Title: Effects of walking in different tree-dominated environments on autonomic response in adults over 50: a randomized crossover trial

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1. Study Design

This study was designed as a randomized crossover field trial to evaluate the physiological and psychological responses associated with exposure to different environments during a leisurely walk.

Each participant was exposed to a total of four experimental conditions, consisting of three tree-lined outdoor environments with different environmental and ecosystem characteristics (A, B, and C) and a control condition in an indoor environment (D).

Each condition constituted an independent period of the crossover design and was conducted on different days.

The research staff was responsible for implementing the protocol, following a prior internal training process aimed at ensuring the standardization of procedures, the proper handling of devices, and consistency in the instructions provided to participants. Each group of participants was accompanied at all times by two researchers.

2. Intervention Sequences and Randomization

Randomization was applied to the assignment of participants to complete exposure sequences, not to individual conditions.

Three intervention sequences were defined:

- ABCD
- BCAD
- CABD

Assignment was performed using a computerized procedure with R (randomizeR package), with a 1:1:1 ratio between sequences.

Each participant was assigned to a sequence after signing the informed consent form and being assigned a unique identifier.

Condition D (in-house control) was always performed in the last period, for methodological and logistical reasons related to the standardization of walking speed on the treadmill. This aspect was considered in the analysis and interpretation of the results as a possible period effect.

3. Blinding

It was not possible to blind either the participants or the staff responsible for the intervention.

However:

- the participants were unaware of the assigned sequence

- they were not informed of the nature of the conditions or the existence of a control condition

Three individuals outside the research team participated exclusively in the blood pressure and heart rate measurements for M1 and M4. These evaluators remained blinded to the setting of each session through physical and functional separation from the rest of the team and through the exclusive use of the participant's identifier during data collection. No breaches of blinding were recorded.

4. Participants

The study included 16 healthy adult participants aged 50 years or older, residing in the vicinity of the study area and with sufficient functional capacity to complete a leisurely 3-km walk independently and safely.

Inclusion Criteria

Participants had to meet all of the following criteria:

1. Age 50 years or older.
2. Ability to independently perform a low-intensity walk.
3. No prior diagnosis of serious cardiovascular, respiratory, neurological, musculoskeletal, or psychiatric conditions that could interfere with participation or the interpretation of results.
4. No severe allergies related to exposure to outdoor environments during the study period.
5. Commitment to completing the four planned experimental sessions.
6. Attendance at an in-person informational meeting prior to the start of the study.
7. Signing of a written informed consent form prior to any procedure.
8. Availability of a mobile device compatible with the Kubios HRV Daily Readiness app and equipped with Bluetooth connectivity.

The following were excluded:

1. Individuals with regular use of narcotic or non-narcotic drugs.
2. Pregnant women.
3. Individuals with any clinical condition or functional limitation that could compromise safety during the protocol or the validity of the measurements.

Recruitment was conducted via self-selection, through advertisements posted on social media, posters on the university campus, and the university's institutional channels.

Interested individuals initially completed an online screening form. Those who met the preliminary criteria were invited to an in-person informational meeting

where eligibility was verified, questions were answered, and inclusion was formalized through the signing of the informed consent form.

Recruitment took place between July 28 and September 4, 2025. The in-person informational meeting was held on September 4, 2025, and the experimental sessions took place between September 9 and 22, 2025.

5. Ethical Considerations

The study was approved by the Ethics Committee of the University of León (Spain) under code ETICA-ULE-087-2025, with an approval date of July 25, 2025.

The study was registered on ClinicalTrials.gov with identifier NCT07114146 on July 27, 2025.

The research was conducted in accordance with the Declaration of Helsinki and applicable regulations regarding research involving human subjects and data protection.

All participants received verbal and written information regarding the study's objectives, procedures, risks, benefits, and their right to withdraw at any time without penalty, and signed the corresponding informed consent form before the start of any study procedures.

6. Study Conditions

The study was conducted in the city of Pontevedra (Spain), in a peri-urban setting near the A Xunqueira University Campus.

Four experimental conditions were evaluated:

- Setting A: Peri-urban wooded area located on the outskirts of the University Campus
- Environment B: A humid semi-natural environment located in a marshland area, dominated by marsh and halophilic vegetation adapted to saturated soils
- Environment C: Peri-urban riparian forest
- Environment D (indoor control): Enclosed room with white walls and a treadmill.

The first three corresponded to forested outdoor environments with distinct environmental and ecosystem characteristics. The fourth corresponded to an indoor control condition in the laboratory.

7. Intervention

All participants walked 3 km under the four experimental conditions.

In outdoor conditions A, B, and C, the walk was performed at a self-paced pace, with the express instruction to maintain a calm and comfortable pace, without imposing a target speed.

In indoor condition D, the walk was performed on a BH HiPower SIRIUS 3.0 treadmill at a preset speed corresponding to the individual average speed obtained by each participant in condition A, calculated in km/h based on the time taken to cover 3 km. This speed could be adjusted downward for safety or tolerance reasons, at the discretion of the principal investigator.

The three outdoor routes had low and comparable gradients, without steep inclines, with the aim of promoting a consistent physical workload.

8. Study Procedure

The three outdoor sessions took place in the morning on September 9, 12, and 15, 2025. Participants were scheduled to arrive at 8:45 a.m. at the Faculty of Physical Therapy on the University Campus. The estimated duration of each outdoor session was until approximately 2:00 p.m.

The indoor condition took place on September 18, 19, or 22, 2025, during time slots adapted to the availability of the participants and the center, with an approximate duration of 2 hours per participant.

8.1 Procedure under outdoor conditions

Upon arrival at the meeting point, measurement M1 was performed. Subsequently, participants were transported as a group to the corresponding location.

- For conditions B and C, transportation was by bus.
- For condition A, and in order to maintain comparable waiting and rest times, participants remained seated for a time similar to the bus ride.
- After the walk, comparable return or waiting times were also applied depending on the condition.

Participants began the walk in staggered intervals every 5 minutes to facilitate individual completion of the route.

8.2 Procedure for the indoor condition

In condition D, participants arrived individually at the School of Physical Therapy at a predetermined time. The same general phases of the protocol were replicated, but without the waiting time associated with the group outdoor walks.

9. Study variables

The following were defined as prespecified study outcomes:

Variables derived from HRV measurement

- RR interval
- RMSSD
- SDNN
- HF power
- LF power

Cardiovascular variables

- Heart rate
- Systolic blood pressure
- Diastolic blood pressure

Psychological variables

- POMS
- TMD

Perceptual variables

- Perceived environmental quality (A, B, C)

10. Measurements

10.1 Heart rate variability (all variables)

The heart rate signal was recorded using a Polar H10 chest strap connected via Bluetooth to the Kubios HRV Daily Readiness mobile app, with subsequent export of the data to Kubios HRV Scientific version 4.1.2.

The signal was recorded continuously starting 5 minutes before the start of the walk, throughout the entire 3-km walk, and up to 5 minutes after its completion.

The following variables were obtained from the continuous recording:

- RR interval
- RMSSD
- SDNN
- HF power
- LF power

The unit of measurement for RR interval, RMSSD, and SDNN was milliseconds (ms). The HF and LF spectral components were expressed in ms^2.

The chest strap was placed according to the manufacturer's recommendations, after moistening the electrodes and adjusting the elastic band to fit the chest. The start and end of the recording were performed by members of the research team.

Signal processing was performed using automatic mean-level noise detection and automatic beat correction. The quality criterion was set to exclude recordings with more than 5% artifacts or corrected beats.

10.2 Blood Pressure and Heart Rate

Systolic blood pressure, diastolic blood pressure, and heart rate were measured using a clinically validated Omron M2 automatic sphygmomanometer.

Measurements were taken on the left arm, with the participant in a seated position, back supported, feet flat on the floor, and arm at heart level. Before each measurement, the participant rested in a seated position for 5 minutes.

At each time point, three consecutive readings were taken, one minute apart. The first reading was systematically discarded, and the final value was calculated as the average of the second and third readings.

Blood pressure was expressed in mmHg and heart rate in beats per minute.

10.3 Mood and Total Mood Disturbance

To measure mood, the 30-item short form of the POMS scale validated in Spanish was used (Andrade et al., 2013). The Profile of Mood States is a questionnaire based on a multidimensional conception of mood, designed to obtain information on fluctuations in emotional states across different contexts. In our study, we used the short version of the POMS in Spanish, which contains 30 items comprising 6 factors, one for each of the main mood factors included in the POMS. Four of these factors are negative in nature—Anger, Fatigue, Tension, and Depressed Mood—and two are positive: Vigor and Friendliness. It is presented as a Likert-type questionnaire with 5 options for each factor, to which values between 0 (None) and 4 (Very Much) are assigned.

Another piece of data obtained from the POMS is the total score, or Total Mood Disturbance (TMD), calculated by summing all the values obtained for each factor, with the exception of the two positive factors, which are subtracted.

10.4 Perceived Environmental Quality

A proprietary instrument called the "Perceived Environmental Quality Scale of the Forest Environment," whose main objective is to quantify the extent to which the perceived attributes and characteristics of forest environments affect a person's experience when coming into contact with the evaluated space.

The scale consists of 41 questions. Thirty-nine of these are scored on a 1-to-5-point Likert scale, where 1 = "none" and 5 = "a lot"; The last two questions on the

scale are referred to as “criterion questions” and are scored on a scale of 1 to 10 points, where 1 = “detestable – none” and 10 = “outstanding – very much.”

11. Physiological Measurement Time Points

Systolic blood pressure, diastolic blood pressure, and heart rate were measured at four time points during each period:

- M1: arrival at the meeting point at the School of Physical Therapy on the University Campus.
- M2: 5 minutes before the start of the walk in the corresponding environment.
- M3: 5 minutes after the walk ended, in the corresponding environment.
- M4: after returning to the meeting point on the University Campus.

The continuous signal for RR interval and HRV was recorded from 5 minutes before the walk until 5 minutes after its completion.

Mood was assessed using the Profile of Mood States (POMS) questionnaire. The POMS was administered at two points during each session:

- immediately after M1
- immediately after M3

The Total Mood Disturbance (TMD) index was subsequently calculated based on these scores.

Perceived environmental quality was assessed using the Perceived Environmental Quality Scale under outdoor conditions A, B, and C. This scale was administered after M3, along with the POMS questionnaire. This measurement was not performed in the indoor control condition.

11. Environmental and Microclimatic Variables

Meteorological and microclimatic data were recorded each study day between 9:00 and 9:30 a.m., before the participants arrived at the environments.

In conditions A, B, and C, meteorological variables were obtained from the Pontevedra-Campolongo station. Noise levels were recorded using a CESVA SC-30 digital sound level meter at the start and end points of the walks.

In condition D, temperature and relative humidity were recorded at the midpoint of the room using a Potermic 72000 multifunction meter with real-time monitoring.

12. Statistical Analysis

The data will be analyzed using statistical models appropriate for repeated-measures designs in a crossover context, taking into account the intra-individual dependence of the observations.

The analysis may include:

- longitudinal modeling of continuous variables
- comparison between experimental conditions
- evaluation of the temporal evolution of the variables
- adjustment for individual covariates (age, sex, weight, and height)
- inclusion of random effects at the participant level

Mixed linear models or other equivalent approaches may be used depending on the nature of each variable.

Analyses will be performed using the statistical software R.

13. Final considerations regarding the scope of the protocol

This protocol describes the entire study, including all variables collected as part of the trial. The results may be published in one or more scientific journals, without this altering the unified nature of the study or the pre-specified nature of the variables and procedures described herein.