothesis, it follows that the activity of the mitochondrial Mg^{2+}-ATPase is connected to and limited by the respiratory control ratio (RCR), i.e. the coupling state between oxidation and phosphorylation. Therefore dinitrophenol (DNP), the classical uncoupler of oxidative phosphorylation which induces a reduction in RCR, stimulates latent ATPase activity (Katyare, Fatterpaker & Sreenivasan, 1971). As reviewed by Meijer & Vloedman (1980), in cases of muscle defect in the oxidative phosphorylation there is a good correlation between biochemical studies with isolated mitochondria and histochemical studies concerning determination of Mg^{2+}-ATPase, i.e. a high level of staining in the ATPase reaction and little or no stimulation of ATPase by DNP.

After fixation (formaldehyde 4%), serial muscle sections (10 μm) were incubated for 50 min at 38 °C with ATP (0·91 mM) in a 0·08 M Tris-maleate buffer (pH 7·2), containing 3·6 mM $(NO_3)_2Pb$ and 5 mM $MgCl_2$. When the enzyme is active, Pb^{2+} ions substitute themselves for Mg^{2+} on the enzyme, and are thereafter revealed by $(NH_4)_2S$. The intensity of staining therefore reflects the enzymatic activity. Maximal decoupling state was obtained with DNP (10^{-3} M) addition in the incubation medium. In addition, oligomycin, a potent inhibitor of the Mg^{2+}-ATPase (Lardy, Johnson & McMurray, 1958) completely inhibited the histochemical reaction. Total inhibition of mitochondrial Mg^{2+}-ATPase was performed with oligomycin (0·02 mg/ml) in addition to DNP. The specificity of the reaction was tested with addition of ouabain (10^{-3} M), a specific inhibitor of Na^+–K^+-ATPase, to the incubation medium. A low Mg^{2+}-ATPase activity associated with a high DNP stimulation indicates tightly coupled mitochondria, whereas high ATPase activity associated with a low DNP stimulation shows loosely coupled mitochondria (Meijer, 1981; Meijer & Vloedman, 1989). As reviewed by Meijer & Vloedman (1980), the activity of monovalent cation transporting, anion transporting, sarcoplasmic reticular Ca^{2+} transporting and myosin ATPases cannot be demonstrated in this histological system.

The histochemical procedure was performed immediately after the sampling and freezing of the muscle, and enzymatic quantification in the 24 h following staining, because the staining intensity depends on the duration of storage of the frozen muscle specimens (Meijer & Vloedman, 1980) and because the staining is labile. As a progressive degradation of the mitochondrial coupling state may explain these results, a standardized method was used from one group to the next. The staining of the histochemical reaction, which is related to the enzymatic activity (Meijer & Vloedman, 1980), was quantified by the mean optical density of the fibre cross-section with an image analysis system (SAMBA 2002 TITN, France) connected to a Zeiss axioplan microscope using a 10 × planachromat objective via a CCD camera. Monochromatic light was obtained through an interference filter (lenda 469 nm, Schott, Glaswerke). Fibre types were identified according to their different areas in both portions of the gastrocnemius muscle by comparison with the classical fibre-type determination performed in parallel. In addition, the more aerobic fibres appeared denser than the less aerobic ones. The non-specific staining corresponding to optical density (OD) of the section with total inhibition of the Mg^{2+}-ATPase reaction by oligomycin, was subtracted from all OD values. After that, OD of the Mg^{2+}-ATPase reaction in one fibre type was expressed as percentage of maximal OD of the reaction after stimulation by DNP. Histochemical quantification was carried out on at least thirty fibres of each type from three to four different cross-sections of the same muscle specimen to take the variations of section thickness into account.

In order to verify experimentally the proportionality between staining intensity and enzymatic activity and to characterize this relationship, we explored the influence of the enzyme activity by changing the quantity of enzyme in the section. Therefore, we measured the OD of various fibre types on serial sections with a thickness ranging from 2 to 16 μm.

Statistics

Two-way analysis of variance was used in some cases. Statistical significance of the difference between means was assessed by the multiple comparison Peritz F test. Differences between values from the same group were assessed by Student's paired t tests. Goodness of fit was assessed by analysis of variance. Data are presented as means ± S.E.M.

RESULTS

Animal and organ mass (Table 1)

Thermoneutral control and cold-acclimated ducklings had the same body mass whereas glucagon-treated ducklings were lighter ($P < 0{\cdot}05$). As the resting metabolic rate was higher in cold-acclimated than in control ducklings (Barré *et al.* 1986*a*) while that of glucagon-treated ducklings did not differ from that of controls (Barré *et al.* 1987*a*), these results suggested an increased food intake in the cold and a decreased food intake during the glucagon treatment by comparison with control ducklings. The total mass of gastrocnemius muscle and its relative proportion to body mass were the same in control and cold-acclimated ducklings while the mass and even the relative proportion to body mass of this muscle were lower in glucagon-treated than in control and cold-acclimated ducklings (Table 1). In addition, the percentage of red gastrocnemius was slightly higher in cold-acclimated and glucagon-treated than in control ducklings ($P < 0{\cdot}05$), while the percentage of white gastrocnemius was concomitantly lower. At the age of 1 week, the higher proportion of the red compared with the white gastrocnemius was already present (53 ± 1 *vs.* 47 ± 1 %), but was not different from that found in 6-week-old control ducklings. Therefore, growth in the cold and growth under glucagon treatment were marked by an increased proportion of the red part of the gastrocnemius muscle.

Muscle fibre type

According to the staining procedure used, the gastrocnemius is a mixed muscle. Two fibre types were found in each part of the muscle. White gastrocnemius consisted of fast oxidative glycolytic (FOG) and fast glycolytic (FG) fibres, whereas red gastrocnemius consisted of FOG and slow oxidative (SO) fibres (Fig. 1). Fast fibres were characterized by their alkali-stable, acid-labile mATPase activity and divided into FOG and FG fibres according to their slightly different acid lability. This was confirmed by their NADH-TR activity, which was higher in FOG than in FG fibres. In white gastrocnemius, concomitant determinations of fibre-type distribution by mATPase or the NADH-TR activity method gave the same results. Slow fibres were characterized by their acid-stable and alkali-labile mATPase activity (Fig. 1). In red gastrocnemius, NADH-TR activity was higher in SO than in FOG fibres but the difference did not allow a precise estimation of the fibre proportions, as did the difference between the FOG and FG fibres.

In white gastrocnemius muscle, FG fibres had a larger cross-sectional area than the FOG fibres in all groups of animals, while in red gastrocnemius, FOG fibres had a larger area than the SO type (Table 2). In white gastrocnemius, the area of each fibre type was not altered by cold acclimation but was considerably reduced ($P < 0{\cdot}05$) by the glucagon treatment. Structural modifications of muscle were induced by each of the experimental procedures with a higher ($P < 0{\cdot}05$) percentage distribution of FG fibres and a concomitantly lower ($P < 0{\cdot}05$) percentage distribution of FOG fibres in glucagon-treated than in control ducklings and even higher ($P < 0{\cdot}05$) than in cold-acclimated ducklings. The variations of fibre-type distribution in cold-acclimated *vs.* control ducklings, although non-significant, were inversed by contrast with those of glucagon-treated ducklings and tended towards a higher proportion of oxidative

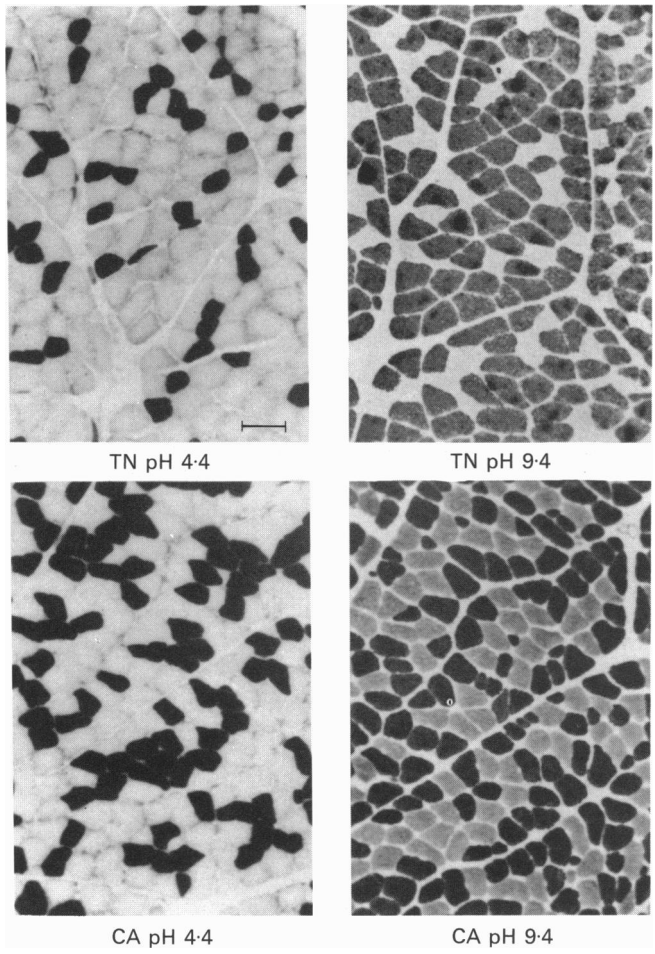

Fig. 1. Serial cross-sections of one thermoneutral (TN) and one cold-acclimated (CA) duckling red gastrocnemius muscle, stained for mATPase, after pre-incubation at pH 4·4 or 9·4. In the control duckling, there is a predominance of FOG fibres, while in the cold-acclimated duckling, there is a significant increase in the percentage of SO fibres. Scale bar 100 μm.

TABLE 1. Body and organ masses of 6-week- and 1-week-old ducklings

	Six weeks old			
	Thermoneutral (8)	Cold-acclimated (8)	Glucagon-treated (8)	One week old (6)
Body mass (kg)	1·69 ± 0·07	1·73 ± 0·04	1·31 ± 0·07*†	0·13 ± 0·01
Gastrocnemius mass (g)	13·56 ± 1·04	14·56 ± 0·70	8·64 ± 0·43*†	0·71 ± 0·03
Gastrocnemius/body mass (%)	0·80 ± 0·03	0·84 ± 0·03	0·66 ± 0·02*†	0·55 ± 0·2
Red gastrocnemius (%)	56 ± 1 (13)	60 ± 1* (14)	61 ± 1* (4)	53 ± 1 (6)

Values are means ± S.E.M. (n) is given in each group. The percentage of red gastrocnemius was calculated from additional ducklings. *$P < 0·05$ *vs.* thermoneutral controls; †$P < 0·05$ *vs.* cold-acclimated ducklings.

TABLE 2. Histochemical characteristics of white and red gastrocnemius muscle in ducklings

	White gastrocnemius			Red gastrocnemius		
	Thermoneutral controls (7)	Cold acclimated (8)	Glucagon treated (8)	Thermoneutral controls (8)	Cold acclimated (7)	Glucagon treated (7)
Fibre type (%)						
FG	66·1 ± 1·7	60·2 ± 2·2	75·5 ± 1·0*†			
FOG	33·9 ± 1·7	39·8 ± 2·2	24·5 ± 1·0*†	66·6 ± 3·6	52·6 ± 3·2*	47·9 ± 5·6*
SO				33·4 ± 3·6	47·4 ± 3·2*	52·1 ± 5·6*
Fibre area (μm^2)						
FG	4662 ± 307	4476 ± 291	2476 ± 186*†			
FOG	1771 ± 174	1754 ± 217	1043 ± 69*†	2603 ± 225	2959 ± 224	1792 ± 172*†
SO				1540 ± 187	2162 ± 181*	1586 ± 80†
Capillary density (mm^{-2})	731 ± 64	899 ± 106	1336 ± 73*†	972 ± 66	1374 ± 174*	1798 ± 124*
Capillary/fibre	2·2 ± 0·1	2·5 ± 0·2	2·9 ± 0·3	2·0 ± 0·1	3·4 ± 0·1*	2·8 ± 0·1*
No. capillaries around a fibre						
FG	6·1 ± 0·1	8·3 ± 0·2*	6·8 ± 0·3†			
FOG	4·8 ± 0·2	5·7 ± 0·2*	4·9 ± 0·2†	5·7 ± 0·3	6·7 ± 0·1*	6·5 ± 0·5*
SO				5·3 ± 0·3	6·8 ± 0·3*	6·9 ± 0·2*

Values are means ± S.E.M. (n). *$P < 0.05$ *vs.* thermoneutral controls; †$P < 0.05$ *vs.* cold-acclimated ducklings.

fibres. In this part of the gastrocnemius muscle, cold-acclimation and glucagon treatment thus appeared to have opposite effects.

In red gastrocnemius, the area of SO fibres was higher in cold-acclimated than in control ducklings, whereas the areas of FOG fibres were not significantly different between the two groups. By contrast, in glucagon-treated ducklings the areas of both

TABLE 3. Histochemical characteristics of white and red gastrocnemius muscle in 1-week-old ducklings

	Gastrocnemius	
	White	Red
Fibre type (%)		
FG	60·8 ± 2·1	—
FOG	39·2 ± 2·2	42·2 ± 4·1
SO	—	57·8 ± 4·1
Fibre area (μm^2)		
FG	—	
FOG	—	658 ± 27
SO	—	392 ± 17

Values are means ± S.E.M. ($n = 5$). The fibre typing in white gastrocnemius muscle was carried out according to the NADH-TR reaction, because FG and FOG fibres could not be clearly distinguished at pH 4·4. Accordingly, the area of these fibres could not be precisely measured, but was in the range of the FOG fibre area in the red part of the muscle.

fibre types were lower ($P < 0·05$) than in cold-acclimated ducklings, and even lower than the control litter-mates for FOG fibres. The structural modification in the sense of an increased proportion of more oxidative fibres was clearer than in white gastrocnemius. Indeed, the percentage distribution of SO fibres was higher ($P < 0·05$) in cold-acclimated and glucagon-treated than in control ducklings, while the percentage distribution of FOG fibres was concomitantly lower. There was no difference between cold-acclimated and glucagon-treated ducklings. In the red part of the gastrocnemius muscle, cold-acclimation and glucagon treatment had similar effects on fibre-type distribution but not on fibre size.

Effect of age

The fibre-type determination showed that the three type of fibres were already differentiated in the gastrocnemius muscle of young ducklings, 1 week after hatching (Table 3). Growth was marked by an increase in muscle mass (Table 1) followed by a rise in fibre area (Tables 2 and 3). The major point arising from these results in young ducklings was the high proportion of oxidative fibres, suggesting a high oxidative metabolism of the gastrocnemius at this age. Indeed, the fibre-type proportions in both parts of the gastrocnemius muscle resembled those of the 6-week-old cold-acclimated ducklings rather than those of the 6-week-old thermoneutral controls. Therefore, muscular growth (at least that of gastrocnemius) at thermoneutrality appeared to be characterized by a differentiation of more glycolytic fibres, whereas cold acclimation during growth inhibited this phenomenon, particularly in the red gastrocnemius. Glucagon treatment also inhibited this normal differentiation in red gastrocnemius, but not in the white part of the muscle.

Capillary supply

In white gastrocnemius, the mean capillary density was not significantly affected by cold acclimation whereas the glucagon treatment induced an increase in capillary density ($P < 0{\cdot}05$, Table 2). Mean capillaries per fibre was not significantly affected

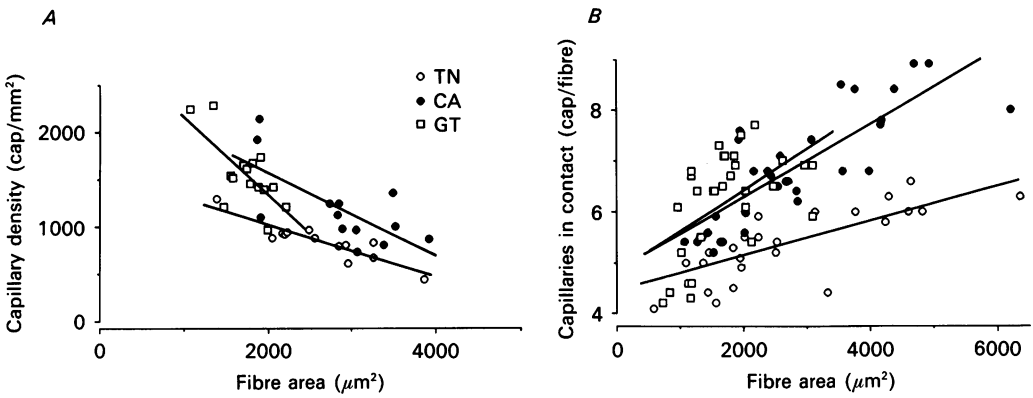

Fig. 2. Relation between capillary density and fibre area (*A*) and between the number of capillaries in contact with fibres and fibre area (*B*) in thermoneutral (TN), cold-acclimated (CA) and glucagon-treated (GT) ducklings. Cold acclimation and glucagon treatment induced an increased capillary supply to the muscle. *A*, regression lines for capillary density *vs.* fibre area. In control ducklings: $y = -0{\cdot}283x + 1586$ ($r = 0{\cdot}91$; $P < 0{\cdot}001$); in cold-acclimated ducklings: $y = -0{\cdot}478x + 2579$ ($r = 0{\cdot}75$; $P < 0{\cdot}01$); in glucagon-treated ducklings: $y = -0{\cdot}843x + 3017$ ($r = 0{\cdot}70$; $P < 0{\cdot}001$). *B*, regression lines for capillaries in contact *vs.* fibre area. In control ducklings: $y = 0{\cdot}000\,35x + 4{\cdot}44$ ($r = 0{\cdot}74$; $P < 0{\cdot}01$); in cold-acclimated ducklings: $y = 0{\cdot}000\,72x + 4{\cdot}84$ ($r = 0{\cdot}81$; $P < 0{\cdot}001$); in glucagon-treated ducklings: $y = -0{\cdot}000\,81x + 4{\cdot}81$ ($r = 0{\cdot}53$; $P < 0{\cdot}01$). Contrary to quadratic coefficients, linear coefficients were significantly different from zero in all cases (at least $P < 0{\cdot}05$).

by either of these experimental procedures despite a small increase. When analysed by fibre type, the capillaries in contact with both FG and FOG fibres were higher in cold-acclimated than in control and glucagon-treated ducklings (see Table 2 for significances).

In red gastrocnemius, the mean capillary density was higher in cold-acclimated and glucagon-treated ducklings than in controls ($P < 0{\cdot}05$), but not significantly different between cold-acclimated and glucagon-treated ducklings (Table 2). The mean number of capillaries per fibre was higher in cold-acclimated and glucagon-treated than in control ducklings ($P < 0{\cdot}05$). The capillaries in contact with both FOG and SO fibres were higher in both cold-acclimated and glucagon-treated than in control ducklings.

As the capillary density and number of capillaries in contact with fibres are greatly dependent on the fibre area, it was of interest to study the relation between capillary supply and fibre size. As shown in Fig. 2, the capillary density decreased as the fibre area increased ($F = 26$; $P < 0{\cdot}001$), but both cold acclimation and glucagon treatment significantly increased the capillary density ($F = 12$; $P < 0{\cdot}001$) in-

dependently of the area factor. Further, the number of capillaries in contact with fibres increased as a function of fibre area but the slope of the relation was steeper in cold-acclimated ducklings than in controls ($t = 3{\cdot}2$; $P < 0{\cdot}01$), while for the glucagon-treated ducklings the difference in slope did not reach the level of significant ($t = 2{\cdot}0$; $P < 0{\cdot}06$).

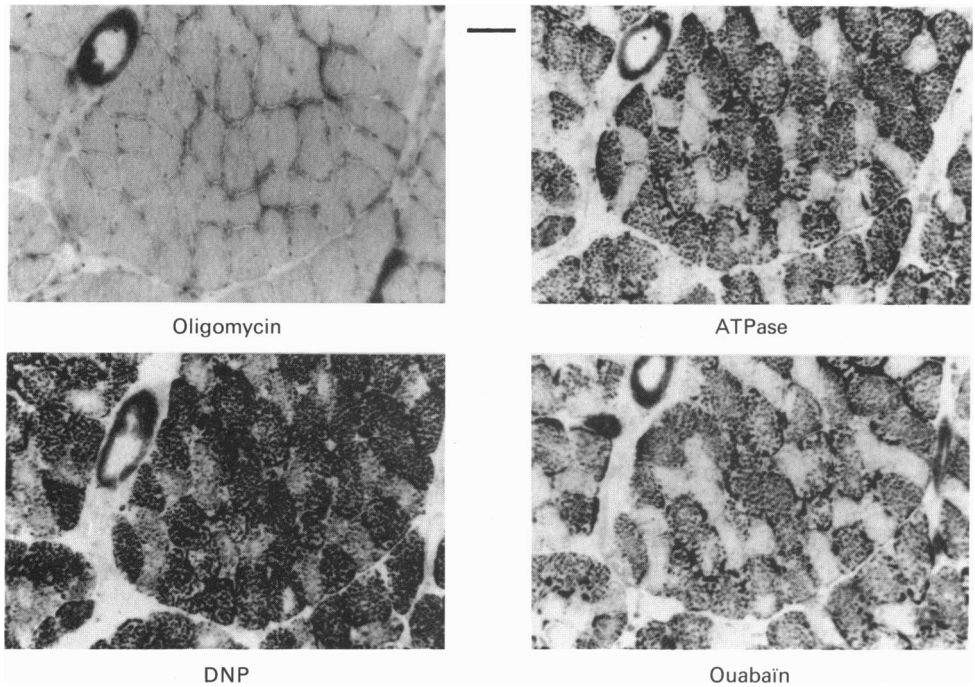

Fig. 3. Serial cross-sections of control duckling white gastrocnemius muscle stained for Mg^{2+}-ATPase, after incubation with oligomycin, ouabain or DNP. The intensity of staining is very low with oligomycin, not significantly different with ouabain and increased with DNP in both FOG and SO fibres. The SO fibres appeared darker than the FOG fibres. Scale bar 50 μm.

Therefore, cold acclimation resulted in an increased capillary supply to the muscle independently of fibre size suggesting an increased ability of oxygen and substrate transport to the cells. This increased capillary supply was reproduced by the glucagon treatment.

Histological detection of the coupling state of skeletal muscle mitochondria

Figure 3 shows the staining characteristics of mitochondrial Mg^{2+}-stimulated ATPase in the presence of either oligomycin, DNP or ouabain in the incubating medium, in the red gastrocnemius of a control duckling. As can be seen in these sections, the Mg^{2+}-stimulated ATPase staining was very low when the enzymatic activity was inhibited by oligomycin, and slightly higher when measured without inhibition of decoupling agent. The staining was strongly increased when oxidative phosphorylations were decoupled by the addition of DNP into the incubating

medium, i.e. when the Mg^{2+}-ATPase activity was no longer slowed down by the coupling state of mitochondria. The addition of ouabain, an inhibitor of Na^{+}–K^{+}-stimulated ATPase, into the incubating medium did not influence the observed staining showing the specificity of the mitochondrial Mg^{2+}-ATPase reaction.

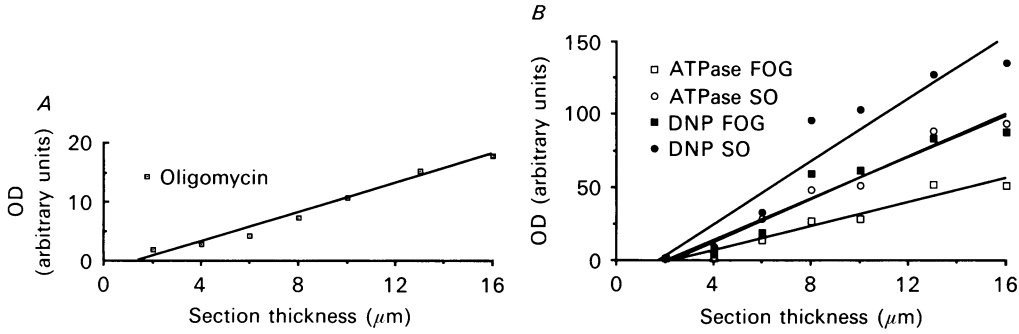

Fig. 4. Relation between optical density (OD) of the histochemical ATPase reaction and thin-section thickness in the presence (*A*) or in absence (*B*) of oligomycin. The OD is linearly related to the section thickness, and therefore to the enzyme capacity. *A*, regression line for OD *vs.* section thickness: $y = 1{\cdot}24x - 1{\cdot}79$ ($r = 0{\cdot}99$; $P < 0{\cdot}01$) represents the non-specific staining intensity. *B*, regression lines for OD *vs.* section thickness. For ATPase in FOG fibres: $y = 4{\cdot}12x - 9{\cdot}47$ ($r = 0{\cdot}97$; $P < 0{\cdot}01$); for ATPase in SO fibres: $y = 7{\cdot}30x - 16{\cdot}66$ ($r = 0{\cdot}98$; $P < 0{\cdot}01$); for DNP in FOG fibres: $y = 7{\cdot}07x - 14{\cdot}33$ ($r = 0{\cdot}96$; $P < 0{\cdot}01$); for DNP in SO fibres: $y = 10{\cdot}79x - 18{\cdot}71$ ($r = 0{\cdot}95$; $P < 0{\cdot}01$). Contrary to quadratic coefficients, linear coefficients were significantly different from zero in all cases (at least $P < 0{\cdot}05$).

These visual observations were confirmed by the quantification performed with the image analysis system. The optical density (OD) was very low in oligomycin-incubated sections, was increased in Mg^{2+}-stimulated ATPase reactions, and even more increased when DNP was added.

The first stage was to characterize the relation between the measured OD and the corresponding enzymatic activity. The OD of the reaction was measured on muscle serial sections with a thickness ranging from 2 to 16 μm. This relation is shown in Fig. 4*A*. The non-specific OD (with oligomycin) increased linearly with the section thickness ($r = 0{\cdot}99$), allowing us to subtract this non-specific OD from the OD measured in Mg^{2+}-stimulated ATPase and with DNP at each section thickness. The relation between the OD of the ATPase reaction with or without DNP and section thickness was also linear (Fig. 4*B*). It can therefore be reasonably postulated that for the same quantity of enzyme on the fibre section, the enzyme activity is almost linearly related to the OD measured.

The corrected OD of the Mg^{2+}-ATPase reaction, and thus, the Mg^{2+}-ATPase activity was higher in FOG than in FG fibres, and both were significantly increased by DNP (Fig. 5). No differences existed between the Mg^{2+}-stimulated reaction and the ouabain-inhibited reaction. The same observations could be made in all groups of animals in white and red gastrocnemius, except that in this last case, the FOG fibres had a lower ATPase activity than the SO fibres.

In cold-acclimated ducklings, the Mg^{2+}-ATPase activity was higher than in control ducklings in all types of fibres of both the white and red gastrocnemius (Fig. 5). The values in glucagon-treated ducklings were not significantly different from the controls, but were lower than in cold-acclimated ducklings for FG fibres in white and

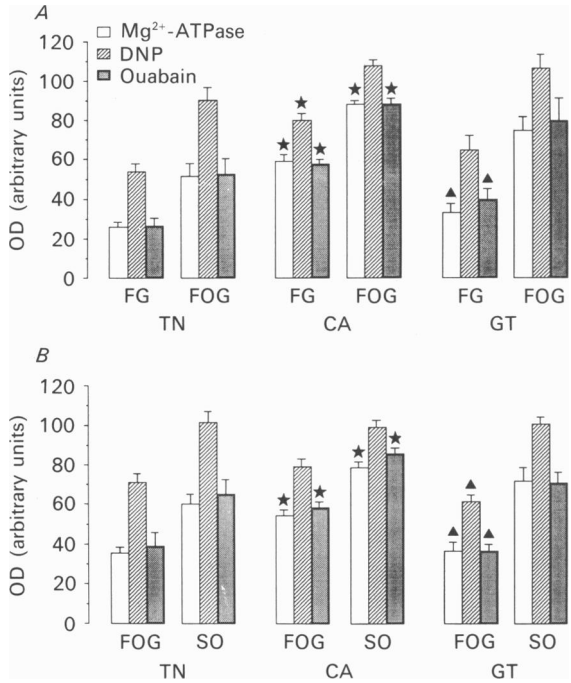

Fig. 5. Intensity of Mg^{2+}-ATPase reaction per fibre type in thermoneutral (TN), cold-acclimated (CA) and glucagon-treated (GT) ducklings in white (*A*) and red gastrocnemius (*B*). Either dinitrophenol (DNP) for maximal decoupling of oxidative phosphorylation or ouabain for inhibition of Na^+–K^+-ATPase were added in the incubation medium. DNP significantly increased the OD in all fibres. The effect of either cold acclimation or glucagon treatment was tested. ★ $P < 0{\cdot}05$ *vs.* TN; and ▲ $P < 0{\cdot}05$ *vs.* CA. Means ± S.E.M.

FOG fibres in red gastrocnemius. By contrast, the Mg^{2+}-ATPase activity in the presence of DNP was similar in all groups. Two exceptions can be given to this statement: FG fibres of white gastrocnemius in cold-acclimated ducklings had a higher enzymatic activity than in controls, and FOG fibres of red gastrocnemius in glucagon-treated ducklings had a lower enzymatic activity than in the cold-acclimated ducklings.

The coupling state of muscle mitochondria was estimated by the ratio (as a percentage) of the enzyme activities in Mg^{2+}-stimulated to DNP-stimulated ATPase reactions. This ratio characterized the increase in enzyme activity to obtain a complete decoupling of mitochondria in response to DNP, and therefore characterized the degree of decoupling of muscle mitochondria *in situ*. This ratio was higher for all fibres of the gastrocnemius muscle in cold-acclimated than in control ducklings (Fig. 6), while it was significantly higher only for FOG fibres of white and

for SO fibres of red gastrocnemius in glucagon-treated than in control ducklings. This ratio was not different between cold-acclimated and glucagon-treated ducklings except for FG fibres of white gastrocnemius which was higher in cold-acclimated than in glucagon-treated ducklings. These results showed a more loose-coupled state

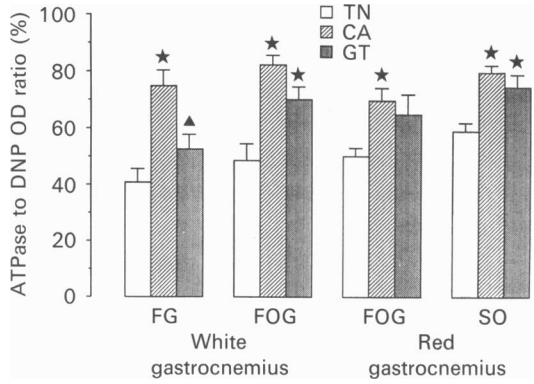

Fig. 6. Mg^{2+}-ATPase to DNP-stimulated ATPase OD ratio in the different fibres of thermoneutral (TN), cold-acclimated (CA) and glucagon-treated (GT) ducklings. This ratio allowed an estimation of the decoupling state of muscle mitochondria in the different fibres *in situ*. FG, fast glycolytic; FOG, fast oxidative glycolytic; SO, slow oxidative fibres. ★$P < 0{\cdot}05$ *vs.* control; ▲ $P < 0{\cdot}05$ *vs.* cold acclimated. Means ± S.E.M.

of muscle mitochondria *in situ* in all fibres of cold-acclimated than in control ducklings and in the most oxidative fibres of both *red and white* gastrocnemius in glucagon-treated birds compared with control ducklings.

DISCUSSION

As assessed by the pH sensitivity of mATPase activity (Guth & Samaha, 1970; Brooke & Kaiser, 1970), which is correlated with the speed of muscle contraction (Melichna, Gutmann & Syrovy, 1974), both fast (alkali-stable/acid-labile mATPase) and slow (acid-stable mATPase) fibres are present in gastrocnemius muscle of ducklings as in adult ducks (Butler & Turner, 1988). With regard to the fibre-type distribution in skeletal muscle, the present results in gastrocnemius muscle of ducklings showed compartmentalization (regional variation) of fibre types with a white part composed of FG and FOG fibres, and a red part composed of SO and FOG fibres. This finding has already been reported in avian (Rosser, Davis, Brocklebank & George, 1987) and particularly, in duck gastrocnemius muscle (Butler & Turner, 1988), and can be a common feature within vertebrate muscles indicating different functional areas within a muscle (Armstrong & Laughlin, 1985).

Effect of cold acclimation

Cold acclimation resulted in a higher oxidative metabolism of skeletal muscle than in thermoneutral controls as reflected by a higher proportion of more oxidative fibres in both white and red gastrocnemius muscle. These results together with those in 1-week-old ducklings suggest that a transformation of fibre type from FOG to FG

fibres in white gastrocnemius, and mainly from SO to FOG fibres in red gastrocnemius did not occur during growth in the cold. The higher proportion of red gastrocnemius in cold-acclimated and glucagon-treated than in control ducklings also suggested a higher proliferation of oxidative fibres without transformation from one type to the other. By contrast, during growth at thermoneutrality, a differentiation of oxidative into glycolytic fibres after hatching is observed. This phenomenon has already been reported in young chicks reared at thermoneutrality (Ashmore & Doerr, 1971).

The increase in the percentage of oxidative fibres and concomitant decrease in glycolytic fibres has been reported previously in cold-acclimated growing mammals (Ratzin Jackson, Sillau & Banchero, 1987; Dauncey & Ingram, 1988, 1990). This higher proportion of more oxidative fibres in gastrocnemius muscle of cold-acclimated ducklings may explain part of the increased oxidative capacity (measured as cytochrome oxidase activity) observed in this muscle after cold acclimation (Barré *et al.* 1987*b*).

No differences in fibre area existed in FOG and FG fibres between cold-acclimated and control ducklings. This finding is in accord with results of Ballantyne & George (1978) on gastrocnemius muscle of cold-acclimated pigeons. The slightly higher area of SO fibres in cold-acclimated than in control ducklings could be explained, at least in part, by differences in the degree of contraction of skeletal muscles when the samples were frozen (Mathieu-Costello, 1987).

The higher capillary supply in cold-acclimated than in control ducklings was due to an increase in both capillary density and capillaries in contact with the fibres. These variations were more marked in red than in white gastrocnemius. Such an increase in capillary density of skeletal muscle has also been reported in cold-acclimated mammals (Ratzin Jackson *et al.* 1987) and mainly in red muscles (Heroux & Saint Pierre, 1957), but has not been associated with NST. Generally, highly oxidative fibres are surrounded by more capillaries than glycolytic ones, but the size of the fibre is also a determinant since the average number of capillaries in contact with fibres increases linearly with fibre cross-sectional area (Hudlicka, 1985) in order to maintain a low diffusion distance for oxygen. The relation illustrated in Fig. 2 is in accord with these results and shows that the cold-induced increase in capillary density and number of capillaries in contact with fibres is independent of the size factor. Therefore, the increases in capillaries in contact with fibres in white and red gastrocnemius could be related to the effect of cold acclimation in order to increase the oxygen and substrate supply to muscle cells for maximal aerobic metabolism of skeletal muscle. The size effect could nevertheless explain why capillaries in contact were higher in glycolytic than in aerobic fibres.

Effect of glucagon treatment

Chronic glucagon treatment was performed to mimic the hyperglucagonaemia induced by cold acclimation (Barré, Geloën, Miahle & Rouanet, 1986*b*). This treatment reproduces some effects of cold acclimation and in particular induces a true NST in these ducklings in response to a glucagon-test injection (Barré *et al.* 1987*a*). In the present study, glucagon-treated ducklings developed similar muscle changes to cold-acclimated ducklings such as a higher proportion of oxidative fibres in red gastrocnemius and higher capillary density than in control ducklings.

However, some differences appeared between cold-acclimated and glucagon-

treated ducklings, firstly in fibre-type distribution in white gastrocnemius, in which glucagon treatment induced an increase in the percentage of FG fibres contrary to cold-acclimated ducklings, and secondly in fibre area, which was smaller in glucagon-treated than in both control and cold-acclimated ducklings. The amplitude of the difference in fibre area cannot be explained only be different degrees of muscle contraction before freezing but could be related, at least in part, to the catabolic effect of glucagon and also to a lower food intake in glucagon-treated than in control and cold-acclimated ducklings. As discussed previously (Barré *et al.* 1987*a*), the dose chosen probably induced higher levels of plasma glucagon than that produced endogenously by cold acclimation (Barré *et al.* 1986*b*). Therefore, the anorectic effect of glucagon, particularly at supraphysiological doses (Howes & Forbes, 1987), might be responsible in part for the differences in body mass and muscle fibre size between the glucagon-treated and the cold-acclimated ducklings. Particularly, in red gastrocnemius, the smaller size of fast fibres and the similar size of slow fibres in glucagon-treated *vs.* control ducklings could be related to the fact that undernutrition induces a selective reduction in the size of fast fibres whereas the size of slow fibres is better preserved (Siek, Lewis & Blanco, 1989; Henriksson, 1990). However, all the modifications observed in the glucagon-treated birds cannot result entirely from a reduced food intake, since undernutrition induces either no effect or a decrease in muscle oxidative capacity in mammals kept at temperatures near thermoneutrality (Dauncey & Ingram, 1988; Siek *et al.* 1989).

As previously demonstrated (Barré *et al.* 1987*a*), the present results underline the fact that glucagon treatment is not directly equivalent to cold acclimation. This could be related to the dose of glucagon used for the treatment and to the mode of administration. Ideally glucagon would have been administered frequently over the 24 h period since its secretion is thought to be pulsatile (Weigle & Goodner, 1986). Osmotic pumps would not have been the complete answer since (1) glucagon delivery would have been continuous, and (2) preliminary studies showed that pumps implanted in ducklings were rapidly embedded in connective tissue which blocked the pumps. Two daily injections were therefore preferred to a single massive dose in order to reduce the stress of multiple injections and to optimize the effects of glucagon.

The dissimilarity between glucagon treatment and cold acclimation suggests that other hormones could act synergistically with glucagon in the process of cold acclimation and NST development in birds. Nevertheless, these results also suggest that glucagon, at least at high doses, may be an important factor in the regulation of capillary growth and fibre-type differentiation in ducklings. Consequently, some of the cold-induced changes might result partly from the cold-induced hyperglucagonaemia.

Loose coupling of skeletal muscle mitochondria in situ

The image analysis system that we developed, improved the Meijer & Vloedman technique because it enabled a quantitative estimation of the intensity of staining. In addition, as the relation between the intensity of the reaction and the enzymatic capacity, and presumably the enzyme activity, was linear, it follows that this system allows a quantification in terms of Mg^{2+}-ATPase activity.

This quantification showed a lower stimulation effect of DNP on the Mg^{2+}-ATPase

activity in all fibres of cold-acclimated ducklings, in FOG and SO fibres of glucagon-treated ducklings than in the control litter-mates. These results clearly suggest a lower coupling of oxidation and phosphorylation in mitochondria of these muscle fibres and confirm those obtained with isolated mitochondria in glucagon-treated ducklings (Barré *et al.* 1989). The present study did not allow a differentiation between subsarcolemmal and intermyofibrillar mitochondria as was the case in this earlier study on isolated mitochondria. However, the present results clearly indicate a mechanism of loose coupling of skeletal muscle mitochondria *in situ* induced by either cold acclimation or glucagon treatment in ducklings. In addition, they enabled a differentiation between mitochondria from different fibre types. The mitochondria of all fibres were more loose coupled in cold-acclimated than in control ducklings, whereas only the mitochondria of the more oxidative fibres in both parts of the muscles were more loose coupled in glucagon-treated ducklings than in controls.

These findings were obtained with an experimental procedure that did not require mitochondrial extraction, and therefore, a possible experimental lesion of mitochondrial integrity. However, the freezing of mitochondria can also induce some partial loose coupling of isolated mitochondria (Meijer & Vloedman, 1980). This may explain why the loose coupling of mitochondria *in situ* was so pronounced in control ducklings. Even if this artifact is taken into account, the loose coupled state of muscle mitochondria in cold-acclimated and glucagon-treated ducklings exceeded that of controls, indicating a persistent physiological loose coupled state of these mitochondria *in situ*.

Skeletal muscle changes and non-shivering thermogenesis

Both cold-acclimated and glucagon-treated ducklings can exhibit NST, either after cold exposure for cold-acclimated ducklings (Barré *et al.* 1986*a*) or after a glucagon-test injection in glucagon-treated ducklings both in a cold and a warm environment (Barré *et al.* 1987*a*). This additional capacity for thermogenesis parallels the increased capacity for oxidative metabolism in muscle (Barré *et al.* 1987*b*, 1989; present study). In addition, this additional oxidative capacity is available to the organism because the capillary network increases in parallel, to make the blood supply commensurate to the O_2 demand.

The question arises as to whether these changes are developed for shivering or for non-shivering thermogenesis. During the first days of cold exposure, young ducklings resort mainly to shivering for heat production, but when the cold exposure is prolonged, NST is developed (Barré *et al.* 1986*a*). Therefore, shivering could play a part in morphological changes of skeletal muscle observed in cold-acclimated ducklings. An increase in oxidative capacity of skeletal muscles has also been found in intermittently cold-exposed bantam chicks in relation to shivering thermogenesis (Aulie & Grav, 1979). Artificial long-term electrical stimulation of muscles or endurance exercise training, situations that could be compared to continuous shivering stimulation, also induce an increase in oxidative capacity of working muscles in mammals (Hoppeler, 1986) and in birds (Butler & Turner, 1988). Further, a transition of fibre type from fast to slow also occurs in rabbits in response to long-term electrical stimulation (Pette, 1984) and in exercise-trained ducks (Butler & Turner, 1988). In addition, an increased muscle capillarization was found in these trained ducks (Butler & Turner, 1988).

However, other arguments suggest that muscle changes are also developed for NST. Indeed, the histochemical changes occurring mainly in the postural part of the gastrocnemius muscle are not in favour of shivering. Further, almost the same adaptations of skeletal muscles are reproduced by the glucagon treatment at thermoneutrality which excludes the influence of shivering. In addition, glucagon injection appeared to inhibit the shivering activity (Barré *et al.* 1987*a*). It should be noted that shivering, recorded in the gastrocnemius muscle itself (Barré *et al.* 1986*a*), was absent at the temperature of acclimation in cold-acclimated ducklings. In cold-acclimated pigs also, the elevated respiratory enzyme activities were unlikely to be related entirely to shivering since they were also found in muscle from the diaphragm (Dauncey & Ingram, 1990). Moreover, in cold-acclimated ducklings, the loose coupling of mitochondria in muscle fibres is a strong argument for an adaptation of muscle to NST, since one fundamental adaptation to endurance exercise is an increase in ATP supply for contraction with tightly coupled mitochondria (Holloszy & Booth, 1976). Finally, another indirect argument in favour of NST is that the Mg^{2+}-ATPase capacity in gastrocnemius muscle, as suggested by maximal DNP staining intensity, is not increased by either cold acclimation or glucagon treatment. If this capacity is representative of ATP synthase capacity, therefore, the increased oxidative capacity of skeletal muscle in cold-acclimated and glucagon-treated ducklings (Barré *et al.* 1987*a*, 1989) is not used for ATP synthesis, and may be available for heat production. Such a low phosphorylative index has already been suggested by a decrease in muscle mitochondrial creatine kinase activity of glucagon-treated ducklings despite a large increase in cytochrome oxidase activity (Barré *et al.* 1989).

In conclusion, the present results clearly showed that in parallel with the development of NST in both cold-acclimated and glucagon-treated ducklings, skeletal muscle developed structural and functional changes in terms of increased oxidative capacity. This was indicated by an increased proportion of more oxidative fibres and an increased capillary supply. In cold-acclimated ducklings, some structural changes, such as in fibre type or capillarization, could also be related to shivering thermogenesis, which precedes the development of a true NST in the process of cold acclimation. In the glucagon-treated ducklings, the nearly similar changes induced by the hormonal treatment at thermoneutrality can be related to the development of NST in muscle. The histochemical detection of the coupling state of mitochondria *in situ* avoided the artifacts inherent in the mitochondrial isolation procedure and demonstrated a more loose-coupled state after both cold acclimation or glucagon treatment. This altered coupling of muscle mitochondria argues for a muscular non-shivering thermogenesis in cold-acclimated or glucagon-treated ducklings.

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