Modulating energy metabolism: pathophysiological aspects and novel interventions

Straat, M.E.

CHAPTER 8

Cold-induced thermogenesis shows a diurnal variation, that unfolds differently in males and females

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ABSTRACT

Context

Cold exposure mobilizes lipids to feed thermogenic processes in organs, including brown adipose tissue (BAT). In rodents, BAT metabolic activity exhibits a diurnal rhythm, which is highest at the start of the wakeful period.

Objective

To investigate whether cold-induced thermogenesis displays diurnal variation in humans, and differs between males and females.

Design

Randomized crossover study.

Participants

Twenty-four young and lean males (n=12) and females (n=12).

Intervention

2.5-hour personalized cooling using water-perfused mattresses in the morning (7:45 AM) and evening (7:45 PM), with one day in between.

Main outcome measures

Energy expenditure (EE) and supraclavicular skin temperature in response to cold exposure.

Results

In males, cold-induced EE was higher in the morning than in the evening (+54±10% *vs.* +30±7%, P=0.05). By contrast, cold-induced EE did not differ between the morning and the evening in females (+37±9% *vs.* +30±10%, P=0.42). Additionally, only in males, supraclavicular skin temperature upon cold increased more in the morning than in the evening (+0.2±0.1°C *vs.* -0.2±0.2°C, P=0.05). In males, circulating free fatty acid (FFA) levels were increased after cold in the morning, but not in the evening (+90±18% *vs.* +9±8%, P<0.001). In females, circulating FFA (+94±21% *vs.* +20±5%, P=0.006), but also triglycerides (+42±5% *vs.* +29±4%, P=0.01) and cholesterol levels (+17±2% *vs.* 11±2%, P=0.05) were more increased after cold exposure in the morning, than in the evening.

Conclusions
Cold-induced thermogenesis is higher in the morning than in the evening in males, however, lipid metabolism is more modulated in the morning than in the evening in females.

INTRODUCTION

The Earth rotates around its own axis in approximately 24 hours, resulting in daily changes in an organism's environment. In order to adapt to these environmental changes, organisms have acquired an internal time-keeping system that regulates an approximate 24 hour rhythm in cellular and behavioral processes, referred to as the circadian rhythm. Circadian rhythms are endogenous and self-sustained, however, they are entrained by external cues. The most important cue is light input on the retina, which is received by the master clock in the suprachiasmatic nucleus (SCN) of the hypothalamus. These circadian rhythms are finetuned via other external cues, such as food intake and temperature changes (1, 2). Disruption of the circadian rhythm, via night-shift work or artificial light exposure at night, increases cardiometabolic disease risk factors in humans (3, 4). This is illustrated by associations between shift work and incidence of metabolic syndrome, type 2 diabetes mellitus and cardiovascular diseases (5-8). To prevent cardiometabolic diseases and develop optimal treatment regimes, knowledge on how circadian rhythms are regulated in metabolic processes is of high importance.

The application of cold exposure to improve metabolic health has gained interest over the last decade, as it increases the metabolic rate via sympathetic activation of thermogenesis in skeletal muscle and brown adipose tissue (BAT) (9). Acute cold exposure increases the uptake and oxidation of fatty acids (FA) and glucose by BAT, associated with an increase in energy expenditure in humans (EE) (10, 11). In addition, cold acclimation improves peripheral insulin sensitivity in humans (12, 13), likely explained by a combination of increased thermogenesis in skeletal muscle (12) and BAT (13, 14). Compared to rodents, humans possess limited amounts of BAT (approx. 1.5 % of total body mass), which is mainly located in the supraclavicular area (15), and shows higher presence in females than in males under non-cold-challenged conditions (16). Nonetheless, the presence of metabolically active BAT on ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography (PET)/computed tomography (CT) is associated with less visceral and liver fat (17), and a lower prevalence of T2DM, dyslipidaemia and cardiovascular diseases (16), underlining the potential of BAT activation to improve cardiometabolic disease.

Interestingly, metabolic activity of murine BAT displays a pronounced circadian rhythmicity, with the highest activity at the onset of the dark phase (*i.e.*, start of the active period for mice), determining the rate at which postprandial lipids can be cleared from the circulation (18). In humans, the postprandial lipid response is also the lowest at the start of their active period (18, 19), suggestive of higher BAT activity in the morning. Taken together, we hypothesize that a circadian rhythm in BAT activity coincides with its responsiveness to therapeutic approaches (*e.g.*, cold exposure), and that timed-therapy may thus be beneficial for activation strategies. Therefore, the aim of this study was to assess whether cold-induced thermogenesis, as determined by the increase in energy expenditure and supraclavicular skin temperature, and cold-induced changes in markers of lipid metabolism differ between the morning and evening in healthy lean males and females.

METHODS

Study design

This study was a randomized, crossover study, using personalized cooling as an intervention, conducted at the Leiden University Medical Center (LUMC) between December 2019 and December 2020. The study was approved by the Medical Ethical Committee of the LUMC and undertaken in accordance with the principles of the revised Declaration of Helsinki. Written informed consent was obtained from all participants prior to participation. The clinical trial is registered at ClinicalTrials.gov NCT04406922.

Participants

Twenty-four participants, namely twelve lean men (BMI: 18-26 kg/m^2, age: 18-31 years old) and twelve lean women (BMI: 18-26 kg/m^2, aged 18-29 years old), were included in this study. Eligibility to participate in the study was tested during a screening that consisted of anthropometry, a questionnaire on medical history, and an overnight fasted blood sample. Exclusion criteria were the presence of any active endocrine, renal or hepatic disease, the use of medication known to influence glucose and/or lipid metabolism or BAT activity, smoking, abuse of alcohol or other substances, pregnancy, recent weight change or a disturbed day-night-rhythm in the last two weeks (*e.g.,* working in night shifts or having a jetlag). Participants were asked to withhold from vigorous exercise 48 hours preceding the study days and to not drink alcohol or drinks with caffeine 24 hours preceding the study days. In addition, they were instructed to eat a standardized meal (prepared supermarket meal including pasta or noodles, ranging from 450-600 kcal) in the evening (between 09:00-10:00 PM hours) and in the morning (between 09:00-10:00 AM hours) before the morning and the evening experiment,

respectively, and not to eat or drink (with an exception for water) anything afterwards until completion of the study day.

Randomization

Participants were exposed to a personalized cooling procedure twice: once in the morning (starting at 07:45 AM) and once in the evening (starting at 07:45 PM), with a single day between these two study days (**Figure 1**). Participants were randomized to determine which experimental study day they would have first: the morning or the evening. Due to COVID-19-related restrictions many participants that were initially included and randomized for the study did eventually not participate. Newly included participants were randomized again, which led to unequal distribution over the two arms (both males and females: 5 in the morning-evening group and 7 in the evening-morning group).

Anthropometric measurements

At the start of the study visit, body weight (measured with a digital balance; E1200, August Sauter GmBH, Albstadt, Germany), height, waist circumference and hip circumference were obtained. Waist-hip-ratio (WHR) was calculated as: 'waist circumference'/'hip circumference'. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m^2). Body surface area (BSA) was calculated according to the Bois formula ($0.007184 * height\ (cm)^{0.725} * weight\ (kg)^{0.452}$) (20). In addition, body composition (fat mass and fat percentage) was estimated using bioelectrical impedance analysis (InBody720, InBody CO., Ltd., CA, USA).

Cooling procedure

After anthropometric measurements were performed, wireless iButton temperature loggers (iButton®, Maxim Integrated Products, Inc, San Jose, CA, USA) were attached to 17 positions to measure skin temperature (21), and an infrared thermographic image (IRT) of the upper thorax/neck region was made (see below for further details). Next, an intravenous cannula was placed in the antecubital vein to sample blood at six time points during the study procedure (**Figure 1**). Then, participants laid down in bed, between two mattresses filled with water (Blanketrol® III, Cincinnati Sub-Zero Products, Inc, Cincinnati, Ohio, USA) with an initial temperature of 32°C (considered thermoneutrality). After 15 minutes of thermoneutrality, energy expenditure (EE) assessment by indirect calorimetry was started as described below. After 45 minutes of thermoneutrality a personalized cooling protocol was applied (22). Water temperature was lowered with 5°C every 10 minutes until shivering occurred, or until the minimum temperature of 9°C was reached. At this moment, the first EE assessment was stopped and the water temperature was increased with 2-3°C. In case of shivering, the water

temperature was further increased with steps of 1-2°C until shivering just stopped. Shivering was defined as an involuntary contraction of the muscles as reported by the participant and visually checked by the researcher. The following 'stable cold'-phase lasted for another 90 minutes, the last 30 minutes of which EE was assessed again. Finally, a second IRT image of the upper thorax/neck region was made.

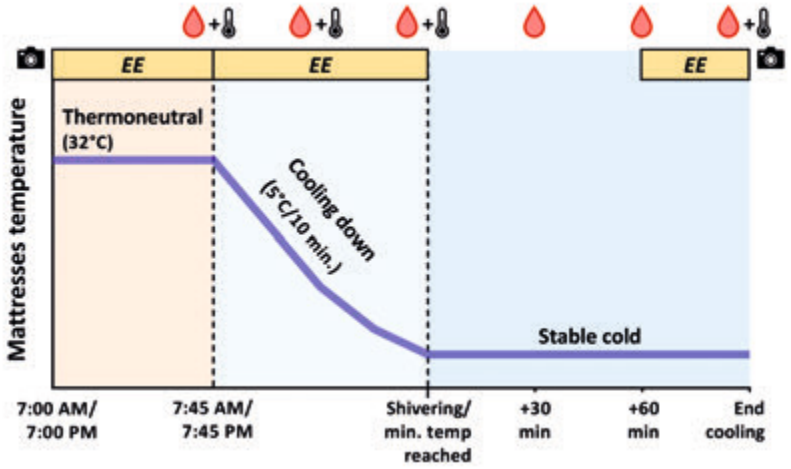

Figure 1. Time line of the study procedures performed both days.
Participants laid in bed between two water perfused mattresses with an initial temperature of 32°C (considered thermoneutrality; mattresses temperature is indicated with the purple solid line). After 45 minutes temperature was gradually decreased with 5°C until shivering occurred or until the minimum temperature of 9°C was reached. Then, the stable cold phase started. Before and after cold, an infrared thermographic picture was made. Blood was drawn at the end of thermoneutrality (indicated with the drop icon), after 15 minutes of cooling down, at the onset of shivering and at 3 time points during the stable cold phase. Energy expenditure (indicated with the yellow rectangle) was assessed during the thermoneutral phase, during cooling down and during the last 30 minutes of the stable cold phase (from +60 min until the end of cooling) using indirect calorimetry. Skin temperatures were measured every minute using iButtons. For analyses concerning skin temperature, the last 5 minutes of the thermoneutral phase, the 5 minutes after the first 10 minutes of cooling down, the 5 minutes before shivering, and the last 5 minutes of the stable cold phase were averaged (indicated with the thermometer icon).

Energy expenditure

EE was assessed by indirect calorimetry using a metabolic cart (Vyntus™ CPX, Carefusion, Hochberg, Germany) equipped with a ventilated hood system to collect total carbon dioxide production (VCO2) and oxygen consumption (VO2) every 10 seconds. EE was estimated with the Weir formula (ignoring urinary nitrogen excretion): EE (kcal/day) = (3.941*VO_2(L/min)) + (1.106*VCO_2(L/min))*1440. The respiratory exchange ratio

(RER) was determined by: RER = VCO2 / VO2. EE and RER were calculated for four phases throughout the experiment: the thermoneutral phase, the cooling down phase, the stable cold phase (60-77.5 min after shivering started; *i.e.*, +60 min) and at the end of cooling (77.5-90 min after shivering started; *i.e.*, end cooling; **Figure 1**). The first 5 minutes gas exchange data of every new recording was discarded (*i.e.*, the first 5 min of the thermoneutral measurement and the first 5 min of the stable cold measurement). Thereafter, for the thermoneutral and the two stable cold phases, the most stable 5 minutes were selected for further analyses, as previously described (23). For the cooling down phase, from the measured gas exchange, the first 5 minutes and the last 5 minutes data were excluded to avoid possible artefacts produced by the cooling down protocol (e.g., VO_2 and/or VCO_2 peaks). Then, from the remaining gas exchange data, the first 15 minutes period and the last 15 minutes period were averaged to estimate the midpoint during the cooling down phase.

Skin temperature

Skin temperature of 17 positions of the body was measued using 1 minute intervals with wireless iButton temperature loggers (21). We assessed supraclavicular skin temperature and used previously described equations to estimate the mean (21, 24), proximal (21) and distal (21, 25) skin temperatures and to calculate a peripheral temperature gradient (left forearm-left fingertip) as a proxy of peripheral vasoconstriction (26). All data were analyzed using Temperatus® software (http://profith.ugr.es/temperatus) (21). The averages of the last 5 minutes of thermoneutrality, the 5 minutes after the first 10 minutes of cooling down, 5 minutes before shivering, and the last 5 minutes of the stable cold phase (*i.e.*, 'end cooling') were taken for analyses. Additionaly, once at the start of the study visit and once after cold exposure, IRT images were made (FLIR T450sc, FLIR systems inc., Wilsonville, Oregon, USA) from the upper-thorax/neck region. We used an open-source IRT toolbox software for image alignment, non-rigid image registration and region of interest (ROI) segmentation using the image before cold in the morning as reference to obtain mean skin temperatures of the supraclavicular region.

Blood samples

Blood was collected after a fasting period of 10 h prior to the start of the study protocol. Blood was drawn from the antecubital vein and obtained with Vacutainer® SST™ II Advance tubes. After a clotting time of at least 30 min, samples were centrifuged to obtain serum. Samples were aliquoted and stored at -80°C until batch-wise analyses. Commercially available enzymatic kits were used to measure serum concentrations of triglycerides (TGs), total cholesterol, high-density lipoprotein cholesterol (HDL-C) (all Roche Diagnostics, Woerden, the Netherlands), free fatty acids (FFA) (Wako chemicals, Nuess, Germany) and glucose (Instruchemie, Delfzijl, the Netherlands).

Questionnaires

In the week prior to the first study day, participants filled in two questionnaires, the Munich Chronotype questionnaire (MCTQ) (27) and the Pittsburgh Sleep Quality Index (PSQI) (28), to determine their chronotype and their sleep quality, respectively. Chronotype was defined as the midpoint between sleep onset and wake up on work-free days, corrected by sleep deprivation on workways (MSFsc). Correction for sleep deprivation was only performed for participants who slept longer on work-free days than on workdays. The correction was calculated by subtracting half of the oversleep on work-free days from the average sleep duration across the entire week. This method is described in detail elsewhere (29).

Statistical analysis

Statistical analyses were performed with SPSS® Statistics (version 25, IBM® Corporation, Armonk, NY, USA). Normal distribution of the data was tested using the Shapiro-Wilk test, visual histograms, and Q-Q plots. Non-normally distributed variables (serum TG) were log10-transformed before further analysis. Baseline characteristics were compared between sexes using two-tailed unpaired Student's *t*-test. For the comparison in the morning *vs.* in the evening of the effect of cold on EE, skin temperatures and serum markers, a general linear model with repeated measures was used with two within-subject factors: moment of the day (morning *vs.* evening) and the cooling phase (*i.e.*, thermoneutral, cooling down, stable cold +60 min and end of cooling). For the comparison of skin temperatures we added 'shivering' instead of 'stable cold +60 min'. For the serum markers we added 'shivering' and 'stable cold +30 min'. *P*-values are shown for main effects and interactions. Percentual changes in EE (hereafter cold-induced EE) and serum markers after cold exposure were calculated using the following formula: ('end cooling'-'thermoneutral')/'thermoneutral'*100%. Absolute changes in skin temperature after cold exposure (deltas; Δ) were calculated as: 'end cooling'-'thermoneutral'. To compare the changes after cold exposure between the morning and the evening two-tailed paired Student's *t*-tests were used. A *P*-value of $P \leq 0.05$ was considered statistically significant. All data are presented as mean ± standard error of the mean. All figures were prepared with Prism 9 for Windows (version 9.0.1, 2021, GraphPad Software, LLC, San Diego, California, USA). All supplemental figures are located in a digital research materials repository (30).

RESULTS

Participant characteristics

One female participant withdrew from the trial after the first study day because of back pain during the cooling experiment and was replaced by another female participant. In

total, we enrolled 24 young lean white Caucasians (**Table 1**), namely 12 males (age: 22.8 ± 1.0 years; BMI: 22.0 ± 0.6 kg/m^2) and 12 females (age: 21.4 ± 0.9 years; BMI: 22.5 ± 0.6 kg/m^2). For one male, values of waist and hip circumference were missing, leaving n=11 for those two outcomes. Males were taller (1.84±0.02 m *vs.* 1.74±0.01 m, *P*<0.001), had a larger BSA (2.0±0.0 m^2 *vs.* 1.8±0.0 m^2, *P*=0.009), a lower waist-to-hip ratio (0.76±0.01 *vs.* 0.78±0.01, *P*<0.001) and a lower fat percentage (13.7±1.0 % *vs.* 26.7±1.2 %, *P*<0.001) compared to females. There were no differences in self-reported wake time in the week prior to the study days between males and females (7:58 ± 00:30 hours *vs.* 8:08 ± 00:13 hours, *P*=0.76), nor in chronotype as estimated using the corrected midpoint of sleep on work-free days (04:58 ± 00:17 hours *vs.* 05:02 ± 00:13 hours, *P*=0.85) (31), nor in sleep quality as assessed with the PSQI (6.9 ± 1.2 points *vs.* 8.9 ± 1.0 points, *P*=0.21).

Table 1. Participant characteristics

	Male (n=12)	**Female (n=12)**
Age, years	22.8 ± 1.0	21.4 ± 0.9
Height, m	1.84 ± 0.02	1.74 ± 0.01 ***
Weight, kg	74.1 ± 2.9	68.2 ± 1.2
BMI, kg/m^2	22.0 ± 0.6	22.5 ± 0.6
BSA, m^2	2.0 ± 0.0	1.8 ± 0.0 **
Waist circumference, cm	79.3 ± 1.8	74.1 ± 0.7 *
Hip circumference, cm	98.4 ± 1.7	100.0 ± 1.1
Waist-to-hip ratio	0.76 ± 0.01	0.78 ± 0.01 ***
Fat mass, kg	10.3 ± 1.0	18.0 ± 0.9 ***
Fat percentage, %	13.7 ± 1.0	26.7 ± 1.2 ***
Self-reported wake time, hh:mm	7:58 ± 0:30	8:08 ± 0:13
MSFsc, hh:mm	04:58 ± 00:17	05:02 ± 00:13
PSQI, total score	6.9 ± 1.2	8.9 ± 1.0

Values are presented as mean ± standard error of the mean; the self-reported wake time indicates the average of the 7 days prior to the first study day; P-values are obtained from unpaired Student's t-test male vs. female. * P-value <0.05; ** P-value <0.01 *** P -value <0.001. BMI: Body mass index, BSA: Body surface area, MSFsc: Midpoint of sleep on work-free days, corrected for sleep deprivation (correction for sleep deprivation was only performed for participants who slept longer on work-free days than on workdays. The correction was calculated by subtracting half of the oversleep on work-free days from the average sleep duration across the entire week), PSQI: Pittsburgh Sleep Quality Index.

Females have a longer time to shivering and lower shivering temperature in the morning than in the evening

In males, the time from the start of cooling down until shivering tended to be longer in the morning than in the evening (52±3 min *vs.* 46±4 min, *P*=0.07, **Figure 2A**). Yet, their shivering temperature did not differ between the morning and the evening (11±1°C *vs.*

13±1°C, *P*=0.17), and neither did the water temperature at the end of the stable cold period between morning and evening (17±1°C *vs.* 18±1°C, *P*=0.38). In females, the time from the start of cooling down until shivering was significantly longer in the morning than in the evening (46±4 min *vs.* 35±4 min, *P*=0.04), accompanied by a lower shivering temperature in the morning than in the evening (13±1°C *vs.* 16±1°C, *P*=0.03) and a lower water temperature at the end of the stable cold period (morning: 13±1°C *vs.* evening: 19±1°C, *P*=0.001) in the morning compared to the evening (**Figure 2B**).

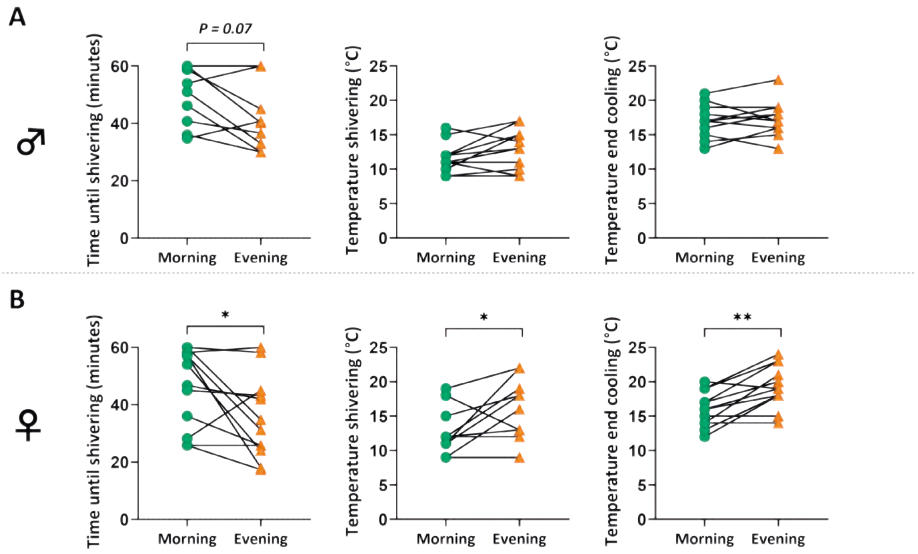

Figure 2. The time until shivering and the shiver- and stable cold temperatures during cold exposure in the morning vs. the evening in males and females.
From left to right: the time from the start of the personalised cooling until shivering occurred, the water temperature at the moment of shivering, and the water temperature at the end of cooling, in males (A) and in females (B). A paired Student's t-test was used to compare the morning and the evening. *P<0.05, **P<0.01.

Cold-induced energy expenditure is higher in the morning than in the evening in males only

To investigate whether diurnal variation is present in cold-induced EE, we compared the course of the change in EE during cold exposure between the morning and the evening. The gas exchange measurement failed for one male and one female due to technical issues, leaving a total of 11 males and 11 females for these analyses. In males, the increase in EE during cold exposure differed between morning and evening (*P interaction*=0.04). More specifically, cold-induced EE was higher in the morning than in the evening (+54±10 % *vs.* +30±7 %, *P*=0.05, **Figure 3A**). In females, the increase in EE during cold exposure was similar between morning and evening (*P interaction*=0.21, +37±9% *vs.* +30±10%,

P=0.42, **Figure 3B**). In males, RER did not change after cold exposure in the morning (thermoneutral [TN]: 0.81±0.10 *vs.* stable cold: 0.79±0.09, *P*=1.00) nor in the evening (TN: 0.77±0.10 *vs.* stable cold: 0.79±0.07, *P*=1.00). Also in females, RER did not change after cold exposure in the morning (TN: 0.79±0.09 *vs.* stable cold: 0.79±0.05, *P*=1.00) nor in the evening (TN: 0.75±0.08 *vs.* stable cold: 0.76±0.04, *P*=1.00).

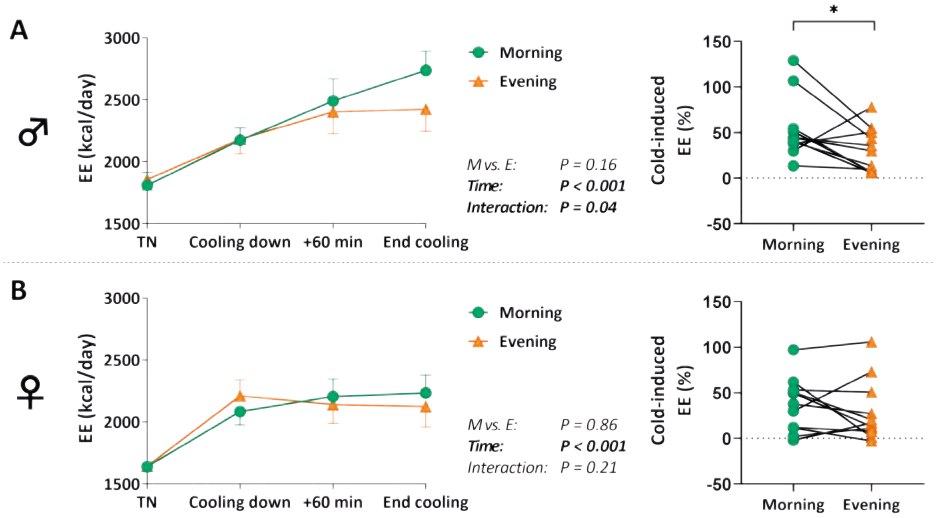

Figure 3. Increase in energy expenditure during cold exposure in the morning vs. the evening in males and females.

Energy expenditure (EE) was assessed during the thermoneutral phase, the cooling down phase and the stable cold phase (the latter was divided into two parts: the first part starting at +60 min and the second part until the end of cooling). The cold-induced change in EE was calculated as the percentual change in EE from thermoneutrality to the end of cooling. Top panel shows results in males (A), bottom panel shows results in females (B). General linear model with repeated measures was used to test for an interaction between the effect of cold over time and the moment of the day (i.e., 'M vs. E': morning vs. evening), a paired Student's t-test was used to compare the cold induced change in EE between the morning and the evening. TN=thermoneutral.

Supraclavicular skin temperature increases following cold exposure in the morning and decreases in the evening in males only

As an indirect proxy of thermogenesis by BAT we determined the change in temperature of the supraclavicular skin area. One male and one female participant were excluded from all iButton analyses, due to a problem with the data acquisition. In males, we observed a different change in supraclavicular skin temperature during cold exposure in the morning compared to the evening (*P interaction*=0.04, **Figure 4A**). More specifically, supraclavicular skin temperature was more likely to increase in the morning, but to decrease in the evening (+0.2±0.1 °C *vs.* -0.2±0.2 °C, *P*=0.05, **Figure 4A**). In females, the

change in supraclavicular skin temperature during cold exposure was similar between the morning and the evening (*P*=0.07, **Figure 4B**). More specifically, supraclavicular skin temperature increased equally in the morning and in the evening (+0.3±0.2 °C *vs.* +0.2±0.1 °C, *P*=0.51, **Figure 4B**). The direction of the changes in supraclavicular skin temperatures as measured with iButtons were confirmed using IRT (**Supplementary figure S1A** and **S1B** (30)). Furthermore, in males, proximal, distal and mean skin temperatures decreased and the peripheral vasoconstriction gradient increased similarly in the morning and the evening (*P interaction*=0.78, 0.15, 0.38, 0.35, respectively; **Supplementary figure S2A-D** (30)). In females however, mean skin temperature (morning: -6.3±0.4°C *vs.* evening: -4.9±0.5°C, *P*=0.02) and distal skin temperature decreased more in the morning than in the evening (-8.5±0.7°C *vs.* -5.6±0.7°C, *P*<0.001), and peripheral vasoconstriction gradient (*i.e.*, forearm-fingertip) increased more in the morning than in the evening (+6.7±1.2 *vs.* +1.4±1.4, *P*<0.001, **Supplementary figure S2F-H** (30)). Curiously, this was not observed for the proximal skin temperature (*P interaction*=0.45, **Supplementary figure S2E** (30)).

Circulating FFAs continuously increase during cold in the morning, but reach a top in the evening in both sexes

Since cold exposure modulates lipoprotein metabolism (32), we aimed to assess whether cold-induced effects on plasma lipids were different in the morning compared to the evening. For two females the blood sample of one timepoint was missing due to constricted veins during cold exposure, leaving n=10 for the analyses in females. In males, cold exposure increased circulating FFA levels only in the morning, but not in the evening (+90±18% *vs.* +9±8 %, *P*<0.001; **Table 2**), while cold-induced effects on TG, total cholesterol and HDL-C did not differ. Circulating FFA levels did initially increase during cold exposure in the evening, but decreased after shivering had occurred, while levels continued to increase in the morning (*P interaction*<0.001, **Supplementary figure S3A** (30)). In females, cold exposure increased circulating FFA (+94±21 % *vs.* +20±5 %, *P*=0.006), TG (+42±5 % *vs.* +29±4 %, *P*=0.01), total cholesterol (+17±2 % *vs.* 11±2 %, *P*=0.05) and HDL-C (+14±2 % *vs.* +9±1 %, *P*=0.03) more in the morning compared to the evening (**Table 2, Supplementary figure S3E-H** (30)). For both males and females, the relative and absolute changes in lipids did not correlate with the relative and absolute changes in cold-induced energy expenditure, respectively (data not shown).

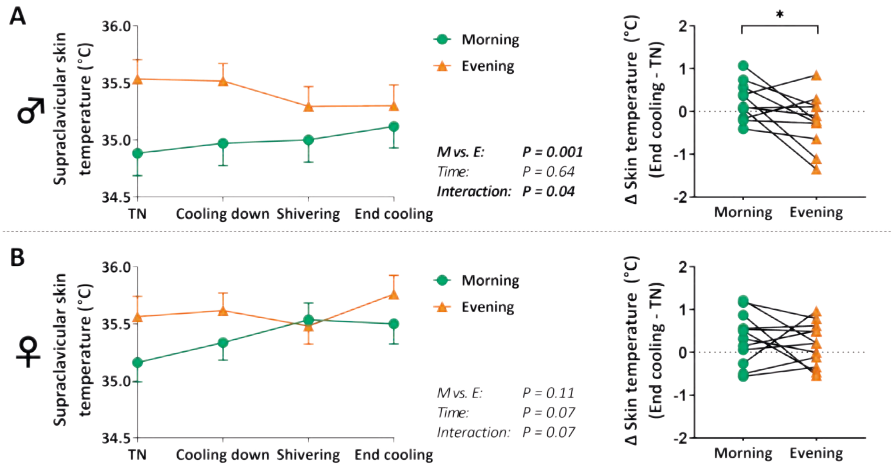

Figure 4. Changes in supraclavicular skin temperature during cold exposure in the morning vs. the evening in males and females.

Supraclavicular skin temperature was measured using wireless iButtons during the last 5 minutes of the thermoneutral phase, during the cooling down phase, right before shivering occurred and during the last 5 minutes of the stable cold phase ('end cooling'). The Δ temperature was calculated as the change in supraclavicular temperature from thermoneutrality to the end of cooling. Top panel shows results in males (A), bottom panel shows results in females (B). General linear model with repeated measures was used to test for an interaction between the effect of cold over time and the moment of the day (i.e., 'M vs. E': morning vs. evening), a paired Student's t-test was used to compare the cold-induced EE in the morning vs. in the evening. TN=thermoneutral.

Table 2. The effect of cold exposure on measures of lipoprotein metabolism and glucose in the morning and the evening in males and females.

Thermoneutral		Morning			Evening			
		End of cooling	Percentual change (%)	Thermoneutral	End of cooling	Percentual change (%)	*P*-value	
Males (n=12)	Triglycerides, mmol/L	0.54±0.09	0.68±0.10	+41±14**	0.44±0.06	0.61±0.08	+40±7***	*P*=0.95
	Free fatty acids, mmol/L	0.33±0.03	0.59±0.04	+90±18***	0.67±0.04***	0.72±0.05	+9±8	***P*<0.001**
	Total cholesterol, mmol/L	3.41±0.19	3.93±0.22	+16±2***	3.48±0.20	3.95±0.22	+14±1***	*P*=0.49
	HDL-C, mmol/L	1.05±0.07	1.19±0.07	+14±2***	1.07±0.06	1.20±0.06	+12±1***	*P*=0.50
	Glucose, mmol/L	5.62±0.08	5.61±0.15	-0.2±1.9	5.08±0.08***	5.26±0.09	+3.7±1.3*	*P*=0.09
Females (n=12)	Triglycerides, mmol/L	0.52±0.08	0.74±0.11	+42±5***	0.43±0.04	0.56±0.07	+29±4***	***P*=0.01**
	Free fatty acids, mmol/L	0.40±0.04	0.74±0.09	+94±21***	0.85±0.05***	1.00±0.04	+20±5**	***P*=0.006**
	Total cholesterol, mmol/L	4.10±0.16	4.71±0.16	+17±2***	4.35±0.19**	4.83±0.21	+11±2***	***P*=0.05**
	HDL-C, mmol/L	1.46±0.09	1.67±0.10	+14±2***	1.55±0.10*	1.68±0.10	+9±1***	***P*=0.03**
	Glucose, mmol/L	5.29±0.06	5.30±0.08	+0.3±1.1	4.82±0.10**	4.84±0.13	+0.5±1.8	*P*=0.92

Values are presented as mean ± standard error of the mean; Significance levels indicated with asterisks are obtained from paired Student's t-test comparing thermoneutral in the morning vs. evening, and comparing thermoneutral vs. end of cooling in the morning and the evening. P-values in the last column are obtained from paired Student's t-test of the relative change from thermoneutral to the end of cooling in the morning vs. the evening. ** P-value <0.01 *** P -value <0.001. HDL-C: High-density lipoprotein cholesterol.

DISCUSSION

In this study we showed that cold-induced thermogenesis, as assessed with cold-induced EE and supraclavicular skin temperature, follows a diurnal variation in males, with a higher activity in the morning than in the evening. In females we did not observe diurnal variation in cold-induced thermogenesis, although females reached a colder shivering temperature in the morning than in the evening, suggestive of a better cold tolerance in the morning. In both sexes, serum FFA levels increased more upon cold in the morning than in the evening, but TGs, cholesterol and HDL-C also increased more in the morning than in the evening in females only. Collectively, this may support that thermogenic tissues, such as BAT, are subjected to a diurnal rhythm and that this unfolds differently in males and females. The diurnal variation should be considered when targeting thermogenic tissues to improve cardiometabolic health.

The finding that cold-induced thermogenesis is higher in the morning than in the evening, at least in males, coincides with earlier pre-clinical and clinical studies. In mice, BAT is the main contributor to cold-induced thermogenesis. Previously, we have shown that the uptake of TG-derived fatty acids by BAT in wild-type male mice follows a diurnal rhythm, with the highest uptake at the onset of the dark phase (*i.e.*, the active period of mice) (18). Similarly, glucose uptake by murine BAT was shown to peak at the end of the light phase, approx. 3 h before the start of the active period in both male and female mice (33). A very recent paper suggests that cold-induced thermogenesis in humans is higher in males with high BAT activity compared to males with low BAT activity as assessed by ^{18}F-FDG-PET/CT scans, but only in the morning (34). To the contrary, another recent study did not find a diurnal variation in cold-induced thermogenesis (35). This seeming discrepancy is possibly explained by the fact that cold exposure in that study only lasted for 65 minutes and data from males and females were pooled, whereas we observe the diurnal variation after more than 60 minutes with differences between the two sexes. The lower shivering temperature in females together with a stronger peripheral vasoconstriction (as estimated using the peripheral temperature gradient, *i.e.* temperature forearm - fingertip) upon cold in the morning compared to the evening, suggests that women could tolerate cold temperatures better in the morning. Interestingly, increased peripheral vasoconstriction is associated with higher BAT activity in humans (36). Nevertheless, we did not observe differences in cold-induced EE between the morning and the evening in females.

In this study we could not directly assess BAT activity using ^{18}F-FDG PET/CT scans, as this widely accepted method is accompanied with radiation burden that restricts its use for repeated measures. Therefore, we used cold-induced EE and supraclavicular skin

temperature as proxies. We acknowledge that skeletal muscles are additional important contributors to cold-induced EE in humans (37, 38), and that – for example – thickness of the skin may cause bias to IRT (39). Nevertheless, rather strong correlations between ^{18}F-FDG uptake by BAT on PET/CT scans and the increase in EE (40, 41) and the change in supraclavicular skin temperature after cold exposure (42-44) have been reported. Irrespective of the magnitude of the contribution of BAT to cold-induced thermogenesis, our data suggest that its metabolic activity exhibits diurnal variation.

We found that the increase in circulating FFA concentration from baseline to the end of cooling was higher in the morning compared to the evening in both males and females. Notably, this difference was caused by a drop in circulating FFAs during the last part of cold exposure in the evening, rather than by a steeper increase during cold exposure in the morning (**Supplementary figure S3A and S3E** (30)). An increase in FFAs after cold exposure has been observed in other human studies (32, 45-48) and is caused by activation of the sympathetic nervous system that stimulates lipolysis in WAT (49, 50), likely to mobilize FFAs for oxidation in thermogenic tissues. Circulating FFA concentration is therefore a reflection of both FFA release and FFA uptake by thermogenic tissues. Importantly, inherent to the research design, participants arrived at the research center at 6:30 AM for the morning session, which is notably earlier than the regular self-reported wake time. Sleep deprivation can result in an increase in early-morning circulating FFA and noradrenaline levels (51), which may partly explain the higher increase in circulating FFA concentration in the morning than in the evening. Moreover, the oxidative capacity of skeletal muscle is shown to be higher the evening than in the morning (52). Hypothetically, activation of thermogenic processes in skeletal muscles lead to a higher consumption of FFAs in the evening than in the morning, which might explain the drop in FFAs at the end of cold exposure in the evening, but not in the morning. In addition to FFAs, albeit only in females, TG, cholesterol and HDL-C concentrations increased more in the morning compared to the evening after cold exposure. We (22, 32) and others (53) have previously demonstrated that cold exposure increases circulating TG levels. This is probably related to the fact that cold exposure increases FFA supply (54, 55) as well as sympathetic outflow (56) towards to the liver to enhance the synthesis and release of TG-rich very-low-density lipoproteins (VLDL) from the liver into the circulation (32, 54-56). Moreover, cold exposure increases cholesterol levels, which we previously attributed to increased production of large VLDLs and increased small low-density lipoproteins (LDLs) due to enhanced lipolysis (32). Besides, cold exposure increases the enrichment of cholesterol in small HDL particles, likely resulting from increased production of HDL precursors during lipolysis of VLDL, which induce release of cholesterol from tissues into the circulation (32). How the diurnal

variation in the effect of cold exposure on lipid metabolism as specifically observed in females relates to the various metabolic tissues warrants further investigation.

The diurnal variation in the thermogenic response likely associates to variation in core body temperature, that reaches its lowest point in the middle of the night (57, 58). The activation of thermogenesis might be important to increase body temperature at the start of the day, causing BAT to be in a more active state in the morning. Hypothetically, when BAT is in its active state it is better able to increase thermogenesis further in response to cold. The results of the present study indicate that the diurnal variation in cold-induced thermogenesis is of importance when applying cold exposure to improve whole-body metabolism, although this needs to be confirmed using longer studies with repeated cold and a larger study population. For instance, chronotherapy (*i.e.*, the administration of medication or interventions at specific times of the circadian cycle) might be useful when targeting thermogenic organs, such as BAT, to improve cardiometabolic health. Yet, similar to our results with cold-induced thermogenesis, diet-induced thermogenesis (*i.e.*, the increase in metabolic rate after food intake, also associated to BAT activity (59)) has been shown to be higher in the morning than in the evening (60-62). Moreover, in mice as well as humans, postprandial lipid excursions are lower and postprandial fatty acid oxidation is higher in the morning than in the evening (18, 19, 34). These studies show the potential of time-restricted feeding specifically with an early time-frame that is aligned with the circadian clock (63, 64), suggesting that the same could be true when applying cold exposure to improve whole-body lipid metabolism.

One of the main strengths of our study is that we used a randomized crossover design, in which participants were randomly assigned to have the morning or the evening measurement first. Therewith, the influence of acclimation to cold exposure was prevented and participants served as their own control, minimizing the effect of confounding covariates. Moreover, for all participants there was only a single day between the morning and evening experiment, minimizing an effect of seasonal changes or outdoor temperatures on cold-induced thermogenesis. Another strength of this study is the use of the personalized cooling protocol, that allows us to determine diurnal variations in cold tolerance. However, the longer duration until shivering occurred in the morning in females inherently caused exposure to a lower temperature in the morning compared to the evening in females, thus possibly stronger stimulating the thermogenic response. Nonetheless, cold-induced thermogenesis was not higher in the morning than in the evening in females. Other limitations of our study are that the study was not powered to compare males *vs.* females or to compare participants with an early chronotype *vs.* those with a late chronotype, as it was powered for within-

subject comparisons. In addition, only young, healthy white Caucasians were included, inquiring further research to test applicability on a broader population (*e.g.*, older or overweight individuals, patients with cardiometabolic diseases, or other ethnic groups).

In conclusion, we show a diurnal variation in cold-induced thermogenesis, as assessed by cold-induced EE and supraclavicular skin temperature, with a higher activity in the morning than in the evening in males, but not in females. On the other hand, females reached a lower shivering temperature in the morning than in the evening, indicating a higher cold tolerance, together with a stronger modulation whole-body lipid metabolism. Together, this suggests that the application of cold exposure to improve cardiometabolic health has more potential in the morning than in the evening.

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SUPPLEMENTAL DATA

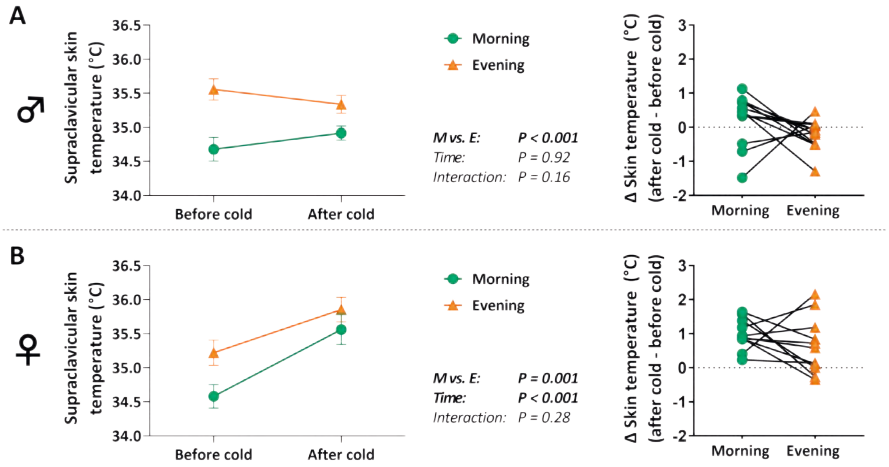

Figure S1. Changes in supraclavicular skin temperature after cold exposure as measured with infrared thermography in the morning vs. the evening in males and females.
Supraclavicular skin temperature was measured using infrared thermography before cold exposure and after cold exposure in the morning and the evening. Top panel shows results in males (A), bottom panel shows results in females (B). General linear model with repeated measures was used to test for an interaction between the effect of cold over time and the moment of the day (i.e., 'M vs. E': morning vs. evening), a paired Student's t-test was used to compare the morning and the evening.

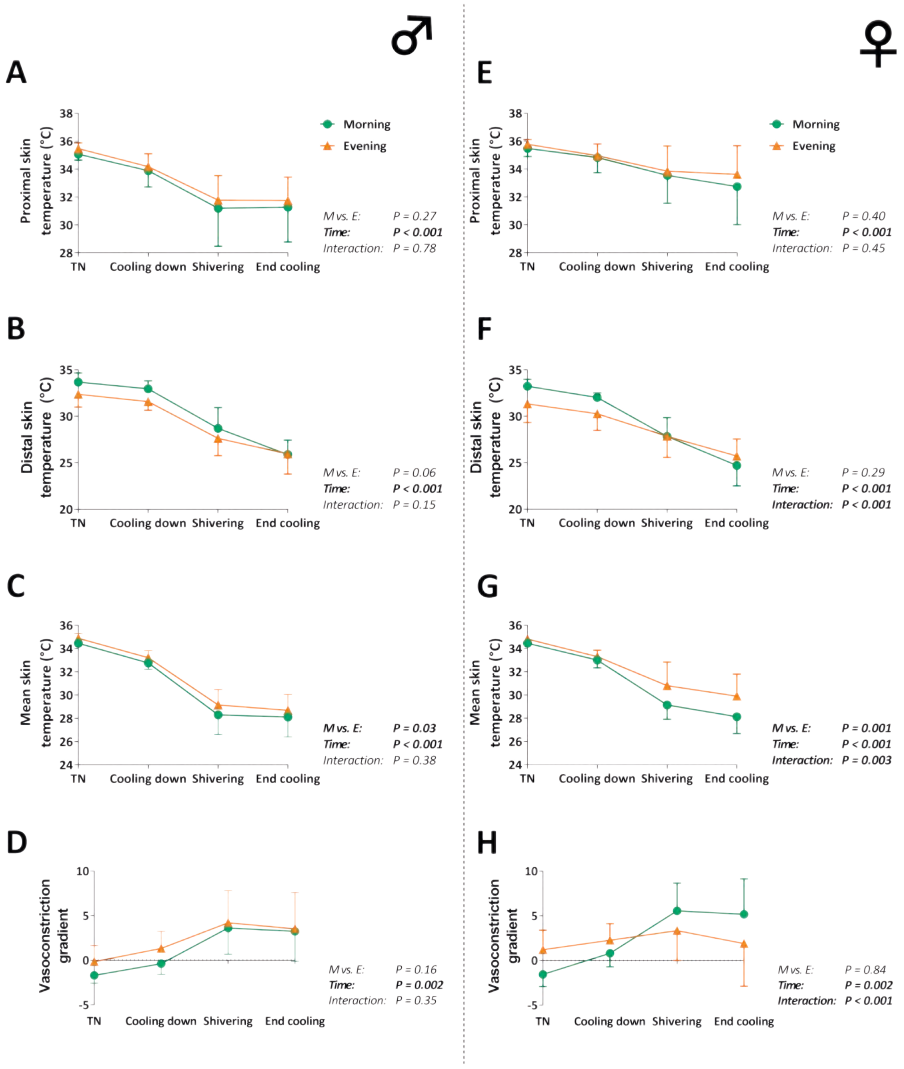

Figure S2. Changes in skin temperatures during cold exposure in the morning vs. the evening in males and females.

Proximal (A+E), distal (B+F), mean (C+G) skin temperatures and vasoconstriction gradient (D+H) were determined using wireless iButtons during the last 5 minutes of the thermoneutral phase, during the cooling down phase, right before shivering occurred and during the last 5 minutes of the stable cold phase ('end cooling'). The left panel (A-D) shows the results in males, the right panel (E-H) shows the results in females. General linear model with repeated measures was used to test for an interaction between the effect of cold over time and the moment of the day (i.e., 'M vs. E': morning vs. evening). TN=thermoneutral.

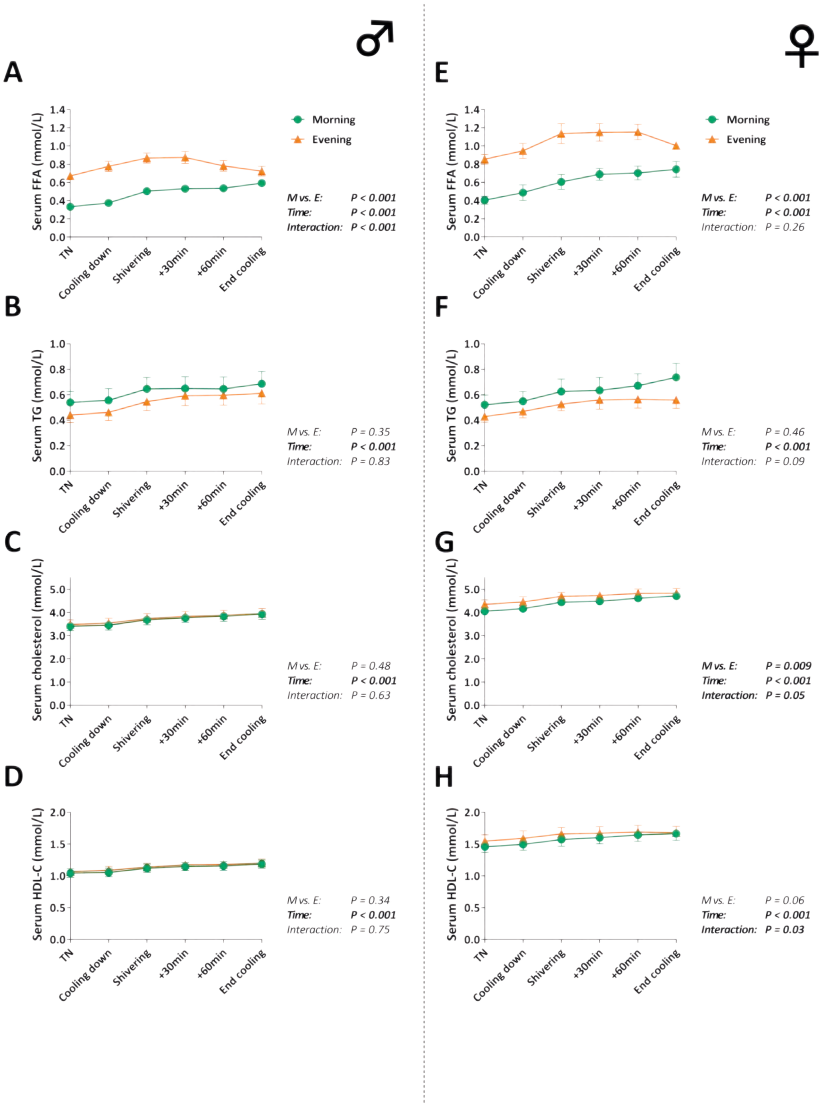

Figure S3. The effect of cold exposure on measures of lipid metabolism in the morning vs. the evening in males and females.

Serum free fatty acids (A+E), triglycerides (B+F), cholesterol (C+G) and HDL-cholesterol (D+H) were measured during the thermoneutral phase, the cooling down phase, when shivering started and 30, 60 and 90 minutes later ('end cooling). The left panel (A-D) shows the results in males and the right panel (E-H) shows the results in females. Data are mean ± standard error of the mean. General linear model with repeated measures was used to test for an interaction between the effect of cold over time and the moment of the day (i.e., 'M vs. E': morning vs. evening). FFA: free fatty acid, HDL-C: high-density lipoprotein cholesterol, TG: triglycerides.