Electronic Supplementary Material

TITLE: Differences in mitochondrial efficiency explain individual variation in growth performance

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RUNNING TITLE: ATP/O ratio explains growth performance

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Whole-body protein content

Three supplementary groups of nine fish were used to determine the initial and final protein content of the experimental fish for the calculation of whole-body protein growth rates. These supplementary groups were of the same origin, age and size distribution, and were maintained simultaneously and in exactly the same manner, as the experimental fish described in the main manuscript. The fish in each of the 3 groups were maintained on the intermediate ration for 5 to 10 weeks. One group was then culled and frozen to determine the relationship between whole-body protein content and wet weight in fish on this intermediate ration (body mass range 2.95-20.31 g). This relationship was used to estimate the initial protein content of the experimental animals. The two other groups were switched to the low or high ration for 14 days and then culled and frozen to determine the relationship between whole-body protein content and wet weight in fish on low and high rations (3.28-15.04 g and 4.91-20.84 g respectively). Variation in fish body mass within each food treatment covered the range of masses for the experimental fish (those used for the analyses of

the physiological assays). These relationships between whole-body protein content and wet weight in fish were used to estimate the final protein content of the experimental animals.

All fish were fasted for 22h prior to culling, and were then weighed and and kept at -70°C for subsequent extraction and quantification of the whole-body protein content [1]. Thawed fish were cut into pieces in lysis buffer (1g tissue per 10 mL of 1x RIPA) supplemented with SDS (2%) and urea (2M). The contents were incubated for an hour at 60°C and shaken every twenty minutes. After this incubation, the tissue lysates were homogenized with an electric homogenizer (Kinematica Polytron PT 1200) until no visible piece of tissue remained. The homogenates were incubated another hour at 60°C, followed by centrifugation at 15,000 g for 10min at 4°C. The supernatants were collected and stored at -70°C until protein content was determined. The protein concentration of the lysates was determined with the Pierce bicinchoninic acid Protein Assay Kit following the manufacturer's specifications (Sigma Aldrich, Dorset, UK). The protein concentration was determined based on the standard curve using bovine serum albumin supplemented with SDS (2%) and urea (2M). Reagent was added to the lysates and incubated at 60°C for 15 minutes. The protein assays of samples and standards were run in duplicate. The absorbance was read at 562 nm. This allowed us to create a calibration regression relating fish body mass to fish whole body protein content in each food treatment (figure S1). Separate regression analyses were performed on data obtained from each food ration to predict the whole-body protein content as a function of body mass:

- Intermediate ration: Whole-body protein content = $0.1026W + 0.0657$
- High ration: Whole-body protein content = $0.1228W - 0.1206$
- Low ration: Whole-body protein content = $0.1226W - 0.0623$

where W is the wet weight of the fish.

For each ration group the protein content determined by protein extraction was highly correlated with the body mass of the fish ($0.92 \leq r^2 \leq 0.97$, figure S1). The estimated protein content as predicted by the body mass measurement thus appeared to be a robust technique to evaluate the whole protein content of the experimental fish whose organs were needed for other assays (fractional rate of protein synthesis and mitochondrial properties).

Validation of the fractional rate of protein turn-over assay.

Use of the flooding dose technique developed by Garlick, McNurlan [2] is based on meeting the following criteria: 1) that the rate of protein synthesis is not affected by high intracellular concentrations of the amino acid tracer (here phenylalanine or phe); 2) that the flooding dose

injection results in a rapid elevation of tracer in the body free amino acid pools that remains elevated and stable, or shows a slow linear decline over time, and 3) that the incorporation of the amino tracer into body protein is linear over time. With regards to criteria 1, previous work has indicated that although high doses injections of some amino acids (e.g. valine and leucine) may affect protein synthesis rates, phenylalanine is not thought to do so [3]. With regards to criteria 2 and 3, although previous work has validated an intraperitoneal flooding dose injection of D_5-Phe to measure rates of protein synthesis [4-6], we conducted a time course validation experiment for the conditions (e.g. temperature, animal model, animal size and stage of life) in this study.

Twenty fish received an intraperitoneal injection of 150 mM phenylalanine containing 50% deuterated phenylalanine (ring-D_5-phenylalanine, Cambridge Isotope Laboratories) dissolved in an aqueous solution of 154 mM NaCl at a dosage of 10 µL per gram of body mass. Fish were immediately returned to their individual tanks and, following an incubation period of 30, 60, 120 or 240 min (n = 5 fish per time point), the fish were culled, and their tissues sampled and stored at -80°C until further analysis. After extraction of the phenylalanine isotopes in liver and muscle tissues, the levels of D_5-Phe and the natural version of phenylalanine in both the free amino acid pool and in the protein pool were determined by gas chromatography coupled with mass spectrometry (GC model 7890B and MS model 5977B, Agilent Technologies Inc.) as described in [4, 7]. Peak detection and integration was performed using MassHunter software (Version B07.01 SP2, Agilent).

The D_5-Phe enrichment in the free pool of phenylalanine in both tissues was elevated and stable for 240 min (figure S2). In the liver, the slope of the free pool in relation to time did not significantly deviate from zero. However, in the white muscle it decreased at a rate of -0.037 % min^{-1} (regression analysis p = 0.001). Although statistically significant, this represents a decrease of 2.2% over the one hour incorporation period and is considered negligible. The phenylalanine enrichment of the protein pool increased linearly with incorporation time (regression analyses - Liver: $r^2 = 0.937$, $P < 0.001$; Muscle: $r^2 = 0.755$, $P < 0.001$; figure S2). From these results, we elected to use an incorporation period of 60 minutes as it allowed sufficient time for the tracer to incorporate into the protein pool while limiting the decrease in level of D_5-Phe in the free pool of amino acids in muscle.

Measurement of mitochondrial properties

The liver was shredded using micro-dissecting scissors to obtain a homogenous solution with a particle size less than 0.5 mm (tested by pipetting through 1 ml tip) in 1 mL of respirometry buffer as in [8, 9]. The shredded homogenate was then diluted further in respirometry buffer to obtain the

desired final concentration (mean ± SE: 5.00 ± 0.26 mg liver mL^{-1}). The liver homogenization was carried out on ice, and the procedure was completed within 30 min of the fish being culled.

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). The oxygen electrodes were calibrated at two points: air-saturated respirometry buffer (daily) and zero oxygen after sodium dithionite addition (fortnightly). Stepwise additions of $MgCl_2$ at each run were performed for calibration of the fluorescent signal into Mg^{2+}. The two binding affinity (K_d) values of ATP and ADP for Mg^{2+} were determined in absence of tissue in [10] ; the values were $K_{d\text{-ATP}}$ = 0.1545 mM and $K_{d\text{-ADP}}$ = 2.1333 mM.

Tissue homogenate from each fish was added to one of the two measurement chambers immediately following preparation; fish from a processing pair were measured in parallel. After addition of homogenate to the respiration chamber at 12˚C, pure oxygen gas was added to reach a concentration of 650 µM (Figure S3). Magnesium green (2.2 µM) was added to the respirometry chambers to allow us to detect changes in $[Mg^{2+}]$ and so measure the rate of ATP production (Figure S3).

Mitochondrial properties were measured as in [10]. We used a protocol for estimating the ATP/O ratio that simultaneously measures both oxygen consumption and ATP production 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 the concentration of free magnesium [11]. ATP production is calculated from the rate of change in $[Mg^{2+}]$ and is based on the differential affinities of ATP and ADP for Mg^{2+} [12]. 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).

A sequential substrate/inhibitor protocol as in [10] was run simultaneously for each fish. Figure S3 displays representative traces of oxygen flux and magnesium fluorescence in homogenized (a) liver and (b) muscle. The rate of oxygen consumption to support ATP production 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 raw rate of ATP production was also measured in this condition. The rate of ATP hydrolysis was then measured after inhibition of mitochondrial ATP synthesis (with 4 µM carboxyatractyloside). The rate of ATP hydrolysis was then added to the raw rate of ATP production to obtain the corrected 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 all other oxygen consumption values. Finally, cytochrome c oxidase (COX) respiration, a measure of the mitochondrial density of the tissues [13], was measured by adding ascorbate (8 mM) and N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride (0.5 mM).

The muscle trial was identical to the liver trial but an inhibitor of adenylate kinase (25 µM P^1,P^5-di(adenosine-5') pentaphosphate) was added to the measurement chamber with the subsample of muscle that was kept on ice (Figure S3b). The final concentration for muscle homogenate was higher (mean ± SE: 18.53 ± 1.03 mg wet muscle mL^{-1}) since the oxidation and phosphorylation fluxes were smaller. The titration protocol of the muscle was completed within 4 h of the fish being culled. Each fish's liver and muscle were run in the same measurement chamber. Every second day, the measurement chamber associated to a treatment group was reversed to control for any inter-respirometry chamber difference in readings. No effect of the choice of measurement chamber on mitochondrial properties was detected.

Sample sizes for statistical analyses

The data from a muscle mitochondrial assay for one fish were excluded from the experiment because the addition of ADP led to a very small elevation in the respiration rate, indicating that either the mitochondrial preparation might have failed or this fish was not "healthy". This fish ate its entire ration during the growth trials but lost body mass. The pattern of results of the analyses of growth performance were the same whether or not this individual was included in the models, so models including data from this fish (excluding those for its muscle mitochondrial properties) are reported in the text. The data from the muscle ATP/O ratio assay for three fish were excluded because the fluxes of ATP production were too low to be detected. The data on the fractional rate of protein synthesis in the liver and in the muscle from three and one fish, respectively, were also removed or missing for the following reasons: extremely high values for the enrichment of phenylalanine in the free amino acid pool (in the muscle of one fish and liver of another) indicated that the tissues had likely been contaminated during sampling, one liver sample for the Ks assay was lost, and the D_5-Phe enrichment of the protein pool value in the liver of one fish was extremely high, indicating a potential error during the extraction steps. Thus, differences in the sample sizes between analyses reflect missing values (Table S1).

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Table S1. Statistics of indices of growth performance, mitochondrial properties (ATP/O ratio and cytochrome *c* oxidase [COX] activity), and fractional rate of protein synthesis (Ks) in brown trout fed different rations. Linear mixed models were used to determine the effect of food intake (high or low) on growth performance, mitochondrial properties of the liver and/or muscle and the fractional rate of protein synthesis. Processing batch was included as a random effect to control for the order in which fish were processed.

	Low Food Intake				**High Food Intake**				**Statistics**		
	N	Min.	Max.	Mean ± SE	N	Min.	Max.	Mean ± SE	*F*	d.f.	*P*
Growth Performance											
Specific Growth Rate (% day^{-1})	30	-0.06	1.02	0.63 ± 0.04	29	1.01	2.70	1.83 ± 0.08	237	1,29	< 0.001
Growth Efficiency (gain in body mass per mass food intake)	30	-0.13	2.23	1.39 ± 0.09	29	0.73	2.16	1.47 ± 0.07	29.0	1, 29	0.422
Specific Protein Gain (% day^{-1})	30	-0.47	1.28	0.79 ± 0.08	29	-0.20	2.94	1.79 ± 0.14	54.2	1, 29	< 0.001
Mitochondrial density											
Liver COX activity (pmol O_2 s^{-1} mg^{-1} wet weight of tissue)	30	23.55	37.95	31.13 ± 0.70	29	20.31	35.32	28.01 ± 0.68	19.6	1, 29	< 0.001
Muscle COX activity (pmol O_2 s^{-1} mg^{-1} wet weight of tissue)	29	10.83	19.68	14.03 ± 0.37	29	9.47	19.20	14.36 ± 0.41	0.43	1, 29	0.519
Mitochondrial Efficiency											
Liver ATP/O ratio	30	0.78	1.41	1.04 ± 0.03	29	0.87	1.45	1.10 ± 0.03	2.50	1, 57	0.119
Muscle ATP/O ratio	26	0.48	3.62	1.60 ± 0.16	29	0.05	3.53	1.36 ± 0.16	1.02	1,53	0.318
Fractional rate of Protein Synthesis											
Liver Ks (% day^{-1})	29	2.84	13.44	7.79 ± 0.37	27	3.27	11.00	6.98 ± 0.42	3.09	1, 28	0.090
Muscle Ks (% day^{-1})	30	0.62	1.65	0.97 ± 0.04	28	0.65	1.69	1.06 ± 0.05	4.03	1, 28	0.055

Table S2. Coefficients of the correlations between physiological traits of the liver and of the white muscle from the same fish. Measured traits were mitochondrial efficiency (ATP/O ratio), mitochondrial density (measured as cytochrome oxidase (COX) activity) and fractional protein synthesis rate (Ks); n = 55 fish for ATP/O ratio, n = 58 fish for COX activity, n = 55 fish for Ks respectively.

	Pearson's *r*	***p***
ATP/O ratio	-0.093	0.499
COX activity	-0.005	0.972
Ks	0.090	0.514

Figure S1. The relationships between whole-body protein content as determined by protein extraction and measurement of whole-body wet mass, for juvenile brown trout maintained on different food rations (Low, Intermediate and High). N = 9 fish per food level.

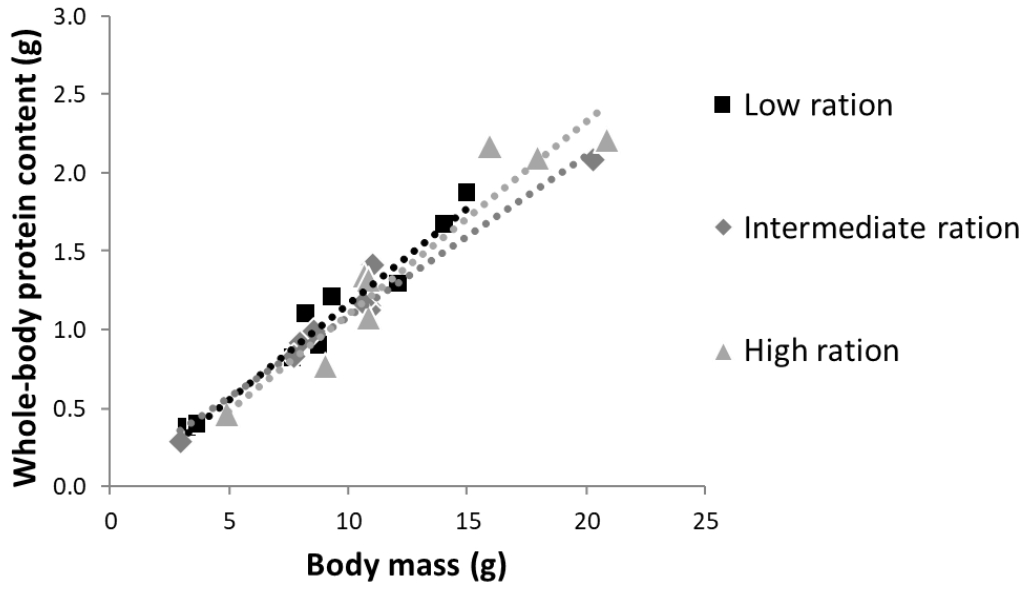

Figure S2. Specific enrichment of the free D_5-phenylalanine pool (squares) and protein D_5-phenylalanine pool (circles) of liver (a) and white muscle (b) in relation to time after injection of the tracer in brown trout (mean ± SE, n = 5).

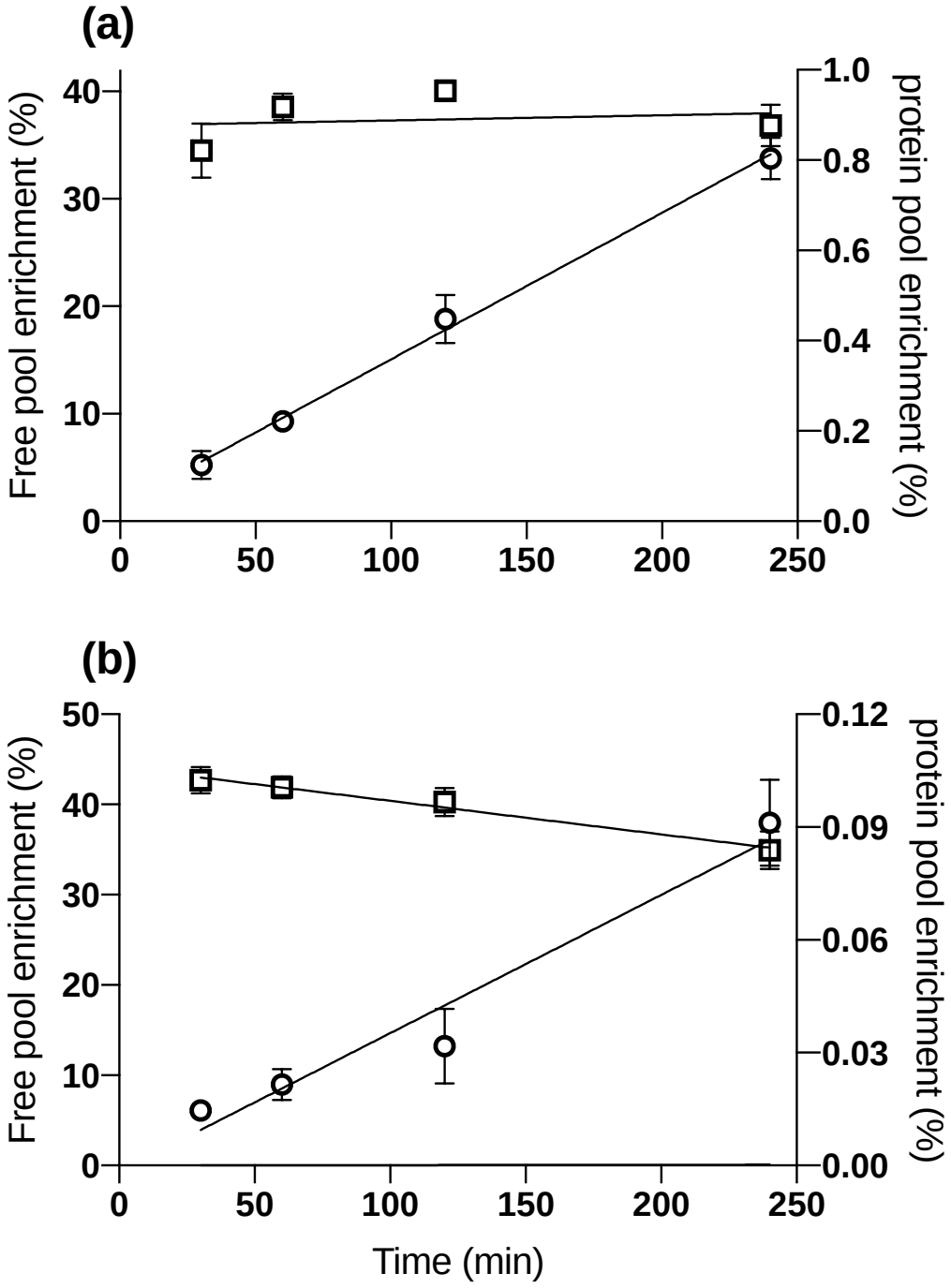

Figure S3. Output from a representative experiment, using homogenized brown (a) trout liver and (b) muscle, showing changes in magnesium green (MgG) fluorescence (black line) and oxygen concentration (blue line). See section "Measurement of mitochondrial properties" for details. An inhibitor of adenylate kinase, P^1,P^5-di(adenosine-5') pentaphosphate, was added to the measurement chamber with the homogenate of muscle. Arrows show sequential titrations of pyruvate malate (Pyr/Mal), succinate (Succ), MgG, EDTA, EGTA, $MgCl_2$, ADP, carboxyatractyloside (cATR), Antimycine and Rotenone (Ant/Rot) and ascorbate and N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride (Asc/TMPD).