Reproducibility of $F_{\text{max}}$ and Fat Oxidation Rates during Exercise in Recreationally Trained Males

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Abstract

Aerobic exercise training performed at the intensity eliciting maximal fat oxidation ($F_{\text{max}}$) has been shown to improve the metabolic profile of obese patients. However, limited information is available on the reproducibility of $F_{\text{max}}$ and related physiological measures. The aim of this study was to assess the intra-individual variability of: a) $F_{\text{max}}$ measurements determined using three different data analysis approaches and b) fat and carbohydrate oxidation rates at rest and at each stage of an individualized graded test. Fifteen healthy males (body mass index $23.1 \pm 0.6$ kg/m$^2$), maximal oxygen consumption ($VO_{2\text{max}}$) $52.0 \pm 2.0$ ml/kg/min) completed a maximal test and two identical submaximal incremental tests on ergocycle (30-min rest followed by 5-min stages with increments of 7.5% of the maximal power output). Fat and carbohydrate oxidation rates were determined using indirect calorimetry. $F_{\text{max}}$ was determined with three approaches: the sine model (SIN), measured values (MV) and 3rd polynomial curve (P3). Intra-individual coefficients of variation (CVs) and limits of agreement were calculated. CV for $F_{\text{max}}$ determined with SIN was 16.4% and tended to be lower than with P3 and MV (18.6% and 20.8%, respectively). Limits of agreement for $F_{\text{max}}$ were $-2 \pm 27$% of $VO_{2\text{max}}$ with SIN, $-4 \pm 32$ with P3 and $-4 \pm 28$ with MV. CVs of oxygen uptake, carbon dioxide production and respiratory exchange rate were <10% at rest and <5% during exercise. Conversely, CVs of fat oxidation rates (20% at rest and 24–49% during exercise) and carbohydrate oxidation rates (33.5% at rest, 8.5–12.9% during exercise) were higher. The intra-individual variability of $F_{\text{max}}$ and fat oxidation rates was high (CV >15%), regardless of the data analysis approach employed. Further research on the determinants of the variability of $F_{\text{max}}$ and fat oxidation rates is required.

Introduction

Carbohydrate and fat are the two main sources of energy that sustain oxidative metabolism. Their relative utilization during aerobic exercise depends largely on exercise intensity [1,2]. The whole-body carbohydrate oxidation rate ($CHO_{\text{ox}}$) increases with the workload, whereas the whole-body fat oxidation rate ($F_{\text{ox}}$) increases from low to moderate exercise intensities, and then markedly declines at high intensities. The exercise intensity at which the maximal fat oxidation (MFO) rate occurs has been defined as $F_{\text{max}}$ [3]. Aerobic exercise training performed at $F_{\text{max}}$ has the potential to increase $F_{\text{ox}}$ and insulin sensitivity in obese patients [4] and in individuals with metabolic syndrome [5]. In patients with type 2 diabetes, aerobic training targeted at $F_{\text{max}}$ was shown to have a greater effect on body composition and glucose control than high intensity interval training [6].

To determine $F_{\text{max}}$, a submaximal graded exercise test using indirect calorimetry is performed, and data is analyzed with two main steps. First, $F_{\text{ox}}$ and $CHO_{\text{ox}}$ at each stage of the test are calculated from indirect calorimetry measures [oxygen consumption ($VO_2$) and carbon dioxide production ($VCO_2$)] by means of the stoichiometric equations [7]. Subsequently, $F_{\text{ox}}$ values are plotted as a function of exercise intensity and $F_{\text{max}}$ is identified with one of the following four commonly used methods: a) the determination of the maximal value of measured $F_{\text{ox}}$ reached during each stage of the graded exercise test and identification of the corresponding intensity (measured values approach, MV) [3,8–10], b) the construction of a 3rd polynomial fitting curve (P3) [11], c) the Sine model (SIN) [12] and d) the non-protein “respiratory quotient technique” [13].

Knowledge of the reproducibility of $F_{\text{max}}$ is necessary for establishing its usefulness as a parameter for training prescription and for adequately interpreting outcomes from research studies. To date, there has been limited research into the reproducibility of $F_{\text{max}}$ and findings to date are conflicting and have methodological limitations [8,13,14]. Achten et al. [8] found a coefficient of variation (CV) for $F_{\text{max}}$ of 9.6% in 10 endurance athletes tested on three occasions and concluded that $F_{\text{max}}$ measurements are reliable. Perez-Martín et al. [13] tested 10
Limited information is available on the reproducibility of \( \text{Fat}_{\text{max}} \) and on the reproducibility of \( \text{CHO}_{\text{ox}} \) and \( F_{\text{ox}} \) at each stage of a graded test. It was therefore the aim of this study to assess the intra-individual variability of: a) \( \text{Fat}_{\text{max}} \) measurements determined using three different data analysis approaches (SIN, P3 and MV), and b) \( \text{CHO}_{\text{ox}} \) and \( F_{\text{ox}} \) at rest and in response to each stage of an individualized graded test. A further aim was to investigate how the CVs of \( \bar{V}\text{O}_2, \bar{V}\text{CO}_2 \) and RER are related to the CV of \( F_{\text{ox}} \).

Methods

Ethics Statement

The study was conducted in accordance with ethical principles of the 1964 World medical Declaration of Helsinki and was approved by the human research ethics committee of the University of Lausanne (Switzerland). All test procedures, risks and benefits associated with the experiment were fully explained, and written informed consent was obtained from all participants.

Subjects

Fifteen healthy, moderately trained male volunteers (see Table 1 for anthropometric and physical characteristics) were recruited to participate in this study. All participants were of normal weight according to the World Health Organization (Body Mass Index < 25 kg·m\(^{-2}\)), non-smokers and disease-free. They were not taking regular medications and were screened for the absence of electrocardiographic abnormalities at rest and during exercise.

General Design

Each participant completed three test sessions. In the first session anthropometric measurements (i.e., stature, body mass and body composition) were taken and a maximal incremental test on a cycle ergometer was performed. In the remaining two sessions the subjects performed an identical submaximal incremental test (Test 1 and Test 2). The two tests were performed in the morning (start of exercise between 7 and 8 am) after a 10-hour overnight fast. They were separated by 3 to 7 days and performed at the same time of day to avoid circadian variance. The volunteers were asked to fill in a 1-day food diary on the day before Test 1 and to repeat this diet before Test 2. Furthermore, participants were asked to refrain from vigorous exercise and alcohol and caffeine consumption in the 24 hours prior to testing. Participants were familiarized with the equipment prior to testing.

Anthropometric Measurements

Body composition (fat mass and percentage of body fat) was estimated from skin-fold thickness measurements at four sites according to the methods of Durnin and Womersley [20].

Maximal Exercise Test

A maximal incremental test on a cycle ergometer (Ebike Basic BPlus, General Electric, Niskayuna, NY, USA) to determine maximal oxygen uptake (\( \bar{V}\text{O}_2_{\text{max}} \)) and maximal aerobic power output (\( W_{\text{max}} \)) was performed. After a 5-min rest period and a 5-min warm-up at 60 W, output was increased by 30 W every minute until volitional exhaustion. \( \bar{V}\text{O}_2 \) was considered as maximal when at least three of the following four criteria were met [21]: 1) a plateauing of \( \bar{V}\text{O}_2 \) (defined as an increase of no more than 2 mL·kg\(^{-1}\)·min\(^{-1}\) with an increase in workload) during the latter stages of the exercise test, 2) an HR >90% of the age-predicted maximum (220-age), 3) an RER >1.1 and 4) an inability to maintain the minimal required pedaling frequency (i.e. 60 rpm) despite maximum effort and verbal encouragement.

\[ \bar{V}\text{O}_2_{\text{max}} = \text{Fat}_{\text{max}} \text{ CV} + \text{CHO}_{\text{ox}} \text{ CV} + F_{\text{ox}} \text{ CV} \]

| Table 1. Subject characteristics. |
|----------------------------------|
| **n=15**                       |
| Age (years) 27.4±4.0           |
| Height (cm) 180±5              |
| Weight (kg) 74.2±7.6           |
| BMI (kg·m\(^{-2}\)) 23.1±2.3  |
| Body fat (%) 14.4±2.9          |
| Fat-free mass (kg) 63.7±5.9    |
| \( V\text{O}_{2\text{max}} \) (mL·kg\(^{-1}\)·min\(^{-1}\)) 52.0±7.7 |
| HR\(_\text{max}\) (beats·min\(^{-1}\)) 185±11 |
| \( W_{\text{max}} \) (Watts) 322±51 |

Values are means ± SD, \( n \), number of subjects; BMI, body mass index; \( V\text{O}_{2\text{max}}, \) maximal oxygen uptake; HR\(_\text{max}\), maximal heart rate; \( W_{\text{max}}, \) maximal aerobic power output.

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healthy males on two occasions, reported a CV for \( \text{Fat}_{\text{max}} \) of 11.4% and suggested this to be a “satisfactory result”. Conversely, Meyer et al. [14] studied 21 recreationally trained men and women who completed the test twice. The limits of agreement (LoA) for oxygen consumption at \( \text{Fat}_{\text{max}} \) corresponded to a heart rate (HR) difference of 35 bpm between the two tests, which lead them to conclude that the intra-individual variability in \( \text{Fat}_{\text{max}} \) measurements is too large to recommend using this parameter for clinical purposes. However, some studies have evaluated the intra-individual variability of the \( \text{Fat}_{\text{max}} \) and on the reproducibility of \( \text{CHO}_{\text{ox}} \) and \( F_{\text{ox}} \) at each stage of a graded test. It was therefore the aim of this study to assess the intra-individual variability of: a) \( \text{Fat}_{\text{max}} \) measurements determined using three different data analysis approaches (SIN, P3 and MV), and b) \( \text{CHO}_{\text{ox}} \) and \( F_{\text{ox}} \) at rest and in response to each stage of an individualized graded test. A further aim was to investigate how the CVs of \( \bar{V}\text{O}_2, \bar{V}\text{CO}_2 \) and RER are related to the CV of \( F_{\text{ox}} \).
calculated as the average $\dot{V}O_2$ over the last 20 seconds of the last stage of the test.

**Submaximal Graded Exercise Tests (Test 1 and Test 2)**

Test 1 and Test 2 were characterized by two phases: a pre-exercise resting phase (rest) and a submaximal incremental exercise test. They were carried out under identical circumstances with an identical protocol. Data from these two tests were subsequently employed for reliability calculations.

In the pre-exercise resting phase (rest), participants were seated for 30-min on the cycle ergometer and respiratory measures were collected during the last 15-min of this sitting period. Subsequently, a submaximal incremental exercise test to determine whole-body $F_{\text{ox}}$ kinetics was performed. After a 10-min warm-up at 20% $W_{\text{max}}$, the power output was increased by 7.5% $W_{\text{max}}$ every 5-min until RER was $>1.0$ during the last minute of the stage.

**Indirect Calorimetry and Calculations**

Oxygen uptake ($\dot{V}O_2$), carbon dioxide output ($\dot{V}CO_2$) and ventilation ($V_e$) were measured continuously using a breath-by-breath system (Oxycon Pro, Jaeger, Würzburg, Germany). Before each test the gas analyzers were calibrated with gases of known concentration (16.00% $O_2$ and 5.02% $CO_2$), and the volume was automatically calibrated at two different flow rates (0.2 L·s$^{-1}$ and 2 L·s$^{-1}$). The HR was recorded continuously using an HR monitor (S810i, Polar Electro OY, Kempele, Finland).

During Test 1 and Test 2, HR and gas exchange data ($\dot{V}O_2$, $\dot{V}CO_2$) collected during the final 5-min of the pre-exercise resting phase and during the last 2-min of each stage of the submaximal incremental exercise test were averaged and used for calculations. RER was calculated as the ratio between $\dot{V}CO_2$ and $\dot{V}O_2$, while $F_{\text{ox}}$ and $\text{CHO}_{\text{ox}}$ were calculated using stoichiometric equations [7], with the assumption that the urinary nitrogen excretion rate was negligible:

$$F_{\text{ox}}(\text{g·min}^{-1}) = 1.67\dot{V}O_2(\text{L·min}^{-1}) - 1.67\dot{V}CO_2(\text{L·min}^{-1})$$ (1)

$$\text{CHO}_{\text{ox}}(\text{g·min}^{-1}) = 4.55\dot{V}CO_2(\text{L·min}^{-1}) - 3.21\dot{V}O_2(\text{L·min}^{-1})$$ (2)

(1-RER) was also calculated given that the equation to calculate $F_{\text{ox}}$ can be simplified to:

$$F_{\text{ox}}(\text{g·min}^{-1}) = 1.67(1\text{-RER})\dot{V}O_2.$$ (3)

$F_{\text{ox}}$ as a function of exercise intensity is reflected by two different linear relationships: a progressive decrease of (1-RER) and a linear increase of $\dot{V}O_2$ as power output is increased. The percentages of total energy expenditure derived from fat ($\%\text{ENE}_{\text{fat}}$) and CHO ($\%\text{ENE}_{\text{CHO}}$) were calculated [22]:

$$\%\text{ENE}_{\text{fat}} = [(1 - \text{RER})/0.29] \times 100$$ (4)

$$\%\text{ENE}_{\text{CHO}} = [(\text{RER} - 0.71)/0.29] \times 100$$ (5)

**Data Analysis Approaches to Determine $F_{\text{ox}}_{\text{max}}$**

$F_{\text{ox}}$ values obtained at each stage of the submaximal graded exercise test (which was terminated when RER was $>1.0$) were graphically depicted as a function of exercise intensity. Then, $F_{\text{ox}}_{\text{max}}$ and MFO (and subsequently RER, $\%\text{HR}_{\text{max}}$ at $F_{\text{ox}}_{\text{max}}$, $\%W_{\text{max}}$ at $F_{\text{ox}}_{\text{max}}$) were determined using three different data analysis approaches (SIN, MV and P3). The “respiratory quotient technique” was not used in this study since it has been shown to be less accurate than the other methods [12].

**SIN model.** The SIN model [12] was used to model and characterize whole-body $F_{\text{ox}}$ kinetics:

$$\%\text{MFO} = \sin\left(\frac{\pi}{\pi/2+2d}\left(k\cdot\text{RER}_{\text{max}}+d+t\right)^2\right)$$ (6)

### Dilation ($d$), symmetry ($\pi$) and translation ($t$) are the three independent variables representing the main modulations of the curve. $k$ is the constant of intensity and corresponds to $(\pi/100)$. To fit the experimental data (i.e. $F_{\text{ox}}$ rates) and to model the $F_{\text{ox}}$ kinetics, the three variables were independently changed using an iterative procedure by minimizing the sum of the mean squares of the differences between the estimated energy derived from fat based on the SIN model and the energy derived from fat calculated from the raw $F_{\text{ox}}$ data, as described in a previous study [12]. For each subject, $F_{\text{ox}}_{\text{max}}$ was calculated by differentiation of the SIN model equation. The $F_{\text{ox}}_{\text{max}}$ zone was determined as the range of exercise intensities with $F_{\text{ox}}$ rates within 10% of MFO [3].

**P3.** Graphical depiction of $F_{\text{ox}}$ values as a function of exercise intensity was performed by constructing a third polynomial curve with intersection at (0;0) [11]. $F_{\text{ox}}_{\text{max}}$ was calculated by differentiation of the P3 equation, and corresponded to the intensity at which the value of the differentiated equation was equal to zero.

### Measured values.

From the graphical representation of $F_{\text{ox}}$ values as a function of exercise intensity, the stage at which the value of measured $F_{\text{ox}}$ rates was maximal was determined, and the corresponding intensity was identified [3,8–10,23].

### Theoretical Example to Study how the CVs of $\dot{V}O_2$ and $\dot{V}CO_2$ are Related to the CVs of RER and the CV of $F_{\text{ox}}$.

In order to investigate how the CV of $\dot{V}O_2$ and $\dot{V}CO_2$ are linked to the CVs of parameters informing of substrate utilization (RER, $F_{\text{ox}}$, $\text{CHO}_{\text{ox}}$, 1-RER, $\text{ENE}_{\text{fat}}$, 3 theoretical scenarios were created. $\dot{V}O_2$ and $\dot{V}CO_2$ values for Test 1 and Test 2 were generated so that CVs of $\dot{V}O_2$ and $\dot{V}CO_2$ between Test 1 and Test 2 were $\leq$3%. A CV of $\leq$3% for $\dot{V}O_2$ and $\dot{V}CO_2$ was chosen in line with results from previous studies [8,10].

### Statistical Analysis

Data are expressed as the means ± standard deviation (SD) for all variables. Intra-individual CVs and LoA were calculated to test the variability between Test 1 and Test 2 for the following measures: a) $F_{\text{ox}}_{\text{max}}$ and physiological measures at $F_{\text{ox}}_{\text{max}}$ (MFO, RER, $\%\text{HR}_{\text{max}}$ and $\%W_{\text{max}}$) determined with three different data analysis approaches (SIN, MV and P3) and b) gas exchange data, HR and substrate oxidation rates at rest and during the first six stages of the submaximal incremental tests (from 20% to 57.5% of $W_{\text{max}}$). Intra-individual CVs were calculated for the physiological variables studied in the three theoretical scenarios.

Two-factorial analysis of variance for repeated measures (RMANOVA) was carried out to test for systematic changes in:
Table 2. Average values, limits of agreement and CVs for Fatmax and physiological measures at Fatmax determined with three approaches: SIN, P3 and MV.

|          | SIN     | P3      | MV      |
|----------|---------|---------|---------|
| Fatmax   |         |         |         |
| (% VO2max) |        |         |         |
| Test 1   | 46.9±9.0 | 44.2±10.2 | 45.7±9.0 |
| Test 2   | 48.9±12.2 | 48.6±13.1 | 49.6±12.6 |
| LoA      | −29.7, 25.7 | −36.7, 28.0 | −32.0, 24.0 |
| CV (%)   | 16.4    | 20.8    | 18.6    |
| MFO      |         |         |         |
| Test 1   | 0.28±0.08 | 0.28±0.08 | 0.29±0.08 |
| Test 2   | 0.29±0.13 | 0.29±0.13 | 0.30±0.12 |
| LoA      | −0.27, 0.24 | −0.25, 0.23 | −0.26, 0.26 |
| CV (%)   | 25.3    | 22.8    | 26      |
| RER Fatmax|         |         |         |
| Test 1   | 0.91±0.02 | 0.91±0.02 | 0.91±0.02 |
| Test 2   | 0.91±0.02 | 0.91±0.02 | 0.91±0.02 |
| LoA      | −0.05, 0.04 | −0.06, 0.04 | −0.06, 0.05 |
| CV (%)   | 1.6     | 1.7     | 1.6     |
| %HRmax Fatmax |        |         |         |
| Test 1   | 60.9±8.3 | 58.7±9.3 | 58.8±8.9 |
| Test 2   | 63.0±10.0 | 62.7±10.5 | 63.3±11.0 |
| LoA      | −23.9, 19.7 | −30.0, 22.2 | −29.4, 20.4 |
| CV (%)   | 10      | 12.8*   | 12.8*   |
| %Wmax Fatmax |        |         |         |
| Test 1   | 34.9±8.9 | 32.4±10.4 | 39.0±10.6 |
| Test 2   | 36.7±11.8 | 36.3±12.8 | 32.0±11.7 |
| LoA      | −26.4, 22.6 | −33.4, 25.6 | −18.1, 32.1 |
| CV (%)   | 19.8    | 26.4*   | 24.9*   |

Values are means ± SD. LoA, limits of agreement; CV, coefficient of variation; SIN, sine model; MV, measured values; P3, 3rd polynomial curve; Fatmax, exercise intensity at which maximal fat oxidation rate occurs; MFO, maximal fat oxidation rate; RER Fatmax, respiratory exchange ratio at Fatmax; %HRmax Fatmax, % maximal heart rate at Fatmax; %Wmax Fatmax, % maximal aerobic power output at Fatmax.

*P<0.05 between SIN and the other approaches (P3 and MV).

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Results

Fatmax and Physiological Measures at Fatmax Determined with SIN, P3 and MV

Fatmax and physiological measures at Fatmax determined with three data analysis approaches (SIN, P3 and MV) are presented in Table 2. For all parameters, average values (n = 15) obtained from Test 1 and Test 2 were not significantly different (i.e. for Fatmax: P = 0.37 for factor test and P = 0.20 for factor interaction between test and approach), indicating that no habituation or training effects occurred between testing sessions. Average values for Fatmax and related measures obtained with the three different approaches were also not significantly different (i.e. for Fatmax: P = 0.13 for factor approach).

On the other hand, the within-individual CVs for Fatmax determined with SIN was 16.4% and tended to be lower (P = 0.10) than with P3 and MV (20.8% and 18.6% respectively). Similarly, the intra-individual CVs of % HR at Fatmax and % Wmax at Fatmax determined with SIN were significantly lower than with the other approaches (P = 0.043 and P = 0.05, respectively).

The Bland–Altman scatterplots for Fatmax and MFO (Figure 1) reveal considerable intra-individual variability. The LoA for Fatmax were −2±27% of VO2max with SIN, −4±32% with P3, and −4±28% with MV. For MFO they were −0.01±0.25 g/min with SIN, 0.01±0.24 g/min with P3, and 0±0.26 g/min with MV (Table 2). A large between-individual difference in the variability between Test 1 and Test 2 was also seen. Accordingly, the CV at Fatmax ranged from 0 to 48%. For seven subjects it was under 10%, for two subjects it was between 10 and 15%, while for six subjects it was over 20%. However, the size of the difference between Test 1 and Test 2 appeared to be independent of the average value between the two measurements.

The difference in the HR at Fatmax between test 1 and 2 was <10 bpm in six participants, between 10 and 25 bpm in eight participants and >25 bpm in one. In both tests, the range of HR frequencies corresponding to the Fatmax zone was broad (it was 38±8 bpm, and ranged from 95±16 to 133±20 bpm).
Figure 1. Bland-Altman plots of \( \text{Fat}_{\text{max}} \) and MFO determined with SIN, P3 or MV. SIN, sine model. P3, polynomial 3rd degree; MV, measured values; \( \text{Fat}_{\text{max}} \), exercise intensity at which maximal fat oxidation rate occurs; \( \dot{V}O_{2\text{max}} \), maximal oxygen uptake; MFO, maximal fat oxidation rate; Biases (solid lines) and 95% limits agreement (dashed lines).

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Physiological Measures at Each Stage of a Submaximal Graded Test

The course of average $\dot{V}O_2$, $\dot{V}CO_2$, RER, $F_{\text{ox}}$ and $\text{CHO}_{\text{ox}}$ in response to two identical submaximal graded test performed on separate days (Test 1 and Test 2) is presented in Figure 2. There was no significant difference between Test 1 and Test 2 in any of the parameters assessed. Average values of most physiological variables (except $F_{\text{ox}}$) significantly increased with exercise intensity ($P<0.001$).

CVs of $\dot{V}O_2$, $\dot{V}CO_2$, HR and RER were <10% at rest and <5% during exercise (Table 3). For instance, CVs for $\dot{V}O_2$ and $\dot{V}CO_2$ and RER were 7.5%, 9.1% and 3.8% at rest and were, on average, 3.1%, 3.0% and 2.5% during exercise. In contrast, CVs for $\text{CHO}_{\text{ox}}$ and $F_{\text{ox}}$ were markedly higher. The CV for $F_{\text{ox}}$ was 20.6% at rest and ranged from 24.1 to 49.2% during exercise, while for $\text{CHO}_{\text{ox}}$ it was 33.5% at rest and ranged from 8.5% to 12.9% during exercise. Interestingly, although the CV of RER was <4% under each condition, the CV of 1-RER was markedly higher (>20%), and was equal to the CV of the % of ENE fat. LoA between Test 1 and Test 2 are presented in Table 4.

### Theoretical Example to Study how the CVs of $\dot{V}O_2$ and $\dot{V}CO_2$ Are Related to the CVs of RER and the CV of $F_{\text{ox}}$

Three theoretical scenarios in which the CVs of $\dot{V}O_2$ and $\dot{V}CO_2$ were ≤3% are presented in Table 5 and additional results with mathematical explanations are presented in appendix S1. In case scenario 1 and 2, the CVs of $F_{\text{ox}}$ from were markedly different (3.1% vs. 38.2%) despite the CVs of $\dot{V}O_2$ and $\dot{V}CO_2$ being identical (3%) [see Appendix S1, eq. 7, 8 and 9]. Further, the CV of 1-RER was higher than the CV of RER, and was equal to the CV of %ENE fat. This difference was particularly apparent in case 2, where the CV of RER was 6%, while the CVs of $F_{\text{ox}}$ and 1-RER were 38.2 and 35.3%, respectively.

From the analysis of the three theoretical scenarios (as well as from the analysis of the whole dataset of 15 participants) we also observed that the CV of $F_{\text{ox}}$ can be calculated from sum or subtraction of the CV of 1-RER and the CV of $\dot{V}O_2$ (Appendix S1, eq. 10 and 11). For example, in case 2, the CV of $F_{\text{ox}}$ was 38.2% and was the sum of the CVs of 1-RER (35.3%) and $\dot{V}O_2$ (3.0%). In case 3, the CV of $F_{\text{ox}}$ was 15.3%, and equaled the CV of 1-RER (15.3% ± CV $\dot{V}O_2$ (0.0%).

### Discussion

In this study we assessed the reproducibility of $\text{Fat}_{\text{max}}$ measurements determined with three different data analysis approaches and of $\text{CHO}_{\text{ox}}$ and $F_{\text{ox}}$ at rest (while sitting) and in response to each stage of an individualized graded test. We observed that the intraindividual variability of $\text{Fat}_{\text{max}}$ was large (CV >16%) regardless of the data analysis approach employed and that $F_{\text{ox}}$ at rest and at each stage of a graded test was also variable (CV >20%), despite the CVs of $\dot{V}O_2$, $\dot{V}CO_2$ and RER being <5%.

The reproducibility of $F_{\text{ox}}$ values at each stage of a graded test, despite being a key aspect in the determination of $\text{Fat}_{\text{max}}$, was previously unexplored. In the current study, the CVs found for the parameters from which $F_{\text{ox}}$ is calculated ($\dot{V}O_2$, $\dot{V}CO_2$ and RER) were in line with previous observations. At rest, the CV for RER was 3.8%, which closely mirrors the CV of 3.5% found by Roffey et al. [17]. In the present study the resting assessment was performed with the individuals in a seated position and this needs to be taken into consideration when making comparisons with studies in which resting metabolism was assessed with participants.

### Table 3. Coefficients of variation (%) for respiratory values and substrate oxidation rates in response to a submaximal graded exercise test.

| Rest | $\dot{V}O_2$ (ml min⁻¹) | $\dot{V}CO_2$ (ml min⁻¹) | RER | $F_{\text{ox}}$ (g min⁻¹) | $\text{CHO}_{\text{ox}}$ (g min⁻¹) | 1-RER | ENE<sub>fat</sub> (%) |
|------|-------------------------|--------------------------|------|-------------------------|-------------------------------|-------|---------------------|
| 7.5  | 9.1                     | 3.7                      | 4.6  | 3.8                     | 3.0                           | 2.8   | 0.001              |
| 9.1  | 9.1                     | 4.4                      | 4.2  | 2.9                     | 2.6                           | 2.6   | 0.001              |
| 20.6 | 20.9                    | 2.0                      | 3.0  | 2.8                     | 2.6                           | 2.6   | 0.001              |

Values are means, $\text{Fat}_{\text{max}}$ maximal aerobic power output, $\dot{O}_2$, oxygen uptake, $\dot{V}CO_2$, carbon dioxide production, RER, respiratory exchange ratio; $F_{\text{ox}}$, fat oxidation rate; $\text{CHO}_{\text{ox}}$, carbohydrate oxidation rate; ENE<sub>fat</sub>, fat energy expenditure ratio; $\text{Fat}_{\text{max}}$, fat energy expenditure derived from fat.
Table 4. Limits of agreement between Test 1 and Test 2 for respiratory values and substrate oxidation rates in response to a submaximal graded exercise test.

| Parameter | Rest | Wmax 20% | Wmax 35% | Wmax 50% | Wmax 65% | Wmax 75% |
|-----------|------|----------|----------|----------|----------|----------|
| VO2 (l min⁻¹) | 142.102 | -139.191 | -132.155 | -128.512 | -124.067 | -118.110 |
| VCO2 (g min⁻¹) | 138.49 | -130.000 | -124.512 | -120.512 | -116.512 | -112.512 |
| RER | 0.57 | 0.50 | 0.45 | 0.40 | 0.35 | 0.30 |
| HR (bpm) | 164.11 | -157.000 | -151.512 | -146.512 | -141.512 | -136.512 |
| CHOox (g min⁻¹) | 0.09 | 0.06 | 0.03 | 0.00 | -0.03 | -0.06 |
| Fatmax (g min⁻¹) | 0.16 | 0.10 | 0.05 | 0.00 | -0.05 | -0.10 |

This shows that even though CHOox and Fatmax are calculated from the same theoretical scenarios, their CVs can be different. The CV of Fatmax determined with the SIN model and its comparison with the CV of Fatmax determined using the respiratory quotient technique, since it has previously been shown to be less accurate [12]. The comparison revealed that the intra-individual CV at Fatmax was higher than 16% with any of the data analysis approaches employed and that there was a relatively small difference between approaches. However, the CVs of Fatmax were similar or lower than those reported in previous investigations [8,13,18,19]. Despite this, the CVs found for Fatmax were higher than 20%. Therefore, in addition to the size of the change in the CV of Fatmax and VCO2, it is crucial to know whether they change in the same or opposite sense between measurements.

The RER is the ratio between VCO2 and VO2 and, therefore, provides information on the relationship between those measurements. However, in the theoretical scenarios the CV of RER remains low (<6%) also when the variability in Fatmax is high. This is because in such conditions, the standard deviation of Fatmax results from the sum of the standard deviations of VO2 and VCO2, multiplied by a factor 1.67. Therefore, in addition to the size of the change in VO2 and VCO2, it is crucial to know whether they change in the same or opposite sense between measurements.

In this study, as well as in other studies investigating the reproducibility of indirect calorimetry measures [8,13,14,16–19], the total variation observed between Test 1 and Test 2 is the sum of both biological and equipment variation. It was beyond the scope of this study to assess the relative contribution of each. However, the average variation of the equipment (gas analysis system) used in this study is known. It was assessed using a portable metabolic simulator (which excludes any biological variability) and the average CV for VO2 and VCO2 was 1.9±0.6% and 1.3±0.5% respectively [18].

In addition to investigating the variability in Fatmax and related parameters at each stage of a graded test, a novel feature of this study was the assessment of the intra-individual variability in Fatmax determined with the SIN model and its comparison with the variability of Fatmax measures obtained using different data analysis approaches. All the approaches to determine Fatmax presented in the literature were compared in this analysis, except the “respiratory quotient technique”, since it has previously been shown to be less accurate [12]. The comparison revealed that the intra-individual CV at Fatmax was higher than 16% with any of the data analysis approaches employed and that there was a relatively small difference between approaches. However, the CVs of Fatmax were similar or lower than those reported in previous investigations [8,13,18,19]. Despite this, the CVs found for Fatmax were higher than 20%. Therefore, in addition to the size of the change in Fatmax and VCO2, it is crucial to know whether they change in the same or opposite sense between measurements.
Figure 2. Course of average $\dot{V}O_2$, $\dot{V}CO_2$, HR, RER, $F_{fat}$ and $CHO_{ox}$ during two identical submaximal incremental tests (mean and SD).

$W_{\text{max}}$: maximal aerobic power output; $\dot{V}O_2$: oxygen uptake; $\dot{V}CO_2$: carbon dioxide production; RER: respiratory exchange ratio; HR: heart rate; $F_{fat}$: fat oxidation rate; $CHO_{ox}$: carbohydrate oxidation rate. *significantly increases with exercise intensity, †rest significantly different than exercise (20–57.5% $W_{\text{max}}$), ‡significantly different than 57.5% $W_{\text{max}}$.

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Table 5. Three case scenario in which CV for VVO and VVCO are 3%.

| Case 1 | Case 2 | Case 3 |
|--------|--------|--------|
| Test 1 | Test 2 | CV (%) | Test 1 | Test 2 | CV (%) | Test 1 | Test 2 | CV (%) |
| 1450   | 1490   | 6.0    | 0.89  | 0.82   | 3.0    | 1.15   | 1.10   | 6.0    |
| 3.0    | 1.27   | 0.87   | 0.42  | 0.52   | 3.0    | 0.15   | 0.16   | 3.0    |
| 19.2   | 35.3   | 0.18   | 0.30  | 0.30   | 3.0    | 0.15   | 0.15   | 3.0    |

The intra-individual variability of Fatmax and related parameters found in this study was in line with those of Meyer et al. [14]. In the present study the LoA for Fatmax determined with SIN were $-2.0 \pm 27.7$ of $\dot{V}O_2_{\text{max}}$, while in the study from Meyer et al. LoA for Fatmax of $-3.9 \pm 27.7$ of $\dot{V}O_2_{\text{max}}$ were observed. Further, also consistent with the results published by Meyer et al. [14], the within-individual variability was markedly different between individuals. On the other hand, the CV for Fatmax observed in this study, on average, was slightly higher than those reported in other studies [8,13,24]. The lower CV found by Achten et al. [8] (9.6%) could be due to the fact that measurements were repeated three times (and the CV generally decreases when the number of measurements increases [25]) and were performed in trained athletes, who may have a less variable response to exercise than individuals with a lower training level. Overall, the differences in the results obtained between studies are difficult to interpret, particularly because most studies only report average results, and do not present “individual responses” and/or ranges. This highlights the need for a better understanding of the determinants of intra-individual variability in Fatmax.

Previous studies in the field considered an intra-individual variability of ±10 bpm in the HR at Fatmax acceptable, since this value reflects a realistic margin in individuals who use HR for the monitoring of training intensity [8,14]. In the present study this target was met by the majority, but not all, participants. However, the range of intensities at which Fmax is within 10% of MFO [Fatmax zone] was broad and this was consistent with previous observations [3,26]. Therefore, despite its variability, training prescription at Fatmax ensures that high rates of $F_{ox}$ are elicited on different days.

The determination of $F_{ox}$ and Fatmax (and therefore the determination of their variability) is influenced by a number of methodological factors including the exercise test design, the data analysis approach and the pre-test conditions. In this study, a robust methodological approach was employed. The submaximal graded exercise was individualized based upon the results of a maximal test. It started at 20% of $W_{\text{max}}$ and the workload was subsequently increased by 7.5% $W_{\text{max}}$ every 5-min. This ensured the reaching of a steady state [27] and allowed to study $F_{ox}$ at several intensities (participants performed at least six exercise stages with an RER<1). Further, the statistical analysis was carried out in accordance with the recommendations for reliability assessment in sport medicine [15].

Pre-test conditions included a 10-hour overnight fast and 24 hours of standardization in diet and physical activity prior to each submaximal graded exercise test. This level of standardization was adopted because it appears to be the most commonly employed approach in our research field [3,8,26–32] and because more rigorous standardization is difficult to achieve both in out-clinic and research settings. Despite the standardization adopted, in some individuals a high intra-individual variability in Fatmax and related variables was found, suggesting that a longer period of standardization (≥2 days prior to testing) might be needed to improve the reproducibility of those measures. However, while more strict pre-test standardization leads to greater internal validity, it also leads to poorer external validity (i.e. harder translation of the results into practice). More generally, while the validity of using a graded exercise tests to determine Fatmax has been better in the present study, the median CV across participants was 3% (IQR 2–5%).

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been reported in a number of studies [3,13,33,34], a recent study questions the usefulness using this approach to prescribe training in populations such as highly trained athletes [35]".

A number of questions on the reproducibility of substrate metabolism during exercise are still to be answered. Further research (a) describes how standardization in physical activity and diet prior to testing impact on reliability of measurements, b) study the determinants of the variability in CHOox and Fox and c) explore the reproducibility in Fatmax in other cohorts including overweight and untrained individuals. In summary, we have shown here that the intra-individual variability in Fatmax is high (CV>16%) and is highly variable between individuals, regardless of the data analysis approach employed. The intra-individual variability at rest and in response to an individualized graded test is high for Fox measures (CV>20% for Fox) although it is low for VO2, VCO2 and RER (CV<5%). The CV of (1-RER) appears to be a more representative measure of the variability in substrate oxidation than CV of RER.

Training prescription at Fatmax can be useful clinically given that, despite its variability, it results in Fatmax rates within 90% of MFO on different days. In a research setting, differences in Fatmax and Fox within and between groups can be detected as long as a sufficiently large number of participants is recruited. Further research in this area is required.

Supporting Information
Appendix S1 (DOCX)

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Author Contributions
Conceived and designed the experiments: IC FB NB RW IH XC DM. Performed the experiments: IC FB XC DM. Analyzed the data: IC FB XC DM. Contributed reagents/materials/analysis tools: IC FB NB RW IH XC DM. Wrote the paper: IC. Revised the manuscript for important intellectual content and approved the final version: IC FB NB RW IH XC DM.

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