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Original article

Estimation of non-shivering thermogenesis and cold-induced nutrient oxidation rates: Impact of method for data selection and analysis

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SUMMARY

Background & aims: Since the discovery of active brown adipose tissue in human adults, non-shivering cold-induced thermogenesis (CIT) has been regarded as a promising tool to combat obesity. However, there is a lack of consensus regarding the method of choice to analyze indirect calorimetry data from a CIT study. We analyzed the impact of methods for data selection and methods for data analysis on measures of cold-induced energy expenditure (EE) and nutrient oxidation rates.

Methods: Forty-four young healthy adults (22.1 ± 2.1 years old, 25.6 ± 5.2 kg/m^2, 29 women) participated in the study. Resting metabolic rate (RMR), cold-induced thermogenesis (CIT), and cold-induced nutrient oxidation rates were estimated by indirect calorimetry under fasting conditions during 1 h of cold exposure combining air conditioning (19.5–20 °C) and a water perfused cooling vest set at a temperature of 4 °C above the individual shivering threshold. We applied three methods for data selection: (i) time intervals every 5 min (5min-*TI*), (ii) the most stable 5-min period of every forth part of the cold exposure (5min-*SS-4P*), and (iii) the most stable 5-min period of every half part of the cold exposure (5min-*SS-2P*). Lately we applied two methods for data analysis: (i) area under the curve as a percentage of the baseline RMR (AUC) and; (ii) the difference between EE at the end of the cold exposure and baseline RMR (Last-RMR).

Results: Mean overall CIT estimation ranged from 11.6 ± 10.0 to 20.1 ± 17.2 %RMR depending on the methods for data selection and analysis used. Regarding methods for data selection, 5min-*SS-2P* did not allow to observe physiologically relevant phenomena (e.g. metabolic shift in fuel oxidation; $P = 0.547$) due to a lack of resolution. The 5min-*TI* and 5min-*SS-4P* methods for data selection seemed to be accurate enough to observe physiologically relevant phenomena (all $P < 0.014$), but not comparable for estimating over-all CIT and cold-induced nutrient oxidation rates ($P < 0.01$). Regarding methods for data analysis, the AUC seemed to be less affected for data artefacts and to be more representative in participants with a non-stable energy expenditure during cold exposure.

Conclusions: The methods for data selection and analysis can have a profound impact on CIT and cold-induced nutrient oxidation rates estimations, and therefore, it is mandatory to unify it across scientific community to allow inter-study comparisons. Based on our findings, 5min-*TI* should be

Abbreviations: BAT, Brown adipose tissue; CIT, Cold-induced thermogenesis; RMR, Resting metabolic Rate; SS, Steady state; TI, Time interval; EE, Energy expenditure; AUC, Area under the curve; CCM, CCM Express (Medgraphics Corp, Minnesota, USA); MGU, Ultima CardiO2 (Medgraphics Corp, Minnesota, USA); DXA, Dual X-ray absorciometry; RER, Respiratory exchange ratio; CHOox, Carbohydrates oxidation; FATox, Fat oxidation; ANOVA, Analyses of variance; 5min-TI, mean values of every consecutive 5-min period; 5min-SS-4P, The most stable 5-min period of every forth part of the cold exposure; 5min-SS-2P, The most stable 5-min period of every half part of the cold exposure.

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considered the method of choice to study dynamics (i.e. changes across time) of CIT and cold-induced nutrient oxidation rates, while 5min-*SS-4P* and AUC should be the method of choice when computing CIT and cold-induced nutrient oxidation rates as a single value.

1. Introduction

In simple terms, obesity results from a positive energy balance (i.e. lower energy expenditure (EE) than energy intake), and thus, weight loss would be easily achieved inducing a negative energy balance. However, many physiological and behavioral adaptations occur in parallel to caloric restriction, making weight loss unsuccessful in long-term [1]. Currently there are no non-invasive successful strategies to achieve sustainable weight loss, and new strategies have to be explored [1]. During the last decade, brown adipose tissue (BAT) activation has been regarded as a possible solution to the obesity problem [2].

BAT was confirmed to be present and active in adult humans in 2009 [3–6]. Since them, BAT has been considered a promising therapeutic target due to its capacity to oxidize glucose and lipids for heat producing purposes, in a process known as non-shivering thermogenesis. In murine models, BAT thermogenesis can account up to 60% of total energy expenditure [7]. In humans, BAT is much more scarce than in murine [8] and there is an open debate on whether BAT activity can significantly influence human energy expenditure [9,10]. Noteworthy, even assuming the most pessimistic views of BAT potential to contribute to energy expenditure [11–14], non-shivering thermogenesis can be mediated by other tissues, such as withe adipose tissue and skeletal muscle [12,13,15], and seems to be relevant enough to be considered a possible solution to the weight loss maintenance problem [1].

Cold-induced thermogenesis (CIT) can be broadly divided into shivering and non-shivering thermogenesis [16], although both processes can occur concomitantly [12]. CIT and the associated changes in nutrient oxidation rates are commonly measured by indirect calorimetry [17]. Indirect calorimetry data are quite variable minute by minute, and methods for data selection based on steady state (SS) periods are often necessary to minimize individuals' and instrument's variability [17–22]. Alternatively, selection of predefined (i.e. not considering data variability) time intervals (TI) is commonly made [18]. Besides how to select data, it is often necessary to compute CIT as a unique value to be used in cross-sectional studies, such as to study the association between BAT and CIT [23]. Therefore, investigators have used area under the curve calculations (AUC) and/or the difference between EE at the end of cold exposure and baseline resting metabolic rate (Last-RMR) [20,24,25]. These methods for data selection (i.e. SS or TI) and analysis (i.e. AUC or Last-RMR) significantly impact on RMR or meal-induced thermogenesis estimations [18–21]. However, the impact of the chosen method for data selection and data analysis on the overall measure of CIT and cold-induced nutrient oxidation rates is largely unknown.

The aim of the present study was to analyze the impact of methods for data selection (TI and SS) and methods for data analysis (AUC and Last-RMR) on measures of cold-induced energy expenditure and nutrient oxidation rates. Despite large scientific interest in cold-induced thermogenesis during the last decade [1,15,26], to our knowledge, there are no studies evaluating the impact of various methodologies on the measurement of cold-induced energy expenditure in healthy humans.

2. Material and methods

2.1. Participants

A total of 63 participants (45 women) participated in the study. The participants were part of the ACTIBATE study, an exercise-based randomized controlled trial (clinicaltrial.gov: NCT02365129) [27]. All participants were young (18–25 years old), healthy, sedentary (<20 min physical activity on <3 days/week), did not smoke or take any medication, had a stable body weight for the past 3 months (<3 Kg change), and were not regularly exposed to cold. The evaluations were performed between October 11th and November 29th, 2016.

The study protocol and informed consent were performed in accordance with the last revision of the Declaration of Helsinki. The study was approved by the Human Research Ethics Committee of the University of Granada (nº924) and of the Servicio Andaluz de Salud (Centro de Granada, CEI-Granada).

2.2. Previous conditions to the study days

Participants came to the lab on two separate occasions (5–7 days apart). They were asked to come by bus or by car, under fasting conditions (at least 6 h), to sleep as usual, to refrain from any moderate (in the previous 24 h) or vigorous (in the previous 48 h) physical activity, and not consume alcoholic or stimulant beverages over the past 6 h. The participants were evaluated between 8.30 and 19.15hrs. For nutrient oxidation rates analysis, only the participants with a fasting time between 6 and 8 h were considered [28,29].

2.3. Shivering threshold test

During the first study day, we determined the individual shivering threshold. The procedure for the shivering threshold determination has been extensively described elsewhere [30,31]. In brief, participants dressed-up with standardized clothes and stayed seated in a warm room (22.1 ± 1.6 °C) for 30 min, before entering the cold (air cooled) room (19.8 ± 0.5 °C), where they were dressed in a temperature-controlled water perfused cooling vest (Polar Products Inc., Ohio, USA) and seated again. The water temperature decreased progressively from 16.6 °C until 3.8 °C or until shivering occurred. Shivering was determined visually and by asking the participants if they were experiencing shivering.

2.4. CIT and cold-induced nutrient oxidation rates determination

In the second day, the participants performed the CIT test at the approximate same time of the day at which the shivering threshold test was performed. They dressed-up with the same standardized clothes as in the shivering threshold test. Later, they were moved into the warm room (23.2 ± 0.7 °C). Before being evaluated, all participants lay down on a reclined bed, in a supine position, and were covered by a sheet for 20 min. They were instructed to breathe normally, and not to talk, fidget, or

sleep. Thereafter, we assessed the participant's RMR maintaining the same standardized conditions [17] during 30 min (Fig. 1).

After assessing RMR, the participants were moved into the cold room (19.7 ± 0.4 °C) and they put on the temperature-controlled water perfused cooling vest (Polar Products Inc., Ohio, USA) set 4 °C above the individual shivering threshold temperature. Once they had the cooling vest on, they lay down on another reclined bed. Indirect calorimetry measurement was performed during two consecutive 30-min periods, separated by a 5-min pause to recalibrate the metabolic cart (Fig. 1).

The indirect calorimetry measurements for both RMR and CIT were performed with the CCM Express (CCM) or with the Ultima CardiO2 (MGU) (Medgraphics Corp, Minnesota, USA), using a neoprene face-mask equipped with a directconnect™ metabolic flow sensor (Medgraphics Corp, Minnesota, USA) [21]. Flow calibration was performed by a 3-L calibration syringe at the beginning of every testing day, and gas analyzers were calibrated using 2 standard gas concentrations following the manufacturer's instructions before every 30 min of indirect calorimetry measurement. We used the same metabolic cart for the RMR and CIT measurements in every participant.

Body composition was measured by a DXA scanner (Discovery Wi, Hologic, Inc., Bedford, MA, USA) and data were extracted from the Hologic APEX 4.0.2. (Hologic, Inc., Bedford, MA, USA) software. Weight and height were measured with a Seca scale and a stadiometer (model 799, Electronic Column Scale, Hamburg, Germany).

2.5. Methods for data selection and analysis

Indirect calorimetry data were averaged every minute and downloaded from the Breeze Suite (8.1.0.54 SP7) software. For RMR, we selected the most stable 5-min period (i.e. the one with the lowest average of coefficients of variance for oxygen consumption, carbon dioxide production, minute ventilation, and respiratory exchange ratio (RER)) [21].

For CIT, we applied different methods for data selection and analysis. Methods for data selection refer to the way of processing the data obtained from the continuous indirect calorimetry instrument. After excluding the first 5 min of every 30-min record [18], we used three different methods for data selection (Fig. 1): i) TI every 5 min (5min-*TI*): mean values of every consecutive 5-min period (i.e. from the 6th to the 10th, from the 11th to the 15th, etc.); ii) The most stable 5-min period of every forth part of the cold exposure (i.e. after dividing the cold exposure into 4 parts equal in length) (5min-*SS-4P*); iii) The most stable 5-min period of every half part of the cold exposure (i.e. after dividing the cold exposure into 2 parts equal in length) (5min-*SS-2P*).

In order to express the CIT as a single value, we used two different methods for data analysis (Fig. 1): i) The AUC following the trapezoidal rule; and ii) the Last-RMR. Both methods for data analysis were expressed as a percentage of the baseline RMR.

Oxygen consumption and carbon dioxide production for each selected data point were used to estimate EE, and carbohydrates (CHOox) and fat oxidation (FATox). EE was estimated through Weir's abbreviated equation, not considering urinary nitrogen concentration [32]. For carbohydrates and fat oxidation estimations, we used Frayn's equation, not considering urinary nitrogen concentration [33].

2.6. Statistical analysis

Results are presented as means ± standard deviation, unless otherwise stated. The analyses were conducted using the Statistical Package for Social Sciences (SPSS, v. 21.0, IBM SPSS Statistics, IBM Corporation), and the level of significance was set at <0.05.

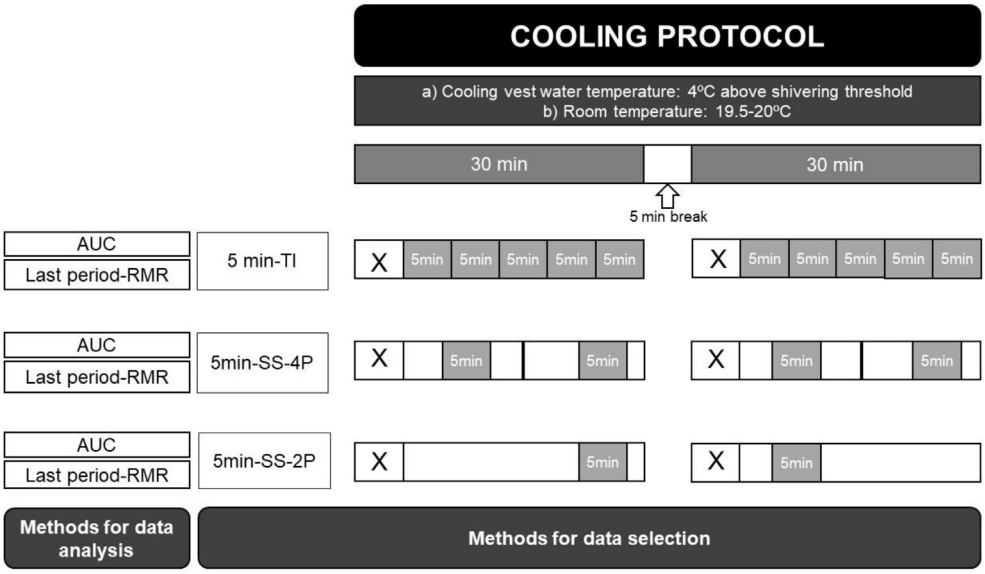

Fig. 1. Cooling protocol, methods for data selection, and methods for data analysis. White rectangles in cooling protocol represent every 30 min of recorded gas exchange. Gray squares represent the 5-min selected period within a specific recorded time (i.e. in the time interval method: average of every consecutive 5-min period; in the steady state method: the 5 min-period with the lowest average of coefficient of variances of: oxygen consumption, carbon dioxide production, respiratory exchange ratio, and minute ventilation). Crosses represent excluded gas exchange data (i.e. first 5 min of every 30-min record). Vertical lines within white rectangles represent divisions of recorded data for the selection of the representative 5-min period. TI: Time interval; SS: Steady State; min: minutes; 4P: Four periods; 2P: Two periods; RMR: Resting metabolic rate; AUC: Area under the curve.

A repeated-measures analysis of variance (ANOVA) was used to test differences in EE and nutrient oxidation rates across the selected data points following the different methods for data selection and analysis. To compare CIT, cold-induced CHOox, and FATox estimations obtained with different combinations of methods for data selection and analysis, we conducted a two-factor (method for data selection * method for data analysis) ANOVA. Bonferroni corrections (automatically performed by the SPSS) were used to perform post hoc comparisons.

3. Results

During the CIT, visually detected and auto-reported shivering was recorded in 17 participants (n = 16 women) who were therefore excluded from further analysis. In addition, participants with RER values higher than 1.1 or lower than 0.7 in any measure point, or a RER higher than 1.0 in RMR assessment, were also excluded from the analysis (n = 2) [17]. Finally, a total of 44 participants were included in the energy expenditure analysis (Table 1). Mean fasting time was 9 ± 3.7 h. Of this sample, a total of 18 (n = 13 women) strictly met the fasting time criterion for assessing nutrient oxidation rates (i.e. a fasting time of 6–8 h) and were included in the nutrient oxidation rate analysis (Table S1).

3.1. Cold-induced thermogenesis

Figure 2 shows the EE dynamics during a mild cold exposure by method for data selection. EE was significantly increased by mild cold exposure, which was detected regardless of the method for data selection used (All $P < 0.001$). Post-hoc comparisons showed that for all methods for data selection, EE was increased just after starting the cold-exposure (i.e. first data point analyzed) and remained unchanged until the end of the mild cold exposure.

Mean overall CIT estimation ranged from 11.6 ± 10.0 to 20.1 ± 17.2 %RMR depending on the methods for data selection and analysis used.

Figure S1 shows the individual data for the over-all estimation of CIT by different combinations of methods for data selection and methods for data analyses. Figure 3 compares the mean overall CIT estimation obtained by the different methods for data selection and analysis. Both main effects (methods for data selection and methods for data analysis) were significant (all $P < 0.01$) and no significant interaction effect (method for data selection * method for data analysis) was found ($P = 0.3$). Mean overall CIT estimation was consistently higher with the Last-RMR than with the AUC in all methods for data selection (all paired comparisons $P \leq 0.043$). No differences in mean over-all CIT estimation were found between different methods for data selection when using the Last-RMR (all $P = 0.6$). However, mean over-all CIT estimation varied across methods for data selection when using the AUC ($P < 0.001$).

Table 1
Descriptive characteristics of the participants included in the energy expenditure analysis.

	All (n = 44)		Male (n = 15)		Female (n = 29)	
Age (years)	22.1	(2.1)	22.4	(2.2)	22.0	(2.2)
BMI (kg/m^2)	25.6	(5.2)	27.9	(6.0)	24.4	(4.4)
Lean mass (kg)	42.7	(10.4)	54.6	(6.8)	36.4	(5.2)
Fat mass (kg)	27.2	(10.6)	29.9	(13.5)	25.8	(8.8)
Fat mass (%)	37.0	(8.0)	32.7	(8.5)	39.2	(6.9)
VO_2 (ml/min)	223	(39)	252	(45)	208	(23)
VCO_2 (ml/min)	192	(33)	216	(37)	178	(20)
RMR (kcal/day)	1564	(277)	1769	(324)	1459	(178)
RER	0.862	(0.054)	0.863	(0.048)	0.861	(0.057)

Data are presented as means (standard deviation). BMI: Body mass index; VO_2: resting oxygen consumption; VCO_2: resting carbon dioxide production; RMR: Resting Metabolic Rate; RER: resting respiratory exchange ratio.

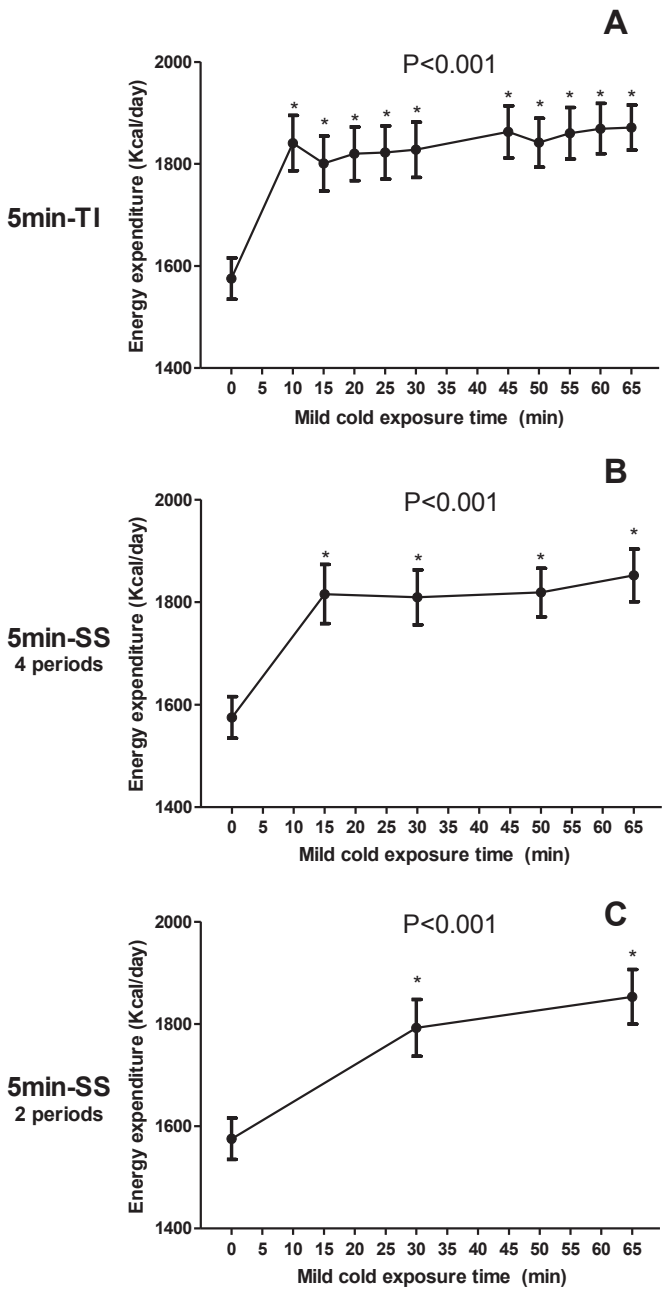

Fig. 2. Energy expenditure (EE) during mild cold exposure by methods for data selection. Represented values are mean ± standard error. Panel A presents data obtained with the 5-min time interval (TI) method; Panel B presents data obtained with the steady state (SS) method after dividing the cold exposure into 4 periods; Panel C presents data obtained with the steady state method after dividing the cold exposure into 2 periods. Min 0 represents the value obtained in the resting metabolic rate (RMR) period (baseline). *: Significantly different to baseline value. P value for repeated measures ANOVA. Min: minutes; Kcal: Kilocalories.

3.2. Cold-induced nutrient oxidation rates

Figure 4 shows CHOox and FATox dynamics during the mild cold exposure by method for data selection. There were

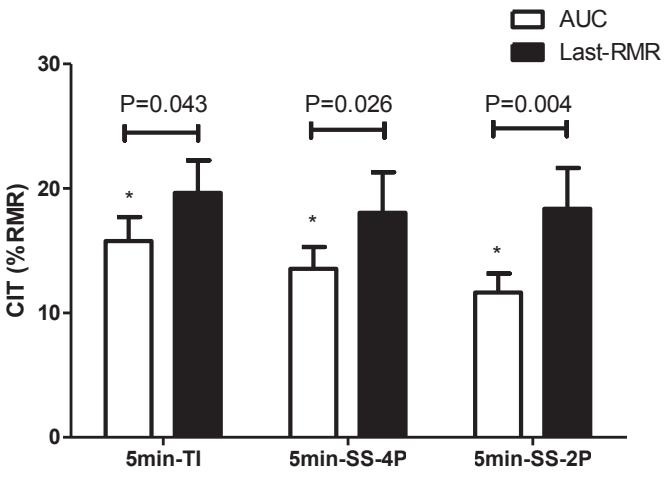

Fig. 3. Comparisons between mean over-all cold-induced thermogenesis (CIT) obtained with different methods for data selection and analysis. Represented values are mean ± standard error. P values for paired *t*-test. *: significant differences with the rest of the AUC results after Bonferroni correction. RMR: Resting metabolic rate; AUC: Area under the curve; Last-RMR: Last period value minus RMR value.5min-TI: mean values of every consecutive 5-min period; 5min-SS-4P: The most stable 5-min period of every forth part of cold exposure; 5min-SS-2P: The most stable 5-min period of every half part of cold exposure.

significant changes in CHOox when selecting the data by 5min-*TI* and 5min-*SS-4P* (all $P < 0.015$), but not with 5min-*SS-2P* ($P = 0.547$). Of note, differences between CHOox at 30 min and at the end of the mild cold exposure were only detected with 5min-*TI* (Figure 4). FATox changes during the mild cold exposure were detected with all methods for data selection (All $P < 0.002$; Figure 4). The highest FATox rate was observed at 30 min with 5min-*TI* and 5min-*SS-4P*, but not with 5min-*SS-2P*. A reduction on FATox after minute 30 was only detected by 5min-*TI*.

Regarding mean overall cold-induced nutrient oxidation rates estimation, no differences were found when comparing the methods for data selection ($P = 0.181$), nor when comparing the methods for data analysis ($P = 0.328$) (Figure S2).

4. Discussion

This study analyzed the impact of methods for data selection (5min-*TI*, 5min-*SS-4P* and 5min-*SS-2P*) in combination with two different methods for data analysis (AUC and Last-RMR) on estimations of cold-induced energy expenditure and nutrient oxidation rates during a 65-min individualized mild cold exposure, designed to elicit maximum non-shivering thermogenesis. The 5min-*TI* and 5min-*SS-4P* methods for data selection seemed to be accurate enough to observe physiologically relevant phenomena, but not comparable for estimating over-all CIT and cold-induced nutrient oxidation rates. Regarding methods for data analysis, the AUC seemed to be less affected for data artefacts and be more representative in participants with a non-stable energy expenditure during cold exposure.

The selection of the method for data selection and analysis influences the estimations of RMR and meal-induced thermogenesis [17–22]. Therefore, it is expected that the selection of the method for data selection and analysis also influences the estimation of CIT and cold-induced nutrient oxidation rates. Regarding the methods for data selection, 5min-*SS-2P* may not be an appropriate method, since, as a consequence of a lack of resolution, it does not allow to detect relevant physiological changes. In contrast, 5min-*TI* allows to detect changes that no other method is able to detect (see Fig. 4). However, for the RMR data selection, there is a consensus on the need of using a method based on the selection of a SS, as it is supposed not to be affected by artefacts, and to ensure a more valid measure [19,21,22]. Therefore, 5min-*SS-4P* could be the method of choice. Our data supports that selection, especially when an over-all CIT estimation is made. In this case, the outcome obtained with the 5min-*TI* method might be affected by artefacts (see Figure S2A), as previously argued [19,21,22]. Indeed, we observed a wider range of CIT values applying 5min-*TI* (−14.9/46.2 %RMR) than 5min-*SS-4P* (−14.8/39.9 %RMR) (see Figure S1). On the other hand, 5min-*TI* might be the method of preference when studying the dynamics (i.e. changes during time of cold-exposure) of CIT or cold-induced nutrient oxidation rates, as it allows a more detailed insight (Figs. 2 and 4). Standardizing the methods for data selection would allow between-studies comparability.

In relation to the methods for data analysis, the AUC resulted in a lower inter-individual variability than the Last-RMR (see Figure S1). We observed a stable EE during the mild cold exposure, and consequently one could expect no differences between the AUC and the Last-RMR in over-all CIT estimation. In contrast, we observed large differences between the AUC and the Last-RMR over-all CIT estimation, with the Last-RMR reporting higher values (Fig. 3). This could be explained by the fact that energy expenditure progressively increases during the mild cold exposure in some individuals while in others did not. This, together with the possibility of the Last-RMR methods to be influenced by artefacts (see outlier in Figure S1) would point to the AUC as the method of choice for over-all CIT estimation.

Observations on humans' CIT have reported huge inter-individual variability [26,34,35]. This is congruent with our results, where some individuals showed negative values of CIT (i.e. lower EE in cold than in RMR) while others even get more than 100% increase over RMR with some methods for data analysis. Many factors have been reported to contribute to inter-individual CIT difference [26]. Here, we show that the method for data selection and analysis could have an important impact on inter-individual CIT variability estimations. This is in line with observations about the impact of the method for data selection and analysis on RMR estimations [17–19,21].

4.1. Limitations

Our results should be considered with caution due to the presence of limitations. Firstly, we did not analyze urine nitrogen excretion, and therefore we could not correct the nutrient oxidation rates for protein oxidation. Although protein oxidation correction would have been desirable, it is not plausible to obtain different urine nitrogen concentration in short intervals such as the periods that we have studied (i.e. ≤60 min). Secondly, although we selected a cooling protocol thought to ensure maximum non-shivering thermogenesis, we cannot be sure of the relative contribution of shivering thermogenesis to CIT [12]. However, we excluded from the analysis participants who reported shivering or whose shivering was visually detected, and therefore it is probable than the contribution of non-shivering thermogenesis is predominant in the included participants. Thirdly, we used two different metabolic carts which are not comparable and have relatively low reliability [36–38]. However,

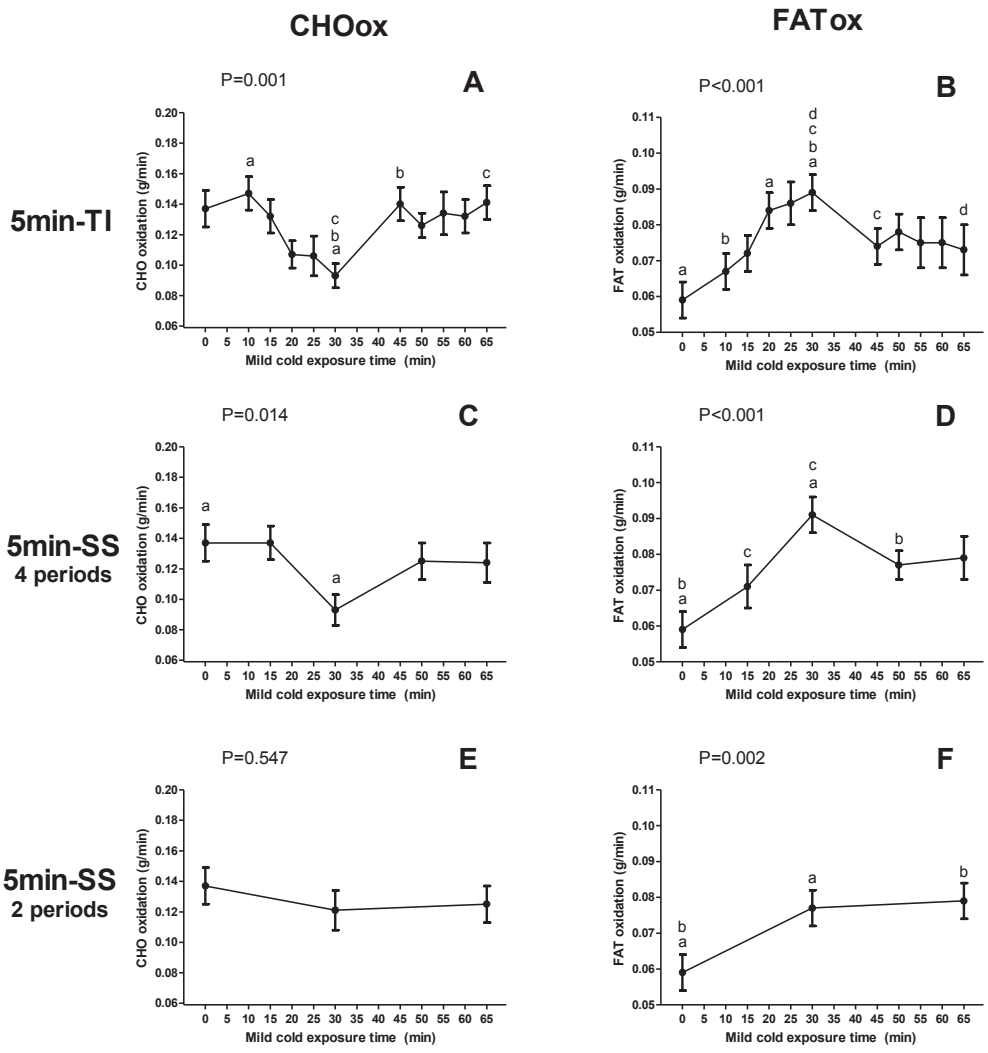

Fig. 4. Nutrient oxidation rates during cold exposure by method for data selection. Panels A and B present data obtained with the 5-min time interval (TI) method; panels C and D present data obtained with the steady state (SS) method after dividing the cold exposure into 4 periods; and panels E and F present data obtained with the steady state method after dividing the cold exposure into 2 periods. Represented values are mean ± standard error. P value for repeated measures ANOVA. Equal lower-case letters indicate significant differences after Bonferroni correction. CHO: Carbohydrates. min: minutes; g: grams.

the within-subject design applied in this study reduce the impact of this limitation. Finally, our results only apply to young healthy individuals, and further studies are needed to confirm whether this also applies to older and unhealthy individuals.

5. Conclusions

The methods for data selection and analysis can have a profound impact on CIT and cold-induced nutrient oxidation rates estimations, and therefore, it is mandatory to unify it across scientific community to allow inter-study comparisons. Based on our findings, 5min-*TI* should be considered the method of choice to study dynamics (i.e. changes across time) of CIT and cold-induced nutrient oxidation rates, while 5min-*SS-4P* and AUC should be the method of choice when computing CIT and cold-induced nutrient oxidation rates as a single value.

Conflict of interest

The authors confirm that there are no conflicts of interest.

Author's contribution

GSD, JMA, and JRR conceived the study; GSD, JMA, ML, IL and JRR designed the study; GSD, JMA, FMA, BMT, FAG and LOA did the data collection; GSD performed the statistical analyses and drafted the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.clnu.2018.09.009.

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