STUDY PROTOCOL AND STATISTICAL ANALYSIS PLAN

Project: Physiological Responses in Young and Older Adults During a Prolonged Simulated Heatwave (H 05-16-07)

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Update note

This document provides an update of the study protocol and statistical analysis plan originally submitted April 13th, 2020. The employed methods and measurements, including the addition of measurements of heart rate variability and acute circulating and intracellular inflammatory and stress markers, have been described in greater detail. The statistical analysis plan has been refined considerably.

Data collection was officially completed April 2nd, 2021. Outcome data was blinded on May 19th, 2021, and data cleaning and analysis was completed on August 11th, 2021. The final data set is still blinded at the time of this update.

Note that, for the sake of readability, the grammatical tense of the document has been kept consistent despite the completion of data collection.

Relevance of the research project

Older adults are among the most at risk of acute injury during extreme heat events due, in large part, to impaired homeostatic responses to heat stress. However, much of our understanding of the effects of extreme heat on physiological responses in older adults has come from studies that have employed short-term (<3 hours) exposure to conditions hotter than those typically experienced during heatwaves. Additionally, there is a dearth of information regarding the physiological impacts of currently recommended heat-mitigation strategies. For instance, the World Health Organization recommends that when home-cooling is unavailable, individuals should visit a cooled location (e.g., a cooling centre) for 1-2 hours in order to cool the body though direct evidence to support this guidance is lacking. Whether this intervention can provide lasting reductions in physiological strain is not clear, but it is likely that reductions in body temperature are only transient as heat will be rapidly gained by the body upon re-exposure to elevated ambient temperatures.

The proposed research will provide important and novel data on i) the effect of prolonged exposure to simulated heatwave conditions on whole-body heat storage and resultant

thermoregulatory, cardiovascular, and inflammatory responses in young and older adults, and ii) the efficacy of a cooling centre intervention for limiting physiological strain.

Research Project Objectives

The objectives of this project are to: i) evaluate the effect of age on whole-body heat storage, body core temperature, and the development of cardiovascular strain and acute inflammation during day-long (9 hours) exposure to simulated heatwave conditions (**Intervention 1**); and ii) determine whether short-duration exposure (2 hours) to an air-conditioned environment following extreme heat exposure results in lasting reductions in physiological strain in older adults upon return to the heat (**Intervention 2**).

The first objective (**Intervention 1**) will be achieved by comparing whole-body heat storage (measured using combined direct and indirect calorimetry) and the accompanying changes in body temperature (i.e., rectal, mean skin temperature), cardiovascular, and circulating and intracellular inflammatory and stress responses between young and older adults during a 9-hour resting exposure to ~40°C and ~10-15% relative humidity conditions (heat index: 37-38°C). These conditions were chosen to simulate peak temperatures experienced during heatwaves and are similar to conditions in recent heatwaves in North American in 2018 (Ottawa, Ontario; 34°C and 58%, heat index: 41°C) and Europe in 2003 (Paris, France; 38°C and 25%, heat index: 38°C). This intervention will thereby allow for the determination of whether ecologically valid heatwave conditions exceed the physiological capacity for heat dissipation in older adults, and how thermal and cardiovascular strain and inflammation develops during long-duration heat exposure.

The second objective (**Intervention 2**) will be accomplished by comparing physiological responses in the older adults in Intervention 1 to a separate age-matched group removed from the heat to spend 2 hours in a cooler environment (23°C, ~50% relative humidity) approximately mid-way (hours 5-6) through the simulated heatwave. Comparing whole-body heat storage and body temperature, cardiovascular, and inflammatory responses during and after the ambient cooling intervention to the non-cooled group will allow for determination of whether short-term cooling confers prolonged reductions in physiological strain or if the cooling-induced physiological alterations occur only transiently.

Hypotheses

Intervention 1*:* Age-related attenuation of the human heat loss responses (cutaneous vasodilation and sweating) means that older adults experience greater elevations in body heat storage and body core temperature compared to younger individuals during heat stress. For example, Kenny et al. (*Temperature (Austin)*. 2016 31;4(1):79-88). observed ~80% greater heat storage (equivalent to a ~0.5°C greater elevation in mean body temperature) in middle-aged-to-older compared to young adults during 3-hours of resting heat exposure. More importantly, the middle-aged-to-older adults did not achieve a state of heat balance (i.e., they were still storing heat within their body at the end of the 3 hours).

Based on this work, our primary hypothesis is that older adults will not achieve a state of heat balance (i.e., rate of heat loss balancing the rate of heat gain; thermal equilibrium) and therefore experience greater heat storage throughout the 9-hour simulated heatwave compared to the younger participants. Consequently, body core temperature will be greater in the older adults and between-group differences will be exacerbated as exposure progresses. We will also explore the secondary hypothesis that differences in body temperature will be paralleled by greater alterations in cardiovascular variables and circulatory and intracellular markers of acute inflammation and stress in the older adults.

Intervention 2*:* Our primary hypothesis is that body heat storage will be exacerbated in the older adults exposed to the cooling centre intervention upon return to the heat (hours 5-6) compared to the older adults from Intervention 1 who remained in the heat. Consequently, body core temperature will be comparable (statistically equivalent) between groups by the end of exposure (i.e., 9 hours, 3 hours after return to the heat). This is because body temperature is regulated through a negative feedback mechanism. Elevations in core and skin temperatures elicit proportional activation of cutaneous vasodilation and sweating to facilitate heat loss and stabilize body temperature. Thus, core and skin cooling will suppress heat loss and, upon return to a hot environment, heat will be rapidly gained until body temperature rises to an extent sufficient to activate heat loss and re-attain thermal equilibrium. We will also evaluate the secondary hypothesis

that, in parallel to body core temperature, any effect of cooling on cardiovascular responses and acute inflammation will be abated by the end of the heat event.

METHODS

Participants

A total of 20 young (age: 18-30 years) and 40 older (age: 64-80 years) adults will be recruited for the project. Young (n=20) and older (n=20) adults will be recruited for **Intervention 1** and a separate cohort of older adults (n=20) for **Intervention 2**. Details on the determination of the sample sizes for each intervention are provided below.

Written and informed consent will be obtained from all volunteers prior to participation. Recruited participants will be homogenous for anthropomorphic characteristics and habitual physical activity levels as verified via standardized questionnaires and each intervention will have an approximately even distribution of males and females. Older participants will be screened for cardiovascular risk factors prior to the heatwave simulations. No participants will be current smokers. The specific inclusion and exclusion criteria for the study are provided below

Inclusion Criteria

- Male or female (non-pregnant) adults with or without a) chronic hypertension (elevated resting blood pressure; as defined by Heart and Stroke Canada and Hypertension Canada), b) type 2 diabetes as defined by Diabetes Canada, with at least 5 years having elapsed since time of diagnosis and or c) obesity as defined by the World Health Organization (Body Mass Index [BMI] greater than or equal to 30).
- Non-smoking.

Exclusion Criteria

- Episode(s) of severe hypoglycemia (requiring the assistance of another person) within the previous year, or inability to sense hypoglycemia (hypoglycemia unawareness).
- Serious complications related to diabetes (gastroparesis, renal disease, uncontrolled hypertension, severe autonomic neuropathy).
- Uncontrolled hypertension – Arterial blood pressure >150 mmHg systolic or >95 mmHg diastolic in a sitting position.

- Restrictions in physical activity due to disease (e.g. intermittent claudication, renal impairment, active proliferative retinopathy, unstable cardiac or pulmonary disease, disabling stroke, severe arthritis, etc.).
- Use of or changes in medication judged by the patient or investigators to make participation in this study inadvisable.
- Cardiac abnormalities identified in your physical health screening forms (adults <65 years of age and older; adults <60 years of age and older with diabetes and or hypertension) or during exercise stress testing as assessed by 12-lead (all adults ≥65 years of age and older).

Sample size calculations

Intervention 1: An *a priori* power analysis determined that a total sample size of 19 young and 19 older adults is required to detect a difference in the rate of whole-body heat storage between groups at the end of each calorimeter session (i.e., hours 3 and 9) with 80% statistical power. In lieu of clinically meaningful data (i.e., what would be considered a clinically meaningful change in whole-body heat storage), the standardized effect size (Cohen's *d* = 1.06) was calculated from the difference in the rate of body heat storage between young and older adults over the final 30-min of a 3-hour heat exposure (young: -2 [26] kJ/hour, older: 43 [54] kJ/hour) in our previous work (Kenny et al. *Temperature (Austin)*. 2016 31;4(1):79-88).

Intervention 2: An *a priori* power analysis determined that a total sample size of 18 older adults in each group is required to confirm whether between-group differences in rectal temperature are within upper and lower bounds of +0.3°C and -0.3°C, respectively, with 80% power. This corresponds to an effect size (Cohen's *d*) of 1.0, based on the pooled-standard deviation of 0.3°C. Equivalence bounds were chosen based on the typical day-to-day variation of body core temperature (Consolazio et al. *Physiological measurements of metabolic functions in man.* 1963:453-480) and suggested meaningful/detectable change in body core temperature in a recent study assessing the influence of personal cooling strategies on physiological strain in young adults (Morris et al. *Annals of Internal Medicine.* 2019;171(9):675-677). The standard deviation for the between group difference was determined from data from our laboratory demonstrating a 0.2°C (SD 0.3) difference in rectal temperature between young and older adults (Kenny et al.

Temperature (Austin). 2016;4(1):79-88) and a 0.0°C (SD 0.3) difference between older adults with and without type 2 diabetes (Poirier et al. *Temperature (Austin).* 2020;7(3):263-269) during 3 hours of rest in the heat (44°C, 30% relative humidity).

Experimental Design

Pre-trial instructions

Participants will be asked to avoid strenuous physical activity and alcohol for 24 hours prior to all preliminary and experimental sessions and to eat a light meal 2 hours before the start of each session. Participants will also be asked to consume a minimum of 500 ml of water the night before and morning of each session to ensure adequate hydration. Adequate hydration will be verified upon arrival to the laboratory on the day of the heat event simulation via urine specific gravity (operationally defined as a urine specific gravity <1.025; Kenefick & Cheuvront. Hydration. *Nutr Rev.* 2012;70 Suppl 2:S137-142). In the event that participants exceed this threshold, ~500 mL of tap water will be provided, and urine specific gravity will be tested again after ~30 min. For all sessions, clothing will be standardized to shorts and sandals (and a summer top for women).

Preliminary screening

All participants will complete one preliminary evaluation a minimum of 7 days before the experimental session. During this session they will be familiarized with all procedures and measurements and complete the Canadian Society for Exercise Physiology Get Active Questionnaire (GAQ) and the American Heart Association Pre-Participation Screening Questionnaire to assess their eligibility to participate. The GAQ will also be used to assess habitual activity levels along with the Kohl Physical Activity Questionnaire to determine the general types of physical activity performed. Arterial blood pressures will then be evaluated in triplicate via manual auscultation (~30 sec between measures). Thereafter, participant physical characteristics will be evaluated. Body height and mass will be determined via a physician stadiometer (Detecto, model 2391, Webb City, MO, USA) and a high-performance weighing terminal (model CBU150X, Mettler Toledo Inc., Mississauga, ON, Canada), respectively. Body mass index and surface area will be calculated from these measures.

Intervention 1: *Physiological responses in young and older adults exposed to a simulated extreme heat event*

Each experimental session will commence between 07:00-09:00. Upon arrival to the laboratory, the participant will provide a urine sample for the assessment of urine specific gravity and insert a temperature probe for the measurement of rectal temperature (Mon-a-therm General Purpose Temperature Probe, Mallinckrodt Medical Inc.). A nude body mass will then be obtained, and the participant will be instrumented with a 5-lead electrocardiogram (Philips DigiTrak XT Holter Monitor, Philips) and skin temperature sensors (DS1922L Thermochron, OnSolution Pty Ltd) at 8 body regions: forehead, right scapula, upper left chest, upper right arm, right forearm, left hand, right anterior thigh, and left calf.

Baseline cardiovascular parameters will then be evaluated via a brief (~45 min) cardiovascular test battery, performed as follows. Brachial arterial systolic and diastolic pressures reconstructed from arterial pressure waveforms measured at the right middle finger using the volume clamp technique (Finometer Pro, Fina-press Medical Systems) and 5-lead echocardiogram recordings (Philips DigiTrak XT Holter Monitor, Philips) will be collected for 10-min while the participant rests quietly (spontaneous breathing). Immediately thereafter, arterial systolic and diastolic pressures will be measured in triplicate via manual auscultation (~30 sec between measures), after which forearm and calf blood flows on the right side of the body will be measured via automated venous occlusion plethysmography (Hokanson AI6, D.E. Hokanson, Inc.). Throughout the short cardiovascular test battery, the participant will be seated with both feet on the floor, except for during the measurements of limb blood flow, where the instrumented limbs will be elevated to facilitate venous drainage. Finally, a venous blood sample and body mass measurement will be obtained.

The participant will then be transferred to the whole-body air calorimeter chamber (a unique device that allows for direct quantification of heat gain/loss by the body), housed within a thermal chamber regulated to ~40°C and ~10-15% humidity (heat index: 37-38°C). These conditions were chosen to simulate peak temperatures experienced during heatwaves and are similar to peak conditions in recent heatwaves in North American in

2018 (Ottawa, Ontario; 34°C and 58%, heat index: 41°C) and Europe in 2003 (Paris, France; 38°C and 25%, heat index: 38°C). The participant will sit quietly for 3-hours (hours 1-3) within the calorimeter chamber while whole-body heat exchange is measured. Metabolic heat production will be evaluated simultaneously via indirect calorimetry (AMETEK model S-3A/1 and CD 3A, Applied Electrochemistry, Bastrop, TX, USA). At the 3-hour mark, participants will exit the calorimeter, and the brief cardiovascular test battery will be performed again, followed by a measurement of body mass.

Hours 4-6 will be spent resting in the heat in the thermal chamber (but outside of the calorimeter). During this time, the participant will be allowed to consume a light (~300 g), self-provided lunch with low water content (e.g., peanut butter sandwich). Tap water will be available *ad libitum* via a self-service insulated water cooler located in the thermal chamber (out of direct sight). Another cardiovascular battery will be performed followed by a measurement of body mass at the end of this period. The participant will then be transferred to the calorimeter where the final 3 hours will be spent resting in the seated position (hours 6-9). At the 9-hour time point, the participant will be removed from the calorimeter, the cardiovascular test battery was conducted, and a venous blood sample and body mass measurement will be procured. The participant will then be provided with water and/or a commercially available sports drink before leaving the laboratory.

Intervention 2: Efficacy of a cooling centre intervention for limiting physiological strain in the elderly

The protocol for Intervention 2 will be identical to that of Intervention 1 except that after the first calorimeter session and the subsequent cardiovascular battery, the participant will be removed from the thermal chamber to spend 2 hours (hours 5-6) seated in an air-conditioned room (~23°C, ~50% relative humidity). Like protocol 1, the participant will be able to consume water (tap) *ad libitum* and eat a small self-provided during this time. The third cardiovascular battery will be performed in the cooled environment. At the end of the 6th hour, participants will re-enter the calorimeter where they will spend the remaining 3 hours of exposure seated in the heat.

Measurements

Primary Outcome measures

Whole-body heat storage and body core temperature

The primary aim of the study is to assess differences in body temperature regulation between young and older adults (Intervention 1) and the efficacy of a cooling centre intervention for limiting hyperthermia in the elderly (Intervention 2) during a 9-hour simulated extreme heat event. Body temperature regulation will be quantified as between-group differences in whole-body cumulative heat storage (i.e., the total amount of heat stored in the body) and body core temperature (rectal temperature, surrogate measure of body heat content) in each intervention.

Whole-body evaporative and dry heat loss will be continuously measured during the first and final 3 hours of exposure via the Snellen air calorimeter, which provides the only standard measure of these metrics. Calorimeter inflow and outflow values of absolute humidity and air temperature will be measured at 8 second intervals using high precision dew point hygrometry (Model 373H, RH Systems) and resistance temperature detectors (Black Stack model 1560, Hart Electronics), respectively. Air mass flow, equivalent to <0.3 m/s where the participant is seated, will be determined via differential thermometry over a known heat source in the effluent air stream. All data will be displayed and recorded on a personal computer with LabVIEW software (Version 7.0, National Instruments). Heat loss via sweat evaporation will be then determined using the outflow–inflow difference in absolute humidity, multiplied by air mass flow and the latent heat of vaporization of sweat (2427 J/g). Dry heat loss will be similarly derived from the outflow–inflow air temperature difference and specific heat capacity of air (1005 J/kg/°C). Ambient temperature (40°C) will be greater than that of the skin (~35-36°C), meaning that a negative dry heat loss will be measured. Dry heat exchange was therefore expressed as a dry heat gain.

Metabolic energy expenditure will be determined using indirect calorimetry. Expired oxygen (O_2) and carbon dioxide (CO_2) content will be measured with electrochemical gas analysers (AMETEK model S-3A/1 and CD 3A, Applied Electrochemistry) from air drawn from a 6 L fluted mixing box located within the calorimeter. Expelled air will be recycled back into the chamber to account for respiratory heat exchange. The gas analyzers and

turbine ventilometer will be calibrated ~30 min prior to each 3-hour calorimetry measurement period. Endogenous metabolic heat production will be assumed to be equivalent to metabolic energy expenditure since no external work will be performed. Cumulative body heat storage will be evaluated as the temporal summation of total heat gain (heat production + dry heat gain) and evaporative heat loss.

Rectal temperature will be monitored using a general-purpose thermocouple temperature probe (Mon-a-therm General Purpose Temperature Probe, Mallinckrodt Medical Inc.) inserted ~12 cm past the anal sphincter with data collected in 15-s intervals using LabVIEW software (Version 7.0, National Instruments).

Secondary Measures

The secondary aim of the project is to evaluate the extent to which age-related alterations in body temperature regulation (Intervention 1) and short-term ambient cooling (Intervention 2) are associated with alterations in physiological responses that can contribute to dysfunction and injury during a prolonged extreme heat event (Meade et al. *Environ Int*. 2020;144:105909). These include whole-body mean skin temperature and clinically relevant cardiovascular responses, as well as circulating and intracellular markers of inflammation and stress.

Mean skin temperature

Skin temperature will be assessed every minute using surface temperature monitors (DS1922L Thermochron, OnSolution Pty Ltd) affixed to 8 body regions, using double-sided adhesives and medical tape, as described in ISO 9886:2004. Mean skin temperature will then be calculated using the provided weightings: forehead (7%), right scapula (17.5%), upper left chest (17.5%), upper right arm (7%), right forearm (7%), left hand (5%), right anterior thigh (19%) and left calf (20%).

Cardiovascular measurements

Electrocardiogram data from Holter monitor (Philips DigiTrak XT Holter Monitor, Philips) will be downloaded and analysed using Philips Zymed Software (Philips Zymed Version 3.0, Andover, MA). R-R interval data will be extracted from the ECG tracing (3 channel at

a sampling rate of 175 Hz). Normal-to-normal beats, as determined by the Zymed annotation algorithm, will be retained for further analysis. Continuous Individualized Variability Analysis (CIMVA) software (http://ohridal.org/cimva/CIMVA-CoreDescription.pdf) will be used to derive absolute heart rate and indices of heart rate variability (5 min windowed analysis with 30 sec time step). The latter includes 1) standard deviation of successive normal-to-normal intervals (SDNN), an index of overall variability; and 2) the root mean squared of the standard deviation (RMSSD) of successive normal-to-normal intervals, which is more reflective of short-term high frequency fluctuations in heart rate (mediated primarily by the parasympathetic nervous system).

Arterial systolic and diastolic blood pressures will be intermittently taken as an average of the three values measured at the brachial artery (~30 s between measures) via manual auscultation. Mean arterial pressure will be calculated as 2/3 diastolic pressure + 1/3 systolic pressure. Rate pressure product, an index of myocardial work, will also be derived as heart rate × systolic pressure.

Brachial arterial blood pressures and cardiac output will be estimated from beat-to-beat recordings of arterial pressure waveform measured at the right middle-finger using the volume-clamp technique (Finometer Pro, Fina-press Medical Systems). Stroke volume will be calculated as cardiac output ÷ heart rate, whilst total peripheral resistance is derived as mean arterial pressure ÷ cardiac output. Cardiac baroreflex sensitivity will be determined via the sequence technique using software provided by Finapres Medical Systems (PRVBRS, Fina-press Medical Systems). Forearm and calf blood flows will be determined as the average of a minimum of four measurements obtained via automated venous occlusion plethysmography (Hokanson AI6, D.E. Hokanson, Inc.).

Circulating and intracellular inflammation and stress markers

Venous blood will be collected for the determination of circulating and intracellular inflammation/stress responses. Samples collected from the antecubital vein at baseline and the end of exposure will be transferred directly into Vacutainer tubes with no additive (1 x 5 mL SST™ Serum Separator Tube, BD) or potassium ethylenediaminetetraacetic acid (1 x 3 mL and 3 x 10 mL K_2EDTA [7.2 mg and 18 mg, respectively], BD).

Non-additive blood will be let to sit for 20 min to clot before centrifugation at 1.38 relative centrifugal force (RCF) for 10 min (room temperature). Separated serum will then be transferred into polypropylene Eppendorf tubes, and frozen and stored at -80ºC. Serum concentrations of tumor necrosis factor alpha (TNF-α, DY210-05, Bio-Techne), interleukin-6 (IL-6, DY206-05, Bio-Techne) and C-reactive protein (CRP, DY1707, Bio-Techne) will be analysed via an enzyme-linked immunosorbent assay (ELISA) kits with provided ancillary reagents (DuoSet ELISA Ancillary Reagent Kit 2, DY008, Bio-Techne) according to the manufacturer's protocol. A 1:2 sample to reagent diluent ratio will be used, and plates will be read on a plate reader at a wavelength of 450 nm (Synergy, Biotek). Circulating protein concentrations will be corrected for the change in plasma volume during heat exposure (described below).

Whole blood (from K_2EDTA tubes) will be immediately layered on histopaque (Histopaque-1077, Sigma-Aldrich) and centrifuged at 0.8 RCF for 30 min (22°C). The peripheral blood mononuclear cell (PBMC) layer will then be separated and washed with phosphate buffered saline (P4417, Sigma-Aldrich) and centrifuged again at 1.1 RCF for 10 min (4°C). Isolated PBMCs will be resuspended in phosphate buffered saline, transferred to 1.5 mL microcentrifuge tubes, and centrifuged a final time at 9.8 RCF for 10 min (4°C). Any remaining supernatant will be aspirated prior to storing the PBMC pellet at -80°C until analysis.

For analysis, the PBMC pellet will be lysed on ice for 30 min in a lysis buffer consisting of Tris-HCL 8.0 pH (15568-025, Invitrogen, Thermo Fisher Scientific), 0.5M EDTA (15575-020, Invitrogen, Thermo Fisher Scientific), 1.5M NaCl (S3014, Sigma-Aldrich), 1% Triton X 100 (X100, Sigma-Aldrich), and freshly added protease (78430, Thermo Fisher Scientific) and phosphatase (7842, Thermo Fisher Scientific) inhibitors. The lysate is then quantified (DC-protein assay, 5000112, Bio-Rad) and normalized to a total protein concentration of 40 µg, which was then electrophoresed on 12% polyacrylamide SDS-PAGE gels.

The proteins are then electrotransferred to nitrocellulose membranes (162-0112, Bio-Rad) then blocked in tris buffered saline (TBST) (150 mM NaCl, pH 8.0) containing 0.2% polysorbate detergent (Tween 20; 170-6531 Bio-Rad) and 5% powdered milk (170-6404,

Bio-Rad) for 60 min at 4°C with gentle agitation. After blocking, nitrocellulose membranes will be incubated at 4°C over-night with primary antibodies (HSP70-inducible form 1:1000, no. 4876S, Cell Signaling Technology; HSP90, Cell Signaling Technology, no. 4875S; TNF-α P300A Invitrogen, Thermo Fisher Scientific; IL-6, PA1-26811, Invitrogen, Thermo Fisher Scientific; β-actin HRP conjugate 1:10000, no. 12262S, Cell Signaling Technology). Following primary antibody incubation, the membranes will be washed repeatedly (3 times, 10 min each) in TBST and incubated with an HRP-conjugated secondary antibody (Anti-rabbit IgG, HRP-linked, no. 7074, Cell Signaling Technology) at room temperature for 1 hour and then washed again 3 times in TBST (10 min per wash). The nitrocellulose membranes will be visualized using the Clarity Max Western ECL Blotting substrate (1705062, Bio-Rad) and C-Digit Blot Scanner (LI-COR). Membranes will be stripped and re-probed with Restore Western Blot Stripping Buffer according to the manufacturer instructions (no. 21063, Thermo Fisher Scientific). Protein bands will be quantified by Image Studio Digits software (LI-COR, version 5.2) and normalized to β-actin, which served as an internal control.

Hydration-related variables

Baseline (start of session) urine specific gravity will be assessed with a hand-held total-solids refractometer. (Reichert TS 400 total solids refractometer, Reichert). Participants presenting to the laboratory with urine specific gravity <1.025 will be considered adequately hydrated to begin the experimental session (Kenefick & Cheuvront. Hydration. *Nutr Rev.* 2012;70 Suppl 2:S137-142). Throughout each intervention, fluid status was monitored via changes in body mass using a high-performance digital weighing terminal (model CBU150X, Mettler Toledo Inc).

Additional whole blood (not used in PMBC extraction) will be used to measure haematological parameters in duplicate (Ac·T diff, Beckman Coulter). Haemoglobin and haematocrit will be used to estimate changes in plasma volume from the start to end of the 9-hour exposure (Dill & Costill. *J Appl Physiol*. 1974;37(2):247-248).

Statistical analysis plan

Intervention 1

Statistical analysis for **Intervention 1** will compare whole-body heat storage and the resultant progression of body temperature, cardiovascular and acute inflammatory responses between the young and older adults during the simulated heatwave. Whole-body heat storage (primary outcome), body temperature responses including rectal temperature (primary outcome) and mean skin temperature (secondary outcome), as well as cardiovascular responses (secondary outcomes) will be evaluated using linear mixed-effects models. Time will be modelled as a repeated within-subject fixed effect (two levels: 3 and 9 hours), and age-group (two levels: young and older adults) will be modelled as a between-subject fixed effect. Pre-heat exposure values of the outcome variable, participant sex, and self-reported weekly physical activity (min/week, as assessed via the GAQ) will be included as covariates. Circulating and intracellular markers of acute inflammation and stress (secondary outcomes) will be evaluated using a similar model but without the fixed effect of time (data was only collected at the end of exposure) and baseline values included as a covariate (where appropriate). Participant identification will be modeled as a random effect in all analyses. Akaike's information criterion will be used to determine random effect and variance/covariance structures.

Post hoc multiple comparisons will be made on model estimated marginal means. Given the small number of comparisons for each variable, multiplicity corrections will not be employed. Homoscedasticity will be evaluated for all models by visual assessment of residual plots. Approximate normal distribution of residuals will be assessed via visual inspection of histograms and Q-Q plots. Data will be log-transformed in the event that the distribution of residuals meaningfully deviates from normality. For all analyses, the level of significance will be set at $P < 0.050$. Descriptive statistics will be presented as means and standard deviations. Comparisons between groups and/or time-points will be presented as means and 95% confidence intervals [lower limit, upper limit].

Intervention 2

Statistical analysis for Intervention 2 will evaluate the acute and lasting impacts of the short-term cooling intervention (i.e., during cooling and following return to the heat, respectively) on physiological responses.

Cumulative heat storage (primary outcome)

The effect of the cooling intervention on cumulative whole-body heat storage over the 3 hours following return to the heat will be evaluated using a linear mixed-effects model with experimental group (two levels: cooling and no-cooling) modelled as a between-subject fixed effect. Cumulative heat storage over the first three hours of the 9-hour exposure will be included as a covariate to account for the influence of any inter- or intra-individual factors (e.g., sex, physical activity levels), measured or unmeasured, impacting whole-body heat exchange and storage. Comparisons of heat storage between groups will be made using model estimated marginal means.

Body core temperature (primary outcome)

Body core temperature, as estimated by rectal temperature, will be analyzed with a linear mixed effects model with experimental group (two levels: cooling and no-cooling) modelled as a between-subject fixed effect and time as a repeated within-subject fixed effect (0-, 1-, 2-, and 3-hours post-cooling intervention). Like the model for heat storage, rectal temperature at the end of the first calorimetry session (i.e., hour 3 of the 9-hour exposure) will be included as a covariate to account for the influence of any measured or unmeasured inter- or intra-individual factors impacting the body temperature responses to resting heat exposure.

Because this study was designed to assess the hypothesis that body core temperature will be similar (equivalent) between groups following return to the heat, the effect of cooling on rectal temperature will be assessed via two one-sided tests (see Lakens. *Soc Psychol Personal Sci*. 2017:8 (4), 355-362) performed on the mixed-effects model derived estimated marginal means at each timepoint. For the equivalence testing, the null hypothesis is taken as a meaningful difference between groups and its rejection therefore

corresponds to an equivalence of means. These are presented alongside the results of a traditional Welch's t-test. This permits evaluation of both statistical differences, and statistical equivalencies in physiological responses. Performing analysis on the marginal means will allow for isolation of the effect of cooling from individual differences in the body temperature response to heat exposure (captured in the 3-hour timepoint covariate). Equivalence bounds will be set to ±0.3°C, which corresponds to the typical day-to-day variation of core temperature (Consolazio et al. *Physiological measurements of metabolic functions in man.* 1963:453-480) and has been suggested to reflect a meaningful/detectable change in body temperatures in a recent study assessing the influence of cooling strategies on physiological strain in young adults (Morris et al. *Annals of Internal Medicine.* 2019;171(9):675-677).

Secondary outcomes

Mean skin temperature as well as cardiovascular responses and circulating/intracellular inflammatory/stress markers will be modeled with linear mixed-effects models. The analyses for mean skin temperature and cardiovascular responses will be performed similar to that for rectal temperature except only two time points will be included (0- and 3-hours post-cooling intervention). Circulating and intracellular changes in inflammatory markers, measured at the start and end of exposure only, will be analyzed with a linear mixed-effects model with age-group (two levels: young and older adults) modelled as a between-subject fixed effect, and participant sex, self-reported and weekly physical activity (min/week, as assessed via the GAQ), included as covariates.

The analysis of secondary variables will focus on whether there was a lasting effect of cooling 3 hours following return to the heat. However, we will also perform tertiary exploratory analysis to evaluate whether the end-exposure responses are similar (equivalent) within pre-specified bounds. Describing clinically meaningful bounds in this context is complex since there is little research regarding how the risk of adverse health events changes with *acute* alterations in cardiovascular responses or inflammatory status during heat exposure (or cooling). For heart rate (± 5 beats/min) and rate pressure product (± 1500 beats/min), the equivalence bounds for these exploratory analyses will be based on meaningful/detectable differences employed in recent studies evaluating the efficacy

of personal cooling strategies in laboratory-based studies (Morris et al. *Annals of Internal Medicine.* 2019;171(9):675-677). For arterial blood pressure responses, we will consider ± 5 mm Hg an appropriate clinically meaningful bound, given the associated 9%, 13%, and 6% reduction in risk of myocardial infarction, stroke, and mortality (Psaty et al. *Arch Intern Med*. 2001;161(9):1183-1192). For the other variables, reasonable equivalence bounds are not forthcoming. We will therefore set the bounds for these outcomes at ±1 SD of the between-group difference (based on our sample size determination).

For all models, participant identification will be modeled as a random effect and Akaike's information criterion will be used to determine random effects and variance/covariance structure. The level of significance will be set at $P < 0.050$. Descriptive statistics will be presented as mean (standard deviation). Both 90% confidence intervals and 95% confidence intervals will be computed to facilitate interpretation of the equivalence testing and null hypothesis significance testing (respectively).

As noted at the start of the document, the statistical analysis plan was submitted after cleaning and analysis of the full data set, but prior to unblinding of the data.

Delimitations and limitations

All participants recruited for this research will be relatively healthy, sedentary, or habitually active but non-endurance trained adults aged 18-80 years. The results may not be directly applicable to different population groups (e.g., children, adolescents or the extremely old [80+ years], the sick). Although an approximately equal number of males and females will be recruited for each intervention to ensure a sample representative of the general population, the experiments performed will not permit the assessment of the interaction of age and sex on thermoregulatory and cardiovascular responses to long-duration heat stress. Finally, behavioral factors in large part determine one’s risk of mortality and morbidity during heatwaves. While the project will provide novel information on the physiological basis of heat tolerance, defining risk is a multi-faceted endeavour and thereby larger in scope than any one series of studies in a given domain.