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Original article

The impact of prolonged fasting on 24h energy metabolism and its 24h rhythmicity in healthy, lean males: A randomized cross-over trial

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SUMMARY

Objective: Human energy expenditure and substrate oxidation are under circadian control and food intake is a time cue for the human biological clock, leading to 24h feeding–fasting cycles in energy and substrate metabolism. In recent years, (intermittent) fasting protocols have also become popular to improve metabolic health. Here, we aimed to investigate the impact of food intake on the 24h patterns of energy metabolism as well as to provide data on the timeline of changes in energy metabolism that occur upon an extended period of fasting.

Research design and methods: In a randomized, cross-over design, twelve healthy males underwent a 60h fast which was compared to a 60h fed condition. In the fed condition meals were provided at energy balance throughout the study. Conditions were separated by a two week period of habitual diet. Volunteers resided in a respiration chamber for the entire 60h to measure energy expenditure and substrate oxidation hour by hour. Volunteers performed a standardized activity protocol while in the chamber. Blood samples were drawn after 12, 36 and 60h.

Results: Immediately following the breakfast meal (in the fed condition), fat oxidation became higher in the fasted condition compared to the fed condition and remained elevated throughout the study period. The initial rapid increase in fat oxidation corresponded with a decline in the hepatokine activin A ($r = -0.86$, $p = 0.001$). The contribution of fat oxidation to total energy expenditure gradually increased with extended abstinence from food, peaking after 51h of fasting at 160 mg/min. Carbohydrate oxidation stabilized at a low level during the second day of fasting and averaged around 60 mg/min with only modest elevations in response to physical activity. Although 24h energy expenditure was significantly lower with prolonged fasting (11.0 ± 0.4 vs 9.8 ± 0.2 and 10.9 ± 0.3 vs 10.3 ± 0.3 MJ in fed vs fasting, day 2 and 3 respectively, $p < 0.01$), the 24h fluctuations in energy expenditure were comparable between the fasted and fed condition. The fluctuations in substrate oxidation were, however, significantly ($p < 0.001$ for both carbohydrate and fat oxidation) altered in the fasted state, favouring fat oxidation.

Conclusions: Energy expenditure displays a day–night rhythm, which is independent of food intake. In contrast, the day–night rhythm of both carbohydrate and fat oxidation is mainly driven by food intake. Upon extended fasting, the absolute rate of fat oxidation rapidly increases and keeps increasing during a 60h fast, whereas carbohydrate oxidation becomes progressively diminished.

Trial registration: www.trialregister.nl NTR 2042.

1. Introduction

Over the course of history, humans have endured periods of famine. Famine has been thought to occur regularly during the hunter and gatherer period (~40 000 years ago), but also within sedentary agricultural societies (10 000–12 000 years ago) [1,2].

Abbreviations: BHB, beta-hydroxybutyrate; En%, percentage of energy; RER, respiratory exchange ratio; SMR, sleeping metabolic rate.

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According to the thrifty genotype hypothesis [3], the capacity to adapt to periods of famine used to be advantageous in earlier days, but can explain why certain populations are prone to obesity and diabetes under current, modern lifestyle with excess of food. At present, deliberately fasting between sunrise and sunset for ~30 days for religious purposes is still common in the Islamic society as part of the Ramadan [4]. The duration of a daily fast can amount up to 16–20h, depending on the location of the country relative to the equator. Earlier studies in healthy, lean individuals have shown that acutely fasting for ≥60h results in an increase in fat oxidation and lowers glucose levels [5–7], which can be favourable for metabolic health. Importantly, repeatedly refraining from food intake for a shorter, more feasible amount of time, i.e. intermittent fasting (including alternate day fasting and 5:2 diets), has gained popularity as a lifestyle intervention to improve metabolic health [7]. Intermittent fasting ranges from prolonging the daily fasting time to 12–18h and can extend to full days (24h) of fasting. These fasting regimes have been shown to result in multiple metabolic health benefits, including improved insulin sensitivity [8] and weight loss [9–13], both in adults with- [9] and without type 2 diabetes [8,10–13].

During prolonged fasting, physiological mechanisms come into play to ensure that tissues which are glucose-dependent (such as the brain) can survive, while employing alternative energy sources to fuel other tissues. Effectively, this means that most energy comes from fat oxidation during fasting [5,14]. The liver plays a pivotal role in the physiological adaptation to fasting, as it is able to maintain blood glucose levels for a certain period of time via processes of glycogenolysis and gluconeogenesis. The major substrates for gluconeogenesis include lactate, glycerol, and glucogenic amino acids. In addition, the liver also produces ketone bodies, which can be used by the brain as an alternative energy source for glucose [15]. Ketone bodies are being formed from ketogenic amino acids or acetyl-CoA. The latter results from an increased β-oxidation of free fatty acids that emanate from white adipose tissue [16,17]. The generation of ketone bodies is thought to be a glucose-sparing strategy, as the glucose reserves in the body are scarce. In addition, insulin sensitivity of peripheral organs is reduced during a prolonged fast [5,18] to push the available glucose towards the glucose dependent organs. The regulation of these changes in substrate metabolism is not completely understood, but may involve fasting-regulated hormones. Indeed, several hormones involved in energy balance shift during a prolonged fast as has been reported for leptin, activin A, follistatin, FGF21 and GDF15 [19–23].

Besides the relevance of the metabolic changes that occur with fasting for surviving longer periods of famine, daily feeding and fasting cycles also align the internal ~24h rhythm of metabolic processes with the external day–night rhythm, posed upon us by the rotation of the earth. In fact, the internal day–night rhythm is a complex interplay between the internal circadian rhythm and different external time cues (*Zeitgebers*), including light, activity, and food intake. These *Zeitgebers* synchronize the internal circadian rhythm to the external 24h day. Thus, our internal circadian clock can optimize metabolic responses to the feeding–fasting cycle leading to a day–night rhythm in metabolic processes [24]. Indeed, we previously showed that healthy lean males exhibit a pronounced day–night rhythm in energy expenditure and substrate oxidation [25]. This rhythm is characterized by a higher respiratory exchange ratio (RER) during the day, indicative of glucose being the main substrate to fuel energy demand, and a lower RER during the night, indicating a more prominent role for fat oxidation. However, this day–night rhythm in energy metabolism most likely reflects food intake. As such, it would be of interest to examine the absence or presence of oscillations in energy metabolism when food intake is ceased as during a prolonged fast.

Here, we retrospectively analysed data from a study [5], in which healthy, young, lean, male volunteers fasted (vs. fed in energy balance) for 60h, while staying in a respiration chamber. We previously reported that 60h of fasting reduced insulin sensitivity and mitochondrial oxidative capacity in skeletal muscle and reported a higher 24h fat oxidation and higher level of circulating free fatty acids [5]. In the current study, we performed an hour-by-hour analysis of energy and substrate metabolism on this data with the aim to investigate the impact of food intake on the 24h patterns of metabolism as well as to provide data on the timeline of changes in energy metabolism that occur upon prolonged fasting.

2. Methods

2.1. Participants

Twelve healthy, lean, male volunteers were recruited in the vicinity of Maastricht. Volunteers did not engage in sports for more than 2h per week. Signed informed consent was obtained from volunteers before they were enrolled in the study. The study protocol was approved by the Medical Ethical Committee of Maastricht University. This study has been registered at www.trialregister.nl with registration number NTR 2042. The primary outcome of this study has been reported previously [5].

2.2. Experimental design

The current study is a secondary analysis from a previously published study [5]. The main outcome of the current study was to examine the existence of a day–night rhythm in energy metabolism in the fed vs fasted state. Research methods have been described in detail elsewhere [5]. Briefly, the study had a randomized controlled cross-over design, where volunteers were randomly assigned to start with either the 60h fed- or the 60h fasted condition, separated by a period of minimally 2 weeks during which volunteers consumed their habitual diet (Fig. 1). A standardized evening meal was provided prior to the start of each intervention. During the fasted condition, volunteers only received calorie-free drinks. During the fed condition, volunteers were provided with breakfast, lunch, dinner and a snack that had a total energy content equal to their energy expenditure. Energy expenditure was estimated using the sleeping metabolic rate measured during the first night multiplied by an activity factor of 1.5, as reported previously [26]. Half (50 En%) of total daily energy intake consisted of carbohydrates, whereas fat and protein provided 35 and 15 % of energy (En%), respectively. All meals (breakfast, lunch and dinner) were of the same macronutrient composition. After the standardized evening meal, volunteers stayed in the respiration chamber from 20.00 onwards, for a total of 60h, and performed a standardized, daily activity protocol as described previously [27]. Blood samples were taken in the morning (8 am), 12, 36, and 60h after entering the chamber and -in case of the fed condition-after an overnight fast.

2.3. Respiration chamber measurement

The respiration chamber was used to measure energy expenditure and substrate oxidation over the 60h time period. The respiration chamber is a small room with a bed, desk, chair, toilet, sink, computer, and television. It measures oxygen consumption and carbon dioxide production continuously by whole-body room indirect calorimetry (Omnical Maastricht Instruments, Maastricht, The Netherlands [28]). Sleeping metabolic rate was determined by taking the lowest 3h of energy expenditure during the night, as calculated using the Weir equation [29]. 24h energy expenditure

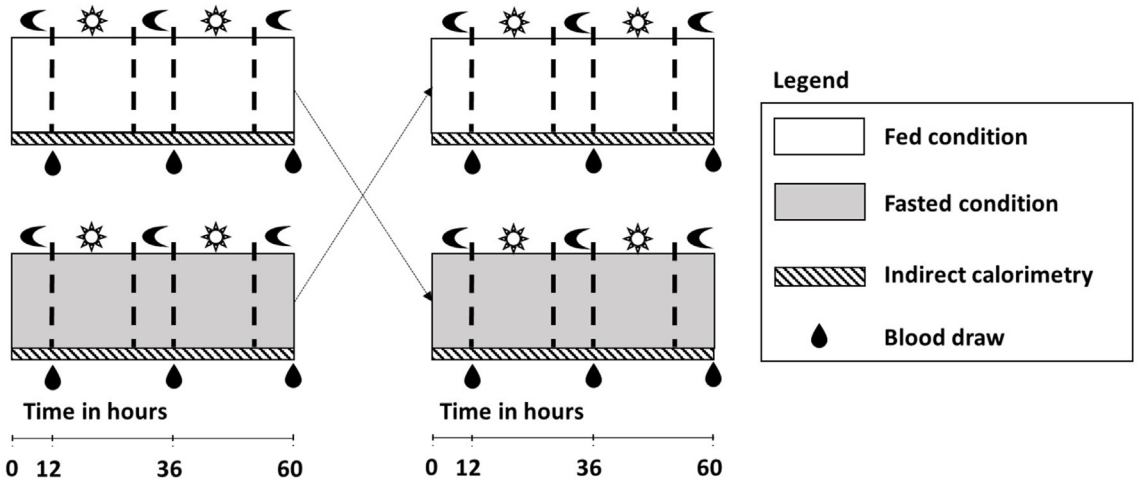

Fig. 1. Overview of the study design of the fasting study.

and substrate oxidation were calculated using the Brouwer equation [30]. Protein oxidation was determined by 24h urinary nitrogen analysis. Daytime was defined as the period in which participants were awake (from 08.00 to 00.00), whereas night time was defined as the period that participants were instructed to sleep.

2.4. Blood analyses

Plasma free fatty acids (Wako Nefa C test kit; Wako Chemicals, Neuss, Germany) and glucose (hexokinase method; Horiba, Montpellier France), were measured with enzymatic assays automated on a Cobas Pentra. Glycerol (Horiba, Montpellier, France) and beta-hydroxybutyrate (Randox, London, England) were measured with enzymatic assays automated on a Cobas Pentra. Insulin concentration was determined using a radioimmunoassay (Linco Research, St. Charles, MO). Activin A, FGF21, follistatin, and GDF15 were measured using the Quantikine ELISA kit (R&D Systems, Minneapolis, USA). For leptin analysis, an ELISA kit was purchased from Biovendor (Heidelberg, Germany).

2.5. Statistical analyses

Data are presented as mean ± SD for participant characteristics, and as mean ± SEM for intervention effects. For energy metabolism, the AUC for the entire 60h fasting period was calculated using the trapezoidal method and compared between fasted and fed condition using the paired t-test. Linear mixed models were run with random effects for volunteers and fixed effects for condition (fasted or fed), time (time of day), and condition*time to test changes in the day–night rhythm per day of fasting. To assess changes in metabolites between the fasted vs fed conditions, the linear mixed model procedure was also used with random effects for volunteers and fixed effects for condition (fasted or fed), duration (fasting duration), and condition*duration. If a significant effect of condition*duration was present, pairwise comparisons were performed to assess after how many hours of fasting the metabolic outcomes differed between the fasted vs fed condition. The Bonferroni method was used to adjust for multiple comparisons. Correlations between metabolites and substrate metabolism were tested using Spearman's correlation analysis. A two-sided p-value <0.05 was considered statistically significant. Analyses were performed using the statistical software packages SPSS 27.0 (IBM Corp, New York, USA) and Prism 9 (GraphPad Software, San Diego, USA).

3. Results

The primary results and subject characteristics of the original study have been reported earlier [5]. Briefly, 12 healthy, lean male volunteers were included (Table 1). Body weight was significantly lower at the end of the 60h fast (79.2 ± 2.9 kg) as compared to before the fast (80.3 ± 2.6 kg, $p < 0.001$), although the change in body weight was not statistically significant between fasted versus fed condition (−1.2 ± 0.2 vs −0.1 ± 0.4 kg, $p = 0.10$). In the fasted condition, a significant decrease in body weight could already be observed after 36h of fasting (weight change −0.8 ± 0.1 kg, $p < 0.001$) and body weight continued to decrease between 36h and 60h of fasting (weight change −0.4 ± 0.2 kg, $p < 0.05$).

3.1. Energy expenditure and substrate metabolism over time

To measure energy expenditure and substrate oxidation, volunteers resided in a respiration chamber for 2 consecutive days. During both days, daytime energy expenditure appeared lower in the fasted versus the fed condition, which likely reflects the lack of any thermogenic effect of food intake (Fig. 2a). The AUC of energy expenditure was on average 8 % lower in response to the 60h fast vs the fed condition (328 ± 9 vs 358 ± 11 AU, $p = 0.01$). As expected, RER was markedly lower in the fasted compared to the fed condition. The lower RER was apparent immediately after the time at which breakfast was served in the fed condition (after ~13 h of fasting) and RER remained lower throughout the fasted condition (Fig. 2b). The AUC of the RER was on average 12 % lower in the fasted condition than in the fed condition (37 ± 0.1 vs 42 ± 0.4 AU, $p < 0.001$). To examine if the lower energy expenditure and RER observed in the fasted state coincided with a lower overall activity, radar counts were plotted (Fig. 2c). During

Table 1
Participant characteristics.

Characteristic	Mean ± SD (n = 12)
Age (years)	23.6 ± 3.6
Body weight (kg)	78.5 ± 8.5
Fat free mass (kg)	65.9 ± 6.3
BMI (kg/m^2)	22.6 ± 1.6

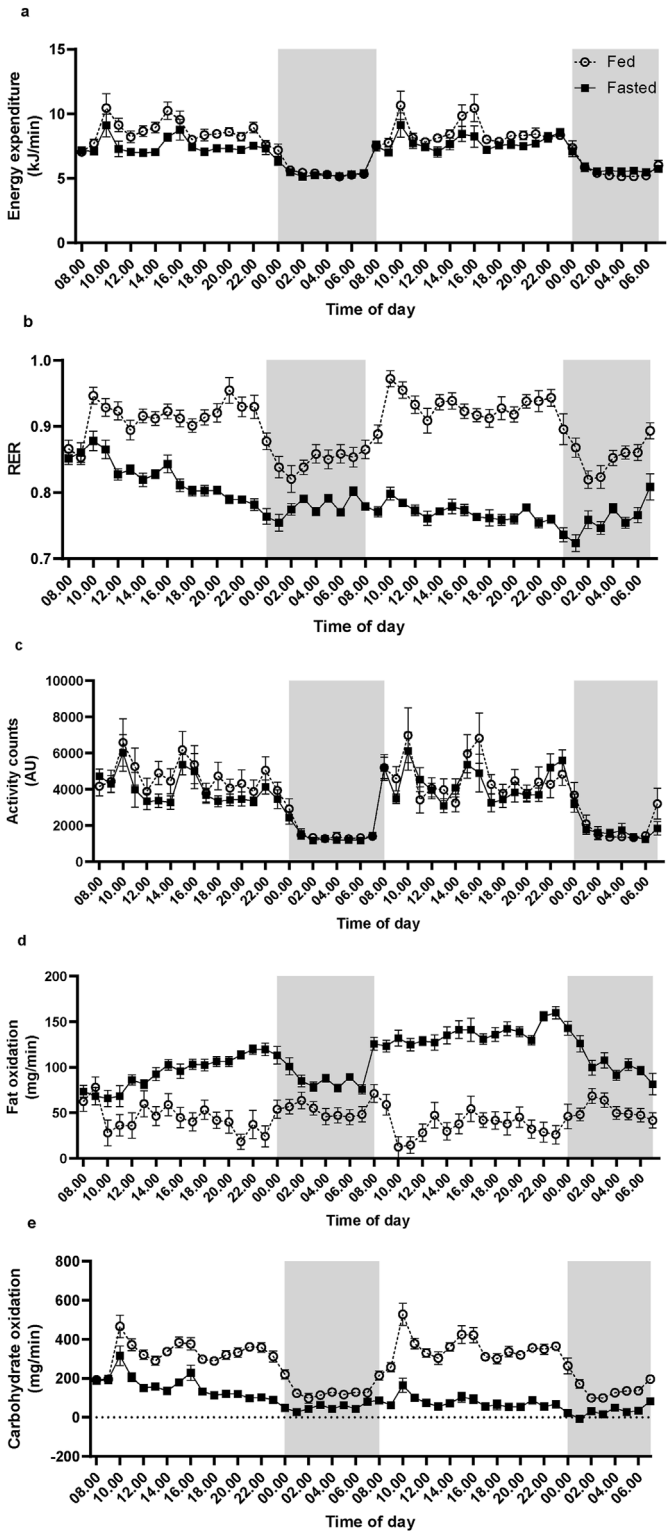

Fig. 2. Energy expenditure (a), respiratory exchange ratio (b), activity counts (c), fat oxidation (d), and carbohydrate oxidation (e) as presented for the last 48h of a 60h fasted (closed squares) or fed (open circles) condition.

daytime, activity was higher in the fed condition for most of the time, whereas activity during the night appeared identical between fasted- and fed condition. Peaks in activity could be attributed to the standardized activity protocol performed inside the chamber. During the fasted period, these peaks coincided with small peaks in RER. This observation indicates that physical activity during a prolonged fast is still able to increase carbohydrate oxidation, although the contribution of fat oxidation to total energy expenditure remains higher. Overall, the AUC calculated over the 60h fast did not show differences in activity between fasted and fed condition (156181 ± 10203 vs 173909 ± 21121 AU, $p = 1.0$).

As exemplified by the changes in RER, fat oxidation gradually increased during the 60h fast as compared to the fed condition, with highest fat oxidation rates being reached at 23:00 on the second day of fasting (after 51h of fasting) with a mean fat oxidation of 160 mg/min. For comparison, highest mean fat oxidation in the fed condition was 80 mg/min and occurred at ~08:30, around breakfast time, in both days in the chamber. The nocturnal decrease in fat oxidation in the fasted condition was mostly related to the decrease in energy expenditure. In contrast, absolute fat oxidation increased during the night in the fed condition, illustrating a typical feeding–fasting cycle. Irrespective of these temporal differences between fed and fasted, nocturnal fat oxidation remained higher in the fasted compared to the fed condition (Fig. 2d). Similarly, on average, the AUC for fat oxidation was 187 % higher for the fasted vs fed condition (5.2 ± 0.2 vs 2.1 ± 0.4, $p < 0.001$).

Carbohydrate oxidation gradually decreased when fasting time was prolonged, with only modest increases due to physical activity (Fig 2c and e). Carbohydrate oxidation reached a stable, low level during the second day of fasting which averaged around 60 mg/min. There were two nadirs in carbohydrate oxidation both occurring at ~01.00 during night 2 and 3 in the fasting condition, when carbohydrate oxidation was negligible. The AUC of carbohydrate oxidation was on average 67 % higher in the fasted condition vs the fed condition (4.3 ± 0.3 vs 12.8 ± 0.5, $p < 0.001$). Over the entire 60h fast, carbohydrate balance (i.e. total carbohydrate intake minus carbohydrate oxidation) was −266.9 ± 18.0 g which probably is related to glycogenolysis. For the first day of fasting, carbohydrate balance was −176.6 ± 10.2 g whereas it reduced to −90.3 ± 9.1 g for the second day of fasting. Fat balance on the first day of fasting was −132.9 ± 3.7 g whereas it increased to −181.3 ± 5.8 g on the second day of fasting indicating that during the prolonged fast progressively more energy was derived from fats vs carbohydrates.

3.2. 24h Energy expenditure and substrate metabolism

We previously reported substrate oxidation data on the last 24h of our study only, and found a significantly lower energy expenditure and higher fat oxidation during the last 24h of the 60h fast (36–60h of fasting) as compared to the fed condition [5]. In the current study, we analysed the respiration chamber data of the entire 60h intervention period and observed that 24h energy expenditure is already significantly decreased from 12 to 36h of fasting ($p < 0.001$, Fig. 3a). Additionally, energy expenditure between 36 and 60h of fasting was significantly lower compared to 12–36h of fasting ($p = 0.002$). Twenty-four-hour fat oxidation is already significantly higher with 12–36h of fasting (Fig. 3b) and increases significantly with 36–60h of fasting ($p < 0.001$); consequently, 24h carbohydrate oxidation during fasting is significantly lower as compared to the fed condition (Fig. 3c). Further, carbohydrate oxidation is significantly lower after 36–60h of fasting compared to 12–36h of fasting ($p < 0.001$). Interestingly, 24h protein oxidation remained comparable between fasted and fed condition, both for the 12–36h and 36–60h periods (Fig. 3d). However, it should be noted that since there is no protein intake in the fasted state, protein balance is still negative by ~75–80 g/day, implicating that the oxidized proteins have to be derived from a non-dietary source.

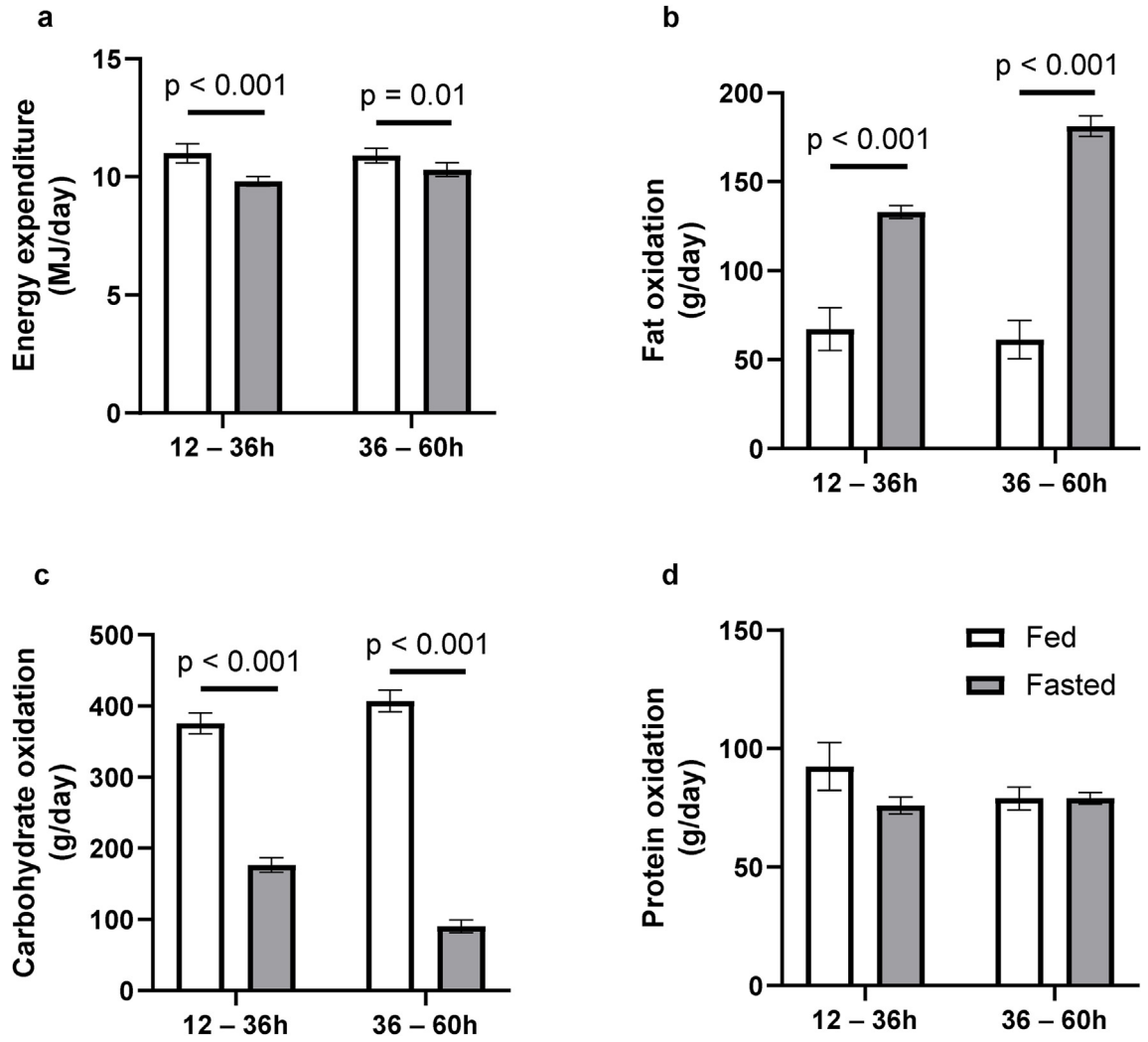

Fig. 3. 24 h Energy expenditure (a) and -substrate oxidation (b, c, d) during 12–36h and 36–60h of fasting compared to the fed control condition.

3.3. Energy expenditure and substrate oxidation during the night

When specifically analysing nocturnal energy expenditure and substrate oxidation, surprisingly, sleeping metabolic rate (SMR) was significantly higher in the fasted vs fed condition, during the third (last) night of the respiration chamber stay (Fig. 4a). To examine if this was due to increased activity during the night, we assessed the activity counts during the SMR. However, the increase in SMR was not accompanied by a higher nightly activity, as reflected by similar activity counts (Fig. 4c). The SMR during the second night in the respiration chamber was comparable between fasted and fed condition (Fig. 4a). The respiratory exchange ratio (RER) during the SMR was lower during the fasted as compared to the fed condition for both the second and the third night in the chamber (Fig. 4b).

Since previous studies have suggested that the RER increases prior to awakening [31,32], probably due to changes in cortisol levels, we examined if this increase in RER persists upon prolonged fasting and could hence reflect a circadian characteristic. For this purpose, we compared the change in RER during the last sleeping hour, just before awakening (from 06.00 to 07.00), in the two conditions. During the fasted condition, RER increased from 06.00 to 07.00 both in the second night (from 0.77 ± 0.00 to 0.80 ± 0.01, $p < 0.001$) as well as the third night (from 0.77 ± 0.01 to 0.81 ± 0.02, $p = 0.02$). During the first night of the fasted condition, the RER remained stable from 06.00 to 07.00 (from 0.84 ± 0.01 to 0.85 ± 0.01, $p = 0.33$). These changes were not accompanied by changes in energy expenditure during this period (energy expenditure: night-1: from 5.3 ± 0.1 to 5.4 ± 0.2 kJ/min, $p = 0.62$; night-2: from 5.3 ± 0.1 to 5.4 ± 0.2 kJ/min, $p = 0.40$; night-3: from 5.5 ± 0.1 to 5.7 ± 0.2, $p = 0.25$). As a control, we tested if the increase in RER was also present in the fed state. This was the case in the third night in the chamber (from 0.86 ± 0.01 to 0.89 ± 0.01, $p < 0.05$), but not in the second night (from 0.86 ± 0.01 to 0.85 ± 0.01, $p = 0.72$). Energy expenditure during the third night in the chamber tended to increase from 06.00 to 07.00 (from 5.2 ± 0.2 to 6.0 ± 0.4 kJ/min) but this did not reach statistical significance ($p = 0.06$). For the second night in the respiration chamber, energy expenditure was similar between 06.00 and 07.00 (from 5.3 ± 0.2 to 5.3 ± 0.2 kJ/min, $p = 0.35$). Therefore, the increase in RER during the third night may be explained by an increase in activity.

3.4. Day-night rhythm in energy expenditure and substrate oxidation

In a previous study, we detected a day–night rhythm in energy expenditure and substrate oxidation when healthy lean males received normal meals over the day [25]. To determine if energy

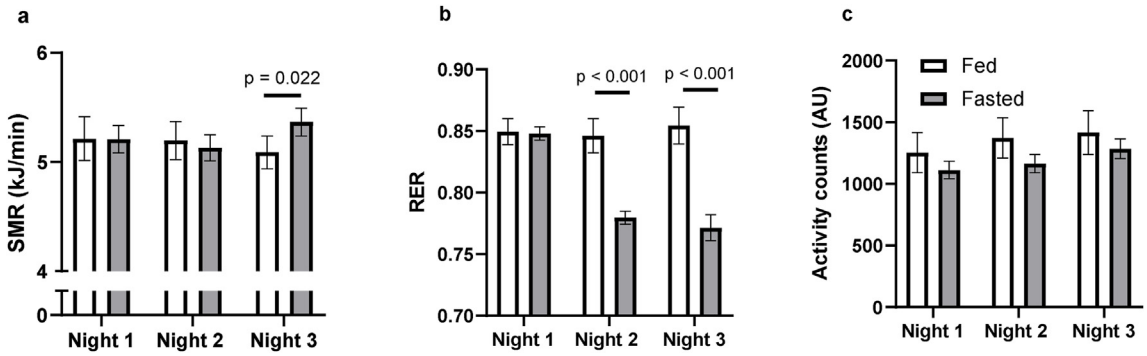

Fig. 4. Energy expenditure (a), substrate oxidation (b), and activity (c) during the night. SMR, sleeping metabolic rate; RER, respiratory exchange ratio.

expenditure and substrate oxidation display a 24h day–night rhythm, independent of food intake, we compared 24h energy metabolism profiles in the fasted and fed condition. For this analysis, we selected 5 time points evenly distributed over the course of 24h. We chose the timepoints such that they were minimally affected by the acute effects of meals in the fed condition (timepoints were selected prior to breakfast, lunch and dinner) and/or by the standardized, low-intensity physical activities (before stepping activity) that were scheduled at fixed time points, as described previously [25].

No interaction effect of condition*time was found for energy expenditure, neither for the first (12–36h, Fig. 5, $p = 0.27$) nor the second (36–60h, $p = 0.10$) 24h period of fasting, indicating that the rhythm in energy expenditure was similar between the fasted and -fed condition. Visual inspection of Fig. 2 shows that the rhythm in energy expenditure follows the rhythm in activity counts, and hence is driven by changes in physical activity. Interaction effects of condition*time were found for respiratory exchange ratio (at both 12–36h and 36–60 h, $p < 0.001$), carbohydrate oxidation (at both 12–36h and 36–60h, $p < 0.001$) and for fat oxidation (at both 12–36h and 36–60h, $p < 0.001$), suggesting that substrate oxidation is mainly driven by food intake. Visual inspection (Fig. 5) indicated that the rhythm in energy expenditure closely follows the rhythm in carbohydrate oxidation in the fed state, indicating that the energy demand is mainly fuelled by carbohydrate oxidation, probably emanating from dietary carbohydrates. In the fasted state, the day–night rhythm of energy expenditure is paralleled by the day–night rhythm of fat oxidation.

3.5. Changes in metabolites in response to fasting

The physiological processes in the body that occur during fasting also affect circulating levels of metabolites. Previously we have shown that the 60h fast compared to a 60h fed condition increased levels of FFA (1873.4 ± 100.4 vs 398.9 ± 55.5 umol/l, $p < 0.001$), and decreased levels of glucose (3.7 ± 0.1 vs 4.7 ± 0.1 mmol/l, $p < 0.001$) and insulin (6.9 ± 0.7 vs 12.2 ± 1.0 uU/ml, $p = 0.001$) [5]. Here, we additionally assessed levels of the ketone body beta-hydroxybutyrate (BHB) and of the gluconeogenic substrate glycerol since these metabolites are related to the glucose-preserving response during a prolonged fast. Furthermore, we also tested the effect of prolonged fasting on levels of hormones that are related to caloric deprivation including leptin, activin A, FGF21, follistatin and GDF15.

Linear mixed models showed a significant interaction of condition*duration ($p < 0.001$) for BHB. Further inspection showed that BHB was significantly higher after 36- and 60h of fasting compared to the fed condition (Fig. 6a, $p = 0.03$ and $p < 0.001$). Furthermore, BHB levels were significantly higher after 60h fasting compared to 36h fasting ($p < 0.001$). An interaction of condition*duration was also found for levels of glycerol ($p < 0.017$). Glycerol levels were also higher with fasting for 36- and 60 h vs the fed condition (Fig. 6b, $p = 0.04$ and $p = 0.01$). Furthermore, glycerol levels after 60h fasting were significantly higher compared to 36h of fasting ($p = 0.01$).

There was also an interaction effect of condition*duration for leptin levels ($p = 0.002$). Leptin levels were numerically lower after 36h fasting and significantly lower after 60h of fasting compared to the fed state (Fig. 6c, $p = 0.06$ and $p = 0.03$). In addition, leptin levels were significantly lower after 60h of fasting compared to 36h of fasting ($p = 0.047$). For activin A levels, a significant interaction between condition*duration ($p = 0.001$) was also observed. After 36- and 60h of fasting levels of activin A were significantly lower in the fasted condition compared to the fed condition (Fig. 6d, $p < 0.001$ after both 36 and 60h of fasting). Activin A levels were similar after 36 and 60h fasting ($p = 0.18$)

An interaction effect between condition*duration ($p = 0.007$) was also found for FGF21 levels. However, further inspection of the data did not show significant differences between the fed and fasted condition neither after 36h or 60h fasting (Fig. 6e). Follistatin levels also showed a significant interaction between condition*duration ($p = 0.001$). Thus, follistatin levels were higher in the fasted condition compared to the fed condition after both 36h of fasting and 60h of fasting (Fig. 6f, $p = 0.006$ and $p < 0.001$). Finally, for the levels of GDF15 no interaction was observed between condition*duration ($p = 0.29$), but a significant effect was observed for condition ($p = 0.022$). Levels of GDF15 were comparable between the fasted and fed condition after 36h of fasting, whereas levels were higher in the fasted condition after 60h of fasting (Fig. 6g).

As an exploratory outcome, we first assessed whether the changes in the measured metabolites were related to each other. We found that the change in follistatin levels from 12 to 36h of fasting correlated with changes in FGF21 (Fig. 6h, $p = 0.026$) and changes in GDF15 (Fig. 6i, $p = 0.001$). Finally, the increase in BHB between 12 and 36h of fasted correlated to changes in FGF21 ($r = 0.74$, $p = 0.009$), follistatin ($r = 0.74$, $p = 0.009$), and GDF15 ($r = 0.79$, $p = 0.004$) during this fasting period. The increase in BHB between 36 and 60h fasting correlated with the change in leptin ($r = -0.65$, $p = 0.042$) and the change in follistatin ($r = -0.67$, $p = 0.017$).

Furthermore, we assessed whether the changes in Activin A, FGF21, follistatin, GDF15 and leptin were related to the changes in energy metabolism during the prolonged fast. The change in activin

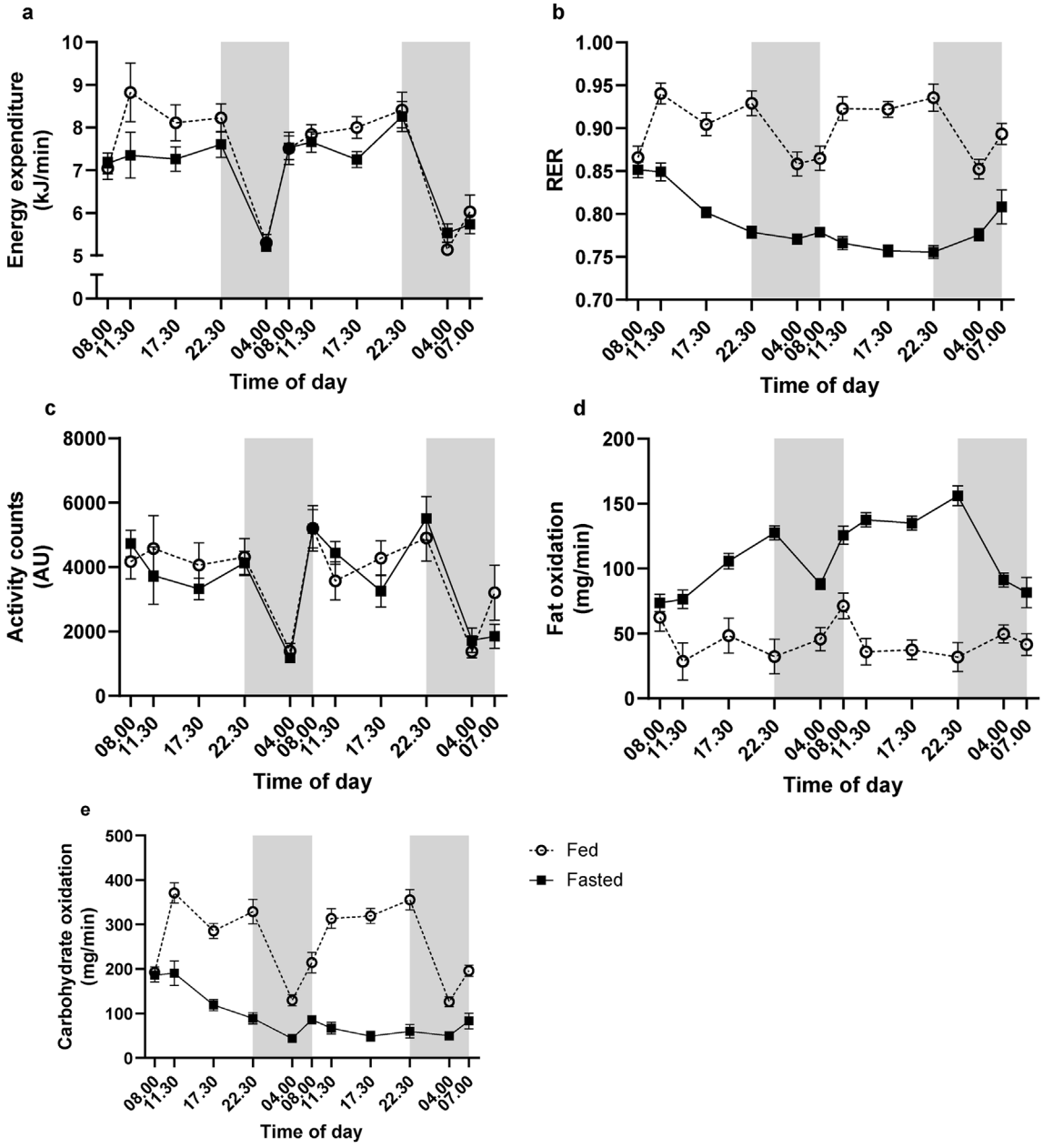

Fig. 5. Day-night rhythm of energy expenditure, level of physical activity, and substrate oxidation for the last 48h of a 60h fasted condition (closed squares) and during the fed (open circles) condition. Five timepoints were selected such that they were minimally affected by the acute effects of meals in fed condition (timepoints were selected before breakfast, lunch and dinner) and/or by the standardized, low-intensity physical activities (before stepping activity). Data are the averages of 60 min per time point.

A from 12 to 36 h of fasting correlated with 24-h fat oxidation during this time period (Fig. 6j, $p = 0.001$). No other significant correlations were observed.

4. Discussion

The metabolic response to fasting is essential for human survival in periods of food scarcity, and fasting for intermittent time periods still serves an important role in religion and in a healthy lifestyle. Previously, we showed that whole-body fat oxidation rates profoundly increase after 60 h of fasting. This increase was accompanied by a reduction in skeletal muscle mitochondrial capacity and insulin sensitivity [5]. In the current study, we retrospectively analysed data from this study and report on the temporal changes in energy expenditure and substrate oxidation during such a prolonged fast. Moreover, since substrate availability is an important time cue for the endogenous day–night rhythm, we examined changes in rhythmicity in energy expenditure and substrate oxidation in absence of food intake, i.e. in response to a prolonged fast. Here we report an early switch (around ~15 h of fasting) from carbohydrate- to fat oxidation during the fast, whereas alternative energy sources were increasingly being used as fasting duration progressed. In contrast, the 24h fluctuations in energy expenditure were similar between the fasted and fed condition.

Examination of the temporal data of energy expenditure and substrate oxidation revealed that the switch to predominantly fat

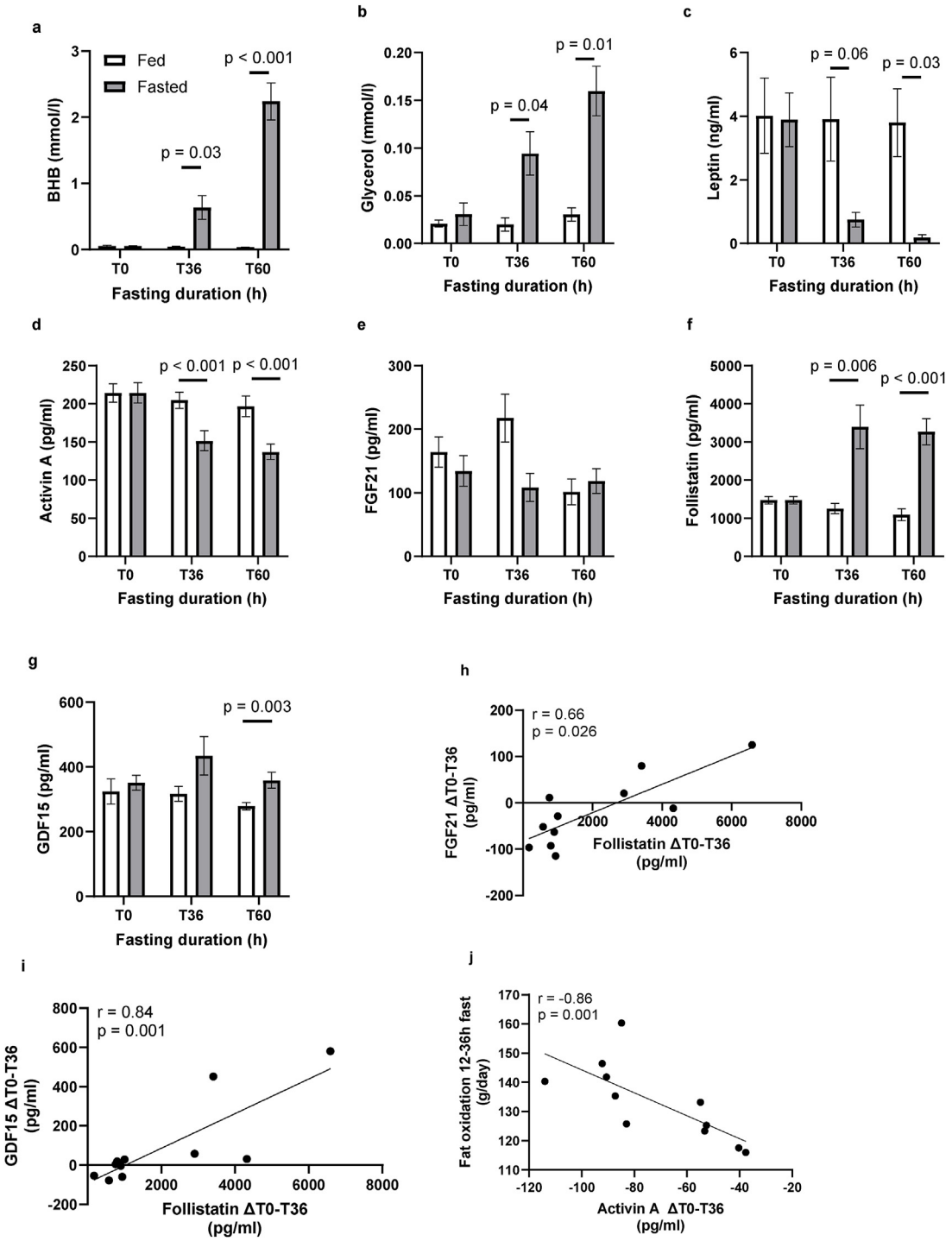

Fig. 6. Changes in levels of metabolites in response to fasting as measured at baseline (T = 0) and after 36 and 60h of fasting (T36 and T60), and compared to the fed condition. BHB: beta-hydroxybutyrate.

oxidation occurs early during prolonged fasting; by default, the RER of the fast and fed condition diverges around 09.00, when dietary carbohydrates become available for oxidation in the fed condition as a result of the breakfast and endogenous carbohydrate stores start to deplete in the fasted condition. This is further supported by the higher 24h fat oxidation and lower 24h carbohydrate oxidation already after 12–36 h of fasting, confirming previous reports [14,33,34]. Despite this early increase in fat oxidation, the contribution of fat oxidation to total energy expenditure continued to increase steadily during the 60h fast, peaking after ~50h of fasting, while the contribution of carbohydrate oxidation became increasingly lower. This change occurs due to increasing utilization and ultimately exhaustion of glycogen stores. Thus, the increase in fat oxidation preserves the limited availability of glucose for glucose-dependent tissues. Our results also showed that although carbohydrate oxidation is profoundly lowered upon prolonged fasting, a minimum level of glucose oxidation remains, at a rate of ~60 mg/min. Probably, this rate reflects the minimum of glucose oxidation

needed by tissues, such as the brain, that are depending on glucose for their fuel utilisation.

Endogenous glucose production during fasting occurs mostly via processes of hepatic gluconeogenesis and glycogenolysis [35]. Previously, it has been shown that hepatic glycogen linearly declines during the first 24h of fasting and only marginally declines further after 40h of fasting [33]. Since 1 g of glycogen is stored with ~3 g of water, loss of glycogen also results in loss of body weight. Indeed, our results show that body weight was rapidly lost during the first day of fasting with less body weight loss during the second day, consistent with the negative glucose balance. In fact, the negative carbohydrate balance of ~180 and 90 g/day in the first and second day of fasting fit well with the change in body weight of ~800 and 400 g/day. With regard to hepatic gluconeogenesis, we found that circulatory levels of glycerol – a precursor for gluconeogenesis - and beta-hydroxybutyrate which can act as an alternative energy source for glucose-dependent tissues were markedly elevated upon fasting. In contrast, protein oxidation was similar between the fasted and fed condition suggesting that the 60h fast did not induce an increase in the breakdown of proteins to provide amino acids for gluconeogenesis, which confirms a previous study [36]. However, since protein oxidation follows protein intake, which is absent with fasting, the observed protein oxidation indicates proteolysis of endogenous proteins. In this perspective, an increase in the release of skeletal muscle phenylalanine and leucine was previously reported in healthy volunteers after a 60h fast, reflecting an increased proteolysis [37]. Thus, it may be possible that during the fast already some endogenous protein was utilized for oxidation.

It should be noted that the increase in gluconeogenesis and ketogenesis during fasting, underscored by higher levels of beta-hydroxybutyrate and glycerol in the current study, poses a challenge for accurately determining substrate oxidation using indirect calorimetry. Only when a substrate is completely converted into CO2 and O2 equations for indirect calorimetry can be applied. The process of gluconeogenesis with alanine as precursor, which is the major non-carbohydrate gluconeogenic precursor [38], requires CO_2 but does not affect O_2 consumption. However, only if the glucose that is formed is not directly oxidized, gluconeogenesis may become problematic for the calculation of whole-body substrate oxidation [39]. Although we did not measure this, it is likely that in our study the formed glucose was directly oxidized resulting in O_2 consumption. Therefore, the amount of substrate oxidation that was calculated in this study should be interpreted as the net rate of utilization, e.g. for carbohydrate oxidation this would be the rate of utilization minus the rate at which glucose is formed [39]. The formation of ketone bodies is an oxygen-consuming process and could also affect the calculated substrate oxidation. Unfortunately, it is impossible to correct for the effect of ketone body utilization on VO_2 and VCO_2 without knowing the metabolic fate of ketone bodies. Thus, both the oxidation and the excretion of ketone bodies affect VO_2 and VCO_2 [39]. The extent to which the formation of ketone bodies has affected the calculation of substrate oxidation in this study is therefore uncertain. The ketogenic response to a 60h fast may be estimated *in vivo* by using tracer studies that use stable isotopes [40], however, these studies are yet to be conducted.

In addition to an elevated glucose production, the liver is also believed to orchestrate energy metabolism during a fast by means of the excretion of hepatokines, including activin A, follistatin, FGF21, and GDF15. Similar to previous studies we found that prolonged fasting resulted in lower levels of activin A and higher levels of follistatin and GDF15 [20,23,41]. Interestingly, the change in follistatin from 12 to 36h fasting was correlated with changes in FGF21 and GDF15 during this same period. These correlations disappeared when fasted for 36–60h. Combined, these results could indicate that follistatin, FGF21 and GDF15 may be involved in the same regulatory pathways that accommodate the change in substrate metabolism during the early phase of a prolonged fast. Indeed, activin A, leptin, and follistatin all changed in the first 24h upon fasting, with only minor changes afterwards. Interestingly, the decrease in activin A between 12 and 36h fasting correlated with fat oxidation during this time period, with a higher decrease in activin A correlating to a higher fat oxidation. This could indicate that the decrease in activin A is involved in the rapid switch to higher fat oxidation in the fasted state, although future studies are necessary to investigate causality.

Sleeping metabolic rate was significantly higher during the third (last) night of fasting compared to the fed condition. The increase in energy expenditure could not be attributed to an increase in activity, but may be related to an increased stress response when fasted for a prolonged time period. Studies have shown that prolonged fasting increases the levels of epinephrine, norepinephrine, and cortisol in healthy adults [42,43]. Specifically an increase in cortisol may underlie the observed higher energy expenditure as cortisol has been shown to increase energy expenditure [44] and cortisol levels increase during the night with the peak during the morning [42]. Moreover, cortisol plays an important role in the protection against hypoglycaemia during fasting by increasing gluconeogenesis in the liver while inhibiting glucose uptake in skeletal muscle and white adipose tissue [45]. In this study, we were unfortunately not able to measure plasma cortisol so we cannot determine if the increase in sleeping metabolic rate was associated with a higher level of cortisol.

Since food intake is an important *Zeitgeber* for the maintenance of a well-aligned day–night rhythm, we analysed the day–night rhythm of energy metabolism in response to fasting based on an analysis of time points that were minimally affected by the acute effects of physical activity and, in case of the fed condition, food intake [25]. This analysis revealed that - besides the absolute changes in substrate oxidation - the 24h fluctuations in glucose and fat oxidation were altered by fasting. The day–night rhythm in energy expenditure, however, remained similar to the rhythm observed in the fed condition, with a peak in energy expenditure at ~22.30 and the nadir in the middle of the night, which is in line with previous reports [25]. This finding is also consistent with studies in healthy volunteers that show that changes in meal timing particularly affect substrate oxidation and to a lesser extent energy expenditure [46–48], suggesting that meal intake -or the absence of meal intake-predominantly affects the rhythmicity in substrate oxidation and not the rhythmicity of energy expenditure.

The novelty of this study includes the assessment of the rhythmicity in energy metabolism in response to a 60h fast. In addition, our study also provides a unique insight into the hour-to-hour changes in energy metabolism upon fasting in human volunteers. A limitation of this study is that it entails retrospective analyses of a prior study that was not designed to examine the mechanisms underlying the temporal changes in energy metabolism. Furthermore, the study was limited to healthy volunteers and therefore cannot conclude on effects of fasting in metabolically compromised individuals.

In conclusion, we here showed that extended fasting leads to a rapid switch to a relatively higher fat oxidation which occurs immediately after skipping the first meal (breakfast). Subsequently, fat oxidation gradually keeps increasing during the prolonged fast with a peak after ~50h of fasting, whereas carbohydrate oxidation diminishes to a minimal level. Furthermore, we report that energy expenditure displays a day–night rhythm that is unaffected by food intake, whereas the day–night rhythm in substrate oxidation is mainly driven by the intake of food.

Author contributions

J.H., M.M., M.K.C.H. and P.S. designed the experiments. N.A.v.H., M.M. and E.M.-K performed the measurements. C.A., D.D., J.H. and P.S. were involved in data analysis. C.A., J.H. and P.S. drafted the manuscript. All authors reviewed and approved the final version of the manuscript. P.S. is the guarantor of this work and, as such, has full access to the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of interest

This clinical trial was granted by Top Institute Food and Nutrition. The authors declare no conflict of interest.

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