

RESEARCH ARTICLE

Alteration of mitochondrial efficiency affects oxidative balance, development and growth in frog (*Rana temporaria*) tadpoles

Karine Salin^{1,*}, Emilien Luquet¹, Benjamin Rey², Damien Roussel¹ and Yann Voituron¹

¹Laboratoire d'Ecologie des Hydrosystèmes Naturels et Anthropisés (U.M.R. CNRS 5023) and ²Laboratoire de Biométrie et Biologie Evolutive (U.M.R. CNRS 5558), Université Claude Bernard Lyon 1, Université de Lyon, 43 Bvd 11 Novembre 1918, F-69622 Villeurbanne Cedex, France

*Author for correspondence (karine.salin@univ-lyon1.fr)

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SUMMARY

Mitochondria are known to play a central role in life history processes, being the main source of reactive oxygen species (ROS), which promote oxidative constraint. Surprisingly, although the main role of the mitochondria is to produce ATP, the plasticity of mitochondrial ATP generation has received little attention in life history studies. Yet, mitochondrial energy transduction represents the physiological link between environmental resources and energy allocated to animal performance. Studying both facets of mitochondrial functioning (ATP and ROS production) would allow better understanding of the proximate mechanisms underlying life history. We have experimentally modulated the mitochondrial capacity to generate ROS and ATP during larval development of *Rana temporaria* tadpoles, via chronic exposure (34 days) to a mitochondrial uncoupler (2,4-dinitrophenol, dNP). The aim was to better understand the impact of mitochondrial uncoupling on both responses in terms of oxidative balance, energy input (oxygen and feeding consumption) and energy output (growth and development of the tadpole). Exposure to 2,4-dNP reduced mitochondrial ROS generation, total antioxidant defences and oxidative damage in treated tadpoles compared with controls. Despite the beneficial effect of dNP on oxidative status, development and growth rates of treated tadpoles were lower than those in the control group. Treatment of tadpoles with 2,4-dNP promoted a mild mitochondrial uncoupling and enhanced metabolic rate. These tadpoles did not increase their food consumption, and thus failed to compensate for the energy loss elicited by the decrease in the efficiency of ATP production. These data suggest that the cost of ATP production, rather than the oxidative balance, is the parameter that constrains growth/development of tadpoles, highlighting the central role of energy transduction in larval performance.

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Key words: ATP/O ratio, energy availability, life history traits, oxidative constraint, amphibian.

INTRODUCTION

Physical constraints and ecological pressures shape an organism's life history. The life history theory, developed in the 1960s, is based on the assumption that the differential allocation of limited resources to various aspects of maintenance, growth and reproduction must be optimized to maximize individual fitness (Cody, 1966; Stearns, 1992). Unravelling the mechanisms underlying the life history trade-off could improve our understanding of the proximal factors driving evolutionary forces. In more recent years, physiological aspects of the trade-off have largely been explored by focusing on energy resource allocation and its genetic and endocrine control (Zera and Harshman, 2001). However, the precise physiological mechanisms that determine the variability in life history traits are still poorly understood (Ackermann et al., 2001; Zera and Harshman, 2001; Alonso-Alvarez et al., 2006).

Although a great number of relevant studies have focused on energy resource acquisition and energy allocation, surprisingly none, as far as we are aware, have yet considered energy flow regulation and conversion at the mitochondrial level. Indeed, energy derived from diet becomes usable only after being converted into high-energy phosphate bonds in adenosine triphosphate (ATP) molecules. The main site of energy conversion is the mitochondria

(90% of cellular ATP) (Lehninger et al., 1993) that extract energy through oxidative reactions during aerobic respiration. An alternative pathway is anaerobic glycolysis, a process that generates ATP from glucose oxidation and lactate as a by-product. As the amount of ATP could limit many vital cellular processes (protein synthesis, cell division and signalling, muscle contractile activity, etc.), the plasticity of mitochondrial functioning would play a central role in the amount of energy that an organism could allocate to its life history traits.

Mitochondrial ATP synthesis is known to have a variable degree of coupling to oxygen consumption (ATP/O ratio). In this sense, the plasticity of mitochondrial energy transduction efficiency strongly varies in a phylogenetic manner and in response to environmental factors, such as season, diet and temperature (Heise et al., 2003; Sokolova, 2004; Sommer and Portner, 2004; Brand, 2005; Emel'yanova et al., 2007; Robert and Bronikowski, 2010). However, according to the above-mentioned theory of limited resources, evolutionary force should have optimized the ATP/O ratio toward better efficiency of the mitochondrial energy transduction system in order to maximize individual fitness. Therefore, the persistence of low ATP/O yield, despite the action of natural selection, suggests that a low efficiency could provide

some evolutionary advantages according to environmental condition (Brand, 2000).

One possible explanation is that mitochondria are also an important source of reactive oxygen species (ROS). Indeed, ROS formation is an unavoidable by-product of aerobic metabolism that greatly depends upon the mitochondrial inner membrane potential, as does the ATP/O ratio (Korshunov et al., 1997; Brand, 2005; Jezek and Hlavata, 2005). These highly reactive molecules will exert oxidative stress on cellular macromolecules. If ROS production is unchecked by antioxidant systems, cumulative oxidative damage to lipids, proteins and nucleic acids will accelerate organism senescence processes (Harman, 1956; Balaban et al., 2005; Barja, 2007). To date, ROS production have been considered as a proximate cost of growth or reproduction investment, supporting the notion that an energy allocation-mediated trade-off may be associated with oxidative stress (Dowling and Simmons, 2009; Metcalfe and Alonzo-Alvarez, 2010) but not necessarily with energy resources or more widely with the energy transduction system. In order to provide new advances for functional studies of trade-offs, we investigated mitochondrial functioning as the keystone between optimization of energy availability for maintenance, reproduction and growth and the cost of ROS production.

The aim of the present study was to investigate whether global mitochondrial functioning (both ATP and ROS production) is associated with life history variation. To test this hypothesis, we chronically exposed frog *Rana temporaria* tadpoles to the mitochondrial protonophore 2,4-dinitrophenol (2,4-dNP) during their larval development. This drug increased the proton leak across the inner mitochondrial membrane, reducing both the efficiency of mitochondrial energy transduction and the rate of ROS production (Brand, 2000; Balaban et al., 2005; Speakman, 2005; Hulbert et al., 2007). Chronic 2,4-dNP exposure at a low dose is expected to induce mild mitochondrial uncoupling (Wallace and Starkov, 2000). Consequently, the treatment could have a beneficial effect on oxidative stress and decrease the cellular availability of ATP (Sibille et al., 1995; Wallace and Starkov, 2000; da Silva et al., 2008; Geelen et al., 2008). There are three main ways to compensate for the reduced mitochondrial ATP production: (i) activation of the glycolytic pathway, (ii) increased mitochondrial biogenesis, and (iii) increased oxidative phosphorylation capacity of individual mitochondria. Therefore, mitochondrial activity as well as whole-body ATP and lactate content and whole-body cytochrome *c* oxidase activity were measured. We also measured different parameters to evaluate the oxidative balance (ROS production, tadpole antioxidant capacity, lipid peroxidation), the energy input (basal metabolic rate and food intake) and the energy output in tadpole performance (growth and developmental rates).

MATERIALS AND METHODS

Animals and experimental design

The present investigation was carried out according to the ethical principles of the French Ministry of agriculture and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Scientific Purposes (Council of Europe, no. 123, Strasbourg).

Spawn clumps of common frog *Rana temporaria* L. were collected from natural ponds located near Lyon (France, 46°50'32"N, 5°33'10"E). Each spawn clump was maintained outdoors in a 75 l tank. When tadpoles reached Gosner stage 25 (free swimming, ~26 days) (Gosner, 1960), tadpoles derived from the spawn clumps were randomly assigned to a group (50 individuals per group) in 20 l aquaria. From this step until the end of experiment, tadpoles

were reared indoors in aquaria continuously supplied with aerated tapwater, at a controlled temperature of 20±3°C, on a 12h:12h light–dark cycle and fed *ad libitum* with spinach.

Tadpoles were reared for 10 days in laboratory conditions (Gosner stage 29) before the 2,4-dNP treatment. Animals were then continuously exposed to 2,4-dNP. Skin absorption is a well-established route of entry following 2,4-dNP exposure in terrestrial and aquatic animals (Agency for Toxic Substances and Disease, 1995). From a pilot study we initially determined that 100 µmol l⁻¹ 2,4-dNP was lethal after a few weeks of chronic exposure whereas a concentration of 1 µmol l⁻¹ 2,4-dNP was without any effect on survival (K.S., personal observation). Hence, 2,4-dNP was introduced at a final concentration of 1 µmol l⁻¹ into the treated tanks.

2,4-dNP lyophilized powder (D7004–Sigma-Aldrich) was first dissolved in absolute ethanol (at a stock concentration of 100 mmol l⁻¹) and 200 µl was added into each of the 20 l aquaria, reaching a final concentration of 1 µmol l⁻¹ 2,4-dNP and 0.001% ethanol. As 2,4-dNP was prepared in ethanol solution, we used an experimental control condition in which tadpoles were maintained in a water tank supplemented with ethanol (0.001%). In the experiment, we also studied whether ethanol alone affected larval performance, by comparing ethanol-treated tadpoles with tadpoles maintained in water. To summarize the data in supplementary material Tables S1 and S2, ethanol treatment (at a final concentration of 0.001%) had no effect on larval performance.

Tadpoles were maintained for 34 days in a water tank containing 1 µmol l⁻¹ 2,4-dNP (experimental group) or in a water tank supplemented with 0.001% ethanol (control group). This treatment was repeated every water change.

Larval performance

After 10 days of acclimation in indoor pools, body mass was measured and the larval stage of tadpoles determined according to Gosner (Gosner, 1960) before the 2,4-dNP treatment began, and again at 16 and 34 days of chronic exposure of 2,4-dNP.

Feeding trial

Tadpole ingestion capacity was measured in 2,4-dNP and control groups. For the experiment, tadpoles were fasting for 48 h followed by a 180 min feeding trial using a colour mixture composed of fluorescent particles (RADGLO[®] PC25-Red, average particle size 3 µm, Radiant Color N.V., Houthalen, Belgium) and herbivore fish granule (Tetra PlecoMin[®], Tetra GmbH, Melle, Germany). The mixture preparation was blended to a ratio of 1 fluorescent particle to 2 fish granules (mass/mass) and was provided *ad libitum* during the experiment. The tadpoles ingested the mixture of coloured particles and food, which resulted in a distinctive colour mark in the gut (Eklöv and Halvarsson, 2000). After 3 h of feeding, tadpoles were killed by an overdose of the anaesthetic phenoxyethanol and dissected. The amount of ingested food was quantified as the ratio between the mouth-to-coloured mark distance and the total length of the gut.

Basal metabolic rate

The basal metabolic rate was estimated by the rate of whole-body oxygen consumption, measured at 20°C in the dark. Prior to the experiment, tadpoles were starved for 2 days to ensure that the digestive metabolism did not affect the results. Oxygen content was monitored with an oxymeter (HQ40 HACH[™], Dusseldorf, Germany), accuracy ±0.01 mg O₂ l⁻¹, before the 125 ml chamber of normoxic water was closed and 5–10 h after the chamber was closed, depending on the tadpole size. The period of 5–10 h was tested to ensure that animals were never in hypoxic water (oxygen content <3 mg O₂ l⁻¹).

Table 1. Effects of 2,4-dinitrophenol treatment on mitochondrial oxygen consumption rate of tadpoles under different conditions

Mitochondrial oxygen consumption (nmol O min ⁻¹ mg ⁻¹ mitochondrial protein)	Control	2,4-Dinitrophenol treated
ADP-stimulated respiration (state 3)	16.4±3.1	32.0±5.2*
Oligomycin-insensitive respiration (state 4)	4.4±0.9	8.5±1.2*
Cytochrome <i>c</i> oxidase activity	24.5±4.8	48.0±6.7*

Conditions: maximal phosphorylating respiration, state 3; basal non-phosphorylating respiration, state 4; and maximal mitochondrial oxidative capacity, the oxygen consumption of isolated cytochrome *c* oxidase (see Materials and methods for details).

Results are means ± s.e.m. (*N*=9–10).

*Significant differences between tadpole groups for *P*<0.05.

Isolation of mitochondria

Animals were rinsed with water and chilled for a few minutes in ice-cold isolation medium (10 mmol l⁻¹ Tris-HCl, 10 mmol l⁻¹ maleic acid, 600 mmol l⁻¹ mannitol, 2 mmol l⁻¹ EGTA, 1 mmol l⁻¹ EDTA, 0.5 mmol l⁻¹ Na₂HPO₄ and 2% free fatty acid bovine serum albumin, pH 7.4 at 4°C). Mitochondria were isolated by differential centrifugation using a Percoll™ density gradient from pooled tadpoles (up to 10 g of tadpoles per preparation), with all steps being carried out at 4°C. Tadpoles were homogenized with a Potter–Elvehjem homogenizer (three passages). Following centrifugation (800 g, 10 min) the pellet was resuspended and recentrifuged (800 g, 10 min). The two supernatants were pooled, and spun at 1000 g for 10 min. The resulting supernatant was filtered through cheesecloth and centrifuged at 8700 g for 10 min. The pellet was resuspended in 15% Percoll™ made up in isolation medium, layered onto a preformed gradient of 23% and 40% Percoll™ as described previously (Sims and Anderson, 2008), and centrifuged at 31,000 g for 5 min. The material at the interface of the 23% and 40% Percoll™ layers was removed by pipette, diluted 1:4 with isolation medium and centrifuged at 16,000 g for 10 min. Supernatant was removed without disturbing the resulting fluffy pellet, and the pellet was diluted 3-fold with isolation medium and centrifuged at 8700 g for 10 min. Finally, the pellet was stored in 200 µl of ice-cold isolation medium. Protein concentration was measured at 540 nm using the Biuret method with bovine serum albumin as a standard. The tadpole mitochondrial preparations contained a dark pigment that absorbed at 540 nm; therefore, the absorbance of the same volume of mitochondria in water containing 0.6% deoxycholate and 3% NaOH was subtracted.

Mitochondrial respiration and oxidative phosphorylation efficiency

Mitochondrial oxygen consumption was measured with a Clark oxygen electrode (Rank Brothers Ltd, Cambridge, UK), in a closed and stirred glass cell of 250 µl volume, thermostatically controlled at 20°C. Tadpole mitochondria (3 mg protein ml⁻¹)

were incubated in a respiratory medium containing 20 mmol l⁻¹ Tris-base, 650 mmol l⁻¹ mannitol, 0.5 mmol l⁻¹ EGTA, 5 mmol l⁻¹ MgCl₂, 5 mmol l⁻¹ KH₂PO₄ and 1% free fatty acid bovine serum albumin (w/v), pH 7.4 at 20°C. Succinate (5 mmol l⁻¹) was used as a respiratory substrate in the presence of 5 µmol l⁻¹ rotenone. The active state of respiration (state 3) was initiated by the addition of 1 mmol l⁻¹ ADP. The basal non-phosphorylating respiration rate (state 4) was obtained by the addition of 2.5 µg ml⁻¹ oligomycin. A second set of experiments was conducted with 0.5 mmol l⁻¹ *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) and 4 mmol l⁻¹ ascorbate as the substrate in order to measure the maximal respiration rate associated with isolated cytochrome *c* oxidase activity. The respiratory control ratio refers to the ratio of oxygen consumed after adding ADP to that consumed in the presence of oligomycin.

The mitochondrial oxidative phosphorylation efficiency was determined from the rate of ATP synthesis and oxygen consumption of isolated tadpole mitochondria (3 mg ml⁻¹) respiring on succinate (5 mmol l⁻¹ plus 5 µmol l⁻¹ rotenone) in the respiratory medium supplemented with an ADP-regenerating system consisting of 1.5 U ml⁻¹ hexokinase, 20 mmol l⁻¹ glucose and two different concentrations of ADP (20 and 200 µmol l⁻¹) as previously described (Rigoulet et al., 1998; Nogueira et al., 2001; Clerc et al., 2007; Salin et al., 2010; Teulier et al., 2010).

Lactate and ATP content in tadpoles

Frozen tadpoles were homogenized with a mixer mill at 50 Hz, 240 s (Retsch MM200, Fisher Bioblock Scientific, Illkirch, France) in perchloric acid solution containing 10% HClO₄ and 25 mmol l⁻¹ EDTA (1 µl of perchloric acid solution mg⁻¹ tadpole). After centrifugation of the denatured protein (21,000 g for 5 min) and neutralization of the resulting supernatant with solution containing 2 mol l⁻¹ KOH and 0.3 mol l⁻¹ Mops, the concentration of lactate and ATP was determined by monitoring the production of NADH in the presence of specific enzymatic reaction buffer at 340 nm. For lactate, samples were incubated in a reaction buffer consisting of 5 U ml⁻¹ lactate dehydrogenase, 0.75 mmol l⁻¹ NAD, 0.63 mmol l⁻¹ glycine and 0.4 mol l⁻¹ hydrate hydrazine, pH 9. For ATP, samples

Table 2. Effects of 2,4-dinitrophenol treatment on oxidative balance parameters

Oxidative balance	Control	2,4-Dinitrophenol treated
Mitochondrial H ₂ O ₂ production (pmol min ⁻¹ mg ⁻¹ mitochondrial protein)	25.2±2.3	18.7±1.5*
Total antioxidant activity (mmol l ⁻¹ trolox g ⁻¹ fresh mass)	79.7±13.5	53.3±5.3
Malondialdehyde content (nmol MDA g ⁻¹ fresh mass)	4.4±0.5	2.3±0.2*

Parameters: mitochondrial pro-oxidant molecule generation; the ability of total antioxidant molecules to prevent the oxidation of reactive oxygen species; and an index of oxidative injury, malondialdehyde (MDA) content, measured in tadpoles.

Results are means ± s.e.m. (*N*=10–18).

*Significant difference between tadpole groups for *P*<0.05.

were incubated in a reaction buffer consisting of 1.5 U ml⁻¹ hexokinase, 0.5 U ml⁻¹ glucose 6-phosphate dehydrogenase, 20 mmol l⁻¹ glucose, 0.5 mmol l⁻¹ NAD, 50 mmol l⁻¹ triethanolamine-HCl, 7.5 mmol l⁻¹ MgCl₂ and 3.75 mmol l⁻¹ EDTA, pH 7.4.

Tissue cytochrome *c* oxidase activity

Frozen tadpoles were homogenized with a mixer mill at 60 Hz, 120 s (Retsch MM200) in a buffer containing 1 mmol l⁻¹ ATP, 50 mmol l⁻¹ Hepes, 100 mmol l⁻¹ KCl, 5 mmol l⁻¹ MgCl₂, 1 mmol l⁻¹ EDTA and 5 mmol l⁻¹ EGTA (pH 7.4). The same volume of buffer containing 10 mg ml⁻¹ lubrol was then added. Cytochrome *c* oxidase activity was determined polarographically at 20°C, with a Clark oxygen electrode (Rank Brothers Ltd), in 500 µl of assay medium containing 30 µmol l⁻¹ cytochrome *c*, 4 µmol l⁻¹ rotenone, 0.5 mmol l⁻¹ 2,4-dNP, 10 mmol l⁻¹ sodium malonate and 75 mmol l⁻¹ Hepes adjusted to pH 7.4 at 20°C. Then, 10 mmol l⁻¹ ascorbate and 0.3 mmol l⁻¹ TMPD were added to start the reaction, and the maximal respiration rate associated with cytochrome *c* oxidase activity was recorded.

Oxidative balance

The peroxide anion (O₂⁻) is the radical oxygen species generated by mitochondria and its dismutation produces hydrogen peroxide (H₂O₂), the molecule that is usually assayed to measure ROS mitochondrial production. The respiratory medium (200 µl) was supplemented with 1 U horseradish peroxidase and 10 µmol l⁻¹ Amplex red reagent, and 1 mg ml⁻¹ mitochondrial protein was given at the start of the detection (Zhou et al., 1997). Succinate (5 mmol l⁻¹) was used as the respiratory substrate. Fluorescence was measured at 20°C using a fluorescence spectrophotometer (Xenius, SAFAS, Monaco) at excitation and emission wavelengths of 563 and 587 nm, respectively. The fluorescent signal was calibrated using a standard curve prepared with H₂O₂ (20 to 400 nmol l⁻¹) at each measurement.

The total antioxidant capacities of tadpoles were determined by using commercially available kits (706001 Antioxidant Assay Kit, Cayman Chemical, Ann Arbor, MI, USA). Frozen tadpoles were homogenized with a mixer mill (30 Hz; 120 s) (Retsch MM200) in a buffer containing 5 mmol l⁻¹ potassium phosphate, 0.9% sodium chloride and 0.1% glucose (pH 7.4). All procedures conformed to the manufacturer's instructions. The capacities of the antioxidants in the sample were expressed as unit equivalent trolox concentration per milligram of fresh mass.

Thiobarbituric acid-reactive substances are an index of lipid peroxidation and oxidative stress. Frozen tadpoles were homogenized with a mixer mill (30 Hz; 120 s) (Retsch MM200) in a buffer containing 100 mmol l⁻¹ KH₂PO₄, 0.05% BSA, 10 mmol l⁻¹ EDTA, 0.13 mmol l⁻¹ butylated hydroxytoluene and 0.13 mmol l⁻¹ deferoxamine (pH 7.4). Tadpole lipid peroxide content was assessed spectrophotometrically at 540 nm, by measuring malondialdehyde (MDA) as an index of thiobarbituric acid-reactive products (TBARS) following a method described previously (Ohkawa et al., 1979). The results are expressed in nmol MDA mg⁻¹ fresh mass of tadpoles.

Statistical analysis

The effects of treatment, exposition duration (16 and 34 days of exposure) and their interaction on larval performance and basal metabolism were analysed using linear models of analyses of variance. Because we measured other variables (biochemical variables) only at 34 days, the treatment effect was analysed using Student's *t*-test. Statistical analyses were performed with JMP software (version 7.0.1).

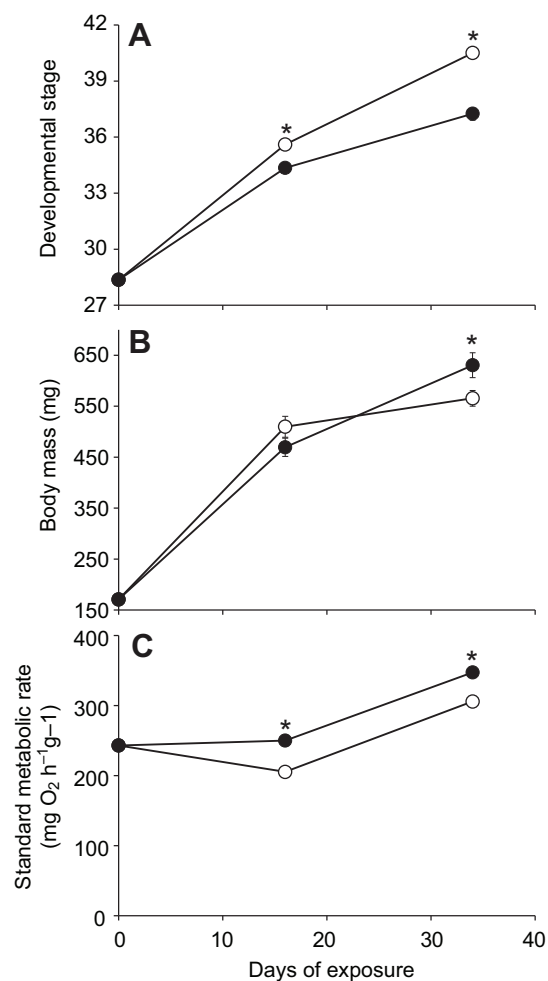


Fig. 1. Effect of 2,4-dinitrophenol (2,4-dNP) treatment on (A) developmental stage, according to Gosner (Gosner, 1960), (B) body mass and (C) basal metabolic rate at 20°C in tadpoles before treatment (0 days of exposure), and after 16 and 34 days of treatment. Results are means \pm s.e.m. ($N=19-20$). Filled circles, 2,4-dNP-treated tadpoles; open circles, control tadpoles. *Significant difference between tadpole groups for $P<0.05$ at equivalent time.

RESULTS

Larval performance

Compared with controls, exposure to 2,4-dNP reduced tadpole developmental rate in a time-dependent manner; the longer the exposure to 2,4-dNP the higher the negative effect on development ($F_{1,76}=17.27$, $P<0.001$, Fig. 1A). After 16 days of treatment, the 2,4-dNP group showed a lower body mass than the control group, whereas at 34 days of treatment, the opposite was the case ($F_{1,76}=27.65$, $P<0.001$, Fig. 1B). These differences in body mass were explained by differences in the developmental rate of tadpoles; no statistically significant effect of treatment on body mass corrected for developmental stage was found ($F_{1,73}=1.74$, $P=0.19$). In other words, the developmental rate is reduced by 2,4-dNP exposure but for the same larval stage, body mass was similar in the two groups. Hence, the observed differences in body mass of 2,4-dNP-treated tadpoles were due to a delay in the rate of development.

Tadpole energy input

Food consumption was not affected by 2,4-dNP treatment ($N=17-20$, 16 days treatment: control $1.47\pm 0.37\%$, 2,4-dNP $1.47\pm 0.35\%$ of

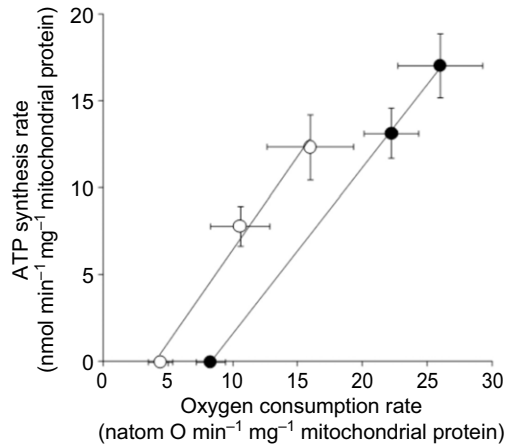


Fig. 2. Oxidative phosphorylation efficiency for 2,4-dNP-treated tadpoles (filled circles) and control tadpoles (open circles). Oxygen consumption and ATP synthesis were measured at different concentrations of ADP: 20, 200 and 200 $\mu\text{mol l}^{-1}$ in the presence of 2.5 $\mu\text{g ml}^{-1}$ oligomycin. The experimental conditions are described in Materials and methods. Results are means \pm s.e.m. ($N=9-10$).

coloured length of gut; 34 day treatment: control $1.69 \pm 0.38\%$, 2,4-dNP $1.68 \pm 0.39\%$ of coloured length of gut, $F_{1,70}=0.00$, $P=0.97$). 2,4-dNP-treated tadpoles displayed a higher rate of oxygen consumption than control tadpoles ($F_{1,75}=45.97$, $P<0.001$, Fig. 1C), irrespective of the duration of exposure ($F_{1,75}=0.07$, $P=0.80$).

Mitochondrial oxidative phosphorylation activity and efficiency

Table 1 reports respiratory characteristics of tadpole mitochondria. Rates of oxygen consumption during maximal ADP-stimulated (state 3) and resting (state 4 in the presence of oligomycin) states were on average 1.8-fold higher in 2,4-dNP-treated than in control tadpoles (state 3, $t_{18}=-2.56$, $P<0.05$ and state 4, $t_{17}=-2.70$, $P<0.05$). The activity of mitochondrial cytochrome *c* oxidase was almost doubled in the 2,4-dNP-treated compared with the control group ($t_{18}=-2.86$, $P<0.05$, Table 1). The respiratory control ratio, i.e. the state 3 to state 4 ratio, was not significantly different between experimental groups (control $=3.6 \pm 0.6$ and 2,4-dNP-treated $=3.9 \pm 0.6$, $t_{16}=-0.37$, $P=0.72$).

Fig. 2 shows the linear relationship between the rates of ATP synthesis and oxygen consumption in tadpole mitochondria working at different steady-state rates of ATP production. After 34 days of 2,4-dNP treatment, the linear relationship concerning the 2,4-dNP-treated group was shifted to the right compared with control. This means that mitochondria from the 2,4-dNP-treated group had to consume a higher quantity of oxygen for any given amount of ATP synthesized; the yield of oxidative phosphorylation was therefore clearly decreased in 2,4-dNP-treated tadpoles. Nevertheless, the slope values of the linear relationships shown in Fig. 2 were slightly but not significantly different between 2,4-dNP-treated (ATP/O $=1.17 \pm 0.06$) and control tadpoles (ATP/O $=1.37 \pm 0.07$, $t_{18}=0.79$, $P=0.44$, Fig. 2). Additionally, and in accordance with the respiratory parameters reported above, maximal rates of oxygen consumption and ATP synthesis, the highest points to the right of the linear relationship, tended to be increased by 2,4-dNP treatment (+63% and +38%, respectively) when compared with corresponding control values ($t_{18}=-2.04$, $P=0.06$ and $t_{18}=-1.70$, $P=0.11$, respectively). In the presence of oligomycin, the non-phosphorylating respiration rate, the lowest point to the left of the

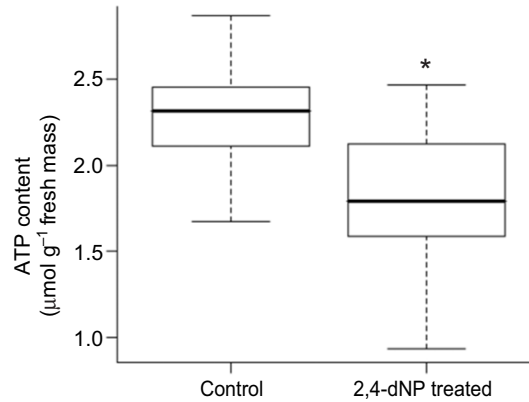


Fig. 3. Whole-body ATP level in control and 2,4-dNP-treated tadpoles. The experimental conditions are described in Materials and methods. Results are means \pm s.e.m. ($N=14$). *Significant difference between tadpole groups for $P<0.05$.

linear relationship, was significantly higher in 2,4-dNP tadpole mitochondria than in control mitochondria (+88%, $t_{18}=-2.50$, $P<0.05$, Fig. 2). Again, respiration control ratios were not significantly different between groups (3.9 ± 0.5 in control and 3.4 ± 0.4 in the 2,4-dNP group, respectively, $t_{18}=0.79$, $P=0.44$).

Whole-body lactate and ATP content

The whole-body lactate content was not significantly different between 2,4-dNP-treated tadpoles (7.33 ± 0.28 nmol lactate mg^{-1} fresh mass) and control tadpoles (7.94 ± 0.49 nmol lactate mg^{-1} fresh mass, $t_{26}=3.29$, $P=0.29$), whereas 2,4-dNP-treated tadpoles displayed a lower ATP content than control tadpoles (-21% , $t_{26}=3.29$, $P<0.05$, Fig. 3). These results indicate that 2,4-dNP-treated tadpoles did not increase their glycolytic pathway activity.

Whole-body cytochrome *c* oxidase activity

As cytochrome *c* oxidase is a mitochondrial inner membrane enzymatic complex, its activity expressed per gram of tadpoles might be proportional to the amount of inner membrane within a tadpole, which can then be viewed as a crude index of mitochondrial biogenesis. In our experimental conditions, whole-organism cytochrome *c* oxidase activity was not significantly different between 2,4-dNP-treated and control tadpoles (42.2 ± 3.2 versus 38.5 ± 3.3 nmol O $\text{min}^{-1} \text{g}^{-1}$ fresh mass, $t_{28}=0.66$, $P=0.42$).

Oxidative balance

Table 2 shows the overall results of the physiological parameters describing the tadpole oxidative balance, including the mitochondrial production of free oxygen radicals (H_2O_2), the total antioxidant capacity and the damage related to oxidative stress (lipid peroxides). The endogenous H_2O_2 generation was lower in 2,4-dNP-treated mitochondria than in controls ($t_{18}=2.39$, $P<0.05$). Tadpole total antioxidant capacity showed a clear tendency to decrease at 34 days of 2,4-dNP exposure in comparison to control ($t_{34}=1.82$, $P=0.08$). Finally, lipid oxidative damage estimated by the malondialdehyde content was 47% lower in 2,4-dNP-treated than in control tadpoles ($t_{27}=3.92$, $P<0.001$).

DISCUSSION

In the present work, we aimed to obtain a better understanding of the proximate mechanisms that underlie life history traits. We experimentally modulated *in vivo* the rate of mitochondrial ROS

generation and the efficiency of ATP synthesis of tadpoles. Our data show that chronic treatment with 2,4-dNP negatively impacted larval development and growth of tadpoles despite an increase in metabolic rate and mitochondrial activity, and a reduction in ROS generation, antioxidant mechanisms and oxidative damage. From these results, we suggest that the mitochondrial oxidative phosphorylation efficiency would be an important proximate factor for constraining life history trajectories.

Most studies on proximal factors of life history trade-offs suggest that ROS production is related to the different components of animal life history, such as the cost of sustained growth (Metcalf and Monaghan, 2003; Alonso-Alvarez et al., 2007; De Block and Stoks, 2008; Costantini et al., 2010; Kim et al., 2011) and the proximate cost of reproduction (Alonso-Alvarez et al., 2004; Metcalfe and Alonso-Alvarez, 2010). Thus, ROS could constitute a primary and universal constraint in life history evolution (Dowling and Simmons, 2009; Monaghan et al., 2009; McGraw et al., 2010). During oxidative phosphorylation processes, the rate of mitochondrial ROS generation is strongly sustained by a reduced state of the electron transport chain and a high inner membrane potential (Boveris et al., 1976; Korshunov et al., 1997; Barja, 2007). Hence, an increased proton flux across the inner membrane *via* the F_0F_1 -ATP synthase (state 3 respiration) or other proton leak pathways such as uncoupling proteins or mitochondrial uncouplers, including the protonophore 2,4-dNP, would attenuate ROS production by lowering the membrane potential (Korshunov et al., 1997; Brand, 2005; Rey et al., 2010). The decreased ROS generation found in 2,4-dNP-treated tadpoles is thus in accordance with previously reported data in isolated mitochondria, organs or whole animals (Okuda et al., 1992; Batandier et al., 2006; da Silva et al., 2008). Interestingly, chronically treated tadpoles exhibited a better oxidative status, i.e. lower generation of ROS and lipid oxidative damage, together with a reduced investment in antioxidant defences. Despite this 'positive antioxidant effect' of 2,4-dNP treatment, the development of treated tadpoles was slower than that in the control group, suggesting that the proximate cost of oxidative balance *per se* was not strong enough to constrain this life history trait, at least in the tadpoles with mild mitochondrial uncoupling used here.

Another aspect of 2,4-dNP-induced mild mitochondrial uncoupling is the increased cost of mitochondrial ATP production that could ultimately decrease the cellular availability of ATP (Sibille et al., 1995; Geelen et al., 2008). In this context, there would be three main cellular ways to compensate for the reduced mitochondrial ATP resulting from 2,4-dNP treatment: activation of glycolysis, mitochondrial biogenesis and/or increase of the oxidative phosphorylation capacity of individual mitochondria. In the present study, 2,4-dNP-treated tadpoles exhibited the same whole-body lactate content, indicating that after 34 days of 2,4-dNP treatment the glycolytic pathway was not up-regulated *in vivo*. We also found no difference in whole-body cytochrome *c* oxidase activity between 2,4-dNP-treated and control tadpoles. On the contrary, the decreased oxidative phosphorylation efficiency of mitochondria from 2,4-dNP-treated tadpoles was balanced by an increase in the rate of oxygen consumption and in mitochondrial cytochrome *c* oxidase activity, when measured on isolated mitochondria. Altogether, these results suggest that 2,4-dNP treatment elicited higher cytochrome *c* oxidase activity per mitochondria but lower mitochondrial biogenesis, i.e. a lower number of mitochondria in whole treated tadpoles. In other words, 2,4-dNP-treated tadpoles exhibited a lower amount of mitochondria with higher oxidative capacity than control animals. Furthermore, this increase in mitochondrial oxidative activity

was associated with a trend towards an increase in the rate of ATP synthesis, showing that the activity of the whole oxidative phosphorylation process was improved by 34 days of 2,4-dNP treatment. As previously reported in cell systems or transgenic animals (Li et al., 1999; Rossmesl et al., 2002; Desquiret et al., 2006; Rohas et al., 2007), it clearly appears here that chronic mild mitochondrial uncoupling has driven metabolic adjustment of tadpoles towards increased oxidative metabolism.

Although such a response would enhance the ability of mitochondria to produce ATP, it failed to provide cells with useable energy *in vivo*, as measured by the lower whole-body ATP content of 2,4-dNP-treated tadpoles (Fig. 3). Thus, the above-reported increase in oxidative phosphorylation activity of mitochondria does not appear to have physiological significance for the overall ATP production capacity in tadpoles. In this context, a decrease in energy availability could well explain the lower rate of growth and development found in 2,4-dNP-treated tadpoles. It is clear that exposure to 2,4-dNP decreased the mitochondrial oxidative phosphorylation efficiency, implying that for any given amount of ATP produced, mitochondria have to consume a higher amount of oxygen (Fig. 2). At the level of tadpoles, all of the cellular energy-demanding processes would therefore require an increased energy input (energetic substrates, oxygen consumption) in the presence of 2,4-dNP, increasing the overall energetic cost of living. This is clearly illustrated by the higher basal metabolic rate of animals chronically exposed to 2,4-dNP for 16 or 34 days (Fig. 1C), demonstrating that the energetic cost of living was indeed increased. In this context, as in 2,4-dNP-treated mice (da Silva et al., 2008), tadpoles with mild mitochondrial uncoupling did not increase their food consumption, and thus failed to compensate for the energy loss elicited by the decrease in efficiency of the ATP production process. In other words, the absence of food intake compensation suggests that inside the organism, the increased respiratory capacity of mitochondria would have been limited by substrate supply, lowering in turn the positive impact of such metabolic adjustment of mitochondria on ATP production in the presence of 2,4-dNP. Hence, the cost of ATP production rather than the rate of oxidative phosphorylation appears to constrain the development of tadpoles in the present study.

Overall, our results indicate that treatment with low doses of 2,4-dNP has beneficial effects on animal oxidative balance and mitochondrial maximal oxidative phosphorylation activity. Yet, the decreased efficiency of mitochondrial energy conversion leads to an increase in the energy cost of living, resulting in a decrease in cellular availability of ATP *in vivo* that would negatively affect development and growth. Contrary to the expectations of numerous studies, oxidative stress, by itself, does not appear to be a physiological constraint to growth rate. The results of the present study therefore suggest that the efficiency of mitochondrial energy transduction is an important parameter that constrains growth and development of tadpoles. In conclusion, mitochondrial oxidative phosphorylation efficiency appears to be a promising way to acquire an in-depth understanding of the proximate factors that may shape the pattern of life history traits. Furthermore, traits such as size at metamorphosis and duration of the larval period are often used as a proxy of the future individual fitness for species with complex life cycles (Altwegg and Reyer, 2003; Ficetola and De Bernardi, 2006; Van Allen et al., 2010). Future studies are needed to clarify whether alteration of mitochondrial efficiency in the adult stage affects the different components of individual fitness, by measuring, for instance, size and age at first breeding, fecundity, and number and size of eggs.

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REFERENCES

- Ackermann, M., Bijlsma, R., James, A. C., Partridge, L., Zwaan, B. J. and Stearns, S. C. (2001). Effects of assay conditions in life history experiments with *Drosophila melanogaster*. *J. Evol. Biol.* **14**, 199-209.
- Agency for Toxic Substances and Disease (1995). *Toxicological Profile for Dinitrophenols*. United States Department of Health and Human Services, Division of Toxicology/Toxicology Information Branch, Atlanta, United States, 1-233.
- Alonso-Alvarez, C., Bertrand, S., Devevey, G., Prost, J., Faivre, B. and Sorci, G. (2004). Increased susceptibility to oxidative stress as a proximate cost of reproduction. *Ecol. Lett.* **7**, 363-368.
- Alonso-Alvarez, C., Bertrand, S., Devevey, G., Prost, J., Faivre, B., Chastel, O. and Sorci, G. (2006). An experimental manipulation of life-history trajectories and resistance to oxidative stress. *Evolution* **60**, 1913-1924.
- Alonso-Alvarez, C., Bertrand, S., Faivre, B. and Sorci, G. (2007). Increased susceptibility to oxidative damage as a cost of accelerated somatic growth in zebra finches. *Funct. Ecol.* **21**, 873-879.
- Altwegg, R. and Reyer, H. U. (2003). Patterns of natural selection on size at metamorphosis in water frogs. *Evolution* **57**, 872-882.
- Balaban, R. S., Nemoto, S. and Finkel, T. (2005). Mitochondria, oxidants, and aging. *Cell* **120**, 483-495.
- Barja, G. (2007). Mitochondrial oxygen consumption and reactive oxygen species production are independently modulated: Implications for aging studies. *Rejuvenation Res.* **10**, 215-223.
- Batandier, C., Guigas, B., Detaille, D., El-Mir, M. Y., Fontaine, E., Rigoulet, M. and Leverage, X. M. (2006). The ROS production induced by a reverse-electron flux at respiratory-chain complex 1 is hampered by metformin. *J. Bioenerg. Biomembr.* **38**, 33-42.
- Boveris, A., Cadenas, E. and Stoppani, A. O. M. (1976). Role of ubiquinone in mitochondrial generation of hydrogen-peroxide. *Biochem. J.* **156**, 435-444.
- Brand, M. D. (2000). Uncoupling to survive? The role of mitochondrial inefficiency in ageing. *Exp. Gerontol.* **35**, 811-820.
- Brand, M. D. (2005). The efficiency and plasticity of mitochondrial energy transduction. *Biochem. Soc. Trans.* **33**, 897-904.
- Clerc, P., Rigoulet, M., Leverage, X. and Fontaine, E. (2007). Nitric oxide increases oxidative phosphorylation efficiency. *J. Bioenerg. Biomembr.* **39**, 158-166.
- Cody, M. L. (1966). A general theory of clutch size. *Evolution* **20**, 174-184.
- Costantini, D., Carello, L. and Fanfani, A. (2010). Relationships among oxidative status, breeding conditions and life-history traits in free-living great tits *Parus major* and common starlings *Sturnus vulgaris*. *Ibis* **152**, 793-802.
- da Silva, C. C. C., Cerqueira, F. M., Barbosa, L. F., Medeiros, M. H. G. and Kowaltowski, A. J. (2008). Mild mitochondrial uncoupling in mice affects energy metabolism, redox balance and longevity. *Aging Cell* **7**, 552-560.
- De Block, M. and Stoks, R. (2008). Compensatory growth and oxidative stress in a damselfly. *Proc. Biol. Sci.* **275**, 781-785.
- Desquiret, V., Loiseau, D., Jacques, C., Douay, O., Malthiery, Y., Ritz, P. and Roussel, D. (2006). Dinitrophenol-induced mitochondrial uncoupling in vivo triggers respiratory adaptation in HepG2 cells. *Biochim. Biophys. Acta* **1757**, 21-30.
- Dowling, D. K. and Simmons, L. W. (2009). Reactive oxygen species as universal constraints in life-history evolution. *Proc. Biol. Sci.* **276**, 1737-1745.
- Eklöv, P. and Halvarsson, C. (2000). The trade-off between foraging activity and predation risk for *Rana temporaria* in different food environments. *Can. J. Zool.* **78**, 734-739.
- Emel'yanova, L. V., Savina, M. V., Belyaeva, E. A. and Brailovskaya, I. V. (2007). Peculiarities of functioning of liver mitochondria of the river lamprey *Lampetra fluviatilis* and the common frog *Rana temporaria* at periods of suppression and activation of energy metabolism. *J. Evol. Biochem. Physiol.* **43**, 564-572.
- Ficetola, G. F. and De Bernardi, F. (2006). Trade-off between larval development rate and post-metamorphic traits in the frog *Rana latastei*. *Evol. Ecol.* **20**, 143-158.
- Geelen, M. J. H., Harris, R. A. and Van den Bergh, S. G. (2008). Enigmatic effect of cellular ATP on fatty acid biosynthesis: Stimulation by moderate decrease and inhibition by increase of cellular ATP. *FEBS Lett.* **582**, 2242-2246.
- Gosner, K. L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* **16**, 183-190.
- Harman, D. (1956). Aging - a theory based on free-radical and radiation-chemistry. *J. Gerontol.* **11**, 298-300.
- Heise, K., Puntarulo, S., Portner, H. O. and Abele, D. (2003). Production of reactive oxygen species by isolated mitochondria of the Antarctic bivalve *Laternula elliptica* (King and Broderip) under heat stress. *Comp. Biochem. Physiol.* **134C**, 79-90.
- Hulbert, A. J., Pamplona, R., Buffenstein, R. and Buttemer, W. A. (2007). Life and death: metabolic rate, membrane composition, and life span of animals. *Physiol. Rev.* **87**, 1175-1213.
- Jezek, P. and Hlavata, L. (2005). Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism. *Int. J. Biochem. Cell Biol.* **37**, 2478-2503.
- Kim, S. Y., Noguera, J. C., Morales, J. and Velando, A. (2011). Quantitative genetic evidence for trade-off between growth and resistance to oxidative stress in a wild bird. *Evol. Ecol.* **25**, 461-472.
- Korshunov, S. S., Skulachev, V. P. and Starkov, A. A. (1997). High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS Lett.* **416**, 15-18.
- Lehninger, A. L., Nelson, D. L. and Cox, M. M. (1993). *Principles of Biochemistry*, 2nd edn. New York, USA: Worth Publishers.
- Li, B., Holloszy, J. O. and Semenkovich, C. F. (1999). Respiratory uncoupling induces delta-aminolevulinic synthase expression through a nuclear respiratory factor-1-dependent mechanism in HeLa cells. *J. Biol. Chem.* **274**, 17534-17540.
- McGraw, K. J., Cohen, A. A., Costantini, D. and Horak, P. (2010). The ecological significance of antioxidants and oxidative stress: a marriage between mechanistic and functional perspectives. *Funct. Ecol.* **24**, 947-949.
- Metcalfe, N. B. and Alonso-Alvarez, C. (2010). Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. *Funct. Ecol.* **24**, 984-996.
- Metcalfe, N. B. and Monaghan, P. (2003). Growth versus lifespan: perspectives from evolutionary ecology. *Exp. Gerontol.* **38**, 935-940.
- Monaghan, P., Metcalfe, N. B. and Torres, R. (2009). Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecol. Lett.* **12**, 75-92.
- Nogueira, V., Piquet, M. A., Devin, A., Fiore, C., Fontaine, E., Brandolin, G., Rigoulet, M. and Leverage, X. M. (2001). Mitochondrial adaptation to in vivo polyunsaturated fatty acid deficiency: increase in phosphorylation efficiency. *J. Bioenerg. Biomembr.* **33**, 53-61.
- Ohkawa, H., Ohishi, N. and Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **95**, 351-358.
- Okuda, M., Lee, H. C., Kumar, C. and Chance, B. (1992). Comparison of the effect of a mitochondrial uncoupler, 2,4-dinitrophenol and adrenaline on oxygen radical production in the isolated perfused rat liver. *Acta Physiol. Scand.* **145**, 159-168.
- Rey, B., Roussel, D., Romestaing, C., Belouze, M., Rouanet, J. L., Desplanches, D., Sibille, B., Servais, S. and Duchamp, C. (2010). Up-regulation of avian uncoupling protein in cold-acclimated and hyperthyroid ducklings prevents reactive oxygen species production by skeletal muscle mitochondria. *BMC Physiol.* **10**, e5.
- Rigoulet, M., Leverage, X., Fontaine, E., Ouhabi, R. and Guerin, B. (1998). Quantitative analysis of some mechanisms affecting the yield of oxidative phosphorylation: dependence upon both fluxes and forces. *Mol. Cell. Biochem.* **184**, 35-52.
- Robert, K. A. and Bronikowski, A. M. (2010). Evolution of senescence in nature: physiological evolution in populations of garter snake with divergent life histories. *Am. Nat.* **175**, 147-159.
- Rohas, L. M., St-Pierre, J., Uldry, M., Jager, S., Handschin, C. and Spiegelman, B. M. (2007). A fundamental system of cellular energy homeostasis regulated by PGC-1 alpha. *Proc. Natl. Acad. Sci. USA* **104**, 7933-7938.
- Rolfe, D. F. S. and Brown, G. C. (1997). Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiol. Rev.* **77**, 731-758.
- Rossmel, M., Barbatelli, G., Flach, P., Brauner, P., Zingaretti, M. C., Marelli, M., Janovska, P., Horakova, M., Syrový, I., Cinti, S. et al. (2002). Expression of the uncoupling protein 1 from the aP2 gene promoter stimulates mitochondrial biogenesis in unilocular adipocytes in vivo. *Eur. J. Biochem.* **269**, 19-28.
- Salin, K., Teulier, L., Rey, B., Rouanet, J. L., Voituron, Y., Duchamp, C. and Roussel, D. (2010). Tissue variation of mitochondrial oxidative phosphorylation efficiency in cold-acclimated ducklings. *Acta Biochim. Pol.* **57**, 409-412.
- Sibille, B., Keriel, C., Fontaine, E., Catelloni, F., Rigoulet, M. and Leverage, X. M. (1995). Octanoate affects 2,4-dinitrophenol uncoupling in intact isolated rat hepatocytes. *Eur. J. Biochem.* **231**, 498-502.
- Sims, N. R. and Anderson, M. F. (2008). Isolation of mitochondria from rat brain using Percoll density gradient centrifugation. *Nat. Protoc.* **3**, 1228-1239.
- Sokolova, I. M. (2004). Cadmium effects on mitochondrial function are enhanced by elevated temperatures in a marine poikilotherm, *Crasostrea virginica* Gmelin (Bivalvia: Ostreidae). *J. Exp. Biol.* **207**, 2639-2648.
- Sommer, A. M. and Portner, H. O. (2004). Mitochondrial function in seasonal acclimatization versus latitudinal adaptation to cold in the lugworm *Arenicola marina* (L.). *Physiol. Biochem. Zool.* **77**, 174-186.
- Speakman, J. R. (2005). Body size, energy metabolism and lifespan. *J. Exp. Biol.* **208**, 1717-1730.
- Stearns, S. C. (1992). *The Evolution of Life Histories*, pp. 1-262. Oxford: Oxford University Press.
- Teulier, L., Rouanet, J. L., Letexier, D., Romestaing, C., Belouze, M., Rey, B., Duchamp, C. and Roussel, D. (2010). Cold-acclimation-induced non-shivering thermogenesis in birds is associated with upregulation of avian UCP but not with innate uncoupling or altered ATP efficiency. *J. Exp. Biol.* **213**, 2476-2482.
- Van Allen, B. G., Briggs, V. S., McCoy, M. W. and Vonesh, J. R. (2010). Carry-over effects of the larval environment on post-metamorphic performance in two hylid frogs. *Oecologia* **164**, 891-898.
- Wallace, K. B. and Starkov, A. A. (2000). Mitochondrial targets of drug toxicity. *Annu. Rev. Pharmacol. Toxicol.* **40**, 353-388.
- Zera, A. J. and Harshman, L. G. (2001). The physiology of life history trade-offs in animals. *Annu. Rev. Ecol. Syst.* **32**, 95-126.
- Zhou, M. J., Diwu, Z. J., PanchukVoloshina, N. and Haugland, R. P. (1997). A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. *Anal. Biochem.* **253**, 162-168.

Table S1: Analyses of covariance of effect of period (after 16 and 34 days of exposure), treatment (water and ethanol) and their interaction on larval development stage, body mass and metabolic rate.

<i>Factors</i>	Stade	VO₂	Mass
<i>Period</i>	F _{1,74} =514.506 <i>P</i> <0.001	F _{1,73} =200.008 <i>P</i> <0.001	F _{1,73} =8.288 <i>P</i> =0.005
<i>Treatment</i>	F _{1,74} =0.755 <i>P</i> =0.387	F _{1,73} =0.194 <i>P</i> =0.661	F _{1,73} =0.0001 <i>P</i> =0.992
<i>Period * Treatment</i>	F _{1,74} =0.590 <i>P</i> =0.448	F _{1,73} =2.022 <i>P</i> =0.159	F _{1,73} =0.0187 <i>P</i> =0.890

Table S2: Statistical analyses of effect of ethanol treatment on tadpole parameters. Variables have been measured only at 34 days of exposure; the treatment effect has been analyzed using Student's t test.

<i>Factors</i>	df	t	P
<i>Mitochondrial oxygen consumption at state 3</i>	16	0.55	0.59
<i>Mitochondrial oxygen consumption at state 4</i>	16	0.00	0.95
<i>Mitochondrial cytochrome c oxidative activity</i>	16	1.44	0.17
<i>Respiratory Control Ratio</i>	16	1.14	0.27
<i>Slope of the linear relation ATP/O</i>	16	1.52	0.14
<i>Mitochondrial oxygen consumption at state 3 (in presence of glucose/hexokinase)</i>	16	0.62	0.54
<i>Mitochondrial oxygen consumption at state 4 (in presence of glucose/hexokinase)</i>	16	0.52	0.61
<i>Mitochondrial ATP synthesis at state 3</i>	16	0.10	0.93
<i>Mitochondrial ROS generation</i>	16	0.72	0.48
<i>Lipid oxidative damage</i>	24	0.56	0.58
<i>Total antioxidant capacities</i>	29	0.55	0.59