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Randomized Control Trials

Diurnal variations of cold-induced thermogenesis in young, healthy adults: A randomized crossover trial

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SUMMARY

Background: Harnessing cold-induced thermogenesis (CIT) and brown adipose tissue (BAT) activity has been proposed as a means of counteracting a positive energy balance, and thus of combating obesity and its related comorbidities. However, it has remained unclear whether CIT and BAT activity show diurnal variation in humans - knowledge that might allow treatments based on these factors to be time-optimized.

Methods: A randomized crossover experiment was designed to examine whether CIT shows morning/evening variation in young, healthy adults (n = 14, 5 women). On the first experimental day, subjects' shivering thresholds were determined following a cooling protocol. After ≈96 h had elapsed, the subjects then returned on two further days (approx. 48 h apart) at 08:00 h or 18:00 in random order. On both the latter days, the resting energy expenditure (REE) was measured before the subjects underwent personalized cold exposure (i.e., according to their shivering threshold). CIT was then assessed for 60 min by indirect calorimetry. In an independent cross-sectional study (n = 133, 88 women), subjects came to the laboratory between 8:00 and 18:00 h and their BAT ^{18}F-fluordeoxyglucose (^{18}F-FDG) uptake was assessed after personalized cold stimulation.

Results: Both the REE and CIT were similar in the morning and evening (all $P > 0.05$). Indeed, 60 min of personalized-mild cold exposure in the morning or evening elicited a similar change in energy expenditure (16.8 ± 12.8 vs. 15.7 ± 15.1% increase above REE, $P = 0.72$). BAT ^{18}F-FDG uptake was also similar in the morning, evening and afternoon (all $P > 0.05$).

Conclusion: CIT does not appear to show morning/evening variation in young healthy adults, with the current study design and methodology. BAT ^{18}F-FDG uptake appears not to change across the day either, although experiments with a within-subject study design are needed to confirm these findings.

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1. Introduction

In simple terms, body weight is determined by the balance between total energy expenditure (TEE) - a multicomponent construct - and energy intake. The REE accounts for some 60–70% of the TEE; the remainder is accounted for by other components such as activity thermogenesis and adaptive thermogenesis (diet and cold-induced thermogenesis) [1]. Theoretically, if not

Abbreviations: AUC, area under the curve; BAT, brown adipose tissue; CHO_{ox}, carbohydrate oxidatino; CIT, cold-induced thermogenesis; FAT_{ox}, fat oxidation; NUT_{ox}, nutrient oxidation rates; PRO_{ox}, protein oxidation; REE, resting energy expenditure; RER, respiratory exchange ratio; TEE, total energy expenditure.

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compensated for by an adaptive mechanism (e.g., an increase in energy intake), changes in any of the previous components could lead to changes in body weight. Inducing such changes might help prevent or treat obesity and its related comorbidities.

Humans are endothermic, i.e., they modulate their heat production to compensate for heat dissipation and *vice versa*. In this way, tight control is maintained over the core temperature during environmental challenges. In cold temperatures, and when changes in the behaviour and skin blood flow are unable to compensate for heat loss, energy expenditure increases - a physiological response known as cold-induced thermogenesis (CIT) [2]. In mice, the REE can be increased by up to 60% under normal animal house temperatures (~22-23 °C) compared to thermoneutral conditions, and may increase 4–5 fold [3,4] when these animals are exposed to an ambient temperature of 4 °C. This reveals the substantial energetic demands of thermoregulatory effectors, and suggests that CIT may be used as a means of inducing a negative energy balance (assuming there are no changes in energy intake), and subsequently, weight loss. In humans, non-shivering CIT is normally related with energy expenditure values of some 15–30% above the REE [5,6]. When shivering reaches its maximum, however, energy expenditure may be five times the REE [7]. Nevertheless, humans have a much lower surface area to volume ratio than small mammals, and have more possibilities of altering their environment to suit their needs; consequently they rely less on CIT to keep their core temperature stable [2]. In addition, human CIT shows high inter-individual variability [6,8]. Hence, it remains unknown whether CIT can be effectively and safely harnessed in humans to help fight against obesity [6,9].

Many mammalian physiological responses are under the control of the circadian system, a network of hierarchically organized structures that regulate the body's temporal relationship with the environment [10–13]. Certainly, heat production - specifically via the REE - in humans fluctuates in a circadian fashion under constant routine and forced desynchrony protocols [14,15]; together with changes in heat loss the core temperature is thus regulated. The circadian system also plays a dominant role in the morning/evening difference in diet induced thermogenesis [16–18] (higher in the morning), which seems to be independent of the influence of the behavioural cycle. However, to our knowledge, there are no studies on whether CIT - or heat loss during cold exposure - obeys a diurnal/circadian rhythm in humans. Interestingly, studies in rodent models seem to indicate an interaction to exist between the biological clock and the thermoregulatory system during cold exposure [19–21]. If the CIT varies at different moments of the day in humans, treatments that harness CIT might be optimized by administering them at specific times, maximizing the effect on the daily TEE.

If CIT were shown to undergo diurnal variation, the component(s) contributing to this would be need to be identified. Brown adipose tissue (BAT), a thermogenic tissue with a unique ability to uncouple mitochondrial respiration through the UCP1 protein, is the major contributor to CIT during mild cold exposure in rodents [22]. Interestingly, both metabolic activity and the formation of BAT have been shown to be under the tight regulation of the biological clock in mice [23–28]. Whether this applies to humans – in which BAT appears to scarcely contribute to CIT [29,30] - needs to be determined.

Therefore, an exploratory controlled crossover experiment was carried out to determine whether CIT shows morning/evening differences in young, healthy adults. To better understand the CIT response, investigations were also made into whether heat loss during cold exposure shows morning/evening variation. In an independent cross-sectional study, the possible variation in BAT ^{18}F-flourdeoxyglucose (^{18}F-FDG) uptake at different times of day was also examined.

2. Materials and methods

2.1. Study subjects and experimental design

The study subjects who took part in the randomized crossover experiment to determine whether CIT shows morning/evening variation were 14 adults (5 women, 26 ± 3 years old; Table 1). Another 133 subjects (88 women, 22 ± 2 years old), all of whom were enrolled in the ACTIBATE study [ClinicalTrials.gov, ID: NCT02365129 [31]], took part in the cross-sectional (between-subjects) study to determine whether BAT ^{18}F-FDG uptake may change at different times of day. Given the novelty/exploratory nature of these experiments, and the lack of previous studies on this topic, no formal calculations of statistical power could be performed. Subjects were mostly recruited via advertisements in electronic media and leaflets. The inclusion criteria were: to be sedentary (self-reported <20 min moderate-vigorous physical activity on <3 days/week), to be a non-smoker, to take no medication that might affect the results obtained, to have had a steady body weight over the last 3 months (change <3 kg), to have no cardiometabolic disease (hypertension, diabetes, etc.), not to be pregnant, and to have no first-degree relative with a history of cancer. All assessments in the CIT study were performed between December 2017 and February 2018; the BAT ^{18}F-FDG study was conducted in October–December of 2015 and 2016 (in 8 waves - 4 per year). All testing was performed in Granada (southern Spain). Written and signed informed consent was obtained from all subjects. All study protocols adhered to the 2013 Declaration of Helsinki, and were approved by the Human Research Ethics Committee of the University of Granada (nº 924), and by the Human Research Ethics Committee of the *Junta de Andalucía* (nº 0838-N-2017).

2.2. Procedures

2.2.1. CIT study

Figure 1 shows the design of this part of the investigation. Testing was performed on three experimental days. On the first, the subjects came to our facilities and were asked to confirm that they had met the pre-study conditions, i.e., i) to arrive having fasted for 6 h, ii) having drunk only water (at least 1 L) in the last 6 h, and iii) to have refrained from moderate-vigorous physical activity in the last 24 h. Female subjects were also asked to report in which phase of the menstrual cycle they were. Subjects were then asked to void their bladder and to dress in standardized clothes (sandals, shorts, and T-shirt, clo: 0.20), and were directed to a quiet, warm (22-23 °C) room where they remained seated for 30 min in order to acclimatise. After this period, they were moved into an air-conditioned cold room (19.5–20 °C) and lay on a bed in the

Table 1
Characteristics of the study participants.

	CIT study (n = 14)	BAT^{18}F-FDG study (n = 133)
Women (%)	5 (36)	88 (66)
Age (years)	26 (3)	22 (2)
BMI (kg/m^2)	23.6 (2.8)	24.9 (4.8)
Lean mass (kg)	45.5 (11.8)	42.2 (10)
Fat mass (kg)	18.3 (4.1)	25.1 (9.2)
Fat mass (%)	28.2 (6.1)	35.9 (7.4)
REE (Kcal/day)	1469 (237)	

Data are presented as mean (standard deviation) and number (percentage) for continuous and categorical variables respectively. For the CIT study, the REE value shown here was calculated as the mean of the REE in the morning and the evening. BAT: brown adipose tissue, BMI: Body mass index, CIT: cold-induced thermogenesis, ^{18}F-FDG: ^{18}F-fluordeoxyglucose, REE: resting energy expenditure.

supine position. Fifteen minutes after entering the cold room, the subjects put on a temperature-controlled water-perfused cooling vest (Polar Products Inc., Stow, OH, USA), which covered the clavicular region, the chest, the abdomen and back. The starting water temperature was 16.6 °C, which was reduced by ~1.4 °C every 10 min until shivering began (self-reported and/or visually detected by the evaluators). This temperature was taken as the individual "shivering threshold", and was used to determine - for each subject - the target temperature of the cooling vest for the following experimental days. The cooling protocol followed has been extensively described elsewhere [5,32].

On the second experimental day (≈96 h after the first day; Fig. 1), and again on the third (≈48 h later), subjects came to the laboratory either at 8:00 or at 18:00 h in a randomized order – this randomization was performed with Excel's Data Randomizer Function (without blocking for any variable or imposing restrictions) by an independent research (i.e., he was not participating in the assessments). Subjects were asked to confirm having strictly met the following required conditions: i) to have fasted for the last 10 h, ii) to have slept as usual (same sleep duration and time frame), iii) to have refrained from any moderate or vigorous physical activity within the last 24 h and 48 h respectively), iv) to have arrived at the research centre by means of motorized transport; and v) to have not consumed any alcoholic or stimulant beverage in the prior 6 h, or drugs that might have affected the peripheral circulation within the last 24 h; as well as to report in which phase of the menstrual cycle they were. They also confirmed having eaten, as instructed, a standardized meal 10 h before each CIT test, consisting of boiled rice, an egg omelette and tomato puré (using olive oil in the preparation of the meal). This meal was individually sized to provide an equivalent 35% of the estimated TEE, which was determined as the product of the estimated REE (with the Harris Benedict equation) by an activity factor (based on the subject's reported weekly activity) [33]. Then, they voided their bladders, dressed as above, and entered the quiet, warm (22-23 °C) room. Twenty DS-1922 L iButton wireless thermometers (Thermochron, Dallas, TX, USA (resolution 0.0625 °C) were attached to the subject's skin in different places (see below), to monitor skin temperature changes during the experiment. The subjects then lay on a bed in the supine position, and were covered by a sheet for 20 min to acclimatise.

The REE was then assessed (approximately 1 h h after the subjects arrived, i.e., at 9:00 or 19:00 h) using a metabolic cart over a 30 min period as recommended [34]. Subjects were instructed to breathe normally and not to talk, fidget, or sleep (keeping their eyes always open). Once data collection was complete, the subjects were moved to a cold room (19.5–20 °C) where they put on the same cooling vest as before, with the temperature set 4 °C above each test subject's previously calculated shivering threshold. All subjects lay on a bed, and CIT measurements were taken over two consecutive 30 min periods, separated by a 5 min pause to recalibrate the metabolic cart gases analysers (see below); during this time the subjects remained cold-exposed. When required, the temperature of the cooling vest water was increased 1 °C to avoid shivering (it was self-reported and/or visually detected by the evaluators) during the CIT experiments. Subjects were continually reminded to breathe normally, and not to talk, fidget, or sleep, which was controlled by the evaluator present in the room.

2.2.1.1. Estimation of gases exchange parameters. For logistic reasons, the indirect calorimetry measurements for energy expenditure, respiratory exchange ratio (RER), and nutrient oxidation rates (NUT_{ox}) were recorded using two breath-by-breath metabolic carts: a CCM Express (CCM) or an Ultima CPX CardiO2 (MGU) metabolic cart (both from the same brand: MGC Diagnostics Corp., St. Paul, MN, USA) [35,36]. Both were equipped with a similar Directconnect™ flow sensor (MGC Diagnostics Corp.). However, each subject's REE and CIT recordings were taken using the same metabolic cart (eight subjects were assessed with the CCM metabolic cart, and the other six with the MGU metabolic cart). A neoprene facemask was used for gas collection. The flow was calibrated using a 3 L calibration syringe at the beginning of every test day. The gas analysers were calibrated using standard gas concentrations before each 30 min bout of gas exchange measurements. All calibrations were performed following the corresponding manufacturer's instructions.

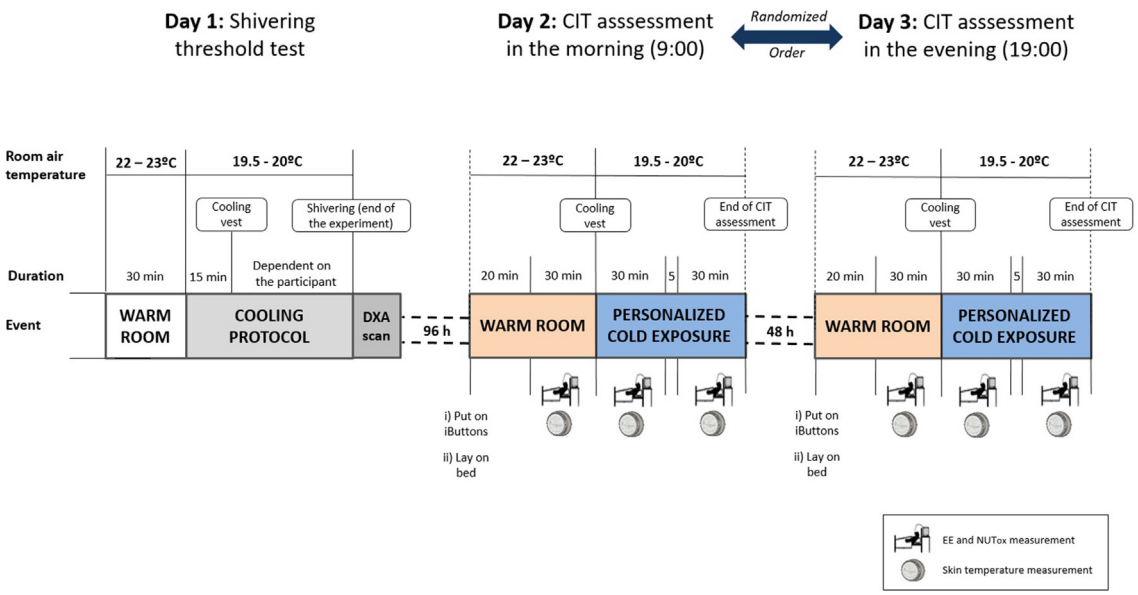

Fig. 1. CIT study design. The man-on-a-bed symbol represents the times during which resting and cold-induced energy expenditure and NUT_{ox} were assessed. The iButton icon (i.e., the object with a button shape), represents the time periods during which skin temperature was monitored during the warm and cold conditions. CIT: cold-induced thermogenesis, DXA: dual-energy X-ray absorptiometry, EE: energy expenditure, NUT_{ox}: nutrient oxidation rates.

Gas exchange data were averaged every minute and exported from the metabolic carts' Breeze Suite (8.1.0.54 SP7) software (MGC Diagnostics Corp.) to Excel for Windows. The volume of oxygen consumption and carbon dioxide production (VO_2 and VCO_2, respectively) for each selected data point were used to estimate energy expenditure, RER, and NUT_{ox} – comprised of carbohydrate, fat and protein oxidation rates (CHO_{ox}, FAT_{ox} and PRO_{ox}, respectively). Energy expenditure was estimated using Weir's abbreviated equation [37]. Frayn's and Westerterp stoichiometric equations [38,39] were used for estimating the CHO_{ox} and FAT_{ox}, and the PRO_{ox}.

$$Energy\ expenditure\ (Kcal/min) = 1.106 * VCO_2 + 3.941 * VO_2 - 2.17 * N$$

$$RER = VCO_2 / VO_2$$

$$CHO_{ox}\ (g/min) = 4.55 * VCO_2 - 3.21 * VO_2 - 2.87 * N$$

$$FAT_{ox}\ (g/min) = 1.67 * VO_2 - 1.67 * VCO_2 - 1.92 * N$$

$$PROox\ (g/min) = 6.25 * N$$

Urinary nitrogen (N) excretion was included in all energy expenditure and nutrient oxidation determinations. For this, urine samples were collected before and after the CIT experiments in two sterilized containers: one was filled with urine produced after the last meal before the experiment (i.e., during the 10 h before the CIT assessment), and the other with urine produced immediately after CIT assessment. Urine volume was recorded and the total urea concentration determined using the Spinreact UREA-37_R1 Kit (Reactivos Spinreact, Girona, Spain). A previously obtained equation was then applied to estimate the N content of the present CIT subjects based on their total urea concentrations. This equation was obtained in an independent cohort of 19 young adult subjects (unpublished), for whom we plotted their determined urea concentrations against urine nitrogen - estimated using the Kjeldahl method – in a linear regression (non-standardized $\beta = 0.007$, adjusted R^2 0.842, $P < 0.001$). The equation obtained from the regression was:

$$N\ (g/l) = 0.0065 \times urea\ (mg/dl) + 1.2598$$

To calculate the REE, the most sable 5 min period (i.e., the one with the lowest mean CV for VO_2, VCO_2, VE, and RER) was selected (excluding the first 5 min of recording) [35,40]. To be considered a stable period, the CV of the above variables had to be <10%, except for the RER (CV<5%). To obtain a mean CIT value, the first 5 min of each 30 min bout of CIT measurement were discarded [34], obtaining two periods of 25 min. Each of these periods was divided into two parts (i.e., 12.5 min each one) [8], and the most sable 5 min period within each of these 12.5 min periods selected. Finally, these four selected 5 min periods, together with the REE, were used to calculate the area under the curve (AUC) (trapezoidal rule), expressing it as a percentage of the REE [8] (i.e., AUC [%REE]). The same procedure was followed to calculate the RER and NUT_{ox} during cold exposure.

2.2.1.2. Changes in skin temperature. Skin temperature during warm and cold conditions were recorded at 1 min intervals, using the above-mentioned 20 iButtons attached to the subject's skin (see the different positions in Supplementary Material). A mean skin temperature for each iButton was first determined for the warm period (i.e., the baseline). For this, the first 20 min of skin temperature readings were discarded, and the mean value for the last 10 min calculated for each iButton (when the subject was acclimatised to the warm room temperature as confirmed from a plateau in skin temperature, data not shown). A mean value for each iButton was then obtained for the skin temperature changes during cold exposure. For this, the first 5 min of skin temperature readings from each period of 30 min during cold exposure (i.e., from minute 0 to 5, and 35 to 40) were discarded, as were those for the time period comprised between minutes 30 and 35 min (i.e., the 5 min pause used to recalibrate the gas analysers). The skin temperature recorded by each button during the cold exposure was then divided into 5 min periods (from minute 5 to 30, and from 40 to 65), to obtain a mean temperature for each button and period (with a total of 10 periods). The mean skin temperature for each button-period was then used, along with the baseline mean skin temperature for each button, to calculate the AUC (trapezoidal rule), expressing each mean for each button as a percentage of the corresponding baseline skin temperature (AUC [%baseline]).

For the analyses of this study, we only used the skin temperature measures recorded by the iButtons of the left hand and right supraclavicular area, as well as the skin temperature recorded by other iButtons which were then used to calculate the overall mean [41] and proximal [42] skin temperatures [see references [5,43] for further information]. These calculations were done based on the following equations:

Overall mean skin temperature = (Forehead*0.07) + (Right Scapula*0.175) + (Left Chest*0.175) + (Right Deltoid*0.07) + (Left Elbow*0.07) + (Left Hand*0.05) + (Right Thigh*0.19) + (Right Gastrocnemius*0.2)

Proximal skin temperature = (Right Thigh*0.383) + (Right Clavicular*0.293) +(Right Abdomen*0.324)

All calculations were performed using Temperatus® software (http://profith.ugr.es/temperatus) [44].

2.2.2. BAT ^{18}F-FDG uptake study

Subjects came to the test facility between 08:00 h and 18:00 h, and were asked to confirm that they had met the same pre-study conditions as above. They then voided their bladders, and dressed with standardized cloths. They stayed in the quiet, warm (22-23 °C) room for 30 min before entering the cold room (19.5–20 °C) where they sat down and wore the same water-perfused cooling vests as above set 4 °C above their individual shivering threshold (determined 48–72 h before BAT assessment). Subjects remained under these conditions for 60 min to induce BAT activation. At the end of this period they were injected with a bolus of ^{18}F-FDG (183.52 ± 12.21 MBq ≈ 2.7 MBq/kg) with the water temperature of the cooling vest raised by ~1 °C for the last 60 min to avoid shivering. After 2 h, all subjects underwent positron-emission tomography/computed tomography (PET/CT) scanning, performed according to current methodological recommendations [45], to determine BAT ^{18}F-FDG uptake and the BAT mean radiodensity. A low dose CT (120 kV) for attenuation correction and anatomic localization was performed prior to each PET scan. Then, we performed a static PET consisting of 2 BED scans (6 min each), approximately from the atlas vertebra to mid chest [46].

2.2.2.1. ^{18}F-FDG-PET/CT analysis. PET/CT scans were analysed using the Beth Israel plug-in for FIJI http://sourceforge.net/projects/bifijiplugins/. To determine BAT ^{18}F-FDG uptake, six regions of interest (ROIs) were outlined from the atlas vertebra to thoracic vertebra 4 using a 3D-axial technique [47]. These ROIs comprised the supraclavicular, laterocervical, paravertebral and mediastinal regions. To determine BAT mean radiodensity, a whole ROI was

outlined covering all the body cross-sectional area (excluding the mouth) in the axial plane, from the atlas vertebra to thoracic vertebra 4. Within these ROIs, the SUV threshold for a voxel to be considered BAT was taken as ≥[1.2/(lean body mass/body mass)]; the radiodensity also had to fall in the range −190 to −10 Hounsfield units (HU) [45]. The mean BAT volume, SUV_{mean} and SUV_{peak}, and the BAT mean radiodensity of all the ROIs were recorded [48,49]. Of note, as a whole (not specific) ROI was drawn to determine BAT mean radiodensity, some voxels belonging to connective tissue, internal organs, glands, etc., were erroneously classified as BAT voxels in some subjects (normally those subjects with a BAT volume below 8 mL). Since, the inclusion of these subjects were likely to bias the BAT mean radiodensity (because they did not have actual BAT voxels) they were not included in this specific analysis. A single slice-ROI was also outlined to determine the SUV_{peak} in the descending aorta (used as the reference tissue). For confirmatory analyses, BAT SUV_{mean} and SUV_{peak} was recomputed as a product of percentage lean body mass (SUV_{LBM}) [47]. The standardized ^{18}F-FDG uptake value (SUV) was calculated as *[^{18}F-FDG uptake (kBq/ml)/(injected dose [kBq]/patient weight [g])]*.

2.3. Body composition and HOMA index

Subject body composition was determined by DXA to obtain absolute values for lean mass, fat mass and percentage body fat. Weight and height were measured using a model 799 scale and stadiometer (Seca, Hamburg, Germany). The HOMA (homeostatic model assessment of insulin resistance) index was determined following the standard method [see information on [50]] extracting the necessary blood samples on non-test days.

2.4. Statistical analyses

Descriptive statistics of the study subjects are shown as mean ± standard deviation unless otherwise stated. Energy expenditure, RER, and NUT_{ox} during resting and cold conditions at different times of day (morning vs. evening) were compared using one-way repeated-measures analysis of variance (ANOVA). One-way repeated-measures analysis of covariance (ANCOVA) was used to compare REE at different times of day, adjusting for lean mass [51]. Linear mixed model analyses were used to examine the kinetics of energy expenditure, RER and NUT_{ox} during cold exposure in the morning vs. the evening. Sex and order of CIT assessment were initially included in the models to examine their influence on the study variables of interest, but neither had a significant influence and were thus removed from the analyses. These analyses (i.e., ANOVA and linear mixed models) were replicated to compare heat loss at different times of day. Differences in BAT ^{18F}-FDG uptake and BAT mean radiodensity in the morning, afternoon and evening (i.e., tertiles of the time at which the PET/CT scanning was performed) were examined by ANOVA. Significance was set at $P < 0.05$. All analyses were performed using the Statistical Package for the Social Sciences v.24.0 (IBM Corporation, Chicago, IL, USA).

3. Results

Table 1 shows the descriptive characteristics of the subjects in the CIT and BAT ^{18}F-FDG uptake studies. In the former, a total of 14 subjects were included in the study (see Flowchart, Fig. S1). The results of all 14 subjects were used for the energy expenditure analyses, whereas those of 8 subjects were used in the RER and NUT_{ox} analyses. The other six subjects returned RER and NUT_{ox} outlying values through the cold exposure, being most of them related to artefacts in VCO_2, but not in the VO_2 – this means that they exerted a minimal (or did not) influence in energy expenditure measures.

3.1. CIT study

3.1.1. Morning/evening differences in CIT

Figure 2 shows the REE and CIT, plus the resting and cold-induced RER and NUT_{ox} assessed in the morning (09:00 h) and evening (19:00 h). Both the REE (also when adjusted for lean mass) and the resting CHO_{ox} were similar at both times of day (all $P \geq 0.18$; Fig. 2, Panels A and C). However, the RER was higher in the morning than in the evening (mean ± standard deviation: 0.856 ± 0.032 vs. 0.812 ± 0.027, $P = 0.01$; Fig. 2, Panel B), whereas the opposite was observed for fat oxidation (FAT_{ox}: 0.027 ± 0.017 vs. 0.053 ± 0.013 g/min, $P = 0.003$; Fig. 2, Panel D), during resting conditions. CIT was similar in the morning and evening (16.8 ± 12.8 vs. 15.7 ± 15.1% above REE, $P = 0.72$, Fig. 2, Panel E). No significant differences (all $P \geq 0.57$) were seen in cold-induced RER, CHO_{ox} and FAT_{ox}, in the morning compared to the evening (Fig. 2, Panels F–H).

Energy expenditure increased during cold exposure (effect of cold exposure; $P < 0.001$; Fig. 2, Panel I) - in the morning from the baseline (i.e., REE) 1 ± 0.19 to 1.24 ± 0.3 Kcal/min at the end of cold exposure ($P = 0.001$), and in the evening from 1.03 ± 0.16 to 1.23 ± 0.3 Kcal/min ($P = 0.005$, data not shown). Neither the time of day nor the interaction *time of the day x cold exposure* had a significant effect on CIT (all $P \geq 0.38$, Fig. 2, Panel I). These results replicated when we only considered the 8 subjects used in the RER and NUT_{ox} analyses (data not shown). During cold exposure, RER and CHO_{ox} were reduced and FAT_{ox} increased (all $P \leq 0.006$) until approximately halfway through the exposure time (minute 30). A reversal then occurred (Fig. 2, Panels J–L). The interaction *time of day x cold exposure* had no influence on RER, CHO_{ox} or FAT_{ox} (all $P \geq 0.26$), although the time of day did have a significant influence on RER and FAT_{ox} (all $P \leq 0.008$), and a trend towards one was seen with respect to CHO_{ox} ($P = 0.09$).

It is noteworthy that the REE and CIT in the morning correlated with their respective measures in the evening (REE: $r = 0.81$, $P < 0.001$; CIT: $r = 0.66$, $P = 0.009$), which supports the accuracy and reliability of these measures (see Fig. S2). All analyses were performed including N excretion in the energy expenditure and nutrient oxidation determinations. However, when N was not included in the determinations, all results replicated (data not shown). Similarly, the findings persisted when the CIT analyses were performed splitting them by sex (see Fig. S3) and the morning/evening order of CIT assessment (data not shown). Of note, 1 of the 5 female subjects reported to be in the luteal phase of her menstrual cycle during the CIT assessment days - and it has been reported that a slight increase of core temperature may happen in this phase [52]. When CIT analyses were replicated excluding this woman, the results did not change (data not shown). Some subjects ($n = 7$) shivered on either both test days or on one test day (despite the cold stress to which they were submitted being similar on both days). Sensitivity analyses excluding these subjects also showed that neither the time of day nor the interaction *time of the day x cold exposure* had a significant effect on CIT (data not shown).

As there is no consensus yet on how to analyse the CIT response [6,8], and given that the high variability of CIT measurements may not allow subjects to achieve a steady-state metabolism, CIT analyses were replicated estimating it as: i) the difference between the mean energy expenditure during the last 5 min of the cold exposure and REE; ii) as the AUC (trapezoidal rule) calculated using the REE, and the mean energy expenditure of the 5-min intervals comprised between the minutes 5–30 and 40–65 of the cold exposure, expressing it as a percentage of the REE [8]; iii) as the whole average of the energy expenditure through the cold exposure (discarding the first 5 min of each 30 min record of indirect calorimetry); and iv) as the mean of the 5 consecutive most stable minutes within the time when energy expenditure appeared to

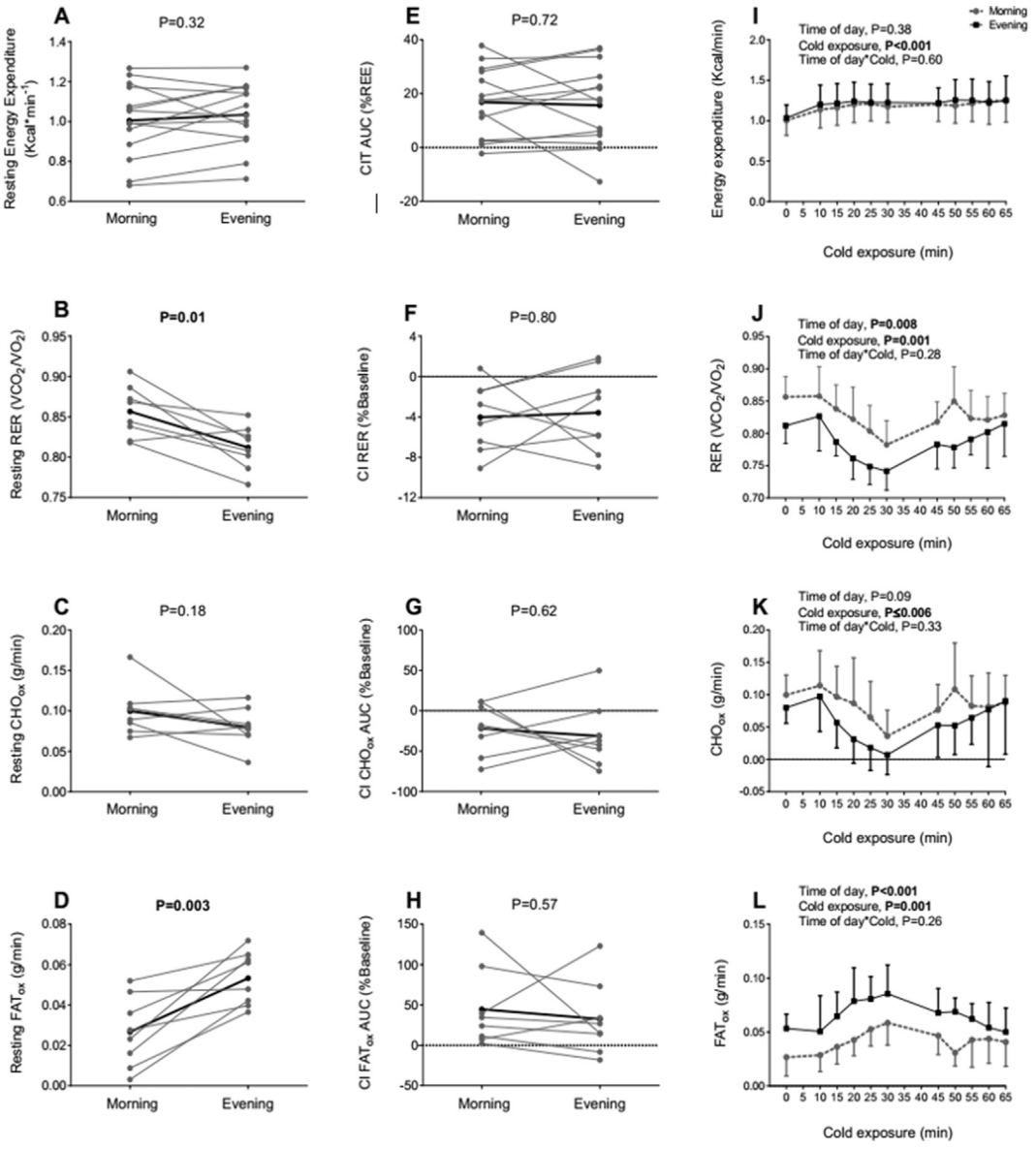

Fig. 2. Morning/evening variation (or lack thereof) in CIT in young, healthy adults. **Panels A, B, C and D** show the REE, RER, CHO_{ox} and FAT_{ox} values at 09:00 and 19:00 h. **Panels E, F, G and H** show the overall changes in CIT and cold-induced RER, CHO_{ox} and FAT_{ox} at these times. **Panels I, J, K and L** show the kinetics of energy expenditure, RER, CHO_{ox} and FAT_{ox} over cold exposure in the morning and evening. Values corresponding to minute 0 represent the REE and resting RER, CHO_{ox} and FAT_{ox}. The black bar in panels A–H shows the mean change in the corresponding variable when comparing morning vs. evening. The sample size for the REE and CIT tests was n = 14, while for RER and nutrient oxidation-related variables it was n = 8. Paired t tests were used to compare all morning vs. evening values. Linear mixed model analysis was employed to compare the morning and evening kinetics of energy expenditure, RER, CHO_{ox} and FAT_{ox} over the cold exposure. AUC: area under the curve, CHO_{ox}: carbohydrate oxidation, CI: cold-induced, CIT: cold-induced thermogenesis FAT_{ox}: fat oxidation, REE: resting energy expenditure, RER: respiratory exchange ratio.

plateau - this was in the minutes 18–30 and 53–65 of cold exposure (see Fig. 2, **Panel I**). Of note, all results remained similar (data not shown). In order to ease the interpretation of data, Fig. S4 shows the PRO_{ox} before and during the cold exposure, and Figs. S5 and S6 the minute ventilation (VE) and the coefficients of variance (CV) of VO_2, VCO_2, RER and VE through the cold exposure (respectively), at both times of day.

3.1.2. Morning/evening differences in skin temperature

To better understand the CIT response, investigations were also made into whether heat loss during cold exposure shows morning/evening variation. The skin temperature of the hand during warm conditions (i.e., the baseline) was similar in the morning and evening (P = 0.69, Fig. 3, **Panel A**), whereas the supraclavicular area, mean, and proximal skin temperatures during warm conditions were higher in the evening than in the morning (all $P \leq 0.009$, Fig. 3, **Panels B, C and D**). However, no significant differences were observed between the morning and evening in terms of cold-induced changes in skin temperature (all $P \geq 0.11$, Fig. 3, **Panels E-H**).

The values for all skin temperatures were reduced by cold exposure (effect of cold exposure: all $P \leq 0.009$) (Fig. 3, Panels I–L), although the supraclavicular skin temperature reached a plateau quickly after the initial decrease. The time of day had a significant effect on the mean and proximal skin temperatures (all $P \leq 0.002$, Fig. 3, **Panels K and L**), which were higher in the evening. However,

the interaction *time of day x cold exposure* had no influence on skin temperature measures (all P ≥ 0.17). Overall, these results replicated when the analyses were performed splitting them by sex (see Fig. S7).

Fig. S8 shows the water temperature of the cooling vest worn by the subjects during the personalized cold exposure (Panels A, C and E), as well as the room temperature to which they were exposed during the warm and cold conditions (Panels B, D and F) in the morning and evening when CIT was assessed. For some subjects there were small differences in the water temperature of the cooling vest during the morning and evening tests. It is unlikely, but it was thought perhaps not impossible, that this could have influenced the skin temperature assessments, especially since some iButtons were in direct contact with the cooling vest. When the analyses were repeated adjusting for these minor differences, the findings persisted (data not shown). Fig. S9 shows the outdoors

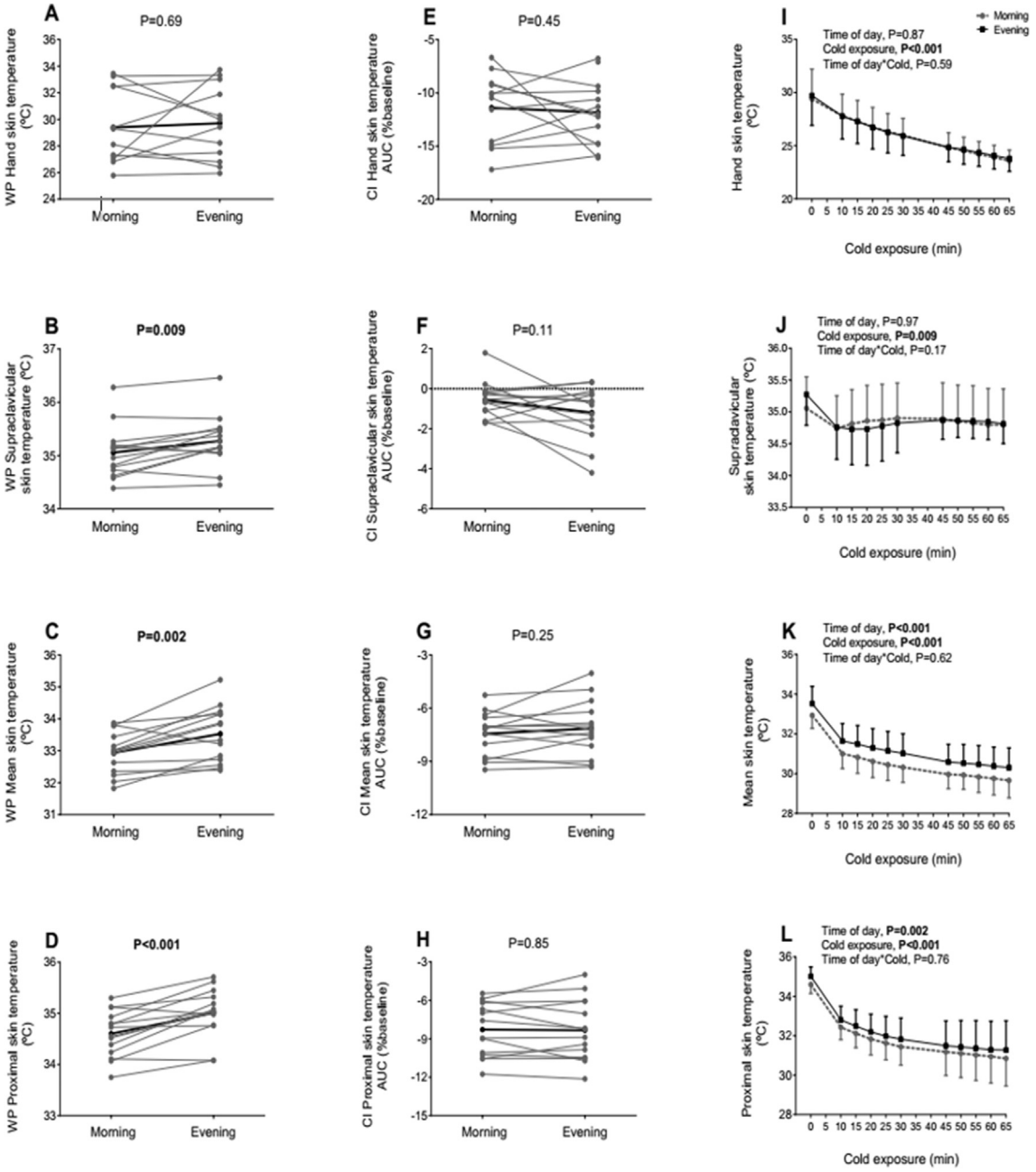

Fig. 3. Morning/evening variation in skin temperature during cold exposure (CIT study). **Panels A, B, C and D** respectively show the left hand, right supraclavicular area, mean, and proximal skin temperatures under warm conditions, at 09:00 and 19:00 h. **Panels E, F, G and H** show the cold-induced reduction in the left hand, right supraclavicular area, mean and proximal skin temperatures at these same times. **Panels I, J, K and L** show the kinetics of the left hand, right supraclavicular area, mean and proximal skin temperatures over cold exposure in the morning and evening. Values corresponding to minute 0 of cold exposure represent the baseline (warm period) skin temperatures. CI: cold-induced, WP: warm period. The black bar in the above panels A–H shows the mean change in the corresponding variable when comparing morning and evening. The sample sizes for the skin temperature measures were: left hand (n = 12), right supraclavicular area (n = 14), mean (n = 14), and proximal skin temperature (n = 14). Paired t tests were used to compare morning and evening values. Linear mixed model analysis was used to compare the morning and evening skin temperature kinetics over the cold exposure. AUC: area under the curve, CI: cold-induced, CIT: cold-induced thermogenesis, WP: warm period.

temperature in the 2 days during which CIT was assessed for each subject.

3.2. BAT ^{18}F-FDG uptake study

3.2.1. BAT ^{18}F-FDG uptake at different times of day

Table S1 shows the descriptive characteristics of the subjects included in the BAT ^{18}F-FDG uptake study, separately for the morning, afternoon and evening groups. Of note, no significant differences in BAT volume, SUV_{mean}, SUV_{peak} or BAT mean radiodensity were seen in the morning, afternoon or evening (all $P \geq 0.3$) (Fig. 4, **Panels A-D**). Indeed, no differences were observed when the above variables were compared across time five time divisions instead of three (data not shown).

Linear regressions were performed to examine the relationships between BAT ^{18}F-FDG uptake-related variables/BAT mean radiodensity and the time of their assessment; no significant associations were found (all $P \geq 0.33$, data not shown). These findings persisted when i) adjustments were individually made for sex, BMI or body fat percentage, the HOMA index, or the date when the PET/CT scan was performed (or when adjusting for all together); ii) the analyses were conducted excluding those subjects who were PET- (i.e., those who showed a BAT volume, SUV_{mean} and SUV_{peak} of zero, [remaining subjects n = 120]); iii) and when BAT SUV_{mean} and SUV_{peak} were calculated as a product of percentage lean body mass (SUV_{LBM}) (data not shown). Finally, no significant relationship with the time of assessment was seen for the descending aorta ^{18}F-FDG uptake (SUV_{peak}) ($P = 0.55$, Fig. S10).

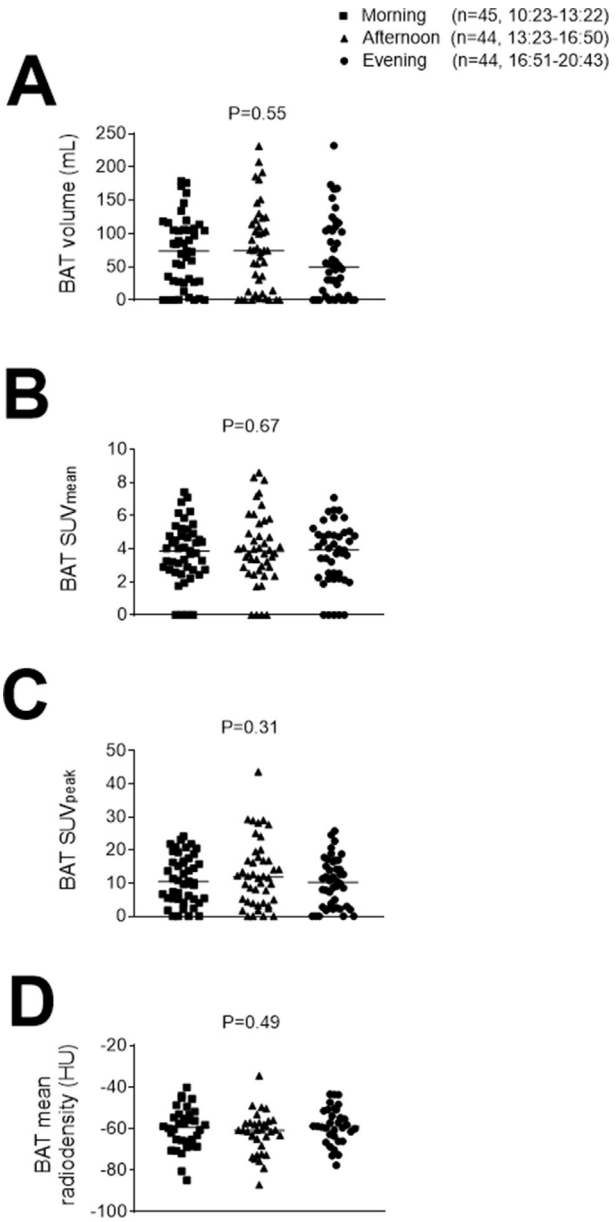

Fig. 4. Brown adipose tissue (BAT) ^{18}F-fluordeoxyglucose uptake (n = 133) and mean radiodensity (n = 101) at different times of day. Analyses of variance (ANOVA) were performed to compare morning, afternoon and evening (tertiles of the time when the PET/CT scan was performed). BAT volume (**Panel A**), mean standardized uptake value (SUVmean, **Panel B**), peak standardized uptake value (SUVpeak, **Panel C**), and BAT mean radiodensity (**Panel D**). Of note, the number of subjects and therefore the tertiles, were different for the BAT mean radiodensity (Morning: n = 33, 10:23–13:04; Afternoon: n = 32, 13:05–16:32; Evening: n = 32, 16:33–20:33). The P-value for the between-subject effect is provided.

4. Discussion

The present results show CIT to exhibit no morning/evening variation in young, healthy adults. Indeed, 60 min of personalized-mild cold exposure in the morning or evening elicited a similar change in energy expenditure, in RER, and in NUT_{ox}. The results also show that BAT ^{18}F-FDG uptake is similar at different times of day, although within-subject studies are needed to confirm this.

Previous studies in humans have shown that, under thermoneutral conditions, the REE and nutrient oxidation rates vary over the day, with REE and FAT_{ox} higher during the evening [14]. These oscillations in heat production occur alongside changes in heat loss needed to regulate the core temperature [15], independent of sleeping/awake status and physical activity-related effects [14]. In addition, the circadian system plays a dominant role in the morning/evening difference in diet induced thermogenesis [16–18], which shares common underlying mechanisms with CIT [54,55] (the sympathetic nervous system being the main effector of both). Further, some areas/nucleus of the hypothalamus (such as the dorsomedial nucleus) involved in the integration of afferent signals and the control of effector responses related to cold, are also involved in the circadian control of body temperature and energy expenditure [2,56]. These different pieces of evidence together led to the hypothesis that the time of the day would have an effect on energy expenditure during cold exposure - although the opposite was found to be the case.

To our knowledge, there have been no prior studies examining whether CIT exhibits diurnal rhythmicity in humans, which precludes any direct comparison with the present work being conducted. However, it has been shown [57–60] that the systemic concentrations of some factors potentially regulating CIT, follow diurnal fluctuations at room temperature [e.g., adiponectin, proANP, FGF21 [61–63]]. It may be speculated that these fluctuations could persist during cold exposure - i.e., the effect of the time of day could interact with the homeostatic response to cold - and help modulate CIT response. Unfortunately, no experiments have tested this. One study [64] reported that mild cold exposure modulated the diurnal rhythm of plasma FGF21 levels in 12 healthy adults. Nevertheless, the facts that: i) a liquid meal was provided to subjects (which could have influenced the kinetics of plasma FGF21 levels); and ii) that the interaction effect of the time of day with the cold exposure was not reported, avoids any comparison with the current work.

Interestingly, whereas CIT showed no morning/evening variation, heat loss during cold exposure at specific body sites did. The fluctuation in heat loss during cold exposure was determined by its baseline, suggesting that there is no interaction between the

circadian and homeostatic mechanisms involved in these thermoregulatory responses. Thus, the higher the baseline mean and proximal skin temperatures in the evening, the greater values measured during cold exposure during the evening – although their overall reduction was similar at both times of day. The REE showed no morning/evening variation - as previously reported in studies with similar characteristics [17,18,66], which may have conditioned the CIT to show no morning/evening variation either.

Humans are endothermic, and can therefore change the difference between heat production and heat loss (i.e., heat content) in order to regulate core temperature [15]. The mild cold exposure to which subjects were subjected is unlikely to have induced changes in the core temperature. However, it has been shown that under controlled but not constant conditions (as in the present study), the core temperature fluctuates, reaching a maximum value around 20:00 h [67]. Importantly, this diurnal rhythm of body core temperature is determined by the diurnal variation in the difference between heat production and heat loss [15,68]. Indeed, the rhythms of heat production and heat loss have nearly identical ranges of oscillation and seem to be in phase with one another [68,69]. Thus, if it is assumed that there was no change in core temperature in response to the cold exposure at any time during the day, and that it peaked in the evening as normal [67,68], one might also expect that an increase in heat loss in the evening would be accompanied by an increase in heat production (i.e., an increase in CIT). However, this was not seen in the present work. This might be explained by the inertia in heat transfer mechanisms and the storage of heat in the tissues leading to a time lag between heat production and heat loss [68] through the day. Hence, during the evening, heat production might be phase-delayed with respect to heat loss, though this was not reflected in the present work because of the short CIT assessment time. Another possibility is that since the body core temperature will progressively decrease as the night comes closer - the core body temperature nadir happens at the late night [15,68] - heat loss (i.e., vasodilation) will increase whereas heat production remains similar. Interestingly, it has been proposed that diurnal variations in heat loss are largely responsible for the range of variation in core body temperature [70], especially during the evening [15]. This might also help explain why heat loss at specific body sites during the cold exposure was greater at this time but was not seen for CIT. Future studies should try to confirm whether CIT shows no diurnal variation. It is noteworthy that the distal skin temperature - one of the sites of major heat exchange - was no different in the morning or evening, but the temperature does, of course, fluctuate over the day, and in fact widely during the night [50].

Neither BAT ^{18}F-FDG uptake not BAT mean radiodensity differed over the day. This seems to contrast with previous evidence from rodent models [23–25] showing that: i) the metabolic function of BAT is under circadian clock regulation; and that ii) BAT receives input from several nuclei and areas of the hypothalamus, shaping the rhythms of systemic glucose and lipids, body temperature and energy expenditure [2,23,71–73]. The evidence suggests that, in rodents, the circadian rhythms of body temperature and metabolism are tightly bound to proper BAT function [74], but evidence for this in humans is lacking. Only one study [75] has suggested glucose utilization by human BAT to be coupled with its heat production (indirectly measured using wireless thermometers) in a circadian manner. Nevertheless, that study has important caveats that call the previous conclusion into question [50]. Hence, whether BAT thermogenesis follows a diurnal/circadian rhythmicity remains a matter of debate, although given its small contribution to daily energy expenditure under mild cold conditions (10 ± 5 Kcal/day) in humans [30], harnessing it at specific times might have no clinical/health benefits.

Together, the results suggest CIT not to show morning/evening variation – even though such variation was observed in specific skin temperatures (indicating heat loss) during cold exposure -, or at least, that the homeostatic heat production responses to cold exposure overshadowed the potential diurnal fluctuations of CIT. That said, the inclusion of more measurements over the day would have allowed for more informative results. In addition, the CIT was assessed as a response to a (relatively short) personalized cold exposure designed to minimize shivering. Whether, CIT may show diurnal variation when subjects are exposed to increased thermal stress (e.g., a protocol with a larger duration or intensity - inducing a CIT increase of 30–500% above REE) is unknown, and should be tested in the future. In addition, the thermoneutral zone for each individual was not determined: this implies the possibility that our subjects were exposed to a light thermal stress during the resting measures, and therefore our REE measures actually represented the REE + a small quantity of energy required for other thermal homeostatic mechanisms (e.g., CIT) [76]. Further, the present study design aimed to examine the fluctuations in heat production under the influence of possible internal and external masking factors. Hence, any change in the rhythm of heat production should be the result of its endogenous rhythmicity, its entrainment to zeitgebers, and the influence of any masking factors. Future studies should examine the endogenous rhythm of CIT (independently of behavioural cycles) using constant routine and forced desynchrony protocols. That said, these protocols cannot reflect the real-world situation, and the results could not be extrapolated to daily life. If CIT were seen to show a diurnal/circadian rhythm when following different protocols, it ought then to be investigated whether harnessing CIT at specific times of the day affects daily energy expenditure and body weight regulation in the long-term.

It should also be noted that, for logistical reasons, two types of metabolic cart - from the same brand and with the same flow sensors - were used (the same type was always used for the same subject). Finally, the possibility exists that REE and CIT do show variations in the morning and evening, but so slight that only metabolic carts with better resolution could detect them [36] (and in which the technical error of the gas analysers is less than the variation the devices are meant to detect). The use of telemetric pills to monitor the body core temperature might also be attempted.

The BAT ^{18}F-FDG uptake study was merely exploratory; there is a need to perform within-subject studies if we are to draw firm conclusions regarding whether BAT activity follows a diurnal/circadian rhythm. These studies should include the simultaneous measurement of CIT, to ascertain to what extent the diurnal variation of CIT is dependent on BAT metabolic activity and its fluctuations throughout the day – which could not be tested in the current study. However, such studies would not be without their difficulty since the several PET/CT scans required would expose subjects to high doses of radioactivity. In the present work, BAT ^{18}F-FDG uptake was quantified using the most extended technique and following updated recommendations [45]. However, static ^{18}F-FDG PET-CT scans suffer several limitations that may prevent a fully accurate estimation of cold-induced BAT metabolic activity [77].

In conclusion, our findings suggest that CIT does not show morning/evening variation in young healthy adults, with the current study design and methodology – or at least, that the effect of the time of day on CIT is so subtle and variable across subjects, that it may not be clinically significant. Our findings also suggest an absence of diurnal variation in BAT ^{18}F-FDG uptake. Altogether, optimizing the time at which CIT (or BAT activity) is elicited does not appear to be an efficient strategy to increase energy expenditure, and potentially face obesity. Future studies should examine if these results replicate under different conditions.

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Authors' contributions

FAM, GSD, BMT, JMAA, JMLLE and JRR designed the study; FAM, BMT, GSD, JMAA, JMLLE conducted the experiments; JRR and JMLLE provided essential reagents and materials; FAM, BMT, GSD and JRR analysed the data and performed the statistical analysis; FAM wrote the manuscript; FAM, BMT, GSD, JMAA, JMLLE and JRR reviewed the manuscript and provided scientific assistance; JRR had primary responsibility for the paper's final content.

Conflicts of interest

None to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2021.08.010.

References

[1] van Marken Lichtenbelt WD, Schrauwen P. Implications of nonshivering thermogenesis for energy balance regulation in humans. AJP Regul Integr Comp Physiol 2011;301(2):285–96.
[2] Tan CL, Knight ZA. Regulation of body temperature by the nervous system. Neuron 2018;98(1):31–48.
[3] Abreu-Vieira G, Xiao C, Gavrilova O, Reitman ML. Integration of body temperature into the analysis of energy expenditure in the mouse. Mol Metab 2015;4(6):461–70.
[4] Cannon B, Nedergaard J. Nonshivering thermogenesis and its adequate measurement in metabolic studies. J Exp Biol 2011;214(2):242–53.
[5] Acosta FM, Martinez-Tellez B, Sanchez-Delgado G, Alcantara JMA, Acosta-Manzano P, Morales-Artacho AJ, et al. Physiological responses to acute cold exposure in young lean men. PloS One 2018;13(5).
[6] Brychta RJ, Chen KY. Cold-induced thermogenesis in humans. Eur J Clin Nutr 2017;71(3):345–52.
[7] Eyolfson DA, Tikuisis P, Xu X, Weseen G, Giesbrecht GG. Measurement and prediction of peak shivering intensity in humans. Eur J Appl Physiol 2001;84(1–2):100–6.
[8] Sanchez-Delgado G, Alcantara JMA, Acosta FM, Martinez-Tellez B, Amaro-Gahete FJ, Ortiz-Alvarez L, et al. Estimation of non-shivering thermogenesis and cold-induced nutrient oxidation rates: impact of method for data selection and analysis. Clin Nutr 2019;38(5):2168–74.
[9] Betz MJ, Enerbäck S. Targeting thermogenesis in brown fat and muscle to treat obesity and metabolic disease. Nat Rev Endocrinol 2017;14(2):77–87.
[10] Garaulet M, Ordovás JM, Madrid JA. The chronobiology, etiology and pathophysiology of obesity. Int J Obes 2010;34:1667–83.
[11] Froy O. Metabolism and circadian rhythms - implications for obesity. Endocr Rev 2010;31(1):1–24.
[12] Garaulet M, Gómez-Abellán P, Madrid JA. Chronobiology and obesity: the orchestra out of tune. Clin Lipidol 2010;5(2):181–8.
[13] Hastings MH, Reddy AB, Maywood ES. A clockwork web: circadian timing in brain and periphery, in health and disease. Nat Rev Neurosci 2003;4(8):649–61.
[14] Zitting KM, Vujovic N, Yuan RK, Isherwood CM, Medina JE, Wang W, et al. Human resting energy expenditure varies with circadian phase. Curr Biol 2018;28(22):3685–90.
[15] Kräuchi K, Wirz-Justice A. Circadian rhythm of heat production, heart rate, and skin and core temperature under unmasking conditions in men. Am J Physiol 1994;267(3):R819–29.
[16] Morris CJ, Garcia JI, Myers S, Yang JN, Trienekens N, Scheer FAJL. The human circadian system has a dominating role in causing the morning/evening difference in diet-induced thermogenesis. Obesity 2015;23(10):2053–8.
[17] Romon M, Edme JL, Boulenguez C, Lescroart JL, Frimat P. Circadian variation of diet-induced thermogenesis. Am J Clin Nutr 1993;57(4):476–80.
[18] Bo S, Fadda M, Castiglione A, Ciccone G, De Francesco A, Fedele D, et al. Is the timing of caloric intake associated with variation in diet-induced thermogenesis and in the metabolic pattern? A randomized cross-over study. Int J Obes 2015;39(12):1689–95.
[19] Watts RH, Refinetti R. Circadian modulation of cold-induced thermogenesis in the golden hamster. Biol Rhythm Res 1996;27(1):87–94.
[20] Machado FSM, Zhang Z, Su Y, de Goede P, Jansen R, Foppen E, et al. Time-of-day effects on metabolic and clock-related adjustments to cold. Front Endocrinol 2018;9(199).
[21] Tokizawa K, Uchida Y, Nagashima K. Thermoregulation in the cold changes depending on the time of day and feeding condition: physiological and anatomical analyses of involved circadian mechanisms. Neuroscience 2009;164(3):1377–86.
[22] Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. Physiol Rev 2004;84(1):277–359.
[23] Hansen SN, Peics J, Gerhart-hines Z. Keeping fat on time: circadian control of adipose tissue. Exp Cell Res 2017;360(1):31–4.
[24] Nam D, Yechoor VK, Ma K. Molecular clock integration of brown adipose tissue formation and function. Adipocyte 2016;5(2):243–50.
[25] Gerhart-Hines Z, Feng D, Emmett MJ, Everett LJ, Loro E, Briggs ER, et al. The nuclear receptor Rev-erbα controls circadian thermogenic plasticity. Nature 2013;503(7476):410–3.
[26] Zhang R, Lahens NF, Ballance HI, Hughes ME, Hogenesch JB. A circadian gene expression atlas in mammals: implications for biology and medicine. Proc Natl Acad Sci Unit States Am 2014;111(45):16219–24.
[27] Zvonic S, Ptitsyn AA, Conrad SA, Scott LK, Floyd ZE, Kilroy G, et al. Characterization of peripheral circadian clocks in adipose tissues. Diabetes 2006;55(4):962–70.
[28] Chappuis S, Ripperger JA, Schnell A, Rando G, Jud C, Wahli W, et al. Role of the circadian clock gene Per2 in adaptation to cold temperature. Mol Metab 2013;2(3):184–93.
[29] u Din M, Raiko J, Saari T, Kudomi N, Tolvanen T, Oikonen V, et al. Human brown adipose tissue [15O]O2 PET imaging in the presence and absence of cold stimulus. Eur J Nucl Med Mol Imag 2016;43(10):1878–86.
[30] Muzik O, Mangner TJ, Leonard WR, Kumar A, Janisse J, Granneman JG. 15O PET measurement of blood flow and oxygen consumption in cold-activated human Brown fat. J Nucl Med 2013;54(4):523–31.
[31] Sanchez-Delgado G, Martinez-Tellez B, Olza J, Aguilera CM, Labayen I, Ortega FB, et al. Activating brown adipose tissue through exercise (ACTIBATE) in young adults: rationale, design and methodology. Contemp Clin Trials 2015;45:416–25.
[32] Martinez-Tellez B, Sanchez-Delgado G, Garcia-Rivero Y, Alcantara JMA, Martinez-Avila WD, Muñoz-Hernandez MV, et al. A new personalized cooling protocol to activate brown adipose tissue in young adults. Front Physiol 2017;8(863).
[33] Roza AM, Shizgal HM. The Harris Benedict equation reevaluated: resting energy requirements and the body cell mass. Am J Clin Nutr 1984;40(1):168–82.
[34] Fullmer S, Benson-Davies S, Earthman CP, Frankenfield DC, Gradwell E, Lee PSP, et al. Evidence analysis library review of best practices for performing indirect calorimetry in healthy and non-critically ill individuals. J Acad Nutr Diet 2015;115(9):1417–46.
[35] Sanchez-Delgado G, Alcantara JMA, Ortiz-Alvarez L, Xu H, Martinez-Tellez B, Labayen I, et al. Reliability of resting metabolic rate measurements in young adults: impact of methods for data analysis. Clin Nutr 2017;37(5):1618–24.
[36] Alcantara JMA, Sanchez-Delgado G, Martinez-Tellez B, Merchan-Ramirez E, Labayen I, Ruiz JR. Congruent validity and inter-day reliability of two breath by breath metabolic carts to measure resting metabolic rate in young adults. Nutr Metabol Cardiovasc Dis 2018;28(9):929–36.
[37] Weir JBDB. New methods for calculating metabolic rate with special reference to protein metabolism. J Physiol 1949;109(1–2):1–9.
[38] Frayn KN. Calculation of substrate oxidation rates in vivo from gaseous exchange. J Appl Physiol 1983;55(2):628–34.
[39] Westerterp KR. Energy balance in motion. Berlin, Heidelberg: Springer; 2013.
[40] Alcantara JMA, Sanchez-delgado G, Amaro-gahete FJ, Galgani JE, Ruiz JR. Impact of the method used to select gas exchange data for estimating the

resting metabolic rate, as supplied by breath-by-breath metabolic carts. Nutrients 2020;12(2).
[41] ISO-standard 9886:2004. Ergonomics – evaluation of thermal strain by physiological measurements. Geneva, Switzerland. Geneva, Switzerland: International Standards Organization; 2004. p. 1–21.
[42] Boon MR, Bakker LEH, van der Linden RAD, Pereira Arias-Bouda L, Smit F, Verberne HJ, et al. Supraclavicular skin temperature as a measure of 18F-FDG uptake by BAT in human subjects. PloS One 2014;9(6):e98822.
[43] Martinez-Tellez B, Sanchez-Delgado G, Acosta FM, Alcantara JMA, Boon MR, Rensen PCN, et al. Differences between the most used equations in BAT-human studies to estimate parameters of skin temperature in young lean men. Sci Rep 2017;7(1):1–12.
[44] Martinez-Tellez B, Quesada-Aranda A, Sanchez-Delgado G, Fernández-Luna JM, Ruiz JR. Temperatus ® software: a new tool to efficiently manage the massive information generated by iButtons. Int J Med Inf 2019;126:9–18.
[45] Chen KY, Cypess AM, Laughlin MR, Haft CR, Hu HH, Bredella MA, et al. Brown adipose reporting criteria in imaging STudies (BARCIST 1.0): recommendations for standardized FDG-PET/CT experiments in humans. Cell Metabol 2016;24(2):210–22.
[46] Martinez-Tellez B, Sanchez-Delgado G, Garcia-Rivero Y, Alcantara JMA, Martinez-Avila WD, Muñoz-Hernandez MV, et al. A new personalized cooling protocol to activate brown adipose tissue in young adults. Front Physiol 2017;8(863).
[47] Leitner BP, Huang S, Brychta RJ, Duckworth CJ, Baskin AS, McGehee S, et al. Mapping of human brown adipose tissue in lean and obese young men. Proc Natl Acad Sci Unit States Am 2017;114(32):8649–54.
[48] Acosta FM, Sanchez-Delgado G, Martinez-Tellez B, Migueles JH, Amaro-Gahete FJ, Rensen PCN, et al. Sleep duration and quality are not associated with brown adipose tissue volume or activity-as determined by 18F-FDG uptake, in young, sedentary adults. Sleep 2019;42(12).
[49] Martinez-Tellez B, Nahon KJ, Sanchez-Delgado G, Abreu-Vieira G, Llamas-Elvira JM, van Velden FHP, et al. The impact of using BARCIST 1.0 criteria on quantification of BAT volume and activity in three independent cohorts of adults. Sci Rep 2018;8(8567).
[50] Acosta FM, Martinez-Tellez B, Blondin DP, Haman F, Rensen PCN, Llamas-Elvira JM, et al. Relationship between the daily rhythm of distal skin temperature and Brown adipose tissue 18F-FDG uptake in young sedentary adults. J Biol Rhythm 2019;34(5):533–50.
[51] Tschöp MH, Speakman JR, Arch JRS, Auwerx J, Brüning JC, Chan L, et al. A guide to analysis of mouse energy metabolism. Nat Methods 2012;9:57–63.
[52] Gonzalez RR, Blanchard LA. Thermoregulatory responses to cold transients: effects of menstrual cycle in resting women. J Appl Physiol 1998;85(2):543–53.
[54] Lowell BB, Spiegelman BM. Towards a molecular understanding of adaptive thermogenesis. Nature 2000;404(6778):652–60.
[55] Wijers SLJ, Saris WHM, Van Marken Lichtenbelt WD. Individual thermogenic responses to mild cold and overfeeding are closely related. J Clin Endocrinol Metab 2007;92(11):4299–305.
[56] Morrison SF, Madden CJ, Tupone D. Central neural regulation of Brown adipose tissue thermogenesis and energy expenditure. Cell Metabol 2014;19(5):741–56.
[57] Villarroya F, Cereijo R, Villarroya J, Giralt M. Brown adipose tissue as a secretory organ. Nat Rev Endocrinol 2017;13(1):26–35.
[58] Sun L, Yan J, Goh HJ, Govindharajulu P, Verma S, Michael N, et al. Fibroblast growth factor-21, leptin, and adiponectin responses to acute cold-induced Brown adipose tissue activation. J Clin Endocrinol Metab 2020;105(3):520–31.
[59] Carper D, Coué M, Nascimento EBM, Barquissau V, Lagarde D, Pestourie C, et al. Atrial natriuretic peptide orchestrates a coordinated physiological response to fuel non-shivering thermogenesis. Cell Rep 2020;32(8):108075.
[60] Janssen LGM, Jauhiainen M, Olkkonen VM, Haridas PAN, Nahon KJ, Rensen PCN, et al. Short-term cooling increases plasma ANGPTL3 and ANGPTL8 in young healthy lean men but not in middle-aged men with overweight and prediabetes. J Clin Med 2019;8(1214).
[61] Parcha V, Patel N, Gutierrez OM, Li P, Gamble KL, Musunuru K, et al. Chronobiology of natriuretic peptides and blood pressure in lean and obese individuals. J Am Coll Cardiol 2021;77(18):2291–303.
[62] Gavrila A, Peng CK, Chan JL, Mietus JE, Goldberger AL, Mantzoros CS. Diurnal and ultradian dynamics of serum adiponectin in healthy men: comparison with leptin, circulating soluble leptin receptor, and cortisol patterns. J Clin Endocrinol Metab 2003;88(6):2838–43.
[63] Yu H, Xia F, Lam KS, Wang Y, Bao Y, Zhang J, et al. Circadian rhythm of circulating fibroblast growth factor 21 is related to diurnal changes in fatty acids in humans. Clin Chem 2011;57(5):691–700.
[64] Lee P, Brychta RJ, Linderman J, Smith S, Chen KY, Celi FS. Mild cold exposure modulates fibroblast growth factor 21 (FGF21) diurnal rhythm in humans: relationship between FGF21 levels, lipolysis, and cold-induced thermogenesis. J Clin Endocrinol Metab 2013;98(1):98–102.
[66] Weststrate JA, Weys PJM, Poortvliet EJ, Deurenberg P, Hautvast JGAJ. Diurnal variation in postabsorptive resting metabolic rate and diet-induced thermogenesis. Am J Clin Nutr 1989;50(5):908–14.
[67] Aschoff J. Circadian rhythms: interference with and dependence on work-rest schedules. In: NIOSH 1981 Jul:13-50. Spectrum Publ; 1981. p. 11–34.
[68] Aschoff J. Circadian control of body temperature. J Therm Biol 1983;8:143–7. Pergamon.
[69] J A, Biebach H, Heise A, Schmidt T. Day-night variation in heat balance. In: Heat loss from animals and man. Butterworths; 1974. p. 147–72.
[70] Aschoff J, Heise A. Thermal conductance in man: its dependence on time of day and on ambient temperature. In: Advances in climatic physiology. Igaku Shoin; 1972. p. 334–48.
[71] van den Berg R, Kooijman S, Noordam R, Ramkisoensing A, Abreu-Vieira G, Tambyrajah LL, et al. A diurnal rhythm in Brown adipose tissue causes rapid clearance and combustion of plasma lipids at wakening. Cell Rep 2018;22(13):3521–33.
[72] Orozco-Solis R, Aguilar-Arnal L, Murakami M, Peruquetti R, Ramadori G, Coppari R, et al. The circadian clock in the ventromedial hypothalamus controls cyclic energy expenditure. Cell Metabol 2016;23(3):467–78.
[73] van der Veen DR, Shao J, Chapman S, Leevy WM, Duffield GE. Diurnal rhythm in glucose uptake in brown adipose tissue inmice revealed by in vito PET-FDG imaging. Obesity 2013;20(7):1527–9.
[74] Kooijman S, van den Berg R, Ramkisoensing A, Boon MR, Kuipers EN, Loef M, et al. Prolonged daily light exposure increases body fat mass through attenuation of brown adipose tissue activity. Proc Natl Acad Sci Unit States Am 2015;112(21):6748–53.
[75] Lee P, Bova R, Schofield L, Bryant W, Dieckmann W, Slattery A, et al. Brown adipose tissue exhibits a glucose-responsive thermogenic biorhythm in humans. Cell Metabol 2016;23(4):602–9.
[76] Kingma BRM, Frijns AJH, Schellen L, van Marken Lichtenbelt WD. Beyond the classic thermoneutral zone: including thermal comfort. Temperature 2014;1(2):142–9.
[77] Schilperoort M, Hoeke G, Kooijman S, Rensen PCN. Relevance of lipid metabolism for brown fat visualization and quantification. Curr Opin Lipidol 2016;27:242–8.