Hydrostatic pressure effects on eel mitochondrial functioning and membrane fluidity.


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INTRODUCTION

The European eel, *Anguilla anguilla* L., is an interesting model for studying hydrostatic pressure (HP) adaptation. This animal, whose ¾ life cycle is spent in shallow freshwater, has to adapt to deep seawater during its spawning migration (6000 km) from the European coasts to the Sargasso Sea. The scale of depth encountered during this journey is not well known because of the difficulties involved in following eels during their trip. However they have been observed up to 2000 meters deep (1), which shows their adaptive capacities to this environmental and thermodynamic factor. At such pressures, cellular and sub-cellular membranes will be particular targets (2). As a consequence, activities of the respiratory chain and oxidative phosphorylation complexes, embedded in the mitochondria inner membrane, could be modified by pressure. Knowing that migration involved long-term swimming which mainly requires aerobic metabolism, pressure will have a potential effect on this physical activity. In non-migrating yellow eels exposed to HP = 10.1 MPa, respiratory chain and oxidative phosphorylation activities have been shown to decrease in the first few hours of exposure (3), together with a decrease in the oxygen consumption of red muscle fibres. This aerobic metabolism alteration has been linked to the decrease in membrane fluidity, and resembles the consequences of histotoxic hypoxia (3). When yellow eels are allowed to stay for a long period under pressure, *i.e.* almost one month, aerobic metabolism reverts...
to normal or supernormal level. There is an improvement in the respiratory chain and oxidative phosphorylation activities due, at least partly, to the restoration of membrane fluidity, after the incorporation of more polyunsaturated fatty acids (4-7). Thus even if eels at the yellow stage do not encounter hydrostatic pressure, they are able to acclimatize to its effects through homeoviscosity. However before departure, eels metamorphose from the yellow stage to the silver stage. As this metamorphosis (1), called the silvering process, is known to prepare the eel for its new environment and activity (absence of solar light, salt water, starvation and sustained swimming). We wondered about the influence of the silvering process in pressure resistance and adaptation mainly in the energy production processes. To answer this query, the aerobic metabolism of eels at the two developmental stages (yellow and silver) was studied in red muscle before and after acclimatization to 10.1 MPa HP. Membrane lipid composition and fluidity in red muscle mitochondria were measured in both yellow and silver eels before any pressure treatment to assess whether silveering induced any homeoviscous adaptation which might be considered as pre-adaptation of mitochondrial membranes to hydrostatic pressure.

METHODS

Animals

Storage

Twelve silver and twelve yellow freshwater European eels, Anguilla anguilla L., were used (body mass, 567 ± 184g and 107 ± 10 g respectively; mean ± S.E.M.). At least 10 days before the experiments, they were stored without feeding in 40 L polyethylene tanks with continuously renewed and aerated tap water. The experiments were performed during the winter. In order to maintain water temperature (14.2 ± 0.1 °C) and to have a photoperiod as close as possible to natural conditions, the tanks were placed in a room open to the outside.

Sampling

The fish were sacrificed by decapitation. Sections of eel, cut backwards from the swimbladder, were frozen in liquid nitrogen and then stored at –80 °C for further measurements of COX (Cytochrome oxidase) activities and nucleotide contents. Thereafter, fresh red muscles were sampled along the lateral line at about 30 % of total length. These samples were then prepared to perform direct oxygen consumption measurements.

Acclimatization to hydrostatic pressure

The eels were placed in an experimental tank (14.9 L), in a hyperbaric chamber and connected to a high-pressure water circulation system (12). The water circulation system allowed a continuous renewal of water (30 L.h⁻¹), so that temperature and oxygen concentration could be controlled.

After 5 days at atmospheric pressure, the hyperbaric chamber was compressed at a rate of 0.2 MPa.min⁻¹ to 10.1 MPa and this pressure was maintained for 21 days. At the time of compression, the pressure at which a strong motor activity began was recorded as the pressure threshold (P₉). During the experiments water oxygen partial pressure and temperature in the experimental tank were regularly monitored. Twice a day, the water flow was stopped for 30 minutes, in order to measure the decrease in water oxygen partial pressure and calculate oxygen consumption (13). After 21 days at this pressure, the chamber was decompressed at a rate of 0.2 MPa.min⁻¹. On reaching atmospheric pressure, the eels were immediately killed and samples taken. The same procedures were used for the control group, which was kept in the same experimental tank under the same environmental conditions.
(water temperature and oxygen content, light, noise, time) but at atmospheric pressure (0.1 MPa).

**Sample preparation**

**Permeabilised muscle fibres**

Freshly removed muscle samples were immediately placed in an ice-cold extraction medium (8). Before oxygen consumption measurements, the muscle fibres were permeabilised using a saponin solution (150 mg.L⁻¹). The technique used was adapted for fish from Veksler et al. (9) and Letellier et al. (10) by Sébert and Theron (8).

**Mitochondria isolation**

Mitochondria were isolated from the lateral line red muscle following a method adapted from Theron et al. (6) and Ohlendieck et al. (11). Dissected muscle was then thinly sliced with scissors, transferred into a potter with 10 mL of buffer (Sucrose 0.25 M, KCl 140 mM, Hepes 40 mM, EDTA 10 mM, MgCl₂ 5 mM, ATP 1 mM and proteases inhibitor cocktail 0.5.10⁻³ v/v; pH 7.4). The homogenate was centrifuged at 1000 g for 10 minutes at 4°C. The supernatant was filtered on gauze and centrifuged at 10,000 g for 10 minutes. Finally the pellet, containing mitochondria, was resuspended in 2 mL of buffer.

**Measurements**

All the measurements were performed at 15 °C.

**Muscle fibre oxygen consumption**

The oxygen consumption of freshly permeabilised muscle fibres was performed according to the technique previously described (6, 8). At atmospheric pressure, respiration was measured in a glass vessel by following its oxygen content decrease, using a Strathkelvin Instrument® O₂ microelectrode (accuracy: ± 0.2 % saturation). The electrodes used were calibrated before the experiment, and then the rates of oxygen consumption were measured using pyruvate plus malate and ADP at saturating concentrations (final concentrations were respectively 12, 6 and 5 mM respectively).

**Cytochrome Oxidase (COX) activity**

The method was adapted from Simon et al. (14). Tissue extracts were prepared from frozen sections of eels. Red muscle samples were dissected, still frozen on a bed of liquid nitrogen. The tissues were homogenized in 100 mg.mL⁻¹ of extraction buffer (Tris, 0.1 M; EDTA, 2 mM; DTE, 2 mM; pH 7.4) at 4 °C, using a Polytron. The obtained extracts were then centrifuged at 11000 g for 20 minutes at 4 °C. Filtered supernatants were directly used for COX activity determination. COX activity was determined by spectrophotometry at 550 nm in sodium phosphate buffer (0.33 M pH 7 at 15 °C) with 50 μmol.L⁻¹ reduced Cytochrome c at saturating concentrations (6).

**Energetic nucleotide contents**

Following the technique used by Sébert et al. (15), energetic nucleotides (ATP, ADP, AMP and IMP) were extracted with the acid solution of Trichloroacetic Acid. The extracts were immediately analysed using a HPLC method: the separation of different nucleotides was performed on a Hypersil ODS 25 cm column. The mobile phase was KH₂PO₄ (0.1 M) dissolved in deionised H₂O and filtered through 0.22 μm before pH adjustment to 6.35 with NH₄OH. The flow rate was 0.8 mL.min⁻¹. Detection was performed by UV spectrophotometry (254 nm) as previously described (16). From the results, Energy Charge was calculated as:

\[
E = \frac{ATP + \frac{1}{2}ADP}{ATP + ADP + AMP}
\]
Membrane fluidity

Membrane fluidity was measured, at 15 °C, by fluorescence polarization of polar hydrocarbon probe (TMA-PH, 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-trien) in mitochondria samples. Increasing values of polarization or anisotropy were taken as indicators of decreasing fluidity. Anisotropy \((r)\) values were measured according to the following formula:

\[
    r = \frac{I_{vv} - G I_{vh}}{I_{vv} + 2G I_{vh}}
\]

where \(I_{vv}\) and \(I_{vh}\) represent the fluorescence intensity parallel and perpendicular to the excitation plane (when set vertically), respectively. \(G\) is a correction factor showing the difference in the transmission efficiency of vertically and horizontally polarised light, and is calculated by \(I_{vh}/I_{hh}\) where \(I_{hh}\) represents the fluorescence intensity parallel to the excitation plane when set horizontally. TMA-DPH was dissolved in tetrahydrofuran to obtain a stock solution of 2 mmol.L\(^{-1}\). Probe penetration into the mitochondrial membrane was monitored by measuring the increasing fluorescence, which reached a steady-state value within 10 to 15 minutes of loading. Measurements were obtained using a SPEX spectrofluorimeter (Jobin-Yvon, Longjumeau, France), equipped with vertical and horizontal polarisation filters. The excitation and emission monochromators were positioned at wavelengths of 350 and 430 nm, respectively, with a slit width set at 10 nm for both excitation and emission modes. Readings were corrected for both the background fluorescence of TMA-DPH and light scatter by the preparation itself.

Membrane composition

- Membrane lipid extraction

Total lipids were extracted from the mitochondria solution using the technique previously described by Bligh and Dyer (17). After solvent evaporation, the lipid samples were solubilized in chloroform (2 volumes)-methanol (1 volume).

- Cholesterol measurement

Before measurements, total lipids were evaporated and solubilized in isopropanol. Total cholesterol measurements (cholesterol esters + free cholesterol) were performed on total lipid extracts in isopropanol using a Sigma kit (Infinity\textsuperscript{TM} Cholesterol liquid stable reagent thermo trace).

- Phospholipids measurement

Phospholipid measurement corresponds to phosphate ion quantification (one phosphate molecule per phospholipid) after mineralization of total lipid extracts in the acid medium following the method of Rouser \textit{et al.} (18).

- Fatty acid composition of membrane phospholipids

Phospholipids were eluted from neutral and glycolipids on a Sep-pak silica column (Labbe \textit{et al.} (19), using different solvents: Chloroform eluted neutral lipids, acetone eluted glycolipids and finally methanol eluted phospholipids. Fatty acid methyl esters (FAME) were obtained after phospholipid transmethylation by the method of Labbe \textit{et al.} (19). Methyl esters dissolved in isooctane were separated using gas chromatography (Chrompack model CP 9000) according to Labbe \textit{et al.}, (19). The vector gas was nitrogen, the 52CB-wax column was 25 meters long with an internal diameter of 0.25 mm. A flame ionization detector allowed the quantification of FAME whose concentration was expressed as molar % of the total FAME population.
**Analysis of data**

The results are expressed as mean ± S.E.M. The statistical significance of the results was evaluated at the 5% level with the Student’s t-test, after having ensured the normality of the distributions and the homogeneity of their variances (Lilliefors test).

**RESULTS**

The effects of compression are evaluated by animal oxygen consumption at the end of compression and by the pressure threshold. Figure 1 shows that yellow eels increase their oxygen consumption substantially and have a lower pressure threshold, showing a higher sensitivity to pressure variations.

After one month under pressure, whatever the stage or the pressure considered, red muscle exhibits a high energy charge (Table 1) i.e. a good energetic state. Under pressure, silver eels exhibit a slight but significantly higher energy charge than yellow eels. Red muscle oxygen consumption (Figure 2A) is decreased by pressure acclimatization in yellow eels and slightly increased in silver eels. Whatever the pressure COX (Cytochrome oxidase) activities are higher in silver eels than in yellow eels, but pressure has the same increasing effect on them in both stages (Figure 2B). Generally speaking, the pressure acclimatization of yellow eels tends to narrow the differences observed at atmospheric pressure between yellow and silver eels (Figure 1 and 2).

**Table 1.** Energy charges in eels at two pressure levels.

<table>
<thead>
<tr>
<th>Energy charge</th>
<th>Yellow eels</th>
<th>Silver eels</th>
</tr>
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<tbody>
<tr>
<td>0.1 MPa</td>
<td>0.89 ± 0.01</td>
<td>0.91 ± 0.01</td>
</tr>
<tr>
<td>10.1 MPa</td>
<td>0.88 ± 0.01</td>
<td>0.92 ± 0.01*</td>
</tr>
</tbody>
</table>

*: p<0.05 compared to yellow eels in the same condition.

**Fig 1.** Compression effects on eels of two silvering stages.

Results at atmospheric pressure are in the full columns and oxygen consumption at the end of compression in the striated columns. P<sub>t</sub>, pressure threshold is expressed in MPa.

*: p<0.05 compared to yellow eels in the same condition.

**Fig 2.** Acclimatization effects on red muscle fibre Oxygen consumption (A) and COX activity (B).

Results are in white for yellow eels and in grey for silver eels; control group (0.1 MPa) are in the full columns, and pressure group (10.1 MPa) in the striated columns. Δ : p<0.05, compared to the 0.1 MPa group. * : p<0.05, compared to yellow eels in the same condition.
Membrane fluidity, which is inversely correlated to anisotropy, is higher in silver eels than in yellow eels (Figure 3). When compared to yellow eels, silver eels have red muscle mitochondrial membranes that contain much less cholesterol and more unsaturated fatty acids (Table 2).

**DISCUSSION**

Metamorphosis, called the silvering process, is known to prepare the eel for its new environment and activity pattern, as it passes through a shallow freshwater environment, with a benthic life style, to a deep seawater environment accompanied by a sustained swimming behaviour (1). The pectoral fin is longer in silver eels, not only to help for swimming but also to stabilize in the water column. The transfer from freshwater to seawater is made easier by the improvement in osmoregulatory capacities and by gill chloride cell multiplication (20). Sustained swimming abilities are improved thanks to an increase in the developed muscular power output (21). Eye size is enlarged (1, 22) and pigments are modified to cope with the lack of solar light at depth (23). This last modification is an argument in favour of migration at depth. This non-exhaustive list of transformations which take place during the silvering process shows its preparatory effects. However no studies have been carried out concerning the metabolic adaptation to the per se hydrostatic pressure effects during silvers. In order to investigate the role of metamorphosis in HP resistance and acclimatization, the two stages of the European eel (yellow and silver) were compared before, during and after pressure acclimatization.

Compression effects show that yellow eels are more sensitive to variations in hydrostatic pressure (great increase in oxygen consumption at the end of compression and lower Pressure threshold). Moreover after three weeks under pressure, yellow eels present slight changes in aerobic capacities whereas silver eels exhibit no change. There is clearly a difference in response to hydrostatic pressure as regards the two stages. The fact that silver eels are scarcely affected by pressure and thus pre-adapted to its effects seems to be logical, bearing in mind that only silver eels are naturally exposed to it. The question lies in how this pressure pre-adaptation is performed.

Simon *et al.* (7) performed an experiment on yellow eels to show the persistence of

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**Table 2.** Composition of mitochondrial membrane extracts from red muscles of yellow and silver eels.

<table>
<thead>
<tr>
<th></th>
<th>Yellow eels</th>
<th>Silver eels</th>
</tr>
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<tbody>
<tr>
<td>Cholesterol/</td>
<td>0.344 ± 0.018</td>
<td>0.109 ± 0.005*</td>
</tr>
<tr>
<td>phospholipids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFA</td>
<td>26.0 ± 1.7</td>
<td>19.9 ± 0.5 ***</td>
</tr>
<tr>
<td>MUFA</td>
<td>15.7 ± 0.8</td>
<td>12.7 ± 0.3 ***</td>
</tr>
<tr>
<td>PUFA</td>
<td>58.3 ± 2.4</td>
<td>67.3 ± 0.5 ***</td>
</tr>
<tr>
<td>UFA(total)</td>
<td>74.0 ± 1.7</td>
<td>80.1 ± 0.5 ***</td>
</tr>
<tr>
<td>SFA/UFA(total)</td>
<td>0.35 ± 0.03</td>
<td>0.25 ± 0.01 ***</td>
</tr>
<tr>
<td>Unsaturation Index</td>
<td>288.1 ± 11.3</td>
<td>336.5 ± 2.6 ***</td>
</tr>
</tbody>
</table>

Yellow (N = 6) and silver eels (N = 12). Lipid results are expressed in percentage of detected phospholipids. *: p<0.05; **: p<0.01; ***: p<0.001.
adaptation effects after decompression. They acclimatized yellow eels for one month to HP = 10.1 MPa, decompressed them and finally the eels were recompressed again four days afterward. They observed that, at the end of compression, the oxygen consumption increase was lower and the pressure threshold higher, showing a less sensitive response after acclimatization. Processes which allow yellow eels to acclimatize have been reviewed (5). It has been shown that alteration in aerobic metabolism observed after a few hours of compression is linked to a decrease in membrane fluidity as enzymatic complexes of the respiratory chain and oxidative phosphorylation are embedded in the inner mitochondrial membrane. After acclimatization, aerobic metabolism reverts to normal values (at least), partly as a result of the restoration of fluidity.

Therefore, it may be hypothesized that the silvering process (metamorphosis) enables the eels to be already pre-adapted to hydrostatic pressure through a modification in membrane fluidity. Our results show that the red muscle mitochondrial membranes of silver eels exhibit higher fluidity together with a higher proportion of unsaturated fatty acids compared to those of yellow eels. As expected, this higher mitochondrial membrane fluidity is also correlated with a lower proportion of saturated fatty acids. Indeed, acyl chains saturation is correlated with an increase in phospholipid order and therefore, the increase in pressure reinforces this effect. By contrast, the unsaturation of acyl chains tends towards a more fluid state with a decrease in the phospholipid order. In this case, the ordering effect of hydrostatic pressure is limited (24). These modifications are accompanied by a three-fold lower cholesterol level in silver eels than in yellow eels.

Cholesterol is known to have modulatory effects: it decreases membrane fluidity in the liquid-crystalline state (25, 26). Therefore, it could be argued that the high level of cholesterol in yellow eel mitochondrial membranes may well lower the pressure rigidifying effects, but the low cholesterol level observed in silver eels helps to maintain high membrane fluidity even in the presence of high hydrostatic pressure. Thus, we propose that at atmospheric pressure, the high membrane fluidity of silver eel mitochondrial membranes (due to efficient fatty acids composition) existing at atmospheric pressure, plays a role in restricting high pressure physical effects and thus to return to normal value, thus optimising the mitochondrial functioning. Several results from the literature tend to confirm this hypothesis. The increase in COX activity during the period under pressure matches the continuous optimisation of membrane fluidity during migration (27). In the same manner fasting, whose influence becomes increasingly important during the migration, increases the proportion of unsaturated membrane phospholipids (28) and limits the incorporation of phospholipids with saturated hydrophobic tails (29). Finally sexual steroids, in particular 17-β-oestradiol which increases during the silvering process (30), can increase membrane fluidity through their insertion between the acyl chains of the phospholipids (31). The fact that the above cited physiological modifications tend to increase membrane fluidity during the migration lead us to believe that eels may well dive deeper and deeper, perhaps in order to ensure optimal reproduction.

ACKNOWLEDGMENTS

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