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Title: The RCR and ATP/O indices can give contradictory messages about mitochondrial efficiency

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Synopsis:
Mitochondrial efficiency is typically taken to represent an animal's capacity to convert its resources into ATP. However, the term mitochondrial efficiency, as currently used in the literature, can be calculated as either the respiratory control ratio, RCR (ratio of mitochondrial respiration supporting ATP synthesis to that required to offset the proton leak) or as the amount of ATP generated per unit of oxygen consumed, ATP/O ratio. The question of how flexibility in mitochondrial energy properties (i.e. in rates of respiration to support ATP synthesis and offset proton leak, and in the rate of ATP synthesis) affects these indices of mitochondrial efficiency has tended to be overlooked. Furthermore, little is known of whether the RCR and ATP/O ratio vary in parallel, either among individuals or in response to environmental conditions. Using data from brown trout *Salmo trutta* we show that experimental conditions affect mitochondrial efficiency, but the apparent direction of change depends on the index chosen: a reduction in food availability was associated with an increased RCR (i.e. increased efficiency) but a decreased ATP/O ratio (decreased efficiency) in liver mitochondria. Moreover, there was a negative correlation across individuals held in identical conditions between their RCR and their ATP/O ratio. These results show that the choice of index of mitochondrial efficiency can produce different, even opposing, conclusions about the capacity of the mitochondria to produce ATP. Neither ratio is necessarily a complete measure of efficiency of ATP production in the living animal (RCR because it contains no assessment of ATP production, and ATP/O because it contains no assessment of respiration to offset the proton leak). Consequently, we suggest that a measure of mitochondrial efficiency obtained nearer to conditions where respiration simultaneously offsets the proton leak and produce ATP would be sensitive to changes in both proton leakage and ATP production, and is thus likely to be more representative of the state of the mitochondria *in vivo*.

Introduction:

Animals often live in variable environments where conditions such as food availability fluctuate considerably. The ability of organisms to adjust their energy metabolism can have important consequences for the rates at which resources are consumed and allocated to whole-organism performance as well as the extent to which animals can cope with deprived environments (Wang et al., 2006; Secor and Carey, 2016). Flexibility in mitochondrial efficiency may be particularly important since mitochondria occupy a pivotal position between resource utilization and whole-organism performance through their primary role in ATP production via oxidative phosphorylation (Salin et al., 2015; Salin et al., 2016a). Indeed, flexibility in the efficiency with which mitochondria produce ATP can set limits on the capacity of an organism to respond to environmental change (Sokolova et al., 2012; Blier et al., 2013).

Mitochondria are responsible for almost 90% of the oxygen consumption of the whole organism (Rolfe and Brand, 1996) and provide up to 90% of cellular ATP (Lehninger et al., 1993), so variation in their efficiency of ATP production may have important consequences for the animal's performance. Oxygen is continually being consumed by the mitochondria, both in order to maintain the proton gradient across the inner mitochondrial membrane (IMM) and to produce ATP. The gradient causes protons to flow back across the IMM to the mitochondrial matrix through the ATP synthase complex, driving the production of ATP; these processes can be measured simultaneously *in vitro* as the state 3 (also known as OXPHOS) respiration rate and the rate of ATP production (Chance and Williams, 1955). However, dissipation of the proton gradient occurs not only during ATP production but also due to the leakage of protons directly across the IMM (Chance & Williams 1955; Brand & Nicholls 2011). This leakage must be continually offset by the activity of the respiratory chain, a compensatory process which consumes a significant amount of both oxygen and substrates even when no ATP is being produced; this process is measured *in vitro* as the state 4 or LEAK respiration rate (Chance and Williams, 1955).

Fasting can change rates of OXPHOS respiration, LEAK respiration and ATP production; these changes are likely to provide energetic benefits to an organism, but substantial energy savings can also be achieved by enhancing the efficiency of the mitochondria (Monternier et al., 2014; Monternier et al., 2015; Monternier et al., 2017). However, while the term "mitochondrial efficiency" may appear to be a relatively straightforward description of the extent to which the yield from oxidative phosphorylation is optimised, in practice it is ambiguous due to alternative definitions of what is meant by "efficiency". In the context of mitochondrial energetics, "efficiency" can refer to: i) the ratio of the amount of energy produced by the system relative to the amount consumed in the form of fuel, or alternatively ii) the proportion of energy that is wasted. The former is measured as the amount of ATP generated per molecule of oxygen consumed during OXPHOS respiration (so that high values indicate "efficient" ATP production), and is termed the ATP/O or effective P/O ratio (Brand, 2005). The latter measure of efficiency is usually calculated as the ratio of the respiration supporting ATP synthesis (OXPHOS) to the respiration wasted to offset the proton leak (LEAK). This ratio, termed the respiratory control ratio (RCR), represents the maximum factorial increase in mitochondrial oxygen consumption that can be achieved above the LEAK oxygen requirement when driving the phosphorylation of ADP into ATP (Brand and Nicholls, 2011). A high RCR indicates that mitochondria have a high capacity for phosphorylating respiration relative to the respiration required to offset the proton leak.

It is increasingly being suggested that variation in mitochondrial efficiency (whichever definition is used) can have important consequences for animal performance (Gnaiger et al., 2000; Conley et al., 2013; Salin et al., 2015; Salin et al., 2016a). Mitochondrial efficiency has been hypothesised to be especially critical during periods of limited food availability (Monternier et al., 2014; Monternier et al., 2015; Monternier et al., 2017), since a higher mitochondrial efficiency is presumed to decrease the energy needed to produce ATP. However, since the RCR and the ATP/O ratio are calculated from different mitochondrial properties (OXPHOS respiration divided by LEAK respiration, and ATP production divided by the OXPHOS respiration, respectively), they can respond differently to changing environmental conditions. If they do not vary in parallel then both the choice of index and its interpretation become critical. However, to date there has been very little evaluation of whether these two indices are showing the same thing.

In the present study we examined the effects of food availability on mitochondrial properties, focusing in particular on the consequences for these alternative measures of mitochondrial efficiency. We determined the mitochondrial responses of brown trout (*Salmo trutta*) to a period of food shortage that simulated the situation periodically faced by these fish in their natural environment (Huusko et al., 2007; French et al., 2016). We chose liver as our tissue to analyse mitochondrial flexibility because liver mitochondria have been shown to be particularly sensitive to fluctuations in food availability (Frick et al., 2008; Trzcionka et al., 2008; Rui, 2014; Chausse et al., 2015). We also place the question of RCR versus ATP/O ratio within the context of food shortage and highlight the varying outcomes that can be obtained depending on which measure of mitochondrial efficiency is used.

Material and methods:

Experimental animal

Twenty four fry brown trout were obtained from a hatchery (Howietoun, UK) in the summer of 2015 and moved to the University of Glasgow. Here the fish were maintained under an 8 h light: 16 h dark photoperiod at 12℃ and fed daily to excess with trout pelleted food (EWOS, West Lothian, UK). In January 2016, fish were moved to individual compartments of a stream system to control the food intake of individual animal while maintaining the same conditions of water quality. Fish were transferred from the stock tank to their individual compartments in batches of two fish per day since the final mitochondrial assay has been conducted on two fish a day. Each processing pair was first acclimated for a week in their individual compartment, during which they were fed daily in excess on the same trout pellets. Fish were then randomly allocated to a diet treatment for 14 days: half of the fish were kept on the same regime of being fed daily in excess, while the other half were deprived of food (N = 12 per treatment group). Fasting led to a reduction in body mass (initial and final mass of the fasted group: 12.14 ± 0.61 g and 10.85 ± 2.14 g), while over the same period the fed trout increased in mass (initial and final mass: 12.00 ± 0.57 g and 16.50 ± 3.80 g).

Mitochondrial homogenate preparation

At the end of the feeding treatment, fish were culled after being deprived of food overnight. A liver aliquot from each fish was immediately weighted and preserved in ice-cold respirometry buffer (0.1 mM EGTA, 15 µM EDTA, 1mM $MgCl_2$, 20mM Taurine, 10mM KH_2PO_4, 20mM HEPES, 110 mM D-sucrose, 60 mM lactobionic acid, 1g L^{-1} bovine serum albumin essentially free fatty acid, pH 7.2 with KOH) to determine mitochondrial properties. Liver was shredded using micro-dissecting scissors, and the shredded solution then homogenized with a Potter-Elvehjem homogenizer (three passages). Validations of the methods are described in (Salin et al., 2016a; Salin et al., 2016b). The homogenate was then diluted further in respirometry buffer to obtain the desired final concentration (mean ± SE: 5.06 ± 0.03 mg mL^{-1}). The entire procedure was carried out on ice, and completed within 30 min of the fish being culled.

Mitochondrial rates of oxidation and phosphorylation

Mitochondrial properties were measured as in Salin et al. (2016c). Briefly, we used a protocol for estimating ATP/O ratio and RCR that simultaneously measures both OXPHOS respiration and ATP production, and then measures LEAK respiration on the same sample. Oxygen consumption was recorded using the Oxygraph-2k high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria). To measure ATP production we used the magnesium-sensitive fluorescent probe, Magnesium Green, to estimate changes in [Mg^{2+}] (Szmacinski and Lakowicz, 1996). ATP production is calculated from the rate of change in [Mg^{2+}] and is based on the unequal affinities of ATP and ADP for Mg^{2+} (Chinopoulos et al., 2009). Oxygen and magnesium green fluorescence signals were detected simultaneously using two respirometry chambers equipped with fluorescent sensors and recorded using DatLab software (Oroboros Instruments, Innsbruck Austria). Part of the liver homogenate from each fish was added to one of the two measurement chambers of an Oxygraph immediately following preparation; fish from a processing pair were measured in parallel. The remaining part of the liver homogenate was preserved on ice for use in a replicate trial. After addition of homogenate to the respiration chamber at 12˚C, pure oxygen gas was added to reach a concentration of 550 µM. The titration protocol was started at 550 µM since it prevented hypoxic conditions developing during the titration (*i.e.* the oxygen levels remained above 100µM throughout each trial). Magnesium green (2.1 µM) was added to the respirometry chambers to allow us to detect changes in [Mg^{2+}] and so measure the rate of ATP production.

A sequential substrate/inhibitor protocol as in Salin et al. (2016c) was run simultaneously for each fish. The rate of OXPHOS was assessed by adding a saturating concentration of ADP (2 mM, Mg^{2+} free) to the chamber containing complex I substrates (5 mM pyruvate and 0.5 mM malate) and complex II substrate (10 mM succinate). The rate of ATP production was also measured in this condition. The LEAK respiration was then measured by inhibiting the ATP-ADP exchanger (Adenylate Nucleotide Translocase) through the addition of carboxyatractyloside (4 µM). The rate of ATP hydrolysis was also measured in this condition, and was then added to the rate of ATP production. Addition of complex I inhibitor (0.5 µM rotenone) and complex III inhibitor (2.5 µM Antimycin A) allowed determination of residual oxygen consumption, which was then subtracted from OXPHOS and LEAK respiration rates.

The second replicated trial was identical to the first one but started two hours later, using the remaining liver homogenate and the other measurement chamber (to control for any inter-chamber

difference in readings). No effect of the choice of measurement chamber on mitochondrial properties was found. We expressed OXPHOS and LEAK respiration as pmoles of O_2 sec^{-1} mg^{-1} wet weight of liver and ATP production as pmoles of ATP sec^{-1} mg^{-1} wet weight of liver for each replicate. Finally, the RCR was calculated as the ratio of OXPHOS respiration to LEAK respiration, and the ATP/O as the ratio of ATP production to two fold OXPHOS respiration; the rate of OXPHOS is doubled since each molecule of oxygen is comprised of two oxygen atoms.

Statistical analysis.

Linear mixed models (LMM) were used to first test whether fed and fasted fish differed significantly in their mitochondrial properties. Each model included one of the mitochondrial parameters (OXPHOS respiration, LEAK respiration, ATP production, RCR, ATP/O ratio) as the dependent variable, diet treatment as the predictor, and processing pair and fish identity as random effects to account for the order in which fish entered the experiment and to control for repeated measurements (the two replicates per fish), respectively. We then used LMMs to examine which of the mitochondrial properties (OXPHOS respiration, LEAK respiration, ATP production) had the greatest impact on variation in the two indices of mitochondrial efficiency (RCR and ATP/O). The models included one of the efficiency indices as the dependent variable, food treatment and mitochondrial property and their interaction as predictors, and processing pair and fish identity as random effects. All analyses were based on a sample size of 12 fish measured in replicate per food treatment, and were performed using IBM SPSS Statistics 21 (SPSS Inc., Chicago, IL, USA); the level of significance was set to $P < 0.05$ and all means are presented ± SE.

Results:

The effect of food treatment on liver bioenergetics differed between the mitochondrial properties. Rate of OXPHOS respiration was significantly higher in the mitochondria of fasted compared to fed fish ($F_{1,11} = 26.93$; $P < 0.001$; Fig. 1a), while the rate of LEAK respiration did not differ between food treatment ($F_{1,22} = 0.19$; $P = 0.67$; Fig. 1b) and the rate of ATP production was slightly – but not significantly – higher in the mitochondria of fasted animals ($F_{1,11} = 6.64$; $P = 0.055$; Fig. 1c). As a result, both indices of mitochondrial efficiency showed a significant difference in the mitochondrial efficiency of fasted and fed fish (RCR: $F_{1,22} = 16.15$; $P = 0.001$; ATP/O ratio: $F_{1,11} = 21.04$; $P = 0.001$; Fig. 2) - but in opposite directions: the RCR of the mitochondria of fasted trout was higher than that of fed trout (15.33 ± 0.65 *vs* 10.34 ± 0.75 in the fasted and fed group, respectively; Fig. 2a), whereas their ATP/O ratio was significantly lower (1.23 ± 0.05 *vs* 1.51 ± 0.07 in the fasted and fed group, respectively; Fig. 2b).

Variation in the indices of mitochondrial efficiency amongst individuals was driven by variation in OXPHOS respiration, LEAK respiration and the rate of ATP production, but not to equal extents, and their relative importance depended on food treatment (Table 1, Fig. 3). OXPHOS and LEAK both influenced RCR (Table 1, Fig. 3a & b), but the effect of OXPHOS on RCR depended on the food treatment (Table 1). While RCR was positively related to OXPHOS in the mitochondria of fed fish ($t = 4.53$; $P < 0.001$; Fig. 3a), there was no relationship between RCR and OXPHOS in fasted fish ($t = 0.24$; $P = 0.815$; Fig. 3a). The RCR in the mitochondria of fasted and fed fish was greatest in mitochondria with a low LEAK (Fasted: $t = -3.84$; $P = 0.005$; Fed: $t = -2.94$; $P = 0.011$; Fig. 3b; Fig. 3b). Interestingly,

there was no relationship between a fish's rate of ATP production and its RCR (Table 1, Fig. 3c). While there was an overall negative relationship between OXPHOS and ATP/O (Table 1), this was driven by a strongly negative relationship in fed individuals (t = -4.70; P < 0.001; Fig. 3d), since in the fasted trout the relationship between OXPHOS and ATP/O was not significant (t = -1.46; P = 0.153; Fig. 3d). In contrast to results obtained for RCR, a fish's ATP/O ratio was not related to its LEAK (Table 1, Fig. 3e) but there was a significant effect of the rate of ATP production such that ATP/O ratio in mitochondria of fasted and fed fish was greatest in mitochondria with a higher rate of ATP production (Fast: t = 2.21; P = 0.033; Fed: t = 2.52; P = 0.016; Fig. 3f). Since OXPHOS respiration is the numerator of the RCR ratio but the denominator in the ATP/O ratio, then in the absence of any counteracting trends in LEAK and ATP production there can be a negative relationship between the two ratios. There was indeed a tendency for ATP/O and RCR values to be negatively related ($F_{1,36}$ = 22.96; P = 0.058; Fig 4), significantly so in the fed fish (t = -6.43; P < 0.001; Fasted fish for comparison: t = -1.82; P = 0.077).

Discussion

Despite the widespread belief that both the RCR and the ATP/O ratio are indicators of efficiency in energy metabolism (Sokolova, 2004; Sommer and Pörtner, 2004; Kayes et al., 2009; Iftikar et al., 2015; Mowry et al., 2016; Brijs et al., 2017), we have shown that they can produce contradictory interpretations of the same dataset. The RCR of the mitochondria of fasted fish was *greater* than that of fed fish (*i.e.* more efficient, since it suggests a decrease in the proportion of oxygen and energy substrates wasted in the proton leak), but the ATP/O ratio was *lower* (*i.e.* less efficient, since less ATP is produced for a given consumption of oxygen and energy substrates). These results suggest that the predicted consequences of mitochondrial efficiency for animal performance depend on which index is being used. Previous studies have also found discrepancies in the indices, for instance showing that ATP/O - but not RCR - was influenced by food availability (Monternier et al., 2015; Monternier et al., 2017; *personnal communication*), and that natural and artificial variation in ATP/O ratio predicted whole-organism growth rate, whereas variation in RCR did not (Salin et al., 2012a; Salin et al., 2012b). However, to our knowledge these differences have not previously been discussed, and so it is clear that more attention should be devoted to the separate relationships between variability in RCR and ATP/O ratio and animal performance.

The contrasting effects of food availability on mitochondrial efficiency indices may arise due to the relative magnitude of changes in mitochondrial properties that are used in the calculation of the efficiency index. Here, we found that the different mitochondrial traits did not respond to the same extent to the experimental treatment. While average rates of OXPHOS respiration and ATP production increased in response to food deprivation (+ 34 % and + 10 %, respectively), LEAK respiration did not differ between food treatment groups. These differential changes in mitochondrial properties in response to environmental stressors corroborate those reported for OXPHOS and LEAK respiration rates in the liver of the Nototheniid fish *Notothenia rossi* under different temperature and pH treatments (Strobel et al., 2013). However, this is unlike the simultaneous change in both OXPHOS and LEAK respiration rates of heart mitochondria of killifish *Fundulus heteroclitus* that occurred in response to temperature changes (Chung et al., 2017). These contrasting results may arise due to a number of factors including differences in the type or

magnitude of environmental change, species- or tissue-specific responses to environmental variation, or variation in the methods of mitochondrial preparation and calculation of the fluxes. While the covariation among OXPHOS and LEAK respiration and ATP production across changing environments remains poorly studied, these results demonstrate at the very least that the separate functional traits of mitochondria can vary independently in response to environmental change.

In order to understand the contrasting responses of the RCR and ATP/O indices to changing food availability we need to consider the mechanisms that are revealed by the experimental conditions under which the mitochondrial properties are measured. The experimental conditions under which OXPHOS respiration and ATP production have been measured were very different to the conditions for measuring LEAK respiration. The maximum rates of OXPHOS respiration and ATP production (i.e. under conditions of unlimited availability of substrates, oxygen and ADP) are usually associated with barely any proton leak, but the transition from OXPHOS to LEAK respiration (i.e. no ATP production) is accompanied by an increase in the mitochondrial membrane potential and a parallel increase in the proton leak (Kadenbach, 2003; Brand and Nicholls, 2011). This may explain why we found no direct support for using RCR alone as a predictor of the rate of ATP production. Rather, our results shown that RCR was influenced by variation in OXPHOS and LEAK respiration. Likewise, we found no relationship between a fish's ATP/O ratio (measured under conditions of minimal proton leak) and its LEAK respiration. This provides the first empirical evidence that it may be necessary to correct for proton leak when calculating mitochondrial efficiency in terms of the ATP/O ratio. *In vivo*, the respiration rates of mitochondria correspond neither to the OXPHOS state (unlimited substrates, oxygen and ADP) not to the LEAK state (total arrest of ATP production), but will be fluctuating around an intermediate state on the OXPHOS-LEAK continuum, reacting to the ATP demand of the cell (Kadenbach, 2003). This highlights the fact that both ATP/O and RCR are measured under conditions that rarely occur in living animals. It would be a more realistic measure of mitochondrial efficiency to estimate the ATP/O ratio when the membrane potential is at an intermediate state, when it might indeed be sensitive to changes in proton leakiness.

We tested whether flexibility in respiratory control ratio and amount of ATP produced per oxygen consumed during periods of food deprivation may be consistent indicators of mitochondrial energy conversion. Flexibility in these traits likely plays an important role in allowing organisms to cope with a diversity of environmental challenges. However, the contrasting trends that we observed in the two commonly used indices of mitochondrial efficiency highlight the need to consider their interpretation carefully, mindful of the conditions under which they are measured. In particular the RCR is not by itself an estimate of ATP production. However, while the ATP/O ratio is more appropriate as an estimate of ATP production, it is not typically measured under conditions directly relevant to the animal. We suggest that a measure of mitochondrial efficiency obtained in conditions nearer to those under which LEAK is measured (for instance under limiting concentrations of ADP) would be sensitive to changes in both proton leakage and ATP production, and is thus likely to be more representative of the state of the mitochondria *in vivo*. Overall, the entire mitochondrial phenotype needs to be considered in order to better understand the mitochondrial response to environmental change.

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Author contributions: KS, CS, CC and NBM conceived the study. KS, EMV and GJA performed the experiments. KS, EMV and CC analysed the data. KS drafted the manuscript; EMV, CS, CC and NBM then revised the manuscript and added some comments. All authors approved the final version of the manuscript.

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411

Table 1: Parameters estimated from linear mixed models of the effects of phosphorylating respiration (OXPHOS, pmol O2 sec^{-1} mg^{-1} liver), leak respiration (LEAK, pmol O2 sec^{-1} mg^{-1} liver) and ATP production (ATP, pmol sec^{-1} mg^{-1} liver) on respiratory control ratio (RCR) and amount of ATP produced per oxygen consumed (ATP/O) of mitochondria in the liver of brown trout under food treatment (fasted *vs* fed). Bold denotes significance.

Dependent variable	Parameter	Estimate ± SE	*t*	*P* value
RCR	Intercept	1.67 ± 1.96	0.85	0.399
	OXPHOS	0.45 ± 0.10	4.53	**< 0.001**
	Treatment	11.90 ± 2.72	4.37	**< 0.001**
	OXPHOS * Treatment	-0.43 ± 0.12	-3.56	**0.001**
RCR	Intercept	17.42 ± 2.54	6.86	**< 0.001**
	LEAK	-3.84 ± 1.30	-2.94	**0.005**
	Treatment	0.84 ± 3.04	0.28	0.783
	LEAK * Treatment	1.52 ± 1.55	0.98	0.332
RCR	Intercept	7.13 ± 2.76	2.58	**0.014**
	ATP	0.05 ± 0.05	1.14	0.265
	Treatment	8.43 ± 3.39	2.49	**0.018**
	ATP * Treatment	-0.08 ± 0.05	-1.14	0.168
ATP/O	Intercept	2.41 ± 0.20	12.16	**< 0.001**
	OXPHOS	-0.05 ± 0.01	-4.70	**< 0.001**
	Treatment	-0.86 ± 0.30	-2.91	**0.007**
	OXPHOS * Treatment	0.04 ± 0.01	2.65	**0.013**
ATP/O	Intercept	1.21 ± 0.27	4.49	**< 0.001**
	LEAK	0.16 ± 0.14	1.16	0.263
	Treatment	-0.17 ± 0.39	-0.43	0.667
	LEAK * Treatment	-0.06 ± 0.20	-0.29	0.774
ATP/O	Intercept	0.74 ± 0.31	2.39	**0.021**
	ATP	0.01 ± 0.01	2.52	**0.016**
	Treatment	0.01 ± 0.37	0.03	0.975
	ATP * Treatment	-0.01 ± 0.01	-0.97	0.337

Figure 1: Comparison of liver mitochondrial properties of fasted and fed groups of Brown trout *Salmo trutta*. (A) Mitochondrial respiration rates to support ATP synthesis (OXPHOS; $F_{1,11} = 26.93$; $P < 0.001$) and (B) to offset the proton leak (LEAK; $F_{1,22} = 0.19$; $P = 0.67$), and (C) rate of ATP synthesis ($F_{1,11} = 6.64$; $P = 0.055$). Data are plotted as mean ± SE. N = 12 fish per treatment group. Some of the data have been extracted from (Salin et al., 2016c).

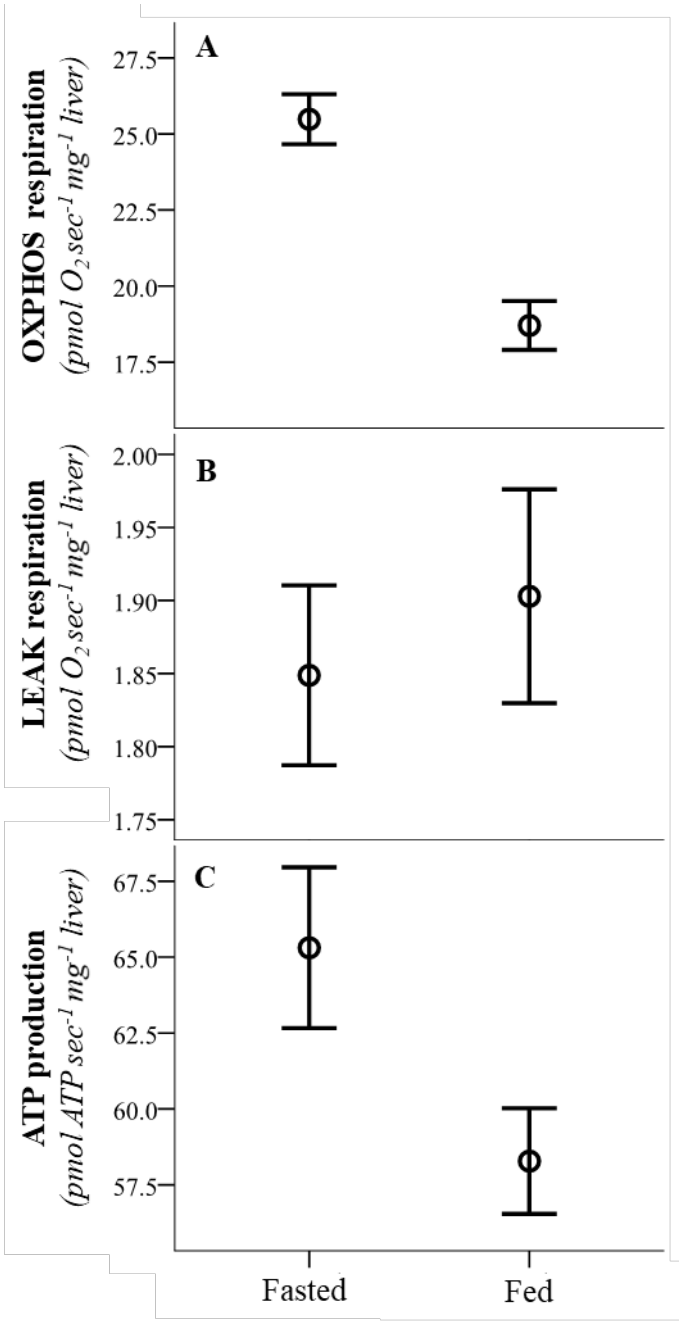

Figure 2: Comparison of liver (A) mitochondrial respiratory control ratio (RCR, $F_{1,22}$ = 16.15; P = 0.001) and (B) amount of mitochondrial ATP produced per oxygen consumed (ATP/O, $F_{1,11}$ = 21.04; P = 0.001) of fasted and fed groups of brown trout *Salmo trutta*. Data are plotted as mean ± SE. N = 12 fish per treatment group. Some of the data have been extracted from (Salin et al., 2016c).

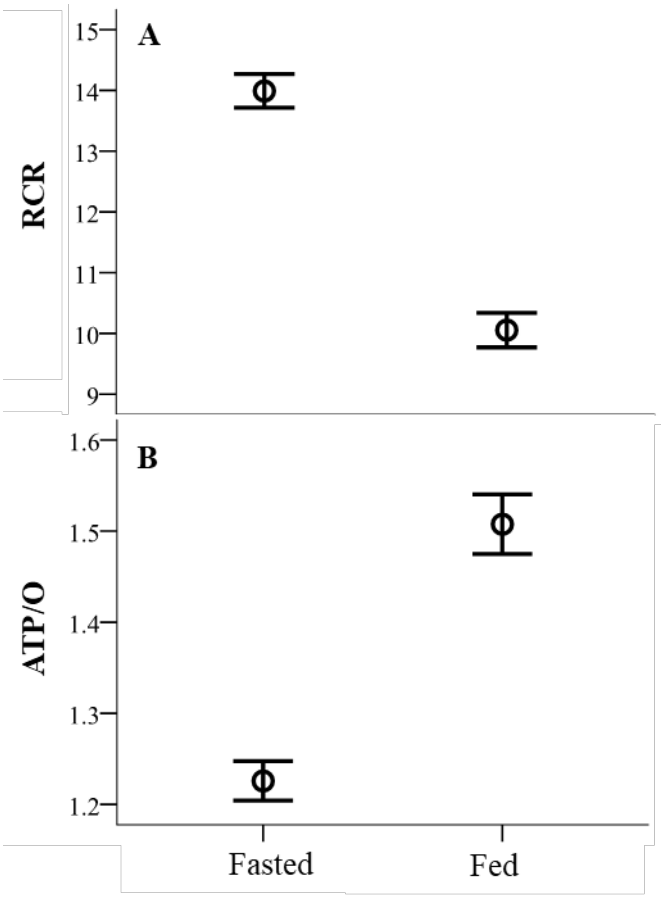

Figure 3: Relationships between the two alternative indices of mitochondrial efficiency (RCR or ATP/O, both plotted on the Y-axis) of fasted and fed groups of brown trout *Salmo trutta* and their mitochondrial properties (plotted on the X-axis). OXPHOS respiration: rate of respiration to support ATP synthesis; LEAK respiration: rate of respiration to offset proton leak; ATP production: mitochondrial rate of ATP synthesis. Data are plotted as N = 12 fish per treatment group, with two replicate measurements of mitochondrial function per fish. Plotted on the graph are regression lines accounting for repeated measurement of two replicate per fish as a random factor of the linear mixed models. See Table 1 for statistical analysis.

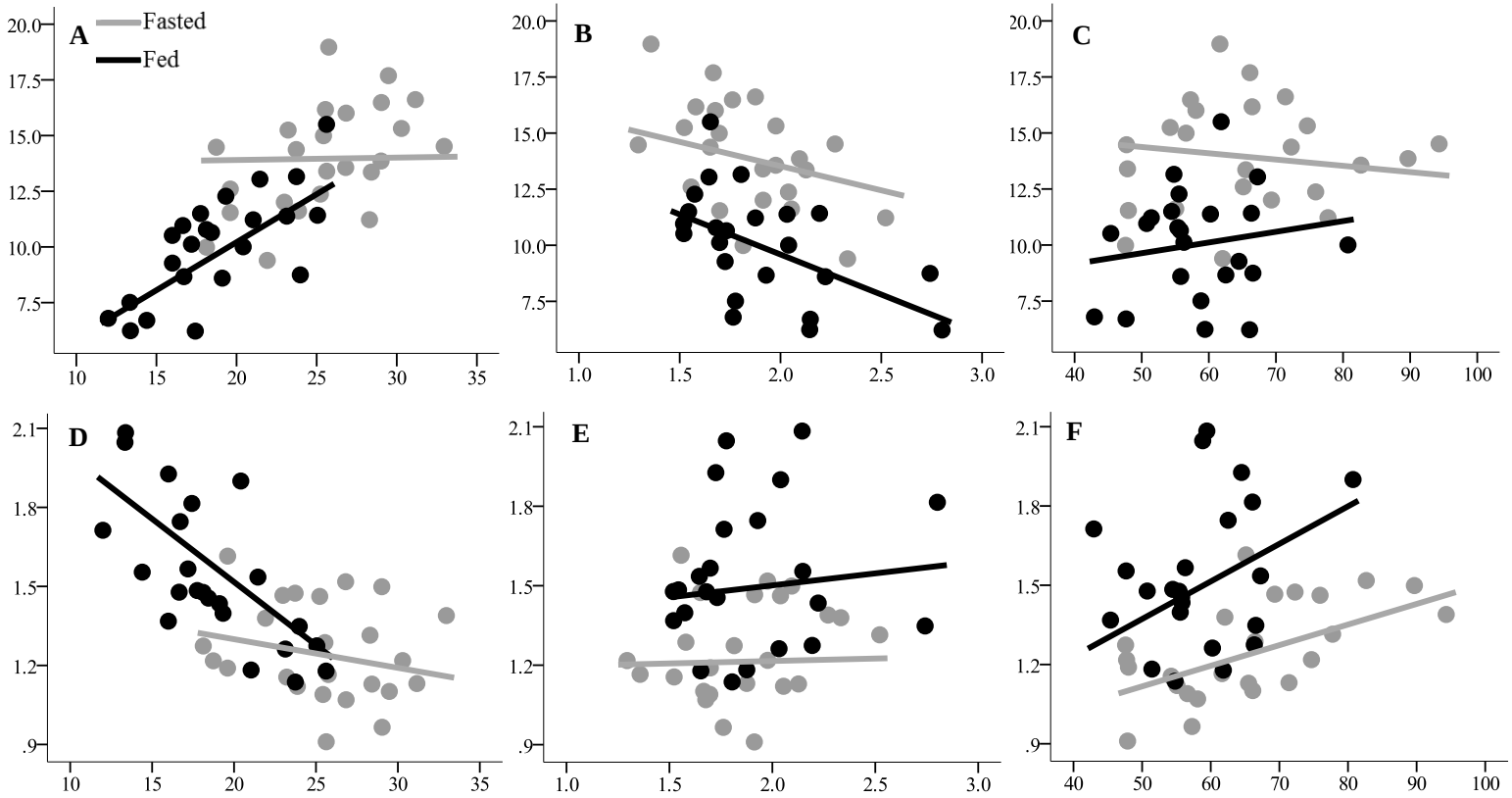

Figure 4: Relationships between the amount of ATP produced per oxygen consumed (ATP/O) in liver mitochondria of fasted and fed groups of brown trout *Salmo trutta* and the respiratory control ratio (RCR). Data are plotted as N = 12 fish per treatment group with two replicate measurements of mitochondrial function per fish. Plotted on the graph are regression lines accounting for repeated measurement of two replicate per fish as random factor of the linear mixed models. See text for statistical analysis.