## ORIGINAL RESEARCH

# Simultaneous measurement of mitochondrial respiration and ATP production in tissue homogenates and calculation of effective P/O ratios

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## Abstract

The use of tissue homogenate has greatly aided the study of the functioning of mitochondria. However, the amount of ATP produced per oxygen molecule consumed, that is, the effective P/O ratio, has never been measured directly in tissue homogenate. Here we combine and refine existing methods previously used in permeabilized cells and isolated mitochondria to simultaneously measure mitochondrial ATP production (JATP) and oxygen consumption ($JO_2$) in tissue homogenate. A major improvement over existing methods is in the control of ATPases that otherwise interfere with the ATP assay: our modified technique facilitates simultaneous measurement of the rates of "uncorrected" ATP synthesis and of ATP hydrolysis, thus minimizing the amount of tissue and time needed. Finally, we develop a novel method of calculating effective P/O ratios which corrects measurements of JATP and $JO_2$ for rates of nonmitochondrial ATP hydrolysis and respiration, respectively. Measurements of JATP and $JO_2$ in liver homogenates from brown trout (*Salmo trutta*) were highly reproducible, although activity declined once homogenates were 2 h old. We compared mitochondrial properties from fed and food-deprived animals to demonstrate that the method can detect mitochondrial flexibility in P/O ratios in response to nutritional state. This method simplifies studies examining the mitochondrial bioenergetics of tissue homogenates, obviating the need for differential centrifugation or chemical permeabilization and avoiding the use of nonmitochondrial ATPase inhibitors. We conclude that our approach for characterizing effective P/O ratio opens up new possibilities in the study of mitochondrial function in very small samples, where the use of other methods is limited.

## Introduction

Mitochondria are responsible for oxidation of food molecules to convert energy in the form of ATP that fuels cell functions (i.e., muscle contraction, protein synthesis, gene replication, ion homeostasis). As mitochondria produce about 90% of the cellular ATP (Lehninger et al. 1993), variation in the amount of ATP produced per unit of oxygen consumed, that is, the effective P/O ratio, have significant consequences at all levels of biological organization. Variation in effective P/O ratio is known to affect energy homeostasis in cells, and thus tissues function (Pham et al. 2014; Layec et al. 2015) and ultimately, disease pathologies and normal aging (Brand 2005; Wu et al. 2007; Kadenbach et al. 2013; Conley 2016). In extreme environmental conditions such as cold, starvation and hypoxia, mitochondrial efficiency has been suggested to be a major physiological adaptation for regulating energy

homeostasis in the organism (Gnaiger et al. 2000; Trzcionka et al. 2008; Monternier et al. 2014). However, while methods of evaluating mitochondrial efficiency have received much attention (Hinkle 2005; Van Bergen et al. 2014), measuring P/O ratios has proved to be challenging (Ferguson 2010).

The mitochondrial preparation required for assaying bioenergetics involves numerous steps encompassing differential centrifugation or bath transfer (Van Bergen et al. 2014), making it technically challenging to obtain a sufficient amount of mitochondria from tissues smaller than those of a mouse (Chinopoulos et al. 2011). This limitation has motivated current efforts to implement the high yield technique of homogenization that is capable of recovering 100% of the tissue sample for use in the mitochondrial analysis (Pecinova et al. 2011; Makrecka-Kuka et al. 2015; Rovenko et al. 2015; Ziak et al. 2015; Salin et al. 2016). Tissue homogenate offers the advantage of studying mitochondrial function close to physiological conditions, therefore differences in mitochondrial properties observed in homogenates may better reflect physiological processes that will occur in the animal (Kuznetsov et al. 2002; Burtscher et al. 2015). However, the problem emerges that homogenates contain ATPases that interfere with the ATP assay.

Techniques used to measure ATP production ($J$ATP) that are compatible with simultaneous measurement of mitochondrial oxygen consumption ($J$O$_2$) have included: (1) estimation of mitochondrial ADP consumption (Estabrook 1967), (2a) direct and (2b) indirect assays of changes in ATP concentration (Ouhabi et al. 1998; Salin et al. 2010a), and (3) measurement of changes in free magnesium concentration, [Mg$^{2+}$] (Chinopoulos et al. 2009). Technique 1 can produce biased measures of $J$ATP when using tissue homogenates due to the presence of nonmitochondrial ATPases. Technique 2b allows measurement of this ATPase activity so that the $J$ATP value can be corrected, but this correction requires twice the amount of tissue and time (Salin et al. 2012a). The presence of ATPase may also affect $J$ATP measurements using technique 3. Techniques 2a and 3 aim to block the action of ATPase so as to prevent ATP hydrolysis (Ouhabi et al. 1998; Chinopoulos et al. 2014), but the efficacy of inhibitors may depend on the tissue, study model or mitochondrial preparation.

Here, we describe a protocol for measuring effective P/O ratios that simultaneously quantifies both $J$ATP and $J$O$_2$ in tissue homogenate, using a combination of techniques 2b and 3. As with technique 3, we use the magnesium-sensitive fluorescent probe, Mg Green, to estimate changes in [Mg$^{2+}$] (Szmacinski and Lakowicz 1996). Mitochondrial $J$ATP is calculated from the rate of change in [Mg$^{2+}$] and is based on the unequal affinities of ATP and ADP for Mg$^{2+}$ (Leyssens et al. 1996). Nonmitochondrial ATPase activity is measured as in technique 2b by inhibiting the mitochondrial ATP flux (Salin et al. 2010a), allowing correction of $J$ATP. Our modified technique facilitates simultaneous measurement of the rates of "uncorrected" ATP synthesis and of ATP hydrolysis, thus minimizing the amount of tissue and time needed. We validate the technique and calculate $J$ATP, $J$O$_2$ and the effective P/O ratio, using liver homogenates from juvenile brown trout (*Salmo trutta*). We compare P/O ratios of fed and starved fish to determine whether our approach was a specific and sensitive assay to detect variation in P/O ratio in tissue homogenate. We chose liver because hepatic mitochondria are known to change their properties in response to food availability. Attempts to assess the role of mitochondria in the regulation of energy homeostasis during fasting have been addressed in mammals (Bobyleva-Guarriero et al. 1984; Brand et al. 1993; Dumas et al. 2004), birds (Bobyleva-Guarriero et al. 1984; Monternier et al. 2015), amphibians (Trzcionka et al. 2008) and fish (Savina et al. 2009; Bermejo-Nogales et al. 2015). Of note, the role of the liver in the control of carbohydrate and lipid homeostasis is essential for providing substrates to others tissues during fasting (Postic et al. 2004). In fish, there is a large body of evidence illustrating adaptive changes in enzymatic activities of the oxidative phosphorylation pathway (Guderley et al. 2003; Frick et al. 2008; Bermejo-Nogales et al. 2015), but whether their effective P/O ratio shows similar flexibility has so far not been determined.

## Methods

### Animal husbandry

Juvenile brown trout were obtained from a hatchery (Howietoun, UK) in summer 2015 and moved to the University of Glasgow. Here, the fish were kept in a communal tank and maintained under an 8 h light: 16 h dark photoperiod at 12°C and fed daily in excess (pellets EWOS, West Lothian, UK). In January 2016, twenty four fish were transferred to individual compartments of a stream tank. Because of the logistical constraints associated with assaying fresh mitochondria, transfers of the fish to individual compartments were staggered over 12 days (two fish per day). All fish were first acclimated for a week and fed daily to excess prior to the start of the food treatments. Half of the fish were randomly allocated to an ad libitum ration, while the other half was deprived of food (initial body mass: $12.14 \pm 0.61$ vs. $12.00 \pm 0.57$ g in the fasted and fed group). Fish were held on these treatments for 2 weeks. At the end of this period, all fish were fasted overnight. The following

morning (mean $\pm$ 1 SE: 09:30 $\pm$ 00:01 h) a pair of fish was culled and their livers collected to determine mitochondrial parameters. Fasting led to reduction in body mass (final mass fasted group: 10.85 $\pm$ 2.14 g). At the same time, the fed trout had an increase in body weight (final mass fed group: 16.50 $\pm$ 3.80 g). All procedures were carried out under the jurisdiction of a UK Home Office project license (PPL 60/4292).

## Estimation of $K_d$ of ATP and ADP for free magnesium (Mg$^{2+}$)

The method for determining the affinity of the magnesium with nucleotides was adapted from (Chinopoulos et al. 2014). The main adjustment of the assay of the $K_d$ is a measurement in the absence of tissue homogenate and ATPase inhibitors (see below). Thus, assays were made in duplicate in a buffer containing 20 mmol/L Taurine, 10 mmol/L KH$_2$PO$_4$, 20 mmol/L HEPES, 110 mmol/L D-sucrose, 60 mmol/L K-lactobionate, 1 g/L BSA fatty acid free, pH 7.2 and at 12°C. The titration protocol was as follows: the buffer was supplemented with pyruvate (5 mmol/L) and malate (0.5 mmol/L). Magnesium green (2.1 $\mu$mol/L), EGTA (0.1 mmol/L) and EDTA (15 $\mu$mol/L) were subsequently added to the chamber. Then, stepwise additions of MgCl$_2$ were performed for calibration of the fluorescent

signal into Mg$^{2+}$ (Fig. 1). Succinate (10 mmol/L) was then added. Stepwise additions of ATP or ADP were then performed to determine the affinity of those nucleotides for Mg$^{2+}$ (25 additions of 0.2 mmol/L ATP or 0.25 mmol/L ADP) (Fig. 1). The two $K_d$ values were determined using the least squares method, described in detail in (9)(9)(8)(8) (7). The binding affinity ($K_d$) of ATP and ADP for Mg$^{2+}$ [3] was calculated, using the method of (Chinopoulos et al. 2014); the values were $K_{d\text{-}ATP}$ = 0.1545 mmol/L and $K_{d\text{-}ADP}$ = 2.1333 mmol/L.

The method for determining the affinity of the magnesium with nucleotides in the presence of mitochondria and ATPase inhibitors (as in Chinopoulos et al. 2014) was not used here due to the insufficient effect of ATPase inhibitors. In particular, there was a lack of stability of the fluorescent signal (Fig. 1) after ATP additions when in the presence of homogenate (5 mg wet mass liver per ml buffer) and inhibitors (25 $\mu$mol/L adenylate kinase inhibitor Ap5A, 2 $\mu$g/mL oligomycin, 4 $\mu$mol/L carboxy- [4] latractyloside, 0.2 mmol/L beryllium sulfate tetrahydrate, 5 mmol/L sodium trifluoride and 30 $\mu$mol/L sodium orthovanadate). We therefore omitted ATPase inhibitors when making measurements of $K_d$, $J$ATP and $J$O$_2$. Thus, the insufficient effect of ATPase inhibitors on liver homogenate motivates us to further develop a method to quantify the rate of ATP hydrolysis.
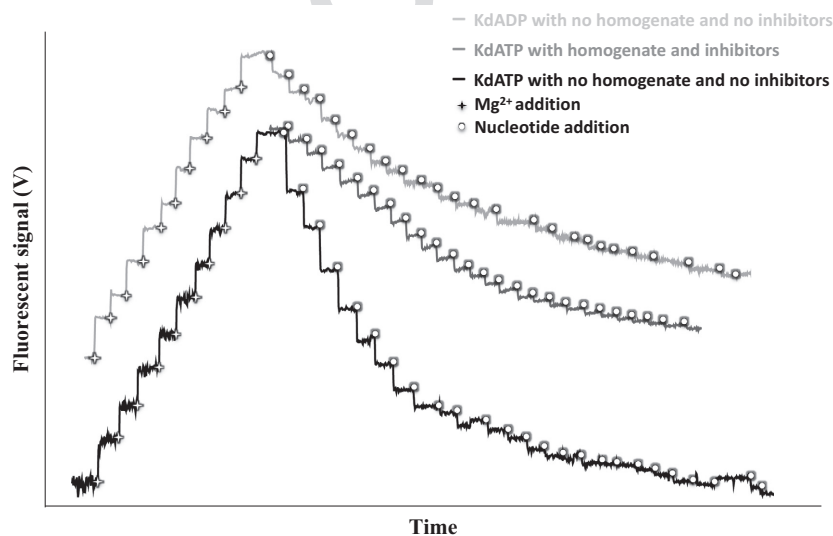


**Figure 1.** Estimation of $K_d$ of ATP and ADP for free magnesium (Mg$^{2+}$). Stepwise additions of MgCl$_2$ (10 times 0.1 mmol/L) were performed to calibrate the relationship between the fluorescent signal and [Mg$^{2+}$]. Stepwise additions of ATP (black line) or ADP (light gray line) were then performed to determine the affinity of those nucleotides for Mg$^{2+}$ (25 additions of 0.2 mmol/L ATP or 0.25 mmol/L ADP). Assays were made with buffer (see main text for composition) and at 12°C, in the presence of magnesium green (2.2 $\mu$mol/L), EGTA (0.1 mmol/L) and EDTA (15 $\mu$mol/L), but in the absence of tissue homogenate and ATPase inhibitors. These were omitted due to the insufficient effect of ATPase inhibitors, as shown by the lack of stability of the fluorescent signal (dark gray line) after ATP additions when in the presence of homogenate and inhibitors (see main text for details).

## Mitochondrial homogenate preparation

Liver homogenate was prepared as in (Salin et al. 2016). Briefly, tissues (mean ± 1 SE: 43.08 ± 2.02 mg) were minced to obtain a shredded solution, which was then homogenized with a Potter-Elvehjem homogenizer in a buffer containing 20 mmol/L Taurine, 10 mmol/L $KH_2PO_4$, 20 mmol/L HEPES, 110 mmol/L D-sucrose, 60 mmol/L K-lactobionate, 1 g/L BSA fatty acid free, pH 7.2. Homogenates were then diluted to obtain a final concentration of 5 mg/mL. The entire procedure was carried out at 4°C and completed within 30 min of the fish being culled. A sample of liver homogenate was then immediately added to one of the two measurement chambers of an Oroboros oxygraph. The leftover preparation of liver homogenate was preserved on ice for use in a replicate trial.

## Mitochondrial rates of oxidation and phosphorylation: measurement

Oxygen and magnesium green fluorescence signals were detected simultaneously, using respirometry chambers equipped with fluorescent sensors and recorded, using DatLab software (Oroboros Instruments, Innsbruck, Austria, as in Chinopoulos et al. 2014). Pure oxygen gas was added to the respirometry chambers to reach a concentration of 550 μmol/L. The titration protocol was as follows: respiration was stimulated by adding pyruvate (5 mmol/L) and malate (0.5 mmol/L). Magnesium green (2.1 μmol/L), EGTA (0.1 mmol/L) and EDTA (15 μmol/L) were subsequently added to the chamber. Then, stepwise additions of $MgCl_2$ were performed for calibration of the fluorescent signal into $Mg^{2+}$. Succinate (10 mmol/L) was then added. State 3 was reached by adding a saturating concentration of ADP (2 mmol/L). In this condition, changes in oxygen and ATP concentrations in the chamber are representative of raw fluxes in oxygen and ATP ($JO_{2-raw}$ and $JATP_{raw}$, respectively). $JO_{2-raw}$ reflects the rate of oxygen consumption by the mitochondria but also by nonmitochondrial reactions; $JATP_{raw}$ is the balance between the rate of mitochondrial ATP production and the rate of ATP disappearance due to the activity of the various ATPase enzymes (phosphorylases, phosphatases and kinases; for further details see Chinopoulos et al. 2014). Addition of carboxyatractyloside (4 μmol/L), an inhibitor of ATP-ADP exchanger, allowed calculation of the rate of ATP
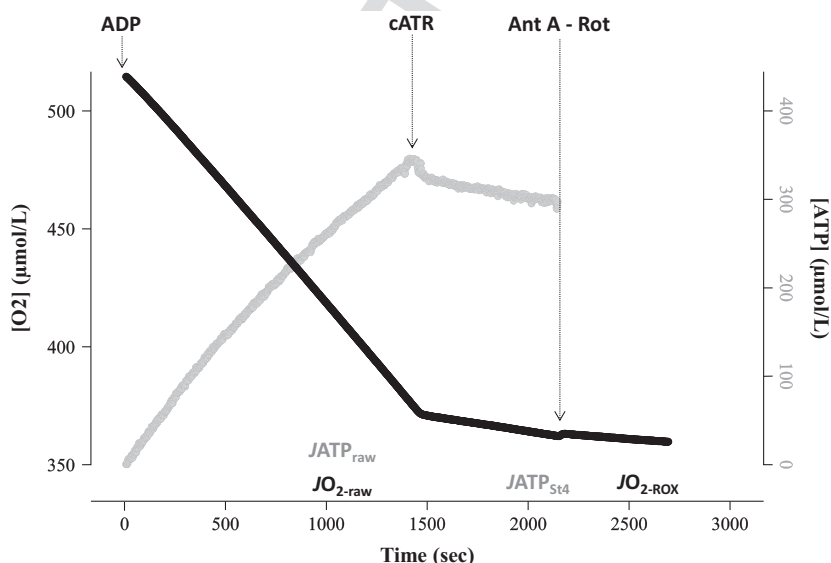


**Figure 2.** Output from a representative experiment, using homogenized brown trout liver, showing changes in oxygen and ATP content as a function of time. The substrates pyruvate, malate and succinate, as well as free magnesium, were present at the start of the experiment. State 3 respiration was initiated by adding 2 mmol/L ADP; the disappearance of oxygen (black line) was matched by the appearance of ATP (gray line). State 3 parameters stabilized 15 min after adding ADP, at which point rates of oxygen consumption ($JO_{2-raw}$) and ATP production ($JATP_{raw}$) were measured for a further 5 min. The rate of ATP disappearance due to the activity of ATPase ($JATP_{St4}$) was quantified by inhibiting the mitochondrial ATP-ADP exchange with carboxyatractyloside (cATR) (note the cessation of ATP production at this point, and reduction in oxygen consumption). Finally, the nonmitochondrial oxygen consumption ($JO_{2-ROX}$) was estimated by inhibiting the respiratory chain with antimycin A (Ant A) and rotenone (Rot). Mitochondrial parameters were measured at 5 mg wet mass liver per ml at 12°C; Oxygen content in the respirometry chambers never dropped below 300 μmol/L, so maintaining the liver above sensitive levels (≈100 μmol/L in our conditions, data not shown).
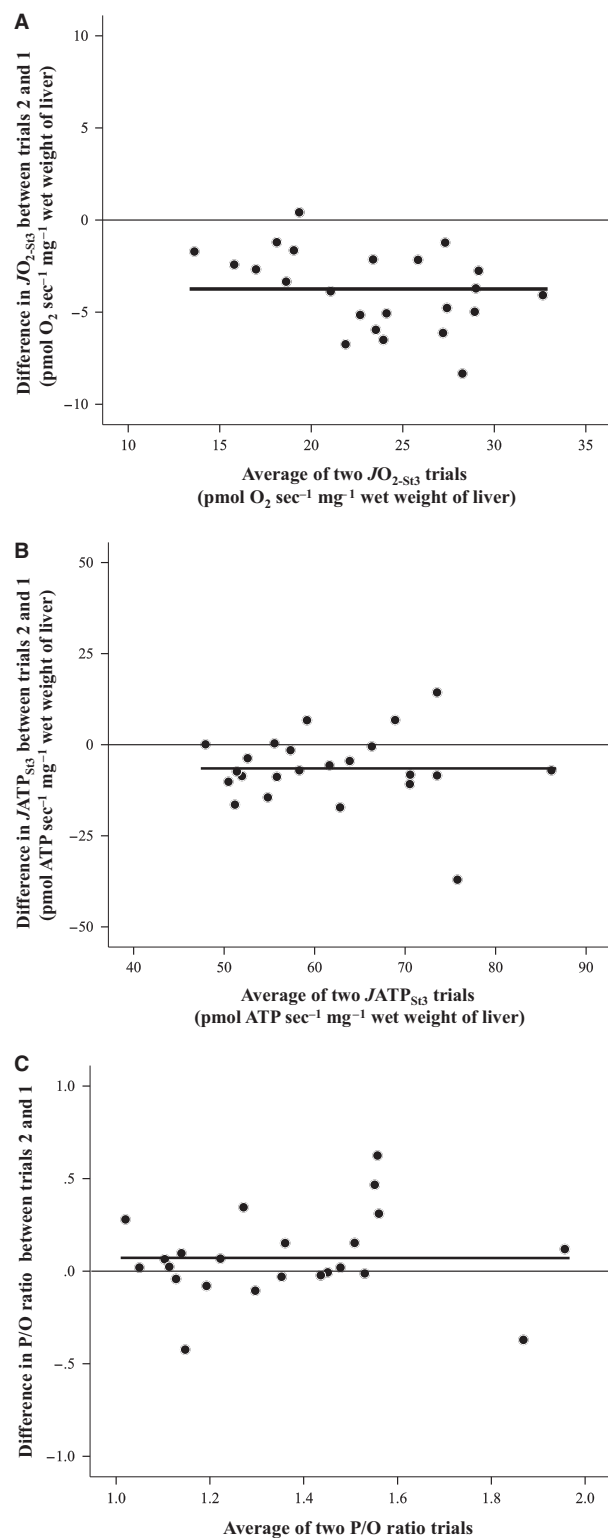
**Figure 3.** Changes in mitochondrial properties as a result of time since liver homogenization, shown as the difference between measurements made immediately versus 2 h after homogenization, and plotted against the mean of the two values. (A) Hepatic mitochondrial oxygen consumption $JO_{2\text{-}St3}$ (drift between trials: paired $t = 8.322$, $P < 0.001$). (B) Liver mitochondrial ATP production $JATP_{St3}$ (drift between trials: paired $t = 3.099$, $P = 0.005$). (C) Liver mitochondrial estimated P/O ratio (drift between trials: paired $t = -1.476$, $P = 0.154$). $N = 23$ fish in all cases. Thick lines represent the mean value of the difference, and the thin line a difference of zero (i.e., no difference in measurement between the two trials).

A (2.5 $\mu$mol/L) determined nonmitochondrial, or residual, oxygen consumption ($JO_{2\text{-}ROX}$). At each step the rates of changes in ATP and/or oxygen were allowed to stabilize. An identical trial was run on the homogenate obtained from the same fish 2 h later, in order to determine the repeatability of measurements and the stability of measurements in relation to time since sample preparation.
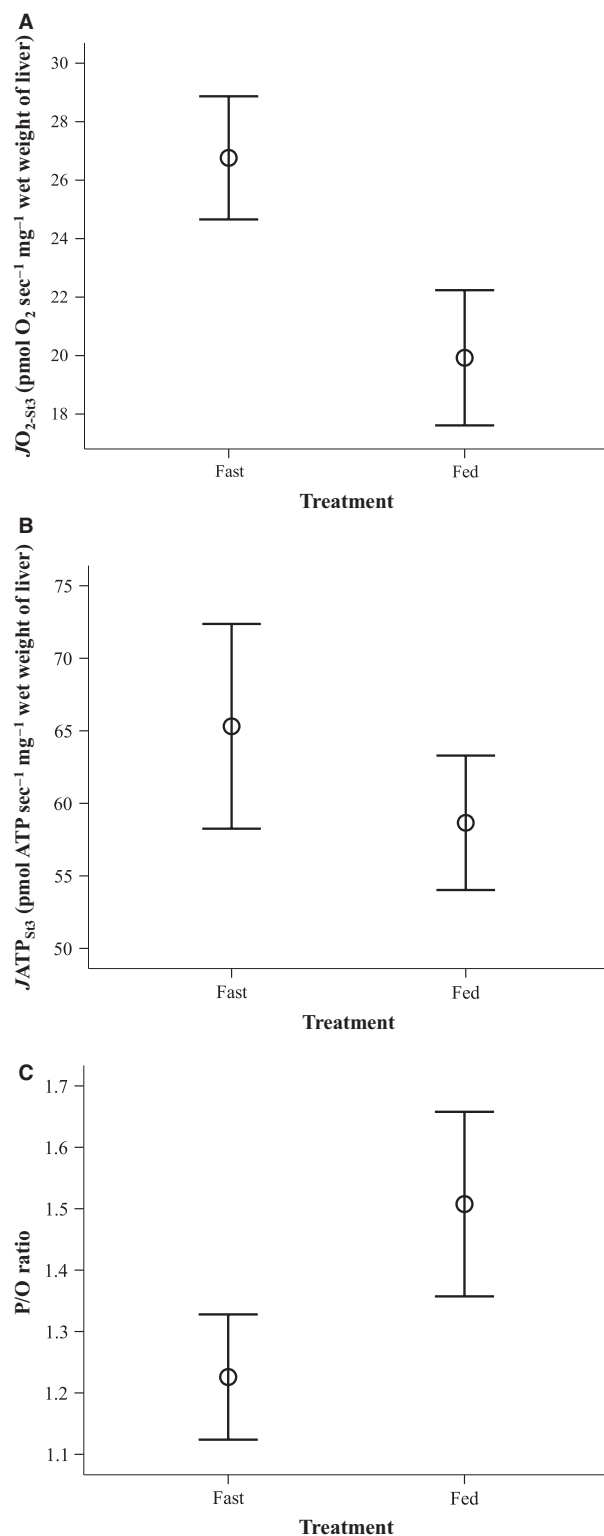
## Analysis of mitochondrial rates of oxidation and phosphorylation

Mitochondrial oxidation and phosphorylation were analyzed with Microsoft Excel 2010. Free $Mg^{2+}$ was converted into extra-mitochondrial ATP concentration, using standard binding equations previously described (Chinopoulos et al. 2014). The rate of ATP production was calculated from the linear regression of ATP concentration as a function of time (Fig. 2); $JATP_{raw}$ was averaged over a 5 min period, starting 15 min post-ADP addition while $JATP_{St4}$ was averaged over a 5 min period after flux stabilisation. $JATP_{St4}$ was then subtracted from $JATP_{raw}$ to obtain the state 3 rate of mitochondrial ATP production ($JATP_{St3}$), which was expressed in pmol ATP/sec/mg wet weight of liver. Changes in oxygen concentration over time were calculated in a similar way (Fig. 2), $JO_{2\text{-}raw}$ being averaged over 5 min, starting 15 min post-ADP addition, while $JO_{2\text{-}ROX}$ was averaged over 1–2 min after flux stabilisation. $JO_{2\text{-}ROX}$ was then subtracted from $JO_{2\text{-}raw}$ to obtain the state 3 respiration rate ($JO_{2\text{-}St3}$), expressed in pmol $O_2$/sec/mg wet weight of liver. Finally, the P/O ratio was calculated as the ratio of $JATP_{St3}$ to twofold $JO_{2\text{-}St3}$; the rate of oxygen consumption is doubled since each molecule of oxygen is comprised of two oxygen atoms.

## Chemicals

Magnesium green was purchased from Thermo Fisher Scientific (Renfrew, UK), ADP was purchase from Merck

disappearance due to ATPase activity under state 4 conditions ($JATP_{St4}$). Addition of complex I inhibitor, rotenone (0.5 $\mu$mol/L) and complex III inhibitor, antimycin

**A**



**B**



**C**



(Hoddesdon, UK), water was purchased from VWR (Lutterworth, UK). All other chemicals were purchased from Sigma-Aldrich (Dorset, UK).

**Figure 4.** Liver mitochondrial $J$ATP and $J$O$_2$ values of fasted and ad libitum-fed groups of brown trout. Food treatment significantly affected (A) mitochondrial oxygen consumption ($J$O$_{2\text{-St3}}$: $F_{1,11} = 28.677$, $P < 0.001$), (B) mitochondrial ATP production to a lower extent ($J$ATP$_{St3}$: $F_{1,11} = 4.805$, $P = 0.051$) and (C) mitochondrial P/O ratio ($F_{1,11} = 20.937$, $P = 0.001$). Shown are average values from the two replicate measurements of mitochondrial traits per fish. Data are plotted as mean $\pm$ 1 SE. $N = 12$ fish per treatment group.

## Statistical analysis

The reproducibility of the estimates of $J$O$_{2\text{-St3}}$, $J$ATP$_{St3}$, and the P/O ratio of the two trials from the same homogenate was assessed, using Intraclass Correlation Coefficients (ICC). Paired *t*-tests were then used to test for any consistent shifts in the values of the measurements between the first and second trial (2 h later). Finally, linear mixed models were employed to test whether fed and fasted fish differed significantly in their hepatic mitochondrial properties (mean value of $J$O$_{2\text{-St3}}$, $J$ATP$_{St3}$, and P/O ratio) while taking into account any effect of testing date (included as a random factor). All statistical analyses were performed in SPSS Statistics 21 (Chicago, IL).

## Results and Discussion

The reproducibility of $J$O$_{2\text{-St3}}$ and $J$ATP$_{St3}$ measurements between the two replicate trials from the same liver homogenate was high ($J$O$_{2\text{-St3}}$: ICC $r = 0.953$, df = 22, $P < 0.001$; $J$ATP$_{St3}$: ICC $r = 0.746$, df = 22, $P = 0.001$). As a result, the P/O ratio was also highly reproducible (ICC $r = 0.778$, df = 22, $P < 0.001$). However, the mitochondrial activity of homogenates declined over time, as indicated by significantly lower $J$O$_{2\text{-St3}}$ and $J$ATP$_{St3}$ measurements in the 2nd assay that was conducted 2 h after the first (Fig. 3A and B). This led to a tendency for the P/O ratio to also change over time, although this change was not significant (Fig. 3C). This lack of a significant change in P/O ratio with time posthomogenization was due to both the numerator and denominator changing in the same direction and at broadly similar rates, but this may not be the case in other tissues or for greater time intervals between assays. The temporal stability of mitochondrial properties should therefore always be assessed when using this method, and delays between sample preparation and measurement kept to a minimum.

The liver mitochondria of trout starved for 14 days showed significant differences in their properties compared to those of fed conspecifics (Fig. 4). The mitochondria from fasted fish exhibited highly significantly higher rates of oxygen consumption and a trend for an increase in rates of ATP production in comparison with those of

fed fish (Fig. 4A and B). As a result of this substantial increase in $JO_2$ but lesser increase in JATP, the P/O ratio of liver mitochondria from food-deprived fish was significantly reduced compared to that of fed fish (Fig. 4C). While an increase in coupling efficiency has been suggested to be a primary means by which animals reduce energy expenditure when faced with harsh conditions (Theron et al. 2000; Monternier et al. 2014; Salin et al. 2015), other studies have proposed that the P/O ratio is a major constraint in optimizing the efficiency of energy utilization (Konstantinov et al. 1976; Bobyleva-Guarriero et al. 1984; Abele et al. 2002; Bottje and Carstens 2009; Salin et al. 2012b; Conley et al. 2013). The effect of starvation on mitochondrial properties is also likely to depend on both the tissue (Trzcionka et al. 2008; Brown et al. 2012; Crescenzo et al. 2012) and the animal species involved (Emel'yanova et al. 2007; Bottje and Carstens 2009). A reduction in the apparent P/O ratio in the liver of the food-deprived fish is consistent with previous studies of chickens (Bobyleva-Guarriero et al. 1984), but contrasts with results found in the skeletal muscle of starving penguins (Monternier et al. 2014) and in the livers of starved rats (Jung and Henke 1997). The degree to which mitochondrial properties change may also depend on the severity of the energetic challenge, for example, the duration of food deprivation (Hung et al. 1997; Salin et al. 2010b). It would be profitable to test whether the effect of fasting on mitochondrial coupling efficiency is consistent across different tissues from the same animal, and whether the direction and magnitude of change in P/O ratio is dependent on the duration of food deprivation.

## Perspectives and significance

The aim of this study was to measure simultaneously JATP and $JO_2$ and hence the P/O ratio in tissue homogenate. Our approach was to use the magnesium-sensitive fluorescent probe, Mg Green, to estimate changes in $[Mg^{2+}]$ and in turn JATP, while correcting for ATPase interference. In our study the average P/O ratio for pyruvate, malate and succinate oxidation was 1.367, which is only slightly lower than maximal theoretical values reported for pyruvate and malate oxidation (2.727) and for oxidation of succinate (1.636) (Brand 2005; Watt et al. 2010). This supports our conclusion that the method is appropriate for measuring effective P/O ratios. In the conditions pertaining in our experiment, inhibitors for ATPase failed to fully inhibit ATP hydrolysis (Fig. 1). This limitation has been overcome by inhibiting the ATP-ADP exchanger with a highly specific inhibitor, the cATR, in order to quantify rates of ATP hydrolysis. This extra step of the protocol does not require any more tissue and only a minimal amount of time for the mitochondria to

reach a steady state 4. Our method can even be used to analyze P/O ratios from permeabilized cells and isolated mitochondria. Thus, P/O ratio comparisons among species, individuals and tissues can be carried out, even across a range of mitochondrial preparations.

The Mg Green method has previously enabled evaluation of ATP production in permeabilized cells and isolated mitochondria (Chinopoulos et al. 2009, 2014). However, such samples are not always appropriate in studies of the energy metabolism of an organism. Moreover, ecology and evolutionary biology requires methods for measuring mitochondrial traits that can be applied to nonmodel organisms that potentially exhibit smaller tissues and a lower mitochondrial density than a laboratory mouse. We have for the first time demonstrated the feasibility of a method for measuring mitochondrial coupling efficiencies on homogenized tissue, showing that our method can be used to calculate effective P/O ratios from tissue samples as small as tens of milligrams. This protocol may facilitate the study of P/O ratios in further models, and shed light on the role of mitochondrial coupling efficiency in influencing fitness-related traits.

## Conflict of Interest

The authors declare they have no conflict of interest.

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# Graphical Abstract

The contents of this page will be used as part of the graphical abstract of html only. It will not be published as part of main.
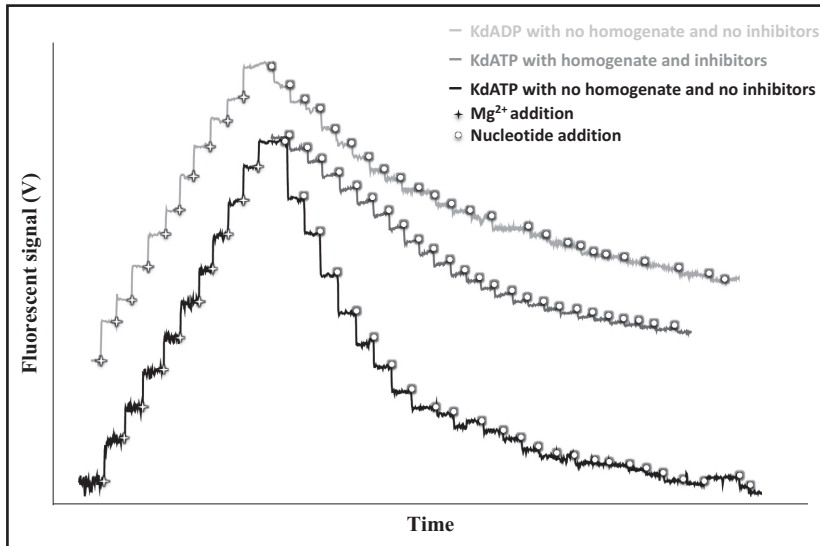


Our study details a new method that simultaneously quantifies both ATP production and oxygen consumption in tissue homogenates, so for the first time allowing the calculation of P/O ratios from tissue samples as small as tens of milligrams. We demonstrate that the method is highly reproducible; we compare data from fed and food-deprived animals to demonstrate that the method can detect mitochondrial flexibility in P/O ratios in response to nutritional state.