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RESEARCH ARTICLE

The effect of heat acclimation on critical environmental limits and rate of rectal temperature change

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Abstract

Quantifying the effect of heat acclimation (HA) on critical wet-bulb globe temperature ($WBGT_{crit}$) and rate of rectal temperature change (vT_{re}) is relevant for developing guidelines with regards to occupational safety while working in warm environments. This study quantified the effect of HA and the period following cessation of the HA protocol on $WBGT_{crit}$ and vT_{re}. Twenty-eight non-acclimatized participants were divided into a HA ($n = 15$) and control (CON; $n = 13$) group. The HA group underwent a warm-humid (35°C, 65% relative humidity) controlled hyperthermia HA protocol (5–9 days of achieving T_{re} ~38.5°C for 60 min) and four progressive heat stress tests (HSTs) to identify $WBGT_{crit}$ and examine vT_{re}: pre-, after 5 and 9 days of HA, and 4 to 8 days of no heat exposure following HA. CON performed two HSTs on average 13 days apart without heat exposure in between. HA increased $WBGT_{crit}$ after nine (28.5 ± 2.7°C vs. 30.5 ± 2.0°C; $P = 0.016$) but not 5 days (28.5 ± 2.4; $P > 0.05$). No effect of HA on vT_{re} was observed ($P > 0.05$). Four-to-eight days post-HA, $WBGT_{crit}$ and vT_{re} did not differ compared with 9 days of HA ($P > 0.05$). However, a reduction in vT_{re} (−0.4 ± 0.3 °C/h) was observed when comparing 4 to 8 days post-HA to pre-HA. In conclusion, our results demonstrate that more than 5 days of HA are required to increase $WBGT_{crit}$ and indicate that 9 days of HA proceeded by adequate recovery reduced vT_{re} during exercise in the heat.

NEW & NOTEWORTHY We assessed the effect of heat acclimation (HA) on critical environmental limits and rate of rectal temperature change. We show that more than 5 days of heat acclimation are required to increase critical environmental limits and that 9 days of HA proceeded by adequate recovery reduces the rate of rectal temperature change. These findings enhance our understanding of heat acclimation's effect on work capacity in the heat and may be used to design occupational guidelines.

compensable heat stress; critical environmental limits; heat acclimation; rate of rectal temperature increase; wet-bulb globe temperature

INTRODUCTION

Heat stress is increasing globally as temperature, specific humidity, and heat wave frequency and intensity are rising (1). These climatic changes are projected to increase human exposure to the "human adaptability limit" representing the theoretical upper limit above which humans cannot dissipate metabolic heat (2). Recent studies revealed that empirically derived compensable heat stress limits [above which heat balance cannot be maintained and body core temperature (T_c) continuously rises] are lower than the previously suggested human adaptability limit of 35°C wet-bulb temperature, even in young, fit individuals performing a low-intensity activity (3). Exposure to uncompensable heat stress will be more frequent and pose a severe threat to human health (4, 5). The presence of protective clothing (6) and higher metabolic rates (7) would further reduce heat stress compensability. In physically demanding jobs, workers perform tasks with high metabolic rates while wearing protective clothing that reduces performance and increases the risk of heat stroke (8, 9).

ISO7243 (10) provides guidelines to estimate heat stress and exposure limits for an 8-h workday to reduce the likelihood of heat-related illnesses. These exposure limits represent an environmental condition that would result in uncompensable heat stress, given the clothing worn and metabolic rate of the activity performed. When the heat stress is uncompensable, additional heat is stored in the body and T_C increases at a higher rate compared with compensable conditions (11, 12). The environmental temperature, humidity, air movement, and (solar) radiation at this tipping point are collectively termed critical environmental limits or critical wet-bulb globe temperature ($WBGT_{crit}$) (10). Research has been performed to establish $WBGT_{crit}$ for

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a variety of age groups and metabolic rates using a humidity step protocol (3, 7, 13–16). In this progressive heat stress protocol, humidity is increased in a stepwise fashion, and T_c data are visually inspected to determine the WBGT-value at which T_c visually deviates from equilibrium. As WBGT is a good indicator of human thermal strain and is easy to determine, knowledge about $WBGT_{crit}$ is implemented in current occupation guidelines (10, 17). In addition to $WBGT_{crit}$, investigating rates of T_c changes (vT_c) may provide additional information as these rates can be used to assess the time needed to reach detrimental T_c and have been proposed to aid in future policy decisions and safety interventions (12). Therefore, combining knowledge regarding $WBGT_{crit}$ and vT_c will provide a more complete understanding into the thermal limits of safe work environments.

Heat acclimation (HA) is used as an effective mitigation strategy to reduce heat strain by inducing physiological adaptations (18, 19) that may increase $WBGT_{crit}$ (18, 20) and/or reduce vT_c (21). Each of these adaptations independently provides a benefit when working in the heat (12). Physiological adaptations shown to occur within the first 5 days of HA include lower resting and exercise T_C, skin temperature (T_{sk}), and heart rate (HR), whereas it takes 7 to 8 days for whole body sweat loss (WBSL) adaptations to occur (18). Some of the underlying mechanisms causing these adaptations include plasma volume expansion that promotes internal heat transfer from core-to-shell, and thus dry heat loss, alongside onset of sweating at lower T_c and increased sensitivity of the sweat glands that enhance evaporative heat loss (22). As such, HA is widely adopted in military, occupational, and athletic settings (8, 9, 22, 23). In addition, HA status is taken into account in ISO7243 (10) with exposure limits being 2.0–3.0°C WBGT higher for acclimatized compared with non-acclimatized individuals for medium-to-high metabolic rates. To the knowledge of the authors, supporting evidence for these adapted limits only includes work from Bernard et al. (20) and Kenney (24). Both studies retrospectively compared the difference in $WBGT_{crit}$ between acclimatized and unacclimatized individuals, without implementing an experimental design consisting of a pre- and post-HA test. Furthermore, both studies showed that $WBGT_{crit}$ in dry-hot environments was higher for participants who executed a constant workload exercise HA protocol for 8 to 10 days (24) or 5 days (20) than for unacclimatized participants. Acclimatized individuals had, on average, 3.4°C higher $WBGT_{crit}$ than unacclimatized individuals independent of metabolic rate (20), but increases were lower for environments with higher water vapor pressure (25). In addition, a recent meta-analysis showed that HA reduced exercising vT_c in most, but not all studies (21). As HA is a time-consuming endeavor, experimentally quantifying the HA-induced gain in thermal limits for safe work environments (i.e., $WBGT_{crit}$ and vT_c) is needed.

To the knowledge of the authors, no studies have investigated the progression of HA-induced changes in $WBGT_{crit}$ and vT_c after the cessation of the HA protocol. Although HA-induced physiological adaptations lessen gradually after removal of the heat exposure (26), some suggest that adaptations such as maximal oxygen uptake (27) or resting and exercising T_{re} (28) show a delayed adaptative response, and therefore, continue to improve after removal of the heat stress. Insight into the progression of $WBGT_{crit}$ and vT_c after the cessation of the HA protocol would be beneficial for effectively scheduling the HA protocol before working in hot environments.

Therefore, the main aim of this study was to quantify the effect of HA on $WBGT_{crit}$ and vT_c. Based on ISO7243 and previous observations (20, 24), First, it was hypothesized that HA would increase $WBGT_{crit}$ by 3.0–3.4°C WBGT and reduce vT_c. Second, we evaluated how many days of HA are needed to see changes in $WBGT_{crit}$ and vT_c. As a large portion of HA-adaptation occurs during the first 5 days of HA (18, 19, 22) and a previous study (20) and ISO7243 (10) indicate that 5 days of HA increase $WBGT_{crit}$, it was expected that 5 days of HA would be sufficient to increase $WBGT_{crit}$ or reduce ΔT_c. Finally, we explored whether the potential HA-induced changes in $WBGT_{crit}$ and ΔT_c would progress or decay following cessation of the HA protocol.

METHODS

Participants

All procedures were approved by the ethics committee of the Faculty of Behavioral and Movement Sciences of the Vrije Universiteit Amsterdam (VCWE-2023-144R1) and conform to the standards set out by the Declaration of Helsinki (2013). Before the study, participants were informed about the procedures and provided verbal and written consent.

Participants in this study were healthy, unacclimatized, regularly active Caucasian individuals. Before the study, potential participants filled in a Physical Activity Readiness Questionnaire (PAR-Q) aiming to gain insight into potential medical complications. Participants were excluded if they had spent 7 days in a hot environment [>25°C dry-bulb temperature (T_{db})] in the last 2 mo preceding the study, if they suffered from cardiovascular complications, experienced heat stroke in the past, smoked, did not commence in any physical activity, or were older than 45 yr for men or 55 yr for women. These exclusion criteria are standard practice for the local ethical committee.

A control group (CON) was included to correct for random variation (29). Participants were divided into groups based on available time and groups were matched for body mass, maximal oxygen uptake ($\dot{V}O_{2max}$), sex, age, and body surface area (BSA) as these individual characteristics might influence thermoregulation or HA adaptation pathways (30–32) (Table 1). Female menstrual cycle was recorded using a questionnaire, but not controlled for during this study. Three females used the combined pill, three used a hormonal intrauterine device, six reported regular natural menstrual cycles (21–44 days), and four opted not the answer the questionnaire.

Study Design

Measurements were executed between January and April (daily mean T_{db} = 8 ± 2°C, range = −3 to 17°C) in the Netherlands to minimize the potential bodily responses due to seasonal warm weather expected for WBGT above 25.2°C (33).

Figure 1 shows the study design. On the first visit, participant's body dimensions were assessed and a graded exercise test in temperate conditions was performed to establish aerobic fitness status, characterized as $\dot{V}O_{2max}$. At least 7 days (7–31 days) later, all participants completed their first heat stress test (HST1) to establish baseline $WBGT_{crit}$ and ΔT_c. The

Table 1. *Participant characteristics of the HA and CON group*

	HA	CON
n	15	13
Sex (male/female)	6/9	6/7
Age, yr	28 ± 7	30 ± 6
Height, cm	174.1 ± 7.5	177.3 ± 8.5
Body mass, kg	69.5 ± 9.1	72.5 ± 11.3
BSA, m^2	1.8 ± 0.1	1.9 ± 0.2
BSA-to-mass ratio, cm^2/kg	265.6 ± 16.2	263.1 ± 16.3
$\dot{V}O_{2max}$, mL/kg/min	45.9 ± 7.9	46.1 ± 6.8

Values are means ± standard deviation. BSA, body surface area; CON, control; HA, heat acclimation; *n*, number of participants; $\dot{V}O_{2max}$, maximal oxygen uptake.

following day, the HA group began their HA protocol. After 5 and 9 HA days, respectively, the HA group performed HST2 and HST3 to assess the effect of 5 and 9 days of HA on $WBGT_{crit}$ and ΔT_c, respectively. Ten of 15 participants, based on participants' availability, revisited the laboratory after 4 days (~10% decay expected) of no heat exposure for HST4, and the other five after 8 days (~20% decay expected) of no heat exposure to assess the evolution of $WBGT_{crit}$ and ΔT_c. CON did not commence in any HA-sessions and only executed HST1 and HST2 at least 2 days (2–21 days, 13 ± 6 days on average) apart in which they were not exposed to heat stress and were allowed to exercise freely apart from 24 h before a HST. All HST sessions were executed at the same time of day (±15 min). All HA and HST sessions were administered in an environmental chamber (b-Cat B.V., Tiel, the Netherlands).

Testing Procedures

Individual characteristics and graded exercise test.

At the first laboratory visit, participant's body mass (platform scale SATEX SA-1 250, Weegtechniek Holland B.V., Zeewolde, The Netherlands) and height (stadiometer Seca 217, Seca, Hamburg, Germany) were obtained. BSA was calculated using the formula proposed by Du Bois and Du Bois (34).

Subsequently, participants performed a graded exercise test on an electrically braked cycled ergometer (Monark LC7TT, Monark Exercise AB, Vansbro, Sweden) using an incremental protocol in temperate conditions [$T_{db} \approx 20°C$, relative humidity (RH) ≈ 30%] to determine $\dot{V}O_{2max}$. Cycling started at an external workload of 0 W for 3 min, after which the external workload increased linearly until participants reached volitional exhaustion. A breath-by-breath analysis using a metabolic cart (Quark CPET, COSMED, Rome, Italy) was used to monitor oxygen uptake ($\dot{V}O_2$) throughout the entire test. Researchers provided participants with strong verbal encouragement.

Heat stress test.

Before each HST and HA-session, participants were asked to drink a sufficient amount of fluid (0.5 L night before and ~10 mL/kg/body mass in the hours before the test) and abstain from exercise (24 h before), caffeine (12 h before), and alcohol intake (24 h before). In addition, participants brought the same sport shoes, socks, cycling shorts, and sports bra (if preferred), and were encouraged to eat the same meal before every HST and HA session. Upon arrival at the laboratory, participants were requested to provide a urine sample to ensure euhydration, defined as urine-specific gravity <1.020 (PAL-10S, Atago Co. Ltd, Tokyo, Japan) (35). If participants were not euhydrated, they were asked to drink 200 mL of water (this occurred 13 times).

An adjusted version of a previously validated incremental heat stress protocol was used to determine $WBGT_{crit}$ during a HST (14). Participants cycled on a cycle ergometer with a heat production (H_{prod}) of 5.5 ± 0.6 W/kg throughout the entire protocol corresponding with moderate exercise intensity (10). This was validated at three timepoints throughout the test by calculating H_{prod} using a breath-by-breath analysis (36). When H_{prod} deviated >0.5 W/kg from the intended H_{prod}, external workload was adjusted during HST1. During subsequent HSTs, participants cycled the same workloads as during HST1 and H_{prod}.

Climatic chamber T_{db} was maintained at 38°C (38.15 ± 0.4°C) with minimal airflow (<0.2 m/s). RH was set to 6% (9.1 ± 2.6%) for the first 30 min (~45 min due to a consistent delay of the climatic chamber). Thereafter, RH was increased in a stepwise fashion by 3% every 5 min. Progressive heat stress continued until rectal temperature (T_{re}) reached the ethically approved limit of 39.3°C, the participant felt unwell, or 140 min had passed. Heat stress during each HST ranged from 22.4 ± 0.9°C WBGT to 33.5 ± 1.3°C WBGT (range: 20.9–35.2°C WBGT; RH: 5%–77%) and test duration was 127 ± 14.8 min (range: 57–140 min).

HA protocol.

An active controlled hyperthermia HA protocol was executed in a climatic chamber set to warm-humid conditions (35°C T_{db}, 65% RH, 31°C WBGT) with minimal airflow of 0.2 m/s. Every session comprised of a constant part (15 min sitting on a chair followed by cycling on an external workload of 1.75 W/kg for 30 min) and subsequent controlled hyperthermia part with the goal to have T_{re} ~38.5°C for 60 min. Participants were allowed to adjust cycling power output and rest to reach this goal.

General Measurements and Calculations

To calculate whole body sweat loss (WBSL) before and immediately after each session, fully equipped and clothed body mass (CBM), and combined clothing and equipment mass (CEM), were obtained. Measurements were done twice (or thrice

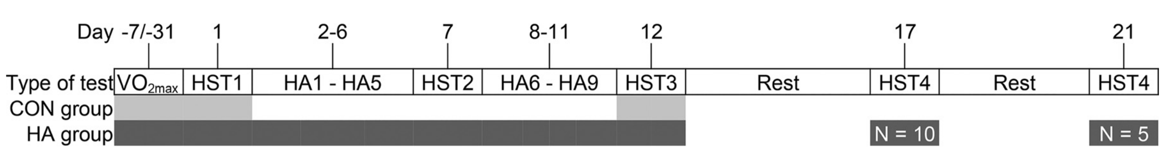

Figure 1. Overview of the study design. Gray bars indicate a lab visit for the corresponding research group. Numbered days represent the timeline for the heat-acclimation group (HA-group). The control (CON, $N = 13$) group executed their two heat stress tests (HST) at least 2 days apart. HA-group executed HST4 after 4 ($N = 10$) or 8 days ($N = 5$) of no heat exposure following the HA protocol. $\dot{V}O_{2max}$, maximal oxygen uptake test.

when the first two measurements were >5 g apart) as recommended (37). WBSL is equal to the sum of changes between CBM and CEM. After weighing, participants were asked to place a rectal probe (MSR, Seuzach, Switzerland; or Yellow Springs Instruments, Yellow Springs, OH) 12 cm past the anal sphincter to monitor T_{re}. In addition, heart rate (HR) (Polar Vantage-M, Kempele, Finland) and T_{sk} on the neck, scapula, hand, and shin (iButtons DS192, Maxim Integrated Products, Inc., San Jose, CA) were monitored throughout the entire protocol. Mean T_{skin} was calculated using weighted averages, see *Eq. 1* (38)

$$\text{Mean } T_{sk} = 0.28 \times T_{sk\_n} + 0.28 \times T_{sk\_sc} + 0.16 \times T_{sk\_h} + 0.28 \times T_{sk\_sh} \quad (1)$$

where, T_{sk} is the skin temperature at the neck (T_{sk_n}), scapula (T_{sk_sc}), hand (T_{sk_h}), and shin (T_{sk_sh}).

Data Analysis

All data were synchronized, formatted, and analyzed using R-software (v.4.3.2, R Foundation for Statistical Computing, Vienna, Austria) in the Rstudio environment (v. 2023.12.1.402, Rstudio, Inc., Boston, MA). Data were reported as means ± standard deviation. The level of statistical significance was set at $P \leq 0.050$. Effect size was reported as partial eta squared (η^2).

In line with previous research, $WBGT_{crit}$ was determined using the current golden standard, namely, via visual inspection by two independent researchers (T.v.d.B., R.M.C.) (method visual) and a segmented linear regression using the visually determined starting point of thermal equilibration (method visual + segmented) (14). If both researchers indicated that an inflection point could not be visually determined using the method visual, data was reported as missing. Differences between researchers were discussed until agreement was reached. The segmented function from the segmented package in R (39) was used to perform segmented linear regression. To explore a method that required no visual input, $WBGT_{crit}$ was also determined using a segmented linear regression (method segmented) using the onset of WBGT increase (time = 40 min) as a start point and 117 min as end point. For each HST, vT_{re} was determined as the slope of a linear model through the data from *minute 0–90*. Starting T_{re} was calculated as the mean during *minute 0–5* and starting T_{sk} as the mean during *minute 20–25* as these timeframes represent the first stable period for these variables. Exercising HR was calculated as the mean HR from *minute 58–62*. $\dot{V}O_{2max}$ was defined as the highest 30-s moving average of $\dot{V}O_2$ during the graded exercise test.

When Mauchly's test indicated a violation of sphericity, Greenhouse–Geisser corrections were applied. The assumption of normality was assessed by visual inspection of the histogram, q-q plot, and the box plot of the data within the groups. *Z* values of skewness and kurtosis, and a Shapiro–Wilks test was also performed on the data. The Shapiro–Wilks and visual inspection indicated that only a limited amount of data was not normally distributed, therefore we used parametric tests. To estimate the degrees of freedom (df) for our statistical analyses, we used the Kendall-Rogers method. To answer our first research question (what is the effect of HA on $WBGT_{crit}$ and vT_{re}), a mixed-model ANOVA with Group (HA, CON) as between-subjects independent variable and HST (HST1, HST3) as within-subjects independent variable was used. A significant interaction effect was further analyzed using a simple planned comparison. To answer our second (if there is an effect of HA, when does it occur) and third (how do $WBGT_{crit}$ and vT_{re} change following cessation of the HA-protocol) research question, we used a one-way ANOVA on the data of the HA group only with HST (HST1, HST2, HST3, HST4) as within-subjects independent variable. Simple planned comparisons were performed and *P* values were corrected using the Bonferroni correction if the ANOVA indicated a significant main effect of HST. Data were pairwise omitted if one set was missing. Finally, these procedures were repeated to assess whether HA induced changes in WBSL, starting T_{re} and T_{sk}, and exercising HR and if the protocol was similarly executed across HSTs and between groups in terms of H_{prod} and test duration.

An a priori power analysis using G*power (40) and a previously reported effect size of 0.4 of HA-induced changes in average exercising body core temperature (41) was executed to determine the required sample size. The analysis suggested that 16 participants (8/group) would yield sufficient statistical power (power > 0.8, $\alpha = 0.05$) to answer our main research question using the mixed-model ANOVA, and 12 participants in the HA-group to answer the second and third research question using the one-way ANOVA.

RESULTS

The Effect of HA on $WBGT_{crit}$

All data reported in this section was obtained through visual inspection by two independent researchers. This method resulted in missing data in instances where an inflection point was not visually determined, which led to the inclusion of a different number of samples for each statistical analysis. See Fig. 2 and Table 2 for the $WBGT_{crit}$ values across HST and groups derived using the "visual" method. See appendix a for an overview of all statistical outcomes for method "visual + segmented" and "segmented."

To test whether $WBGT_{crit}$ was affected by HA, a mixed-model ANOVA with a Group (HA, CON) × HST [1 (pre-HA), 3 (after 9 days of HA)] design was executed. Seven participants for the HA group and six for the CON group who had a $WBGT_{crit}$ value for both HST1 and HST3 were included. There was an interaction effect of Group and HST on $WBGT_{crit}$: $F(1,11) = 8.05$, $P = 0.016$, $\eta^2 = 0.11$. Post hoc comparisons showed that $WBGT_{crit}$ was similar between CON and the HA group during HST1 and was higher by 2.3 ± 2.7°C WBGT when comparing HST3 with HST1, $t(15.1) = -3.35$, $P = 0.0044$, $\eta^2 = 0.65$.

To assess when this effect of HA on $WBGT_{crit}$ occurred, a one-way repeated-measures ANOVA was executed. Six participants had a $WBGT_{crit}$ value for all HSTs. The assumption of sphericity was met ($P > 0.05$). $WBGT_{crit}$ changed significantly across HSTs [$t(3, 15) = 3.696$, $P = 0.036$, $\eta^2 = 0.17$]. $WBGT_{crit}$ was significantly higher for HST3 compared with HST1, $t(15) = 2.62$, $P = 0.05$, $\eta^2 = 0.35$. No significant change was found for HST2 and HST4 compared with HST1 and HST4 compared with HST3 ($P > 0.05$). RH at the $WBGT_{crit}$ for the HA-group was 31 ± 13, 30 ± 12, 40 ± 11, 36 ± 9% for HST1–4, respectively.

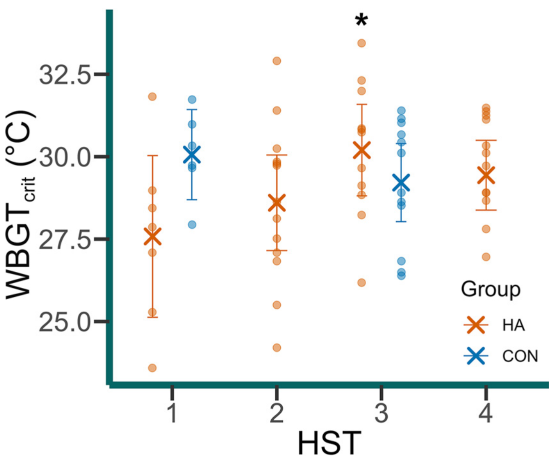

Figure 2. Mean, 95% confidence interval, and individual datapoints of the critical wet-bulb globe temperature ($WBGT_{crit}$) across four heat stress tests (HST). Data are displayed for all visually determined inflection points within the heat acclimation (HA) and control (CON) group pre-HA (HST1, $n = 9/6$), post 5 days of HA (HST2, $n = 15$), post 9 days of HA (HST3, $n = 13/12$), and post 4–8 days of HA cessation (HST4, $n = 14$). *A significant difference with HST1 ($P < 0.05$).

The Effect of HA on vT_{re}

The results are shown in Fig. 3, and mean values are presented in Table 2. There was no significant interaction effect of Group and HST on vT_{re}, $F(1,25) = 0.39$, $P = 0.537$, $\eta^2 = 0.00$. Furthermore, there was a significant main effect of HST on vT_{re} for the HA-group across HST1–4, $F(3,36) = 6.94$, $P = < 0.001$, $\eta^2 = 0.10$. Specifically, vT_{re} was significantly lower for HST4 compared with HST1, $t(36) = -4.11$, $P = < 0.001$, $\eta^2 = 0.32$. Although not significant ($P = 0.09$), there was a downward trend observed as vT_{re} was 0.2 °C/h lower for HST3 compared with HST1. No significant change was found for HST2 compared with HST1 and HST4 compared with HST3 ($P > 0.05$).

Protocol Evaluation

HST.

There was no significant interaction between Group and HST on H_{prod} or test duration ($P > 0.05$). Mauchly's test indicated that the assumption of sphericity was violated for test duration, therefore Greenhouse–Geisser corrections (GGe = 0.53) were applied to the one-way repeated-measures ANOVA outcomes within the HA-group for HST1–4. Test duration was significantly influenced by HST [$F(1.59,20.63) = 4.74$, $P = 0.027$, $\eta^2 = 0.11$], whereas H_{prod} was not ($P > 0.05$). Specifically, test duration was significantly longer in HST2 (128 ± 14 min), HST3 (130 ± 12 min), and HST4 (126 ± 15 min) compared with HST1 (116 ± 22 min) ($P < 0.05$).

HA effectiveness.

There was a significant interaction effect between Group and HST on WBSL [$F(1,26) = 13.79$, $P < 0.001$, $\eta^2 = 0.05$] and starting T_{re} [$F(1,26) = 20.79$, $P < 0.001$, $\eta^2 = 0.05$]. Post hoc comparisons showed that WBSL and starting T_{re} were similar between CON and the HA group during HST1 and significantly increased for WBSL by 165 ± 119 mL/h [$t(26) = -5.38$, $P < 0.001$, $\eta^2 = 0.84$] and decreased for starting T_{re} by 0.44 ± 0.22°C [$t(26) = 7.83$, $P < 0.001$, $\eta^2 = 0.15$] when comparing HST3 with HST1. There was no significant interaction effect for Group and HST on exercising HR and starting T_{sk} ($P > 0.05$). Mauchly's test indicated that the assumption of sphericity was violated for WBSL, therefore Greenhouse–Geisser corrections (GGe = 0.522) were applied for the one-way repeated-measures ANOVA WBSL outcomes. Within the HA-group, there was an effect of HST on WBSL [$F(1.57,20.35) = 15.23$, $P < 0.001$, $\eta^2 = 0.08$], starting T_{re} [$F(3, 39) = 17.42$, $P < 0.001$, $\eta^2 = 0.14$], exercising HR [$F(3,33) = 14.13$, $P < 0.001$, $\eta^2 = 0.19$], and starting T_{sk} [$F(3,36) = 6.50$, $P = 0.001$, $\eta^2 = 0.12$].

Specifically, WBSL was significantly higher and starting T_{re}, starting T_{sk}, and exercising HR were significantly lower

Table 2. *Outcomes of the HST (means ± SD) and the corresponding outcomes of the mixed-models and one-way ANOVA for the HA and CON groups*

Variable	Group	HST 1	HST 2	HST 3	HST 4
$WBGT_{crit}$, °C WBGT	HA	28.5 ± 2.7	28.5 ± 2.4	30.5 ± 2.0#**	29.6 ± 2.0
	CON	30.1 ± 1.3		29.2 ± 1.9	
vT_{re}, °C/h	HA	0.9 ± 0.5	0.8 ± 0.4	0.7 ± 0.4	0.6 ± 0.4***
	CON	0.8 ± 0.3		0.7 ± 0.4	
H_{prod}, W/kg	HA	5.6 ± 0.9	5.6 ± 0.7	5.6 ± 0.6	5.4 ± 0.5
	CON	5.3 ± 0.2		5.4 ± 0.4	
Test duration, min	HA	114 ± 22	128 ± 14**	129 ± 12*	126 ± 14*
	CON	128 ± 7		133 ± 8	
Starting T_{re}, °C	HA	37.2 ± 0.3	36.8 ± 0.4***	36.8 ± 0.4#***	36.9 ± 0.4*$^{+}$
	CON	37.1 ± 0.4		37.0 ± 0.4	
WBSL, mL/h	HA	642 ± 120	755 ± 205***	806 ± 232#***	774 ± 223*
	CON	676 ± 186		676 ± 186	
Starting T_{sk}, °C	HA	35.6 ± 0.5	35.1 ± 0.5**	35.1 ± 0.5**	35.1 ± 0.6*
	CON	35.7 ± 0.6		35.4 ± 0.5**	
Exercising HR, beats/min	HA	125 ± 15	113 ± 15***	106 ± 11***	110 ± 13***
	CON	123 ± 20		113 ± 16**	
Body mass, kg	HA	70.3 ± 9.4	70.4 ± 9.0	70.3 ± 9.2	70.4 ± 9.7
	CON	72.9 ± 11.0		73.1 ± 11.1	

HST1: pre-HA, HST2: post 5 days HA, HST3: post 9 days HA, HST4: 4–8 days post HA cessation. CON, control; H_{prod}, heat production (W/kg body wt); HA, heat acclimation; HR, heart rate (beats/min); HST, heat stress test; T_{sk}, skin temperature (°C); vT_{re}, change in rectal temperature during the first 90 min of the HST (°C/h); $WBGT_{crit}$, critical wet-bulb globe temperature; WBSL, whole body sweat loss (mL/h). #A significant interaction effect according to the mixed-models ANOVA. Significantly different according to the one-way ANOVA compared with HST1. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; $^{+}$a significant difference between HST4 and HST3 according to post hoc tests. CON has no values for HST3 and HST4 as they only performed HST1 and HST2.

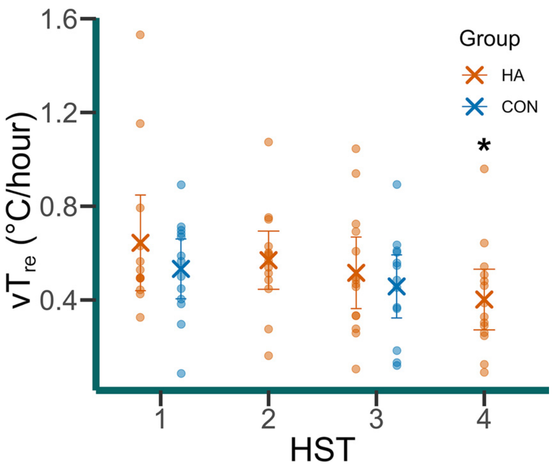

Figure 3. Mean, 95% confidence interval, and individual datapoints of rectal temperature increase (vT_{re}) during the first 90 min of a heat stress test (HST). Data are displayed for the heat acclimation (HA, $n = 15$) and control (CON, $n = 13$) groups. HST1: pre-HA, HST2: post 5 days HA, HST3: post 9 days HA, HST4: post-decay. *A significant difference with HST1 ($P < 0.05$).

in HST2-4 compared with HST1 ($P \leq 0.05$). Starting T_{re} was significantly higher in HST4 compared with HST3, $t(39) = -2.72$, $P = 0.04$. WBSL, HR, and starting T_{sk} were not different between HST3 and HST4 ($P > 0.05$).

DISCUSSION

The main goal of this study was to quantify the effect of HA on $WBGT_{crit}$ and vT_{re}. Results indicate that HA was effective in increasing $WBGT_{crit}$ by 2.3 ± 2.7°C WBGT, but did not alter vT_{re}.

A secondary goal was to assess when these changes occurred. Data show that more than 5 days of HA were necessary to increase $WBGT_{crit}$ and 9 days of HA were insufficient to reduce vT_{re}. Finally, the progression of $WBGT_{crit}$ and vT_{re} following cessation of the HA protocol was explored. We found that the effects of HA on $WBGT_{crit}$ were still present, and vT_{re} reduced 4 to 8 days after cessation of the HA protocol.

Effect of HA on $WBGT_{crit}$

Our experimental data show that 5 days of HA did not increase $WBGT_{crit}$. This is contrary to current occupation guidelines (10) and a recent comparison of HA versus non-HA individuals finding 5 days to be sufficient (+3.4°C WBGT) (20). In addition, 9 days of HA in the current study induced changes in $WBGT_{crit}$ of similar magnitude (2.3 ± 2.7°C WBGT) compared with those reported for 5 days of HA in occupational guidelines (+2.0 to 3.0°C WBGT, for moderate to high metabolic rates) (10, 17). The main difference in research population between our study and Kenney (24) is the inclusion of only female participants in the latter. However, despite differences in absolute $WBGT_{crit}$ values in unacclimatized (15) and acclimatized males and females, $WBGT_{crit}$ increases to a similar magnitude in females compared with males during HA (20). Therefore, we do not expect population differences to explain the different findings in HA-induced changes in $WBGT_{crit}$ between the studies. The HA protocol used in the current study differed from the previously reported HA protocols in terms of the approach, duration, and ambient conditions. First, Bernard et al. (20) and Kenney (24) compared data from acclimated individuals using a constant workload exercise approach and occupational guidelines do not specify the approach, whereas the current study implemented a controlled hyperthermia approach. Second, Kenney (24) (8 to 10 days) and the current study (9 days) implemented a longer HA protocol than the 5-day protocol of Bernard et al. (20) and occupation guidelines. Longer HA protocols are proposed to be more effective compared with shorter protocols, especially when an increasing thermal stimulus is provided, such as with the controlled hyperthermia approach in the current study (18, 19, 22, 42). Third, the ambient conditions in the current study are considered warm-humid (T_{db} 35°C, 65% RH, 31°C WBGT), whereas the HA-protocols mentioned in Bernard et al. (20) [T_{db} 50°C, 20% RH, 35°C WBGT (6, 43)] and Kenney (24) [T_{db} 50°C, 11% RH, 32°C WBGT (44)] are considered hot-dry with slightly higher heat stress in terms of WBGT. Although current literature is inconsistent with regards to the effect of acclimatizing in warm-humid versus hot-dry conditions (22), previous research suggests that the imposed heat stress in terms of °C WBGT determines the magnitude of physiological adaptations (45, 46). All in all, the differences in HA protocol are unlikely to explain the large discrepancy in HA-induced changes in $WBGT_{crit}$ found in the current study compared with previous research (20, 24) and occupation guidelines (10, 17) following 5 days of HA. Our study used a within-subject HA intervention, addressing the large individual variability in $WBGT_{crit}$ presented in this paper (see Fig. 2) and previous studies (e.g., range ±6–10°C WBGT) (7, 20).

By definition, $WBGT_{crit}$ depicts the moment when the required evaporation for thermal balance exceeds the maximal evaporation possible in the environment or when the required evaporation cannot be attained due to an inability to reach sufficiently high sweat levels (11). Consequently, T_{re} will continuously rise. Here, H_{prod} was shown equal across HSTs and T_{db} was higher than T_{sk}. Therefore, evaporative heat loss was most likely the only avenue of heat loss (30) and consequently the only way through which HA could have caused an increase in $WBGT_{crit}$. Evaporation is limited by whole body sweat rate in hot-dry conditions that allow for more evaporation and are characterized by a smaller water vapor pressure gradient than warm-humid conditions (44). Consequently, $WBGT_{crit}$ and the effect of HA-induced physiological adaptations on $WBGT_{crit}$ depend on the environmental conditions (24, 25). RH around $WBGT_{crit}$ for unacclimatized individuals in the current study was 31 ± 13%, and the increased WBSL after 5 days of HA was ineffective in increasing $WBGT_{crit}$. Indeed, HA-induced increases in WBSL have a larger effect on $WBGT_{crit}$ in conditions with lower ambient water vapor pressure versus higher water vapor pressure (7, 13, 24). Similarly, females have lower $WBGT_{crit}$ in hot-dry versus warm-humid conditions, whereas males have similar $WBGT_{crit}$ across conditions as they are able to attain higher maximal sweat rates (15). However, when an increase in WBSL is combined with other sudomotor HA adaptations such as an earlier onset of sweat threshold (19), more uniform sweat distribution (47, 48), and more dilute sweating (19), this increases evaporative heat loss and potentially $WBGT_{crit}$ in both hot-dry and warm-humid conditions. These sudomotor adaptations are shown to occur after more than 7 days of HA (18), which explains the finding of increased $WBGT_{crit}$ after nine, but not five, days of HA in the current study. Although the current study cannot confirm this explanation as these sudomotor adaptations were not measured, it should be noted that these HA-induced sudomotor adaptations have the potential to increase $WBGT_{crit}$ to a greater extent in environments with low water vapor pressure (24, 25) and gently points in the direction of water vapor pressure-dependent occupational guidelines. However, before changing these guidelines, further research should investigate whether $WBGT_{crit}$ increases are indeed larger in hot-dry conditions.

Surprisingly, starting T_{sk} and exercising HR were lower in CON during HST2 compared with HST1. A satisfactory explanation for these physiological changes is lacking as physiological accommodation to the heat (49) and seasonal acclimatization (33) seem unlikely given the wash-out period between HST1 and HST2 (average of 13 ± 6 days) and the maximum environmental dry bulb temperature (17°C) during the measurement period. These changes in CON are aligned with, but less pronounced than, the T_{sk} and HR changes in the HA-group. As the reduction in starting T_{sk} and exercising HR did not influence $WBGT_{crit}$ (and vT_{re}, see *Effect of HA on vT_{re}*) in CON, these unexpected changes do not influence our conclusions.

All in all, the first hypothesis that HA can increase $WBGT_{crit}$ was confirmed, although the increase (+2.3 ± 2.7°C WBGT) is less than expected (+3.4°C WBGT). The second hypothesis (5 days of HA is sufficient to increase $WBGT_{crit}$ and the increase is further augmented following 9 days of HA) was rejected as more than 5 days of HA are necessary to increase $WBGT_{crit}$ in warm-humid conditions. Finally, the third hypothesis ($WBGT_{crit}$ improvements partially decay following cessation of the HA-protocol) was rejected as $WBGT_{crit}$ was similar after 4 to 8 days following cessation of the HA protocol compared with post 9 days HA.

In addition to reporting $WBGT_{crit}$ determined by the "visual" and "visual + segmented" methods, which are previously shown to provide similar results (14) and are commonly used (7, 13–16), we made an effort to incorporate a method without any subjective interpretation. Although other physiological change patterns, such as the anaerobic threshold (50), have been successfully determined mathematically, our objective method for $WBGT_{crit}$ did not yield similar results to the "visual" method. Specifically, $WBGT_{crit}$ determined by the "segmented" method did not increase after 5 or 9 days of HA and correlated poorly with the "visual" method ($r = 0.33$). Visual inspection of the breakpoints identified by the "segmented" method did not always make sense. Therefore, we cannot consider the "segmented" method a viable replacement for the current visual standard. Future research could focus on developing a reproducible objective method for determining $WBGT_{crit}$.

Effect of HA on vT_{re}

Nine days of HA did not significantly change vT_{re}. Research with regards to the effectiveness of HA for reducing vT_{re} is inconclusive. Some studies suggest that HA can reduce vT_{re} (51), whereas other report mixed results (21). A reduction in vT_{re} might be caused by a reduction in T_{re} threshold for skin vasodilation and onset of sweating and increases in sweat rates and skin blood flow, which collectively increases cardiovascular efficiency and heat dissipation mechanisms (21, 51). Other reviews confirm that HA might cause these adaptations (18, 19, 22). Of these adaptations, only WBSL was measured in the current study. Although WBSL increased following 5 and 9 days of HA, this did not reduce vT_{re}. Two potential explanations for this finding are the HA-induced decrease in starting T_{re} and the potential for overreaching (which will be discussed in the next paragraph). Following a decrease in starting T_{re} and starting T_{sk} of similar magnitude (~0.4–0.5°C), the core-to-shell gradient remains similar whereas the skin-environment temperature gradient increases, leading to higher dry heat gain and higher vT_{re} during the early stages of the HST. This could potentially mask improvements in heat loss capacity when looking at vT_{re} with different starting T_{re}. Additional analyses (see appendix b) confirm this notion as vT_{re} of only the first 30 min did not change across HSTs, whereas vT_{re} without the first 30 min was significantly reduced by 0.2°C/h following 9 days of HA. Future research might focus on elucidating the effectiveness of HA on vT_{re} by having starting T_{re} similar pre- and post-HA.

Although not significant, the downward trend in vT_{re} found in the present study following 9 days of HA compared with pre-HA (−0.2°C/h) might be relevant in the work setting. For example, given a starting T_{re} of 37.0°C and using vT_{re} reported in Table 2, it takes 96 min for an unacclimatized individual and 126 min for an acclimatized individual to reach the occupational vT_{re} threshold of 38.5°C (17). All in all, 9 days of HA might cause a workplace-relevant reduction in vT_{re}, but future research is warranted.

Progression of vT_{re} and $WBGT_{crit}$ following Cessation of HA

$WBGT_{crit}$ and vT_{re} did not differ 4 to 8 days following cessation of the HA protocol when compared with immediately post-HA. However, vT_{re} showed a significant reduction 4 to 8 days following cessation of the HA protocol when compared with baseline. In addition, of the HA-induced physiological adaptations, only starting T_{re} showed decay following 4 to 8 days after cessation of the HA protocol. Although common belief is that HA-adaptations decay following removal of the heat stress exposure with a rate of ~2.5% per day, this rate differs per physiological adaptation (26) and studies suggest that some adaptations, i.e., $\dot{V}O_{2max}$ (27) and vT_{re} (28), might be optimized after the cessation of the HA protocol. Our finding of a reduction in vT_{re} 4 to 8 days following cessation of the HA protocol supports this notion. The delayed adaptive response of $\dot{V}O_{2max}$ was attributed to recovery from the strain associated with the HA protocol (27). In line with this is the more recent attention for recovery around HA protocols as daily heat exposure and intense exercise, without adequate recovery, can lead to physiological stress, overreaching, and incomplete adaptation (52, 53). For example, a 5-day high-intensity cycling exercise in the heat caused a reduction in starting T_c, but also a decrease in cycling performance immediately after the HA protocol (54). Compared with recent guidelines (52), the current study avoided unnecessary heat exposure as experiments were conducted in European winter and participants were encouraged to drink and eat sufficiently but did not pay attention to sleep hygiene or incorporate cooling strategies, and participants were unaccustomed to the imposed training loads during the HA protocol (52).

The reduction in vT_{re} 4 to 8 days following cessation of the HA protocol compared with pre-HA (-0.4 ± 0.3°C/h) might be workplace-relevant. Compared with an unacclimatized individual, it takes 54 min longer for an acclimatized individual with recovery following the cessation of the HA protocol to reach the occupational vT_{re} threshold of 38.5°C (17). Although part of this reduction might be attributed to the increase in starting T_{re} found in the current study, the recovery following cessation of HA might also play a role (28). Further research might contribute to the optimization of HA protocols (55) by exploring which adaptations decay and which adaptations might benefit from the removal of the heat stress exposure.

Limitations

First, our findings indicate that more than 5 days of HA was necessary to increase $WBGT_{crit}$. In the current study, $WBGT_{crit}$ increased following 9 days of HA; however, this might not be the minimum number of days required to increase $WBGT_{crit}$. Future research might conduct a HST after a shorter (e.g., 6 or 7 days) HA period to optimize these recommendations. Second, T_{re} was used in the current study as a proxy for T_c. T_c measurement location has been shown to have no influence on $WBGT_{crit}$ results (16) and previous studies have also used T_{re} (6, 43, 56). However, T_{re} is slower to respond compared with other methods, e.g., esophageal, due to thermal inertia, which is particularly large in the poorly perfused rectum when combining exercise with heat stress (57). This potentially limited the ability to determine inflection points in real time. Third, the identified T_c plateaus in the current study are steeper than previously reported (average in warm-humid conditions 0.09 °C/h, range 0.04–0.23 °C/h) (12). This might be contributed to the higher relative and absolute work intensity of the current study compared with Cottle et al. (12) as the rate of T_c increase is higher in compensable (58) and uncompensable (59) conditions for higher work intensities. Finally, the HST in the current study to determine $WBGT_{crit}$ resulted in many missing data, especially when participants were unacclimatized. This indicates that the typical plateau and inflection point required to determine $WBGT_{crit}$ as previously reported (7, 12, 14, 15) was not always present and the findings regarding the effect of HA on $WBGT_{crit}$ only apply to individuals showing the typical T_{re} curve. Future studies are advised to validate the existence of a clear inflection point for the condition of interest (e.g., high work rate) and consider longer steady-state conditions (> 30 min) during the HST before commencing a time-consuming intervention study to minimize missing data.

Conclusions and Recommendations

To the authors knowledge, this study is the first to proactively, and experimentally, evaluate the effect of heat acclimation on critical environmental limits. Results show that *1*) heat acclimation increases critical environmental limits by 2.3 ± 2.7°C wet-bulb globe temperature in warm-humid conditions, *2*) more than 5 days of heat acclimation is necessary to increase critical environmental limits, and *3*) the increase in critical environmental is sustained following 4 to 8 days of cessation of the heat acclimation protocol. Potentially, the warm-humid conditions during the current study explain the smaller than expected increase in critical environment limits after 5 days of heat acclimation due to limited effectiveness of increased sweating. Future research might focus on the effects of heat acclimation on critical environmental limits in hot-dry conditions.

In addition, the rate of change in rectal temperature was unaffected during heat acclimation, but reduced 4 to 8 days post the heat acclimation protocol. This might be caused by the necessity for recovery after exposure to a physically demanding heat acclimation period. Future research might focus on the time path of adaptation following cessation of the heat acclimation protocol.

APPENDIX A

An overview of all statistical outcomes for method "visual+segmented" and "segmented" (Appendix Fig. A1 and Appendix Tables A1, A2, and A3).

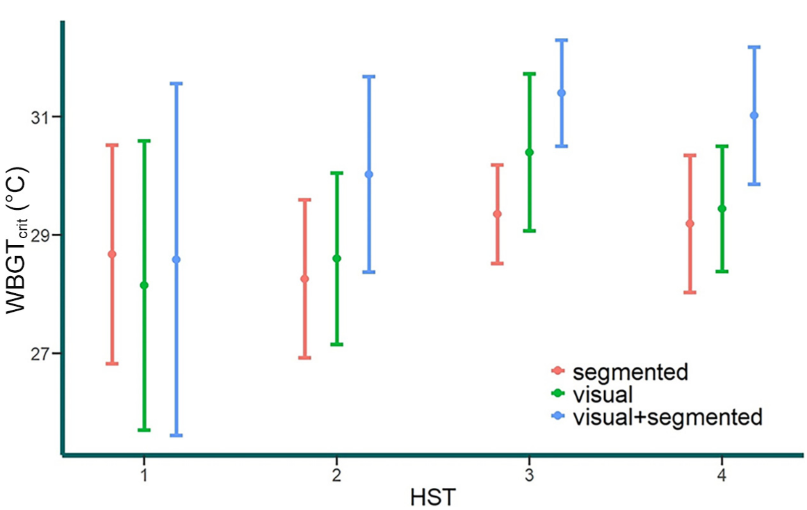

Figure A1. Average and 95% confidence intervals of critical wet-bulb globe temperature ($WBGT_{crit}$) per heat stress test (HST) derived using three different methods for the heat acclimation group. HST1: pre-HA, HST2: post 5 days HA, HST3: post 9 days HA, HST4: post-decay. Only $WBGT_{crit}$ determined using the method visual is significantly higher during HST3 compared with HST1 ($P < 0.05$). No other significant differences were found.

Table A1. *Outcomes of the mixed-models ANOVA analyses*

Method	Group	*n* HST1	*n* HST3	*n* Included	Effect	Statistics
Visual	HA	9	12	7	Interaction	$F(1,11) = 8.05$, $P = 0.016$, $\eta^2 = 0.11$
	CON	6	12	6		
Visual + segmented	HA	9	13	7	HST	$F(1,11) = 5.85$, $P = 0.034$, $\eta^2 = 0.13$
	CON	6	12	6		
Segmented	HA	10	14	10	Group	$F(1,20) = 5.89$, $P = 0.025$, $\eta^2 = 0.12$
	CON	12	12	12		

The number of participants (n) for the heat acclimation (HA) and control (CON) group across two heat stress test (HST) moments are reported alongside the amount of samples included in the analyses (n included). A significant interaction or main effect is mentioned in column "effect" with the corresponding statistics in column "statistics." HST1 = pre-HA, HST3 = post 9 days HA, η^2 = partial eta squared. η^2 indicates the effect size (very small < 0.01, 0.01 ≤ small > 0.06, 0.06 ≤ medium > 0.14, large ≥ 0.14).

Table A2. *Outcomes of the one-way repeated measures ANOVA analyses*

Method	Group	*n* HST1	*n* HST2	*n* HST3	*n* HST4	*n* Included	Effect	Statistics
Visual	HA	9	14	12	13	6	HST	$t(3,15) = 3.696$, $P = 0.036$, $\eta^2 = 0.17$
Visual + segmented	HA	9	14	13	13	6		$t(2,10) = 2.41$, $P = 0.140$, $\eta^2 = 0.15$
Segmented	HA	10	13	14	12	9		$t(2,16) = 0.20$, $P = 0.817$, $\eta^2 = 0.02$

The number of participants (n) for the heat acclimation (HA) group across four heat stress test (HST) moments are reported alongside the amount of samples included in the analyses (n included). A significant main effect is mentioned in column "effect" with the corresponding statistics in column "statistics." HST1 = pre-HA, HST2 = post 5 days HA, HST3 = post 9 days HA, HST4 = post HA and 4 or 8 days of recovery, η^2 = partial eta squared. η^2 indicates the effect size (very small < 0.01, 0.01 ≤ small > 0.06, 0.06 ≤ medium > 0.14, large ≥ 0.14).

Table A3. *Outcomes of the post hoc tests comparing HST 2, 3, and 4 with baseline HST and HST4 with HST3*

Method	Group	*n*	HST2 vs. HST1	HST3 vs. HST1	HST4 vs. HST1	HST4 vs. HST3
Visual	HA	6	$P = 1.00$	$t(15) = 2.84$, $P = 0.050$, $\eta^2 = 0.34$*	$P = 0.06$	$P = 1.00$
Visual + segmented	HA	6	$P = 0.48$	$P = 0.14$	$P = 0.09$	$P = 1.00$
Segmented	HA	9	$P = 1.00$	$P = 1.00$	$P = 1.00$	$P = 1.00$

The number of participants (n) for the heat acclimation (HA) group showing an inflection point across all four heat stress tests (HSTs) were included. Bonferroni corrected P values are reported. Only P values are reported if not significant. HST1 = pre-HA, HST2 = post 5 days HA, HST3 = post 9 days HA, η^2 = partial eta squared. η^2 indicates the effect size (very small < 0.01, 0.01 ≤ small > 0.06, 0.06 ≤ medium > 0.14, large ≥ 0.14). *The significant ($P < 0.05$) results.

APPENDIX B

Rate of rectal temperature change (vT_{re}) was calculated for the first 30 min (vT_{re_30}) and for *min 30–90* ($vT_{re\_30\_90}$) for the HA group. Subsequently, a repeated-measures ANOVA with HST as independent variable and $vT_{re\_30\_90}$ and vT_{re_30} as dependent variable was executed. A significant main effect of HST was succeeded by post hoc comparisons.

See Appendix Figs. B1 and B2 for vT_{re_30} and $vT_{re\_30\_90}$ data, respectively. The assumption of sphericity was met ($P > 0.05$). $vT_{re\_30\_90}$ changed significantly across HSTs [$t(3,36) = 5.11$, $P = 0.005$, $\eta^2 = 0.13$]. Specifically, $vT_{re\_30\_90}$ was significantly lower for HST3 [$t(12) = 2.44$, $P = 0.031$] and HST4 [$t(12) = 2.78$, $P = 0.017$] compared with HST1. No differences were observed for vT_{re_30} ($P = 0.633$).

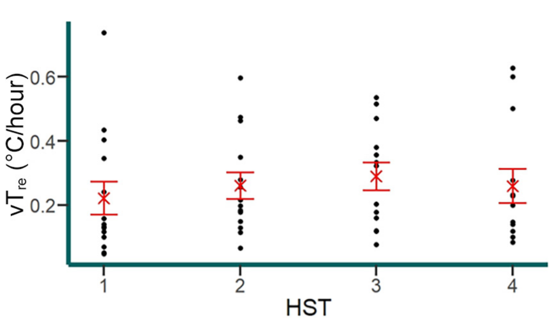

Figure B1. Average and 95% confidence intervals of rate of rectal temperature increase for the first 30 min (vT_{re}) of a heat stress test (HST) for the heat acclimation group. HST1: pre-HA, HST2: post 5 days HA, HST3: post 9 days HA, HST4: post-decay. vT_{re} during the first 30 min did not significantly change throughout the heat acclimation protocol ($P > 0.05$).

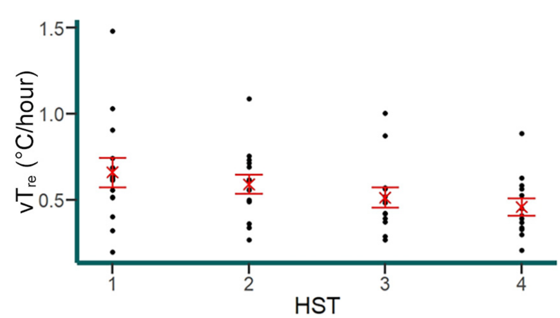

Figure B2. Average and 95% confidence intervals of rate of rectal temperature increase for *minute 30–90* (vT_{re}) of a heat stress test (HST) for the heat acclimation group. HST1: pre-HA, HST2: post 5 days HA, HST3: post 9 days HA, HST4: post-decay. vT_{re} during *minute 30–90* was significantly lower during HST3 and HST4 compared with HST1 ($P < 0.05$).

DATA AVAILABILITY

Source data for this study are openly available at https://doi.org/10.48338/VU01-8GXANQ.

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DISCLAIMERS

The content is solely the authors' responsibility and does not necessarily represent the official views of the listed institutions.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

T.v.d.B., L.K., J.V.E., and H.A.M.D. conceived and designed research; T.v.d.B. performed experiments; T.v.d.B. and R.M.C. analyzed data; T.v.d.B., L.K., J.V.E., and H.A.M.D. interpreted results of experiments; T.v.d.B. prepared figures; T.v.d.B. drafted manuscript; T.v.d.B., L.K., R.M.C., J.V.E., and H.A.M.D. edited and revised manuscript; T.v.d.B., L.K., R.M.C., J.V.E., and H.A.M.D. approved final version of manuscript.

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