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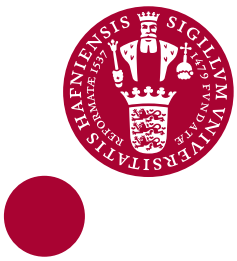

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Plasma Free Fatty Acid concentration is closely tied to whole-body peak fat oxidation rate during repeated exercise

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Running title: Combined fasting and exercise increases peak fat oxidation in a dose dependent manner

Keyword: Fat oxidation rate, Fast, Repeated exercise, Fatmax, Substrate availability.

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Abstract:

Plasma Free Fatty Acids (FFA) are a major contributor to whole body fat oxidation during exercise. However, to what extent, manipulating plasma FFA concentrations will influence whole body peak fat oxidation rate (PFO) during exercise remains elusive. In this study we aimed to increase plasma FFA concentrations through a combination of fasting and repeated exercise bouts. We hypothesized that an increase in plasma FFA concentration would increase peak fat oxidation rate in a dose dependent manner. 10 healthy young (31±6 years) (mean ± SD) well-trained ($\dot{V}O_2$max: 65.9±6.1 ml/min/kg) men performed 4 graded exercise tests (GXT) on one day. The GXT were interspersed by 4 hours of bedrest. This was conducted either in fasted state, or with the consumption of a standardized carbohydrate-rich meal 3½ hours before each GXT. Fasting and previous GXT resulted in a gradual increase in PFO from 0.63±0.18g/min after an overnight fast (10 hours) to 0.93±0.17 g/min after app. 22 hours of fasting and three previous GTX. This increase in PFO coincided with an increase in plasma FFA concentrations (r^2=0.73, p<0.0001). Ingestion of a carbohydrate-rich meal 3½ hour before each GXT resulted in unaltered PFO rates. This was also reflected in unchanged plasma FFA, glucose and insulin concentrations. In this study we show that plasma FFA availability is closely tied to whole-body peak fat oxidation rate and that the length of fasting combined with previous exercise are robust stimuli towards increasing plasma FFA concentration, highlighting the importance for pre-exercise standardization when conducting graded exercise tests measuring substrate oxidation.

New and noteworthy:

We show that peak fat oxidation is increased in close relationship with plasma FFA availability after combined fasting and repeated incremental exercise tests in healthy highly-trained men. Therefore it may be argued that whole body fat oxidation rates measured in most cases after an overnight fast indeed does not represent whole-body maximal fat oxidation rate but a whole-body peak fat oxidation rate within the context of the pre-exercise standardisation obtained in the study design

Introduction

For almost a century it has been known that glucose and fat are the main substrates for human metabolism during rest and exercise (16) and that fat oxidation rates peaks at approximately 45-65% of maximal oxygen uptake and then declines with increasing exercise intensity (1, 7). The advent of novel methodologies, i.e. stable isotopic tracers confirmed these early findings and has revealed that fat oxidized in skeletal muscle originates from different sources; Plasma free fatty acids (FFA), circulating lipoproteins (mainly Very-Low-Density-Lipoproteins, VLDL) and intramuscular triacylglycerol (IMTG) (17, 25). In these more recent studies, it is demonstrated that the main source of fat for skeletal muscle oxidation at low exercise intensities in trained men is plasma FFA derived from adipose tissue lipolysis (17, 25). Fasting (14, 18), high-fat and ketogenic diets (6, 13, 21, 32) and lowering of skeletal muscle glycogen by training twice a day or sleeping with low glycogen concentrations (12, 24, 33) all increase fat oxidation during rest and exercise (23).

A widely used method to determine maximal fat oxidation rate during exercise is the fatmax test. This test was initially validated in healthy well-trained young men by Achten, Gleeson, and Jeukendrup (1) in 2002 where they demonstrated that an incremental exercise test on a cycle ergometer could be used to establish a maximal fat oxidation rate (MFO) and the relative exercise intensity at which MFO occurred. This methodology has since been used in multiple studies investigating maximal fat oxidation rate in relation to gender and exercise mode (29, 30), in various populations from patients to athletes (2, 3, 5, 9, 22, 26) and in several diet and training interventions (4, 19, 20). In addition, we have recently demonstrated a relationship between MFO and ultra-endurance performance in both men and women (10, 31). The majority of incremental exercise tests in these studies are performed under

65 standardized conditions. That is in the overnight fasted state, and with consumption of a standardized diet or the
66 participants' regular mixed macronutrient diet on the day or days before examination together with a request to
67 abstain from vigorous exercise the day before testing. To the best of our knowledge no previous studies has
68 investigated the influence of various length of fasting and prior exercise on maximal fat oxidation rate using this
69 incremental exercise protocol.

70 The primary objective of the present study was therefore to investigate the effect of fasting and repeated
71 incremental exercise bouts on the peak fat oxidation rate and to which extent changes in peak fat oxidation rates
72 could be attributed to changes in plasma FFA availability. We hypothesized that fat oxidation and hence plasma
73 fatty acid availability would increase with increased length of fasting and repeated exercise bouts and that energy
74 and substrate repletion by a carbohydrate-containing meal 3½-hours before exercise would abolish an increase in
75 peak fat oxidation.

Methods:

Participants

We included ten healthy male athletes in the study. The study was approved by the Science ethical committee of the greater region of Copenhagen (H-15017269) and adhered to the Principles of the Helsinki declaration. Subjects received written and oral information about possible risks associated with the study before they volunteered to participate and signed a written informed consent form. The participants were national-level athletes in triathlon, cycling and cross-country skiing.

General design

The participants reported to the laboratory on three occasions. At the first visit baseline characteristics for body composition and maximal oxygen uptake ($\dot{V}O_2max$) were assessed on a cycle ergometer. On the second and third visit four consecutive graded exercise tests on the same cycle ergometer separated by approximately 3½ hours of bed rest were performed in either the fasted or fed state. The order of the second or third visit was randomized and separated by between seven and fourteen days.

The participants were told to refrain from vigorous exercise in 24 hours leading up to the laboratory tests and to eat their habitual diet and thus to maintain their macronutrient composition and energy content. On the test day participants were questioned regarding their nutrition and exercise on the day before the test. Participants reported to the laboratory after an overnight fast between 7 and 8 am. Participants were instructed to arrive at the laboratory

well-rested and requested to commute by car, or public transport. All participants complied with the pre-test instructions.

Experimental design

Pre-test screening: Body composition was determined using dual-energy X-ray absorptiometry (Lunar Prodigy Advance; Lunar, Madison, WI, USA). Participants were allowed water consumption in the morning but emptied their bladder before the DXA-scan. $\dot{V}O_2$peak was established by a graded exercise protocol with 25-watt increases every minute starting at 200 watts after a brief individual warm-up period on a cycle ergometer (Monark E839, Värberg, Sweden). The criteria for achieving $\dot{V}O_2$peak was a leveling off in $\dot{V}O_2$ (<2 ml/min/kg body weight increase in $\dot{V}O_2$ despite continuous increase in ventilation and workload) and a respiratory exchange ratio >1.15 during the final 30 seconds of the test (see figure 1).

Fasting day: Participants attended the laboratory in the morning after an overnight fast. The Graded Exercise Test (GXT) (described in details below) was performed approximately 30 min after reporting to the laboratory. On the first GXT test, 12 mL blood was sampled during the last 30 seconds of each workload. Every fourth hour throughout the day a GXT test was performed, e.g., at 8.00 am., 12.00 am., 16.00 pm, and 20.00 pm. In between tests participants rested in a bed with access only to water ad libitum. Participants were encouraged to drink sufficiently to remain euhydrated. Prior to the second and third GXT test, a 20 mL blood sample was collected at rest immediately before the exercise. Before the fourth and last GXT test blood samples were collected as described for the first GXT test (see figure 1).

Fed day: On the fed day participants were carefully instructed to eat a standardized meal (as described below) 3 hours before meeting in the laboratory (e.g., 5.00 am). Participants were questioned regarding the exact time and composition of the self-prepared meal. All participants adhered to instructions provided.

The fed day design and process were similar to the fasting day described above except for a standardized meal (3219 kJ., Fat = 9.6 E%, Carbohydrate=73.7 E%, and Protein=16.7 E%) where ingestion was initiated 5 minutes after each graded exercise test, i.e., approximately 3½hours before the start of the next graded exercise test. The meal was repeated four times and consisted of oatmeal with skimmed milk and raisins along with a glass of skimmed chocolate milk. The meals were given based on an estimated daily resting metabolic rate of 8000 Kilojoules (kJ/day (16 hours in the laboratory = 5333 kJ) and an exercise energy expenditure of 5120 kJ (approximately 1280 kJ per incremental exercise test, based on pilot study data) amounting to an estimated energy expenditure during each intervention day of 10453 kJ. All meals to every participant consisted of 3219 kJ which sums to total energy intake (12.876 kJ) which is slightly higher than the estimated energy expenditure, and this was intentionally done to ensure that participants were not in energy deficit (see figure 1).

Graded exercise tests (GXT)

Before each cycle ergometer exercise test, participants rested 5 minutes on the cycle ergometer in order to obtain a baseline fat oxidation rate. Subsequently, subjects performed a graded exercise protocol commencing at 95W for 3 minutes followed by 35W increases every 3rd minute. Averaged $\dot{V}O_2$ and $\dot{V}CO_2$ from the last 90 seconds was used to calculate fat oxidation rates (see equation below) and compute a 2nd-degree polynomial regression curve fit with fat oxidation as the dependent and relative exercise intensity (%$\dot{V}O_2$max) as the indendent variable. This

exercise protocol has previously been validated by Achten et al. ($GE_{35/3}$protocol) (1). When the subjects reached a 60 second period with a respiratory exchange ratio (RER)>1.0 no further workload was applied, and the GXT was terminated. The last completed workload before termination of the GXT is denoted LCW throughout the manuscript. Pulmonary $\dot{V}O_2$ and $\dot{V}CO_2$ were measured breath-by-breath with the automated online system (Oxycon Pro system, Jaeger, Würzburg, Germany). The online gas analyzers were carefully calibrated with an automated volume calibration and with a 4.95% CO_2 - 95.05% N_2 gas mixture before each exercise test. Substrate oxidation was calculated using the equation of Frayn with the assumption that the urinary nitrogen excretion rate was negligible (11):

Fat oxidation (g/min) = (1.67 x $\dot{V}O_2$) - (1.67 x $\dot{V}CO_2$)

All data were obtained and analyzed by the same researcher to secure reliability in the data analyses. All pulmonary data were controlled for steady state and no significant differences were found between averaging pulmonary gasses from the last 30, 60 or 90 seconds of each increment (data not shown).

Blood samples

Before each GXT test a 20 mL resting venous blood sample was obtained, from a venflon catheter placed in the antecubital vein of the forearm, and immediately centrifuged at 4000g for 10 minutes at 4^0C. The plasma fraction was collected and stored at -80^0C for later analysis (Centrifuge Hettich Universal 30 RF. Hettich Tuttlingen, Germany). During the first and last GXT test (Fed 1, Fed 4, Fast 1 and Fast 4) of each intervention day, 12 mL blood samples were obtained at each workload during the incremental exercise test. The blood samples obtained

at rest were analysed for plasma glucose, lactate, insulin, β-hydroxybutyrate, triglycerides, glycerol and FFA concentrations, whereas the blood samples obtained during exercise were analyzed for plasma glucose, lactate, β-hydroxybutyrate, glycerol and FFA concentrations using a COBAS 6000 analyzer 501C (Roche, Germany).

Blood samples at rest were obtained from all 10 subjects before every GXT (n=80). During exercise, blood samples were only obtained when RER remained below 1.0. In Table 2 blood sample data in the "LCW" column is data from the last completed workload with a RER≤1.0. Due to technical issues with the blood sampling during exercise in one subject in the Fast 1 and Fed 4 exercise tests all the blood data obtained during exercise from this subject is not included in the data analysis.

Statistics

Two-way analyses of variance (ANOVA) with repeated measures were applied to all resting blood samples and to the last completed workload (LCW). When a significant main effect (state, time) or interaction (state x time) were present a Tukey post hoc test were applied to test for differences in resting plasma concentrations, between nutritional states and GXT (Table 2.) (Sigmaplot 13.0, Systat Software, San Jose, CA, US)

Linear mixed models were conducted on all blood sample data during exercise with (state x workload) as fixed effects with the use of the Maximum Likelihood (ML) estimation process and pairwise comparison in order to establish differences between both fixed effects (Figure 3.) (SPSS statistics Version. 25, IBM software, NY, US)

A linear mixed model was fitted to evaluate the association between peak fat oxidation values and plasma FFA concentrations while adjusting for repeated measurements by means of overall and within-day subject random

effects. Estimation was done using restricted maximum likelihood estimation using R (Figure 4) (R Core Team, 2017).

All figures were constructed in GraphPad Prism 7 (GraphPad Software, La Jolla, CA, US).

All data are presented as mean±SD, and the level of significance was set at $p<0.05$

Results

Fat oxidation. In the fed state peak fat oxidation rate (PFO) decreased from 0.54±0.16 g/min to 0.39±0.11 g/min from the first GXT (Fed 1) to the second GXT (Fed 2) ($p<0.0001$). The 3rd fed GXT (Fed 3) was also different from the first GXT (Fed 1) with a mean PFO rate of 0.44±0.14 g/min ($p<0.05$). The 4th and last fed GXT (Fed 4) were not different (0.46±0.14 g/min) from all other fed GXT, meaning that the 2nd, 3rd, and 4th GXT test in fed state were similar (Figure 1). In the fasted state there was no difference between PFO in the first (Fast 1) (0.63±0.18 g/min) to the second GXT (Fast 2) (0.69±0.19 g/min) ($p=0.51$). The 3rd fasted GXT (Fast 3) (0.81±0.17 g/min) was higher than both the first and second GXT (Figure 2) ($p<0.0001$ and $p<0.01$, respectively). At test 4 PFO rate was 0.93±0.17 g/min and higher than the three other GXT in the fasted state (Fast 4 vs. Fast 1, $p<0.0001$, Fast 4 vs. Fast 2, $p<0.0001$ and Fast 4 vs. Fast 3, $p<0.05$). When PFO rates between nutritional states (Fed vs. Fast) were compared, there was a tendency ($p=0.056$) towards a higher PFO in the fasted state in the first GXT (Fed 1 vs. Fast 1). In all other tests, PFO were significantly higher in the fasted state compared to the fed state (Figure 2, $p<0.0001$).

The relative intensity at which PFO occurred (FATMAX) was 42±5% in the first fed GXT (Fed 1), and decreased from the first to the second fed GXT (42±5% vs. 38±3%, $p<0.01$). On the 3rd and the 4th GXT FATMAX was 39±3% and 40±5% similar to both the first and the second GXT. On the fasting day FATMAX initially occurred at 44±6% during Fast1, similar to the first GXT test on the fed day. On the second, third and fourth GXT in the fasted state FATMAX was 43±5%, 47±4%, and 47±5%, respectively. With a significant difference between the second and third (Fast 2 vs. Fast 3, $p=0.043$) and second and fourth exercise GXT (Fast 2 vs. Fast 4, $p=0.042$) (However, the two-way analyses of variance revealed a significant interaction between nutritional state and exercise test (nutritional state*test, $p=0.002$).

Concentrations of metabolites, substrates and hormones in the resting state

At rest before each GXT, plasma glucose concentrations were not different in the fed condition (Fed 1-4). The resting plasma glucose concentrations were likewise unaltered between the 1st, 2nd and 3rd GXT in the fasted state. However, at rest before the fast 4 GXT plasma glucose concentrations were lower than the fast 1 GXT ($p=0.003$, Table 2). When nutritional states were compared, plasma glucose concentrations were lower in the fasted than the fed condition before the 4th GXT ($p<0.005$, Table 2). At rest, the plasma lactate concentrations were not different before all fed GXT and also all GXT in the fasted state (Table 2). Plasma FFA concentrations were not different between blood samples at rest before any of the GXT performed in the fed state, whereas in the fasted condition the plasma FFA concentrations at the 3rd and 4th GXT were higher than both the 1st and 2nd test (Fast 1. vs. Fast 3, $p<0.0001$, Fast 1 vs, Fast 4, $p<0.0001$, Fast 2 vs. Fast 3, $p<0.001$, Fast 2 vs. Fast 4, $p<0.0001$,Table 2). The plasma FFA concentrations were higher in the fasted state before all four GXT compared to the parallel GXT in the fed

state (Fed 1 vs. Fast 1, $p<0.05$, Fed 2 vs. Fast 2, $p<0.0005$, Fed 3 vs. Fast 3, $p<0.0001$, Fed 4 vs. Fast 4, $p<0.0001$). Plasma glycerol concentrations did not change in the fed state before any GXT. In the fasted condition, plasma glycerol concentrations were elevated in the last two GXT (fast 3rd and 4th) compared to the first GXT (fast 1st) (Fast 1 vs. Fast 3, $p<0.05$, Fast 1 vs. Fast 4, $p<0.0001$, Table 2). Between nutritional states, plasma glycerol concentrations were higher in the fasted compared to the fed state before the 2nd, 3rd and 4th test (Fed 2 vs. Fast 2, $p<0.05$, Fed 3 vs. Fast 3, $p<0.005$, Fed 4 vs. Fast 4, $p<0.0001$, Table 2). Plasma β-hydroxybutyrate concentrations were similar across all tests in the fed condition but increased in the fasted state before the 3rd test compared to both the 1st and 2nd tests (Fast 1 vs. Fast 3, $p<0.0001$, Fast 2 vs. Fast 3, $p<0.0001$, Table 2). Plasma β-hydroxybutyrate concentration in the 4th test in the fasted state was higher than all other tests in the fasted state (Fast 4 vs. Fast 1, $p<0.0001$, Fast 4 vs. Fast 2, $p<0.0001$, Fast 4 vs. Fast 3, $p<0.001$, Table 2). Plasma β-hydroxybutyrate concentrations were also higher before the 3rd and 4th test in the fasted condition compared to the fed condition (both $p<0.001$, Table 2). The plasma insulin concentrations were similar in the resting state before all tests in the fed condition. In the fasted condition plasma insulin concentrations were lower in the 3rd and 4th compared to the 1st and 2nd tests (Fast 1 vs. Fast 3, $p<0.05$, Fast 1 vs. Fast 4, $p<0.005$, Fast 2 vs. Fast 3, $p<0.05$, Fast 2 vs. Fast 4, $p<0.005$, Table 2). Plasma insulin concentrations were lower across all tests in fasted state compared to the same tests in the fed state (all, $p<0.05$, Table 2). In the fed state plasma triglyceride concentrations were higher before the 3rd and 4th tests compared to the two first tests, whereas in the fasted state no changes were observed between the tests (Fed 1 vs. Fed 3, $p<0.0001$, Fed 1 vs. Fed 4, $p<0.0001$, Fed 2 vs. Fed 3, $p<0.001$, Fed 2 vs. Fed 4, $p<0.0001$, Table 2). When the nutritional states were compared, the plasma triglyceride concentrations were significantly elevated during the 2nd, 3rd and 4th test in the fed compared to the fasted state (Fed 2 vs, Fast 2,

223 $p<0.001$, Fed 3 vs. Fast 3, $p<0.0001$, Fed 4 vs. Fast 4, $p<0.0001$, Table 2). In summary, all plasma substrates
224 (glucose, lactate, FFA, and β-hydroxybutyrate) together with insulin remained unchanged in blood samples
225 obtained at rest in the fed state. In the fasted state on the last test (Fast 4) plasma glucose concentrations were
226 lower, and plasma FFA, glycerol and β-hydroxybutyrate concentrations were all significantly elevated.

227 **Concentrations of metabolites, substrates and hormones during exercise (from rest to last completed**
228 **workload)**

229 From rest to the last completed workload (LCW) (RER≥1.0) plasma glucose concentrations decreased during the
230 4^{th} exercise test (Fed 4, Δ0.92±0.12 mmol/l, $p<0.005$, Table 2.) in the fed state. In the fasted state plasma glucose
231 concentrations were unaltered between rest and LCW during the 1^{st} test, whereas it increased during the 4^{th} test
232 from 4.7±0.4 mmol/l at rest to 5.1±0.7 mmol/l at LCW ($p<0.05$, Table 2). Plasma lactate increased significantly
233 from rest to LCW across all test and nutritional status ($p<0.05$, Table 2.) , and the increase was higher in the fasted
234 compared to the fed state in the 4^{th} exercise test ($p<0.05$, Table 2). Plasma FFA concentrations did not change
235 from rest to LCW in any of the tests in the fed state but decreased during both the 1^{st} and 4^{th} test in the fasted state
236 (Fast 1, Δ191±44 μmol/l, $p<0.05$ and Fast 4, Δ459±77 μmol/l, $p<0.001$, Table 2). The plasma glycerol
237 concentrations did not change significantly from rest to LCW in any of the fed tests, but increased markedly in the
238 fasted state in both the 1^{st} and 4^{th} test and more considerable in the latter (Fast 1, Δ56±14 μmol/l, $p<0.05$ and Fast
239 4, Δ119±30 μmol/l, $p<0.05$, Table 2.). Plasma insulin concentrations decreased significantly from rest to LCW in
240 both the first and last GXT in the fed state, (Fed 1, $p<0.05$ and Fed 4, $p<0.05$, Table 2) but not in the fasted state
241 (Fast1 and Fast 4, Table 2). Plasma β-hydroxybutyrate concentration remained low and unchanged in the fed state

242 and the first GXT test in the fasted state but decreased from rest to LCW in the last fasted GXT (Fast 4, $p<0.01$,
243 Table 2). Plasma TG concentrations increased from rest to LCW in both the first and the last GXT in both
244 nutritional states (Fed 1, $p<0.05$, Fed 4, $p<0.005$, Fast 1, $p<0.05$, Fast 4, $p<0.05$, Table 2) , but the concentrations
245 of plasma TG remained lower in the fasted state compared to the fed state in the last GXT (Fast 4 vs. Fed 4,
246 $p<0.0001$, Table 2) at the LCW.

247

248 Concentrations of FFA in plasma were higher across all workloads in the first fasted test compared to the first test
249 in the fed state (Figure 3A). Before the final exercise test in the fasted state (Fast 4, Figure 3B) plasma FFA
250 concentrations were more than 6-fold higher compared to the fed state (Fed 4, Figure 3B). Under both incremental
251 exercise tests in the fed state where blood samples during work were collected, plasma FFA concentrations
252 remained unaltered throughout. However, in the fasted conditions plasma FFA concentrations were lower at
253 workload 235 and 270 watts in the first test compared to the initial 95 watts workload. During the last fasted test
254 (Fast 4) plasma FFA was lower from 165 watts and the rest of the test compared to the initial workload. Plasma
255 glycerol increased with increasing exercise intensities and was higher at the last exercise step (270 watts) across
256 all exercise tests. In the initial tests (Fed1/Fast 1) plasma glycerol concentrations were higher in the fasted state
257 compared to the fed state. In the final tests (Fed4/Fast 4) plasma glycerol concentrations were higher in the fasted
258 state across all workloads (Figure 3C and D). Plasma glucose concentrations were higher in the fasted test 1 from
259 165 watts and the remaining workloads compared to Fed 1 test. Plasma glucose concentrations were additionally
260 higher in the Fast 4 test compare to Fed 4 test from 200 watts and the remaining workloads. The plasma glucose

concentrations were lower at 200 and 235 watts compared to 95 watts in the first fed test. In the Fed 4 test plasma glucose concentrations were lower from 165 watts and onwards compared to the first workload (Figure 3E and F).

When resting plasma FFA concentrations were plotted against PFO rates a strong linear relationship (r^2=0.73) p<0.0001) was seen despite adjusting for the dependency of the previous GXT of the same day (Figure 4). The significant estimated slope of 0.00049 between plasma FFA concentrations and PFO signifies that a 1 µmol/l increase in plasma FFA concentrations amounts to a 0.0005 g/min increase in PFO i.e. 1000 µmol/l increase in plasma FFA concentration may result in 0.5 g/min in PFO.

Discussion

Main findings

The main finding of the present study is that peak fat oxidation rate measured after an overnight fast is indeed not a maximal fat oxidation rate, as often stated, and that interpretation and application towards specific metabolic conditions requires careful standardization. Our data furthermore clearly reinforces the major regulatory importance of exogenous plasma FFA availability for substrate partitioning during exercise hightlighted by the strong linear relationship between plasma FFA concentrations and peak fat oxidation rate..

The relationship between fat oxidation and substrate availability

Studies applying stable isotope tracer methodology have elegantly shown that plasma FFA is a significant contributor to whole-body fat oxidation rates during exercise (17, 25). At rest and during low-intensity exercise (25-40% of $\dot{V}O_2$max) the majority (≈100-57%) of fatty acids contributing to fat oxidation is derived from the plasma FFA pool, and with increasing exercise intensities (40-85% of $\dot{V}O_2$max) approximately 50% of fatty acids are derived from IMTG and circulating VLDL (13, 17, 25). The central role of circulating plasma FFA for substrate provision was underlined by Romijn and colleagues in a study where plasma FFA was increased by intravenous infusion to concentrations between 1-2 mM during exercise at 85% of $\dot{V}O_2$max, and this resulted in a 27% increase in total fat oxidation rate. However, fat oxidation at 85% of $\dot{V}O_2$max with high plasma FFA was still somewhat lower than at 65% $\dot{V}O_2$max suggesting that other factors influence the attenuation of fat oxidation at high exercise

297 intensities. In the present study, the incremental increase in plasma FFA concentrations from 339±187 μmol/l (Fast
298 1) to 811±260 μmol/l before the last exercise test (Fast 4) can probably explain the major part of the 47% increase
299 in PFO from 0.63±0.18 g/min to 0.93±0.17 g/min. This finding is further underlined by the linear relationship
300 between fasting time and elevation in peak fat oxidation rates (Figure 2). In line with this, Montain and colleagues
301 previously found a strong inverse relationship between plasma FFA concentrations and glucose oxidation during
302 exercise (18).

303 Montain and colleagues manipulated plasma substrate and insulin concentrations by altering the time since last
304 meal and they also found a gradual decrease in plasma insulin concentrations in relation to the length of the fasting
305 period. This is in agreement with the finding of the present study, when comparing the two 1st incremental exercise
306 test under different nutritional states (Fed 1 vs. Fast 1, respectively). When subjects ingested a carbohydrate-rich
307 breakfast 3 hours prior to the baseline blood sample, plasma insulin concentrations were significantly higher
308 (57±49 vs. 32±19 μmol/l, $p<0.001$) compared to after an overnight fast which were also reflected in the plasma
309 FFA concentrations which were significantly suppressed in the fed state (148±88 vs. 339±187μmol/l). Although
310 no other differences in plasma metabolites (glucose, lactate, β-hydroxybutyrate, and triglycerides) peak fat
311 oxidation rates were borderline different between Fed 1 and Fast 1 (0.54±0.16 vs. 0.63±0.18 g/min, $p=0.053$,
312 respectively). The near significant relationship can be due to the relatively low number of participants (n=10). The
313 close association between plasma FFA availability and substrate oxidation becomes evident when plotting the 80
314 resting plasma FFA samples against PFO measured immediately after and despite statistically correcting for the
315 dependency due to individuals performing multiple tests we find a strong relationship between PFO and plasma

316 FFA (r^2=0.73, p<0.0001) (Figure 4). This finding highlights that in highly trained men, one of the major
317 determinants for the rate of fat oxidation in the fasted state during incremental exercise tests is the availability of
318 plasma free fatty acids. A number of studies have previously investigated how manipulating plasma FFA
319 concentrations affects whole-body fat oxidation (15, 27, 28). In accordance with the findings of this present study
320 they in general find that increased plasma FFA concentrations increase whole-body fat oxidation and vice versa.
321 The standardized meals resulted in constant plasma insulin and suppressed FFA concentration throughout the fed
322 day and compared to the tests on the fasted day. This is in line with a previous study in which well-trained men
323 ate a carbohydrate-rich meal 4 hours before moderate intensity continuous exercise (105 minutes, 70% of $\dot{V}O_2$max)
324 which resulted in a significant suppression of both plasma glycerol and FFA concentrations despite a normalization
325 of plasma insulin and glucose concentrations compared to an overnight fast. Horowitz and co-workers furthermore
326 found that glucose ingestion one hour prior to low/moderate intensity continuous exercise decreases fat oxidation
327 and that co-infusion of intra-lipids did not re-establish fat oxidation rate to the overnight fasting levels [15]. This
328 implies that rate of lipolysis is an important factor for fat oxidation in the following exercise and this finding by
329 Horowitz and colleagues is in line with the finding of the present study where plasma glycerol concentrations (a
330 surrogate marker for the rate of lipolysis) were similarly suppressed through each exercise test in the fed state,
331 opposite to the continuous increase observed throughout each exercise test on the fasted day. In summary, we find
332 that whole-body fat oxidation rate is closely tied with substrate availability in both the fed and the fasted state. It
333 should however be considered that plasma FFA is only one factor out of several possible altered with fasting.
334 Intramuscular lipolysis could also be affected by insulin and cortisol concentrations thus adding to the variation in
335 whole-body fat oxidation observed with different nutritional states.

Plasma substrate alterations during exercise

In 1977 Costill and colleagues observed that elevated plasma FFA (1.01 mmol/l) 5 hours after the combination of heparin infusion and a high-fat meal resulted in a 44% decrease in plasma FFA concentrations during 30 minutes of continuous exercise at 70% of $\dot{V}O_2$max in well-trained young men (8). This is in line with major decrease in plasma FA during the 4th graded exercise test in the fasted states in this study. It can be contemplated that this decline in plasma FFA concentrations is a result of a high rate of adipose tissue lipolysis in the resting state and when exercise intensity is increased, and the FFA uptake to the working skeletal muscle increases above resting concentrations adipose tissue lipolysis is insufficient to maintain plasma FFA concentrations constant. Our findings imply an extensive skeletal muscle uptake of plasma FFA during incremental exercise after app. 22 hours of fasting, which is presumably contributing to the marked increase in whole-body fat oxidation. Contrarily, plasma glucose concentration was more stable in the fasted state compared to the fed state, which may be an effect of decreased skeletal plasma glucose uptake, despite three previous exercise bouts in the fasted state (Figure 1, Table 2).

Maximal fat oxidation rate or peak fat oxidation rate?

The findings of the present study highlight the importance of careful pre-exercise standardization with regards to the length of the fasting period and time since the previous exercise. Based on our findings it may be argued that whole body fat oxidation rates measured in most cases after an overnight fast indeed does not represent and calculate a whole-body maximal fat oxidation rate but a whole-body peak fat oxidation rate within the context of

355 the pre-exercise standardization obtained in the study design. Even with the findings of the present study it would
356 be questionable to label the whole-body fat oxidation measured after > 20 hours of fast and 4 previous incremental
357 exercise bouts maximal as the linearity of the increase in fat oxidation does not seem to level off, as would be
358 expected if the absolute whole-body maximal fat oxidation was reached. Previous studies conducted in so-called
359 "fat-adapted athletes", athletes adapted to a chronic very low carbohydrate diet have found peak fat oxidation rates
360 on average as high as 1.54 g/min with a single subject eliciting a peak fat oxidation rate of 1.74 g/min during
361 graded exercise on a treadmill, indicating that whole body fat oxidation capacity is dynamic and highly adaptable
362 (32). Whether the capacity to oxidize fat is equally high in athletes not chronically adapted to a high-fat diet after
363 prolonged fasting remains unanswered.

364

365 **Limitations**

366 As we did not obtain skeletal muscle biopsies, it remains unclear to what extent the combination of fasting and
367 prior exercise depleted skeletal muscle glycogen stores and how they may have been restored by the CHO-rich
368 meal and 3½ hours of rest. Subjects were weighed in the morning before the first exercise bout and immediately
369 after the last exercise bout and in the evening. A significant 1.5 kg body weight loss on the fasted day and an
370 insignificant 0.3 kg body weight loss on the fed day (nutritional state x time $p=0.026$) was present. This could
371 indicate that skeletal muscle substrate stores were not degraded during the fed trial. As the subjects were
372 encouraged to drink sufficiently on both days the body weight loss on the fasted days could be an effect of a net
373 skeletal muscle glycogen breakdown. Aditionally we did not measure protein oxidation which potentially could

influence data in particularly in the fasted state. We assume that protein oxidation is negligible and constant which most likely is the case under short term fasting and in the fed state, however to what extent protein oxidation is significantly increased during a GXT after 20 hours of fast is uncertain, but would be worthwile to investigate further.

Conclusion

Peak fat oxidation increased in close relationship with plasma FFA availability after combined fasting and repeated incremental exercise tests in healthy highly-trained men. Carbohydrate-rich meals 3½ hours before repeated incremental exercise bouts abolished the increase in PFO and plasma FFA concentrations observed when fasted, implying that the previous exercise per se did not cause the increase *in* PFO. These findings underline the importance of managing the fasting period rigorously in subjects undergoing incremental exercise tests aiming to establish substrate utilization including peak fat oxidation rate. Furthermore, these findings strongly enforce the major importance of exogenous plasma FFA as a key substrate source for fat oxidation during exercise in highly-trained men.

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Conflict of Interest:

The Authors declare no conflict of interest

Figure 1:

General study design and timeline

Figure 1: DXA-scan (dual-energy X-ray absorptiometry) Fed and Fast trials were conducted in randomized order

Figure 2.

Individual peak fat oxidation rates during the graded exercise tests

Figure 2. Individual (black circle) and mean (box) peak fat oxidation rates during four consecutive exercise tests in the fed state (A) and the fasted state (B). (1,2,3) shows a significant difference from the number in the bracket within the nutritional state. * shows differences between the same incremental exercise but between different nutritional states. Data are shown as mean±SD, (n=10).

Figure 3.

Plasma substrate concentrations during the graded exercise tests

Figure 3. Plasma concentrations during exercise the first (Fed 1, Fast 1) (A, C, E.) and last (Fed 4, Fast 4) (B, D, F) incremental exercise test. Filled circles and lines=fed state, open circles and dashed line=fasted state. FFA=Free Fatty Acids. * shows differences between the same incremental exercise but between different nutritional states. † shows difference from 95W within the same GXT. The number of subjects remaining: Fed 1: n= 9, 9, 8, 8, 7 and 6 at 95, 130, 165, 200, 235 and 270 Watts, respectively. In Fed 4: n=9, 9, 9, 9, 6, 4, in Fast 1, n=9, 9, 9, 9, 9, 6 and Fast 4: n=9, 9, 9, 9, 9, 8 at 95, 130, 165, 200, 235 and 270 Watts respectively. Data are shown as mean±SD

Figure 4.

Relationship between peak fat oxidation rates and plasma FFA concentrations

Figure 4. Linear regression plot of plasma FFA (Free Fatty Acids) at rest with PFO (peak fat oxidation) as the dependent variable of all 80 incremental exercise tests

Table 1.

Participant characteristics

Table:1 Anthropometric data of the participants, BMI= Body Mass Index, Mean±SD, (n=10)

Table 2.

Plasma concentrations at rest and after last completed workload

Table 2. Plasma concentrations during rest and immediately after the last completed workload (LCW). FFA = Free Fatty Acids, TG=triglycerides, (1, 2..) shows a significant difference from the GXT number in the bracket within the nutritional state, * shows difference between the same GXT number but between different nutritional states. † shows difference from Rest to LCW within the same GXT. Data are shown as mean±SD

453

454 **Table 1.**

Variable	*Mean±SD*
Age (yrs)	31±6
Height (cm)	183±4
BMI (kg/m²)	23±2
Weight (kg)	77.7±7.5
Lean body mass (kg)	65.5±5.8
Fat (%)	12.6±3.6
$\dot{V}O_2$ *(ml/min)*	5079±595
$\dot{V}O_2$*peak (ml/min/kg)*	65.9±6.1

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Table 2.

n=10	*Fed 1*		*Fed 2*	*Fed 3*	*Fed 4*	
Variable:	*Rest*	*LCW*	*Rest*	*Rest*	*Rest*	*LCW*
Glucose (mmol/l)	5.1±0.9	4.7±0.8	5.1±0.6	5.3±0.5	5.3±0.4	4.4±0.5 †
Lactate (mmol/l)	1.1±0.2	3.1±1.8 †	1.1±0.3	1.0±0.2	0.9±0.2	2.3±0.9 †
FFA (µmol/l)	148±88	118±62	87±62	99±69	125±116	126±90
Glycerol (µmol/l)	25±10	64±39	18±12	17±12	21±14	56±57
TG (mmol/l)	0.7±0.3	0.9±0.3 †	0.8±0.3	1.0±0.3 (1,2)	1.1±0.3 (1,2)	1.3±0.3†
β-hydroxybutyrate (mmol/l)	0.03±0.02	0.03±0.02	0.02±0.02	0.03±0.02	0.04±0.06	0.03±0.02
Insulin (µmol/l)	57±49	18±18 †	49±43	38±31	43±42	14±10 †
	Fast 1		*Fast 2*	*Fast 3*	*Fast 4*	
	Rest	*LCW*	*Rest*	*Rest*	*Rest*	*LCW*
Glucose (mmol/l)	5.4±0.4	5.2±0.7	5.1±0.3	4.9±0.4	4.7±0.4 * (1)	5.1±0.7 * †
Lactate (mmol/l)	0.9±0.2	3.9±1.8 †	0.9±0.4	0.8±0.3 *	1.0±0.3	4.0±1.5* †
FFA (µmol/l)	339±187 *	148±78 †	405±232 *	688±286 * (1,2)	811±261 * (1,2)	351±104 * †
Glycerol (µmol/l)	31±19	87±56 †	41±25 *	64±48 * (1)	87±49 * (1,2)	206±108 * †
TG (mmol/l)	0.7±0.1	0.8±0.2 †	0.6±0.2*	0.7±0.3 *	0.6±0.2 *	0.7±0.1 * †
β-hydroxybutyrate (mmol/l)	0.10±0.08	0.06±0.03	0.12±0.11	0.28±0.15* (1,2)	0.41±0.23 * (1,2,3)	0.22±0.14 * †
Insulin (µmol/l)	32±19 *	22±17	26±18 *	22±15 * (1,2)	17±14 * (1,2)	9±6

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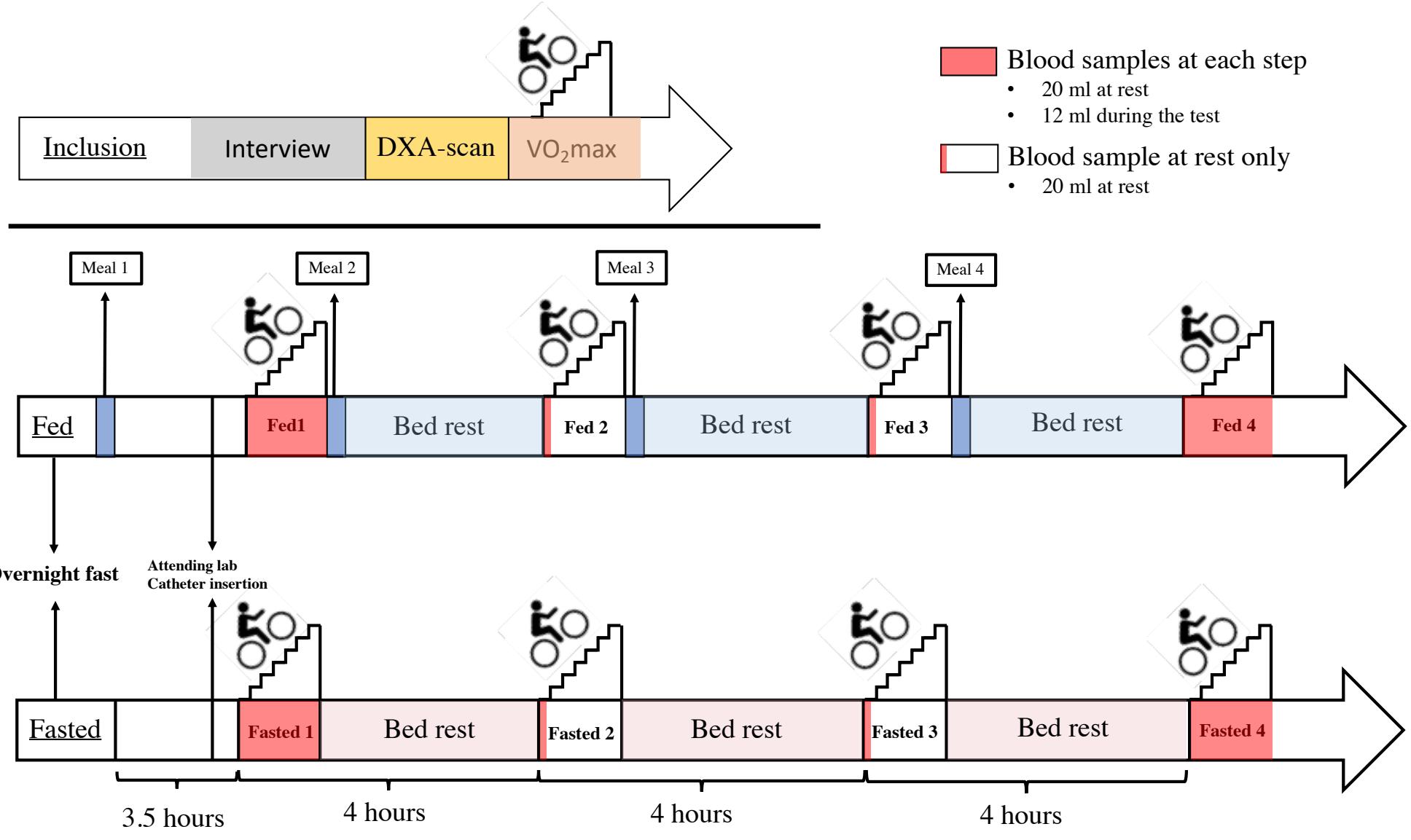
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Blood sample at rest only
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Inclusion
Interview
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Meal 3
Meal 4
Fed
Fed1
Bed rest
Fed 2
Bed rest
Fed 3
Bed rest
Fed 4
Overnight fast
Attending lab
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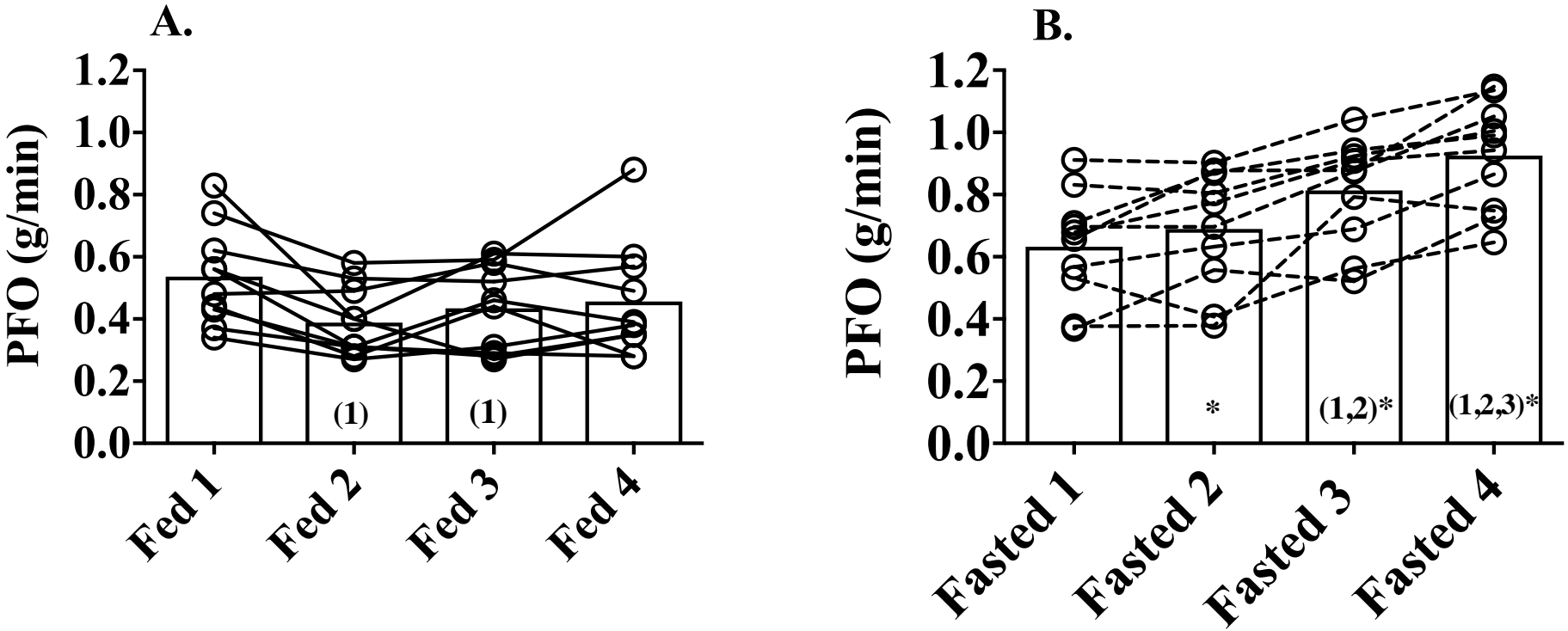
4 hours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PFO (g/min)
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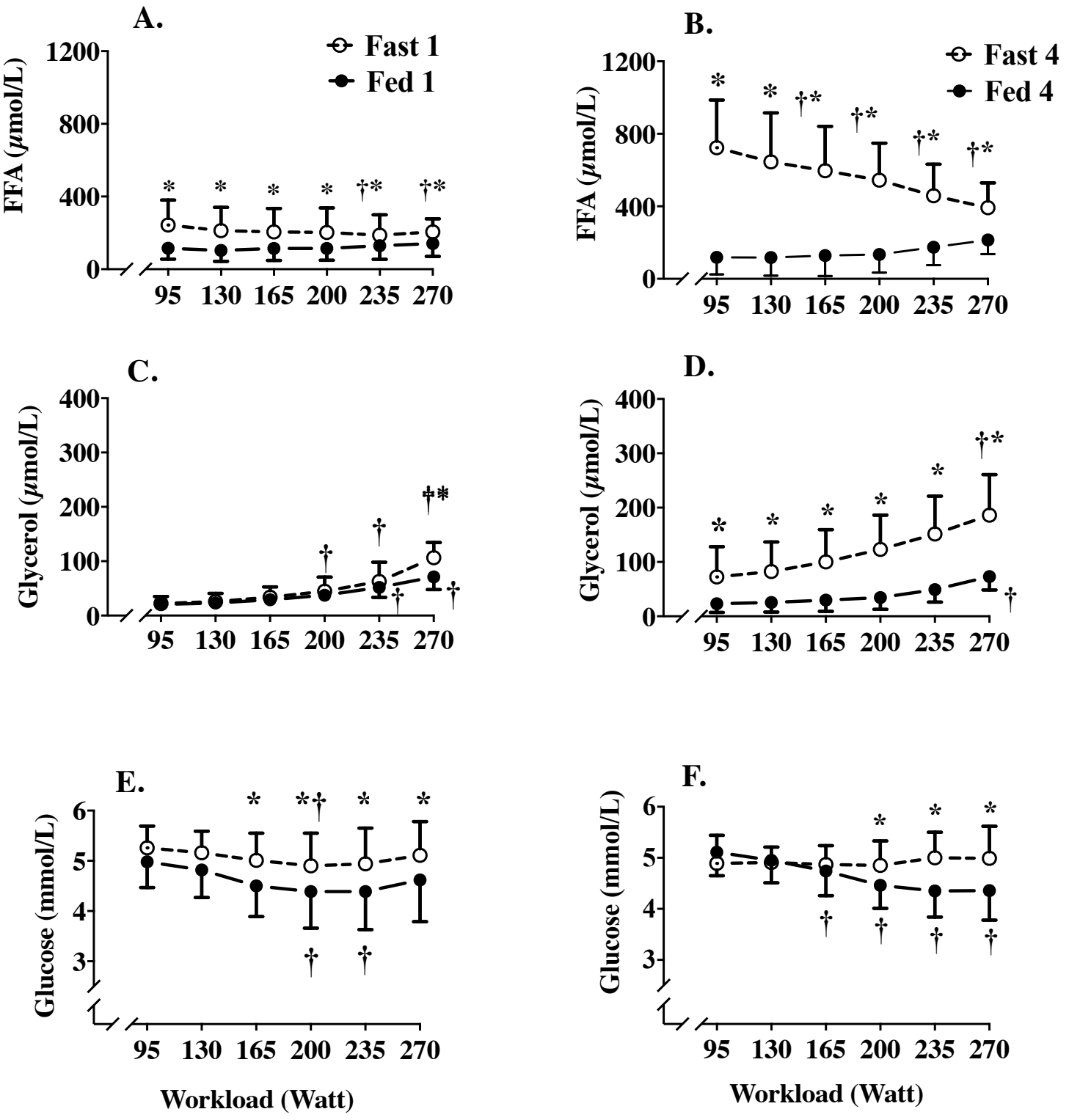
1.0
0.8
0.6
0.4
0.2
0.0
(1)
(1)
Fed 1
Fed 2
Fed 3
Fed 4
B.
PFO (g/min)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
(1,2)*
(1,2,3)*
Fasted 1
Fasted 2
Fasted 3
Fasted 4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Fast 1
Fed 1
FFA (μmol/L)
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800
400
0
95 130 165 200 235 270
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Fast 4
Fed 4
FFA (μmol/L)
C.
Glycerol (μmol/L)
400
300
200
100
D.
Glycerol (μmol/L)
E.
Glucose (mmol/L)
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5
4
3
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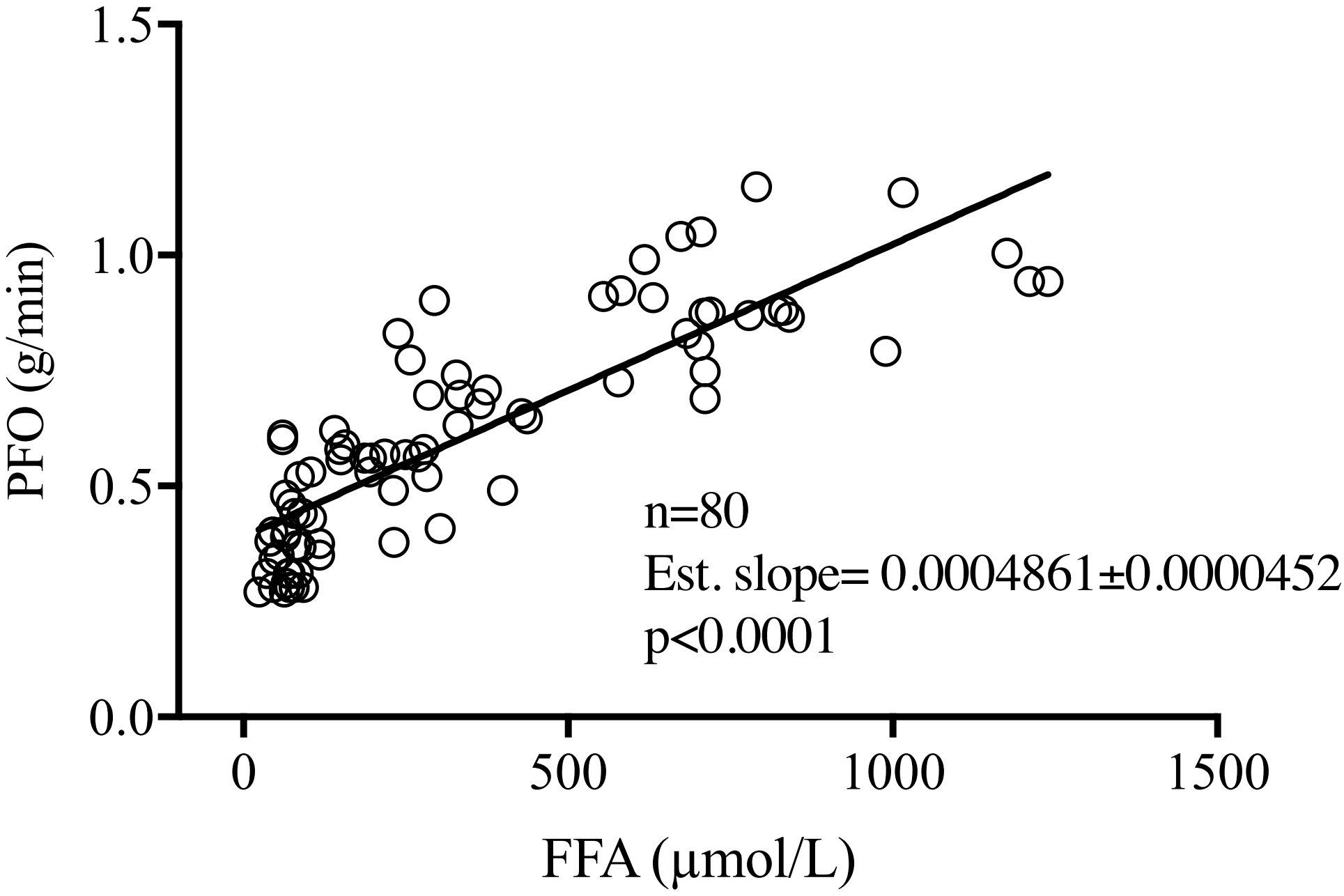
F.
Glucose (mmol/L)
Workload (Watt)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