© 2014. Published by The Company of Biologists Ltd | The Journal of Experimental Biology (2014) 217, 3579-3589 doi:10.1242/jeb.103945

RESEARCH ARTICLE

Mitochondrial uncoupling as a regulator of life-history trajectories in birds: an experimental study in the zebra finch

Antoine Stier[1,‡], Pierre Bize[2,*], Damien Roussel[3], Quentin Schull[4,5], Sylvie Massemin[4,5] and François Criscuolo[4,5]

ABSTRACT

Mitochondria have a fundamental role in the transduction of energy from food into ATP. The coupling between food oxidation and ATP production is never perfect, but may nevertheless be of evolutionary significance. The 'uncoupling to survive' hypothesis suggests that 'mild' mitochondrial uncoupling evolved as a protective mechanism against the excessive production of damaging reactive oxygen species (ROS). Because resource allocation and ROS production are thought to shape animal life histories, alternative life-history trajectories might be driven by individual variation in the degree of mitochondrial uncoupling. We tested this hypothesis in a small bird species, the zebra finch (*Taeniopygia guttata*), by treating adults with the artificial mitochondrial uncoupler 2,4-dinitrophenol (DNP) over a 32-month period. In agreement with our expectations, the uncoupling treatment increased metabolic rate. However, we found no evidence that treated birds enjoyed lower oxidative stress levels or greater survival rates, in contrast to previous results in other taxa. *In vitro* experiments revealed lower sensitivity of ROS production to DNP in mitochondria isolated from skeletal muscles of zebra finch than mouse. In addition, we found significant reductions in the number of eggs laid and in the inflammatory immune response in treated birds. Altogether, our data suggest that the 'uncoupling to survive' hypothesis may not be applicable for zebra finches, presumably because of lower effects of mitochondrial uncoupling on mitochondrial ROS production in birds than in mammals. Nevertheless, mitochondrial uncoupling appeared to be a potential life-history regulator of traits such as fecundity and immunity at adulthood, even with food supplied *ad libitum*.

KEY WORDS: Mitochondria, Uncoupling, Free radical theory of ageing, Oxidative stress, Reactive oxygen species, Life-history trade-off, Bird

INTRODUCTION

For most animals, the main site of energy transduction (i.e. the conversion of acquired nutrients into usable ATP by their cells) is undoubtedly the mitochondria (Stearns, 1992; Nicholls and Ferguson, 2002). Briefly, mitochondria use electrons harvested from oxidizable substrates and O_2 as a final electron acceptor to build up a proton-motive force by pumping protons from the mitochondrial matrix into the intermembrane space. The subsequent backflow of protons to the matrix across the protein complex 'ATP synthase' of the inner membrane drives the synthesis of ATP. This whole process is referred to as oxidative phosphorylation (Williams, 1966; Stearns, 1992; Nicholls and Ferguson, 2002). Hence, one main function of mitochondria is to couple respiration (i.e. O_2 consumption) and nutrient oxidation to ATP synthesis, but interestingly, this coupling (also defined as mitochondrial efficiency) demonstrates a certain degree of plasticity (Brand, 2000; Divakaruni and Brand, 2011; Helle et al., 2012).

According to the idea that limited resources can constrain individual investment toward competing life-history traits (Stearns, 1992), one could suggest that natural selection should favour a tight coupling between O_2 consumption and ATP synthesis (i.e. a high mitochondrial efficiency), in order to maximize resource investments into the different life-history traits (e.g. growth or fecundity) and ultimately the fitness of individuals. However, proton leakage might also have beneficial consequences; for instance, it has been shown that the production of reactive oxygen species (ROS) by the mitochondria is sharply decreased at low membrane potential (Boveris and Chance, 1973; Skulachev, 1996; Korshunov et al., 1997; Nicholls and Ferguson, 2002). ROS are inevitable by-products of aerobic metabolism that are generated when electrons escape the mitochondrial electron transport chain during oxidative phosphorylation and react directly with molecular oxygen (Beckman and Ames, 1998; Nicholls and Ferguson, 2002; Halliwell and Gutteridge, 2007). When ROS production exceeds the capacity of antioxidant defences, it leads to a situation of 'oxidative stress' characterized by the occurrence of oxidative damage to diverse biomolecules such as nucleic acids, lipids and proteins (Sies, 1985). According to the 'free radical theory of ageing' (Harman, 1956), oxidative stress is thought to impair organism functioning and contribute to the ageing process (Beckman and Ames, 1998; Balaban et al., 2005). In this context, mitochondrial proton leak might help to protect the organism against oxidative stress and ageing, as proposed by the 'uncoupling to survive' hypothesis (Brand, 2000).

Because mitochondrial coupling state is a key regulator of ATP and ROS production and because the amounts of both ATP and ROS produced are likely to influence life histories (Brand, 2005; Salin et al., 2012a; Salin et al., 2012b), one hypothesis is that inter-individual variation in mitochondrial coupling state might account for the variations in life-history trajectories frequently observed between individuals. In particular, individuals with a highly uncoupled metabolism are expected to produce fewer ROS and ATP molecules, which in turn could select for greater longevity but also a lower amount of energy to invest into growth, immune function or reproduction. Few studies have addressed the

[1]University of Angers, Groupe Écologie et Conservation des Vertébrés (GECCO), 49045 Angers, Cedex 01, France. [2]Department of Ecology and Evolution, University of Lausanne, Biophore 1015 Lausanne-Dorigny, Switzerland. [3]Laboratoire d'Écologie des Hydrosystèmes Naturels et Anthropisés, CNRS UMR 5023, Université de Lyon, 69622 Villeurbanne Cedex, France. [4]University of Strasbourg, Institut Pluridisciplinaire Hubert Curien, 67037 Strasbourg Cedex, France. [5]Département d'Ecologie, Physiologie et Ethologie (DEPE), CNRS UMR 7178, 23 rue Becquerel, 67087 Strasbourg Cedex 2, France.
*Present address: Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen AB24 2TZ, UK.

‡Author for correspondence (antoine.stier@gmail.com)

Received 5 March 2014; Accepted 17 July 2014

The Journal of Experimental Biology (2014) doi:10.1242/jeb.103945

List of symbols and abbreviations

DNP	2,4-dinitrophenol
GEE	generalized estimating equation
GSH	glutathione
GSSG	oxidized glutathione
MDA	malondialdehyde
NAbs	natural antibodies
PHA	phytohaemagglutinin
RF	red fluorescence
ROM	reactive oxygen metabolite
ROS	reactive oxygen species
UCP2	uncoupling protein 2
$\dot{V}_{O_2}$	oxygen consumption

importance of mitochondria functioning/efficiency in shaping animal life histories. Experiments in which individuals were treated with a mitochondrial uncoupler [i.e. the artificial protonophore 2,4-dinitrophenol (DNP)] demonstrated that mild mitochondrial uncoupling increases metabolic rate in *Drosophila* (Padalko, 2005), *Rana temporaria* tadpoles (Salin et al., 2012a) and laboratory mice (Caldeira da Silva et al., 2008), and concomitantly decreases oxidative stress levels (mice and tadpoles) and extends lifespan (*Drosophila* and mice). However, so far no studies have addressed the impact of such an uncoupling treatment on other life-history traits such as growth rate, reproductive performance or immune responsiveness.

Here, we investigated the impact of a long-term experimental exposure to the mitochondrial uncoupler DNP on the life-history trajectories of a small passerine bird: the zebra finch (*Taeniopygia guttata* Reichenbach 1862). We hypothesized that increasing mitochondrial uncoupling state would lead to elevated metabolic rate, decreased oxidative stress levels and increased longevity, but at the expense of the investment toward other life-history traits such as growth, reproduction and immunity. We chronically treated adult birds with DNP for more than 2 years. We evaluated parameters related to metabolism (body mass dynamics, food consumption and metabolic rate), oxidative stress markers (ROS production, antioxidant defences and oxidative damage) and a full set of life-history traits (survival, reproductive performances and immune competence). Additionally, we investigated the effects of our treatment on the metabolic rate and growth of chicks treated at the same time as their parents. Finally, we also tested the effects of DNP on mitochondrial functioning *in vitro*, in order to compare the effects of DNP on mitochondria isolated from skeletal muscles between the zebra finch and a size-matched mammalian model, the mouse. Indeed, preliminary *in vivo* analyses resulted in contrasted findings between what we were observing in birds (present study; P.B., unpublished results in Japanese quails, *Coturnix japonica*) and results previously reported for mice (Caldeira da Silva et al., 2008), thereby suggesting the need for insights into the effects of DNP at the mitochondrial level.

RESULTS

Phenotypic parameters

Body mass and food consumption

Body mass was not significantly affected by uncoupling treatment (control=14.9±0.2 g versus DNP=14.7±0.1 g, P=0.32) or the time period (P=0.23). Females were significantly heavier than males (males=14.5±0.2 g, females=15.1±0.1 g, P=0.002; Table 1). DNP-treated birds had significantly higher food consumption than control birds (control=3.93±0.10 g day^{-1}, DNP=4.47±0.10 g day^{-1}, P<0.001; Table 1). Daily food consumption was also higher for males and positively correlated with body mass (P=0.040 and 0.009, respectively; Table 1).

Metabolism

DNP-treated adult birds presented overall elevated metabolic rates, either in terms of mean or resting oxygen consumption ($\dot{V}_{O_2}$), but only after the beginning of the treatment as revealed by the significant time × treatment interactions (P=0.045 and 0.004, respectively; Table 2, Fig. 1). Body mass was a significant predictor of mean (P<0.001) and resting $\dot{V}_{O_2}$ (P=0.005; Table 2). Of note, the metabolic chamber in which the bird was measured affected our absolute measurements of $\dot{V}_{O_2}$, and thus we controlled for this effect in our analyses (P<0.001; Table 2) and in our experimental design (see Materials and methods).

The resting metabolism of chicks measured at day 12 post-hatching was affected by the treatment of their parents, with chicks presenting higher resting $\dot{V}_{O_2}$ in the DNP than the control treatment (treatment effect: F=9.90, P=0.021; mass effect: F=10.34, P=0.008; Fig. 1).

Oxidative balance

Mitochondrial superoxide production of adult birds was not significantly affected by treatment [control=0.130±0.040 red fluorescence (RF) min^{-1} versus DNP=0.139±0.047 RF min^{-1}, F=0.60, P=0.44] or sex (F=1.50, P=0.23). Similarly, the plasma concentration of reactive oxygen metabolites (ROMs) was not significantly affected by experimental treatment (F=0.07, P=0.79; Fig. 2A), sex (F=0.17, P=0.68) or time period (F=1.55, P=0.22). We

Table 1. Results of general linear models testing differences between control and DNP treated zebra finches in terms of body mass dynamics and daily food consumption

		Estimate	SE	F	P
Body mass (g)					
Repeated effect	ID	2.55	0.24		
Fixed effects and covariates	**Constant**	14.00	0.25	20565.18	**<0.001**
	Treatment	0.20	0.21	0.99	0.320
	Sex	0.65	0.21	9.98	**0.002**
	Time	0.52	0.29	1.44	0.230
Daily food consumption (g day^{-1})					
Fixed effects and covariates	**Constant**	3.01	0.58	19.58	**<0.001**
	Treatment	−0.54	0.13	17.78	**<0.001**
	Sex	–0.27	0.13	4.42	**0.040**
	Body mass	0.11	0.04	7.43	**0.009**

Body mass was measured both before and several times after the beginning of the treatment. Estimates for fixed factors are given for the following levels: Treatment, control; Sex, female; Time, 6 months (see Materials and methods for details). Significant factors and associated P-values are in bold.

The Journal of Experimental Biology

The Journal of Experimental Biology (2014) doi:10.1242/jeb.103945

Table 2. Results of general linear models testing differences between control and DNP-treated zebra finches in terms of mean and resting metabolic rate ($\dot{V}_{O_2}$)

		Estimate	SE	*F*	*P*
Mean oxygen consumption					
Repeated effect	Time	0.036	0.005		
Fixed effects and covariates	**Constant**	0.73	0.16	23.81	**<0.001**
	Treatment	−0.02	0.05	6.25	**0.014**
	Sex				n.s.
	Time	0.24	0.05	22.28	**<0.001**
	Time × Treatment	−0.14	0.07	4.11	**0.045**
	Cage	0.32	0.05	51.25	**<0.001**
	Body mass	0.04	0.01	13.21	**<0.001**
Resting oxygen consumption					
Repeated effect	Time	0.036	0.004		
Fixed effects and covariates	**Constant**	0.72	0.16	23.09	**<0.001**
	Treatment	−0.02	0.05	10.71	**0.001**
	Sex				n.s.
	Time	0.25	0.05	16.70	**<0.001**
	Time × Treatment	−0.21	0.07	8.71	**0.004**
	Cage	0.30	0.01	46.66	**<0.001**
	Body mass	0.03	0.01	8.13	**0.005**

Measurements of metabolic rates were conducted both before and after the beginning of the treatment (Time). Estimates for fixed factors are given for the following levels: Treatment, control; Time, after treatment; Time × Treatment, after treatment × control; Chamber, chamber no. 1 (see Materials and methods for details). Significant factors and associated *P*-values are in bold.

found similar results for chicks, as we did not find a significant effect of treatment (F=2.67, P=0.13; Fig. 2A), despite a significant effect of age (F=32.18, P<0.001). The plasma antioxidant capacity of adult birds was not significantly affected by treatment (F=0.15, P=0.70; Fig. 2B), sex (F=1.31, P=0.25) or time period (F=2.68, P=0.10), nor was the antioxidant capacity of chicks (treatment effect: F=0.11, P=0.74; Fig. 2B), despite, again, a significant effect of age (F=148.49, P<0.001).

We did not find any significant changes in oxidative stress markers measured in the pectoral muscle and in the heart following the DNP treatment (Table 3). Indeed, the total glutathione content did not significantly differ between groups, neither in the pectoral muscle nor in the heart (Table 3). Accordingly, the proportion of oxidized glutathione (GSSG/GSH) was not significantly affected by the DNP treatment, neither in the pectoral muscle nor in the heart (Table 3). The enzymatic activity of the glutathione reductase and the level of lipid peroxidation were not significantly affected by the DNP treatment (Table 3).

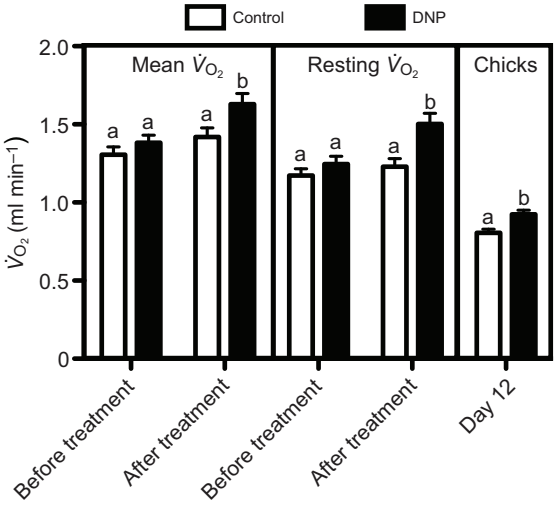

Fig. 1. Effect of 2,4-dinitrophenol (DNP) treatment on metabolic rate ($\dot{V}_{O_2}$) of adult zebra finches (mean and resting $\dot{V}_{O_2}$) and 12-day-old chicks. White and black bars represent control and DNP-treated birds, respectively. Different letters indicate significant differences between groups according to statistical models (see Table 2 and Results for statistics). Means are plotted ±s.e.

Life-history parameters

Survival

Approximately 28% of adult birds (17 of 60) died naturally during our experimental study over a period of 32 months after the beginning of the DNP treatment, yet the mortality rate between DNP-treated and control birds was not significantly different (control=23% versus DNP=33%; Kaplan–Meier survival analysis: χ^2=0.69, P=0.41).

Reproduction

The experimental uncoupling treatment did not significantly affect the number of reproductive events initiated by the breeding pairs during the 8 month period (Wald χ^2=2.05, P=0.15; Fig. 3). However, the total number of eggs produced during this period was significantly lower for the DNP breeding pairs (Wald χ^2=4.05, P=0.044), yet the number of fledged chicks remained unchanged between experimental groups (Wald χ^2=0.02, P=0.88; Fig. 3).

Immunity

DNP treatment significantly reduced the response to a phytohaemagglutinin (PHA) immune challenge (F=6.35, P=0.018; Fig. 4). We did not find any significant effect of the treatment on parameters measured by the haemolysis-haemagglutination assay (lysis score: Wald χ^2=1.63, P=0.20; agglutination score: Wald χ^2=0.01, P=0.91; Fig. 4).

Growth

We did not find significant effects of the DNP treatment on chick growth trajectories measured by their logistic growth rate (K: control=0.426±0.014 versus DNP=0.423±0.019, F=0.05, P=0.83) or their asymptotic mass (A: control=11.98±0.26 g versus DNP=11.98±0.25 g, F=0.00, P=1.00).

The Journal of Experimental Biology (2014) doi:10.1242/jeb.103945

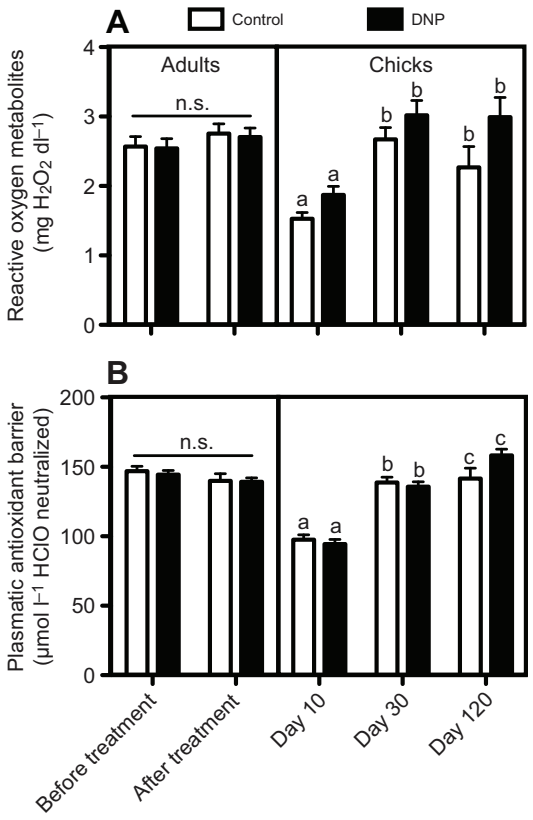

Fig. 2. Effect of DNP treatment on plasmatic oxidative balance parameters of adult zebra finches both before and after the beginning of the treatment (left) and chicks during the growth period (right). (A) Reactive oxygen metabolites; (B) antioxidant capacity. White and black bars represent control and DNP-treated birds, respectively. Different letters indicate significant differences between groups according to statistical models (see Results for statistics). n.s., not significant. Means are plotted ±s.e.

Comparison of DNP impact on mitochondrial functioning measured *in vitro* in zebra finch and mouse

Mitochondrial respiration rate fuelled with pyruvate-malate (Fig. 5A) was not significantly affected by species (F=0.07, P=0.79) or the interaction between species and the concentration of DNP (F=3.10, P=0.06), but was significantly influenced by the increasing concentrations of DNP (DNP: F=19.72, P<0.001). Succinate-fuelled respiration (Fig. 5B) was significantly influenced by species (zebra finch>mouse; F=25.07, P<0.001) and the concentration of DNP (F=36.55, P<0.001), but also by the interaction between these two parameters (species × DNP: F=7.05, P=0.003). Separate analyses for each species revealed that the addition of increasing concentrations of DNP increased respiration (DNP effect: F=28.25, P<0.001 and F=24.90, P<0.001 for mouse and zebra finch, respectively), but apparently to a different extent between species (i.e. significant species × DNP interaction, see above).

When using pyruvate-malate as substrate, mitochondrial H_2O_2 production was significantly different between the two species (mouse<zebra finch, F=105.63, P<0.001, Fig. 5C), H_2O_2 production being also modulated by DNP concentration (F=10.78, P<0.001), but differently according to the species considered (species × DNP: F=5.01, P=0.012). The addition of increasing concentrations of DNP significantly decreased H_2O_2 production for mice (F=63.34, P<0.001), but not for zebra finches (F=2.69, P=0.12; Fig. 8C). Mitochondrial H_2O_2 production using succinate as substrate (Fig. 8D) was significantly decreased by the addition of increasing concentrations of DNP (F=31.39, P<0.001), but was not significantly affected by species (F=2.42, P=0.14) or by the interaction between species and DNP concentration (F=3.20, P=0.06).

DISCUSSION

In the present study, by treating captive zebra finches with the mitochondrial uncoupler DNP over up to 32 months, we aimed to test the importance of mitochondrial coupling state (i.e. efficiency) in determining bird life-history trajectories. As previously reported in the mouse (Caldeira da Silva et al., 2008) and *Rana temporaria* tadpoles (Salin et al., 2012a), and in agreement with the expected pharmacological effects of DNP (for a review, see Harris and Cocoran, 1995), treated birds exhibited significantly higher energy expenditure than control birds. However, we found no evidence that long-term exposure to the uncoupler DNP can help nestling or adult birds to mitigate their exposure to oxidative stress [for positive findings, see Caldeira da Silva et al. and Salin et al. (Caldeira da Silva et al., 2008; Salin et al., 2012a)] and live longer [for positive findings, see Padalko and Caldeira da Silva et al. (Padalko, 2005; Caldeira da Silva et al., 2008)]. Interestingly, our results demonstrate moderate but nonetheless significant effects of elevated mitochondrial uncoupling on life-history trajectories of adult zebra finches, as measured by a reduction in their immune responsiveness to a PHA test and in the total number of eggs produced by females. Additional *in vitro* experiments on isolated mitochondria from adult zebra finches and mice (a size-matched mammalian species) highlight that ROS production might be less sensitive to mitochondrial uncoupling in birds than in mammals. This lower *in vitro* sensitivity to DNP might at least partially explain the

Table 3. Effect of the DNP treatment on different markers of oxidative stress [total glutathione content, proportion of glutathione oxidized, glutathione reductase activity and levels of oxidative damage on lipids (MDA content)] measured in the pectoral muscle and heart of young zebra finches (4 months old)

Oxidative stress marker	Control birds	DNP-treated birds	Treatment effect
Pectoral muscle			
Total glutathione content (mmol mg protein^{-1})	8.24±0.89	7.88±0.42	F=0.13, P=0.72
Proportion of glutathione oxidized (GSSG/total GSH)	0.084±0.001	0.081±0.002	F=1.14, P=0.72
Glutathione reductase activity (mU mg protein^{-1})	0.261±0.019	0.331±0.041	F=2.30, P=0.14
MDA content (nmol mg protein^{-1})	2.28±0.11	2.14±0.18	F=0.43, P=0.52
Heart			
Total glutathione content (mmol mg protein^{-1})	15.12±1.16	16.48±1.22	F=0.70, P=0.41
Proportion of glutathione oxidized (GSSG/total GSH)	0.077±0.002	0.078±0.002	F=0.02, P=0.88
Glutathione reductase activity (mU mg protein^{-1})	0.145±0.027	0.132±0.008	F=0.23, P=0.64
MDA content (nmol mg protein^{-1})	2.59±0.12	2.40±0.17	F=0.81, P=0.38

Mean values ± s.e. are reported for each group, along with the F- and P-values of the general linear models testing the effect of the treatment.

The Journal of Experimental Biology (2014) doi:10.1242/jeb.103945

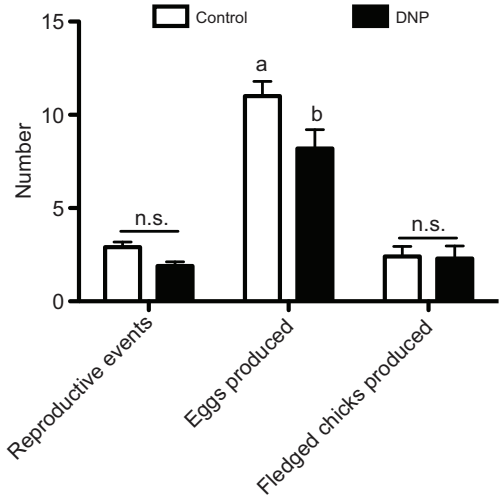

Fig. 3. Reproductive performance (number of reproductive events initiated, number of eggs produced and number of chicks that fledged successfully) of control (white bars) and DNP-treated breeding pairs of zebra finches (black bars). Breeding pairs were randomly constituted and birds were allowed to reproduce freely during a period of 8 months. Different letters indicate significant differences between groups according to statistical models (see Results for statistics). n.s., not significant. Means are plotted ±s.e.

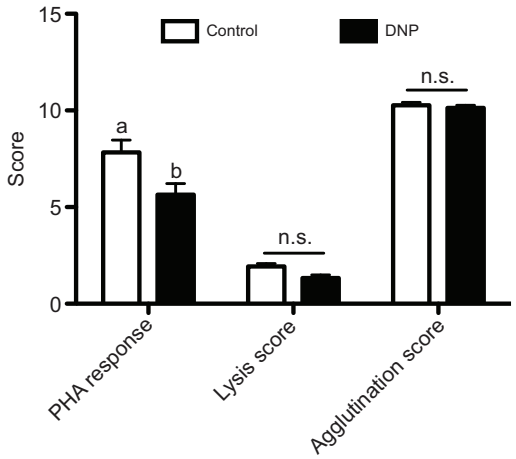

Fig. 4. Immune parameters [response to a phytohaemagglutinin (PHA) challenge and scores from a haemolysis-haemagglutination assay] of control (white bars) and DNP-treated zebra finches (black bars). The PHA response is expressed in tenths of millimetres, for illustration purposes. Different letters indicate significant differences between groups according to statistical models (see Results for statistics and Materials and methods for a full description of the immune assays). n.s., not significant. Means are plotted ± s.e.

discrepancies at the whole-organism level between our results and previous ones in mammals (Caldeira da Silva et al., 2008).

Mitochondrial uncoupling and whole-organism body mass and metabolism

The long-term administration of DNP had no significant effect on body mass dynamics of treated adult birds and chicks compared with control ones. This contrasts with previous findings in mice (Caldeira da Silva et al., 2008) showing a clear reduction in body mass/mass gain of individuals treated with the same molecule. As previously reported in birds (Gleeson, 1986; Dominguez et al., 1993) and many other animals (for a review, see Harris and Cocoran, 1995), our chronic uncoupling DNP treatment led to a moderate but consistent increase in metabolic rates of adult birds and chicks (+22.7% and +20.7% for mean and resting metabolic rate in adults, respectively, and +14.6% for resting metabolic rate in chicks), and we found a similar effect for a sub-group of birds measured a second time (below the thermoneutral zone) 20 months after the beginning of the treatment [25°C: +17.0% in terms of resting $\dot{V}_{O_2}$ (Stier et al., 2014b)]. Because DNP-treated birds increased their food consumption by 13.7% compared with controls, one hypothesis is that a greater energy intake allowed DNP-treated birds to maintain a body mass similar to that of control birds. There was no evidence for feeding compensatory mechanisms in studies on mice (Caldeira da Silva et al., 2008) and frog tadpoles (Salin et al., 2012a). Of note, an increase in mitochondrial biogenesis can also be invoked to compensate the loss of efficiency linked to the DNP treatment, as has been shown for laboratory rats (Schlagowski et al., 2014).

Mitochondrial uncoupling and whole-organism oxidative balance and survival

The 'uncoupling to survive' hypothesis stipulates that mild mitochondrial uncoupling can reduce the production of ROS, and in so doing prevent the accumulation of oxidative damage and allow organisms to live longer (Brand, 2000). Accordingly, laboratory mice with high natural levels of mitochondrial uncoupling were observed to live longer (Speakman et al., 2004), and experimental treatment of laboratory mice with the mitochondrial uncoupler DNP reduced their exposure to oxidative stress and increased their lifespan (Caldeira da Silva et al., 2008) [see also Salin et al. (Salin et al., 2012a) for similar findings on oxidative markers in tadpoles, and Padalko (Padalko, 2005) for similar findings on longevity in *Drosophila*]. Unexpectedly, our uncoupling treatment did not modify significantly the oxidative stress parameters measured in zebra finches, neither in terms of ROS production, nor in terms of oxidative damage and antioxidant defences. However, measuring oxidative damage to DNA and proteins might be necessary to definitively rule out a beneficial impact of the DNP treatment, because multiple biomarkers of oxidative stress might behave differently (Sepp et al., 2012).

At least four different hypotheses can be proposed. First, the DNP dose given to zebra finches could be irrelevant in terms of reducing ROS production (too low or too high). However, this hypothesis seems unlikely because the effect of DNP on energy metabolism was significant but moderate and comparable in magnitude to the effects reported for mice and frog tadpoles (Caldeira da Silva et al., 2008; Salin et al., 2012a). Second, mitochondrial uncoupling has been suggested to be physiologically irrelevant in terms of lowering ROS production *in vivo* (for reviews, see Shabalina and Nedergaard, 2011; Shabalina et al., 2014). In this context, the beneficial effect of the DNP treatment on oxidative stress levels in mice (Caldeira da Silva et al., 2008) could be mediated by the decrease of mass gain (i.e. protection against obesity) rather than a direct uncoupling effect on mitochondrial ROS production. Moreover, the improved oxidative state observed in DNP-treated frog tadpoles (Salin et al., 2012a) may be linked to the decreased developmental rate rather than a direct effect of DNP on mitochondrial ROS production. In contrast, our results on oxidative stress parameters are unlikely to be biased by such confounding effects because body mass dynamics was not modified by the DNP treatment in our study. Third, the beneficial effect of mitochondrial uncoupling at the mitochondrial level could be obscured at the cellular and/or organismal level by an increase in mitochondrial density. As mentioned above, an increase

The Journal of Experimental Biology (2014) doi:10.1242/jeb.103945

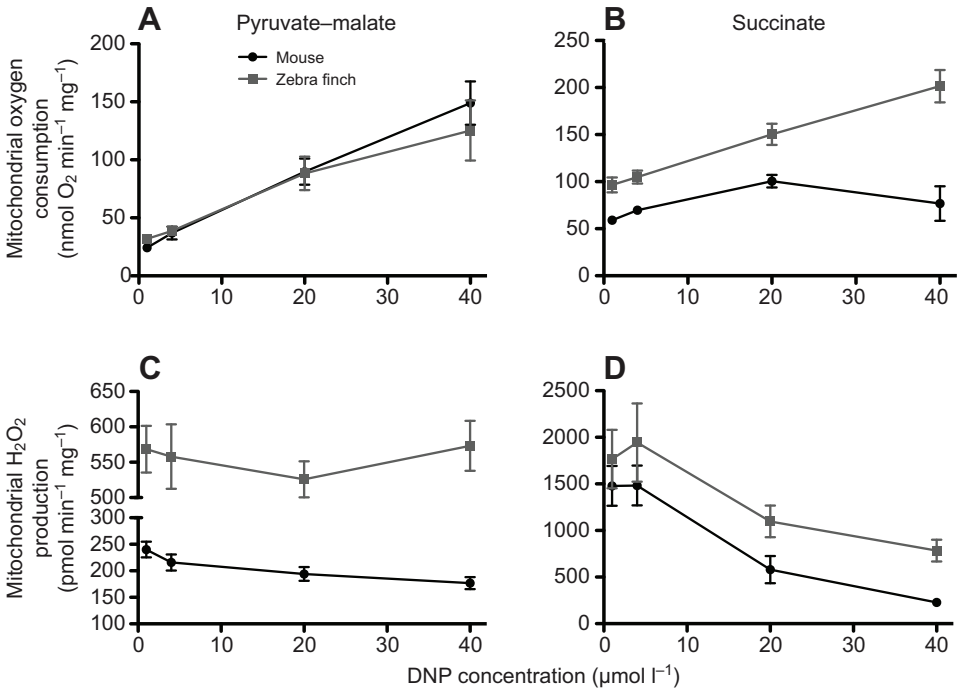

Fig. 5. *In vitro* responses to a progressive artificial uncoupling (increasing concentrations of DNP) of state 4 mitochondria isolated from zebra finch (grey) and mice (black) skeletal muscles. (A,B) Mitochondrial oxygen consumption; (C,D) reactive oxygen species production. The measurements represented in A and C were conducted using 2.5 mmol l^{-1} of pyruvate–malate as substrate, whereas the measurements in B and D were obtained using 5 mmol l^{-1} of succinate as substrate (see Results for statistics). Means are plotted ±s.e.

in mitochondrial biogenesis and/or density might be an adaptive response to an uncoupling treatment (Schlagowski et al., 2014), even if we cannot ascertain that such compensation occurred in our particular study. Consequently, even if the DNP treatment might reduce ROS production at the mitochondrial level, an increase in mitochondria density might counterbalance this beneficial effect at the organismal level. Finally, avian mitochondria could be less sensitive than other taxa to mitochondrial uncoupling in terms of ROS production (present study; P.B., unpublished results in Japanese quail). Avian mitochondria are known to differ from mammalian ones in some characteristics, such as ROS production (Ku and Sohal, 1993; Lambert et al., 2010; but see Montgomery et al., 2011) and mitochondrial membrane fatty acid composition (Pamplona et al., 1999; Montgomery et al., 2011). According to this idea, our *in vitro* results suggest that the ROS production of zebra finch mitochondria is less sensitive to mitochondrial uncoupling than the ROS production of mouse mitochondria, at least when the respiration is fuelled with pyruvate-malate (Fig. 5C). Our data are in accordance with previous findings relating a less sensitive H_2O_2 production by heart mitochondria to changes in ΔpH for pigeon than for rat (Lambert et al., 2010). Our results also suggest that mitochondrial uncoupling is an effective way of reducing *in vitro* ROS production in mice with complex I substrate, contrary to previous conclusions (Shabalina and Nedergaard, 2011; but see Starkov and Fiskum, 2003; Clarke and Porter, 2013). At first glance, the higher (Fig. 5C) or similar (Fig. 5D) ROS production of the zebra finch compared with the mouse might appear contradictory to the initial idea that avian mitochondria produce fewer ROS than mammalian mitochondria. However, this idea has recently been seriously questioned (Montgomery et al., 2011), and our results are in accordance with the results of two recent studies comparing birds and mammals (Montgomery et al., 2011; Kuzmiak et al., 2012).

The lower *in vitro* sensitivity of zebra finch mitochondria could be linked to different factors, but the non-linearity of the relationship between mitochondrial membrane potential and ROS production could be a key element in our understanding of this phenomenon (Korshunov et al., 1997). If we consider the hypothetical case where zebra finches have intrinsically ‘mild’ uncoupled mitochondria [i.e. in accordance with their high metabolic rate/body temperature compared with other taxa (Holmes et al., 2001)], an artificial uncoupling treatment will probably be ineffective in further reducing ROS production *in vivo*. Data coming from the comparison between rat and pigeon kinetics of proton leak could be in agreement with this idea (Brand et al., 2003). Nonetheless, proper comparative studies on proton leak and mitochondrial membrane potential remain to be done for our study models as well as to be extended to a larger set of bird–mammal species comparisons. In this context, determining the exact mechanisms explaining the differential impact of mitochondrial uncoupling on ROS production between mammals and birds will be an essential step toward a better understanding and a potential generalisation of the ‘uncoupling to survive hypothesis’.

In addition, it is conceivable that maintaining a high mitochondrial uncoupling state might bring benefits by preventing major ROS outburst during stress situations, even if it does not noticeably affect baseline oxidative stress levels as shown in this study. For instance, a recent study conducted on mice demonstrated the importance of mitochondrial uncoupling in maintaining low levels of oxidative stress in response to cold exposure (Stier et al., 2014a), and data about the oxidative stress response to acute cold exposure in zebra finches also support this idea (Stier et al., 2014b).

Mitochondrial uncoupling and whole-organism reproduction and immunity

Despite the increased metabolic rate induced by the DNP treatment, few life-history traits were significantly affected in zebra finches. First, growth performances were not affected by the DNP treatment, contrary to observations made on frog tadpoles (Salin et al., 2012a). As mentioned earlier, the fact that DNP-treated birds showed increased food consumption could be the main explanation for the limited impact of the treatment found on life-history traits. Moreover, by increasing their nutrient supply, the treated birds were probably able to compensate for the decreased mitochondrial efficiency in most cases. We did not find a significant effect of the DNP treatment on survival pattern, however, which is in accordance

with the lack of effect observed for oxidative stress markers. Approximately 70% of the birds were still alive at the end of the experimental period, which precludes definitive conclusions about the impact of DNP on longevity before having data on the lifespan of the remaining birds. The reproductive capacity of birds was slightly reduced by the uncoupling treatment, although this result was significant only for the total number of eggs produced. The compensation of mitochondrial inefficiency by increased food intake could be insufficient in periods of high energy demand such as egg production [+22% of RMR in female zebra finch (Vézina and Williams, 2005)]. Finally, regarding the immune competence, the downregulation of the immune response to a PHA test exhibited by DNP-treated birds may have at least two distinct causes. First of all, treated animals could be unable to allocate enough energy toward immune defences (as mentioned above for reproduction) because of their increased resting metabolic rate. According to this idea, the immune response has been shown to be energetically costly [+28.8% of resting metabolic rate in *Passer domesticus* submitted to a PHA test (Martin et al., 2003)]. However, following this hypothesis, we should have observed a similar effect of DNP for immune parameters evaluated via the haemolysis-haemagglutination assay [i.e. natural antibodies (NAbs) and complement]. Therefore, we propose an alternative hypothesis to explain this result, which is based on the fact that mitochondrial coupling state might be important in determining the inflammatory responsiveness of individuals (Emre and Nübel, 2010). It has been shown that mice deficient for uncoupling protein 2 (UCP2) have a more effective immune response against *Toxoplasma gondii*, thanks to a higher ROS production by macrophages (Arsenijevic et al., 2000). In wild-type mice, UCP2 content in macrophages is downregulated consecutively to an immune challenge with lipopolysaccharide, to allow the build-up of an oxidative burst used to neutralize pathogens (Kizaki et al., 2002). This suggests that if UCP2 is involved in ROS production, the disappearance of any uncoupling activity in mitochondria might be a prerequisite to the triggering of the immune response. In this case, the lower inflammatory response toward the PHA injection might then be explained by the lower capacity of DNP-treated birds to build up an adequate oxidative burst, because of their loosely coupled mitochondria.

Conclusions

Altogether, our results suggest that birds (at least zebra finches) might behave differently than other taxa in response to an experimental uncoupling treatment. Indeed, one major point of this study is that a moderate mitochondrial uncoupling has no significant beneficial impact on various oxidative stress markers, contrary to results observed in both mammals (Caldeira da Silva et al., 2008) and amphibians (Salin et al., 2012a). According to this idea, we have shown here that zebra finch mitochondria behave differently than mouse mitochondria in terms of ROS response to an artificial uncoupling. More work is now required to understand the origins of such differences, and to test the possible generalisation of this pattern to other bird–mammal pairs (e.g. rat–pigeon). According to our study, the impact of mitochondrial inefficiency on life-history trajectories seems limited when food is provided *ad libitum* to animals. Therefore, a logical extension of this study should be to test the impact of mitochondrial uncoupling on life-history trajectories with limited food supply, or under conditions increasing the effort required to obtain food (Koetsier and Verhulst, 2011). Finally, important insight on the role of mitochondrial efficiency in life-history evolution should also arise from field studies (e.g. Salin et al., 2012b), even if there are obvious difficulties in implementing mitochondria measurements in field conditions and/or with wild animals [but see Stier et al. (Stier et al., 2013) for a possible method of measuring mitochondrial functioning based on blood samples in non-mammalian vertebrates].

MATERIALS AND METHODS

Animals and experimental design

The study complied with the 'Principles of Animal Care' publication no. 86-23, revised 1985 of the National Institutes of Health, and with current legislation (L87-848) on animal experimentation in France. The experiment was performed on 60 primiparous adult zebra finches (30 females and 30 males) approximately 6 months of age from our own husbandry. Birds were housed at 24°C on a 13 h:11 h light:dark light cycle and provided with food (commercial mix of seeds for exotic birds enriched with vitamins and eggs) *ad libitum*.

We divided our experiment into four successive steps. The first step of the experiment (2 months) served to measure baseline values for body mass, metabolic rate and plasma oxidative balance before the beginning of the DNP treatment.

In the second step (6 months), half of the birds were kept with water *ad libitum*, whereas the other half was switched to the DNP treatment (20 mg l^{-1} of DNP diluted in drinking water). This second step was conducted to assess the effects of our treatment on body mass (after 1, 3 and 6 months of treatment), food consumption, metabolic rate, immune competence and oxidative stress markers.

In the third step (8 months), the treatment remained unchanged but birds were allowed to reproduce. Birds were kept as unisex pairs in cages of 0.57×0.31×0.39 m during steps 1 and 2, whereas they were kept as breeding pairs in step 3. We randomly formed breeding pairs within each experimental group, provided them with a nestbox and nesting material, and allowed animals to freely breed repeatedly. Chicks were weaned 35 days after hatching, and they were subsequently kept on water or DNP solution, depending on the experimental group of their parents (i.e. chicks were then continuously maintained under control or DNP treatment from embryonic stage onwards). We also conducted measurements on chicks coming from this experiment to determine metabolic rate, growth rate and oxidative stress markers (at 10, 30 and 120 days).

In the last step (18 months), we reconstituted unisex pairs and followed birds over a period up to 32 months after the beginning of the treatment (step 2) to assess their survival. Treatment remained unchanged, with birds being fed with either water or DNP according to their treatment attributed in step 2.

The DNP dose [~4 mg kg^{-1} day^{-1} for an average bird weighing 15.0 g and drinking 3.0 ml of water per day (Calder, 1964)] was chosen after a pilot experimentation on 15 birds (see Appendix for details), demonstrating that a lower dose (5 mg l^{-1}) was insufficient to noticeably affect the metabolic rate, whereas the chosen dose (20 mg l^{-1}) moderately increased the metabolic rate of birds (~20%) without apparent deleterious effect. We excluded the use of a higher dose (50 mg l^{-1}) because it was associated with potentially deleterious effects (i.e. moderate hyperthermia and decreased water consumption). Drinking water was replaced twice a week, and the DNP solution was freshly prepared each time.

Food consumption

We measured food consumption 2 weeks after the beginning of the treatment (step 2). We monitored food consumption for each bird during a period of 10 days during which the birds were kept in individual cages. We weighed bird feeders every 2 days at 10:00 h to determine the amount of seeds consumed by each bird, and we used the average food consumption of these five measurements to determine the daily food consumption (g day^{-1}).

Metabolism

For adult birds (N=60), oxygen consumption ($\dot{V}_{O_2}$; ml O_2 consumed per minute) was determined both 1 month before and 1 month after the beginning of the treatment (i.e. in steps 1 and 2), at thermoneutrality (30°C). We recorded O_2 consumption with an open-flow respirometry system (Sable Systems, Henderson, NV, USA) during 4 h (08:00 to 12:00 h) after one night of acclimation (without food, but with water or

DNP solution *ad libitum*). We equilibrated our experimental groups between the four metabolic chambers of the respirometry system, and each bird was measured in the same metabolic chamber both before and after the start of the treatment. We included the metabolic chamber as a co-factor in the statistical analysis to take into account variations between our four metabolic chambers. We analysed both the average of these 4 h values (mean $\dot{V}_{O_2}$) and the average of the three lower values (resting $\dot{V}_{O_2}$), which is a better indicator of resting metabolism. Birds were weighed at the start and at the end of the metabolic measurement, and we included average body mass as a covariate in statistical model to control for the effect of body mass on metabolism.

We also determined the resting $\dot{V}_{O_2}$ (30°C) for a sub-sample of chicks 12 days after hatching (control: N=14; DNP: N=20). We recorded O_2 consumption (in the dark to reduce the chicks' movements and stress) for 30 min with a portable open-flow respirometry system (FOXBOX, Sable Systems). We defined the resting $\dot{V}_{O_2}$ as the lowest consecutive 2 min within the 30 min recording.

Oxidative stress measurements

The production of superoxide by blood cells mitochondria was determined for adult birds (N=60) following the protocol fully described elsewhere (Stier et al., 2013), 6 to 8 weeks after the beginning of the treatment (i.e. in step 2 but not in step 1). Briefly, we used the specific fluorescent probe MitoSOX Red (Molecular Probes, Life Sciences) to assess the production of mitochondrial superoxide, which was detected using a flow cytometer (FACScalibur, BD Bioscience). A T_0 acquisition was carried out for each sample, and after 30 min of incubation at 40°C, a second acquisition (T_{30}) was made to evaluate the change in mitochondrial superoxide content. We expressed superoxide production as the increase in red fluorescence per minute (RF min^{-1}).

Plasma antioxidant capacity and the concentration of reactive oxygen metabolites (ROMs) were measured using the OXY-Adsorbent (5 µl of 1:100 diluted plasma) and d-ROMs tests (5 µl of plasma, Diacron International, Italy), respectively, following the manufacturer's protocols (for details, see Stier et al., 2012). We measured these two parameters in adult birds (N=60) both before (step 1) and approximately 3 months after the beginning of the treatment (step 2), and in chicks at day 10 (control: N=30; DNP: N=29), day 30 (control: N=22; DNP: N=21) and day 120 (control: N=9; DNP: N=13). The OXY-Adsorbent test allows us to quantify the ability of the plasma antioxidant components to buffer a massive oxidation through hypochlorous acid, while the d-ROMs test measures hydroperoxides, as a marker of global early oxidative damage. Antioxidant capacity is expressed as µmol l^{-1} of HClO neutralized per millilitre, and ROMs as mg H_2O_2 equivalents dl^{-1}. Intra-individual variation based on duplicates was low (CV=4.21±0.60% for the OXY test and CV=3.90±0.56% for the d-ROMs test) as was inter-plate variation based on a standard sample repeated over plates (CV=8.46% for OXY and 6.95% for d-ROMs).

We also measured different parameters of oxidative stress in tissues (pectoral muscle and heart) in a sub-sample of chicks at day 120 (control: N=12; DNP: N=12). Birds were culled by cervical dislocation, and tissues were immediately collected and snap-frozen in liquid nitrogen. Glutathione (GSH) content and the proportion of oxidized glutathione (GSSG) were determined using the DetectX® Glutathione fluorescent detection kit (Arbor Assays), following the manufacturer's instructions. We evaluated the total GSH content as an indicator of endogenous antioxidant protection and the proportion of GSSG (ratio GSSG/GSH) as an indicator of the oxidative challenge (i.e. the pro-oxidant power buffered by the glutathione system). Values are expressed as nmol total glutathione mg^{-1} protein, and as a ratio of GSSG/GSH [0 meaning that all glutathione is free GSH, and 1 meaning that all glutathione is oxidized (GSSG)], respectively. Intra-individual variation based on duplicates was low (CV=1.76±0.43%). We also determined the enzymatic activity of the glutathione reductase using the DetectX® Glutathione Reductase fluorescent kit (Arbor Assays), following the manufacturer's instructions. Glutathione reductase plays an indirect but essential role in the prevention of oxidative damage within the cell by helping to maintain appropriate levels of intracellular free GSH. Enzymatic activity is expressed as mU mg^{-1} protein. Intra-individual variation based on duplicates was low (CV=5.86±1.61%). Finally, we determined oxidative damage on lipids (lipid peroxidation) by measuring the levels of malondialdehyde (MDA), using a protocol adapted for microplate analysis (lipid peroxidation microplate assay kit, Oxford Biomedical Research). MDA levels are expressed as nmol mg^{-1} protein. Intra-individual variation based on duplicates was 8.87±2.28%.

Immune parameters

We subjected a sub-sample of birds (males, control: N=15; DNP: N=15) to a phytohaemagglutinin (PHA) skin-swelling test, 5 months after the beginning of the treatment (i.e. during step 2). This test assays the pro-inflammatory response to a foreign plant protein (PHA), which is mainly linked to the accumulation of small lymphocytes and the infiltration of macrophages (Smits et al., 1999; Vinkler et al., 2010). To conduct the PHA test, we measured the right wing web of each bird three times with a digital micrometer (±0.02 mm; Mitutoyo, Japan) to obtain an average pre-swelling measurement and then injected this area with 100 µg of PHA-P (Sigma-Aldrich, St Louis, MO, USA) diluted in 20 µl of phosphate-buffered saline. The birds were measured a second time 24 h after the injection, and we present the results as the difference between mean post-injection swelling and mean pre-injection swelling (i.e. wing web swelling index).

We also evaluated the constitutive innate humoral immunity for the same sub-sample of birds, using a haemolysis-haemagglutination assay described extensively by Matson et al. (Matson et al., 2005). Briefly, such a test allows determination of two elements of the innate immunity, namely the NAbs and the complement, using the ability of plasma samples to provoke lysis and/or agglutination of rabbit erythrocytes *in vitro*. We evaluated lysis and agglutination scores according to Matson et al. (Matson et al., 2005). The lysis score reflects the interaction of complement and NAbs, whereas the agglutination score reflects NAbs only.

Growth analysis

Body mass of chicks was recorded every morning from hatching to day 30, using an electronic balance (0.1 g precision). Body mass growth was fitted for chicks surviving up to 30 days (control group: N=22, DNP group: N=21), with the following logistic equation:

$$Y(x) = \frac{A}{[1+\exp(-Kx-B)]}, \quad (1)$$

where $Y(x)$ represents the body mass (g) of a chick at age x, A is the asymptotic final mass, K is the growth rate constant (an increase in K implies an increase in the rate at which mass increases from initial value to asymptotic value) and B is a constant linked to the initial mass. Growth fitting was performed with the nonlinear regression procedure in SPSS (SPSS 20.0© 1989-2011 SPSS Inc., USA) for each chick, and the minimum R^2 for model fitting was 0.96 across all chicks.

In vitro mitochondrial respiration and ROS production in adult zebra finches versus laboratory mice

We conducted *in vitro* experiments to investigate the effects of artificial uncoupling on ROS production by zebra finch mitochondria, compared with a size-matched mammalian model, the mouse. Nine individuals of each species (male, 3-month-old C57BL/6 mice, and 8-month-old zebra finches) were killed by cervical dislocation, and skeletal muscles (hind feet for mice and a mix of hind feet and pectoral muscle for zebra finches) were immediately collected and rinsed in ice-cold isolation medium (100 mmol l^{-1} sucrose, 50 mmol l^{-1} KCl, 5 mmol l^{-1} EDTA, 50 mmol l^{-1} Tris-base and pH 7.4). All of the following steps were carried out at 4°C. Briefly, muscles were cut up finely, diluted 1:10 (w/v) in ice-cold isolation medium and homogenized with a Potter-Elvehjem homogenizer. Samples were subsequently treated with protease (1 mg g^{-1} muscle wet mass) for 5 min, then diluted 1:2 in isolation medium and homogenized a second time. The mixture was centrifuged (10 min at 1000 ***g***) and the resulting supernatant containing muscle mitochondria was filtered through cheesecloth and centrifuged two times at 8700 ***g*** for 10 min. We determined the protein concentration of mitochondrial suspensions in duplicates using a Biuret method with BSA as standard.

Oxygen consumption was recorded with a Clark oxygen electrode (Rank Brothers Ltd, UK) in 1 ml of respiration buffer (120 mmol l^{-1} KCl,

The Journal of Experimental Biology (2014) doi:10.1242/jeb.103945

5 mmol l^{-1} KH_2PO_4, 1 mmol l^{-1} EGTA, 2 mmol l^{-1} $MgCl_2$, 0.3% BSA, 3 mmol l^{-1} Hepes and pH 7.4). Muscle mitochondria (0.25 mg protein ml^{-1}) were incubated either with 5 mmol l^{-1} succinate or 5 mmol l^{-1} pyruvate and 2.5 mmol l^{-1} malate as substrate. We used these two different substrates because mitochondrial ROS production is likely to differ in magnitude (succinate>pyruvate–malate), in origin (mainly reverse electron transport with succinate) and in response to uncoupling (higher response with succinate) according to the type of substrate used (Votyakova and Reynolds, 2001). We added increasing concentrations of DNP to induce a progressive uncoupled state (i.e. 4, 20, 40 µmol l^{-1}; higher concentrations having deleterious effects), in order to compare the response of state 4 mitochondria of the zebra finch and the mouse to this chemical uncoupling. The DNP stock solution (8 mmol l^{-1}) was prepared with absolute ethanol, which implies a final concentration of ethanol of between 0.05 and 0.5% in the respiratory buffer, depending on final DNP concentration. Classical mitochondrial respiration measurements (states 2, 3 and 4) were conducted with pyruvate–malate as substrate in order to ensure that mitochondrial preparations were of sufficient quality. Respiratory control ratio values (mouse=6.51±0.57 versus zebra finch=7.71±0.96) were in the expected range and did not significantly differ between the two species (non-parametric Wilcoxon test: Z=–0.93, P=0.35).

In addition, we determined ROS production by isolated mitochondria using an H_2O_2-based detection, under the exact same conditions as those used for mitochondrial respiration measurements (i.e. state 4 followed by increasing concentrations of DNP). Briefly, the respiratory medium (1 ml) was supplemented with 1 U ml^{-1} horseradish peroxidase and 10 µmol l^{-1} Amplex® red reagent (Invitrogen, USA). Amplex® red fluorescence was quantified using a fluorescence spectrophotometer (Xenius, Safas, Monaco) at excitation and emission wavelengths of 563 and 587 nm, respectively. The fluorescent signal was converted into H_2O_2 equivalents (pmol min^{-1} mg^{-1}) using a standard curve prepared with H_2O_2.

Oxygen consumption and ROS production have been recorded at 37°C for both species in order to run one sample of each species in every run, thereby avoiding inter-run bias between the two species. However, the body temperature of zebra finches is higher than 37°C [i.e. 40.2°C (McNab, 1966)], and therefore we applied a Q_{10} correction to take into account this issue. We used a Q_{10} value of 2.09, which is a standard value for birds (White et al., 2006). Still, analyzing raw data instead of Q_{10}-corrected data does not qualitatively change our results (data not shown).

Statistics

We tested the effect of the DNP treatment on the variables collected on adult birds using either general linear models (for parameters measured only one time) or repeated general linear models (for parameters measured repeatedly over time). The treatment was included as a fixed factor, and we included bird ID as the repeated factor (when needed) and sex as a co-factor.

We specifically analyzed data on reproduction (i.e. number of reproductive events, clutch size and brood size) using generalized linear models following a Poisson distribution, with the nest as the statistical unit. We also analyzed scores coming from the haemolysis-haemagglutination assay (i.e. also count data) using similar statistical models.

We analyzed data on chicks using linear mixed models, with the treatment as a fixed factor and nest as a random factor to take into account that chicks coming from one nest are not independent statistical units.

We analyzed data of the *in vitro* comparison between mouse and zebra finch mitochondrial respiration rate and H_2O_2 production using repeated general linear models. Species was included as a fixed factor and the concentration of DNP (0, 4, 20, 40 µmol l^{-1}) was included as the repeated factor. When a significant species × DNP interaction was detected, we subsequently analyzed each species separately.

General linear and linear mixed models were fitted with a normal error distribution (SPSS 20.0), and data were tested for normality and homoscedasticity. All tests were two-tailed and P-values ≤0.05 were considered significant. Means are always quoted ± s.e.

APPENDIX

Pilot study to determine DNP dosage

Methods

Fifteen individuals were chosen randomly from our captive population to participate in a preliminary experiment designed to evaluate the appropriate dose of DNP to be used in our long-term experiment. Each bird was measured once before the beginning of the treatment, and was then allocated randomly into one of three groups: 5, 20 or 50 mg ml^{-1} of DNP in drinking water. The different parameters (mass, resting metabolic rate, body temperature, food consumption and water consumption) were subsequently monitored for 3 weeks. We only present an average value for the 3 weeks of

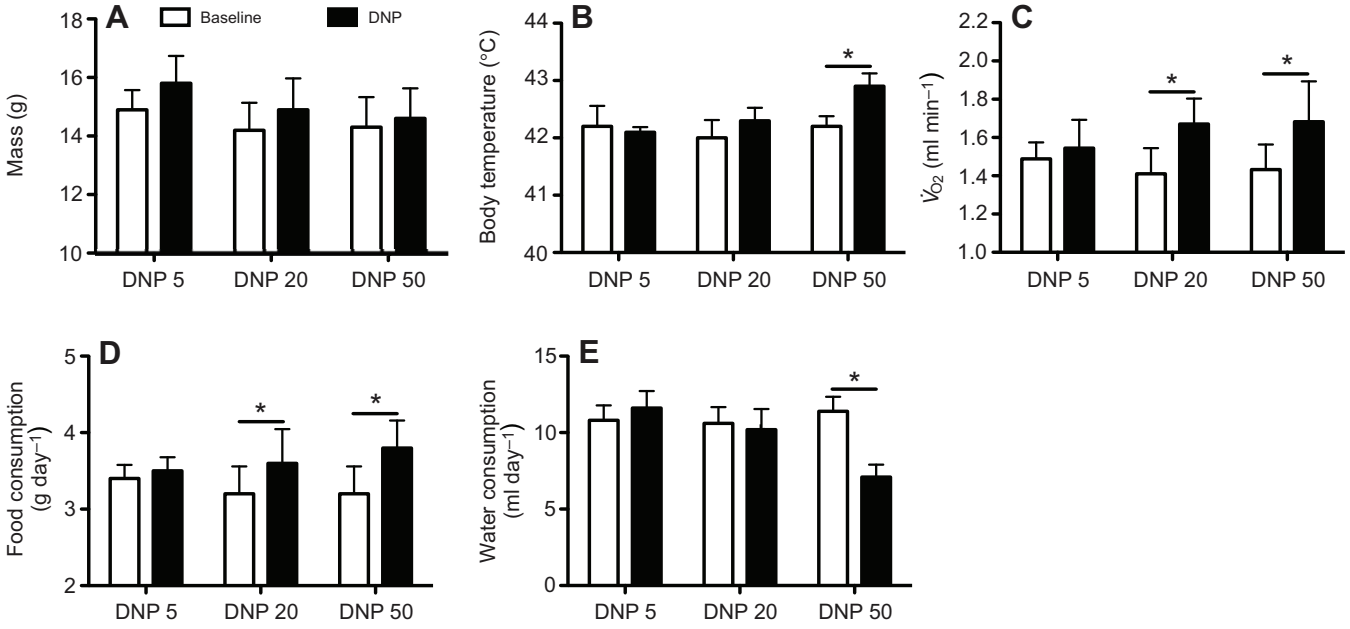

Fig. A1. Preliminary experiment examining the optimal dosage of the DNP treatment in drinking water (5, 20 or 50 mg ml^{-1}). (A) Body mass, (B) body temperature, (C) resting metabolic rate, (D) food consumption and (E) water consumption. Each individual was measured both before the treatment (baseline/white bars) and during three consecutive weeks of treatment (DNP/black bars). N=5 per group, means are plotted ± s.e., and asterisks indicate a significant difference between baseline and DNP value for a given group according to *post hoc* tests of GEE models.

treatment because we did not find a significant effect of the post-treatment time (i.e. 1, 2 or 3 weeks).

Results

Body mass significantly increased after the treatment [generalized estimating equation (GEE): χ^2=26.1, d.f.=1, P<0.001; Fig. A1A], but was not significantly affected by the DNP dose (GEE: χ^2=0.7, d.f.=2, P=0.70) or the interaction between DNP dose and treatment (GEE: χ^2=3.7, d.f.=2, P=0.16).

Body temperature significantly increased after the treatment (GEE: χ^2=5.8, d.f.=1, P=0.02; Fig. A1B), but was also marginally affected by the interaction between DNP dose and treatment (GEE: χ^2=5.5, d.f.=2, P=0.06). *Post hoc* analyses revealed a significant increase in body temperature only for the group treated with 50 mg ml^{-1} DNP (P=0.001).

$\dot{V}_{O_2}$ significantly increased after the treatment (GEE: χ^2=10.0, d.f.=1, P=0.002; Fig. A1C), but was also marginally affected by the interaction between DNP dose and treatment (GEE: χ^2=5.1, d.f.=2, P=0.08). *Post hoc* analyses revealed a significant increase in $\dot{V}_{O_2}$ only for the groups treated with 20 (P<0.001) and 50 mg ml^{-1} DNP (P=0.02).

Food consumption significantly increased after the treatment (GEE: χ^2=26.5, d.f.=1, P<0.001; Fig. A1D), but was also significantly affected by the interaction between DNP dose and treatment (GEE: χ^2=18.1, d.f.=2, P<0.001). *Post hoc* analyses revealed a significant increase in food consumption only for the groups treated with 20 (P=0.006) and 50 mg ml^{-1} DNP (P<0.001).

Water consumption significantly decreased after the treatment (GEE: χ^2=10.9, d.f.=1, P=0.001; Fig. A1E), but was also significantly affected by the interaction between DNP dose and treatment (GEE: χ^2=29.6, d.f.=2, P<0.001). *Post hoc* analyses revealed a significant decrease in water consumption only for the group treated with 50 mg ml^{-1} DNP (P<0.001).

Acknowledgements

We are grateful to C. Romestaing for the help provided with *in vitro* measurements on isolated mitochondria, to A. Hranitzky for support with bird maintenance, to S. Reichert for editing the English, and finally to three anonymous reviewers for their helpful comments.

Competing interests

The authors declare no competing financial interests.

Author contributions

F.C. and A.S. designed the study. A.S. and Q.S. collected the data. A.S., F.C., P.B. and S.M. took part in data analyses and interpretations. D.R. and A.S. managed the measurements on isolated mitochondria. A.S., P.B. and F.C. wrote the paper. All authors have read and approved the final version of the manuscript.

Funding

We are grateful to the CNRS (PICS, grant no. 5296), the French Ministry of Research and the University of Strasbourg for funding. P.B. was funded by the Swiss National Research Foundation (grant no. 31003A_124988).

References

Arsenijevic, D., Onuma, H., Pecqueur, C., Raimbault, S., Manning, B. S., Miroux, B., Couplan, E., Alves-Guerra, M. C., Goubern, M., Surwit, R. et al. (2000). Disruption of the uncoupling protein-2 gene in mice reveals a role in immunity and reactive oxygen species production. *Nat. Genet.* **26**, 435-439.

Balaban, R. S., Nemoto, S. and Finkel, T. (2005). Mitochondria, oxidants, and aging. *Cell* **120**, 483-495.

Beckman, K. B. and Ames, B. N. (1998). The free radical theory of aging matures. *Physiol. Rev.* **78**, 547-581.

Boveris, A. and Chance, B. (1973). The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem. J.* **134**, 707-716.

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.

Brand, M. D., Turner, N., Ocloo, A., Else, P. L. and Hulbert, A. J. (2003). Proton conductance and fatty acyl composition of liver mitochondria correlates with body mass in birds. *Biochem. J.* **376**, 741-748.

Caldeira da Silva, 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.

Calder, W. A. (1964). Gaseous metabolism and water relations of the zebra finch, *Taeniopygia castanotis*. *Physiol. Zool.* **37**, 400-413.

Clarke, K. J. and Porter, R. K. (2013). Uncoupling protein 1 dependent reactive oxygen species production by thymus mitochondria. *Int. J. Biochem. Cell Biol.* **45**, 81-89.

Divakaruni, A. S. and Brand, M. D. (2011). The regulation and physiology of mitochondrial proton leak. *Physiology* **26**, 192-205.

Dominguez, S. E., Menkel, J. L., Fairbrother, A., Williams, B. A. and Tanner, R. W. (1993). The effect of 2,4-dinitrophenol on the metabolic rate of bobwhite quail. *Toxicol. Appl. Pharmacol.* **123**, 226-233.

Emre, Y. and Nübel, T. (2010). Uncoupling protein UCP2: when mitochondrial activity meets immunity. *FEBS Lett.* **584**, 1437-1442.

Gleeson, M. (1986). Respiratory adjustments of the unanaesthetized chicken, *Gallus domesticus*, to elevated metabolism elicited by 2,4-dinitrophenol or cold exposure. *Comp. Biochem. Physiol.* **83A**, 283-289.

Halliwell, B. and Gutteridge, J. (2007). *Free Radicals in Biology and Medicine*. Oxford: Oxford University Press.

Harman, D. (1956). Aging: a theory based on free radical and radiation chemistry. *J. Gerontol.* **11**, 298-300.

Harris, M. O. and Cocoran, J. J. (1995). *Toxicological Profile for Dinitrophenols*, pp. 262. Atlanta, GA: Agency for Toxic Substances and Disease Registry. Available at: www.atsdr.cdc.gov/toxprofiles/tp64.pdf.

Helle, H., Koskela, E. and Mappes, T. (2012). Life in varying environments: experimental evidence for delayed effects of juvenile environment on adult life history. *J. Anim. Ecol.* **81**, 573-582.

Holmes, D. J., Flückiger, R. and Austad, S. N. (2001). Comparative biology of aging in birds: an update. *Exp. Gerontol.* **36**, 869-883.

Kizaki, T., Suzuki, K., Hitomi, Y., Taniguchi, N., Saitoh, D., Watanabe, K., Onoé, K., Day, N. K., Good, R. A. and Ohno, H. (2002). Uncoupling protein 2 plays an important role in nitric oxide production of lipopolysaccharide-stimulated macrophages. *Proc. Natl. Acad. Sci. USA* **99**, 9392-9397.

Koetsier, E. and Verhulst, S. (2011). A simple technique to manipulate foraging costs in seed-eating birds. *J. Exp. Biol.* **214**, 1225-1229.

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.

Ku, H.-H. and Sohal, R. S. (1993). Comparison of mitochondrial pro-oxidant generation and anti-oxidant defenses between rat and pigeon: possible basis of variation in longevity and metabolic potential. *Mech. Ageing Dev.* **72**, 67-76.

Kuzmiak, S., Glancy, B., Sweazea, K. L. and Willis, W. T. (2012). Mitochondrial function in sparrow pectoralis muscle. *J. Exp. Biol.* **215**, 2039-2050.

Lambert, A. J., Buckingham, J. A., Boysen, H. M. and Brand, M. D. (2010). Low complex I content explains the low hydrogen peroxide production rate of heart mitochondria from the long-lived pigeon, *Columba livia*. *Aging Cell* **9**, 78-91.

Martin, L. B., 2nd, Scheuerlein, A. and Wikelski, M. (2003). Immune activity elevates energy expenditure of house sparrows: a link between direct and indirect costs? *Proc. Biol. Sci.* **270**, 153-158.

Matson, K. D., Ricklefs, R. E. and Klasing, K. C. (2005). A hemolysis-hemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds. *Dev. Comp. Immunol.* **29**, 275-286.

McNab, B. K. (1966). An analysis of the body temperatures of birds. *Condor* **68**, 47-55.

Montgomery, M. K., Hulbert, A. J. and Buttemer, W. A. (2011). The long life of birds: the rat–pigeon comparison revisited. *PLoS ONE* **6**, e24138.

Nicholls, D. G. and Ferguson, S. J. (2002). *Bioenergetics*. London: Academic Press.

Padalko, V. I. (2005). Uncoupler of oxidative phosphorylation prolongs the lifespan of *Drosophila*. *Biochemistry* **70**, 986-989.

Pamplona, R., Portero-Otín, M., Riba, D., Ledo, F., Gredilla, R., Herrero, A. and Barja, G. (1999). Heart fatty acid unsaturation and lipid peroxidation, and aging rate, are lower in the canary and the parakeet than in the mouse. *Aging* **11**, 44-49.

Salin, K., Luquet, E., Rey, B., Roussel, D. and Voituron, Y. (2012a). Alteration of mitochondrial efficiency affects oxidative balance, development and growth in frog (*Rana temporaria*) tadpoles. *J. Exp. Biol.* **215**, 863-869.

Salin, K., Roussel, D., Rey, B. and Voituron, Y. (2012b). David and Goliath: a mitochondrial coupling problem? *J. Exp. Zool. A* **317**, 283-293.

Schlagowski, A. I., Singh, F., Charles, A. L., Gali Ramamoorthy, T., Favret, F., Piquard, F., Geny, B. and Zoll, J. (2014). Mitochondrial uncoupling reduces exercise capacity despite several skeletal muscle metabolic adaptations. *J. Appl. Physiol.* **116**, 364-375.

Sepp, T., Sild, E., Blount, J. D., Männiste, M., Karu, U. and Hõrak, P. (2012). Individual consistency and covariation of measures of oxidative status in greenfinches. *Physiol. Biochem. Zool.* **85**, 299-307.

Shabalina, I. G. and Nedergaard, J. (2011). Mitochondrial ('mild') uncoupling and ROS production: physiologically relevant or not? *Biochem. Soc. Trans.* **39**, 1305-1309.

Shabalina, I. G., Vrbacký, M., Pecinová, A., Kalinovich, A. V., Drahota, Z., Houštěk, J., Mráček, T., Cannon, B. and Nedergaard, J. (2014). ROS production in brown adipose tissue mitochondria: the question of UCP1-dependence. *Biochim. Biophys. Acta* [Epub ahead of print] doi:10.1016/j.bbabio.2014.04.005.

Sies, H. (1985). *Oxidative Stress*. Orlando, FL: Academic Press.
Skulachev, V. P. (1996). Role of uncoupled and non-coupled oxidations in maintenance of safely low levels of oxygen and its one-electron reductants. *Q. Rev. Biophys.* **29**, 169-202.
Smits, J. E., Bortolotti, G. R. and Tella, J. L. (1999). Simplifying the phytohaemagglutinin skin-testing technique in studies of avian immunocompetence. *Funct. Ecol.* **13**, 567-572.
Speakman, J. R., Talbot, D. A., Selman, C., Snart, S., McLaren, J. S., Redman, P., Krol, E., Jackson, D. M., Johnson, M. S. and Brand, M. D. (2004). Uncoupled and surviving: individual mice with high metabolism have greater mitochondrial uncoupling and live longer. *Aging Cell* **3**, 87-95.
Starkov, A. A. and Fiskum, G. (2003). Regulation of brain mitochondrial H_2O_2 production by membrane potential and NAD(P)H redox state. *J. Neurochem.* **86**, 1101-1107.
Stearns, S. C. (1992). *The Evolution of Life Histories*. Oxford: Oxford University Press.
Stier, A., Reichert, S., Massemin, S., Bize, P. and Criscuolo, F. (2012). Constraint and cost of oxidative stress on reproduction: correlative evidence in laboratory mice and review of the literature. *Front. Zool.* **9**, 37.
Stier, A., Bize, P., Schull, Q., Zoll, J., Singh, F., Geny, B., Gros, F., Royer, C., Massemin, S. and Criscuolo, F. (2013). Avian erythrocytes have functional mitochondria, opening novel perspectives for birds as animal models in the study of ageing. *Front. Zool.* **10**, 33.
Stier, A., Bize, P., Habold, C., Bouillaud, F., Massemin, S. and Criscuolo, F. (2014a). Mitochondrial uncoupling prevents cold-induced oxidative stress: a case study using UCP1 knockout mice. *J. Exp. Biol.* **217**, 624-630.
Stier, A., Massemin, S. and Criscuolo, F. (2014b). Chronic mitochondrial uncoupling treatment prevents acute cold-induced oxidative stress in birds. *J. Comp. Physiol. B.* doi:10.1007/s00360-014-0856-6.
Vézina, F. and Williams, T. D. (2005). The metabolic cost of egg production is repeatable. *J. Exp. Biol.* **208**, 2533-2538.
Vinkler, M., Bainová, H. and Albrecht, T. (2010). Functional analysis of the skin-swelling response to phytohaemagglutinin. *Funct. Ecol.* **24**, 1081-1086.
Votyakova, T. V. and Reynolds, I. J. (2001). ΔΨm-Dependent and -independent production of reactive oxygen species by rat brain mitochondria. *J. Neurochem.* **79**, 266-277.
White, C. R., Phillips, N. F. and Seymour, R. S. (2006). The scaling and temperature dependence of vertebrate metabolism. *Biol. Lett.* **2**, 125-127.
Williams, G. (1966). Natural selection, the costs of reproduction, and a refinement of Lack's principle. *Am. Nat.* **100**, 687-690.