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Journal of Sport and Health Science

Original article

Sexual dimorphism on the acute effect of exercise in the morning *vs.* evening: A randomized crossover study

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Received 26 April 2024; revised 29 July 2024; accepted 20 September 2024
Available online 22 December 2024

Abstract

Background: Mammalian cells possess molecular clocks, the adequate functioning of which is decisive for metabolic health. Exercise is known to modulate these clocks, potentially having distinct effects on metabolism depending on the time of day. This study aimed to investigate the impact of morning *vs.* evening moderate-intensity aerobic exercise on glucose regulation and energy metabolism in healthy men and women. It also aimed to elucidate molecular mechanisms within skeletal muscle.

Methods: Using a randomized crossover design, healthy men ($n = 18$) and women ($n = 17$) performed a 60-min bout of moderate-intensity aerobic exercise in the morning and evening. Glucose regulation was continuously monitored starting 24 h *prior to* the exercise day and continuing until 48 h post-exercise for each experimental condition. Energy expenditure and substrate oxidation were measured by indirect calorimetry during exercise and at rest before and after exercise for 30 min. Skeletal muscle biopsies were collected immediately before and after exercise to assess mitochondrial function, transcriptome, and mitochondrial proteome.

Results: Results indicated similar systemic glucose, energy expenditure, and substrate oxidation during and after exercise in both sexes. Notably, transcriptional analysis, mitochondrial function, and mitochondrial proteomics revealed marked sexual dimorphism and time of day variations.

Peer review under responsibility of Shanghai University of Sport.
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https://doi.org/10.1016/j.jshs.2024.101021
Cite this article: Sevilla-Lorente R, Marmol-Perez A, Gonzalez-Garcia P, et al. Sexual dimorphism on the acute effect of exercise in the morning *vs.* evening: A randomized crossover study. *J Sport Health Sci* 2025;14:1–17.

Conclusion: The sexual dimorphism and time of day variations observed in the skeletal muscle in response to exercise may translate into observable systemic effects with higher exercise-intensity or chronic exercise interventions. This study provides a foundational molecular framework for precise exercise prescription in the clinical setting.

Keywords: Circadian rhythms; Continuous glucose monitor; Energy metabolism; Mitochondria; Sex-differences; Transcriptomics

1. Introduction

Mammalian cells and tissues possess molecular clocks that work as a coupled network to coordinate physiology.[1] Cues such as light/dark, food, and physical activity interact with the molecular clocks to generate approximately 24-h oscillations (i.e., circadian rhythm) in the function of thousands of genes.[1] Adequate functioning of this circadian machinery is decisive for optimal metabolic health.[2] In humans, its disruption through shifted sleep patterns or an inadequate eating window has been demonstrated to increase the risk of obesity, type 2 diabetes mellitus, and cardiovascular diseases.[2]

Exercise is a powerful and well-recognized modulator of the skeletal muscle metabolism.[3] Indeed, aligning the timing of exercise (i.e., the moment of the day when exercise is performed) with the individual's circadian rhythms could be an effective approach to maximize the positive influence it has on metabolic function and overall health.[4] Epidemiological studies underscore the relevance of the time of exercise for inducing health benefits,[5–7] but the results are not conclusive regarding glucose homeostasis[8] or lipid metabolism.[9] Some studies have reported that fat oxidation[10–12] and energy expenditure (EE)[11] were higher in the evening, whereas others did not observe any discernible differences in these outcomes based on the time of day (morning *vs.* evening).[13–17] Crucially, the majority of these studies have been focused on men. Women remain disproportionately underrepresented in exercise medicine research despite significant biological differences.[18] Given the need for action to redress this sex gap in research and the growing interest surrounding comprehension of women's physiology over recent decades, there exists a noteworthy scientific, clinical, and practical interest in ascertaining the molecular and physiological mechanisms explaining the potential exercise-induced diurnal variation differences between sexes and their effects on health-related metabolic parameters.

This study aimed to determine the differential impact of an acute exercise session performed in the morning *vs.* evening on glucose regulation, energy metabolism, and metabolic health in healthy men and women, and to explore the underlying skeletal muscle molecular mechanisms. Investigating the optimal time for exercise is a matter of clinical and public health importance with respect to the prevention and treatment of metabolic diseases. Exploring distinct responses in both men and women is imperative for addressing the sex gap and creating inclusive guidelines. Further, examining molecular mechanisms can be of interest for implementing precision medicine in the clinical setting.

2. Methods

2.1. Subject details

Thirty-five healthy adults (men, $n = 18$; women, $n = 17$) completed the current trial. Participants mostly presented intermediate and moderate chronotypes. Recruitment was done via advertisements on social networks and through different faculties of the University of Granada, Spain. Inclusion criteria were (a) age between 18 and 50 years, (b) body mass index (BMI): 18.5−27 kg/m^2, and (c) practicing exercise fewer than 5 days per week. Exclusion criteria were (a) history of disease (i.e., major adverse cardiovascular event, kidney failure, cirrhosis, eating disorder, weight control surgery, human immunodeficiency virus/acquired immunodeficiency syndrome, rheumatoid arthritis, Parkinson's disease, active cancer treatment in the past year, diabetes mellitus), (b) use of drugs or medications that may affect the results, (c) unstable body weight for 3 months before the start of the study (>4 kg weight loss or gain), (d) pregnancy and breastfeeding, (e) active tobacco abuse or illicit drug use or a history of alcohol abuse treatment, (f) on a special diet or prescribed for other reasons (e.g., celiac disease). Sample size was calculated based on the results of a previous pilot study conducted in our laboratory. A total of 17 participants are needed to observe statistically significant differences between conditions (morning *vs.* evening) in fat oxidation during exercise (∼10%−15%) (80% statistical power and an α of 0.05). In order to conduct the analyses in men and women separately, we finally recruited 18 men and 17 women.

2.2. Study design

The present study is a randomized crossover trial. In a randomized and counterbalanced order, each participant performed a single bout of aerobic exercise at 2 different times of day: (a) morning (i.e., 11:30 a.m.) and (b) evening (i.e., 6:30 p.m.) with a washout period of at least 3 days in between. This trial strictly followed CONSORT guidelines (http://www.consort-statement.org/consort-statement) and was registered at the https://clinicaltrials.gov (NCT05369715).

2.3. Study approval

All procedures were performed according to the Declaration of Helsinki, and the study was approved by the Human Research Ethics Committee of the University of Granada and the Provincial Human Research Ethics Committee (Junta de Andalucía, Ref. 1288-N-20). All participants gave their oral and written informed consent. Data for each participant were

collected over 4 visits. Fig. 1 shows the overall design of the study.

2.4. Visit 1: Preliminary examination

Sociodemographic, lifestyle, and body composition data were registered, and a medical screening was performed. Participants completed the HÖME Morningness−Eveningness questionnaire[19] to determine individual's chronotype (i.e., morningness−eveningness) and the short version of the International Physical Activity Questionnaire (IPAQ)[20] to assess their physical activity levels. Height and weight were measured (no shoes and light clothing) using a Model 799 Seca scale and stadiometer (Seca, Hamburg, Germany). Body fat mass and percentage, lean body mass, and visceral adipose tissue (VAT) mass were evaluated by dual X-ray absorptiometry (Discovery Wi; Hologic, Bedford, MA, USA) and analyzed by APEX software (Discovery Wi). The device was calibrated each day using a lumbar spine phantom. Participants were asked to remain still while being scanned in the supine position, as per guidelines from the International Society of Clinical Densitometry.[21] BMI, lean mass, and fat mass indexes were expressed as kg/m^2.

2.5. Visit 2: Cardiorespiratory fitness

Previous physical activity and diet were controlled as cofounder variables. Participants were asked to complete the following pre-experimental conditions: (a) to refrain from vigorous physical activity the previous 48 h and from moderate physical activity the previous 24 h, (b) to avoid caffeine ingestion 12 h before, and (c) to sleep with normality the night before. In case of women, the phase of their menstrual cycle was registered. Room temperature was controlled to maintain a range between 20°C−24°C. Participants attended the laboratory in fasting conditions (4 h) after the ingestion of a complete self-selected meal.

Cardiorespiratory fitness was assessed through a maximal effort test in an Ergoselect 200 cycle ergometer (Ergoline GmbH, Lindenstrasse, Germany). A Polar OH1 photoplethysmographic heart-rate monitor (Polar Electro Oy, Kempele, Finland) was placed on the forearm to measure heart rate. After a resting period of 10 min, the maximum effort test started with a 3-min stage at 20 watts (W) as a warm-up, followed by an increase in increments of 20 W every 3 min until the respiratory exchange ratio (RER) was ≥1 for at least 30 s.[22] At this point, further increments of 20 W every 1 min were implemented (with no interruptions) until (a) volitional exhaustion was reached, or (b) participants had to stop because of peripheral fatigue. Through the maximum effort test, participants' fatigue perception was assessed using a rating of perceived exertion (RPE) scale (Borg CR10 Scale®).[23] Respiratory gas exchange was measured during the entire exercise test by indirect calorimetry (Quark CPET, COSMED, Rome, Italy) and collected with a facemask (Hans Rudolph, Shawnee, KS, USA). According to the manufacturer's recommendations, volume was calibrated using a 3 L calibration syringe and the

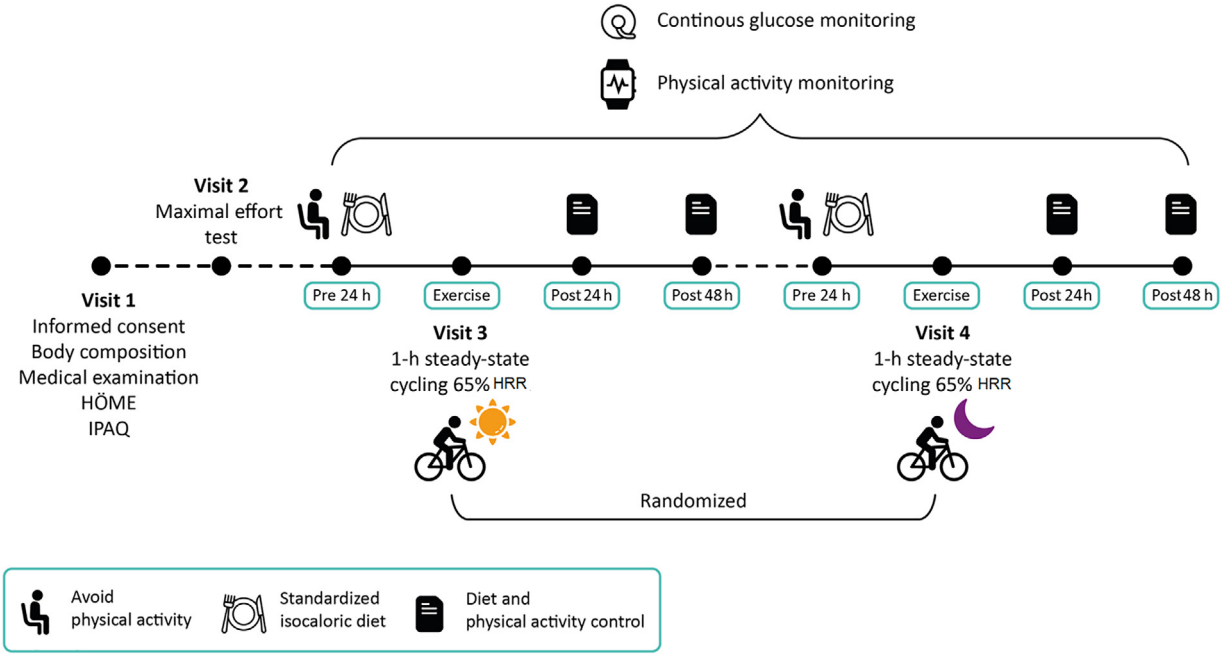

Fig. 1. Overall procedures used in the study. Visit 1: preliminary examinations and body composition; Visit 2: maximal effort test and start monitoring interstitial glucose (continuous glucose monitor) and physical activity (accelerometer); Visits 3 and 4: 1-h steady-state test on cycle-ergometer at 65% HRR, randomly at 11:30 a.m. or 6:30 p.m. Every exercise protocol was separated by at least 3 days. Participants were asked to follow a standardized isocaloric diet and refrain from moderate and vigorous physical activity the previous day. The 1st day and 2nd day after Visit 3, participants registered every meal and all physical activity and replicated same after Visit 4. Each black circle represents 1 day. Continuous lines connect consecutive days; dashed lines connect days that may not be consecutive. Created with BioRender.com. HÖME = HÖME Morningness−Eveningness questionnaire; HRR = heart rate reserve; IPAQ = International Physical Activity Questionnaire.

gas analyzer was calibrated using standard gas concentrations (O_2 = 16%, CO_2 = 5%) immediately before each trial. Resting and maximum heart rate were used to calculate exercise intensity in subsequent visits. Gas exchange data were exported from the metabolic cart to an Excel spreadsheet in a sample frequency of 5 s. Maximal volume of oxygen consumption (VO_{2max}) was defined as a respiratory exchange ratio of ≥1.1, once a VO_2 plateau was reached, and having attained a heart rate value within 10 beats/min of the individuals' age-predicted maximum ($209 - 0.73 \times age$)[24] during the maximal effort exercise test. When participants did not achieve the VO_{2max} criteria, VO_{2peak} was used as the highest VO_2 value that was not an artifact (we screened the data set from the 2nd to the 10th subsequent largest VO_2 uptake value). This value was calculated relative to body mass.

2.6. Visits 3 and 4: Morning and evening moderate-intensity aerobic exercise

On the 3rd and 4th visits (Fig. 2), participants performed a 60-min steady-state exercise bout on the same cycle ergometer used for the effort test at an intensity of 65% of their heart rate reserve (HRR) either at 11:30 a.m. or 6:30 p.m. The conditions (morning or evening, respectively) were randomized, and visits were separated by a range of 4−25 days (median = 7 days).

2.6.1. Continuous glucose monitoring

At least 24 h before the 3rd visit, participants started to wear a continuous glucose monitor (CGM; FreeStyle LibrePro; Abbot, Alameda, CA, USA) on the non-dominant upper-arm, and this was maintained until the end of the study. We calculated hourly values and defined daytime interstitial glucose levels as the mean glucose concentration from 06:00 a.m. to 11:59 p.m. and nocturnal glucose as the mean glucose concentration from 00:00 a.m. to 05:59 a.m., as recommended by current guidelines.[25] For both morning and evening conditions, mean, area under the curve (AUC), and coefficient of variation (CV) were calculated for the 24 h previous to exercise day (i.e., Pre 24 h), the 24 h of the exercise day (i.e., Exercise), and the 2 days subsequent to exercise (i.e., Post 24 h and Post 48 h, respectively; Fig. 1).

2.6.2. Physical activity and sleep

At least 24 h before the third visit, participants were fitted with a non-dominant wrist-worn triaxial accelerometer (ActiGraph GT3X+; ActiGraph, Pensacola, FL, USA), which they wore until the end of the study. The devices were initialized to collect raw accelerations at a frequency of 100 Hz for at least 7 consecutive days (24 h/day). In addition, participants were instructed to register their bedtime and wake-up time every day. Raw data from the accelerometers were downloaded using the ActiLife software (ActiGraph) and then processed with the open-source GGIR R package, Version 3.0-0.[26] In brief, the Euclidean Norm of the raw accelerations Minus One g with negative values rounded to 0 (ENMO) was calculated over 5-s epochs; non-wear time periods were identified from the magnitude and variability of the raw accelerations measured at each accelerometer axis[27] and imputed when appropriate by the average ENMO at the same time interval during the rest of the recording days; sleep and awake periods were identified using an automated algorithm based on the variability of the arm posture and guided by the sleep times reported by the participants. Then, sedentary (<30mg), light (30−99mg), and moderate-to-vigorous (≥100mg) activities were classified using the Hildebrand et al.[28,29] cut-points.

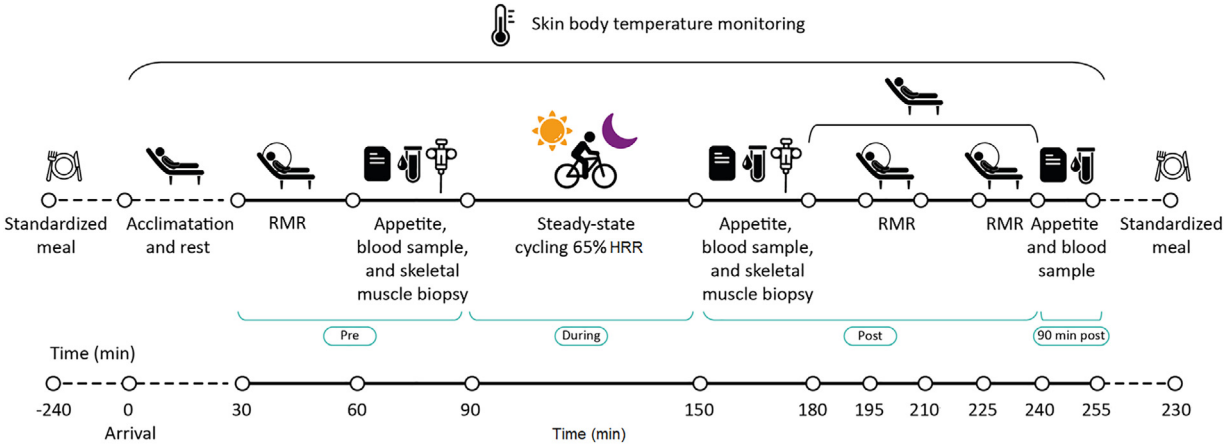

Fig. 2. Detailed procedures on Visits 3 and 4, where exercise (time point = 90 min) was performed either at 11:30 a.m. or 6:30 p.m., in random order. A standardized meal (i.e., bread, cheese, olive oil, and fruit) was consumed 4 h before arrival (−240 min). At arrival (0 min) a heart rate monitor was placed on the forearm and 16 thermal iButtons™ were attached to different skin points. After an acclimatation period, gas exchange at rest was measured (30 min) and a 1st blood sample was collected (60 min). Then, gas exchange was measured during 60 min of steady-state exercise at 65% HRR (90 min). A second blood sample after the test (150 min) was collected. They lay down again for 60 min of rest, wherein gas exchange was measured for 30 min in 2 separate 15-min stages (195 min and 225 min). To finish, a 3rd blood sample was completed (240 min). Participants were asked to consume a standardized meal within 1 h after leaving the laboratory (i.e., salad with lettuce, tomato, sweet corn, carrot, tuna and olive oil, and potato omelette with egg, potato, and olive oil). A skeletal muscle micro-biopsy was obtained from a subsample of 6 men and 8 women before and after exercise (60 min and 150 min). Created with BioRender.com. HRR = heart rate reserve; RMR = resting metabolic rate.

Finally, moderate-to-vigorous physical activity was considered when the activity above the moderate-to-vigorous threshold lasted at least 5 min, allowing for a maximum of 1 min below the threshold. During the sleep period time, min-by-min classification of sleep *vs.* awake status was conducted, and then total sleep time, total time awake after sleep onset,[30] and sleep efficiency were calculated for analytical purposes.

2.6.3. Energy metabolism assessment

2.6.3.1. Resting period before exercise. Participants arrived at the laboratory (Fig. 2, time point = 0 min) and lay down on a stretcher, staying relaxed for 30 min (acclimatation period), a time during which they were instructed not to move, talk, sleep nor cross their arms and/or legs. Then (Fig. 2, time point = 30 min), following the same instructions, gas exchange at rest was collected and measured using a ventilated canopy hood attached to the Quark resting metabolic rate (RMR) metabolic cart for 30 min. The same procedures were followed on the 2nd visit.

2.6.3.2. Steady-state exercise bout. Participants were equipped with a facemask for collecting gas exchange. The gas exchange measurement started 1 min before the beginning of the steady-state test with participants sitting on the cycle ergometer without pedaling and connected to the Quark RMR cart. After 1 min of recording gases in resting conditions, the steady-state test at 65% HRR intensity started (Fig. 2, time point = 90 min; i.e., Pre) and continued (constant intensity) until completing a total of 60 min, the moment at which the test finished (Fig. 2, time point = 150 min; i.e., Post). Gas exchange was continuously measured. Every 5 min, participants were asked to report their fatigue perception using the RPE scale.

2.6.3.3. Resting period after exercise. Participants lay down for 60 min, and resting gas exchange was measured (using a canopy hood for collection) for 30 min in 2 separate 15-min stages (Fig. 2, time point: 195−210 min and 225−240 min; i.e., Post) corresponding to 45−60 min and 75−90 min after exercise, respectively.

2.6.3.4. Gas exchange data. Gas exchange data were downloaded at a sample frequency of 5 s, and VO_2 and volume of carbon dioxide production (VCO_2) were used to estimate EE and respiratory exchange ratio (RER = VCO_2/VO_2). EE was estimated using Weir's abbreviated equation[31] considering 0 nitrogen excretion.

$$\mathrm{EE}\ (\mathrm{kcal/min}) = (1.106 \times \mathrm{VCO_2}) + (3.941 \times \mathrm{VO_2}) \quad (1)$$

For gas exchange data at rest (i.e., Resting), the first 5 min of measurement were discarded.[32] Then, average VO_2, VCO_2, RER, and EE Pre- and Post-exercise were calculated. For gas exchange data during exercise, continuous data (every 10 s) from VO_2, VCO_2, RER, and EE were used to examine potential point-by-point kinetic differences between morning and evening exercise. Average baseline (i.e., Pre) values were used to adjust gas exchange data during exercise to account for circadian variability in resting gas exchange.

2.6.4. Heart rate

The same heart-rate monitor described in Visit 2 was placed on the forearm to measure heart rate during the entirety of Visits 3 and 4.

2.6.5. Skin temperature

A total of 16 thermal iButtons™ (iButtons DS 1922 L; Maxim, Dallas, TX, USA) were attached to the skin in different spots upon arrival at Visits 3 and 4 (i.e., forehead, left pectoralis, left elbow region, left index fingertip, left forearm, rear neck central area, right clavicula, right deltoid, right shinbone, right sub-clavicular area, right supra-clavicular area, right thigh, and upper breastbone). Skin temperature measurements were taken every 60 s (resolution = 0.0625°C).[33] The programming of iButtons™ (Maxim), as well as the downloading and initial processing of raw data, were performed using the Temperatus™ software.[34] Following the completion of the experiment, the data for each iButton were downloaded every 60 s and saved in a CSV file. Anomalies in the data were eliminated by excluding time points where the rate of change from the previous value exceeded the interquartile range between Quartiles 1 and 3 (corresponding to percentiles 25 and 75, respectively) for the entire dataset.[35] Subsequently, the data were divided into 5-min blocks and the average value calculated, resulting in 12 mean values (1 for each 5-min block during the 60-min steady-state test). Finally, the overall mean temperature[36] as well as the proximal[37] and distal skin temperatures were determined using the Temperatus software,[38,39] which is a valid and reliable tool for that purpose.[35,40] The equation used for calculating overall mean temperature was:[38]

Overall mean skin temperature

$$\begin{aligned} = {} & (\mathit{forehead} \times 0.07) + (\mathit{right\ scapula} \times 0.175) \\ & + (\mathit{left\ chest} \times 0.175) + (\mathit{right\ deltoid} \times 0.07) \\ & + (\mathit{left\ elbow} \times 0.07) + (\mathit{left\ hand} \times 0.05) \\ & + (\mathit{right\ thigh} \times 0.19) + (\mathit{right\ gastrocnemius} \\ & \times 0.2). \end{aligned} \quad (2)$$

2.6.6. Blood samples

Intravenous blood samples from the antecubital vein were taken before (Fig. 2, time point = 60 min; i.e., Pre), immediately after (Fig. 2, time point = 150 min; i.e., Post) and 90 min after (Fig. 2, time point = 240 min; i.e., 90 min post) the exercise bout. Two tubes were collected per sample (Vacutainer serum separator tube 5 mL; BD Medical, Franklin Lakes, NJ, USA). The tubes were left to rest for 45 min at room temperature then centrifuged at 1300 relative centrifugal force for 10 min. The first was stored at 4°C for 24−72 h until analysis. The second

was used to prepare serum aliquots and stored at −80°C until analysis.

Serum parameters including glucose, triacylglycerol lipase, creatine kinase, lactate dehydrogenase (LDH), triglyceride, total cholesterol, C-reactive protein (CRP), C3 complement, and C4 complement were determined in a Beckman Coulter AU5832 analyzer (Beckman Coulter, Brea, CA, USA). Insulin, cortisol, thyrotropin, free triiodothyronine, and thyroxine were determined using chemiluminescent immunoassay of Beckman Coulter with a DxI analyzer (Beckman Coulter).

2.6.7. Muscular biopsies

Muscular biopsies were taken from the distal part of the vastus lateralis (quadriceps) in a sub-sample (6 men and 8 women) before (Fig. 2, time point = 60 min; i.e., Pre) and immediately after (Fig. 2, time point = 150 min; i.e., Post) the steady-state exercise bout. Biopsies were performed by an experienced surgeon using microbiopsy needles (Achieve Automatic Needle (16 Gauge × 15 cm; CareFusion Manufacturing, LLC of San Diego, CA, USA), after previous local anaesthesia with 2% lidocaine (Merck, Darmstadt, Germany), obtaining ∼30 mg per biopsy. From each time point (Pre and Post), 4 skeletal muscle samples were collected. The first was treated fresh to study mitochondrial respiration, and the rest were immersed in liquid nitrogen and stored at −80°C until further analysis (i.e., transcriptomics, proteomics, and mitochondrial supercomplexes).

2.6.8. Mitochondrial oxygen consumption

Fresh skeletal samples were preserved 6 h in BIOPS medium (10 mM Ca-ethylene glycol tetraacetic acid (EGTA) buffer (Sigma-Aldrich, St. Louis, MO, USA), 0.1 μM free calcium (Sigma-Aldrich), 20 mM imidazole (Sigma-Aldrich), 20 mM taurine (Sigma-Aldrich), 50 mM 2-(N-Morpholino) ethanesulfonic acid (Sigma-Aldrich), 0.5 mM dithiothreitol (Sigma-Aldrich), 6.56 mM $MgCl_2$ (Merck & Co., Inc., Rahway, NJ, USA), 5.77 mM ATP (Sigma-Aldrich), 15 mM phosphocreatine (Sigma-Aldrich), pH = 7.0).[41] Skeletal muscle samples (∼10 mg) were submerged in 1 mg/mL proteinase K solution (P-6556; Sigma-Aldrich) for 60 s. Then, muscle was homogenized (1:10, w/v) in isolation buffer (250 mM sucrose (Sigma-Aldrich), 2 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich), 10 mM Tris (Panreac Applichem, Barcelona, Spain), 0.5% free fatty acids albumin (Sigma-Aldrich), pH = 7.4) at 800 rotations per minute (rpm) at 4°C with a glass−teflon homogenizer (Thermo Fisher Scientific, Waltham, MA, USA). The homogenate was centrifuged twice at 1000*g* for 5 min at 4°C, and the supernatant was centrifuged at 23,000*g* for 10 min at 4°C in an Allegra 64R centrifuge (Beckman Coulter, Inc., Brea, CA, USA). Then the mitochondrial pellet was resuspended in 100 μL of isolation buffer, and a 10 μL aliquot was used for protein determination. The remaining sample was washed with 900 μL of isolation buffer and centrifuged at 13,000*g* for 3 min at 4°C. The final crude mitochondrial pellet was resuspended in 90 μL mitochondrial assay solution (MAS) 1 × medium (70 mM sucrose (Sigma-Aldrich), 220 mM mannitol (Sigma-Aldrich), 10 mM KH_2PO_4 (Thermo Fisher Scientific), 5 mM $MgCl_2$ (Merck & Co., Inc.), 2 mM 4-(2-hydroxyethyl)−1-piperazineethanesulfonic acid (HEPES; Sigma-Aldrich), 1 mM EGTA (Sigma-Aldrich) and 0.2% (w/v) free fatty acids albumin (Sigma-Aldrich), pH = 7.2). Mitochondrial respiration was measured using an Xfe24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA, USA).[42] Mitochondria were first diluted to the needed concentration required for plating in cold 1× MAS (1 μg/μL). Next, 50 μL of mitochondrial suspension was delivered to each well (except for background correction wells) while the plate was on ice. The plate was then centrifuged at 2000*g* for 10 min at 4°C. After centrifugation, 450 μL of 1× MAS + substrate (10 mM succinate for complex II or 2 mM malate, 2 mM glutamate, and 10 mM pyruvate for complex I) was added to each well. Respiration by the mitochondria was sequentially measured in a coupled state with substrate present (basal respiration or State 2), followed by State 3o (phosphorylating respiration, in the presence of ADP and substrates); State 4 (non-phosphorylating or resting respiration) was measured after the addition of oligomycin when all ADP was consumed, and then maximal uncoupler-stimulated respiration (State 3 u). The respiration control ratio (RCR) was calculated as State 3o/State 4. Injections were as follows: Port A, 50 μL of 40 mM ADP (4 mM final); Port B, 55 μL of 30 μg/mL oligomycin (3 μg/mL final); Port C, 60 μL of 40 μM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP; 4 μM final); and Port D, 65 μL of 40 μM antimycin A (4 μM final). All data were expressed in pmol/min/μg protein.[42,43]

2.6.9. Mitochondrial proteomics analysis

Frozen skeletal muscle samples (∼10 mg) were homogenized with 5 strokes in a Potter-Elvehjem tissue grinder attached to a rotating drill (∼1000 rpm) in 1 mL Solution B (1 mM EDTA, 220 mM mannitol, 20 mM HEPES-KOH (pH = 7.6), 70 mM sucrose, 0.5 mM phenylmethylsulfonyl fluoride and spun at 1000*g* for 5 min at 4°C. The supernatant was further spun at 12,000*g* for 10 min at 4°C, and the ensuing pellet was stored frozen at −80°C for subsequent proteomics analysis.[44] The pellet was transferred into a 2 mL centrifuge tube, containing 2 steel beads, 1× cocktail with lysis buffer L3 (7 M urea, 2 M thiourea, 0.2% sodium dodecyl sulfate (SDS), 20 mM tris, pH: 8.0−8.5) and 0.2 M EDTA. After 5 min in ice, 10 mM dithiothreitol was added in each tube. Samples were homogenized by using a grinder (60 Hz and 2 min), centrifugated at 25,000*g* for 15 min at 4°C for 15 min. The supernatant was collected and incubated in a water bath at 56°C for 1 h. Then 55 mM of iodoacetamide was added and the sample was placed in a dark room for 45 min. Cold acetone was added to the protein solution at a ratio of 1:5, and the resultant solution was placed in a refrigerator at −20°C for 30 min. The sample was then centrifuged at 25,000*g* at 4°C for 15 min. The resultant pellet was air-dry precipitated by adding lysis buffer (without SDS L) and homogenate in a use grinder (60 Hz and 2 min) to promote protein solubilization. Then the sample was centrifugated at 25,000*g* for 15 min at 4°C. The resultant supernatant

was identified as protein solution for mass spectrometry (MS) experiments.

Next, 100 μg of each protein sample was centrifuged at 12,000*g* for 20 min at 20˚C. Then 100 μL 0.5 M triethylammonium bicarbonate (TEAB) was added to the resultant pellet and centrifuged at 12,000*g* for 20 min at 20˚C. Trypsin enzyme (Sigma-Aldrich) was added to a protein in a trypsin/protein ratio of 1:20, and the sample was incubated for 4 h at 37˚C. After that, the sample was centrifugated at 12,000*g* for 15 min at 20˚C. The peptide solution after enzymatic digestion was collected at the bottom of the tube. Then 100 μL of 0.5 M TEAB was added to the ultrafiltration tube and centrifuged 12,000*g* for 15 min at 20˚C. After protein digestion, each peptide sample was resuspended in a 200-μL volume of Buffer A (2% acetonitrile with 0.1% formic acid (FA)), centrifuged at 20,000*g* for 10 min, and the resultant supernatant was taken for injection.

Separation was performed by Thermo UltiMate 3000 UHPLC (Thermo Fisher Scientific). The sample was first enriched in a trap column and desalted, and then inyected onto a tandem self-packed C18 column (75 μm internal diameter, 3 μm column size, and 25 cm column length) and separated at a flow rate of 300 nL/min through the following effective gradient: 0−5 min, 5% mobile phase B (98% I and 0.1% FA); 5−90 min, mobile phase B linearly increased from 5% to 26%; 90−100 min, mobile phase B increased from 26% to 35%; 100−108 min, mobile phase B rose from 35% to 80%; 108−113 min, 80% mobile phase B; 113.0−113.5 min, mobile phase B decreased to 5%; 113.5−120.0 min, 5% mobile phase B. The nanoliter liquid phase separation end was directly connected to the mass spectrometer. The peptides separated by liquid phase chromatography were ionized by a nano electrospray ionization (nanoESI) source and then passed to a tandem mass spectrometer Q-Exactive HF X (Thermo Fisher Scientific) for Data Dependent Acquisition mode detection. The main parameters were set: ion source voltage was set to 1.9 kV, MS1 scanning range was 350−1500 m/z; resolution was set to 60,000; MS2 starting m/z was fixed at 100; resolution was 15,000. The ion screening conditions for MS2 fragmentation: charge 2+ to 6+, and the top 30 parent ions with the peak intensity exceeding 10,000. The ion fragmentation mode was high-energy collisional dissociation, and the fragment ions were detected in Orbitrap (Thermo Fisher Scientific). The dynamic exclusion time was set to 30 s.

The raw data was identified using the software MaxQuant (Version 1.5.3.30; https://www.maxquant.org/). At the spectrum level, filtering was performed with PSM-level false discovery rate (FDR) < 1%, and at the protein level, further filtering was performed with protein-level FDR < 1%. WoLF PSORT software (https://www.genscript.com/wolf-psort.html) was used to filter the results by subcellular localization. Only proteins that the software predicted to be located in mitochondria were further analyzed. Functional analysis was developed by using GO, KOG, and KEGG databases. Based on the quantitative values obtained from each protein in multiple replicates of the sample, fold-change was obtained in the comparative groups. Statistical significance was set at $p < 0.05$ for the *t* test in the comparison between groups. MitoCarta 3.0[45] was used to group the proteins in mitochondrial pathways.[46]

2.6.10. Transcriptome analysis by RNA-sequence

RNA from skeletal muscle was extracted using Trizol (Thermo Fisher Scientific). The RNAs were precipitated, and their quality and quantity assessed using an Agilent Bioanalyzer 2100 and an RNA 6000 chip (Agilent Technologies, Santa Clara, CA, USA). Subsequently, cDNA libraries were constructed using the Hieff NGS™ Ultima Dual-mode mRNA Library Prep Kit (Yeasen, Shanghai, China), and their quality was checked using an Agilent Bioanalyzer 2100 with a DNA 1000 chip (Agilent). The libraries were then subjected to Paired End 150 sequencing in a DNBSEQ-G400 system (MGI, Shenzhen, China) with a target of 40 M reads per sample. The quality of the resulting sequencing reads was evaluated using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The GCF_000001405.39_GRCh38.p13 reference human genome was obtained from the NCBI database. Filtering was performed using SOAPnuke (https://github.com/BGI-flexlab/SOAPnuke), developed independently by BGI Research (Shenzhen, China), with the following criteria: removal of reads containing adaptor pollution; removal of reads with an N content greater than 5%; and removal of low-quality reads, defined as reads with bases having a quality score less than 15, and with the proportion of total bases in the reads greater than 20% considered as low-quality reads.

The filtered "clean reads" were saved in FASTQ format. Subsequently, HISAT (https://daehwankimlab.github.io/hisat-genotype/) was utilized to align reads to each genomic locus. Bowtie2 (https://bowtie-bio.sourceforge.net/bowtie2/index.shtml) was employed to map the clean reads to the reference gene sequence (transcriptome), and RSEM (http://deweylab.github.io/RSEM/) was used to calculate the gene expression level for each sample.

2.6.11. Evaluation of supercomplexes formation by blue-native gel electrophoresis (BNGE)

Supercomplexes levels were analyzed in isolated mitochondria from human muscle biopsies by BNGE.[47] Mitochondrial proteins were solubilized with 10% digitonin (4 g/g; D5628; Sigma-Aldrich) and run on a 3%−13% gradient blue native gel. The gradient gel was prepared in 1.5-mm glass plates using a gradient former connected to a peristaltic pump. After electrophoresis, the gels were further processed for Western blotting. After BNGE, proteins were electroblotted onto polyvinylidene difluoride (PVDF) transfer membrane (Immobilon-FL 0.45 μm, IPFL00010; Merck Millipore, Mumbai, IN, USA) for 1 h at 100 V (complex IV) (anti-cytochrome C oxidase I; Invitrogen), complex III (anti-cytochrome B-C1 complex subunit 2, 14742-1-AP; Proteintech, Rosemont, IL, USA) and voltage-dependent anion-selective channel 1 (VDAC1) (ab15895; Abcam, Cambridge, UK). The secondary antibodies were goat anti-rabbit IgG (H + L), Alexa Fluor 680 conjugate from Life Technologies (A-21076; Life Technologies, San Jose, CA, USA), and anti-mouse DyLight 800

(Invitrogen SA5-10176; Life Technologies), and acquired with the Odyssey Infrared Imaging System (LICORbio, Lincoln, NE, USA) were incubated for 45 min at room temperature.

2.6.12. Control of cofounding variables (Visits 3 and 4)

Participants were asked to complete the same pre-experimental conditions described in Section 2.5., control of cofounding variables (Visit 2). For women the phase of their menstrual cycle was registered. Room temperature was controlled to maintain a range between 20°C−24°C. Participants followed a standardized diet during the previous day (i.e., Pre 24 h) (Fig. 1). The ingredients were adequately defined (balanced menu with approximately 55% carbohydrates, 27% fat, and 18% proteins) and quantities were chosen by the participants ad-libitum. On exercise day they consumed a standardized meal (i.e., white bread, cheese, olive oil, and apple or similar fruit) 4 h before exercise (Fig. 2, time point = −240 min). The subsequent meal was again standardized and consumed within 1 h after leaving the laboratory (i.e., salad with lettuce, tomato, sweet corn, carrot, tuna and olive oil, and potato omelette (egg, potato, and olive oil)) (Fig. 2, time point = 300 min). They were provided with a template for the register of their food consumption, and compliance was checked by the researchers after finishing the study. Similarly, they were instructed to replicate, as closely as possible, the physical activity they engaged in at Post 24 h and Post 48 h after Visit 3, aiming to reproduce it after Visit 4. Data from accelerometers were utilized to corroborate this. Details about their eating schedule outside of study visits (normal daily routine of participants) were not collected.

2.7. Statistics

Descriptive statistics of the study subjects are shown as mean ± SD. Continuous data obtained by specific outcomes related to indirect calorimetry (i.e., VO_2, VCO_2, RER, and EE every 10 s during exercise) were analyzed using the Statistical Parametric Mapping 1-dimension (SPM1D)[48] package available for Matlab (V.0.4), which allows for conducting conventional statistical tests on 1-dimensional data.[48] Previously, smooth data function in Matlab was applied to all curves to smooth noisy data. Smooth data returns a moving average of the force curve using a fixed window length that is determined heuristically. We performed paired *t* tests in SPM1D to compare VO_2, VCO_2, RER, and EE data curves during morning *vs.* evening exercise.

Normal mixed models using random intercepts were used to analyze changes in the following outcomes: interstitial glucose, resting energy metabolism (i.e., VO_2, VCO_2, EE, and RER), physical activity and sleep (i.e., moderate-to-vigorous physical activity, low physical activity, sedentary behavior, sleep, total sleep time, wakefulness after sleep onset (WASO), times awake), mitochondrial respiration (i.e., RCR_N = Respiratory Control Ratio with NADH-linked substrates like pyruvate or glutamate; RCR_F = Respiratory Control Ratio with $FADH_2$-linked substrates like succinate; State 3_N = State 3 respiration with NADH-linked substrates; State 3_F = State 3 respiration with $FADH_2$-linked substrates; State $4o_N$ = State 4 respiration with NADH-linked substrates; State $4o_F$ = State 4 respiration with $FADH_2$-linked substrates), mitochondrial super complexes (SC) (i.e., SC/VDAC), temperature and blood parameters. The models included fixed effects for time and condition (2 levels; morning *vs.* evening) as well as the unique patient identifier as a random effect separately for both men and women. The use of normal mixed models was chosen to preserve the number of data points and avoid listwise exclusion of data. A paired *t* test was used for comparing morning *vs.* evening sleep parameters data. Time was described as a continuous variable. Significance was set at $p < 0.05$. These analyses were performed with R Version 3.5.2 (https://community.chocolatey.org/packages/microsoft-r-open/3.5.2) using packages "lme4" and "lmerTest". GraphPad Prism Version 9.4.1 (GraphPad Software, San Diego, CA, USA) was used to plot the figures.

For transcriptomic analyses, we observed the number of genes changing their expression at Pre in the morning, Post in the morning, Pre in the evening, and Post in the evening. Changes in expression levels of genes related to glycogen degradation, cytokines, and growth factors were calculated as Post−Pre in the morning, and separately in the evening. Changes in expression levels of circadian genes were calculated as Morning−Evening at Pre, and separately at Post. The DESeq2 method[49] was applied to detect differentially expressed genes. The transcripts that fulfilled the inclusion criteria were then subjected to gene classification using a databank based on hand-curated literature. The list of differentially expressed genes (DEG) was obtained by analysis of variance filtering with FDR correcting with a cut-off of adjusted $p < 0.05$. The fold changes are represented as log2FC (base-2 logarithm of the fold change). The general canonical pathways implicated for the significantly changed transcripts were generated by Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA, USA) before being evaluated, and $p < 0.05$ were considered significant. IPA was also used for generating the gene expression heatmaps.[48]

3. Results

3.1. A single bout of moderate-intensity aerobic exercise in the morning and evening demonstrates similar effects on systemic glucose regulation in both men and women

In order to study the potential influence of diurnal variations on glucose regulation in response to exercise, a group of healthy men and women (Table 1) performed separate bouts of moderate-intensity aerobic exercise, once in the morning (11:30 a.m.) and once in the evening (6:30 p.m.), while wearing a CGM (Figs. 1 and 2). Interstitial glucose levels during the 24 h before exercise day, the exercise day, and 2 days subsequent to exercise exhibited a similar response in both morning and evening conditions (Fig. 3A−3F). When examining diurnal (Fig. 3G−3L) and nocturnal (Fig. 3M−3R) glucose levels separately, no distinct differences were observed between morning and evening conditions. However, in men the coefficient of variation of glucose during the

Table 1
Descriptive data of the study subjects.

General characteristics	Men ($n = 18$)		Women ($n = 17$)	
	n	Mean ± SD	*n*	Mean ± SD
Age (year)	18	27.3 ± 7.2	17	25.8 ± 6.9
Chronotype	17		17	
Definitely morning type	0		0	
Moderately morning type	4		3	
Neither type	9		12	
Moderately evening type	3		1	
Definitely evening type	1		1	
Anthropometry and body composition				
Body mass index (kg/m^2)	18	24.3 ± 2.4	17	22.2 ± 1.9
Lean body mass (kg)	18	57.6 ± 8.7	17	38.5 ± 4.0
Lean mass index (kg/m^2)	18	18.2 ± 1.6	17	14.0 ± 1.3
Fat mass (kg)	18	15.3 ± 4.9	17	19.5 ± 3.8
Fat mass index (kg/m^2)	18	4.9 ± 1.6	17	7.1 ± 1.2
Fat mass (%)	18	20.2 ± 5.1	17	32.2 ± 4.3
Visceral adipose tissue mass (g)	18	273.7 ± 148.3	17	204.2 ± 96.7
Apendicular lean mass (kg)	18	25.8 ± 5.2	17	15.8 ± 2.1
Apendicular lean mass index (kg/m^2)	18	8.1 ± 1.1	17	5.8 ± 0.8
Cardiorespiratory fitness and physical activity habits				
VO_{2peak} (mL/min)	18	3172.7 ± 509.3	15	2073.8 ± 361.8
VO_{2peak}/lean mass (mL/kg/min)	18	55.6 ± 9.5	15	54.1 ± 8.4
Low physical activity (min/week)	18	533.3 ± 974.0	16	356.3 ± 264.3
Moderate physical activity (min/week)	17	329.7 ± 454.0	15	173.0 ± 230.4
Vigorous physical activity (min/week)	17	204.4 ± 192.0	14	128.6 ± 113.1
Sedentary activities (min/week)	16	2366.9 ± 1213.3	14	2180.0 ± 989.1

Note: Missing values in VO_{2peak} are due to technical issues with the equipment, and missing chronotype or self-reported physical activity data is due to incomplete questionnaires or "don't know" responses from participants.
Abbreviation: VO_{2peak} = peak volume of oxygen.

diurnal phase follows a different pattern when considering both the exercise stimulus (changes in time) and time of day (changes in condition), with a greater increase at 24 h post-exercise in the evening (time × condition, $p = 0.04$, Fig. 3I). Other blood parameters (i.e., plasma glucose and insulin) were assessed pre- and post-exercise and 90 min post-exercise, resulting into no differential effect of exercise and time of day (Supplementary Tables 1 and 2). Free living physical activity levels objectively assessed by accelerometery were similar between morning *vs.* evening exercise conditions the 24 h preceding exercise, on the exercise day, and in the days subsequent to exercise (Supplementary Fig. 1).

As an overview, the behavior of glucose in response to exercise is similar in the morning and evening for both men and women. However, in men there appears to be greater variability in interstitial glucose levels the day after evening exercise.

3.2. A single bout of moderate-intensity aerobic exercise in the morning and evening has similar effects on energy metabolism in men and women

We also investigated EE and substrate oxidation pre-, during, and post-exercise in both morning and evening conditions. EE (Fig. 4A–4D) and substrate oxidation (approximated by the RER; Fig. 4E–4H) during exercise were similar in the morning and the evening in both men and women. As expected, the increase in EE induced by exercise was maintained at rest at post-exercise, with no different effect of exercise by time of day in men (Fig. 4I) and women (Fig. 4N). Likewise, resting RER was similar in both men and women (Fig. 4J and 4O).

We did not observe a differential effect of exercise by time of day in men with respect to blood lipids or thyroid hormones (Supplementary Table 1). Results were similar in women, except for blood triglycerides, which did not achieve statistical significance (time × condition, $p = 0.07$) but did decrease at 90 min post-exercise more markedly in the evening than in the morning (Supplementary Table 2).

Skin body temperature exhibited a different pattern between men and women. In men, temperature exhibited a distinct progression from 30 min of exercise onwards, showing a greater increase at this point in the morning compared to evening (time × condition, $p = 0.01$, Fig. 4S). Meanwhile, women started exercising with a higher temperature in the evening, which decreased during exercise, while in the morning it remained stable (time × condition, $p = 0.046$, Fig. 4U).

In the skeletal muscle, we examined mitochondrial respiration through the calculation of the RCR by either complex I (RCR_N) (Fig. 4K and 4P) and complex II (RCR_F) (Fig. 4L, Fig. 4Q and Supplementary Fig. 2), and total mitochondrial SC/VDCA through densitometric analysis (Fig. 4M, Fig. 4R, and Supplementary Fig. 3). We found no relevant changes in men or women when considering the exercise stimulus and time of day. In women, a slightly different response (showing a trend towards significance) was observed in RCR_F, which

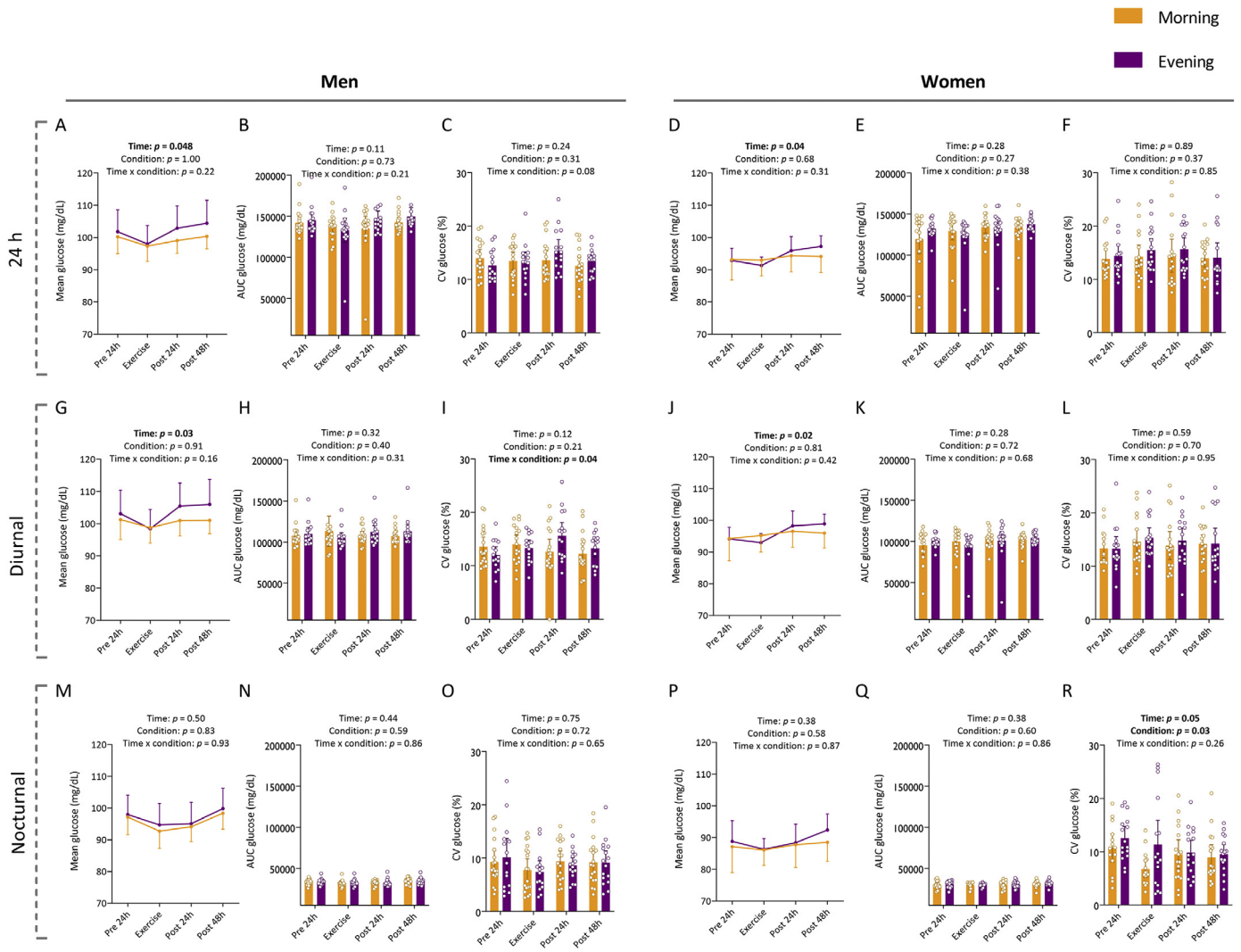

Fig. 3. Interstitial glucose levels in response to exercise in the morning and evening conditions. Interstitial glucose levels were assessed during the 24 h before exercise day (Pre 24 h), the exercise day (Exercise), and 2 days subsequent to exercise (Post 24 h and Post 48 h, respectively). (A−F) show average data from 24 h each day; (G−L) show average data from diurnal glucose (6:00−23:59) each day; and (M−R) show average data from nocturnal glucose (00:00−5:59) each day. Data from men (A, B, C, G, H, I, M, N, and O) and women (D, E, F, J, K, L, P, Q, and R) are presented separately. Normal mixed models are used to calculate the effect of time, condition, and time and condition interaction (time × condition). Values are presented as means and 95%CI. Data in bold are of significant difference. 95%CI = 95% confidence interval; AUC = area under the curve; CV = coefficient of variation.

decreased post-exercise in the evening and increased in the morning (time × condition, $p = 0.07$, Fig. 4Q).

In summary, at a systemic level, energy metabolism during and after a single session of moderate aerobic exercise did not display significant variations between morning and evening conditions in both men and women. However, there were contrasting responses between sexes in terms of skin body temperature and mitochondrial respiration in the skeletal muscle.

3.3. A single bout of moderate-intensity aerobic exercise impacts sleep parameters differently according to time of day in women but not men

We used accelerometers to study the impact of morning *vs.* evening exercise on sleep parameters the night immediately following exercise (Supplementary Table 3). In men, total sleep time, WASO, sleep efficiency, and time awake were not affected differently by morning or evening exercise, although there was a trend towards significance in total sleep time favoring evening ($p = 0.07$). In women, we observed a longer total sleep time after evening exercise ($p < 0.001$) and less WASO at this time of day, with the latter showing a trend towards significance ($p = 0.09$); however, no remarkable morning *vs.* evening variations were noted in sleep efficiency and time awake.

To summarize, exercise had comparable effects on sleep parameters regardless of time of day in men, while women experienced longer sleep after evening exercise.

3.4. Transcriptomic analysis in the skeletal muscle reveals sex and time-of-day differences in response to a single bout of moderate-intensity aerobic exercise

To further evaluate local adaptations in response to acute exercise, we conducted a transcriptomic analysis in the skeletal

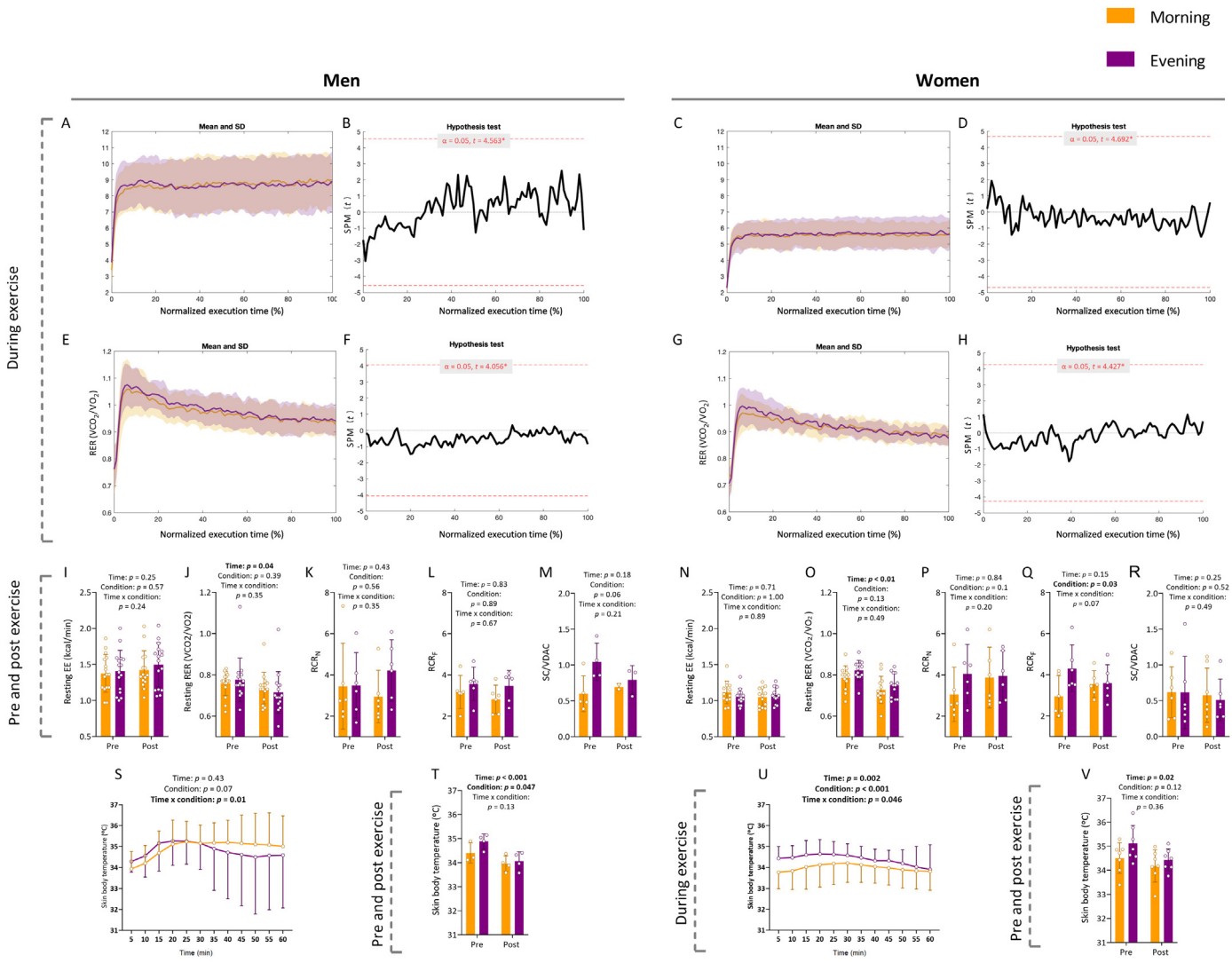

Fig. 4. Energy metabolism pre-, during, and post-exercise in the morning and evening conditions. EE and RER (calculated from VO_2 and VCO_2 exchange) were assessed during exercise and pre- and post-exercise at rest (Resting). (A, C, and E, G) SPM1D-analysis for EE and RER during exercise, respectively (solid lines represent mean and shaded areas SD); (B, D, F, and H) SPM{t} from each corresponding A, C, E, and G; (I, M and J, N) EE and RER at rest, respectively; mitochondrial function was assessed pre- and post-exercise, (K and O) mitochondrial RCR from complex I (generation of NAD^+); (L and P) mitochondrial RCR from complex II (generation of FAD); (Q and T) densitometric analysis of the total supercomplexes amount normalized for the VDCA1 signal; skin body temperature was assessed pre, during, and post-exercise, (R and T) average temperature values during exercise; (S and V) average temperature values at pre- and post-exercise. Data from men (A, B, E, F, I, J, K, Q, R, and S) and women (C, D, G, H, M, N, O, P, T, U, and V) are presented separately. Normal mixed models are used for I−V (time, condition, and time and condition interaction). (I−V) values are mean and 95%CI. Data in bold are of significant difference. 95%CI = 95% confidence interval; AUC = area under the curve; EE = energy expenditure; FAD = flavin adenine dinucleotide (oxidized form); $FADH_2$ = flavin adenine dinucleotide hydroquinone form; NAD^+ = nicotinamide adenine dinucleotide (oxidized form); NADH = nicotinamide adenine dinucleotide; RCRF = respiratory control ratio with $FADH_2$-linked substrates like succinate; RCRN = respiratory control ratio with NADH-linked substrates like pyruvate or glutamate; RER = respiratory exchange ratio; SPM{t-value} = the trajectory of Student's t statistic or, equivalently, the mean difference curve normalized by sample-size normalized variance; SPM1D = statistical parametric mapping 1-dimension; VCO_2 = carbon dioxide volume; VDCA1 = voltage-dependent anion-selective channel 1; VO_2 = oxygen volume.

muscle. Overall, men had fewer differentially expressed genes than women post-exercise (1093 *vs.* 3343 genes, respectively) (Supplementary Fig. 4A and 4B). In men, the number of genes modified by exercise only in the morning predominated over those modified only in the evening or commonly in the morning and evening (Supplementary Fig. 4A). Contrary, in women, the highest number of genes modified by exercise were observed in the evening (Supplementary Fig. 4B). A closer examination of the common exercise-induced modification in the morning and evening revealed different metabolic pathways in men (Supplementary Fig. 4C) and women (Supplementary Fig. 4D). Interestingly, the study of metabolic pathways revealed that genes involved in glycogen and glucose degradation changed their expression only in the evening in both men (Fig. 5A and Supplementary Fig. 5C) and women (Fig. 5A and Supplementary Fig. 5D). In men, these genes were generally repressed post-exercise at the evening session, while an overexpression was seen in women (Fig. 5A).

In signaling pathways, within the common changes in the morning and evening, we observed a strong activation of

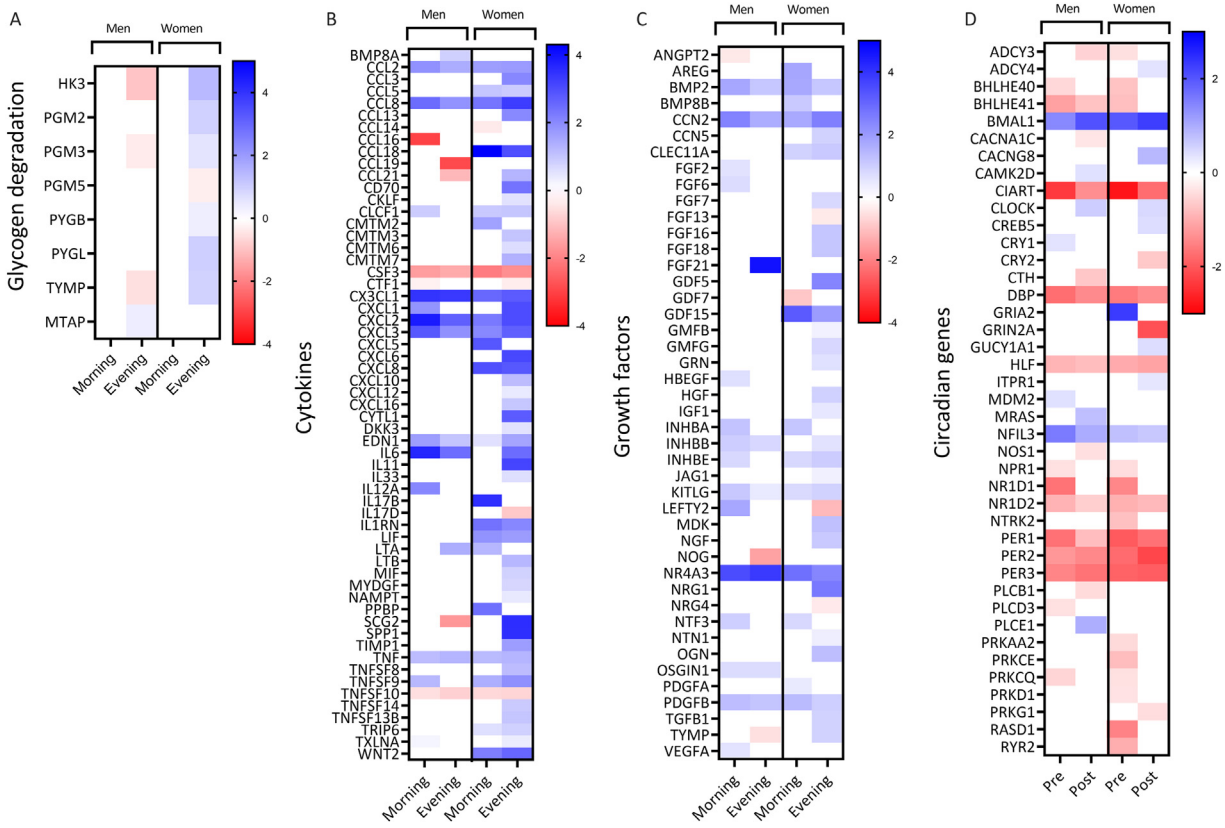

Fig. 5. Muscle gene expression profile pre- and post-exercise in the morning and evening conditions. Representative heatmaps of the changes in the expression level from pre- to post-exercise of the genes related to (A) glycogen degradation, (B) cytokines, and (C) growth factors in the morning and the evening and in men and women; (D) changes in the expression level comparing morning *vs.* evening of the genes related to circadian rhythm separately pre- and post-exercise and in men and women. Abbreviations are listed in Supplementary material: List of abbreviations in Fig. 5.

pathways linked to inflammation both in men (Supplementary Fig. 4E) and women (Supplementary Fig. 4F). Interestingly, these were further activated in the evening in women (Supplementary Fig. 5F and 5H), but no relevant time of day variations were observed in men (Supplementary Fig. 5E and 5 G). Additionally, changes in cytokines were higher in women than in men at both times of day (Fig. 5B). We analyzed the expression of growth factors, which are signaling proteins frequently involved in the regulation of the metabolism and the inflammation process (Fig. 5C). Remarkably, *growth differentiation factor 15* (*GDF15*)—a myokine associated with the regulation of mitochondrial metabolism[50]—was upregulated only in women independently of the time of day (Fig. 5C). In contrast, there was an overexpression of *fibroblast growth factor 21* (*FGF21*), which is also involved in the control of mitochondrial metabolism,[51] only in men post-exercise in the evening (Fig. 5C).

Changes in clock genes after exercise notoriously differed between men and women. *Cryptochrome circadian regulator 2* (*CRY2*), *period circadian regulator 2* (*PER2*), *etinoic acid receptor-related orphan receptor alpha* (*RORα*), and *peroxisome proliferator-activated receptor alpha* (*PPARα*) were repressed only after evening exercise in women, while *PER1* was induced by exercise in men, particularly in the evening (Supplementary Fig. 6). When taking into consideration only the morning *vs.* evening differences without the exercise stimulus (Supplementary Fig. 7), both women and men differentially expressed genes related to circadian rhythms signaling (Supplementary Fig. 7E and 7F). Further, independently of the exercise stimulus in both men and women (Supplementary Fig. 8), *brain and muscle ARNT-like 1* (*BMAL1*) was mainly overexpressed in the evening, while *circadian associated repressor of transcription* (*CIART*), *D-box binding PAR BZIP transcription factor* (*DBP*), *hepatic leukemia factor* (*HLF*), *nuclear receptor subfamily 1 Group D Member 2* (*NR1D2*), *PER1, PER2*, and *PER3* were mainly repressed in the evening (Fig. 5D).

Summarizing these findings, gene expression in the skeletal muscle revealed sex and time-of-day differences in metabolic and signaling pathways. These differences can be explained, at least partially, by the different expression of myokines and other growth factors together with the time-of-day dependent expression of the genes regulating circadian rhythms.

3.5. *Sex and time-of-day differences in response to a single bout of moderate-intensity aerobic exercise are observed in the mitochondrial proteome*

In men, the pattern shown in the mitochondrial proteome revealed a general decrease in the mitochondrial proteins post-exercise, which was more evident in the evening (Fig. 6). On the contrary, a general increase in the mitochondrial proteins

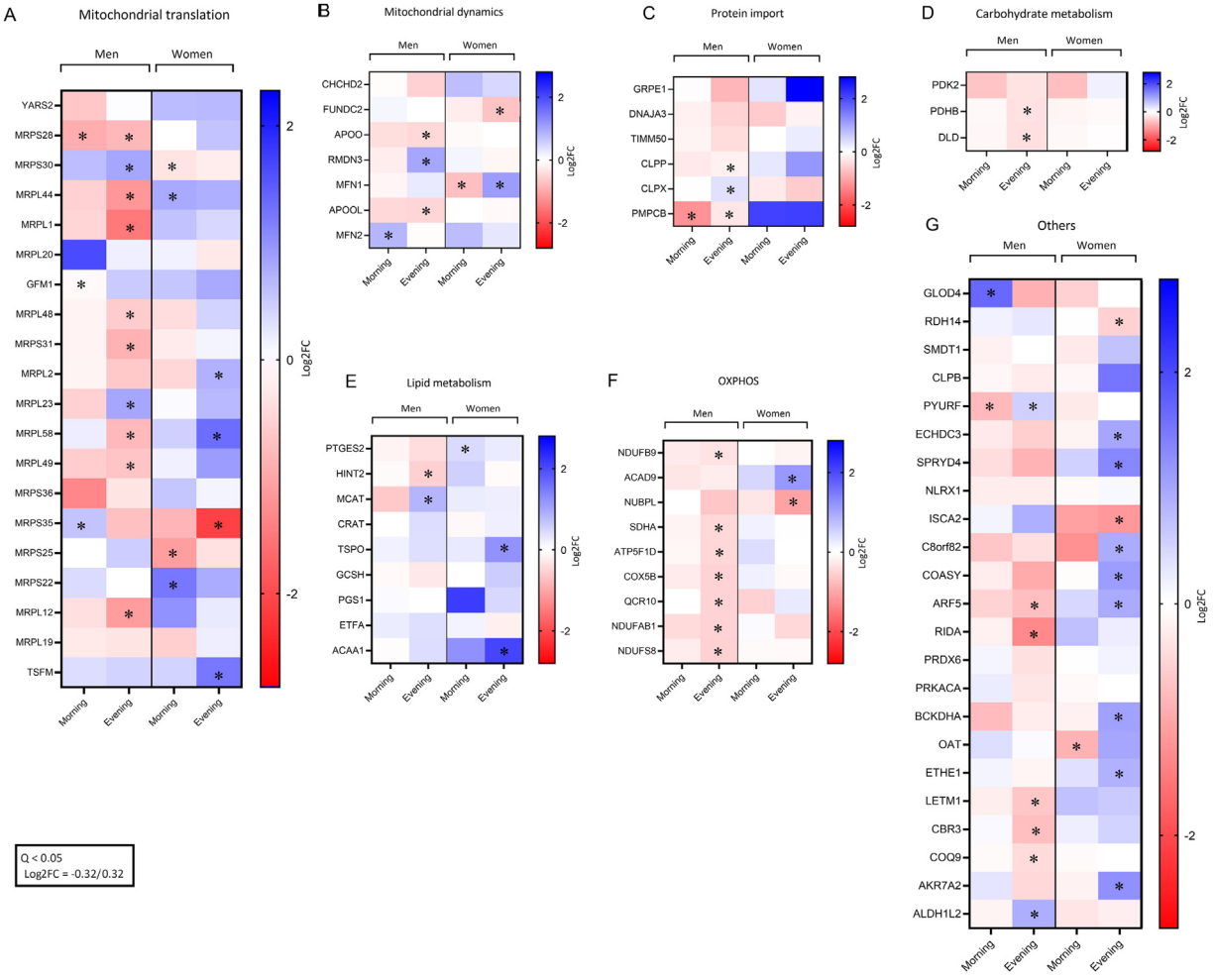

Fig. 6. Mitochondrial proteome pre- and post-exercise in the morning and evening conditions. Representative heatmaps of the fold change of the protein's levels comparing pre-exercise data *vs.* post-exercise data in the morning (Morning) and pre-exercise data *vs.* post-exercise data in the evening (Evening) in men and women. Proteins are classified according to their functions in (A) mitochondrial translation, (B) mitochondrial dynamics, (C) protein import, (D) carbohydrate metabolism, (E) lipid metabolism, (F) the OXPHOS system, and (G) others. Mitochondrial proteomics was performed in isolated mitochondria from muscle biopsy. * $p < 0.05$, significant difference between pre- and post-exercise. Abbreviations are listed in Supplementary materials: List of abbreviations in Fig. 6.

post-exercise was noted in women and was also more evident in the evening (Fig. 6). These changes were reflected in proteins involved in mitochondrial translation (Fig. 6A), mitochondrial dynamics (Fig. 6B), protein import (Fig. 6C), carbohydrate metabolism (Fig. 6D), lipid metabolism (Fig. 6E), the OxPhos system (Fig. 6F), and some other mitochondrial functions (Fig. 6G). Considering only the time-of-day differences (Supplementary Fig. 9), our analysis revealed a diffused pattern regarding the sex differences. However, higher levels of mitochondrial proteins in the evening were seen in women, particularly post-exercise.

In summary, these results suggest that the mitochondrial proteome is different in response to a single bout of moderate-intensity aerobic exercise attending to the sex and the time of day.

4. Discussion

A single bout of moderate-intensity aerobic exercise, whether in the morning or the evening, overall manifests similar systemic effects on glucose regulation and energy metabolism in both men and women. However, in the skeletal muscle, transcriptional analysis, mitochondrial function, and mitochondrial proteomics revealed a marked sexual dimorphism and time-of-day variations in response to this bout of exercise. These results emphasize the importance of accounting for sex-specific differences in the implementation of exercise interventions and the need to conduct separate analyses for both men and women. This study lays the groundwork for a detailed molecular framework, providing a basis for research on precise exercise prescription in clinical settings.

Considering the influence of molecular clocks in orchestrating glucose and energy metabolism, it is biologically plausible to anticipate distinct responses when exercising in the morning compared to the evening. The present findings, together with other comparable studies in healthy men[52–54] and women,[55] revealed a similar systemic response to a single bout of moderate-intensity aerobic exercise in both interstitial and blood glucose independently of the time of day and sex. Similarly, in line with analogous studies,[56,57] we observed

comparable EE and substrate oxidation response to morning and evening exercise.

Upon detailed examination, our systemic-level findings suggest that men and women could exhibit differential metabolic responses to exercise influenced by the time of day. This sexual dimorphism had already been suggested by previous studies conducted in our laboratory.[10,58,59] In women, we identified substantial changes (showing proximity to statistical significance) in outcomes such as blood triglyceride levels, which exhibited a greater reduction following evening exercise, and skin body temperature, which was higher in the evening. Higher temperature during exercise has been reported to stimulate endogenous carbohydrate metabolism.[60] Additionally, in women's skeletal muscle, a contrasting pattern of mitochondrial respiration was observed between morning and evening sessions, indicating an increase in respiration through complex II post-exercise in the morning and a decrease in the evening. Lastly, genes involved in glycogen and glucose degradation were upregulated post-exercise in the evening, accompanied by increased cytokine expression at this time of day. Higher cytokine expression post-evening exercise indicates that, even though the exercise protocol is consistent at both times of day, it may represent a more robust stimulus in the evening.[61] In light of this information, our findings suggest that moderate-intensity aerobic exercise in women induces a more oxidative response in the morning, plausibly with increased fat utilization, while in the evening a distinct metabolic response with heightened carbohydrate utilization is evident. At first contradictory, mitochondrial proteome expression in women was especially increased in the evening. Nevertheless, it may be reasonable to assume that a negative feedback loop regulation is occurring, wherein translation regulators monitor mitochondrial function and use this information to upregulate or downregulate protein synthesis.[62]

Dissimilar results were observed in men. They did not exhibit noteworthy systemic-level variations according to time of day, except for a greater increase in body skin temperature in the morning, starting from Minute 30 of the exercise bout. In male skeletal muscle, changes in glycogen and glucose degradation genes were fewer than in women and they were repressed after evening exercise. Neither was important time-of-day differences found in cytokines. Lastly, mitochondrial proteome expression was particularly downregulated post-exercise in the evening. Based on this data, it could be hypothesized that evening exercise, more so than morning exercise, promotes mitochondrial function while inhibiting carbohydrate utilization. Yet the comprehensive dataset for men does not evidence clear exercise-induced morning and evening variations. Importantly, diverse response to exercise according to sex could be shaped by the distinct expression of the myokines *GDF15* and *FGF21* in men and women, which are important regulator myokines of mitochondrial metabolism.[50,51]

It is important to emphasize that when exercise stimulus was not considered, the expression of clock genes (e.g., *BMAL1, PER1, PER2*, or *PER3*) consistently changed between morning and evening in both of sexes. This serves as a proof of concept and substantiates the methodological integrity of the study. However, time-of-day and sex differences revealed by our molecular data were not reflected at the systemic level, which may be because (a) we employed a single bout of aerobic exercise (b) performed at a moderate intensity and (c) in a healthy population. Longer term exercise interventions applying (a) different methodologies (e.g., strength training) (b) at different intensities (e.g., high-intensity interval training) in (c) patients with cardiometabolic disturbances (e.g., metabolic syndrome) could potentially reveal whether the molecular alterations noted in skeletal muscle have broader systemic impacts. Remarkably, the molecular characteristics we identified in the skeletal muscle of women align with intervention studies reporting increased fat loss with morning exercise in this population.[63,64] Additionally, one of the studies also reported sex-based differences, with men presenting higher fat oxidation after evening exercise.[63] Higher exercise-intensity would also be expected to translate into more pronounced and observable physiological effects on glucose and energy metabolism, as suggested previously.[4]

It should be mentioned that genetic data from mouse models suggest sexual dimorphism and time-of-day variation with respect to the effects of exercise on metabolic parameters. Male mice experienced the most striking response to exercise in their active phase, similar to the higher global changes in genes we observed in men in the morning.[65,66] In female mice, late training demonstrated greater beneficial anti-atherosclerotic effects.[67] However, the sex of the mice did not impact time-of-day dependent exercise capacity.[68] The interplay between sex, time-of-day, and exercise therefore warrants further exploration.

We may expect different results if a similar experiment were carried out in populations with metabolic diseases.[69,70] Some studies have reported that exercising in the evening potentially prevents hyperglycemic events in patients with type 2 diabetes.[71–74] In contrast, a recent exercise intervention study in middle-aged adults with metabolic syndrome has shown that morning exercise improves fasting insulin levels to a higher extent than afternoon exercise, yet no differences were noted in fasting glucose levels between morning *vs.* afternoon exercise.[75] Of note, these studies analyzed the data in men and women together, which hampers a determination of the sex-specific exercise-induced effects. The mixed results underscore the importance of a comprehensive exploration into the temporal aspect of exercise to reveal potential differences in outcomes among diverse populations. Future research should explore the impact of metabolic disease and sex differences in the diurnal variation of the physiological and molecular effects of exercise.

5. Conclusion

The present study shows that there are distinct molecular responses to exercise in skeletal muscle based on the time of day and sex, although a single session of moderate-intensity aerobic exercise is not enough to reflect systemic adaptations. Our results illustrate that the time of day of exercise has a

more significant impact on women, for whom exercise is a stronger stimulus in the evening, at which time it produces a metabolic response with a higher reliance on carbohydrates. It would be premature to make clinical recommendations based on these findings, but here we provide a molecular framework for future research. By identifying and understanding the molecular underpinnings of exercise responses, exercise professionals will be able to make more targeted, personalized prescriptions and potentially optimize therapeutic benefits.

Authors' contributions

RSL contributed to data curation, formal analysis, investigation, project administration, resources (patients), validation, visualization, and writing (original draft); AMP contributed to data curation, formal analysis, investigation, resources (patients), validation, and writing (review and editing); PGG and JMMG contributed to data curation, formal analysis, investigation, validation, and writing (review and editing); NRM contributed to investigation and resources (patients); BRG, JAV, and JGC contributed to investigation; PMG and JMAA contributed to data curation, formal analysis, and writing (review and editing); SC contributed to data curation, investigation, validation, methodology, visualization, resources, supervision, and writing (review and editing); LMS contributed to investigation and resources; JRH contributed to resources, methodology, supervision, and funding acquisition; LCL contributed to data curation, formal analysis, resources, methodology, validation, visualization, supervision, funding acquisition, and writing (original draft and review and editing); JRR and FJAG co-conceived the study, and contributed to resources, methodology, project administration, validation, visualization, supervision, funding acquisition, and writing (review and editing). All authors have read and approved the final version of the manuscript, and agree with the order of presentation of the authors.

Competing interests

The authors declare that they have no competing interests.

Data availability

RNA-Seq data were generated as described above. The files have been uploaded to the repository Sequence Read Archive. The accession number is PRJNA1073746. All data can be found at https://www.ncbi.nlm.nih.gov/sra/PRJNA1073746. Any additional data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments

We extend our sincere gratitude to all the participants who generously contributed to this study, making it possible to advance our understanding in the field. Your dedication and cooperation have been invaluable. Special thanks to our colleagues from the PROFITH Group and the MiTo LAB Group at the University of Granada. Although not listed as authors, their unwavering support and valuable insights significantly enriched the research process. We would also like to express our appreciation to the staff and administration at San Cecilio Hospital for their cooperation and assistance throughout the duration of the study. This study was funded by the Spanish Ministry of Culture and Sport, Consejo Superior de Deportes, Plan de Recuperación, Transformación y Resiliencia, Unión Europea, Next Generation EU; Ayudas Europeas a Proyectos de Investigación Aplicada a la Actividad Física Beneficiosa para la Salud y la Medicina Deportiva (EXP_77437); by the Spanish Ministry of Science and Innovation (PID2022-141506OB-I00) and the European Regional Development Funds (ERDF); by the University of Granada Plan Propio de Investigación-Excellence actions: Unit of Excellence on Exercise Nutrition and Health (UCEENS); RSL and AMP are supported by the Spanish Ministry of Education, Culture and Sport (FPU19/03745 and FPU20/05530, respectively); JMAA is supported by the Juan de la Cierva-Formación Grant FJC2020-044453-I funded by Ministerio de Ciencia e Innovación/Agencia Estatal de Investigación MCIN/AEI/10.13039/501100011033 and European Union NextGeneration, Plan de Recuperación, Transformación y Resilencia (EU/PRTR); SC is a recipient of a "Ramón y Cajal fellowship 2013−2017" funded by MCIN/AEI /10.13039/501100011033 and "El Fondo Social Europeo invierte en tu futuro". Research in Sara Cogliati lab is partially supported by the Grant PID2020-114054RA-I00 1001100482, funded by MCIN/AEI/10.13039/501100011033. Research in LCL lab is supported by a grant from the MCIN/AEI/10.13039/501100011033, Spain, and the ERDF (PID2021-126788OB-I00).

Supplementary materials

Supplementary materials associated with this article can be found in the online version at doi:10.1016/j.jshs.2024.101021.

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