Title: Influence of repeated daily menthol exposure on human temperature regulation and perception

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Author contributions

D. Jason Gillis; research question, protocol, data collection, analysis, interpretation, manuscript

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Habituation to menthol

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Highlights

- A single skin surface exposure to menthol enhances cool sensations and heat storage
- Heat storage by menthol is mediated by a vasoconstrictor response
- Repeated menthol exposure causes an habituation of cool sensation but not heat storage
- 0.2 % menthol activation of thermoreceptors equals a 0.5°C fall in skin temperature

Abstract (250 words)

A single exposure to menthol can, depending on concentration, enhance both cool sensations and encourage body heat storage. This study tested whether there is an habituation in either response after repeated-daily exposures. Twenty-two participants were assigned to one of three spray groups: Control (CON; n=6), 0.05% l-menthol ($M_{0.05\%}$; n=8), 0.2% l-menthol ($M_{0.2\%}$; n=8). On Monday (20°C, 50% rh) participants were sprayed with 100mL of solution and undertook 40-minutes of cycling at 45% of their peak power (Ex_1), from Tuesday to Thursday (30°C, 50% rh) they were sprayed twice daily whilst resting (R_1 to R_6), Friday was a repeat of Monday (Ex_2). TS, thermal comfort, perceived exertion, irritation, rectal and skin temperature (T_{sk}), skin blood flow (SkBF) and sweat rate were monitored. A two-way ANOVA (alpha=0.05) compared responses from the beginning (Ex_1, R_1) and end (Ex_2, R_5) of the testing week. $M_{0.2\%}$ induced significantly (P<0.05) cooler TS at the beginning of the week (Ex_1, R_1) compared to the end (Ex_2, R_5), indicating habituation of TS; this was not observed in $M_{0.05\%}$. No other perceptual or physiological responses habituated. 0.2% menthol caused a heat storage response, mediated by vasoconstriction, at the beginning *and* end of the week, suggesting the habituation of TS occurred in a pathway specific to sensation. In summary, the cooling influence of 0.2% menthol habituates after repeated-daily exposures, but with no habituation in heat storage.

Key words

habituation; perception; thermoregulation; menthol; cold receptor TRPM8

Introduction

Menthol ($C_{10}H_{20}O$; molecular weight, 156) is a cyclic terpene alcohol produced from mint oils or prepared synthetically (Eccles, 1994). It is found in many active forms, but the L isomer is most commonly used in commercial products because it produces the strongest cooling effects and is nontoxic to humans (Eccles et al., 1988). Both menthol and temperatures below 28 °C activate the transient receptor potential melastatin-eight (TRPM8) family of ion channels, which are embedded in the terminals of primary afferent nerve endings (McKemy, Neuhausser & Julius, 2002; Piere et al., 2002). These thermo-sensitive neurons are thought to project to the somatosensory cortex, where temperature is perceived (Craig, 2002) and towards the hypothalamus, where body temperature is regulated (Morrison & Nakamura, 2011). In this way, menthol is thought to influence both human perception and temperature regulation.

A growing number of studies support the notion that menthol influences human temperature regulation; an elevation in deep body temperature can be observed after it is applied to the skin surface of resting and exercising humans (Gillis, House & Tipton, 2010; Kounalakis et al., 2010; Lee et al., 2011), but it is not yet clear whether this heat storage response is driven by a reduction in skin blood flow at rest and/or a withdrawal of sudomotor function during exercise. The magnitude of body heat storage is probably influenced by the size of the surface area stimulated by menthol, and the dose of menthol used, although this requires clarification. Regardless, when a stimulus is strong enough to induce such a change in homeostasis, adaptation theory suggests that the physiological outcome (*i.e.* heat storage) resulting from the forcing function (*i.e.* menthol exposure) progressively reduces with repeated exposures (*i.e.* it habituates) (Tipton et al., 2008). This often follows from a shift in the deep body temperature threshold for vasoconstriction, vasodilation and sweating (Tipton et al., 2008). Therefore, repeated exposure to menthol may attenuate the heat storage response, perhaps through a withdrawal of vasoconstrictor tone, and an increase in skin blood flow; but this has not been tested.

Although there is a large body of research describing menthol's perceptual influence, most studies are psycho-physical in nature and assess perceptual responses to small applications of menthol on the forearm of resting participants. Far fewer studies have applied menthol to larger body surface areas, especially during exercise, so its influence on more global measures of perception, like thermal comfort or perceived exertion, is not well understood. The findings from these few studies are in general agreement with the psychophysical literature in that menthol elicits cool sensations (Barwood, Corbett & White, 2014; Barwood et al., 2011; Gillis, House & Tipton, 2010; Schlader et al., 2011) and irritation (Gillis, House & Tipton, 2010; Lee et al., 2011) when applied to large body surface areas, but it is not clear whether menthol improves thermal comfort during rest or exercise (Gillis, House & Tipton, 2010), lowers perceived exertion during exercise (Gillis, House & Tipton, 2010; Lee et al., 2011), or improves exercise performance (Barwood, Corbett & White, 2014; Barwood et al., 2011).

The influence of repeated menthol exposure on perception has received little attention, and those studies which have been conducted have separated menthol exposures (oral cavity) by minutes, not hours or days (Cliff & Green, 1996; Cliff & Green, 1994). Given the paucity of research in this area, studies assessing cold adaptation in humans might give clues about the repeated influence of menthol on thermal sensation. A single exposure to menthol is comparable to a single cold exposure in that each gives rise to cool sensations. The distinction being that menthol achieves this by direct stimulation of the TRPM8 cold receptor (McKemy, Neuhausser & Julius, 2002; Peier et al., 2002) without changing skin temperature (Gillis, House & Tipton, 2010), whilst a cold exposure achieves this sensation by first lowering skin temperature, which increases the firing rates of cold receptors and brings about cool sensations. With this distinction in mind, repeated exposures to either cold air (Bruck, Baum & Schwennicke, 1976; Leppaluoto, Korhonen & Hassi, 2001; Makinen et al., 2006) or cold water (Golden & Tipton, 1988; Smolander et al., 2004) have been shown to cause an habituation of cool sensations and/or thermal discomfort. These findings suggest that repeated exposure to menthol may result in an habituation of thermal sensation, but this has not been tested.

The aim of this experiment was to examine whether the perceptual (*i.e.* cool sensations) and/or thermoregulatory (heat storage) responses that accompany menthol exposure undergo any habituation after repeated exposures. It was hypothesised that there would be no habituation in either response following repeated exposure to menthol.

Methods

This experiment received ethical approval from the BioSciences Research Ethics Committee at the University of Portsmouth.

Participants

Twenty-two participants volunteered for this study. They were assigned to their testing condition according to the order in which they were enrolled, such that participant one through four were assigned to CON, $M_{0.05\ \%}$, $M_{0.2\ \%}$ and CON, respectively. This pattern continued until groups filled. Participant characteristics are shown in Table 1.

Table 1. Mean (SD) participant age, height and weight

Spray group	Age	Weight (kg)	Height (m)
Water (*n*=6)	21.6 (1.3)	78.8 (5.5)	1.80 (0.05)
0.05% menthol (*n*=8)	19.6 (0.9)	70.5 (6.5)	1.78 (0.08)
0.2% menthol (*n*=8)	19.7 (1.5)	76.7 (15.3)	1.82 (0.09)

There were no significant differences in participant mass or height between conditions ($P > 0.05$).

General design

Participants were divided into one of three groups; Control (CON, $n = 6$), 0.05 % menthol ($M_{0.05\ \%}$, $n = 8$), and 0.2 % menthol ($M_{0.2\ \%}$, $n = 8$). Prior to testing all participants completed a peak power-output test (PO_{peak}). Testing always began on Monday with a pre-intervention exercise test (Ex_1) and ended on a Friday, with a post-intervention exercise test (Ex_2). On Tuesday, Wednesday and Thursday participants underwent two resting exposures each day ($R_{1\text{-}6}$), once in the morning and once in the afternoon, each separated by three hours. The testing schedule is displayed in Table 2.

Table 2. Participant testing schedule.

	Mon	Tue	Wed	Thu	Fri
am	Ex_1	R_1	R_3	R_5	Ex_2
pm		R_2	R_4	R_6	

Exercise sessions (Ex_1 and Ex_2)

Prior to the testing week, participants performed an incremental test until exhaustion on a Monark cycle ergometer. Peak O_2 uptake ($\dot{V}O_{2peak}$) was defined as the highest O_2 uptake attained during the test, analysed retrospectively from the gas collected in Douglas bags, provided that the participant also attained either their age-predicted maximal heart rate during the test, or they reached a respiratory exchange ratio of greater than 1.1 (Hale, 2003).

Exercise testing was undertaken on Monday (Ex_1) and Friday (Ex_2). Each participant entered the environmental chamber (20 °C; 60 % relative humidity [rh]) wearing a long sleeve breathable shirt, shorts, training shoes and socks and remained seated at rest on a cycle ergometer for 10 minutes. Participants then underwent either 0.05 % or 0.2 % menthol spraying or water spraying, and remained seated for five additional minutes. At the 15th minute they began to cycle at 45 % of their previously determined peak power ($PO_{45\%}$), until T_{re} rose by 0.5 °C. At this point the test was terminated. Expired gas was collected both at rest (6th minute) and again just prior to the termination of exercise. The timeline for Ex_1 and Ex_2 is displayed in Fig. 1.

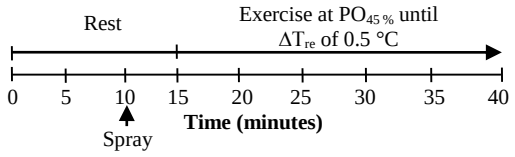

Fig. 1. Experimental timeline for Ex_1 and Ex_2.

To minimize potential deterioration in performance due to dehydration, participants were instructed to drink 500 mL of water before going to bed the previous evening before testing, and 500 mL two hours before arrival at the laboratory. They were provided with tap water throughout the test. Participants arrived at the laboratory, were weighed naked (before and after testing) and equipped with a heart rate monitor (Team System Polar, UK). They then self-inserted a calibrated rectal thermistor (Grant Instruments (Cambridge) Ltd., Royston, UK) 15 cm beyond their anal sphincter. Eight calibrated skin thermistors (Grant Instruments, Cambridge, UK) were secured by single pieces of adhesive tape (Tegaderm™ Film, 3M, UK) at eight different sites (left chest, right scapula, left biceps, left dorsal hand, right vastus medialus, left hamstring, right tibalis anterior, right dorsal foot). Mean skin temperature ($\overline{T}_{msk}$) was calculated using an eight site weighted formula developed by Olesen (1984), and mean body temperature ($\overline{T}_b$) was calculated using Burton's formula (Burton, 1935). Participants were further instrumented with one ventilated sweat capsule (with a surface area of 0.787 cm^2, and flow rate of 60 mL·min^{-1}) on the lower back (Q-Sweat Quantitative Sweat Measurement System, Model 1.0, WR Medical Electronics Co., MN, USA). Ventilated sweat capsule data were recorded four times a second and averaged by the minute. Upon entering the chamber, participants were instrumented with laser Doppler fibre optic probes to measure skin blood flow (SkBF) at the left index finger (Moor Instruments Ltd., Axminster, England, UK). Laser Doppler data were recorded once per second, but as flux data can be highly variable within and between participants, attempts were made to smooth and normalise it. First, an average of the highest 60 values from the entire data set was taken to serve as a 100 % value. All per second data were then normalised to this 100 % value, and averaged by the minute. These data were then displayed relative to their lowest point during each test, which occurred immediately after spraying (equating to a state of vasoconstriction). Skin and rectal temperatures were recorded on an electronic data logger (Squirrel 1000/1250 series, Grant Instruments [Cambridge] Ltd., Royston, UK) each minute during testing.

Laminated paper scales for thermal sensation (TS) and thermal comfort (TC) (Zhang, 2003), rating of perceived exertion (RPE [Borg, 1982]) and irritation (IRR [Green, Shaffer & Gilmore, 1993]) were held atop the handle bars of the cycle ergometer, directly in front of participants every 5th minute throughout the test.

Resting sessions (R_1 through R_6)

To provide a stimulus for an habituation whilst avoiding any training effect from multiple exercise sessions, all groups underwent six resting exposures over three days to either a water spray, 0.05 % or 0.2 % menthol spray. Perceptual and physiological measures were only taken on the first (R_1) and fifth (R_5) resting exposures. Measures were taken at R_5 rather than R_6, as R_5 took place in the morning, so any comparison between R_1 and R_5 should not be influenced by circadian variations in body temperature. Rectal, skin, mean skin, and mean body

temperatures, heart rate and skin blood flow (but not sweat rate) were recorded as described earlier for Ex_1 and Ex_2, along with the perceptual measurements, excluding RPE. Each participant entered the environmental chamber (30 °C; 55 % rh) wearing a long sleeved breathable shirt, shorts, training shoes and socks and remained seated at rest on a stool for 30 minutes. Participants then underwent either 0.05 % or 0.2 % menthol or water spraying and remained seated for an additional 30 minutes. At this time the test was terminated. The timeline for each resting session is displayed in Fig. 2.

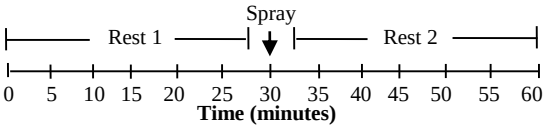

Fig. 2. Experimental timeline for resting tests (R_1 to R_6).

Description the Control and menthol sprays

The Control spray contained 3 g (3 %) of surfactants mixed in 100 mL of water, while the experimental sprays contained a concentration of either 0.05 % (0.05 g) or 0.2 % (0.2 g) l-menthol suspended in 100 mL of water with 3 g (3 %) of surfactants, which suspended menthol in the solution. When sprayed on the upper body (excluding head and neck), which represents 55 % of the total surface area (Yu, Lin & Yang, 2010), 0.2 % and 0.05% menthol equated to approximately 2.1 mg and 0.52 mg · 100 cm^{-2}, for the average male with a body surface area of 1.76 m^2. All solutions were stored at room temperature and transferred into the environmental chamber three hours before testing, where they remained until they were applied. All solutions were applied using a manual spray bottle. Participants were given protective glasses and a mask during spraying to prevent any of the sprays coming into contact with the eyes, nose or mouth. To standardize the method of application, the same investigator sprayed the solutions during every test. Spraying always took place from left to right and from top to bottom. The bottle was held approximately 15 cm from the participant with each spray around the torso (the spray bottle was held closer during arm spraying to avoid wastage). The spray bottle was set to 'mist' and spraying was repeated until the entire upper body was covered evenly.

Statistical analysis

Habituation of a response was judged to occur when it diminished over the testing week. Evidence of habituation would be found if Ex_1 or R_1 was significantly lower than Ex_2 or R_5, respectively. All data were tested for distribution normality using the Kolmogorov-Smirnov test for small sample size (six or less), while the D'Agostino and Pearson omnibus normality test was used for normality testing in larger groups. Parametric data were assessed using a two-way repeated measure ANOVA by spray group (CON, $M_{0.05\,\%}$, $M_{0.2\,\%}$) and time (Ex_1 vs. Ex_2, or R_1 vs. R_5), with an interaction assessed between the two factors. Non-parametric data were analysed using the Wilcoxon matched-pairs sign rank test within each spray group (*e.g.* CON Ex_1 vs. CON Ex_2), with a correction for multiple comparisons, with median (range) scores shown. The alpha level was set at 0.05, unless otherwise specified. Minute-by-minute data were not analysed; instead, either a single mean score, or a change (Δ) in an outcome measure over time (*e.g.* mean thermal sensation, or the change in T_{re} during exercise), were calculated from the raw data and subsequently analysed. For the exercise sessions (Ex_1 and Ex_2), all data were displayed and analysed up to the 40th minute, as all participants experienced a ΔT_{re} of at least 0.5 °C by this time. Resting data (R_1 and R_5) were compared over the last 30 minutes of testing. All statistical testing was performed using GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego California USA).

Results

This section is divided in two parts; Part A includes data from the exercise sessions (Ex_1 *vs.* Ex_2), and Part B, resting data (R_1 *vs.* R_5).

Part A. Exercise sessions (Ex_1 *vs.* Ex_2)

Environmental conditions (Ex_1 vs. Ex_2)

There was no difference in mean (SD) dry air (19.6 °C [0.6] °C) or globe (19.7 °C [0.6] °C) temperatures between Ex_1 and Ex_2, or by spray group, and no interaction ($P > 0.05$). Wet bulb temperature differed by spray group ($P = 0.0002$) and between Ex_1 and Ex_2 ($P = 0.016$), with no interaction ($P > 0.05$). *Post-hoc* testing showed that the wet bulb temperature in both Ex_1 and Ex_2 were warmer in CON compared to $M_{0.05\,\%}$ and $M_{0.2\,\%}$, by 2 °C ($P < 0.05$). As such, rh also differed by spray group ($P = 0.001$) and between Ex_1 and Ex_2 ($P = 0.002$), with no interaction ($P > 0.05$). Again, *post-hoc* testing showed that rh in Ex_1 and Ex_2 was higher in CON compared to $M_{0.05\,\%}$ and $M_{0.2\,\%}$, by 12 % rh ($P < 0.05$).

Measures of work-rate (Ex_1 vs. Ex_2)

Neither the mean (SD) $\dot{V}O_{2peak}$ (48.2 [6.8] mL · kg^{-1} · min^{-1}) nor PO_{peak} (322.1 [48.9] w) differed by spray group ($P > 0.05$). Similarly, mean $\dot{V}O_2$ measured just prior to exercise termination did not differ between Ex_1 and Ex_2, or spray group, with no interaction ($P > 0.05$). The mean (SD) $\dot{V}O_2$ at exercise termination was 32.1 (3.5) mL · kg^{-1} · min^{-1} across all conditions. Heart rate did not differ between Ex_1 and Ex_2, or by spray group, with no interaction ($P > 0.05$). During rest, heart rate remained stable around 73 (10.3) beats · min^{-1} across conditions, but rose to 147 (14.8) beats · min^{-1} by the end of exercise. RPE was described as 'very light' to 'light' at the onset of exercise across conditions, and 'heavy' by the end of exercise. The mean RPE during exercise did not differ between Ex_1 and Ex_2, or by spray group, with no

interaction ($P > 0.05$). Mean (SD) RPE during 25 minutes of exercise for CON, $M_{0.05\ \%}$ and $M_{0.2\ \%}$ was 13.0 (2.5), 12.8 (2.0) and 12.0 (2.7) respectively.

Thermoregulatory responses (Ex_1 vs. Ex_2)

Fig. 3 shows thermoregulatory responses measured during Ex_1 and Ex_2 by spray condition. The ΔT_{re}, $\Delta \overline{T}_{msk}$ and $\Delta \overline{T}_b$ from minute 15 to 40 did not significantly differ between Ex_1 and Ex_2, or by spray group, with no interaction ($P > 0.05$).

The ΔSR did not differ between Ex_1 and Ex_2, nor by spray condition, with no interaction ($P > 0.05$). There were no significant differences in onset of sweating time (minutes), or those measures coinciding with the onset of sweating, including; $\overline{T}_{msk}$, T_{re}, ΔT_{re}, $\overline{T}_b$, or $\Delta \overline{T}_b$ between Ex_1 and Ex_2, by spray group, nor was there any interaction ($P > 0.05$), respectively. The change in finger SkBF did not differ between Ex_1 and Ex_2, nor by spray group, with no interaction ($P > 0.05$). There were no significant differences in time of onset of vasodilation (minutes), or the coinciding hand skin temperature between Ex_1 and Ex_2, by spray group, nor any interaction ($P > 0.05$).

Perceptual responses (Ex_1 vs. Ex_2)

Participants across all conditions felt 'just comfortable' to 'comfortable' prior to spraying. After spraying and with the onset of exercise, TC fell across all conditions such that participants felt 'just uncomfortable' by the end of exercise. Thermal comfort did not differ between Ex_1 and Ex_2, nor by spray group, with no interaction ($P > 0.05$). Eight participants (four in each menthol spray group) noted some irritation in the intensity range of 'barely detectable' to 'weak'. Of these eight, five reported greater irritation during Ex_1 compared to Ex_2; however, a non-parametric Wilcoxon test showed no difference ($P > 0.05$) in the averaged irritation score between Ex_1 and Ex_2.

Fig. 4a shows upper body thermal sensation by spray group for Ex_1 and Ex_2, Fig. 4b shows the mean TS score during exercise, from minute 15 to 40. Participants across all conditions felt 'neutral' prior to spraying. After spraying and with the onset of exercise, TS fell across all conditions such that participants felt 'cool' by the 15th minute (start of exercise). All participants felt warmer as exercise continued, but participants in CON appeared to feel warmer than those sprayed with 0.05 % menthol, who in turn felt warmer than those sprayed with 0.2 % menthol. Thermal sensation differed significantly between Ex_1 and Ex_2 ($P = 0.017$) and by spray group ($P = 0.047$), with an interaction ($P = 0.015$), suggesting that the scores in Ex_1 and Ex_2 were influenced differently by each spray condition. *Post-hoc* testing showed that 0.2 % menthol spraying induced significantly cooler sensations than Control spraying during Ex_1 ($P < 0.01$), but not during Ex_2 ($P > 0.05$), indicating an habituation of thermal sensation after repeated exposure to 0.2 % menthol.

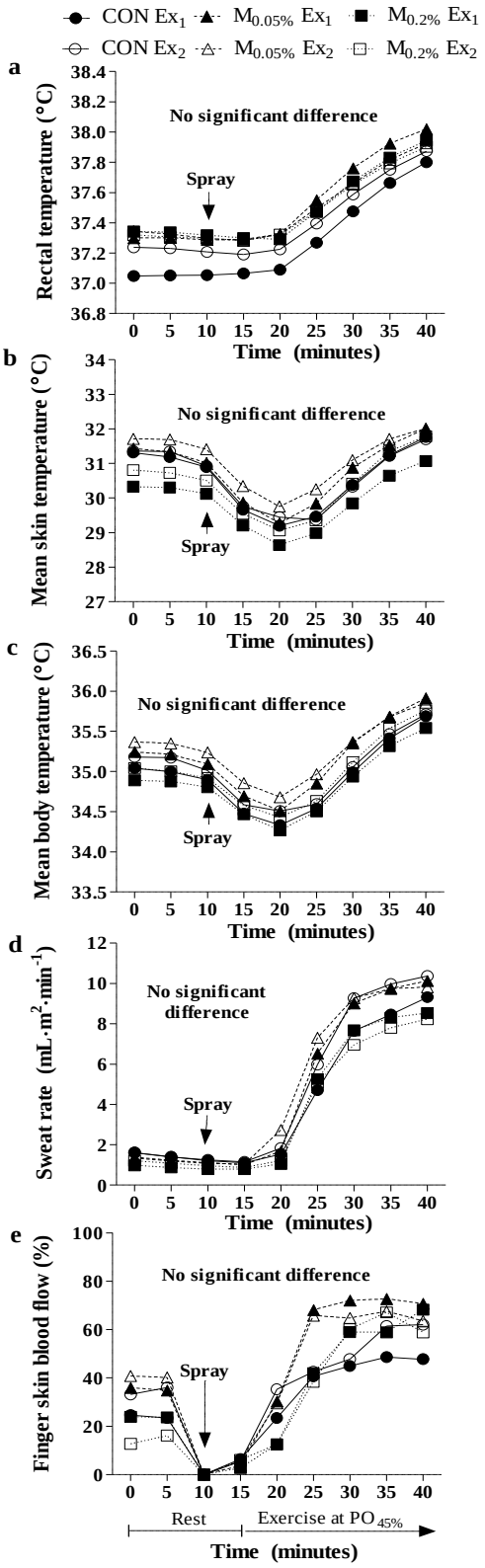

Fig. 3. Mean rectal (a), mean skin (b), mean body temperature (c), lower back sweat rate (d), and finger skin blood flow (e), by spray group (CON [$n = 6$], $M_{0.05\ \%}$ [$n = 8$], $M_{0.2\ \%}$ [$n = 8$]) and exercise condition (Ex_1, Ex_2).

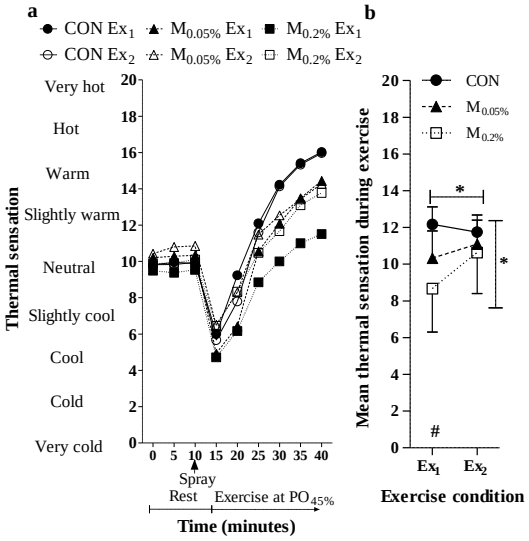

Fig. 4. Upper body thermal sensation during rest and exercise (a) and mean (SD) upper body thermal sensation from the 15th to the 40th minute (b) by spray (CON [n = 6], $M_{0.05\,\%}$ [n = 8], $M_{0.2\,\%}$ [n = 8]) and exercise (Ex_1, Ex_2) condition. Significant difference (* $P < 0.05$) between Ex_1 and Ex_2 (⊢⊣) and by spray condition (⊥). *Post-hoc* test: Significant difference between CON and $M_{0.2\,\%}$ (#, $P < 0.01$).

Part B. Resting sessions (R_1 *vs.* R_5)

Environmental conditions (R_1 vs. R_5)

There was no difference in the mean dry bulb (29.1 [0.5] °C), globe bulb (28.9 [0.5] °C), or wet bulb (22.3 [1.4] °C) temperatures, or rh (54.0 [4.6] %) by spray group, or between R_1 and R_5, and no interaction ($P > 0.05$).

Thermoregulatory responses (R_1 vs. R_5)

Fig. 5a shows the mean T_{re} scores by spray group for R_1 and R_5. The ΔT_{re} in the 30 minutes post-spraying did not differ between R_1 and R_5 ($P > 0.05$), but did significantly differ by spray group ($P = 0.007$), with no interaction ($P > 0.05$). *Post-hoc* testing showed that 0.2 % menthol spraying induced a significant elevation in T_{re} compared to CON and $M_{0.05\,\%}$, during both R_1 ($P < 0.05$) and R_5 ($P < 0.05$), indicating a menthol-mediated heat storage response following a single exposure to 0.2 % menthol, and no habituation of this heat storage after repeated exposure to 0.2 % menthol. Fig. 5b shows $\overline{T}_{msk}$ scores by spray group for R_1 and R_5 from minute 30 to 60. The fall in $\overline{T}_{msk}$ in this period did not differ between R_1 and R_5, nor by spray group, with no interaction ($P > 0.05$). Fig. 5c shows $\overline{T}_b$ scores by spray group for R_1 and R_5 from minute 30 to 60. The fall in $\overline{T}_b$ in this post spraying period did not differ between R_1 and R_5, nor by spray group, with no interaction ($P > 0.05$). Fig. 5d shows finger SkBF by spray group for R_1 and R_5 from minute 30 to 60.

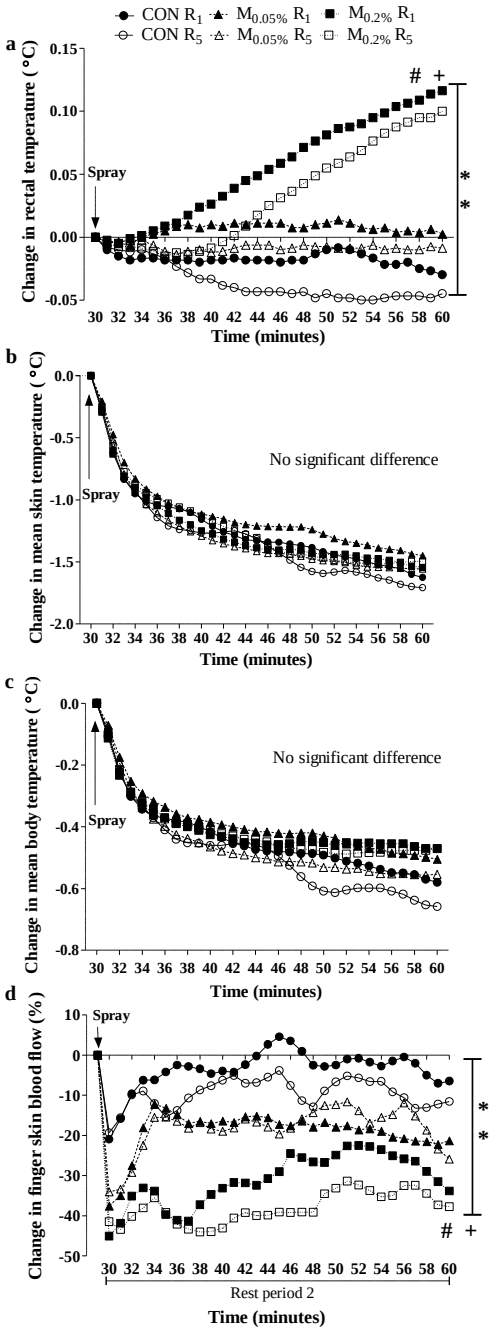

Fig. 5. Mean rectal (a), mean skin (b), mean body temperature (c), and finger skin blood flow (d), from minute 30 to 60, by spray group (CON [n = 6], $M_{0.05\,\%}$ [n = 8], $M_{0.2\,\%}$ [n = 8]) and resting condition (R_1, R_5). ** ⊥; Significant difference by spray group $P < 0.01$). *Post-hoc testing:* Fig. 5a (T_{re}), significant difference between CON and $M_{0.2\,\%}$ (#, $P < 0.05$) and between $M_{0.05\,\%}$ and $M_{0.2\,\%}$ (+, $P < 0.05$). Fig. 5d (finger SkBF); significant difference between CON and $M_{0.2\,\%}$ (#, $P < 0.05$) and between $M_{0.05\,\%}$ and $M_{0.2\,\%}$ (+, $P < 0.05$).

Mean SkBF over this post-spraying period did not differ between R_1 and R_5 ($P > 0.05$), but did significantly differ by spray group ($P = 0.002$), with no interaction ($P > 0.05$). *Post-hoc* testing showed that 0.2 % menthol spraying induced a significant reduction in finger SkBF compared to CON during both R_1 ($P < 0.01$) and R_5 ($P < 0.01$), and compared to $M_{0.05\,\%}$ in R_5 ($P < 0.05$). Neither the onset of vasodilation, nor the coinciding increase in skin temperature measured on the back of the hand differed between R_1 and R_5, or by spray group, with no interaction ($P > 0.05$).

These findings show a menthol-mediated vasoconstriction after a single exposure to 0.2 % menthol, and no habituation of the enhanced vasoconstrictor response following repeated 0.2 % menthol spraying.

Perceptual responses (R_1 vs. R_5)

Participants across all conditions felt 'just comfortable' to 'comfortable' prior to spraying. After spraying, TC fell across all conditions, albeit more so with either menthol spray, such that comfort reduced, but did not reach discomfort. Thermal comfort did not differ between R_1 and R_5, nor by spray group, with no interaction ($P > 0.05$).

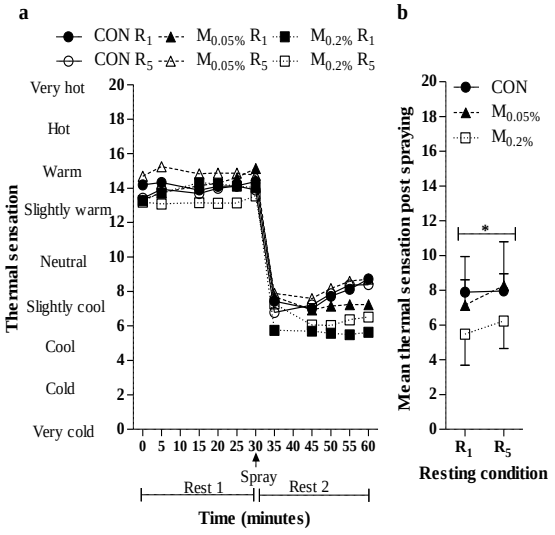

Fig. 6. Mean upper body thermal sensation during 60 minutes of rest (a) and its mean (SD) score over the last 30 minutes (b) by spray (CON [$n = 6$], $M_{0.05\,\%}$ [$n = 8$], $M_{0.2\,\%}$ [$n = 8$]) and resting (R_1, R_5) condition. Significant difference (* $P < 0.05$) between R_1 and R_5 (⊢⊣). *Post-hoc* test: Significant difference between CON and $M_{0.2\,\%}$ (#, $P < 0.05$) and between $M_{0.05\,\%}$ and $M_{0.2\,\%}$ (+, $P < 0.05$).

Fig. 6a shows upper body thermal sensation by spray group for R_1 and R_5, Fig. 6b shows the mean (SD) TS score during the last 30 minutes of rest, post spraying. Participants across all conditions felt 'slightly warm' to 'warm' prior to spraying. After spraying, TS fell across all conditions such that participants felt 'slightly cool' to 'cool' by the 35th minute. Participants in CON appeared to feel warmer than those sprayed with 0.05 % menthol, who in turn felt warmer than those sprayed with 0.2 % menthol. Thermal sensation differed significantly between R_1 and R_5 ($P = 0.017$), but not by spray group ($P = 0.08$), with no interaction ($P > 0.05$); the direction of effect could not be statistically determined with *post-hoc* testing.

Nine participants out of 16 exposed to menthol noted irritation (five in $M_{0.2\,\%}$ and four in $M_{0.05\%}$) in the intensity range of 'barely detectable' to 'weak'. Of these nine, six noted greater irritation during R_1 than R_5; however, a non-parametric Wilcoxon test showed no difference ($P > 0.05$) in the averaged irritation score between R_1 and R_5.

Discussion

This study examined whether the perceptual or physiological effects of menthol habituate after repeated 0.05 % or 0.2 % menthol solution spraying.

Menthol, perception, and habituation

That 0.2 % menthol spraying resulted in significantly cooler sensations than Control spraying during Ex_1, but not during Ex_2, suggests that repeated exposure to 0.2 % menthol results in an habituation of thermal sensation. Over the testing week, cool sensations diminish by two units on the TS scale, which, by the end of the exercise, equated to a perceptual shift from feeling 'neutral' in Ex_1 to 'slightly warm' in Ex_2. Although not significant, the 0.05 % menthol group also underwent a shift, whereby cool sensations diminished by one TS unit over the week. That $M_{0.05\,\%}$ did not induce significantly cooler sensations than CON during Ex_1 is in contrast to other studies (Gillis, House & Tipton, 2010), but probably can be attributed to increased variability accompanying a between participant study design. As a result, it remains to be clarified whether 0.2 % or 0.05 % menthol still induces cool sensations that are significantly (statistically) cooler than a Control spray, after an habituation has occurred. It is likely that cool sensations would still prevail even after an habituation to 0.05 % menthol spraying, as Gillis, House & Tipton (2010) has shown that thermal sensation was improved by four units on the TS scale, so losing one TS unit by habituation may still allow for a 3 TS unit improvement.

A number of reasons may explain why thermal sensation did not undergo a significant habituation from R_1 to R_5. By the time participants had completed R_1 they had already undergone one menthol exposure in Ex_1, suggesting the habituation occurred after one exposure. Also, there was less of a forcing function between R_1 and R_5 because participants underwent five menthol exposures between R_1 and R_5, and eight from Ex_1 to Ex_2.

These findings suggest that repeated exposure to menthol results in an habituation of thermal sensation. The

observation that T_{re} and finger SkBF were altered both before and after repeated menthol exposure suggests that the adaptation was not at the peripheral receptor and not physiological in nature. The adaptation was probably located more centrally in higher brain structures, and indicative of a perceptual adaptation, but the underlying mechanisms are not clear.

A single menthol exposure results in activation of the TRPM8 receptor (McKemy, Neuhausser & Julius, 2002; Peier et al., 2002), which triggers neuronal activations that ascend to higher brain structures, possibly terminating in the somatosensory cortex (perhaps the insular cortex) by way of the thalamus (Craig, 2002). The menthol-mediated perceptual habituation might occur in any of these higher structures. This assertion is not new, and is reminiscent of the conclusions drawn by physiologists studying human adaptation to cold. But unlike cold habituation, the menthol induced habituation of TS occurs without a change in any physiological variable measured in this study, and although repeated exposures to either cold air (Bruck, Baum & Schwennicke 1976; Leppaluoto, Korhonen & Hassi 2001; Makinen et al., 2001) or cold water (Smolander et al., 2004; Tipton et al., 2008) have been shown to cause an habituation in cool sensations and/or thermal discomfort, the underlying mechanisms driving the habituation may not be comparable.

The habituation in TS might also be described using psychological theories in adaptation, which attribute the perceptual habituation to altered expectations and reduced attentional focus on a once novel and unfamiliar stimulus (Veitch & Arkkelin 1995; Wohlwill 1975).

There was no measurable habituation in thermal comfort during the exercise or resting sessions. Further, irritation did not reduce after repeated exposure to menthol. It is important to note that only eight participants in either menthol group perceived irritation. Although five of these individuals noted greater irritation at the beginning of the week than at its end, these findings primarily support the notion that there is a large individual difference in the perception of irritation with menthol exposure.

Menthol, body temperature regulation, and habituation

The combination of cycle ergometry and heat stress employed in Ex_1 and Ex_2 was sufficient to induce a cardiovascular and thermoregulatory challenge. Each group was similar in $\dot{V}O_{2peak}$ and PO_{peak}, and all participants maintained a comparable HR, $\dot{V}O_2$ and RPE across conditions. Air temperature was also similar across conditions. Although rh was 12 % higher in CON, compared to $M_{0.05\ \%}$ and $M_{0.2\ \%}$, this should not have reduced the capacity for evaporative heat loss in CON because it only amounts to a difference in ambient water vapour pressure of 0.2 Kpa. Furthermore, this study was primarily concerned with comparing the change in response from the beginning to the end of the week *within* each spray group; so the elevation in rh observed in CON is of little consequence, particularly as a significant difference was not observed in any of the physiological or perceptual responses in CON. Although T_{re} appeared to be greater in CON Ex_2 compared to CON Ex_1 (Fig. 3a) there was no difference in the ΔT_{re} between the two during exercise. Similarly, there was no difference in the ΔT_{re} observed during exercise within $M_{0.05\ \%}$ or $M_{0.2\ \%}$ from Ex_1 to Ex_2, nor was there any difference in $\Delta \overline{T}_{msk}$, $\Delta \overline{T}_b$, finger SkBF, sweat rate, and the respective measures coinciding with the onset of either thermoeffector over this period. Given that 0.2 % menthol has previously been shown to increase ΔT_{re} by 0.2 °C compared to Control spraying (Gillis, House & Tipton), a complete habituation should see a similar reduction from Ex_1 to Ex_2; but the reduction seen in $M_{0.2\ \%}$ over this time (0.03 °C) was smaller than that in CON (0.05 °C); further emphasising there was no habituation in the 0.2 % menthol-mediated heat storage response.

That 0.2 % menthol spraying did not induce a significant increase in heat storage compared to CON in Ex_1 is in contrast to the findings of Gillis, House & Tipton(2010), and other studies with menthol (Kounalakis et al., 2010; Lee et al., 2011). When comparing between groups in this study, the influence menthol exerted over T_{re} during exercise was likely clouded with participant differences, whereas previous studies were more sensitive to the effect of menthol because subjects served as their own controls. It is also possible that environmental factors or individual differences in exercise-induced metabolic heat production increased variability.

Metabolic heat production was lower in the resting sessions, which allowed menthol to exert a more measurable effect. When 0.2 % menthol was sprayed on the skin at the 30th minute of the resting sessions, a reduction in skin blood flow followed that was greater than that observed in CON. The enhanced vasoconstrictor tone was not mediated by a fall in $\overline{T}_{msk}$, but instead most probably by activation of the TRPM8 receptor (Mckemy, Neuhausser & Julius 2002; Peier et al., 2002). These data suggest that TRPM8 may function as a kind of comparator such that when activated by menthol (or by skin temperatures below 27 °C), warming or heat conservation responses are observed. The role of TRPM8 as a comparator, or as a 'thermostat of the skin' as described by Tajino et al., (2007) is a topic of debate. Thermoreceptors located within the body convey thermal information to higher brain structures; this information is then integrated in the hypothalamus (Romanovsky, 2007). Cold and heat defense responses are driven by two distinct areas in the hypothalamus (Morrison & Nakamura, 2011), but it is not clear how the hypothalamus integrates the information and triggers these responses. One theory suggests that the neural pathways for cold and heat defense communicate with each other whereby activation of one inhibits the other in a process referred to as reciprocal cross inhibition (Sherrington, 1906; Bazett, 1949; Bligh, 1998); but it is also possible that each pathway is independent

(Kobayashi *et al.,* 2006). Given that the present experiment did not observe a menthol-mediated withdrawal of sudomotor function, it is difficult to confirm or refute the importance of reciprocal cross inhibition in thermoregulatory function at the systems level.

In any case, observations made on resting (Savage & Brengelmann, 1996) and exercising (Franks *et al.,* 1996) humans suggest that the regulated variable in the whole system is an integrated mean body temperature, which is probably derived from the cumulative input from thermoreceptors located within the body (Werner *et al.*, 2008). Building upon this premise, menthol-mediated activation of TRMP8 cold receptors enhanced the proportional afferent output arising from cold receptors in the skin, such that higher brain structures received a cold input that would have been interpreted as a fall in skin temperature. As a result, and because individuals were in the thermoneutral zone, the hypothalamus attempted to stabilise $\overline{T}_b$ by allowing T_{re} to drift up. But because the additional vasoconstriction mediated by menthol was independent of, and not due to, a fall in skin temperature, mean body temperature rose with rectal temperature.

Given that the regulation of $\overline{T}_b$ at rest is characterised by an inverse relationship between skin and deep body temperature (Savage & Brengelmann, 1996), it is possible to estimate the reduction in mean skin temperature required to offset the menthol-mediated rise in T_{re}. For example, if $\overline{T}_b$ is maintained around 35.1 °C (as it was in CON R_1, end of the resting session), a 0.15 °C menthol-mediated elevation in T_{re} (equating to 37.1 °C in $M_{0.2\ \%}$, R_1) would need to be offset with a mean skin temperature of 31.48 °C according to Burton's formula (Burton, 1935) ($[T_{re} \cdot 0.65] + [\overline{T}_{msk} \cdot 0.35]$). However, the actual mean skin temperature value in the 0.2 % menthol spray condition was 0.5 °C warmer than this (32 °C). This suggests that the menthol-mediated increase in neuronal output arising from peripheral cold thermoreceptors was equivalent to a 0.5 °C fall in $\overline{T}_{msk}$ and the body reacted by regulating $\overline{T}_b$ as described.

The 0.2 % menthol-mediated activation of cold receptors was associated with an enhanced vasoconstriction and a lower skin blood flow in a warm (30 °C) environment compared to a Control condition. During rest in a thermoneutral environment, $\overline{T}_b$ is regulated by altering skin blood flow (Savage & Brengelmann 1996). In this zone, maximal states of vasoconstriction and vasodilation are primarily influenced by neuronal activity arising from thermoreceptors. Of course, thermoreceptor activity is most often influenced by skin temperature, which can be influenced by a number of factors, including ambient temperature (Mekjavic & Eiken, 2006) or water spraying (Savage & Brengelmann 1996). But this experiment has shown that the activity arising from thermoreceptors in the thermoneutral zone (TNZ) can also be influenced by menthol, as depicted in Fig. 7.

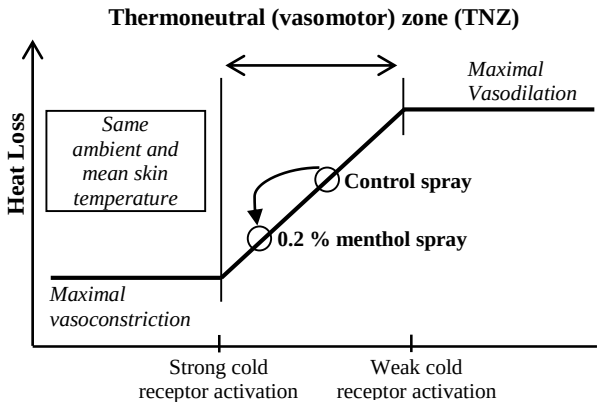

Fig. 7. The influence of menthol on the thermoneutral zone (TNZ).

Menthol-mediated vasoconstriction, as shown in Fig. 7, is independent of skin and ambient temperature. For this reason, labelling its horizontal axis with the skin temperatures associated with thermoneutrality (*i.e.* 33 °C and 35 °C; [Savage & Brengelmann 1996]) is misleading, as is labelling it with ambient temperature. Although Fig. 7 is an over-simplification of the neuronal input driving vasomotion in the TNZ, its purpose is to focus on the neuronal drive arising from thermoreceptors as an input to thermoregulatory centres, rather than skin or ambient temperature.

0.2 % menthol spraying represented a sufficient forcing function to perturb thermal homeostasis upon a single exposure (Fig 5a), but the heat storage response did not undergo an habituation after repeated exposure, a finding which is counter-intuitive to adaptation theory (Tipton et al., 2008). Although 0.2 % menthol spraying resulted in an elevation in T_{re} above the Control condition, there was no significant elevation in $\overline{T}_b$, indicating that thermal balance was achieved. This may suggest that the added heat storage encountered with 0.2 % menthol spraying, at least during rest, is more statistically relevant than practically. But it remains to be determined whether a larger dose or greater surface area exposed to menthol might increase the forcing function such that an habituation might be observable after repeated exposures. Further research is required to clarify this question.

Conclusions

The enhanced vasoconstrictor tone that followed menthol spraying appeared to contribute to a heat storage response, and there is no habituation of this response. Thermal sensation underwent an habituation, most significantly after repeated 0.2 % menthol spraying. Given the menthol-mediated vasoconstrictor response was evident before and after repeated 0.2 % menthol sprayings, the peripheral receptor is not likely to have been the site of the habituation, as its activation is thought to be causal in initiating the heat storage response. This suggests that the habituation in thermal sensation was located more centrally, in higher brain structures. Given

the modest sample size, and between-group design employed in the present experiment, further work will be necessary to evaluate the relative durability of the autonomic versus sensory effects of menthol application.

The hypothesis that there will be no habituation of the heat storage response following repeated 0.2 % menthol spraying cannot be rejected. This experiment supports the hypothesis that after repeated exposure to menthol, thermal sensation undergoes an habituation.

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Author contributions

Gillis DJ: research question, protocol, data collection, analysis, interpretation, manuscript. Weston N: analysis, interpretation, manuscript. House JR: protocol, research question, analysis, interpretation, manuscript, Tipton MJ: funding, research question, protocol, analysis, interpretation, manuscript.

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