

## RESEARCH ARTICLE

# Physiological adaptations to reproduction. II. Mitochondrial adjustments in livers of lactating mice

Nicolas Pichaud<sup>1,2,\*</sup>, Michael Garratt<sup>1</sup>, J. William O. Ballard<sup>2</sup> and Robert C. Brooks<sup>1</sup>

<sup>1</sup>Evolution and Ecology Research Centre and School of Biological, Earth and Environmental Sciences, The University of New South Wales, Sydney, New South Wales 2052, Australia and <sup>2</sup>School of Biotechnology and Biomolecular Sciences, The University of New South Wales, Sydney, New South Wales 2052, Australia

\*Author for correspondence (pichaud.nicolas@wanadoo.fr)

### SUMMARY

**Reproduction imposes significant costs and is characterized by an increased energy demand. As a consequence, individuals adjust their cellular structure and function in response to this physiological constraint. Because mitochondria are central to energy production, changes in their functional properties are likely to occur during reproduction. Such changes could cause adjustments in reactive oxygen species (ROS) production and consequently in oxidative stress levels. In this study, we investigated several mechanisms involved in energy production, including mitochondrial respiration at different steps of the electron transport system (ETS) and related the results to citrate synthase activity in the liver of non-reproductive and reproductive (two and eight pups) female house mice at peak lactation. Whereas we did not find differences between females having different litter sizes, liver mitochondria of reproductive females showed lower ETS activity and an increase in mitochondrial density when compared with the non-reproductive females. Although it is possible that these changes were due to combined processes involved in reproduction and not to the relative investment in lactation, we propose that the mitochondrial adjustment in liver might help to spare substrates and therefore energy for milk production in the mammary gland. Moreover, our results suggest that these changes lead to an increase in ROS production that subsequently upregulates antioxidant defence activity and decreases oxidative stress.**

Key words: reproduction, metabolism, mitochondrial respiration, liver, citrate synthase.

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### INTRODUCTION

In mammals, lactation is the most energetically demanding period of a female's life and is characterized by a dramatic increase in the energy and nutrient requirement of the organism for milk production (Gutgesell et al., 2009). It is well known that several metabolic adaptations occur in the different tissues of a lactating animal (Williamson, 1980; Smith and Grove, 2002; Gutgesell et al., 2009). For example, rates of fatty acid oxidation and ketogenesis, which predominantly occur in the liver, are reduced during lactation and this reduction helps to spare fatty acids for milk production in the mammary gland (Whitelaw and Williamson, 1977). Reproduction also requires particularly high levels of metabolism. In lactating laboratory mice, for example, an elevated food intake as well as a metabolic rate increase of 400% have been observed when compared with non-reproductive mice (Hammond and Diamond, 1992; Hammond, 1997; Cretegy and Genoud, 2006).

Although food consumption can increase dramatically during lactation, a point is reached where this intake is maximized and females appear unable, or unwilling, to increase their energy intake any further (Speakman, 2008). Several theories have been put forward to explain limits on the maximum rate at which animals can ingest and expend energy (Hammond and Diamond, 1992; Hammond et al., 1994; Speakman and Król, 2005; Speakman and Król, 2010; Speakman and Król, 2011). The original 'central limitation' hypothesis suggests a limitation in the capacity of the alimentary tract to assimilate energy (including the liver), although

this has been refuted by several studies (Król and Speakman, 2003a; Król and Speakman, 2003b; Król et al., 2003; Wu et al., 2009). The 'peripheral limitation' hypothesis states that the sustained energy intake is set by the energy-consuming organs such as the mammary glands during lactation (Hammond et al., 1994; Speakman and Król, 2005; Zhao and Cao, 2009; Speakman and Król, 2011; Zhao, 2011). Alternatively, the 'heat dissipation limit' theory postulates that the sustained energy intake is driven by the capacity of animals to dissipate heat (Król and Speakman, 2003a; Król and Speakman, 2003b; Król et al., 2003; Speakman and Król, 2011).

Both hypotheses are likely to be important in all animals, but to different extents (Speakman and Król, 2011; Zhao, 2011), and suggest that energy intake and expenditure are central to the ability of animals to reproduce. Additionally, the 'saturated neural control' hypothesis suggests that there may be limits in the ability of peripheral signals such as leptin to stimulate factors in the brain that regulate the intake of food, and contribute to energy intake during lactation (Woodside et al., 2000; Mercer and Speakman, 2001; Denis et al., 2003; Speakman and Król, 2011). The abilities of animals to increase metabolic rates and food intake have therefore been fairly well investigated. However, the cellular changes that occur during periods of high energy demand such as lactation have received less attention.

Mitochondria are fundamental for energy production and their ability to generate ATP in relation to tissue or cell demands could potentially dictate reproductive investment. Cellular adaptation to

physiological processes such as reproduction necessitates the control of mitochondrial respiration to be fine-tuned in its response to changes in energy demand and substrate delivery (Benard et al., 2010). Consequently, mitochondrial energy production has to be modulated to fulfil the requirements of the organism depending on its physiological status. This might be accomplished by several regulatory events occurring at different levels. Such events include but are not limited to electron transport system (ETS) activity, proton leak, uncoupling of mitochondria, organization and stoichiometry of the different complexes of the ETS as well as mitochondrial density (Benard et al., 2010). Changes in one or several of these parameters would potentially indicate an adjustment of the cell at the level of mitochondria to cope with the increased energetic demand.

As a feature of energy production, mitochondria produce reactive oxygen species (ROS), which can cause oxidative stress and are thought to be a major cause of senescence and aging (Harman, 1956). Although their role in cellular function is most commonly associated with damage to macromolecules, ROS are also involved in the regulation of several cellular processes such as mitochondrial biogenesis and increased activation of antioxidant defences (St-Pierre et al., 2006; Rasbach and Schnellmann, 2007; Han et al., 2008; Siegel et al., 2011). Several studies have predicted that oxidative stress could be an unavoidable consequence of reproduction (Costantini, 2008; Speakman, 2008; Dowling and Simmons, 2009; Monaghan et al., 2009). However, empirical evidence for this hypothesis is still scarce. Moreover, in contrast to this prediction, it has been shown that several markers of oxidative damage indicate lower oxidative stress in the liver of reproductive mice (Garratt et al., 2011; Garratt et al., 2013), as well as in kidneys and muscles of breeding bank voles (Oldakowski et al., 2012). While these changes indicate an alteration in redox state during lactation, they do not support the hypothesis that oxidative damage to tissues is the proximate mechanism of reproduction costs (Garratt et al., 2011; Oldakowski et al., 2012). Several antioxidant defences have been shown to be upregulated during reproduction, but changes to the functional properties of mitochondria have not been examined. This is quite surprising considering that mitochondria are the main producers of both cellular energy and ROS.

The aim of this study was to investigate the possibility of cellular and metabolic adaptations by adjustment of the functional properties of mitochondria during the lactation period. For this purpose, we manipulated female energy demand during lactation by altering female litter size at birth to either two or eight pups, which represents a large and small litter size for wild-derived female mice

(Berry, 1981) and compared these two treatments with non-reproductive females. We evaluated mitochondrial respiration and analysed the results according to citrate synthase (CS) activity. Specifically, we measured properties of mitochondria in permeabilized livers of non-reproductive and reproductive females having small and large litters. This method allows a physiologically relevant assessment of mitochondrial functions *in situ* with a few milligrams of tissue, maintaining mitochondria in their normal intracellular position and assembly and preserving essential interactions with other organelles (Kuznetsov et al., 2002; Kuznetsov et al., 2008; Gnaiger, 2009; Horan et al., 2012). Using high-resolution respirometry, we measured oxygen consumption at different steps of the ETS (Gnaiger, 2009) (Table 1) to assess basal proton leak (state 2'), activity of the different complexes of the ETS (state 3), maximum capacity of the ETS (Unc) and complex IV activity as a proxy of ATP production (COX). We then normalized the results to CS activity, which is considered to be one of the most accurate biomarkers of mitochondrial density (Larsen et al., 2012). This allowed us to investigate whether processes involved in reproduction (comparison of reproductive and non-reproductive females) or the relative investment in lactation (comparison between reproductive females having different litter size) elicit changes in mitochondrial functions.

## MATERIALS AND METHODS

### Animals

Animals were adult captive-bred female house mice (*Mus musculus domesticus* Linnaeus 1758) that were derived from breeding 30 mice caught at a chicken farm in the northwest of Sydney, Australia. We used wild-derived mice as opposed to a laboratory strain to ensure that levels of reproductive investment were representative of natural populations. Experimental females were weaned at the age of 28 days and remained with their female siblings until the beginning of the experiment. Males that were used to breed with females were housed singly after weaning at 28 days old. All animals were housed in cages (48×11.5×12 cm) lined with Corn Cob Absorb substrate (Gordon's Speciality Stockfeeds, Yanderra, NSW, Australia) and had shredded newspaper and tissue added for bedding. All animals had *ad libitum* access to water and to a maintenance rodent feed from Gordon's Speciality Stockfeeds (Yanderra, NSW, Australia). Mice were maintained on a 12h:12h reverse light cycle and experimental procedures were conducted under dim red light during the dark phase.

All experimental procedures were approved by the University of New South Wales Animal Ethics Committee (approval no. 11/45A).

Table 1. Physiological significance of the different oxygen fluxes measured in the livers of non-reproductive and reproductive (small and large litters) females of *Mus musculus domesticus*

Oxygen flux measured	Additions to the respiration medium	Physiological significance
State 2'	Substrates for Complex I (glutamate, pyruvate and malate) and permeabilized livers	Resting state of non-phosphorylating respiration, basal proton leak
CI	ADP	Partial State 3, ADP-activated state, Complex I capacity for oxidative phosphorylation
CIc	Cytochrome c	Partial State 3 (see above) with cytochrome c to test for integrity of mitochondria
CIc+CII	Substrate for Complex II (succinate)	State 3, ADP-activated state, maximum (Complexes I and II) capacity for oxidative phosphorylation
Unc	FCCP	Uncoupled respiration, measure of the electron transport system capacity
Rot	Rotenone	Inhibition of Complex I
Rox	Antimycin A	Inhibition of Complex III, residual oxygen consumption due to residual oxidative side reactions
COX	Ascorbate and TMPD	Activity of Complex IV, oxygen consumption by the cytochrome c oxidase

### Breeding protocol

Six different females were allocated to each of the three following treatments: non-reproductive, small litter (two pups) and large litter (eight pups). Reproductive females (small litter and large litter treatments) were randomly allocated a male to breed with. Three days prior to breeding, females were given a small handful of bedding taken from the cage of the male they were to breed with and were housed adjacent to that male's cage for the next 3 days to allow familiarization. Males and females were then housed in the same cage and allowed to breed for 18 days, after which the male was removed. Non-reproductive females were each paired with a novel female after an identical familiarization period with a male. Females under different treatments were of equivalent ages (age at pairing with a male: non-reproductives=105±11 days old; large litter=109±13 days; small litter 97±7 days; differences between treatments:  $F_{2,15}=0.31$ ,  $P=0.73$ ).

Within 24 h of giving birth, the natural litter of each experimental female was removed and replaced with either a litter of two pups (small litter) or eight pups (large litter). The cross-fostered pups were born within 24 h and each litter was derived from litters from two different females. Females were allowed to nurse these pups for 14 days, which represents the peak of the lactation period (Johnson et al., 2001) and were then culled by cervical dislocation. Non-reproductive females were culled in the same manner and over the same period as reproductive females. Immediately after being culled, livers from females were dissected and weighed. A sample of the liver was immediately used for mitochondrial respiration measurement and the remaining was flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for subsequent analysis of CS activity and total protein content.

### Preparation of permeabilized liver

All procedures were conducted on ice. Fresh liver samples were immediately transferred into Petri dishes containing 2.5 ml of mitochondrial respiration medium consisting of 200 mmol l<sup>-1</sup> sucrose, 0.5 mmol l<sup>-1</sup> EGTA, 3 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 20 mmol l<sup>-1</sup> taurine, 10 mmol l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 20 mmol l<sup>-1</sup> HEPES and 1 g l<sup>-1</sup> fatty acid free bovine serum albumin adjusted to pH 7.1 with KOH at 37°C. Mechanical permeabilization was performed following the procedure of Kuznetsov et al. (Kuznetsov et al., 2002). Briefly, two pairs of sharp forceps were inserted centrally into the sample, and the tissue was repeatedly torn apart in different directions until 0.1 mm sized fragments were obtained in the form of a loosely connected network (Kuznetsov et al., 2002). Permeabilized tissues were then incubated in new mitochondrial respiration medium and gently shaken for 3 min. After the samples were blotted and weighed using a Sartorius CP2P Electronic Micro Precision Balance (Sartorius, Göttingen, Germany), they were immediately transferred into an Oxygraph-2K respirometer (Oroboros Instruments, Innsbruck, Austria) filled with air-saturated respiration medium.

### High-resolution respirometry

Oxygen electrodes of the respirometer were calibrated with air-saturated respiration medium at 37°C, and zero oxygen measurements were taken after sodium dithionite addition. O<sub>2</sub> solubility for the respiration medium was calculated at 37°C according to Rasmussen and Rasmussen (Rasmussen and Rasmussen, 2003). Glutamate, pyruvate and malate (10 mmol l<sup>-1</sup> each) were injected in the oxygraph chambers just before transferring the samples. To avoid limitation of oxygen diffusion at low oxygen concentration occurring in permeabilized tissue (Gnaiger et al., 1998), oxygen levels were maintained above air saturation (at

~500 nmol ml<sup>-1</sup>). Intermittent reoxygenation was achieved by partial opening of the oxygraph chambers and oxygen transfer from the gas phase, avoiding any bubble formation (Kuznetsov et al., 2002). All measurements were expressed as mean ± s.e.m. respiration rates (in pmol O<sub>2</sub> consumed s<sup>-1</sup> mg<sup>-1</sup> permeabilized liver).

### Quality control of the preparations

After stabilization of the signal, state 2' respiration rate (Table 1) (Gnaiger, 2009) was monitored. Injections of the following substrates were then performed (Table 1): ADP (5 mmol l<sup>-1</sup>, to reach state 3 respiration for complex I, CI), cytochrome *c* from equine heart (15 μmol l<sup>-1</sup>, as an index of functional integrity of the outer mitochondrial membrane, CIc). These states of respiration allowed for the calculation of the respiratory control ratio (RCR=CI/state 2') and of the cytochrome *c* effect (CIc/CI). Both parameters were used as quality control of mitochondria in the permeabilized tissue.

### O<sub>2</sub> flux measurements

After the state 2', CI and CIc respiration were measured, the protocol was followed by injections of succinate (10 mmol l<sup>-1</sup>, to monitor maximum state 3 respiration with convergent electron flow from complexes I and II, CIc+CI), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (uncoupled respiration, optimum concentration reached between 0.5 and 1.25 μmol l<sup>-1</sup>, Unc), rotenone (1 μmol l<sup>-1</sup>, inhibitor of complex I, Rot) and antimycin A (2.5 μmol l<sup>-1</sup>, inhibitor of complex III, residual oxygen consumption, Rox) (Table 1). The inhibition of complexes I and III allowed us to measure the residual oxygen consumption due to residual oxidative side reactions occurring in permeabilized tissues (Gnaiger, 2009), which was used to correct all the O<sub>2</sub> fluxes.

### Cytochrome *c* oxidase activity

Cytochrome *c* oxidase (COX) activity (complex IV; Table 1) was measured after inhibition of complexes I and III by injecting *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) and ascorbate (0.5 μmol l<sup>-1</sup> and 2 mmol l<sup>-1</sup>, respectively). Because of autoxidation of TMPD, ascorbate and cytochrome *c*, chemical backgrounds were evaluated at the end of each experiment after inhibition of COX and were subtracted from the activity (Gnaiger et al., 1998).

### Citrate synthase activity

CS is a key enzyme of the tricarboxylic acid cycle located in the matrix of mitochondria. After dissection, fresh livers were immediately flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . CS activity was measured after the livers were homogenized. Homogenates were transferred in 100 mmol l<sup>-1</sup> imidazole-HCl (pH 8), 0.1 mmol l<sup>-1</sup> 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 0.1 mmol l<sup>-1</sup> acetyl CoA and 0.15 mmol l<sup>-1</sup> oxaloacetate. Activities were determined by following the increase in absorbance due to the reduction of DTNB at 412 nm (Pichaud et al., 2010). Protein concentration was determined using Coomassie Blue (Bradford, 1976) and results were expressed as U mg<sup>-1</sup> proteins, where U is 1 μmol of substrate transformed per minute.

### Analysis of mitochondrial metabolism

To understand the variations of mitochondrial activity and the amount of functional mitochondria, measurement of oxygen fluxes in the oxygraph chambers (pmol O<sub>2</sub> consumed s<sup>-1</sup>) were first normalized per mg wet mass. This allows the evaluation of mass-specific flux of permeabilized livers, integrating mitochondrial quality and quantity (Pesta and Gnaiger, 2012). Intrinsic changes are most often determined by normalizing the functional measure

of interest (in this case the oxygen fluxes) to a marker of mitochondrial content (Larsen et al., 2012). For this purpose, we used CS to express the oxygen fluxes per CS activity using the same mice. Moreover, the integrity of the ETS is reflected in the stoichiometry of respiration rates driven by the different mitochondrial complexes (Picard et al., 2010). We therefore calculated different ratios of complexes such as CIc+CI/CIc, COX/CIc and COX/CIc+CI to emphasize the possible quantitative adjustment of the complexes of the ETS in each treatment.

### Chemicals

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

### Statistical analyses

All statistical analyses were performed with SAS software (9.1.3, SAS Institute, Cary, NC, USA). After testing for homogeneity of variances, differences among the three treatments were tested by one-way ANOVA using a general linear model procedure followed by a *posteriori* least significant difference (LSD) comparison analysis for specific determination of differences between the three treatments. Significance was defined at  $P < 0.05$ .

### RESULTS

Results from one-way ANOVA for RCR, mass-specific O<sub>2</sub> fluxes, COX activity, CS activity, O<sub>2</sub> fluxes normalized to CS and ratios of complexes (CIc+CI/CIc, COX/CIc and COX/CIc+CI) are presented in Table 2.

#### Quality control of the preparations

Both RCR and cytochrome *c* confirmed high quality and functionality of mitochondria in all preparations used for the measurement of mitochondrial respiration. RCRs were calculated for complex I as CI/state 2'. In all experiments, RCRs obtained using substrates for complex I were above 5.0 for each treatment (Table 3), indicating

Table 2. Results of ANOVAs for respiratory control ratio, mass-specific oxygen fluxes, citrate synthase activity, oxygen fluxes normalized to citrate synthase and ratios of complexes

	$F_{2,15}$	$P$
Respiratory control ratio	0.22	0.803
Citrate synthase activity	2.78	0.094
Mass-specific O <sub>2</sub> fluxes		
State 2'	2.57	0.110
CI	1.83	0.195
CIc	0.79	0.470
CIc+CI	3.32	0.640
Unc	4.68*	0.026
Rot	0.56	0.582
COX	10.51*	0.001
O <sub>2</sub> fluxes/CS		
State 2'	3.92*	0.043
CI	3.05	0.077
CIc	1.99	0.171
CIc+CI	3.98*	0.041
Unc	4.22*	0.035
Rot	3.62	0.052
COX	4.6*	0.028
Ratios of complexes		
CIc+CI/CIc	0.43	0.658
COX/CIc	0.35	0.711
COX/CIc+CI	0.16	0.855

\*Significant effect of treatment.

Table 3. Values of respiratory control ratio (RCR=CI/state 2') and cytochrome *c* effect (CIc/CI) in permeabilized livers of female mice

	Non-reproductives	Small litters	Large litters
Respiratory control ratio	5.17±0.52	5.10±0.75	5.60±0.36
Cytochrome <i>c</i> effect	0.99±0.02	1.09±0.05	1.01±0.02

Data are means ± s.e.m.

well-coupled respiration in every preparation. No significant effect of treatment was detected on RCR values ( $F_{2,15}=0.22$ ,  $P=0.803$ ; Table 2). Cytochrome *c* addition was used as a test for intactness of the mitochondrial outer membrane (Kuznetsov et al., 2008). As expected, respiration rates before and after cytochrome *c* additions were very similar in all preparations (Table 3).

#### O<sub>2</sub> flux measurements

Mass-specific O<sub>2</sub> fluxes were measured in permeabilized livers and results are presented in Table 2. Within all the parameters measured, only uncoupled respiration (Unc) showed a significant treatment effect ( $F_{2,15}=4.68$ ,  $P=0.026$ ; Table 2). Using the LSD analysis, significant differences were detected between the non-reproductive and the small litter treatments ( $P=0.008$ ), but not between the non-reproductive and the reproductive females having a large litter ( $P=0.135$ ) or between the reproductive females having a small and a large litter ( $P=0.161$ ).

#### Cytochrome *c* oxidase activity

COX activity represents the maximal efficiency of mitochondrial complex IV to consume oxygen and is therefore a good indicator of ATP production capacity. It was evaluated in the permeabilized livers and was expressed as mass-specific O<sub>2</sub> fluxes induced by injection of ascorbate and TMPD after inhibition of complexes I and III. Overall, the observed differences suggest that in reproductive females complex IV is less efficient at reducing O<sub>2</sub> to H<sub>2</sub>O. A significant effect of treatment was detected ( $F_{2,15}=10.51$ ,  $P=0.001$ ; Table 2). Moreover, the non-reproductive females had significantly higher COX activity than the reproductive females ( $P < 0.001$  for the small litter treatment and  $P=0.009$  for the large litter treatment).

#### Citrate synthase activity

Considering the six mice per treatment we used in this study for mitochondrial respiration experiments, we did not detect a treatment effect on CS activity ( $F_{2,15}=2.78$ ,  $P=0.094$ ; Table 2). Reproductive females have greater mean CS activity ± s.e.m. compared with non-reproductive females (90.20±12.03, 108.72±15.77 and 67.86±7.61 Umg<sup>-1</sup> proteins for small litter, large litter and non-reproductive females, respectively). The fact that this difference was not significant was due to a lack of power, rather than there being no real difference. As this enzyme can be measured in frozen tissue, we were able to increase the power of this analysis by examining a further sample of mice that had been through identical treatments (see Garratt et al., 2013). With this increased power we were able to detect highly significant differences between treatments [results reported in the accompanying paper (Garratt et al., 2013)], with reproductive females having a greater CS activity than the non-reproductive females.

#### Analysis of mitochondrial metabolism

O<sub>2</sub> fluxes were normalized to CS activity (Fig. 1). Adjustments of both mitochondrial content and functional properties of mitochondria are occurring in reproductive females compared with non-reproductives. An effect of treatment was detected with one-way

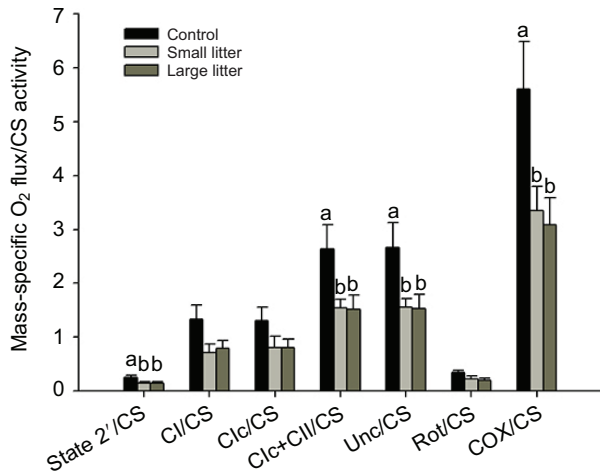


Fig. 1. Oxygen consumption normalized to citrate synthase (CS) activity. Mass-specific O<sub>2</sub> fluxes were divided by CS activity measured in the liver of non-reproductive and reproductive (small and large litter) mice. For all the parameters measured (state 2', CI, Clc, Clc+CII, Unc, Rot and COX), results are presented as means  $\pm$  s.e.m. for six different preparations for each treatment. Significance was set at  $P < 0.05$ ; different letters denote significant differences between treatments.

ANOVA for state 2'/CS, Clc+CII/CS, Unc/CS and COX/CS ( $F_{2,15}=3.92$ ,  $P=0.043$ ;  $F_{2,15}=3.98$ ,  $P=0.041$ ;  $F_{2,15}=4.22$ ,  $P=0.035$ ; and  $F_{2,15}=4.60$ ,  $P=0.028$ , respectively; Table 2) whereas CI/CS, Clc/CS and Rot/CS were not significant. Moreover, LSD comparison shows that the non-reproductive females had higher values for each parameter than both the small litter ( $P=0.026$ ,  $P=0.029$ ,  $P=0.025$  and  $P=0.026$  for state 2'/CS, Clc+CII/CS, Unc/CS and COX/CS, respectively) and the large litter ( $P=0.032$ ,  $P=0.026$ ,  $P=0.022$  and  $P=0.015$  for state 2'/CS, Clc+CII/CS, Unc/CS and COX/CS, respectively) treatments.

Ratios of complexes were evaluated to determine whether one of the different complexes of the ETS was particularly affected by reproduction and/or litter size. None of the ratios calculated (Clc+CII/Clc, COX/Clc and COX/Clc+CII) were significantly different between treatments (Fig. 2, Table 2).

## DISCUSSION

Reproduction has significant costs to animals and changes in cellular structure and function are likely to occur as a result. We show that in mammals at peak lactation, mitochondrial functions adjust in the liver. Specifically, after examining several mechanisms involved in mitochondrial energy production, we found that the differences observed between reproductive and non-reproductive females are mainly due to a combination of changes in the functional properties of the ETS and of mitochondrial density in the liver. However, we did not find differences between reproductive females having either two or eight pups. These results suggest that experiencing reproduction could have triggered physiological and metabolic changes in mitochondria (comparison between reproductive and non-reproductive females) and that the degree to which animals are investing in lactation may not have a significant influence in the changes we observed in reproductive females (comparison between small and large litters). However, we suggest that adjustments of mitochondrial functions may occur to spare substrates for milk production in other tissues such as the mammary gland [in accordance with the 'peripheral limitation' hypothesis (Hammond et al., 1994)] and to maintain a balance between energy and ROS production.

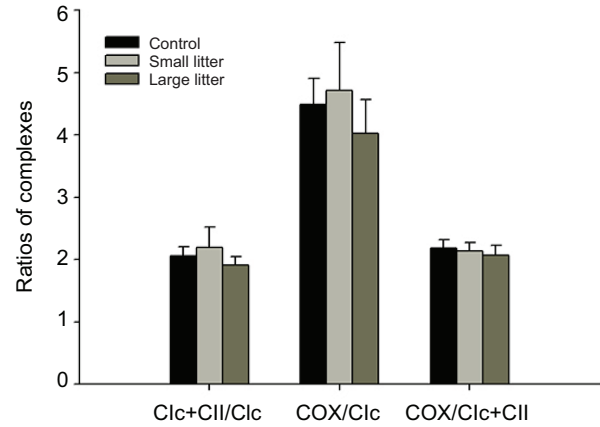


Fig. 2. Ratios of complexes. For Clc+CII/Clc, COX/Clc and COX/Clc+CII, ratios were calculated according to the mass-specific O<sub>2</sub> flux measured.

One way that mitochondrial energy production can be regulated is through changes in basal proton leak across the mitochondrial inner membrane (Parker et al., 2009; Jastroch et al., 2010). State 2' is a resting state of non-phosphorylating respiration when oxygen flux is maintained mainly to compensate for the proton leak (Gnaiger, 2009) and can thus be used as an indicator of basal proton leak in the liver of mice (Table 1). We found no differences between treatments in mass-specific state 2' oxygen flux (Table 2), suggesting that the basal proton leak in liver mitochondria is not significantly affected at peak lactation.

Regulation of mitochondrial energy production can also occur by adjusting the functional properties of the different complexes of the ETS. The state 3 monitored at different steps of the ETS (Clc, Clc+CII; Table 1), is not affected by the treatments (Table 2). However, both uncoupling respiration triggered by injection of FCCP (Unc) and COX were higher in non-reproductive females (Table 2). These results may indicate that non-reproductive females have a higher ETS capacity than the small litter females and a higher ATP production than the two reproductive treatments. These changes may be related, at least in part, to a difference in fatty acid utilization during lactation. It has been shown that in tissues with high rates of fatty acid catabolism such as the liver, downregulation of Ppar $\alpha$  and its coactivator (involved in fatty acid uptake, fatty acid oxidation and thermogenesis) is responsible for the reduced utilization of fatty acids in liver during lactation in mice (Gutgesell et al., 2009). Because fatty acid oxidation generally leads to high ATP production, our results on COX activity are in line with these previous observations and suggest that in the livers of lactating mice lower oxidation of fatty acids is associated with lower ATP production; these substrates are probably diverted to other tissues such as the mammary gland for milk production.

When O<sub>2</sub> fluxes were normalized to CS activity, state 2', Clc+CII, Unc and COX rates were higher in non-reproductive when compared with reproductive females (Fig. 1). It is therefore likely that CS activity and thus mitochondrial density has an effect on the oxygen consumption of reproductive mice (see also Garratt et al., 2013). This adjustment in mitochondrial number, combined with an adjustment in ETS capacity and in ATP production, may explain the differences observed between treatments.

It is, however, possible that other factors are impacting the regulation of mitochondrial energy production. Across the phyla of bacteria, higher plants, fungi and mammals, complex I always seems to be associated in supercomplex with one or two units of complex

III comprising between zero and four units of complex IV (Lenaz and Genova, 2010). In bovine heart mitochondria, higher enzymatic activities of complex I (2.3-fold) and complex III (16.5-fold) were found when they were organized in the I<sub>1</sub>III<sub>2</sub>IV<sub>1</sub> form than in the I<sub>1</sub>III<sub>2</sub> form (Schäfer et al., 2006). Moreover, it has been shown that supercomplexes comprised of various stoichiometries of complexes I, III and IV declined significantly in heart mitochondria of aging rats (Gómez and Hagen, 2012). Changes in the channelling of respiratory chain intermediate substrates *via* differences in the organization and content of supercomplexes may thus be another factor influencing mitochondrial respiration in lactating mice. We evaluated the ratios of different complexes and observed no differences between the different females, suggesting that the stoichiometry of the ETS complexes is maintained during peak lactation in mice and that the differences observed were not due to quantitative adjustments of any particular complex.

The adjustments we observed in reproductive females may have several different consequences in terms of maximal investment in reproduction but also in terms of aging. ROS are generated by mitochondria as an unavoidable drawback of energy production. These have the potential to cause oxidative stress, which might mediate the trade-off between reproduction and lifespan (Dowling and Simmons, 2009; Monaghan et al., 2009). Although this is still a topic of great debate, several hypotheses have tried to link mitochondrial activity and ROS production (Brand, 2000; Balaban et al., 2005; Speakman, 2008; Neretti et al., 2009; Siegel et al., 2011). Two different hypotheses can be put forward to relate our results to ROS production.

First, the lower mitochondrial respiration we detected in reproductive females could lead to a decrease in ROS production (Balaban et al., 2005; Neretti et al., 2009). Second, the higher respiration rates observed in non-reproductive females is mainly due to a higher mitochondrial futile proton cycle, which decreases the generation of ROS according to the ‘uncoupling to survive’ theory (Brand, 2000). In our study, non-reproductive females had greater oxygen consumption when an uncoupler was used (Unc; maximum ETS capacity; Table 1). Such agents lower proton-motive force by increasing its consumption and decrease the ROS production as a consequence (Brand, 2000). Thus, it suggests that reproductive females are producing more ROS due to a lower ETS capacity.

Several studies have shown lower oxidative stress in reproductive females of different species (Garratt et al., 2011; Oldakowski et al., 2012) using different markers of oxidative damage. One explanation for these results is that ROS production is decreased in reproductive females. ROS have long been considered a by-product of mitochondrial respiration, which cause damage to macromolecules and cellular dysfunction. However, more recent studies have now demonstrated that ROS are potential regulators of a number of intracellular pathways (St-Pierre et al., 2006; Han et al., 2008; Rasbach and Schnellmann, 2007; Siegel et al., 2011; Finkel, 2012; Ray et al., 2012). Recently, using the same set of mice, we demonstrated that livers of reproductive females showed higher activity of superoxide dismutase (SOD) relative to non-reproductive females, as well as decreased protein oxidation (Garratt et al., 2013). Altogether, these results suggest that the adjustment of mitochondria we observed here might lead to higher ROS production. In turn, this triggers upregulation of antioxidant defences such as SOD that detoxify the cell from ROS and thus decrease oxidative damage.

Surprisingly, we failed to find any differences between reproductive females with small and large litters. A possible explanation could be that changes occur in mitochondria in response to reproduction in general (pregnancy and/or lactation), but are not related to the relative amount animals are investing

in lactation. Another explanation would be that manipulating female investment during lactation by altering female litter size to the degree conducted in this study does not cause sufficient cellular changes to be detected in the livers of lactating mice. An interesting alternative explanation would be that other peripheral signals are eliciting these changes, as stipulated by the ‘saturated neural control’ hypothesis. For example, during lactation, there is a profound reduction in circulating leptin levels (Kunz et al., 1999; Woodside et al., 2000), which potentially stimulates food intake (Speakman and Król, 2011). In accordance with our results, it has been shown that an increase in leptin levels is associated with reduced mitochondrial volume density and altered substrate oxidation kinetics in liver of mice (Singh et al., 2009). Therefore, it is possible that during lactation the leptin levels cannot be reduced under a specific rate, regardless of the number of pups that can be assigned to a female, explaining why we did not find differences within the reproductive females. Further studies linking food consumption, leptin levels and changes in mitochondrial functions might be worth investigating in reproductive mice with different litter sizes.

We demonstrated in this study that reproduction, one of the most physiologically constraining and energetically demanding periods in a female’s life, results in cellular and metabolic adaptations at the mitochondrial level. Liver mitochondria of reproductive females showed lower ETS activity and an increase in mitochondrial density. We suggest that this mitochondrial adjustment in the liver might help to spare substrates and therefore energy for milk production in the mammary gland. Moreover, our results suggest that these changes lead to an increase in ROS production (even if we did not directly measure ROS production) that subsequently upregulates antioxidant defence activity and decreases oxidative stress. Therefore, reproduction could correlate with higher ROS production but not necessarily oxidative stress, at least in liver. Further investigations focusing on regulation of mitochondrial energy production at other levels (such as inducible proton leak by expression of uncoupling proteins) in the liver and in other tissues as well as measurement of ROS production *in vivo* (Cochemé et al., 2011) would greatly improve our knowledge on the metabolic and cellular adaptations occurring during reproduction.

#### LIST OF ABBREVIATIONS

CI	complex I state 3 respiration
CIc	complex I state 3 respiration after injection of cytochrome <i>c</i>
CIc+CII	complexes I and II state 3 respiration
COX	complex IV
CS	citrate synthase
ETS	electron transport system
FCCP	carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
LSD	least significant difference
RCR	respiratory control ratio
ROS	reactive oxygen species
Rot	rotenone-inhibited respiration
Rox	residual oxygen consumption
SOD	superoxide dismutase
TMPD	<i>N,N,N',N'</i> -tetramethyl- <i>p</i> -phenylenediamine
Unc	uncoupled respiration

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#### AUTHOR CONTRIBUTIONS

N.P. and M.G. designed and executed the experiments, and analysed and interpreted the data. N.P. wrote the first draft and revised the manuscript. M.G., J.W.O.B. and R.C.B. helped with the writing of the first draft.

## COMPETING INTERESTS

No competing interests declared.

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