

UNIVERSIDAD POLITÉCNICA DE MADRID
Facultad de Ciencias de la Actividad Física y del Deporte



Lactate: influence of its transporter, sex, temperature and nutritional status

DOCTORAL THESIS

Submitted for the degree of Doctor by:

José Antonio Benítez Muñoz

Máster Universitario en Rendimiento Físico y Deportivo
Graduado en Ciencias de la Actividad Física y del Deporte

Madrid, 2024



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Under the supervision of:
Dr. Rocío Cupeiro Coto

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Title: Lactate: influence of its transporter, sex, temperature and nutritional status

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Doctoral Programme: Ciencias de la Actividad Física y del Deporte

Thesis Supervision:

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External Reviewers:

Thesis Defense Committee:

Thesis Defense Date:

This thesis has been partially supported by the project Erythrocyte Transport of Lactate During Exercise (“Transporte Eritrocitario de Lactato en Ejercicio”, TELE project) provided by Universidad Politécnica de Madrid (grant number PINV18ENW9ML92B2287R). Moreover, this thesis has been partially supported by the project “Prestación de servicios científicos: evaluación y control del rendimiento” (PSC191100471). José Antonio Benítez Muñoz was supported by a grant provided by Universidad Politécnica de Madrid.

A mi familia: mi hermana, mi madre y mi padre.

Acknowledgement

Lo que me dice el corazón.

En primer lugar, me gustaría agradecer a Rocío, diría que es mi tutora, pero sería injusto porque esa palabra se le queda corta. Tú dices que una buena directora también debe traspasar el plano académico, y vaya si lo has conseguido, tú has entrado de lleno en el plano de la amistad. Da una tranquilidad tremenda saber que vas de la mano de una persona que no te va a soltar cuando tropieces.

Pedro, siempre te estaré agradecido por todo lo que has hecho por mí, no voy a enumerarlas porque no quiero ser más cansino de lo que he sido estos años. Sobre todo, me gustaría darte las gracias por la capacidad que tienes para transmitir que todo va a salir bien cuándo tú estás presente, desde organizar una cena en el casino de Tomelloso hasta sacar adelante el Simposio de Fuerza.

Ana, siempre recordaré el día que al final de clase de kine te pregunté si podía colaborar en el laboratorio de fisiología. Desde ese día sentí el ánimo que nos das a todas las personas que queremos aventurarnos al mundo de la academia. Desde luego que has conseguido que el laboratorio sea una pista de atletismo. Gracias.

Miguel, gracias por hacernos reír con tus chistes, aunque algunos merecían tarjeta amarilla de lo malo que eran. Una de las cosas que más alegran el día es llegar al laboratorio y ver que tú estás ahí. Siempre en mi equipo, por la capacidad que tienes de transmitir buen rollo.

Nuria, Víctor, Bea... gracias por labrar el terreno. Hicisteis que mis primeros años en el laboratorio fueran una lluvia de aprendizaje y diversión, hasta me dieron ganas de hacer el doctorado así que imaginaos... Después se incorporó Isa, qué largos se me hacían los días cuando venías al laboratorio, pero más largos se me hacían cuando no estabas... gracias por hacerme pasar tantos buenos momentos juntos. Estoy super orgulloso de lo que estás consiguiendo tanto en lo académico como en lo personal.

Jacobo y Domingo, sois el mejor ejemplo que podemos tener los que acabamos de empezar, gracias.

Gracias a las mejores médicas que el laboratorio podía tener. Mercedes, era ilusionante pasar las tardes contigo haciendo pruebas de esfuerzo. María, me encantan los debates que tenemos que me hacen exprimirme al máximo.

Bodoque, Jaime y Estrella, gracias por hacer que la vuelta a Madrid después del verano cobrara sentido. Si algo he conseguido en estos años es a vosotros. Ahora me tendréis que aguantar para siempre porque no os pienso dejar escapar. No os voy a engañar, tengo miedo, el mismo miedo que sentía cuando acabamos la carrera a que el resto de nuestra amistad no sea como hasta ahora, pero confío en vosotros para que os quedéis junto a mí.

Claudia, gracias por el apoyo que me demuestras todo el tiempo independientemente de la distancia, esa es una de las mejores muestras de amor. La ilusión con la que vives las cosas nos recuerda que tenemos que aprovechar hasta el más mínimo detalle. Las ganas de superarte en los diferentes aspectos de tu vida son admirables. Y, sin duda, ser buena persona es tu mayor virtud, gracias por hacer de este mundo un mundo mejor.

Gema, o como la llamamos en mi familia, Gemita o La niña. Gracias por enseñarme lo importante de verdad, y aunque esto lo tuvimos que aprender por las malas, lo importante eres tú. Lo admirable de una persona no es lo que consigue, sino lo que se esfuerza, y tú eres el mejor ejemplo de ello. Y para ello hay que ser muy fuerte para darse cabezazos contra el muro una y otra vez, aunque ahora hemos aprendido que hay veces que hay que parar si el muro es más duro que nuestra cabeza. Y también hay que ser valiente, cualidad que a ti te sobra, porque no sabes lo que hay detrás del muro, pero para eso está la positividad.

Mamá, gracias por enseñarme a vivir cargado de alegría. Pocas cosas me hacen más feliz que verte “jartarte” reír. Nos recuerdas que la vida está cargada de buenos momentos, si sabes identificarlos y aprovecharlos. Aunque a veces me desespero cuando te enseño a hacer algo con el móvil o a colgar una estantería, en esos momentos admiro las ganas que tienes de seguir aprendiendo.

Papá, gracias por decirme “adelante” cada vez que tomaba una decisión. Tú, y el campo, me habéis enseñado que para recoger castañas durante apenas unas semanas, hay que estar todo el año podando, desbrozando, regando... es decir, me has enseñado desde pequeño lo que cuesta conseguir las cosas, gracias a eso ahora valoro lo que tengo...

En definitiva, por encima de todo, me quedo con las personas que forman parte de mi vida.

Abstract

Lactate has three crucial functions: it is an energy substrate, the main gluconeogenic precursor, and it plays a signalling role in multiple processes. This makes measuring this metabolite extremely useful in both health and disease. However, to correctly interpret lactate, it is necessary to understand its transport in the body and the factors that influence it. Therefore, the aim of this thesis is to study the influence on lactate of its main transporters, sex, temperature and nutritional status.

First, a systematic literature review was conducted to understand the effect of exercise on the levels of the muscle proteins MCT1 and MCT4, which are the main lactate transporters (study I). Then, a project was carried out with participants having TT and AA genotypes of the T1470A SNP of the MCT1 gene. They performed an interval protocol while venous blood samples were taken to measure plasma and erythrocyte lactate concentrations (study II). In the same project, males and females performed an incremental protocol to determine power and heart rate at the first and second lactate thresholds, in both absolute and relative terms, to see if there were sex differences (study III). In another project, both males and females performed an incremental protocol in three different situations (control, heat, and glycogen depletion) to observe differences in carbohydrate and fat oxidation and lactate concentration between sexes (study IV). Additionally, this project compared the correlation between fat oxidation and lactate concentration in the three situations mentioned, and whether changes in both variables produced by these situations were proportional (study V). Finally, the same project determined heart rate, power, and perceived exertion at the first and second ventilatory thresholds to identify which variable remained most stable across the three situations (study VI).

The results of the systematic review in study I showed that MCT1 protein levels increased with both a single exercise session and a training program. MCT4 increased after a training program, although its responsiveness was less than that of MCT1. In study II, it was observed that lactate concentration in plasma and erythrocytes is very similar regardless of the T1470A SNP genotype. Study III found that females have lactate thresholds at a higher percentage of peak power and maximum heart rate than males. In study IV, it was reported that fat oxidation was higher in females than in males during an incremental protocol, regardless of the situation (control, heat, and glycogen depletion). Study V showed

a close relationship between fat oxidation and lactate concentration in the three mentioned situations. However, the greatest change in lactate concentration was observed between the control and heat situations, while the greatest change in fat oxidation was observed between the control and glycogen depletion situations. Lastly, study VI revealed that heart rate and perceived exertion at ventilatory thresholds were very similar across situations, while power at the thresholds differed between situations.

In conclusion, exercise appears to be an effective strategy to increase MCT1 and MCT4 levels. The presence of the T1470A SNP does not seem to alter lactate transport between plasma and erythrocyte. Establishing intensity as a percentage of peak power or maximum heart rate may create a different metabolic situation between sexes. Females tend to obtain energy from fats rather than carbohydrates to develop the same absolute intensity as males. Lactate concentration does not appear to be the main regulator of fat oxidation, as changes between the two are not proportional. Finally, it is recommended to set intensity through heart rate or perceived exertion when thresholds are determined in a different situation from that in which the training is conducted.

Resumen

El lactato tiene tres funciones cruciales: es un sustrato energético, es el principal precursor gluconeogénico y desempeña una función señalizadora en múltiples procesos. Esto hace que medir este metabolito sea útil tanto en salud como en enfermedad. Sin embargo, para interpretar correctamente el lactato, es necesario conocer su transporte en el cuerpo y los factores que lo influyen. Por tanto, el objetivo de esta tesis es estudiar el transporte del lactato a través de sus principales transportadores y evaluar el efecto del sexo, la temperatura y el estado nutricional.

Primero, se realizó una revisión sistemática de la literatura para conocer el efecto del ejercicio en los niveles de proteínas de los transportadores de lactato MCT1 y MCT4 (estudio I). Luego, se llevó a cabo un proyecto con participantes con genotipos TT y AA del SNP T1470A del gen MCT1, quienes realizaron un protocolo interválico mientras se les extraían muestras de sangre para medir las concentraciones de lactato plasmáticas y eritrocitarias (estudio II). Además, hombres y mujeres realizaron un protocolo incremental para determinar la potencia y la frecuencia cardíaca en los umbrales de lactato, evaluando diferencias entre sexos (estudio III). En otro proyecto, hombres y mujeres llevaron a cabo un protocolo incremental en tres situaciones (control, calor y depleción de glucógeno) para observar diferencias en la oxidación de carbohidratos y grasas y en la concentración de lactato (estudio IV). Además, se comparó la correlación entre la oxidación de grasas y la concentración de lactato en estas situaciones y si los cambios en ambas variables eran proporcionales (estudio V). Finalmente, se evaluó la frecuencia cardíaca, la potencia y el esfuerzo percibido en los umbrales ventilatorios para determinar qué variable permanecía más estable entre las tres situaciones (estudio VI).

Los resultados del estudio I mostraron que los niveles de MCT1 aumentaban tanto con una sola sesión de ejercicio como con un programa de entrenamiento. El MCT4 aumentó tras un programa de entrenamiento, aunque su respuesta era menor que la del MCT1. En el estudio II, se observó que la concentración de lactato en plasma y eritrocitos es similar independientemente del genotipo del SNP T1470A. El estudio III reveló que las mujeres tienen los umbrales de lactato a un mayor porcentaje de la potencia pico y de la frecuencia cardíaca máxima que los hombres. En el estudio IV, se encontró que la oxidación de grasas es mayor en mujeres que en hombres durante un protocolo incremental, independientemente de la situación.

El estudio V mostró una relación estrecha entre la oxidación de grasas y la concentración de lactato en las tres situaciones. Sin embargo, el mayor cambio en la concentración de lactato se observó entre la situación control y la de calor, mientras que el mayor cambio en la oxidación de grasas se observó entre la situación control y la de depleción de glucógeno. El estudio VI reveló que la frecuencia cardíaca y el esfuerzo percibido en los umbrales ventilatorios eran similares en todas las situaciones, mientras que la potencia en los umbrales variaba entre situaciones.

En conclusión, el ejercicio parece ser una estrategia eficaz para aumentar los niveles de MCT1 y MCT4. La presencia del SNP T1470A no altera el transporte de lactato entre plasma y eritrocito. Establecer la intensidad como un porcentaje de la potencia pico o la frecuencia cardíaca máxima puede crear una situación metabólica diferente entre sexos. Las mujeres tienden a obtener energía de las grasas en lugar de los carbohidratos para la misma intensidad absoluta que los hombres. La concentración de lactato no parece ser el principal regulador de la oxidación de grasas, ya que los cambios entre ambos no son proporcionales. Finalmente, se recomienda establecer la intensidad a través de la frecuencia cardíaca o el esfuerzo percibido cuando los umbrales se determinen en una situación diferente a la del entrenamiento.

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Abbreviations and Acronyms

UPM	Universidad Politécnica de Madrid
%HR _{max}	Percentage of maximal heart rate
%VO _{2max}	Percentage of maximal oxygen consumption
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BMI	Body mass index
CHO	Carbohydrate
CHox	Carbohydrate oxidation
CHox/FFM	Carbohydrate oxidation relative to fat free mass
CI	Confidence interval
CPT1	Carnitine Palmitoyl Transferase 1
D	Days
DXA	Dual-energy X-ray absorptiometry
EE/FFM	Energy expenditure normalized to fat free mass
Fatmax	Intensity corresponding to maximal fat oxidation
Fatmin	Intensity corresponding to minimal fat oxidation
FATox	Fat oxidation
FATox/FFM	Fat oxidation relative to fat free mass
FFM	Fat free mass
GLUT	Glucose transporter
GPR81	Orphan G protein-coupled receptor
HIE	High Intensity Exercise
HR	Heart rate
ICC	Intraclass correlation coefficient
IPAQ	international physical activity questionnaire
LMIE	Low/Moderate-intensity exercise

LT1	Lactate threshold 1
LT2	Lactate threshold 2
MCT	Monocarboxylate transporter
MCT1	Monocarboxylate transporter 1
MCT4	Monocarboxylate transporter 4
MFO/FFM	Maximal fat oxidation normalized to fat-free mass
MLSS	Maximal lactate steady state
MLSSc	Lactate concentration at maximal lactate steady state
MLSSw	Power output at maximal lactate steady state
MPC	Mitochondrial pyruvate carrier
mRNA	Messenger ribonucleic acid
N.S.	Not specified
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide + hidrogen
OBLA 4	Intensity at a blood lactate concentration of 4 mmol/L
PAR-Q	Physical Activity Readiness Questionnaire
PO	Power output
PO/FFM	Power output relative to fat free mass
PRISMA	Preferred Reported Items for Systematic Reviews and Meta-Analysis
PROSPERO	International Prospective Register of Systematic Review
PYGM	phosphorylase gene
RPE	Rate of perceived exertion
S/WKS	Sessions per week
SD	Standard deviation
SEE	Standard error of estimate
SLC16A1	Solute carrier 16A1
SLC16A3	Solute carrier 16A3
SNP	Single-nucleotide polymorphism
VCO ₂	Carbon dioxide production
VE	Ventilation
VLamax	Maximal lactate accumulation rate

VO ₂	Oxygen consumption
VO ₂ max	Maximal oxygen consumption
VPE	Voluntary physical exercise
VT1	Ventilatory threshold 1
VT1	Ventilatory threshold 2
WK	Week
η ² p	Partial eta-squared

1. Introduction

One of the substrates used by humans to obtain energy is carbohydrates, specifically glucose. This molecule can circulate freely throughout the body via the bloodstream, and cells can uptake it through transporters called GLUT (in the case of muscle fibers, they are called GLUT4), either for storage or oxidation (1). Glucose, being a monosaccharide, is stored in the human body as glycogen, a polysaccharide. Therefore, glycogen is the union of several glucose molecules through the process called glycogenesis, which is catalyzed by the enzyme glycogen synthase (2). This union is reversible, with a glucose molecule being able to detach from glycogen, facilitated by the enzyme glycogen phosphorylase in the process called glycogenolysis (2). Whether taken from the bloodstream as free glucose or derived from glycogen breakdown, the cell can extract energy from this molecule through its oxidation (3). The metabolic pathway by which a glucose molecule is degraded is known as glycolysis or the Embden-Meyerhof pathway (3). This pathway involves the degradation of one glucose molecule (6 carbons) into two molecules of lactate (3 carbons), resulting in 4 ATP and 2 NADH. However, if pyruvate converts to Acetyl CoA (inside the mitochondria) instead of lactate (in the cytoplasm), the balance will be 2 ATP and 4 NADH (2). Figure 1 illustrates the glycolysis pathway.

Early discoveries about lactate concluded that it was produced only under conditions of oxygen absence (anaerobiosis) and returned to resting levels when oxygen levels were restored, implying lactate production due to oxygen lack. Hence, glycolysis was determined to be an anaerobic pathway (4,5). In subsequent years, this simple and intuitive deduction that lactate was caused by hypoxia was established (4). However, it was later discovered that this reaction occurs even in the presence of sufficient intracellular oxygen (2-3 Torr), with ≈ 0.5 Torr being the minimum intracellular oxygen pressure for maximum mitochondrial functioning (6,7). No correlation has been found between intracellular oxygen pressure and lactate concentration (7). It is now known that lactate is always the final product

of glycolysis for two main reasons (5): a) the equilibrium constant between lactate and pyruvate greatly favours lactate production ($1,62 \times 10^{11} \text{ M}^{-1}$) and b) the lactate/pyruvate ratio at rest in skeletal muscle cells is 10:1, meaning the amount of lactate is ten times higher than that of pyruvate.

Lactate formed in the cytoplasm as the final result of glycolysis can produce energy through oxidative phosphorylation (Krebs cycle and electron transport chain), similar to pyruvate (8–12). However, when glycolysis accelerates, leading to increased lactate production, there comes a threshold where mitochondria cannot oxidize all the lactate being produced. As a consequence of this imbalance between glycolysis acceleration and oxidative phosphorylation acceleration, there is an increase in lactate concentration inside the cell (13).

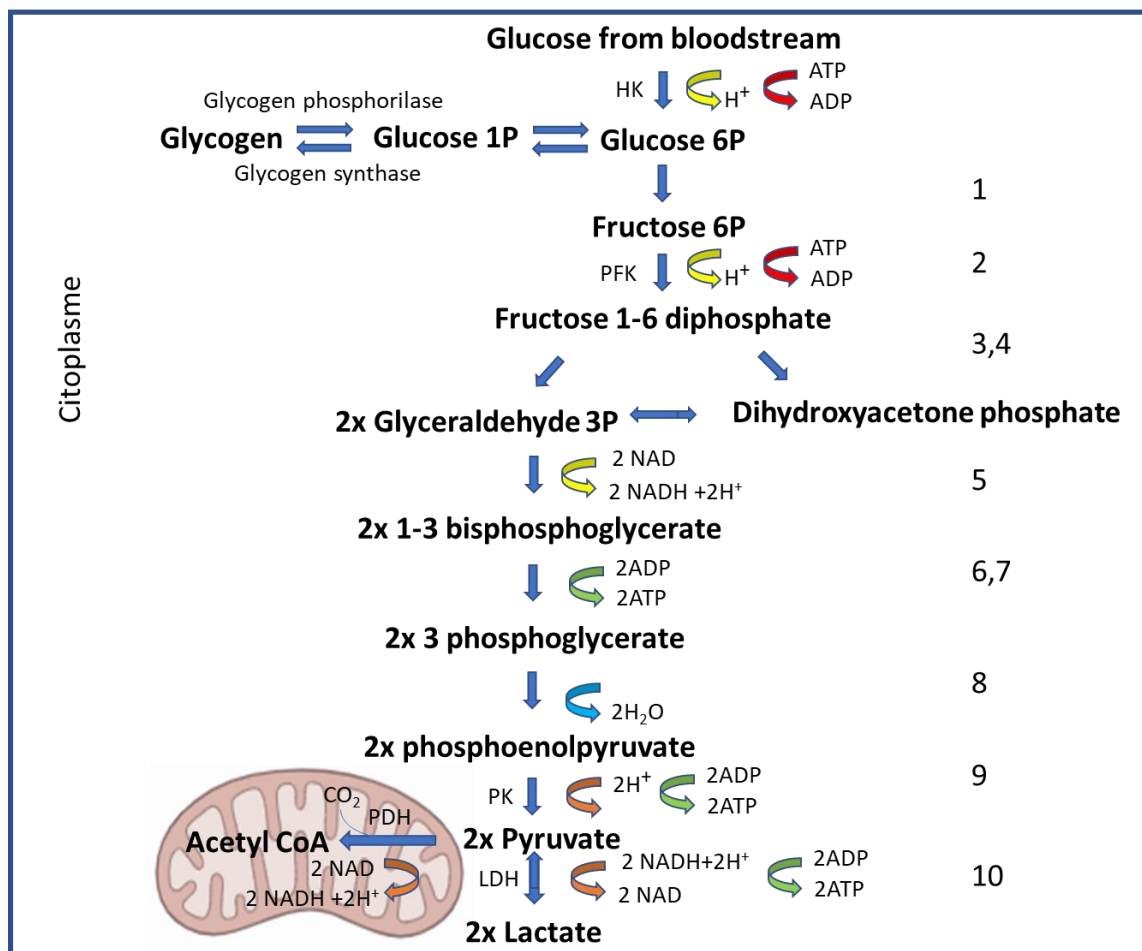


Figure 1 Glycolysis pathway (modified from (Boron & Boulpaep, 2012))

Factors influencing blood lactate concentration during exercise.

Blood lactate concentration is one of the most often measured parameters during clinical exercise testing as well as during performance assessment of athletes (14). Its frequent measurement is due to the valuable information provided by this metabolite, fulfilling three crucial functions in the body: 1) a major energy source, 2) a gluconeogenic precursor and 3) a signalling molecule (15). These functions are possible thanks to the proper transport of lactate through the cells membranes by the MonoCarboxylate Transporters (MCTs) (16). MCTs cotransport monocarboxylates (such as lactate) coupled with a proton in a 1:1 ratio. This cotransport of lactate and protons is passive and bidirectional, requiring a gradient difference between two compartments separated by a membrane (17). According to the lactate shuttle theory (15), during exercise, lactate is released from the producing cells (mainly active muscles cells) to the surroundings cells and circulation. In the blood, lactate is distributed between plasma and erythrocytes and it is transported through the whole body (18). Once in the circulation, it is taken up by the consuming cells (less active muscles cells, adipose tissue, heart, brain, liver or kidney). That means lactate is highly interchangeable between the producer and the consumer cells (Figure 2) (19). Therefore, it is important to have in mind when we are measuring lactate that the concentration of lactate in the blood is the balance between the production and the consumption (19).

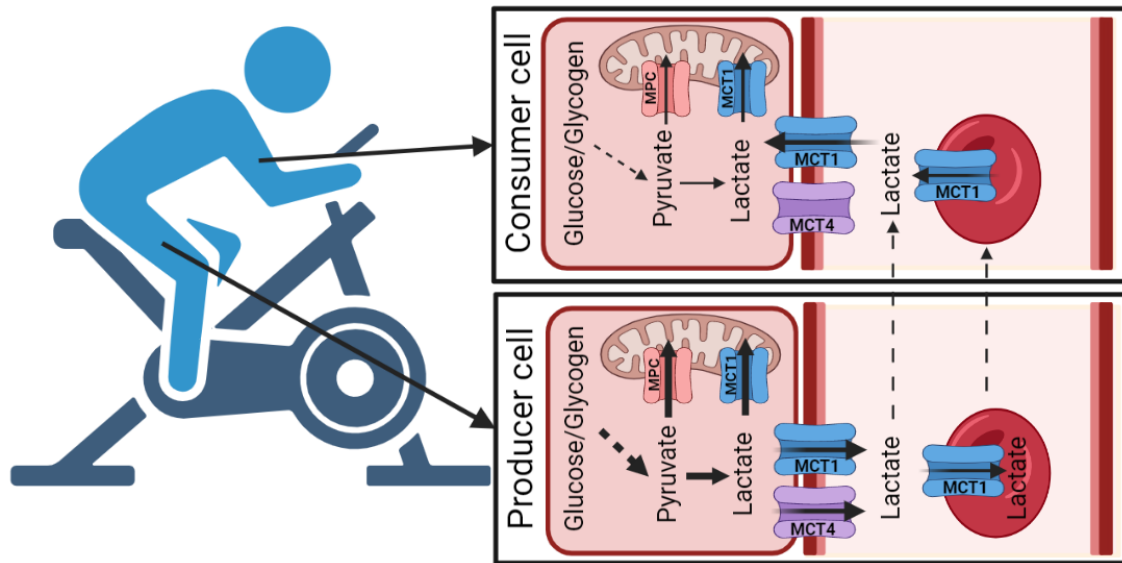


Figure 2 Representation of the lactate shuttle theory. The width of the arrow represents the relevance of a specific process. MPC: mitochondrial pyruvate carrier; MCT1 and 4: monocarboxylate transporter 1 and 4, respectively.

Literature combined with our experience investigating lactate metabolism have showed that several factors modify lactate concentration. It is crucial to be aware of these factors to prevent misinterpretation.

Methodological factors

Those factors independent of the person were classified as methodological factors. These factors can be controlled independently of the individual.

Exercise intensity

Exercise intensity is one of the most famous aspects affecting lactate concentration. During an incremental test, blood lactate concentration remains stable during the first minutes. However, a slow initial increment in blood lactate concentration occurs once a certain intensity is reached. As intensity continues to increase, it is produced a second exponential increase in lactate concentration. The first and the second breakpoints in blood lactate concentration plotted against intensity are called lactate threshold 1 (LT1) and 2 (LT2), respectively (14,20). Below LT1, mainly type I fibres are recruited. These fibres are primarily oxidative, so lactate production is minimal (21). Above LT1, type IIa fibres begin to be recruited, which have an intermediate oxidative-glycolytic metabolism increasing

lactate production inside the cell (21). Lactate starts to increase in the cytoplasm because the production of lactate in the glycolysis is faster than the consumption by the mitochondria. As lactate accumulates in the cytoplasm, it is released to the blood by MCTs resulting in the increment of lactate in the blood causing the LT1 (22). Finally, when the intensity is very high, type IIx fibres are recruited, which are mainly glycolytic increasing lactate production considerably inside the cell. Subsequently, lactate will be released from the cell by MCTs increasing blood lactate concentration abruptly causing the LT2 (21). It is important to note that once lactate is released into the blood it begins to be taken up by the consuming tissues through MCTs modifying the blood lactate concentration. Hence, LTs are indicators of the lactate production/consumption ratio, specially of the active muscle cells (Figure 3). It is noteworthy to say that these increments in lactate concentration are transient rather than sudden, making difficult to establish specific points. Despite its difficulty, different concepts and exercise protocols exist to identify LT1 and LT2, delivering different results (20,23,24). Given the wide variety of concepts and protocols available to calculate LT1 and LT2, we recommend to always use the same methodology when assessing the evolution of a subject or comparing different subjects.

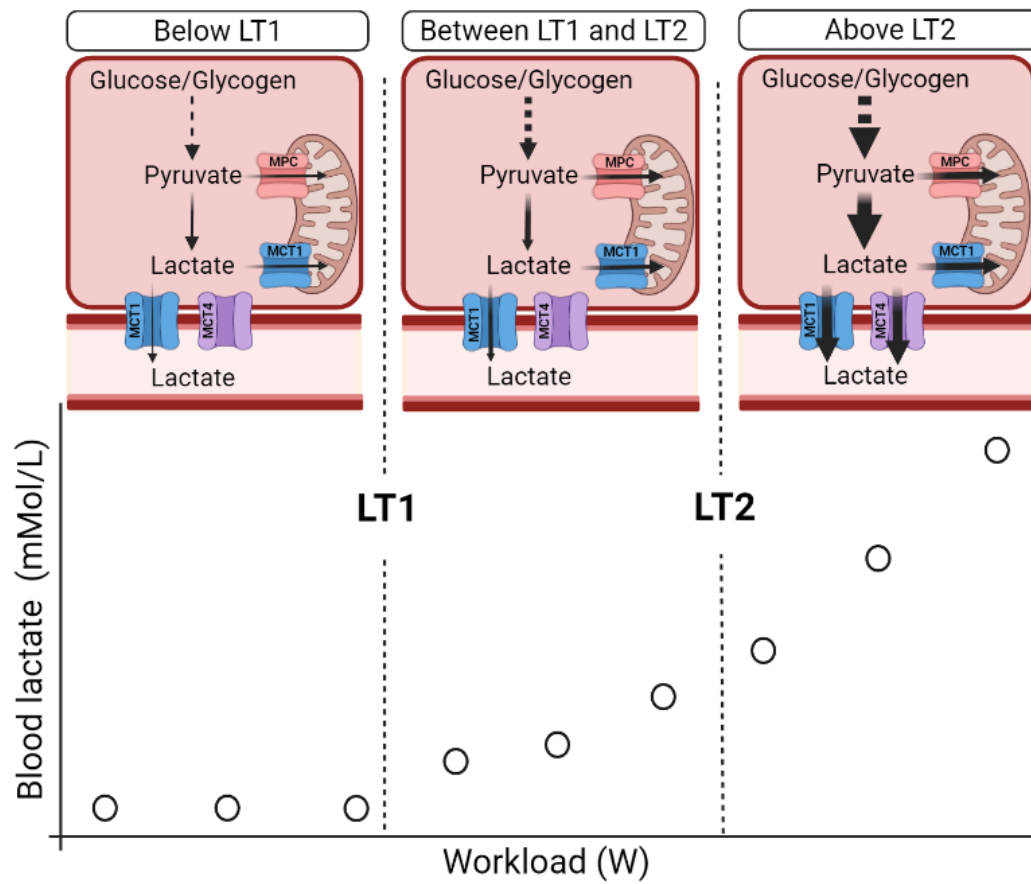


Figure 3 Representative illustration of the lactate threshold 1 (LT1) and 2 (LT2). The width of the arrow represents the relevance of a specific process. MPC: mitochondrial pyruvate carrier; MCT1 and 4: monocarboxylate transporter 1 and 4, respectively.

During constant tests at low intensity, lactate remains stable close to baseline values. The highest intensity during a constant test that can be maintained without an increase in blood lactate concentration above baseline levels coincides with LT1, previously explained. Hence, the first spectrum of intensities is characterized by a basal lactate concentration. If the intensity is higher, it is reached a specific intensity at which lactate concentration increases above resting levels remaining stable during the test. Therefore, the second spectrum of intensities is defined by a stable lactate concentration above baseline values. If intensity is even higher during a constant test, it is reached a specific intensity at which lactate starts to increase during the test despite intensity is constant. Thereby, the third spectrum of intensities is indicated by a gradual increase in lactate concentration despite intensity is constant. The highest intensity during a

constant test that can be maintained with a stable blood lactate concentration above baseline values is defined as the maximal lactate steady state (MLSS) (14,25–28) (Figure 4). In this aspect, it is important to differentiate between the blood lactate concentration (MLSSc) and the workload (MLSSw) obtained at this intensity (25). Several methodologies have been proposed to identify the MLSS (for further review see (20)). A stable blood lactate concentration during a constant test means that the release of lactate by the producer cells is similar to the removal by the lactate consumer cells (28). Since the main destination of blood lactate during exercise is being used as an energy source or gluconeogenesis (9,11,15), a stable lactate concentration would imply most of the energy is coming from oxidation (29). However, if blood lactate starts to increase during a constant test means that the release is higher than the removal (28). A gradual increase in blood lactate concentration during a constant test suggests that energy coming from glycolysis of the type II fibres is becoming more relevant (29), since lactate is the end product of glycolysis (5). Since, MLSS is the intensity at which the release of lactate is similar to the consumption by the whole body (25), MLSS could be used as an indirect marker of the intensity eliciting the maximal whole-body oxidation during a particular exercise without a great contribution of the glycolytic pathway.

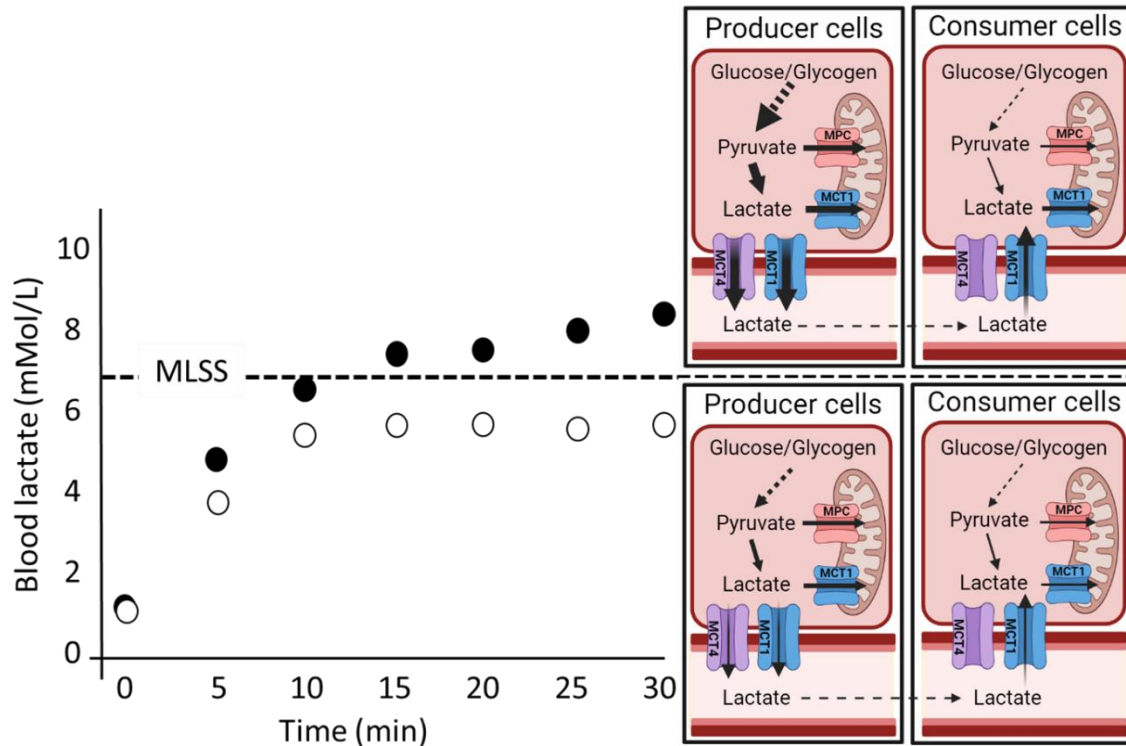


Figure 4 Blood lactate concentration during a constant test performed below (white circles) and above (black circles) maximal lactate steady state (MLSS). The width of the arrow represents the relevance of a specific process. MPC: mitochondrial pyruvate carrier; MCT1 and 4: monocarboxylate transporter 1 and 4, respectively.

During a maximal effort of short duration (~30-120s), it is possible to measure maximal lactate concentration (14). Blood lactate concentration at the end of the exercise test, final lactate, is not the highest. In fact, the highest blood lactate concentration, maximal lactate, is observed a few minutes later (3-8 min) (14). This is because there is a delay between lactate production in the active myocyte and its distribution in the blood through the body. Therefore, it is important to differentiate between final and maximal blood lactate concentration. Maximal lactate accumulation rate (V_{Lmax}) is typically used as an indicator of the glycolytic capacity (30). However, this interpretation should be taken with caution because blood lactate concentration is the balance between the release and the removal, it is not just the result of lactate production (14,15,19).

During a recovery after an exercise intensity above LT1 (blood lactate above baseline levels), a greater amount of lactate is clarified the closer the intensity is

to the LT1 (31), decreasing blood lactate concentration. In fact, the metabolic clearance rate of lactate reaches an apex just below the LT1 (32). Blood lactate concentration decreases faster as the intensity approach to LT1 because active muscles cells uptake lactate to use it as an energy source, considering the other lactate consuming tissues remove the same amount of lactate independently of the intensity of the recovery (33).

Type of blood

Since lactate is allocated through the whole body, it is important to consider the type of blood analysed (venous, capillary or arterial blood). Previous studies have found different lactate concentration comparing the types of blood (34–39). According to the lactate shuttle theory (14,15,19), the different lactate concentration depending on the type of blood is a result of the lactate consumption/production balance. Producer cells will release lactate to capillary blood, moving to venous blood where it will travel until the heart. This type of blood is the most representative of muscle metabolism (36–39). Subsequently, it will make a round journey from the heart to the lungs. Finally, it will be distributed from the heart through the body in arterial blood. Once lactate is released by the producer cells, it can be removed by the consumer cells (40). Therefore, lactate will be lower the more it has circulated through the different tissues because of the removal of the consumer cells. That means that the higher lactate concentration is found in the capillary and venous blood coming from active muscles, and the lower lactate concentration will be found in arterial blood returning to the active muscles (36–38). It is crucial to consider the type of blood analysed and the circulation of lactate from its production until its measurement (Figure 5). Based on that, lactate arterio-venous difference of the active muscles during exercise could be used as an indirect indicator of lactate clearance by the consumers cells.

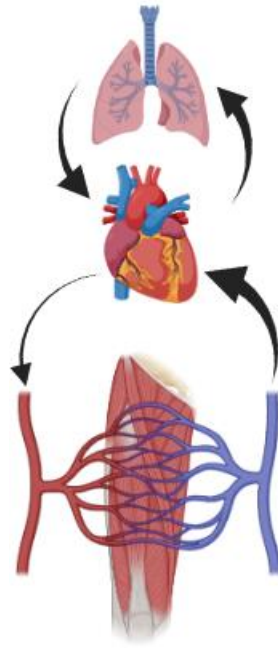


Figure 5 Representative illustration of the lactate circulation through the body. The width of the arrow indicates the expected amount of lactate in the blood.

Blood compartment

It is well known that blood can be divided in two main compartments: plasma and erythrocytes. Lactate can be transported through membrane erythrocyte by three different pathways: 1) free diffusion of the undissociated acid, 2) exchange with inorganic anions (band three system) and 3) MCT1 (41). MCT1 is the specific carrier in erythrocytes, being responsible for >90% of the transport (38).

Red blood cells are purely glycolytic cells as they lack mitochondria (42). Hence, red blood cells cannot use lactate as an energy source, they can only transport it (Figure 2). Despite being glycolytic cells, they have MCT1 which is a bidirectional transporter with a high affinity for lactate specialized for uptake (17). This shows that the exchange of lactate across the erythrocyte membrane is very relevant, especially for two reasons. On the one hand, lactate transport in both plasma and erythrocytes increases the total capacity of the blood to transport lactate. This would allow a higher distribution of lactate through the body to be used as an energy source and to meet its signalling functions. On the other hand, in capillaries surrounding active myocytes, the transport of lactate from plasma to erythrocytes reduce the lactate concentration in plasma. That increases the gradient between

the active myocyte and plasma allowing for a higher release of lactate from the active myocyte (43).

Previous studies performing a test of constant or incremental intensity have observed lower lactate concentration in erythrocytes compared to plasma (35,38,44–48). To our knowledge, only one study performing an intervallic test showed higher lactate levels inside the erythrocyte after two series at the velocity of 95% of maximum running speed (18). The explanation of these apparent contradictory results could reside in the different methodologies of the studies. Presumably, during an incremental or a constant test above LT1, active muscles cells produce and release lactate to the capillary and venous plasma continuously. Once in the plasma, lactate will be taken by erythrocytes. Therefore, if the sample is analysed in the venous blood coming from active muscles, lactate will be higher in plasma compared to erythrocytes. As occurs in those studies finding a higher lactate concentration in plasma than erythrocytes (35,38,44–48). Thereafter, this venous blood coming from active muscles will circulate through the body and consumer cells will remove lactate from plasma reducing lactate concentration in plasma. Consequently, lactate will leave erythrocytes as plasma lactate declines. Then, if the sample is analysed in the blood once has circulated through the body, the difference between plasma and erythrocytes will be reduced (38). Even, lactate concentration can be higher in erythrocytes compared to plasma. This may be found when the consumption of lactate is higher than the production, i.e., during the rests of an intervallic protocol or during a final recovery. As occurs in the study finding a higher lactate concentration in erythrocytes compared to plasma (18).

It is remarkable to take in mind the blood processing by the analyser. Since some of them analyse plasma lactate, while others lyse erythrocytes and measure plasma and erythrocytes lactate together (whole blood). In this sense, if plasma lactate is higher than erythrocyte lactate, lysing erythrocytes will result in a lower lactate concentration measuring whole blood. Conversely, if plasma lactate is lower than inside erythrocyte, lysing erythrocytes will result in a higher lactate concentration measuring whole blood.

Type of exercise

Considering the ability of the non-muscle consuming tissues to clearance lactate is unaffected by the type of exercise, the amount of muscle mass involved during exercise is the predominant source of lactate turnover during exercise (33,49). Accordingly, different MLSSc are obtained in different sports (rowing, cycling and skating) (50). In fact, some authors have concluded that the different MLSSc may correspond to the sport-specific mass of working muscle (50). Aligned with that, the amount of muscle mass involved in a specific exercise plays a major role in blood lactate production, the subsequent release to the blood and its removal (51). Exercise involving a low amount of muscle mass implies fewer lactate producing muscle fibres and a low lactate production. Therefore, there is a considerable amount of muscle fibres able to uptake lactate reducing blood lactate concentration. In contrast, whole-body exercises involve a considerable amount of muscle mass, i.e., a greater lactate producing muscle fibres and a high lactate production. Hence, lactate-consuming muscle fibres able to reduce lactate will decrease. Therefore, skeletal muscle mass involved during exercise should be considered when measuring blood lactate concentration. Concretely, when comparing different types of exercise, participants with different body composition or the evolution of a person suffering changes in body composition.

Blood flow restriction

It is very frequent to measure blood pressure during exercise testing. Blood pressure measurement implies to occlude blood flow of the brachial artery causing local hypoxia in the specific arm. Since hypoxia increase blood lactate concentration (see *hypoxia* section), the local hypoxia produced in the arm by blood pressure measurement will increase lactate concentration in this arm. Additionally, blood flow restriction prevents the distribution of venous blood from the arm to the body (52). This would reduce lactate removal increasing lactate concentration. These two aspects are especially important if the blood pressure measurement is very long. Therefore, it is highly recommended to prevent measuring blood lactate concentration in the same arm as blood pressure.

Another common strategy causing blood flow restriction is the use of blood tourniquet during blood extraction. As occurs with blood pressure measurement,

prolonged use of the tourniquet may reduce arterial blood flow producing local hypoxia in the arm increasing lactate production, and prevent distribution of venous blood from the arm to the body reducing lactate removal (52). This will increase lactate concentration inevitably. This should be especially considered for blood extraction with difficulties whose measurement takes longer than usual.

Amount of blood

Finally, we would like to mention an often overlooked and under-researched factor, which is the amount of blood. It is common to highlight the benefits of a low amount of blood, such as faster or easier sample collection. However, a low amount of blood presents some drawbacks too. In our experience, we have noted that those analysers using a low amount of blood are more easily contaminated, likely with sweat. When we analyse a blood sample with a determined amount of a contaminant agent (e.g., sweat), the lower the amount of blood the higher the impact of the contaminant agent. Unfortunately, to the best of our knowledge, there is no study investigating the impact of the amount of blood on lactate concentration, what prevents to recommend a specific amount of blood. This leads us to suggest the amount of blood should be investigated to continue improving blood lactate concentration measurements.

Biological factors

Modifiable factors

Those factors inherent to the individual were classified as biological factors. These factors are totally dependent on the individual. This does not mean these factors cannot be controlled methodologically.

Training status

It is well known the effect of training on blood lactate concentration are 1) a reduction in blood lactate concentration at a given external work (49,53,54), or 2) a higher external work at a given blood lactate concentration (53). This is because endurance training decreases whole body and working-muscle lactate production and increases clearance by active muscle (49). The effect of training on blood

lactate concentration is commonly observed during an incremental exercise test. Hence, the predictable effect of training on blood lactate is a rightward shift on the lactate-load relationship (54,55). This is manifested as a higher workload achieved at LT1 and LT2 (55). In line with that, a huge difference has been found on blood lactate concentration between participants with different training status, observing lower lactate concentrations the higher the training status at the same absolute workloads (56). It is hypothesized this different lactate response across populations with widely ranging metabolic capabilities are mainly due to their different mitochondrial characteristics (56,57). During exercise, lactate produced in the glycolysis is oxidized in mitochondria as it is an important mitochondrial substrate. Thus, a premature increase in blood lactate during exercise would indicate a poor ability of the mitochondria to oxidise lactate (57) (Figure 6). Subjects with the highest blood lactate concentration before training present the most marked reduction during the physical training period (54). This suggests that the lower the training status of the participants, the higher the effectiveness of training reducing blood lactate.

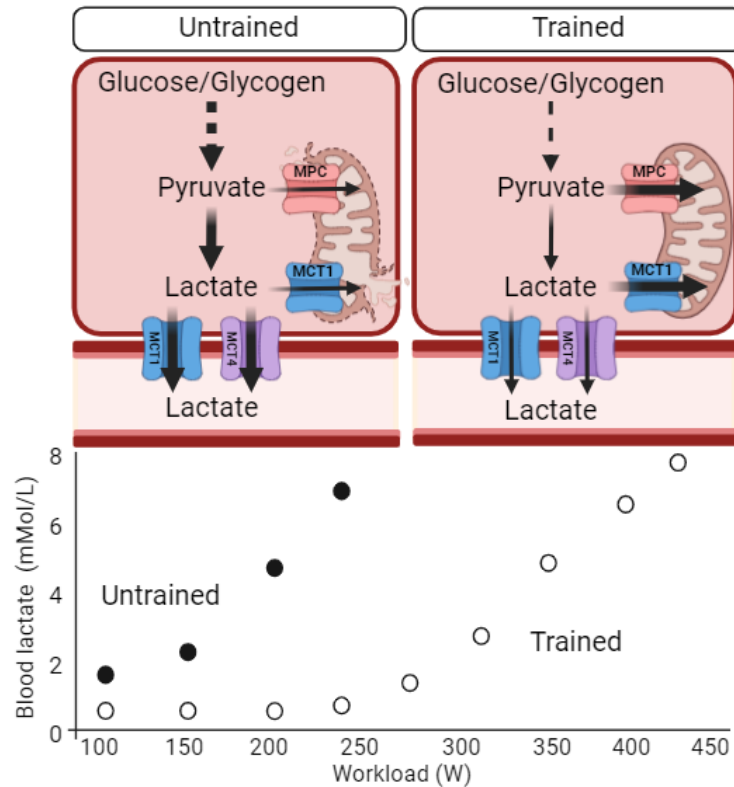


Figure 6 Blood lactate concentration difference between a trained and untrained person.

The width of the arrow represents the relevance of a specific process. MPC: mitochondrial pyruvate carrier; MCT1 and 4: monocarboxylate transporter 1 and 4, respectively.

The effect of training is usually evaluated through constant tests determining MLSS as well. The goal is to achieve a stable blood lactate concentration above baseline levels at a higher workload (i.e. a higher MLSSw) (25,58). This is desirable due to the relationship between MLSSw and performance (25). A higher MLSSw would imply an enhance on intensity eliciting the maximal whole-body oxidation during a particular exercise without a great contribution of the glycolytic pathway. In contrast, MLSSc present a high inter-individual variability not being changed by training or related to performance (25,58,59).

Regarding maximal lactate concentration, it appears that training increases this value (54,60). The main reason could reside in the higher glycolytic flux observed with training allowing to achieve a higher workload (60). In accordance, VLamax is positive correlated to performance (60).

Nutrition

It is reasonable to assume that as one of the sources of lactate is muscle glycogen (5), the lower the level of muscle glycogen the lower the production of lactate. Several studies have modified the previous glycogen levels combining diet and exercise showing lower lactate concentrations with low glycogen levels during an incremental test (61–67), during a constant test (68,69) and during recovery after a constant test (70). The maximal lactate concentration has also been reduced after an incremental test with low glycogen levels (65–67). These consequences just mentioned caused by a low glycogen level state on lactate response could be wrongly understood as an improvement in performance because training produce similar adaptations (20,28). The mainly response differing between a low glycogen level state and an adaptation to training is the maximal lactate production because this parameter increases with training (54,60) and decreases with low glycogen levels (65–67). Therefore, it is highly recommended to measure the maximal lactate concentration while testing to differentiate between the adaptations to training and a low glycogen level situation.

However, not all studies have found different lactate response with different glycogen levels. In the study of Hargreaves (71), subjects performed a 75s all out exercise in two different conditions: after exercise and a high carbohydrate diet (80% carbohydrates) or after exercise and a low carbohydrate diet (25% carbohydrates). Maximal lactate did not differ between conditions despite of muscle glycogen content was reduced after exercise and a low carbohydrate diet. However, net glycogen utilisation was not significantly different between conditions. This could be the reason why maximal lactate concentration did not differ between conditions. Mikulski et al (72) exposed the subjects to four incremental test on different visits. On the first visit, it was performed the control incremental test. In the following three visits, in the previous day, it was performed a test of 90 min at 70% maximal heart rate to reduce muscle glycogen content. 2h before the subsequent three incremental tests subjects were given a high-carbohydrate meal (4% proteins, 1% fat, 95% carbohydrates), a low-carbohydrate meal (35% proteins, 64% fat, 1% carbohydrates) or remained fasted,

respectively. No differences in lactate concentration during the tests and LT were found. Probably, performing 90min at 70% maximal heart rate the previous day and a meal before the test is not enough to reduce muscle glycogen content and affect lactate concentration. This is because exercise protocol designed to reduced muscle glycogen include high intensity interval exercise in addition to low moderate intensity exercise (73).

Studies trying to reduce glycogen levels with diet exclusively have opposites results. In the study by Quirion et al. (74), the following protocol was administered: 48-h period of mixed dieting (53% carbohydrates, 30% lipids, 17% proteins) preceding the first incremental test, immediately followed by a 48-h period of either a high carbohydrate diet (68% carbohydrates, 23% lipids, 9% proteins) or a high fat diet (19% carbohydrates, 57% lipids, 26% proteins) leading to the second incremental test and separated from the third incremental test by a 12-days period. The results showed that these dietary modifications had no significant effect on lactate response. Most likely, a dietary alteration during 48h without previous exercise is not enough to affect glycogen levels and the consequent lactate production. To our knowledge, in the only previous study (75) that has used a similar diet without exercise to reduce glycogen levels, the diet was modified for 4 days finding differences in the lactate response. In this study (75), subjects were exposed to three different diets: a mixed diet for 3 days (50% carbohydrates, 30% fat, and 20% protein), a low carbohydrates diet for 4 days (30% carbohydrates, 50% fat and 20% protein) and a high carbohydrates diet for 3 days (70% carbohydrates, 20% fat, and 10% protein). The results revealed that absolute and relative VO_2 decreased at 4 mmol/L of blood lactate after a high carbohydrate diet, indicating a leftward shift of the lactate- VO_2 relationship in this situation. It would be interesting to know which nutrition protocol can reduce glycogen levels by itself, with the intention of avoiding such an intense exercise session and the consequent fatigue as conducted in other studies.

It has been found a close relationship between adrenaline (66) or noradrenaline (67) and lactate concentration and between adrenaline threshold and LT (66). Catecholamines binds to β -adrenergic receptors on the skeletal muscle membrane

initiating a cascade of events leading to glycogen breakdown which ultimately increases lactate production (66). Lower catecholamines concentration and the subsequent lower lactate concentration was found with low glycogen levels (66), suggesting that the glycogen levels could have an impact on catecholamines response. Gollnick et al. (73) were one step further when their subjects performed two-legged cycle ergometer exercise in which one leg was glycogen depleted and the another one was normal. Glycogen depletion of one leg was produced by one-legged exercise on a previous day followed by the consumption of a low carbohydrate diet. The results of this experiment showed that the low glycogen leg extracted lactate from the blood and the normal leg released lactate to the blood. That means that lactate transport through the membrane cell by the MCTs is dependent of glycogen levels. These results were observed having the same catecholamines concentration in both legs suggesting that glycogen levels per se have a greater impact on lactate concentration than catecholamines.

Hypoxia

The most common intervention to study the effects of hypoxia is altitude. In this aspect, it is important to differentiate between an acute and a chronic response due to acclimation. In the first hours in hypoxia, a leftward shift in the lactate-load relationship (76–80) and in the lactate-oxygen consumption relationship (7,81) has been observed without changes in the maximal lactate concentration (7,79,82,83). With acclimation, it is produced a re-establishment of the lactate-load relationship (76,77,84) and in the lactate-oxygen consumption relationship (81) approaching normoxia levels with a concomitant decrease in maximal lactate concentration (82–86). In acclimatized subjects, an inverse relationship between hypoxia and maximal lactate concentration has been reported (87). It is noteworthy to say that the reduced maximal lactate concentration due to acclimation to hypoxia is not observed in phosphagen dependent efforts like high intensity exercise with a short duration (≈ 10 s), while it is observed in more glycolysis-dependent efforts like high intensity exercise with a longer duration (≈ 30 s) and in more oxidative phosphorylation-dependent efforts like incremental exercise test (86). This occurs

because the contribution of the glycolysis in short duration efforts (≈ 10 s) is less relevant.

However, not all studies have found an acclimation of lactate to hypoxia showing that the leftward shift in the lactate-load relationship persists with acclimation (78,88) and the concentration of lactate during a constant test is still elevated in chronic hypoxia, similar to acute hypoxia, compared to normoxia (89,90). It is important to emphasize that in these studies not showing an acclimation of lactate to hypoxia it was produced a decrease in body weight because the diet was not controlled. This reduction in body weight is probably accompanied by a reduction in muscle mass. As mentioned in the *type of exercise* section, the amount of muscle mass is a methodological aspect to consider. Since muscle mass is a major consumer of lactate (37), this reduction in the weight could have reduced muscle mass diminishing the uptake of lactate. The reduced lactate removal would counteract the expected rightward shift of the lactate-load relationship with acclimation to hypoxia. Another explanation could be that the relative intensity of each muscle fibre is higher if there is a loss of muscle mass because a less amount of muscle fibres would be developing the same absolute work. This would produce more lactate due to the close lactate-load relationship (76), preventing the rightward shift of the lactate-load relationship expected with chronic altitude. However, it cannot be overlooked that one study found the rightward shift of the lactate-load relationship with chronic altitude despite reduction in body weight (84), suggesting it is possible to find adaptations to hypoxia despite weight loss. On the other hand, regarding maximal lactate concentration, some contradictory results can be found in the literature too. A study have shown that it increases with acclimation returning to normoxia values (83), others do not find differences between acute or chronic hypoxia and normoxia (78,88) and another reports an increase with acute hypoxia compared to normoxia (91).

Some studies have observed that total oxygen consumption (36,80,81,84,89,90,92–98), arterial oxygen delivery to the legs (7,81,95) and leg oxygen uptake (7,36,90) do not differ between acute, chronic altitude exposure or sea level at the same absolute submaximal intensity. This phenomenon gives rise to the lactate paradox.

It was considered paradoxical as the changes in lactate with acclimation to hypoxia occurs despite the finding that oxygen response does not differ between hypoxic conditions. However, it is currently known that lactate production is independent of intracellular oxygen (7), which means that the lactate paradox does not have to be paradoxical. Although, some studies have observed a lower oxygen consumption in hypoxic conditions (7,91,92).

Since the concentration of lactate depends on removal and production, it is important to know which of the two factors are affected by hypoxia. Whole body lactate oxidation and lactate oxidation by the legs is increased with chronic hypoxia compared to normoxia (90). Also, metabolic clearance rate of lactate is enhanced in acute and chronic hypoxia compared to normoxia, being higher in the former (96). Moreover, chronic hypoxia increases MCT1 content in erythrocytes enhancing the transport capacity of lactate by erythrocytes (43). This improves the distribution of lactate through the body increasing lactate uptake by the consumer cells. All these results suggest that lactate removal is improved with hypoxia, most notably chronic hypoxia. On the other hand, lactate released by the legs (7,36,78,88,92,94), rate of appearance lactate (94,96) and muscle lactate (92) is higher in acute hypoxia compared to normoxia what suggests that the production of lactate increases with acute hypoxia. Regarding chronic hypoxia, some studies have shown a lactate released by the legs (92,94), rate of appearance lactate (94,96) and muscle lactate (92) close to normoxia. This indicates that the production of lactate approaches normoxia values with chronic hypoxia. On the contrary, some studies have not observed this acclimation in the production of lactate with chronic hypoxia (78,88). These are the same studies mentioned above that did not observed a re-establishment of the lactate-load relationship with acclimation. As previously explained, a considerable weight loss was produced in these studies. A body weight reduction could imply a loss of muscle mass increasing the relative intensity of each muscle fibre if the same absolute intensity is performed. This higher relative intensity of the muscles would produce more lactate due to the close relationship between lactate and intensity (76).

Some studies have tried to investigate the mechanisms behind the lactate kinetics in hypoxia. In an incremental maximal test breathing oxygen-enriched gas it was reversed the typical leftward shift observed in the lactate-load relationship in acute altitude (77,80,81,84,88). That suggests that the lack of oxygen is the responsible of the leftward shift in the lactate-load relationship at acute altitude. With acclimation to altitude, the lactate-load relationship was very similar between chronic altitude, altitude breathing oxygen-enriched gas and sea level (77,84). This indicates that adaptations to chronic altitude have reversed the effects of the lack of oxygen on the lactate-load relationship at acute altitude. On the contrary, maximal lactate was similar between breathing oxygen-enriched gas at altitude and one, three or five weeks at altitude, all being lower than at sea level (84). This reduction in the maximal lactate at altitude was not counteracted breathing oxygen-enriched gas. Therefore, maximal lactate does not seem to be oxygen-dependant. Another possibility explaining the effect of altitude on lactate response is the different workload achieved in each situation. However, performing an incremental maximal test after one and five weeks at altitude breathing oxygen-enriched gas increased peak power output compared to acute altitude without changes in maximal lactate (84). This suggests that the peak workload is not responsible for the decrease in maximal lactate.

Hypoxia has been shown to increase adrenal epinephrine existing an inverse relationship between arterial oxygen saturation and epinephrine concentration (98). During an incremental test in normoxia and acute hypoxia it has been observed a close relationship between adrenaline (7,77,91) or noradrenaline (77,91) and lactate concentration. Also, this relationship between adrenaline or noradrenaline and lactate concentration has been observed in chronic hypoxia (77). Pooling acute and chronic hypoxia values of adrenaline, it is also found a close relationship between adrenaline and lactate concentration. Regarding adrenaline at acute altitude, most of the studies have observed an increased concentration of adrenaline (7,36,78,89,90,96–98), but not all (88,91). At chronic altitude, some studies have showed a diminished adrenaline concentration compared with acute altitude (89,96–98), while others have observed an increment (78,88). Regarding

noradrenaline, at acute altitude, most studies have observed an increased concentration (78,89–91,97,99), but others have not (36,88,96). With chronic altitude, an increased (78,88,96,97,99) or a similar (89) noradrenaline concentration has been found compared to acute altitude, with no studies showing a reduction. It has been suggested that during acute altitude, epinephrine would produce a higher activation of the glycolysis rising lactate production. The return of adrenaline to sea level values with chronic hypoxia would explain the reduction in lactate production (97). Moreover, studies with β -blocked subjects preventing the rise in epinephrine have shown a decrease on lactate concentration at sea level, in acute altitude and in chronic altitude compared to a control group (98). However, β -blockade did not completely prevent the rise in blood lactate on acute altitude suggesting additional mechanisms are involved in this process (98).

Temperature

Exercise in the heat in unacclimated individuals results in a greater reliance on carbohydrate metabolism (100,101). Since lactate is the end product of glycolysis (5), the greater reliance on carbohydrates metabolism in a hot environment will produce an increase in lactate production and a subsequent higher blood lactate concentration (100–108). In addition, it has been observed a relationship between muscle temperature and muscle lactate (109). Furthermore, heat acclimation with the intention to decrease the rise in temperature in hot conditions has been effective to reduce muscle lactate concentration (106,110,111). Also, using cooling strategies during exercise in a thermoneutral environment (109) decreases lactate concentration by preventing the rise in temperature.

Different mechanism could explain the increased lactate production during heat exercise. Catecholamines seems to play a key role in this mechanism. Heat increases circulating epinephrine enhancing the glycolytic rate rising lactate production (100,112,113). It has been observed that epinephrine infusion at 20°C to similar levels observed during exercise at 40°C increases glycogen use and the subsequent lactate production without changes in muscle temperature compared to control (114). Furthermore, heat acclimation decreases epinephrine levels reducing lactate concentration (100). All these studies demonstrate the close

relationship between lactate and catecholamines in the heat. On the other hand, it has been suggested that the activity of the key enzymes involved in carbohydrate metabolism, such as glycogen phosphorylase, phosphofructokinase and pyruvate dehydrogenase, are affected by temperature *per se* via a Q_{10} effect (106,115). Therefore, it could be that the mentioned enzymes could increase their activity in heat condition rising lactate production and the subsequent blood lactate concentration.

Regarding cold situations, in the study by No et al., participants cycled for 20 minutes at 60% VO_{2max} . After resting for 10 minutes, exercise intensity was increased at a rate of 0.5 kp/2 min until exhaustion in three different environmental conditions (10 °C, 22°C and 35°C). Blood lactate concentrations were lower at 22°C than at 10°C or 35°C, at rest and during 20 min at 60% VO_{2max} suggesting cold and heat increase blood lactate concentration. Contrary, blood lactate concentration was higher at 22°C during maximal exercise, likely due to the higher time to exhaustion in this situation (105). In line with that, it has been reported a higher lactate concentration during a constant test at 70% of the VO_{2max} after immersing the legs for 45 min in a stirred water bath at 12°C compared to control (116). In agreement, another study (117) showed that whole body skin surface cooling wearing a water-perfusion suit (water at 10°C) during 1h before and during an incremental exercise test at an ambient temperature of 10°C increases lactate accumulation. Contrary, this effect was not observed at an ambient temperature of 25°C. This might be because only prior cooling combined with a low ambient temperature was effective reducing skin temperature raising noradrenaline before exercise, what increases lactate production during the subsequent exercise (117).

A possible explanation for the higher lactate concentration at low temperatures could reside on the effect of cold on muscle fibre recruitment pattern. Studies in animals have showed a greater recruitment of fast twitch fibres at low temperature (118,119). In line with that, studies in humans have shown a higher amplitude of electromyography after cold water immersion (120). This suggests that a decrease in temperature could reduce the conduction velocity of the nerve and the muscle

fibres. This could be compensated with an increase in muscle fibres recruitment (120). Since fast twitch fibres are more glycolytic, the increased recruitment of these fibres in a cold condition could increase lactate production.

Contrary, not all studies have observed a higher lactate concentration with cold. It has been observed a lower lactate concentration during an incremental test at -2°C compared to 24°C (121). These results are supported by another study of the same research group showing a lower lactate concentration during an incremental test at 10°C compared to 30°C (122). Finally, a previous study compared the LT during an incremental test in a thermoneutral situation (20°C) with a cold situation (0°C), showing a higher power output at 0°C indicating a rightward shift of the lactate-load relationship with cold (123). Therefore, based on the present studies, apparent contradictory results are found regarding cold and lactate concentration. Likely, due to the different acclimation protocols used before exercise and the broad ranges of ambient temperatures studied.

Hydration

Hydration state has been proposed to influence muscle metabolism and the vast majority of studies have concluded that dehydration increase lactate concentration (113,124–131). It is well known the close relationship between dehydration and temperature. However, most of the studies have not observed the effect of isolated hydration matching temperature (102,113,124–130,132). Therefore, they usually suggest that the elevated muscle temperature is the most plausible mechanism to explain the increased muscle glycogenolysis and the subsequent lactate production during dehydrated exercise (129).

On the contrary, some studies have managed to dissociate the effect of temperature and hydration on lactate concentration. England et al. (131) developed a study where subjects performed an incremental test in a 5% weight loss hypohydration state and in a normal hydration state. Previous hypohydration was induced by sauna exposure and the test was performed when the rectal temperature returned to the pre-sauna baseline value. In both situations, the rectal temperature was the same during the test avoiding the confusing effect of this variable. Lactate

concentrations were higher in the hypohydration situation compared to the normal hydration situation suggesting that dehydration *per se* increase lactate concentration. Conversely, several studies have observed opposite results. González-Alonso et al. compared 30min at 72% $\text{VO}_{2\text{max}}$ in heat (35°C) and cold (8°C) situations with different hydration conditions (euhydration, 1.5%, 3% and 4.2% dehydration). They found similar blood lactate concentration in the cold situations with different hydrations conditions, indicating hydration status *per se* has no effect on lactate concentration. In line with that, in the study by Papadopoulos et al (102), subjects underwent four maximal incremental test in four different conditions: in a hypohydrated conditions ($\approx 2.5\%$ body weight loss with previous exercise) at room temperature ($\approx 22^\circ\text{C}$) and in a warm chamber ($\approx 37^\circ\text{C}$) and in a euhydrated conditions at room temperature ($\approx 22^\circ\text{C}$) and in a warm chamber ($\approx 37^\circ\text{C}$). They found that during the test in the hot condition, the LT occurred at a significantly earlier stage and with a lower oxygen consumption compared to the thermoneutral condition independently of the hydration state. Finally, Armstrong et al (132) compared participants in a euhydrated situation and 5% hypohydration situation (refraining the previous day from any fluid intake and performing a 1h run at 75% $\text{VO}_{2\text{max}}$). They found a similar immediately post lactate concentration between experimental conditions after running 10 min at 70% and 85% $\text{VO}_{2\text{max}}$.

Due to the close relationship between temperature and hydration, the vast majority of studies have not observed the effect of isolated hydration matching temperature. (102,124–130,132). However, those studies trying to isolate the effect of hydration have observed that hydration *per se* seems not to affect blood lactate concentration.

Sleep

Sleep seems to affect many physiological functions in the body. To understand the interaction between sleep and lactate, it is necessary to pay attention to the effect of sleep on the relationship between workload and lactate concentration. During submaximal exercise at the same absolute intensity, a partial sleep loss of $\approx 3\text{h}$ seems to increase lactate concentration compared to a reference night (133,134).

However, blood lactate concentration does not differ between the control situation and partial sleep deprivation condition when a submaximal self-selected intensity is performed (135). Nevertheless, the velocity was lower in the partial sleep deprivation condition, what means that the same blood lactate concentration is found at a lower exercise intensity with partial sleep deprivation. Another study found similar lactate concentration during an intermittent-sprint protocol after 30h sleep deprivation compared to a control night. It is important to highlight that it was observed a lower performance after sleep deprivation, indicating that the same blood lactate concentration was reached at a lower workload after sleep deprivation (136).

Regarding maximal lactate concentration, in the studies by Mejri et al (137) and Romdhani et al (138), it was found that partial sleep deprivation ($\approx 3\text{-}4\text{h}$) reduces maximal lactate concentration in a maximal effort. This reduction in maximal lactate concentration was accompanied by a decline in the maximal workload achieved after partial sleep deprivation (137). Similar results were found by Romdhani et al. (138), who found a lower final lactate concentration associated with a lower performance during a repeated sprint ability test after 4h of partial sleep loss. Also, Keramidas et al. found that maximal lactate concentration was lower following a constant test until exhaustion performed after a military expedition where subjects slept $\approx 5\text{h}$ distributed in 51h compared to before the expedition. This reduction in the lactate concentration could be explained by the reduction in the time to exhaustion observed after expedition compared to before (139). On the other hand, Mougin et al (134) showed that the maximal lactate concentration was higher in a 3h sleep deprived night compared to the control night. Maximal workload was similar between conditions indicating that the lactate concentration was higher in the sleep deprived night at the same workload compared to the control night (134). In another study by Mougin et al (133), maximal lactate concentration did not differ between conditions. However, maximal workload was lower in the partial sleep loss condition compared with the reference night. This indicates that the maximal lactate concentration found during the partial sleep loss condition was produced at a lower workload (133). In

agreement with that, Souissi et al (140) exposed the subjects to four Wingate tests after two different conditions (reference night vs sleep deprivation night). After the reference night, subjects performed the Wingate tests 1h after and 13h after 7h of sleep. After the sleep deprivation night, the Wingate tests was performed after 24h and 36h of sleep deprivation. Lactate concentration was not different in the four different Wingate tests. On the contrary, mean power and peak power was lower after 36h of sleep deprivation, indicating that the same lactate concentration was reached at a lower absolute workload after 36h of sleep deprivation.

On the contrary, not all studies have showed an effect of sleep on lactate response and performance. Goodman et al. (141) found that time to exhaustion during an incremental test and maximal lactate concentration did not differ after 60h of sleep deprivation. Also, Symons et al. (142) found no significant differences in performance and blood lactate concentration in a Wingate test after 60h of sleep deprivation. In line with this, Mougin et al (143) did not observed an effect of ≈ 4 h of sleep restriction on either exercise performance or lactate concentration during a Wingate test. These studies showing no effect of sleep on lactate concentration do not observe changes in performance either. This support the idea that the effect of sleep on lactate response could be mediated by the changes in performance.

As it has been shown, the effect of sleep on lactate concentration seems to be mediated by performance. Thus, it is important to observed changes in performance when evaluating the effect of sleep on lactate concentration.

Circadian rhythms

The circadian rhythms refer to the daily fluctuations in some parameters suffered by the organisms (144). That means the behaviour of different variables is different depending on the moment of the day.

Most of the studies have shown a higher lactate concentration in the evening than in the morning (145–152). This increased lactate concentration in the evening compared to morning is accompanied with a higher temperature (145,146,148–152). As previously explained, the higher temperature reached during evening could affect the higher lactate concentration. In addition, the higher lactate

concentration during evening than morning is coupled with a higher performance (higher peak power output (145,146,148,150), higher time to exhaustion (147,152), higher distance covered (149) or lower time trial performance (151)). In this sense, due to the close relationship between load and lactate concentration, the higher lactate concentration observed during evening could be due to the higher performance at this moment of the day as well.

However, some studies have not shown an effect of time of the day on lactate concentration. In a study (153) performing a 1km cycling time trial it was not observed a different lactate concentration in the morning compared to evening despite finding differences in other variables (such as a lower insulin, cortisol and free and total testosterone and a higher growth hormone and plasma glucose in the evening). In this study, performance was higher in the evening, so the different load between situations could be affecting the results due to the close lactate-load relationship. Something similar was found in another study developing a Wingate test in the evening and in the morning (154), where similar lactate concentration were found. However, a higher performance, temperature and oxygen consumption was found in the evening, all of these variables being closely related to lactate. Therefore, changes in these variables could be confounding the effect of time of day on lactate. Finally, something to consider is the habitual time of training of the participant. For example, a study performing a 15 min time trial (155) found that lactate and performance was similar at different times of the day, although temperature was higher in the evening. It is noteworthy to say that subjects in this study usually trained in the morning. This could modify the effects of the time of the day, since adaptations to training are greater at the time of day at which training is regularly performed (156). The same occurred in a study (157) where subjects usually trained at the same time of the day (3:30-5:30pm), showing a similar lactate concentration between morning, afternoon and early evening after a resistance training session. On the contrary, it was found a time of the day effect for creatine kinase, testosterone and cortisol.

Unmodifiable factors

Sex

Although sex-based differences on substrate oxidation have been extensively studied, lactate concentration has received less attention. No sex differences were found in blood lactate concentration at 40% $\text{VO}_{2\text{max}}$ during 2 h (158). However, exercise intensity was too low to increase lactate concentration above baseline levels considerably and it is not known whether both sexes had similar training status. Accordingly, no sex differences were found in blood lactate concentration during 90 min at 58% $\text{VO}_{2\text{max}}$ matching both sex by training status (similar $\text{VO}_{2\text{max}}$ relative to lean body mass) (159). In contrast, comparing both sexes during an incremental test at the same power outputs, we observed that lactate concentration was higher in females than males. However, final lactate concentration was similar between sexes. Unfortunately, both sexes were not matched by training status. In a following study, we compared lactate concentration during an incremental test at the same power outputs between sexes matching both sex by training status (similar $\text{VO}_{2\text{max}}$ relative to fat free mass). We showed that lactate concentration was similar between sexes at the same power outputs.

Hence, sex does not seem to affect blood lactate concentration when training status is matched between sexes.

Age

Aging affects several physiological functions, being lactate concentration one of them. Importantly, the effect of age on lactate concentration at a specific power output seem to be influenced by training status (160). In this sense, lactate concentrations were similar at different power outputs between highly trained master athletes and young athletes (160). In contrast, lactate concentrations were higher in untrained elderly participants than in young athletes (160). Regarding final lactate concentration, highly trained master athletes and untrained elderly participants showed a lower lactate concentration than young athletes (160). Aligned with that, a curvilinear relationship with an inverted u-shape was found between blood lactate concentration analysed at the end of a sprint events (100, 200 and 400 m) and age (161), indicating age reduces maximal blood lactate concentration specially at advanced ages. In line with that, it was found that

lactate concentration after an incremental test until exhaustion was lower the higher the age (162). Hence, there is compelling evidence that final or maximal lactate concentration is reduced with age.

Genetic

McArdle disease (glycogenosis type V) is an autosomal recessive condition caused by a total inherited deficiency of the muscle isoform of glycogen phosphorylase (myophosphorylase), an enzyme encoded by the phosphorylase (PYGM) gene (163,164). Given that myophosphorylase catalyses the breakdown of glycogen, the absence of glycogen breakdown in these patients results in a very low lactate concentration since glycogen is one of the main precursors of lactate, as mentioned above. This phenomenon is observed in several studies (164–166).

On the other hand, it has been identified the presence of a single-nucleotide polymorphism (SNP) in the gene that codifies MCT1, T1470A (rs1049434), resulting in an aspartic acid to-glutamic acid change in codon 490 (167). This genetic variant results in a deficiency of lactate transport, although no clinical effects have been reported (167). However, this genetic modification could be functionally relevant during exercise at high intensity due to the high gradient difference of lactate between muscles and plasma and between plasma and erythrocytes. There is compelling evidence that the polymorphism T1470A affect lactate concentration (33,168–172), due to the lower lactate transport through the MCT1 with the T allele. Although this is not a universal finding because some studies have observed no significant differences (173,174).

Perspective

According to the lactate shuttle theory, blood lactate concentration depends on production, transport and consumption. Therefore, it is important to take in mind that many methodological and biological factors affect these processes and the subsequent measurement of lactate concentration (Figure 7). Since lactate it is usually measured in research, medical and training testing, it is important to understand these factors to avoid misinterpretation. The main recommendation is to control all these factors when measuring lactate concentration and perform the

measurement in the same conditions when monitoring the evolution of a specific person or comparing different individuals.

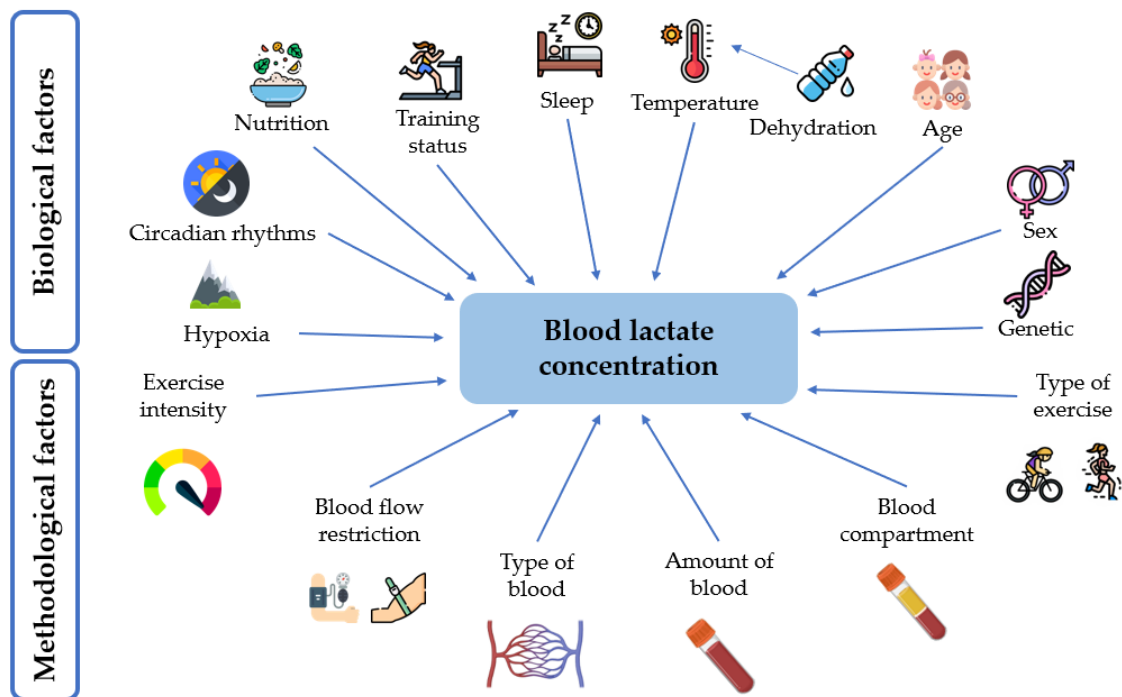


Figure 7 Schematic illustration of the identified factors affecting blood lactate concentration.

Contextualization of the present thesis

As previously mentioned, lactate has three fundamental functions in the body: it serves as an energy substrate, it is the main gluconeogenic precursor, and it is a signalling molecule (15). For all these functions to occur, lactate needs to travel through the body. This transport is thanks to the MCTs, as they transport lactate across different cell membranes.

In the case of skeletal muscle, the most abundant are MCT1 and MCT4. There is only one review explaining the effect of exercise on MCT1/MCT4 protein levels (175), carried out several years ago. This study was not a systematic review, and many studies have been published since then. Hence, we do not know what the studies published to date say about the effect of exercise on MCT1 and MCT4 protein levels.

In case of erythrocytes, most (>90%) of the lactate is transported across the erythrocyte membrane by MCT1 (41). Hence, measuring lactate concentration inside and outside the erythrocyte is an excellent model to study MCT1 function. On the other hand, the existence of the single-nucleotide polymorphism rs1049434 of the MCT1 gene has been reported to affect blood lactate transport and concentration (168–171,176). Nevertheless, we do not know whether this genetic variant affects the functionality of the MCT1 of the erythrocyte.

Apart from the specific transporters, different factors influence lactate concentration, as reviewed in the previous section. Specifically, this thesis will focus on the influence of sex, temperature, and heat. We know that lactate concentration changes with intensity in a non-linear fashion, presenting two breakpoints: LT1 and LT2. Previous studies have found that females have the ventilatory threshold 1 and maximum lactate steady state closer to maximal heart rate compared to males (177). However, males and females have these thresholds at the same percentage of maximum power output (177). But ventilatory threshold 1 and maximum lactate steady state are not the same as LT1 and LT2, respectively. Therefore, we do not know the differences in lactate thresholds between the sexes.

Continuing with the influence of sex on lactate, it was observed in males that the worse the training status, the sooner the lactate concentration increased in the same incremental test. Furthermore, it was also observed that the worse the training status, the earlier fat oxidation decreased. It was established that those individuals with an earlier increase in lactate concentration and an earlier decrease in fat oxidation presented worse metabolic flexibility (56). Furthermore, a very strong relationship was found between lactate concentration and fat oxidation (56). On the other hand, in another study carried out in females, lactate concentration was very similar regardless of fitness level, whereas fat oxidation was higher the better the training level for the same incremental test (178). Therefore, in the case of females it seems that lactate is not effective in detecting differences in metabolic flexibility while fat oxidation is effective in detecting differences in metabolic flexibility. However, we still do not know the differences

in lactate concentration and fat oxidation in the same incremental test (indicators of metabolic flexibility) between sexes.

Regarding the influence of nutritional status and heat, as mentioned in the previous section, glycogen depletion (64) and heat (102) affect lactate concentration. On the other hand, there is a very close correlation between lactate concentration and fat oxidation (179). This association holds among people with different metabolic states (56). Nevertheless, we still do not know whether changes in lactate concentration, due to glycogen depletion or heat, are accompanied by changes in fat oxidation.

Finally, heart rate or power output value are variables used to prescribe training intensity, in order to achieve a specific metabolic situation, being the metabolic thresholds [i.e. lactate thresholds (LT1 and LT2) or ventilatory thresholds (VT1 and VT2)] common reference points. As we have just seen, heat and glycogen depletion affect the metabolic response, but is still unclear how these two situations affect different variables at the thresholds, such as heart rate or power output.

2. Objectives

2.1. General aim

The general aim of this thesis is to study the influence on lactate of its main transporters, sex, temperature and nutritional status.

2.2. Specific aim

Specific aims to study the influence of the main lactate transporters:

Study I

To know the effect of exercise considering exercise intensity and duration of the intervention (single-session vs training program) on protein levels of the MCT1 and MCT4 in the skeletal muscle.

Study II

To determine whether changes in lactate concentration induced by exercise, in plasma and erythrocytes, differed between genotypes for the polymorphisms of the MCT1 gene.

Specific aims to study the influence of sex:

Study III

To compare the differences in absolute and relative heart rate and power output at lactate thresholds 1 and 2 between sexes.

Study IV

To explore the differences in lactate concentration and fat oxidation at the same exercise intensity, as indicators of the metabolic flexibility, between sexes.

Specific aims to study the influence of nutritional status and heat:

Study V

To assess whether changes in lactate concentration, due to glycogen depletion or heat, are accompanied by changes in fat oxidation during an incremental test.

Study VI

To compare heart rate, rate of perceived exertion (RPE), and power output at VT1, VT2 and at maximal effort in different situations: control, heat (36°C), and glycogen depletion.

3. Materials and methods

3.1. Experimental overview

The present thesis is composed of a systematic review, and two projects: Erythrocyte Transport of Lactate During Exercise (“Transporte Eritrocitario de Lactato en Ejercicio”, TELE project) and Lactate Profile and Fat Oxidation During Exercise (Lac-Fat project). The systematic review was carried out with the intention of satisfying specific objective I, resulting in study I. The TELE project was carried out to respond to specific objectives II and III, resulting in studies II and III. Finally, the Lac-Fat project was developed to meet specific objectives V, VI, and VII, resulting in studies V, VI, and VII.

Table 1: Experimental overview of the present thesis.

Systematic	Study I	Purpose: To know the effect of exercise considering exercise intensity and duration of the intervention (single-session vs training program) on protein levels of the MCT1 and MCT4 in the skeletal muscle.	
		Title: Exercise influence on monocarboxylate transporter 1 (MCT1) and 4 (MCT4) in the skeletal muscle: A systematic review.	
TELE Project	Study II	Purpose: To determine whether changes in lactate concentration induced by exercise, in plasma and erythrocytes, differed between genotypes for the polymorphisms of the MCT1 gene.	Influence of the main lactate
		Title: Differences in plasma and RBC lactate concentration induced by intervallic exercise between genotypes for the T1470A SNP (rs1049434) on the MCT1 (SLC16A1) gene.	
	Study III	Purpose: To compare the differences in absolute and relative heart rate and power output at lactate thresholds 1 and 2 between sexes.	Influence of sex
		Title: Greater relative first and second lactate threshold in females compared to males: Consideration for exercise prescription.	
Lac-Fat project	Study IV	Purpose: To explore the differences in lactate concentration and fat oxidation at the same exercise intensity, as indicators of the metabolic flexibility, between sexes.	Influence of sex
		Title: Females have better metabolic flexibility in different metabolically challenging stimuli.	
	Study V	Purpose: To assess whether changes in lactate concentration, due to glycogen depletion or heat, are accompanied by changes in fat oxidation during an incremental test.	Influence of heat and nutritional status
		Title: Changes in lactate concentration are accompanied by opposite changes in the pattern of fat oxidation: Dose-response relationship?	
	Study VI	Purpose: To compare heart rate, rate of perceived exertion (RPE), and power output at VT1, VT2 and at maximal effort in different situations: control, heat (36°C), and glycogen depletion.	Influence of heat and nutritional status
		Title: A comparison between heart rate, RPE and power output to prescribe exercise intensity in control, low glycogen availability and heat.	

3.2. Systematic review (Study I)

The methodological process was based on the recommendations indicated by the guidelines of the Preferred Reported Items for Systematic Reviews and Meta-Analysis (PRISMA) (180). The study was preregistered in the International Prospective Register of Systematic Review (PROSPERO) with the following registered number: CRD42021238997. A comprehensive database search was systematically conducted (PubMed and Web of Science) up to January 13, 2023 and performed independently by two authors obtaining the same results. The following combination terms was used: “MCT” OR “monocarboxylate transporter” OR “monocarboxylic acid transport” OR “SLC16A1” OR “muscle lactate transport”. The Boolean operator “AND” was used to combine these descriptors with: “Exercise” OR “train” OR “training” OR “sport” OR “physical activity”. The search results were downloaded and imported into Reference Manager EndNote (version X7, Clarivate Analytics, Philadelphia, PA, USA).

Two authors carried out the study selection independently. Disparities found were discussed by the authors to reach a consensus. Research studies were included if (1) studies examined the effect of single exercise session or training program on MCT1/MCT4 protein levels or *SLC16A1/SLC16A3* mRNA; (2) humans or rodents were analysed; (3) studies were published in English, Spanish or Portuguese; and (4) an exercise intervention was carried out. Research studies were excluded if (1) it was a review; (2) it was not an original investigation; (3) the study was in vitro; (4) information about pre-post proteins levels or mRNA was lacking; (5) for human studies, the sample population was not between 18 and 65 years of age; (6) for human studies, men and women were mixed for the analysis or the sex of the sample was not specified; (7) training characteristics (volume, intensity, frequency, type of exercise...) were not properly specified; (8) protein or mRNA measurements were not properly specified; (9) rodents were genetically modified to alter the MCTs protein content or the mRNA expression of their respective genes; (10) MCT proteins were not measured with western-blot; (11) studies in animals that were not rodents; and (12) an hypoxia stimulus was included.

The main outcome variables of the present study were MCT1 protein levels, MCT4 protein levels, *SLC16A1* mRNA expression and *SLC16A3* mRNA expression. MCT1 and MCT4 protein levels were assessed because they are the two main muscle transporters of lactate and protons during exercise. On the other hand, *SLC16A1* and *SLC16A3* mRNA were evaluated because they are usually used to predict changes on MCT1 and MCT4 protein levels.

3.3. TELE Project (Study II and III)

Participants

The project included a total of 24 trained females (12 females with the AA genotype and 12 females with the TT genotype) and 20 trained males (10 males with the AA genotype and 10 males with the TT genotype). Only subjects who have one of the two required genetic profiles (AA or TT) were selected, and subjects with the TA genotype were discarded. All participants were informed of the tests to be performed, their risks and benefits, and will sign the informed consent. The inclusion criteria were as follows:

- Have a body mass index (BMI) between 18 and 25 kg/m².
- Be between 18 and 30 years old.
- Not being a smoker (18).
- In the case of trained participants, perform at least 5 hours per week of training, of which at least 3 must be aerobic/anaerobic endurance. Have passed the Faculty of Physical Activity and Sports Sciences of the Polytechnic University of Madrid entrance exams with the corresponding presentation of the medical certificate (or present a medical certificate certifying their fitness to perform intense exercise), in addition to having a low risk assessed through the PAR-Q questionnaire (answer all the questions in the questionnaire negatively) (181).
- Do not present any pathology that may prevent the correct performance of the tests, or may influence the main variables of study.

- For both the incremental test and the interval test, volunteers went to the Exercise Physiology Laboratory meeting the following requirements: having eaten their last meal at least two hours in advance, abstaining from alcohol, caffeine and intense exercise for at least 24 hours prior to the test (4).

This study was approved by the ethics committee of Universidad Politécnica de Madrid (2019-044). Clinical trials: NCT06104150.

First visit

This first visit was used to sign the informed consent. In order to know the genotype of the *SLC16A1*, and to make the selection based on this genotype, all those interested in participating went through an initial assessment that consisted of:

Antropometric variables

The anthropometric variables analyzed were weight, height and BMI. A Detecto® scale (Lafayette Instruments Company, Lafayette, Indiana, United States) was used to measure the weight, with a measuring range between 0.0 and 150.0 kg and an accuracy of 100 g. Periodic calibrations were carried out, as well as zero adjustments prior to each measurement. For the measurement of height, the distance between the vertex and the soles of the subject's feet was taken as a reference. It was measured using a conventional rack-and-pinion stadiometer with triangular plane of head drill bit (Holtain Limited, Crymych, UK), whose measurement range spans from 63 to 213 cm with a measurement error of ± 0.1 mm. Based on weight and height, BMI was calculated using the following formula:

$$\text{BMI (kg/cm}^2\text{)} = \text{Weight (kg)} / \text{Talla}^2 \text{ (cm)}$$

Genetic analysis

Capillary blood collection was performed at the Physiology Laboratory of the Faculty of Physical Activity and Sports Sciences of the Polytechnic University of Madrid (INEF-UPM). To extract capillary blood, a lancet was pricked into the pulp of a finger. Two circles of the filter paper cards were then filled with dried blood. The genetic study was carried out in the Paediatrics Laboratory of the Department

of Medical and Surgical Sciences of the University of Cantabria in Santander. For genetic analysis, DNA was extracted from filter paper cards with dried blood, using the commercial *Qiaamp DNA Mini Kit* from Qiagen (Hilden, Germany) following the manufacturer's instructions. Once purified, the DNA samples were stored at -20°C until further analysis.

The genetic analysis was performed by real-time PCR genotyping using the corresponding *TaqMan SNP Genotyping Assays* from Applied Biosystem (Foster City, United States) on a *StepOne Real Time PCR System* from Applied Biosystem (Foster City, United States), including the corresponding quality controls on each plate.

Subjects were classified into three groups based on the genetic profile of MCT1, following an additive model: genotype AA, genotype TA, and genotype TT. Heterozygous subjects, i.e., those who carried the polymorphism in only one of the two alleles (AT group) were discarded in order to compare the two most genetically differentiated alleles. This discarding was done because we expect an additive effect of the polymorphism, based on previous studies (168,170,176,182,183), and therefore the greatest differences, if any, are expected to be found in genotypes AA and TT.

PAR-Q

Once we knew the genetic profiles of the subjects who went to participate in the project, they are digitally given a PAR-Q (Physical Activity Readiness Questionnaire) (181) in the presence of one of the investigators to assess the risks of physical activity.

Second visit

First, physical activity levels will be determined. Subsequently, an incremental test will be carried out.

Determination of Physical Activity

The IPAQ (international physical activity questionnaire, abbreviated version) was used to estimate the level of physical activity (184). Participants were given a

detailed explanation of it and asked to fill it out. A researcher remained close to the participants during the IPAQ completion period to dispel any doubts. The primary objective of the IPAQ is to determine the total amount of walking, moderate physical activity, and vigorous physical activity and to generate a total score for weekly energy expenditure from physical activity, expressed in METs. Total physical activity was calculated assuming metabolic equivalents of 3.3, 4.0, and 8.0 METs for low, moderate, and intense activities, respectively. A low level of physical activity represents less than 599 MET/min/week, a moderate level of physical activity represents between 600 MET/min/week and 3000 MET/min/week, and a high level of physical activity represents more than 3000 MET/min/week (185).

Incremental Test

A maximal incremental test was performed on a cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). The ergometer was fitted to the ergonomics of each participant. Heart rate was measured every 5 s with a Polar RS800sd device (Polar Electro Oy, Kempele, Finland) with a chest strap. The test started with a warm-up of 5 min at 75 W, followed by 35 W increments every 3 min until task failure, which was defined as the inability to maintain a cadence above 70 rpm for longer than ~5 s or the participant's voluntary withdrawal despite strong verbal encouragement. The cadence had to be kept stable between 70 and 90 rpm. Participants had to be seated during the whole test. Capillary blood samples were obtained from the earlobe and lactate concentrations were measured using a portable lactate analyser (Lactate Plus; Nova Biomedical, Cheshire, UK) at rest, in the last 30 seconds of each step, as well as immediately after the end of the test.

Third visit

Interval Test

At the third visit, an interval test was performed (Figure 8). The cadence of the protocol was the same as during the incremental test. It started with 1 min of rest followed by 5 min of warm-up at an intensity equal to 80% of the LT1. This was

followed by 4 sets of 1 min 30 sec at an intensity 20% above LT2, with recovery intervals of 5 min at 80% LT1. After the 4 sets, a final recovery of 20 min was performed at 80% of the LT1. During the interval protocol, capillary blood were drawn from the earlobe, venous blood extractions from a vein in the forearm were performed, the perceived exertion scale 6-20 was passed, and the perceived recovery scale was passed as indicated in Figure 8.

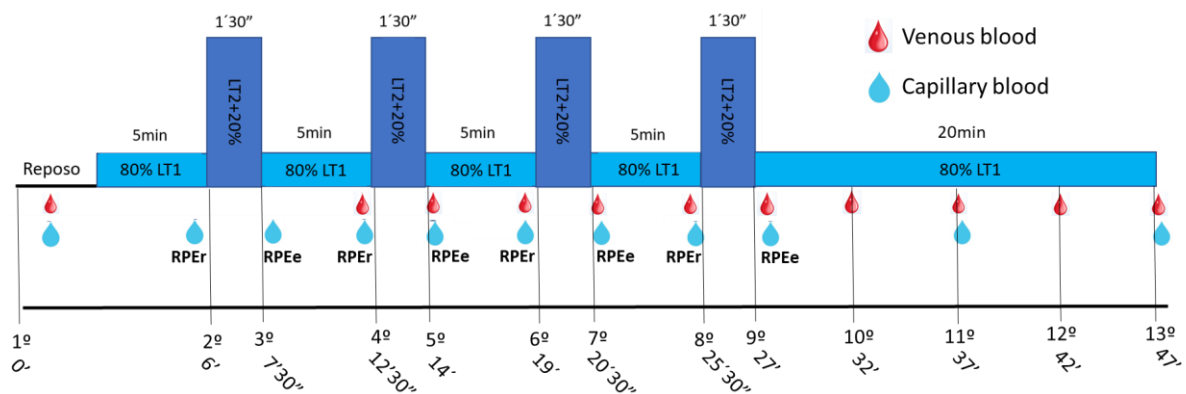


Figure 8 Interval test protocol with sampling moments. LT2+20%: intensity 20% above LT2; 80% LT1: intensity equal to 80% of the LT1; RPEe: rate of perceived exertion; RPEr: rate of perceived recovery.

3.4. LAC-FAT project (Study IV, V and VI)

Participants

The project included a total of 12 females and 11 males. The exclusion criteria in the project will be as follows:

- Be under 18 years old or over 40 years old.
- Suffering from any previous disease, disorder or ailment that may affect the result of the study variables (pay attention to polycystic ovary syndrome).
- Smoke.
- Endurance training between 3 hours and 12 hours/week.
- Be a user of any type of hormonal contraceptive.

- Having irregular menstrual cycles.
- Suffering from dysmenorrhea, assessed through a visual pain scale. In addition, they will be asked about the need to take anti-inflammatories during menstruation, ruling out those who cannot perform the tests without taking them.
- Having heavy menstrual bleeding, detected with the menstrual bleeding questionnaire (186)
- The volunteers will go to the Physiology of Effort Laboratory fulfilling the following requirements: be rested, have eaten their last meal at least two hours in advance, abstain from drinking alcohol, ingesting caffeine, taking any medication and performing intense exercise for at least 24 hours prior to the test.

Ethical authorization for this study was obtained from the Research Ethics Committee of the University where the research was conducted (ID: DATOS-20220524-RCC-LacFat).

Study design

This crossover study consisted of four situations carried out on five visits to the laboratory on five different days (Figure 9). The first visit included informed consent, an assessment of body composition by total body dual-energy X-ray absorptiometry (DXA) and individualized nutritional plan. Afterwards, in the following appointments, the same maximal incremental tests were performed in three different experimental situations (control, glycogen depletion and heat). Tests were performed at the same time of day (± 2 hours). In the case of females, all tests occurred during the early-mid follicular phase of the menstrual cycle (day 4 ± 1.67). The first test was in all cases a maximal incremental test in the standard situation (control situation). The order of the two remaining incremental tests (glycogen depletion or heat situations) was randomized. In order to prepare for the glycogen depletion test, 24 hours before the incremental test, an exercise protocol was performed to completely deplete muscle glycogen stores. In addition, from breakfast on the same day of the glycogen depletion exercise protocol until the maximal incremental test, a low-carbohydrate diet was followed. On the contrary,

in control and heat situations, from breakfast of the previous day of the tests, participants followed a high-carbohydrate diet. During both control and glycogen depletion situations, the ambient temperature was maintained at ~21°C. Finally, during the heat situation participants were placed in a heat chamber designed to maintain an ambient temperature between 36-38°C for 30 min prior the incremental test, and the incremental test was performed at the same temperature (187). In all the situations, body weight loss was less than 2%. Clinical trial registration: NCT05703100.

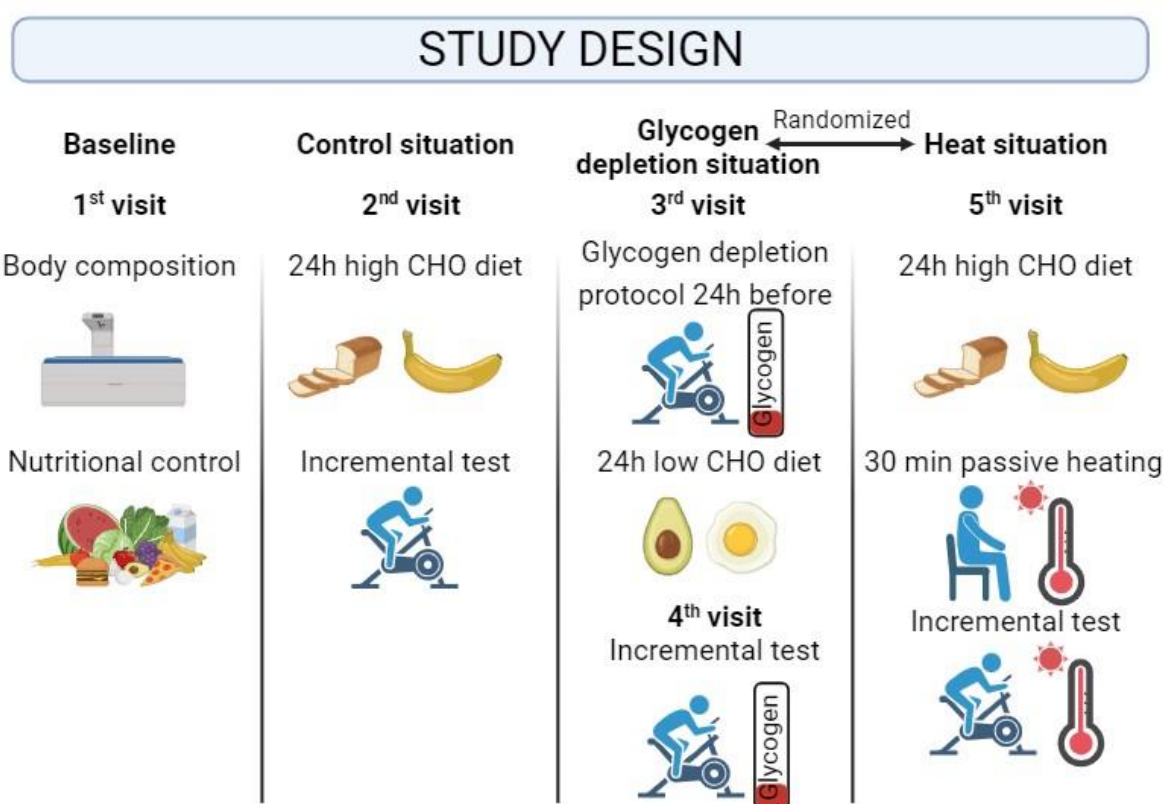


Figure 9 Overview of the study design. CHO: Carbohydrates.

Body composition assessment

Body composition was measured by a DXA scan (Version 6.10.029GE Encore 2002, GE Lunar Prodigy; GE Healthcare, Madison, WI, USA) to obtain the fat mass and fat-free mass (FFM). All participants performed the test wearing light sportswear (e.g., top and shorts). In addition, they must be with their body and hands in a supine position and their feet joined by a strap. Participants were instructed not

to move or speak while the scanner performed the measurements. The scan was calibrated every other day using the phantom supplied by the manufacturer.

Nutritional intervention

High-energy, high-carbohydrate diet

Participants followed a diet at least 24 hours before the incremental test in the control and heat situations to ensure adequate energy and carbohydrate intake to avoid performance limitations during testing. The macronutrient distribution was standardized for all participants at an approximate ratio of 65% carbohydrate, 15% protein and 20% fat. Energy and carbohydrate intake were determined for all participants in relation to their FFM (energy: $60 \text{ kcal} \cdot \text{kg} \cdot \text{FFM}^{-1}$; CHO: $9.8 \text{ g} \cdot \text{kg} \cdot \text{FFM}^{-1}$) (188) (see example in Supplementary Material of the study IV in Annexes).

High-energy, low-carbohydrate and high-fat diet

This diet matched the energy intake of the high carbohydrate diet ($60 \text{ kcal} \cdot \text{kg}^{-1} \cdot \text{FFM} \cdot \text{day}^{-1}$). However, carbohydrate intake was severely restricted ($<50 \text{ g} \cdot \text{day}^{-1}$) and protein and fat intake were 20% and 75-80%, respectively (188). The most difficult nutritional objective to achieve was to reach the previously established daily caloric intake without exceeding the carbohydrate limit. Therefore, a medium-chain triglycerides supplementation was used to achieve this energy objective without contributing to increase carbohydrates (see example in Supplementary Material of the study IV in Annexes).

Exercise protocol

Incremental exercise test

The incremental test performed in the three different situations was carried out on a cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). The cadence throughout the test was constant, remaining between 70-90 rpm. The protocol started with 1 min of rest followed by 5 min of warm-up at 30W. Subsequently, power was increased by 30 W every 3 min until maximum effort was reached, considering this when a reduction in cadence of 10 rpm was observed.

Glycogen depletion protocol

The glycogen depletion protocol consisted of a 60-minute continuous cycling exercise at the power associated with 60% of VO_2max calculated from the power- VO_2 ratio obtained during the maximal incremental cycling test performed in the control situation. The pedalling cadence was set at 70 rpm. After that, six 30-second sprints were performed at the maximum possible intensity with a 7.5% body weight load with a 4-minute recovery between bouts. This protocol has been carried out in previous studies with the same objective, having already demonstrated its efficacy in the depletion of glycogen stores (189).

4. Results and discussion

4.1. Study I: Exercise influence on monocarboxylate transporter 1 (MCT1) and 4 (MCT4) in the skeletal muscle: A systematic review

APPROACH TO THE PROBLEM

The solute carrier 16 (SLC16) gene family has fourteen members. It should be noted that only four genes of the human family (*SLC16A1*, *SLC16A3*, *SLC16A7*, and *SLC16A8*) have been shown experimentally to encode proteins that transport monocarboxylates (MCT1, MCT4, MCT2, and MCT3, respectively) (190), which play an important role in metabolic communication between cells (191). They are found in diverse tissues exhibiting different selectivity and affinity for their substrates, with lactate being quantitatively the most important (17). MCTs 1 to 4 cotransport protons and monocarboxylates passively through the plasma membrane (190). The direction of transport depends on the gradient of the predominant substrate and protons, and the net rate of transport is determined by the difference between the influx and efflux. In thermodynamic equilibrium, the ratio inside/outside the cell is similar in the concentration of monocarboxylates and protons (190). Therefore they play a key role in the regulation of pH between cells (190). MCTs also are capable to influence the redox state of the different cells of the body. Most of the reactions involving monocarboxylates (pyruvate, lactate, β -hydroxybutyrate and acetoacetate) modify the NADH/NAD⁺ state inside the cell. Thus, due to the close equilibrium between monocarboxylates and NADH/NAD⁺ state, the transport of one of these monocarboxylates from one cell to another through MCTs will modify the redox state of these cells as well (17). The importance of lactate in the redox balance is reflected in the absence of MCT1 in the beta cells of the pancreas to prevent insulin release triggered by an increase in blood lactate due to exercise (192).

The distribution of the different MCTs varies among tissues, fitting in part with the different functionality of each member. MCT1 is ubiquitous (except in pancreatic β -cells) and has the most diverse selection, as it transports lactate, pyruvate, ketone bodies, acetate, butyrate, and propionate (193). MCT2 is mainly present in the brain (neurons), liver, kidney and spermatogonia transporting pyruvate, lactate, and ketone bodies (193). MCT3 is only found in retinal pigment epithelium transporting lactate (193). Finally, MCT4 is present in the muscle (white fibers), brain (astrocytes) and white blood cells transporting lactate and ketone bodies (193). Although MCT1 is ubiquitous, in the skeletal muscle is predominant in the oxidative fibers and the transport direction depends on the metabolic situation of the cell (194). On the other hand, MCT4 is predominant in the glycolytic fibers of the muscles and is specialized in lactate efflux (194). MCT4 has a low affinity for pyruvate (MCT4 K_m for pyruvate is 153mM) to avoid efflux out of the cell (17). This is because active white skeletal muscles cells require the conversion of pyruvate to lactate to regenerate cytosolic NADH from NAD^+ to produce energy. At the same time, the low affinity of MCT4 for lactate (MCT4 K_m for lactate is 28mM) results in its accumulation inside the cell. This prevents large amounts of H^+ from being expelled out of the cell, which would lower the blood pH and have disastrous consequences (17). According to the lactate shuttle theory, in high-glycolytic environments (like glycolytic tumor cells or muscle fibers) lactate and protons are released from these glycolytic cells by MCT4 (17,195). Once lactate and protons are out of the cell, they travel through plasma or into erythrocytes. The transport of lactate and protons between plasma and erythrocytes is mediated mainly by MCT1 (41). MCT1 protein is highly expressed in sarcolemma and mitochondrial membranes of type I and type IIA fibers and other oxidative cells and plays a role in cellular lactate uptake and oxidative disposal (195). These complementary functions between the two transporters play an important role in many scenarios in which the role of lactate is relevant, like intermediary metabolism (196), glucose homeostasis (197), or cancer (195). In fact, the consequences of the partial invalidation of MCT1 have been already described in a knocking mouse for the *SLC16A1*, leading to paramount changes in body composition and energy regulation (198).

MCT1 and MCT4 protein content seems to be modified in some situations. In some conditions, such as cancer, an overexpression of MCT1 and MCT4 occurs due to the high glycolytic activity of tumor cells (Warburg effect) (199). Additionally, obese patients present a higher MCT4 protein content in the muscles and, interestingly, this content was reduced following weight loss. The higher MCT4 protein content of the muscle in obesity could reflect the need to release greater amounts of lactate from these fibers characterized by a greater dependence on glycolytic metabolism due to the low capillarization causing hypoxia (200). In fact, hypoxia itself seems to increase *SLC16A3* mRNA mediated by hypoxia-inducible factor 1 α (HIF-1 α) (199). In addition to being modified in various diseases, MCTs content may also be modified with training status, being reported a higher MCT1 protein content in well-trained subjects compared to less trained subjects (201).

It is suggested that exercise would modify MCT1/MCT4 protein levels or *SLC16A1/SLC16A3* mRNA in the muscle. In the literature, there is a wide variety of exercise protocols studied. However, it is well known that the management of the exercise variables will modify the physiological response. Thereby, it would be useful to know in more detail the effect of exercise and its characteristics (such as intensity and duration) on these transporters, to determine which stimulus will provide the most benefit. MCT1 and MCT4 are the most studies related to exercise due to their localization in the muscles and their effect on co-transporting lactate and protons (194). It is very common to develop this type of studies in rodents, and therefore it is necessary to check if the results in rodents are in line with the results in humans.

There is only one review explaining the effect of exercise on MCT1/MCT4 protein levels or *SLC16A1/SLC16A3* mRNA (175) carried out several years ago. This study was not a systematic review, and many studies have been published since then. In addition, this previous study did not compare the results in humans with those in animals to see whether the two models provide aligned results. Hence, given the importance of MCT1 and MCT4 in metabolism, exercise physiology and health, it is necessary to bring together all these findings to get a clear picture of how exercise and its characteristics affect the two main muscle MCTs.

METHODS

Design

The methodological process was based on the recommendations indicated by the guidelines of the Preferred Reported Items for Systematic Reviews and Meta-Analysis (PRISMA) (180). The eligibility criteria were established by the authors. The study was preregistered in the International Prospective Register of Systematic Review (PROSPERO) with the following registered number: CRD42021238997.

Data sources and searches

A comprehensive database search was systematically conducted (PubMed and Web of Science) up to January 13, 2023 and performed independently by two authors obtaining the same results. The following combination terms was used: “MCT” OR “monocarboxylate transporter” OR “monocarboxylic acid transport” OR “SLC16A1” OR “muscle lactate transport”. The Boolean operator “AND” was used to combine these descriptors with: “Exercise” OR “train” OR “training” OR “sport” OR “physical activity”. The search results were downloaded and imported into Reference Manager EndNote (version X7, Clarivate Analytics, Philadelphia, PA, USA).

Selection criteria

Two authors carried out the study selection independently. Disparities found were discussed by the authors to reach a consensus. Research studies were included if (1) studies examined the effect of single exercise session or training program on MCT1/MCT4 protein levels or *SLC16A1/SLC16A3* mRNA; (2) humans or rodents were analysed; (3) studies were published in English, Spanish or Portuguese; and (4) an exercise intervention was carried out. Research studies were excluded if (1) it was a review; (2) it was not an original investigation; (3) the study was in vitro; (4) information about pre-post proteins levels or mRNA was lacking; (5) for human studies, the sample population was not between 18 and 65 years of age; (6) for human studies, men and women were mixed for the analysis or the sex of the sample was not specified; (7) training characteristics (volume, intensity, frequency, type of exercise...) were not properly specified; (8) protein or mRNA measurements

were not properly specified; (9) rodents were genetically modified to alter the MCTs protein content or the mRNA expression of their respective genes; (10) MCT proteins were not measured with western-blot; (11) studies in animals that were not rodents; and (12) an hypoxia stimulus was included.

Outcome variables

The main outcome variables of the present study were MCT1 protein levels, MCT4 protein levels, *SLC16A1* mRNA expression and *SLC16A3* mRNA expression. MCT1 and MCT4 protein levels were assessed because they are the two main muscle transporters of lactate and protons during exercise. On the other hand, *SLC16A1* and *SLC16A3* mRNA were evaluated because they are usually used to predict changes on MCT1 and MCT4 protein levels.

Study selection and data extraction

Two authors independently extracted data from the included studies in a spreadsheet. The following information was extracted: 1) study (authors, title, year, design and duration of the intervention (single-exercise session or training program)); 2) sample (size, age, weight, height, training experience and disease); 3) training (duration of intervention, type of training, type of exercise, training frequency, session characteristics (intensity, duration, rests...)); 4) biopsy (timing, type of tissue, analysis method and amount of tissue); 5) MCT1/MCT4 proteins levels and *SLC16A1/SLC16A3* mRNA. If insufficient information was reported, the corresponding authors of the included studies were contacted to obtain missing information. In case results were reported in figures, values were extracted from the studies' figures using an online software (WebPlotDigitizer 4.6, California, USA).

Risks of bias assessment

The articles involving human sample of this review were evaluated with the Cochrane collaboration tool for risk of bias assessment (202). The following criteria were assessed: 1) random sequence generation; 2) allocation concealment; 3) blinding of participants and personnel; 4) blinding of outcome assessment; 5) incomplete outcome data and 6) selective reporting. For each study, each source of

bias was described as having either a low risk of bias, an unclear risk of bias, or a high risk of bias. Each item evaluated as "High" was scored with 1 point. The score for each study was calculated by adding the score for each item. A punctuation lower than 3 was classified as low, between 3 and 4 as medium and higher than 4 as high.

RESULTS

Study selection

The initial search identified 1476 articles from the database. After removing duplicates, 1108 manuscripts were screened for eligibility based on their title and abstract. 983 of records were excluded after reading the title and/or abstract, remaining 125 articles. 18 additional articles were identified from other sources for eligibility. Also, four additional articles were found for eligibility in the last update. A total of 147 were assessed as full texts, excluding 106 articles based on the exclusion criteria. The flow diagram of the selection criteria is shown in Figure 10. This qualitative analysis review includes 41 studies: 18 articles with a human sample and 23 with samples in rodents. The results of the studies in rodents will be analyzed to compare them with the results in humans to know if the rodent model is useful to infer the human model. In addition, some methodological aspects of the human's studies will be analyzed with the intention to improve future studies on this topic. The main characteristics of the included studies are presented in Tables 1-11. Due to the large variation in study design and outcomes, quantitative analysis was not deemed appropriate and, therefore, the results have been presented in a narrative form.

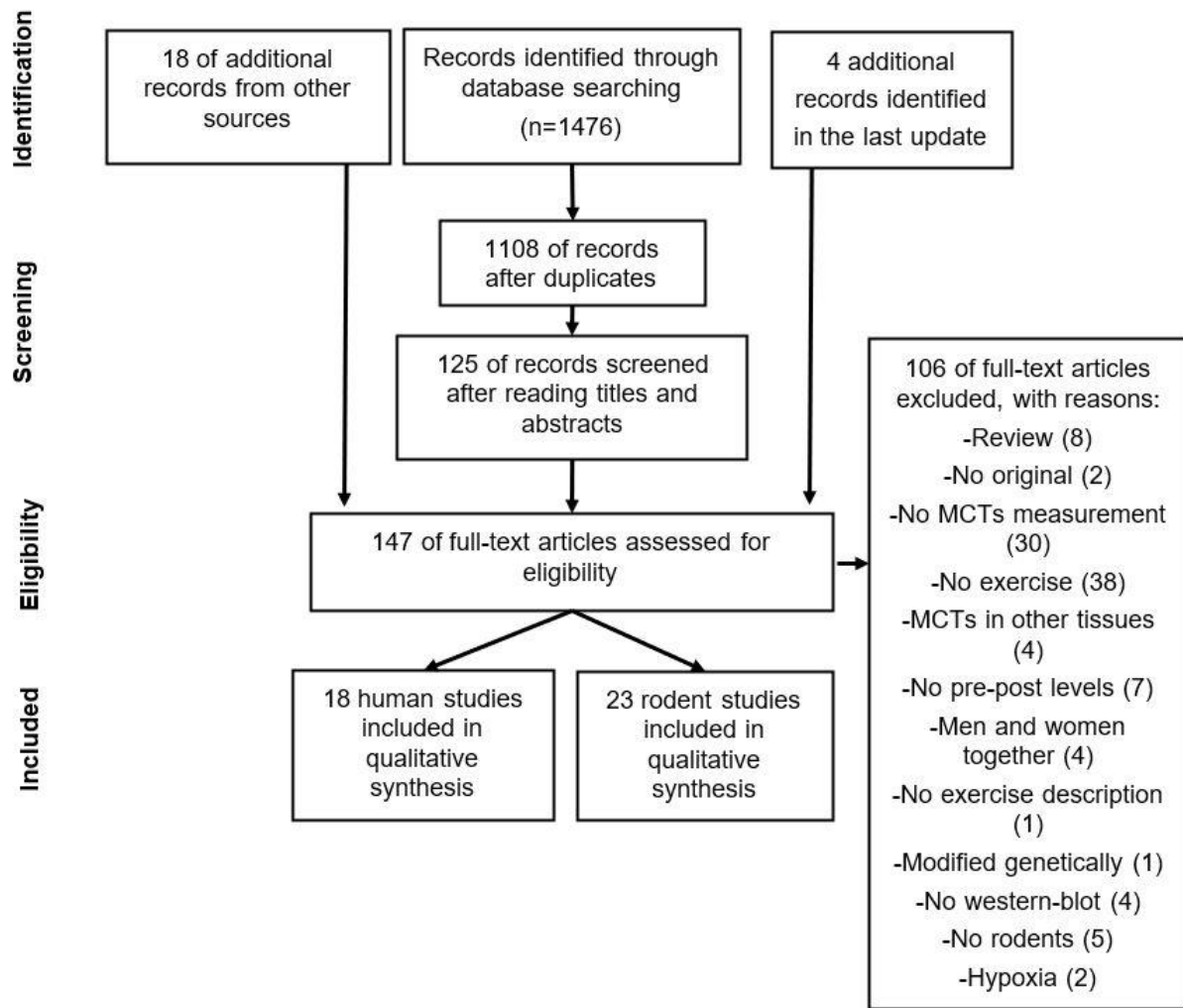


Figure 10 Flowchart showing screening process and search results.

Risks of bias

The risk of bias assessment of each study is presented in supplementary material of the study I in Annexes. The mean score on the Risk of Bias was 2.77 out of a possible 7 points (range 0-4). This score is explained because: a) Eight studies were evaluated with a high risk of bias of random sequence generation bias because the study only included one group (exercise group); b) eight studies were evaluated with a high risk of allocation concealment bias because only included one group; c) seventeen studies were classified as a high risk of blinding participant and personnel bias because exercise intervention cannot be blinded to the participant; d) Fifteen studies were evaluated with a high risk of incomplete outcome data bias, one of them reported the data in a graph with an exponential axis making it

difficult to extract the data and fourteen of them reported the data without a measure of dispersion in the values before exercise.

Characteristics of the sample and exercise

All the studies investigating exercise effect on MCT1/MCT4 protein levels or *SLC16A1/SLC16A3* mRNA (n=41) included a total of 201 humans (10% women) with ages between 19-62 years, and a total of 572 rodents (no sex specification).

The modulation of high-intensity exercise response on protein levels or *SLC16A1/SLC16A3* mRNA comprised of 119 humans (22% women) (n=12), and 16 rodents (no sex specification) (n=2). The duration of interventions used in human studies were a single-session (n=4) or a program training (n=10), whereas the rodent studies were a single-session (n=1), or a program exercise (n=1).

The studies examining the changes in MCT1/MCT4 protein levels or *SLC16A1/SLC16A3* mRNA after low/moderate-intensity exercise included 30 human volunteers (0% women) (n=4) and 379 rodents (no sex specification) (n=15). The duration of interventions used in human studies were a single-session (n=) or a training program (n=3), whereas the rodent studies were a single-session (n=6), or a training program (n=9).

In addition, several studies investigated the effect of other types of exercise on MCT1/MCT4 protein levels or *SLC16A1/SLC16A3* mRNA in humans, such as the combination of high and low/moderate intensity exercise in 24 participants (n=2) or resistance exercise in 34 humans (n=1). In both cases, intervention was conducted as a training program over several weeks. In rodents, the other types of exercise used were electrostimulation in 78 rodents (n=4), voluntary physical exercise in 114 rodents (n=2) and a combination of low/moderate exercise intensity and low physical activity in 40 rodents (n=1). These interventions were carried out as a training program except one that was carried out in a single session.

Characteristics of the study design in human studies

Methodological characteristics of the studies will be reported only for human studies since studies in rodents were used only to observe if their results are in

line with human studies. In addition, several methodological differences exist between studies in humans and rodents making impossible to compare them in this aspect. Only five studies included a control group (203–207), while the remaining studies were designed with only the experimental group. Control groups among studies were heterogeneous: one study did not extracted a biopsy of the control group (203); one study trained one leg and used the untrained leg as a control (204); in one study the experimental and the control group developed an exercise intervention (205); in another study the experimental group trained in hypoxia and the control group trained without hypoxia, which was used to extract the data (206); and the control group in one study continued the training as during the period before the study (207).

Characteristics of the biopsy and the measurement of *SLC16A1/SLC16A3* mRNA and MCT1/MCT4 protein levels in humans

In humans, all the studies extracted a homogenized sample from the vastus lateralis (203–218), except one study in which the sample origin was not specified (219). The amount of tissue extracted in the biopsies varied among studies, from 4-5 mg dry wt until 150 mg, being the most common 30 mg. More specifically, the amounts extracted in each study were: 4-5 mg dry wt (207), 10-50 mg (204), 20 mg (213), 20-50 mg (205), 25 mg (214,215), 30 mg (208,211,216,219,220), 30-40 mg (218), 40 mg (217), 40-150 mg (210), 50 mg (212), 50-80 mg (209) and two studies did not specify the amount of tissue (203,206). The timing of the biopsy after exercise was highly heterogeneous varying from immediately post exercise until a week after training (Tables 1-11) and it was not clearly specified in four studies (207–210).

SLC16A1/SLC16A3 mRNA was measured with real-time PCR in all the studies (206,214,215). The control used as a standard was GAPDH (214), cyclophilin (206) and β -actin (215). On the other hand, to measure MCT1/MCT4 proteins levels, all the studies included carried out a western blot. The control used as a standard was bovine serum albumin in most of the studies (205–211,215–220); one study used GAPDH (213), and three studies did not specify the control used (203,204,212).

SLC16A1/SLC16A3 mRNA after exercise were expressed relative to mRNA before exercise in two studies (214,215) and relative to cyclophilin proteins in one study (206). On the other hand, MCT1/MCT4 proteins levels after exercise were expressed relative to bovine serum albumin standard in five studies (206,211,216,219,220), relative to protein levels before exercise in most studies (203,205,207–209,212,213,215,217,218), relative to the control group in one study (204) and relative to RBC membranes proteins in one study (210).

Reported findings on the effect of high-intensity exercise on *SLC16A1/SLC16A3* mRNA and MCT1/MCT4 protein levels

Two studies examined the effect of a single high-intensity session on mRNA in men (214,215) (Table 2). Although they used different types of exercise (leg extension vs. running), none of them reported changes in expression in any of the genes (*SLC16A1* and *SLC16A3*). In contrast, when a high-intensity ≈6 weeks intervention is applied in men the results are not conclusive: Bickham et al. (215) observed a decrease in mRNA of both *SLC16A1* and *SLC16A3* using exercise to exhaustion, while Nordsborg et al. (214) found no change with an interval leg-extension exercise training (Table 2). Studies in rodents with high-intensity exercise are scarce, with only one study using a single session as an intervention (221). They reported increases in the intervention compared to the control group at 5 h for *SLC16A1* mRNA and at 18h for *SLC16A3* mRNA (221) (Table 3). To date, we have not found any study that analyzes the expression of mRNA after implementing a high-intensity training program in rodents.

The results of protein level changes after a single session of high-intensity training point to different responsiveness of MCT1 compared to MCT4. In men, increases in both proteins have been reported after high-intensity interval training at 79% of the maximal power (216). However, a higher MCT1 protein content and a no changes in MCT4 protein content were reported when the exercise was performed until exhaustion (215). On the contrary, the only study that included exclusively women in its sample reported a decrease in the levels of both transporters after a high-intensity session (217) (Table 4). If the exercise stimulus is a training

program instead of only one session it appears to produce an increase in the level of MCT1, MCT4 or both proteins (7 out of 9 studies observed increases) (203–205,208,211,215,220). MCT1 seems to be slightly more responsive than MCT4, as in 4 studies (205,208,211,220) MCT1 protein levels increase at more time points compared to MCT4. Of the remaining 5 studies, 1 found MCT4 protein increases in more timepoints (203), 2 found similar increments in both transporters (204,215) and the other 2 found no changes for these proteins (207,209). Again, one of the studies finding no difference is the one carried out by Bishop et al. (209), where the sample was composed only of women (Table 4). The only study with rodents using high-intensity training program reported increments for both MCT1 and MCT4 protein contents, 48h after 6 weeks of training when red gastrocnemius was analyzed, but only for MCT4 when white gastrocnemius was analyzed (222) (Table 5).

Reported findings on the effect of low/moderate-intensity exercise on *SLC16A1/SLC16A3* mRNA and MCT1/MCT4 protein levels

No studies investigating the effect of low/moderate intensity on mRNA in humans were included in the present review. In rodents, the use of low/moderate intensity has been more common than high-intensity exercise, observing an increase in *SLC16A1* mRNA at some point in most cases (221,223–226), except one study (227). Of the 9 exercise sessions (applied by the 6 studies), 5 of them produced increases in *SLC16A1* mRNA, and 4 produced increases in *SLC16A3*. Interestingly, the two studies carrying out a training intervention reported different results depending on fiber composition: an increase of *SLC16A1* mRNA while a decrease of *SLC16A3* mRNA in an oxidative muscle (red gastrocnemius) when compared to the control group (226); and a rise of *SLC16A3* mRNA plus a reduction of *SLC16A1* mRNA in a glycolytic muscle (white gastrocnemius) when compared to baseline (227) (Table 6).

The effect of low/moderate-intensity exercise on protein levels in humans has been only studied in men. Only Green et al. investigated the effect of a single session, reporting increases for both proteins after 2, 4, and 6 days of a 5-6h cycling session at 60% VO₂peak (218) (Table 7). The results after a training program showed that

MCT1 appeared to be more affected than MCT4 since 2 of the 3 studies observed increments only in MCT1 protein content with respect to baseline values (210,212). The third study observed increases in MCT4, but not in MCT1, compared to baseline values (213). This is the only one of the 3 works whose sample is composed of cyclists with extensive training experience (Table 7). In rodents, three studies used what we can consider single-session interventions with low/moderate intensity. One finding an increase after 2 h of exercise consisting of 30 min exercise flowed by 30 mi rest, another found no changes in MCT1 levels (228) after running 30 min, whereas Kim et al (229) included exercise until fatigue and reported increases in levels of both proteins in similar quantities: almost doubling its presence after the session (Table 8). For multiple-sessions interventions, similar results as those seen in men were observed in rodents for MCT1 protein content, since 5 of 9 training programs found increments when compared to the control group (226,230–233). Although the percentage of positive results is not as high as in men, for MCT1 the significant results are all positive (increments in MCT1 protein content) and therefore more homogeneous than for MCT4 protein content. Considering the studies investigating MCT4 protein changes after a low/moderate-intensity training program, 3 of 7 training programs reported a decrease at some point (226,234,235), 2 of them increases (231,233) and two found no differences (230,236) in all cases compared to control groups (Table 8).

Reported findings on the effect of other types of exercise on *SLC16A1/SLC16A3* mRNA and MCT1/MCT4 protein levels

This section includes all the studies that use exercise programs that either combine high and moderate intensity, use a strength training protocol (or electrostimulation in the case of rodents), or are programs that cannot be classified in the previous sections. We will try to present the results in a clustered manner, grouping those programs that are similar to each other.

In humans, not many studies investigate the MCT1/MCT4 proteins or *SLC16A1/SLC16A3* mRNA responses using other exercise apart from high-intensity or low/medium-intensity exercises, and all of them use a training program as intervention (no single-session studies). Two of them employed exercise

combining high-intensity with low/medium-intensity exercise, either by merging them in the same session (206) or alternating sessions of each intensity throughout the week (213). No changes in mRNA or protein content were seen after training program intervention merging both intensities (206). Only an increase in MCT4 protein levels was reported by Neal et al (213) after a 6 weeks-program of polarized training in experienced male cyclists (Table 9-11). The third study applied a 6-week lower limb strength training program in healthy and diabetic older (61-62 years old) men (219). This intervention resulted in an increase in the levels of both MCT1 and MCT4 protein transporters in the healthy participants, whereas it caused an increase only in MCT1 protein levels in type 2 diabetes participants, in both cases compared to control groups (219) (Table 11).

Four studies investigated the effect of electrostimulation in rodents (237–240). For single-session interventions, we only have the results reported by Tonouchi et al (2002), with MCT1 and MCT4 proteins levels decreasing after the session, compared to control groups (Table 12). For longer interventions, either with separate sessions of chronic muscle stimulation (i.e., 24 h/day), results indicate increases in *SLC16A1* mRNA on white and red muscles, as well as a transient decrease in *SLC16A3* mRNA also for both types of muscle (white and red). These lowered mRNA values came back to control levels after 3 weeks of intervention (238) (Table 9). For protein levels, electrostimulation program interventions resulted in increments of MCT1 protein levels in two studies (238,239) and no differences with control in only in one study (240). For MCT4 protein levels, results are scarce and inconclusive, finding increases in one study (240) and no changes in the other (238) (Table 12).

Finally, studies applying a voluntary running program (i.e., exercise *ad libitum*) found increases in *SLC16A1* mRNA in gastrocnemius (241) (Table 9), and MCT1 protein levels in plantaris and anterior tibialis muscles, but only after 6 weeks (242)(Table 11). Moreover, the only study recording the level of physical activity reported no changes in proteins levels, of even a decrease of MCT4 protein levels when low physical levels were combined with a low/moderate-intensity exercise program (243) (Table 12).

Table 2 Effect of high-intensity exercise on SLC16A1 and SLC16A3 (MCT1 and MCT4 genes) mRNA expression in humans.

	Study	N (women)	Duration	Exercise	Biopsy after exercise	SLC16A1 mRNA		SLC16A3 mRNA	
						PRE	POST	PRE	POST
Single-session intervention	Bickham, D.(2006)(215)	7	single-session	HIE before 6 wks of training (Running: 110% VO2max until exhaustion)	2h	0.99	0.77 ± 0.34	0.99	0.76 ± 0.9
			single-session	HIE after 6 wks of training (Running: 110% VO2max until exhaustion)	2h	0.45 ± 0.24	0.58 ± 0.19	0.36 ± 0.36	0.55 ± 0.17
	Nordsborg, N.(2003)(214)	6	single-session	HIE before 5.5 wks of training (Leg extension: 15 x 60s at 150% VO2max; Rest: 180s)	0h	1	1.5 ± 3.8	1	1.8 ± 8.7
					1h		1.1 ± 4.41		2.2 ± 58.79
					3h		1.5 ± 2.33		1.5 ± 3.18
					5h		1.5 ± 1.84		2 ± 5.27
					24h		0.9 ± 2.82		1.3 ± 3.55
			single-session	HIE after 5.5 wks of training (Leg extension: 15 x 60s at 150% VO2max; Rest: 180s)	0h	1.1 ± 1.59	1 ± 2.57	1.5±2.94	1.8 ± 7.23
					3h		0.8 ± 1.59		1.3 ± 5.27
Training program intervention	Bickham, D.(2006)(215)	7	6 wks	HIE (Running: 3 s/wk; 4 x (14:30 x 40-100m; Density: 1:5/3); Rest: 5min)	48h	1	0.24 ± 0.24↓	1	0.36 ± 0.36↓
	Nordsborg, N.(2003)(214)	6	5.5 wks	HIE (Leg extension: 4.2 s/wk; 15 x 60s at 150% VO2max; Rest: 180s)	2h	1	1.1 ± 1.59	1	1.5 ± 2.94
HIE: High Intensity Exercise; wk: week; s/wks: sessions/week; d: days; ↓; Significant decrease compared to Pre.									

Table 3 Effect of high-intensity exercise on SLC16A1 and SLC16A3 (MCT1 and MCT4 genes) mRNA expression in rodents.

	Study	N per group	Animal	Duration	Exercise	Muscle region	Biopsy after exercise	SLC16A1 mRNA	SLC16A3 mRNA
Single-session intervention	Takimoto, M. (2013)(221)	6	Sprague-Dawley rats	single-session	Control	Epitrochlearis and triceps		0.98 ± 0.23	0.99 ± 0.33
					HIE (swimming: 15 x 20s 18% body weight)		5h	2.04 ± 0.46↑	1 ± 0.21
							18h	1.4 ± 0.61	1.47 ± 0.09↑
HIE: High-Intensity Exercise; ↑: Significant increase compared to the control group.									

Table 4 Effect of high-intensity exercise on MCT1 and MCT4 protein content in humans.

	First author	N (women)	Duration	Exercise	Biopsy after exercise	MCT1 protein content		MCT4 protein content	
						PRE	POST	PRE	POST
Single-session intervention	McGinley, C. (2016a)(216)	16	single-session	HIE (Cycling: 7 x 120s at 79% maximal power; Rest: 120s)	0h	0.65 ± 0.5	1.19 ± 0.32↑	0.81 ± 0.77	1.00 ± 0.69↑
					3h		1.23 ± 1.4↑		1.05 ± 0.88↑
					9h		1.19 ± 1.36↑		0.99 ± 0.62↑
					24h		1.52 ± 1.65↑		1.05 ± 1↑
					48h		1.56 ± 1.65↑		1.05 ± 0.85↑
					72h		1.56 ± 2.48↑		1.13 ± 1.35↑
	Bickham, D. (2006)(215)	7	single-session	HIE before 6 wks of training (Running: 110% VO2max until exhaustion)	2h	1	1.5 ± 0.5↑	1	1.1 ± 0.2
			single-session	HIE after 6 wks of training (Running: 110% VO2max until exhaustion)	2h	1.52 ± 0.24	1.74 ± 0.84	1.1 ± 0.18	1.23 ± 0.3
	Bishop, D. (2007)(217)	6 (6)	single-session	HIE (Cycling: 45s at 200% power output at VO2peak)	Immediately after	100	75.94 ± 6.20↓	100	74.08 ± 7.5↓
Training program intervention	Bickham, D. (2006)(215)	7	6 wks	HIE (Running: 3 s/wk; 4 x (14-30 x 40-100m; Density: 1:5/3); Rest: 5min)	48h	1	1.52 ± 0.24↑	1	1.09 ± 0.18↑
	Burgomaster, K. (2007)(203)	8	1 wks	HIE (Cycling: 3 s/wk; 4-6 x 30s ; Rest: 240s)	~72h	100	157.65 ± 53.67	100	139.40 ± 26.23↑
			6 wks				226.42 ± 162.68↑		153.81 ± 39.61↑
	Juel, C. (2004)(208)	6	2 wks	HIE (Leg extension: 3-5 s/wk; 15 x 60s at 150% VO2max; Rest: 180s)	N.S.	100	108 ± 10	100	98 ± 9
			4 wks				138 ± 19		112 ± 7

			7 to 8 wks				115 ± 5↑		111 ± 11
	McGinley, C. (2016b)(220)	8	2 wks	HIE (Cycling: 3 s/wk; 5-15 x 120s at 71% peak power; Rest: 60s)	2-3 days	0,7	0.87↑	1.07	1.08
			4 wks		3 days		1.75↑		1.5↑
		8	2 wks	HIE (Cycling: 3 s/wk; 4-10 x 120s at 98% peak power; Rest: 60)	2-3 days	0,7	0.75↑	1.13	1.19
			4 wks		3 days		1.43↑		1.19
	McGinley, C. (2017)(211)	7(7)	4 wks	HIE (Cyling: 3 s/wk; 4-10 x 30s 121-186% peak power; Rest: 60s)	3 Days	1.05	1.25↑	1.03	1.04
			10 wks				1.40↑		0.86
		7(7)	4 wks	HIE (Cyling: 3 s/wk; 4-10 x 30s 121-186% peak power; Rest: 300s)		1.24	1.15↑	1.04	1.02
			10 wks				1.40↑		0.78
	Mohr, M. (2007)(205)	6	8 wks	HIE (Running: 3-6 s/wk; 15 x 6s at 95% max speed; Rest: 60s)	End of the test	0	29.96 ± 22.88↑	0	10.31 ± 25.26
		7	8 wks	HIE (Running: 3-6 s/wk; 8 x 30s at 130% VO2max; Rest: 90s)		0	28.21 ± 33.46↑	0	12.45 ± 25.22
	Pilegaard, H. (1999)(204)	4	8 wks	Control			100		100
				HIE (Leg extension: 3-5 x 30-60s at 50-120N; Rest: 120s)	48h		170 ± 64↑*		133 ± 20↑*
	Bangsbo, J. (2009)(207)	5	6 to 9 wks	Control		100	95 ± 223.61	100	86 ± 44.72
8 to 12		HIE (Running: 3-4 s/wk; 8-12 x 30s at 95% max speed; Rest: 180s)		N.S.	100	98 ± 37.95	100	90 ± 88.54	

	Bishop, D. (2008)(209)	6(6)	5 wks	HIE (Cycling: 3 s/wk; 6- 12 x 120s at 100% VO2max; Rest: 60s)	0s or 60s	100	96 ± 12	100	119 ± 21
HIE: High-Intensity Exercise; Wks: Weeks; s/wk: sessions per week; N.S.: Not Specified; ↑: Significant increase compared to Pre; ↓: Significant decrease compared to Pre; ↑*: Significant increase compared to control.									

Table 5 Effect of high-intensity exercise on MCT1 and MCT4 protein content in rodents.

	Study	N per group	Animal	Duration	Exercise	Muscle region	Biopsy after exercise	MCT1 protein content	MCT4 protein content
Training program intervention	Horii, N. (2017)(222)	10	Sprague-Dawley rats	6 wks	Control	Red gastrocnemius		1.01 ± 1.42	0.98 ± 0.49
					HIE (Swimming: 4 s/wk; 14 x 20s with 16% body weight; Rest: 10s)		48h	1.68 ± 0.89↑	1.24 ± 0.87↑
					Control	White gastrocnemius		0.66 ± 0.42	1.26 ± 0.74
					HIE (Swimming: 4 s/wk; 14 x 20s with 16% body weight; Rest: 10s)		48h	0.76 ± 0.36	1.61 ± 0.68↑
HIE: High-Intensity Exercise; wks: weeks; s/wk: sessions per week; ↑: Significant increase compared to the control group.									

Table 6 Effect of low/moderate-intensity exercise on SLC16A1 and SLC16A3 (MCT1 and MCT4 genes) mRNA expression in rodents.

	Study	N per group	Animal	Duration	Exercise	Muscle region	Biopsy after exercise	SLC16A1 mRNA	SLC16A3 mRNA
Single-session intervention	Coles, L. (2004)(225)	3 to 13	Sprague-Dawley rats	single-session	Baseline	Red gastrocnemius		100	100
					LMIE (running: 30 min of exercise followed by 30 min rest until 2-h of exercise at 21m/min 15% grade)		0	154.01↑*	120.92 ± 23.53
							5h	167.61 ± 12.28↑*	120.33 ± 74.72
							10h	167.02 ± 14.32↑*	240.89 ± 43↑*
							24h	209.57 ± 75.74↑*	159.92 ± 44.02↑*
					Baseline	White gastrocnemius		100	100
					LMIE (running: 30 min of exercise followed by 30 min rest until 2-h of exercise at 21m/min 15% grade)		0	197.75 ± 25.58↑*	84.27
							5h	134.51 ± 29.68	87.82 ± 20.47
							10h	142.19 ± 23.55	86.64 ± 24.56
							24h	250.94 ± 39.92↑*	116.19 ± 16.38
	de Araujo, G. (2015)(224)	5	C57BL/6J mice	single-session	Control	Red gastrocnemius		1.15 ± 0.19	0.94 ± 0.1
					LMIE (swimming: 25 min at 100% maximal lactate steady state)		Immediately	1.03 ± 0.14	0.89 ± 0.14
							5h	1.04 ± 0.28	0.96 ± 0.18
							10h	0.97 ± 0.05	0.88 ± 0.04
					Control	White gastrocnemius		0.35 ± 0.09	0.7 ± 0.19
					LMIE (swimming: 25 min at 100% maximal lactate steady state)		Immediately	0.62 ± 0.13↑	0.69 ± 0.19
							5h	0.23 ± 0.05	0.46 ± 0.16
							10h	0.24 ± 0.05	0.62 ± 0.175
					Control	Soleus		0.36 ± 0.18	0.52 ± 0.14
					LMIE (swimming: 25 min at 100% maximal lactate steady state)		Immediately	1.09 ± 0.06↑	0.95 ± 0.09↑
							5h	1.18 ± 0.19↑	0.47 ± 0.07
							10h	1.2 ± 0.22↑	0.82 ± 0.17↑
	Forte, L. (2022)(223)	10	Wistar rat	single-session	Control	Gastrocnemius		100 ± 28.28	99.74 ± 38.04
					LMIE (swimming: 37,5 min at 80% of the anaerobic threshold)		4h	120.32 ± 25.71	115.78 ± 52.31

					LMIE (swimming: 33,3 min at 90% of the anaerobic threshold)			202.43 ± 133.69↑	121.8 ± 88.77
					LMIE (swimming: 30 min at 100% of the anaerobic threshold)			160.97 ± 69.41	130.82 ± 53.89
					LMIE (swimming: 27,3 min at 110% of the anaerobic threshold)			186.17 ± 53.99	130.82 ± 31.70
					LMIE (swimming: 25 min at 120% of the anaerobic threshold)			156.91 ± 51.42	119.79 ± 28.53
					Control			99.45 ± 32.22	97.29 ± 30.77
					LMIE (swimming: 37,5 min at 80% of the anaerobic threshold)		Soleus	97.41 ± 15.46	107.02 ± 112.82
					LMIE (swimming: 33,3 min at 90% of the anaerobic threshold)			96.6 ± 58	237.83 ± 280.33
					LMIE (swimming: 30 min at 100% of the anaerobic threshold)			90.89 ± 20.62	89.72 ± 44.44
					LMIE (swimming: 27,3 min at 110% of the anaerobic threshold)			113.31 ± 25.77	74.59 ± 44.44
					LMIE (swimming: 25 min at 120% of the anaerobic threshold)			100.27 ± 30.93	127.56 ± 88.88
					Control		Epitrochlearis and triceps	0.96 ± 0.3	1.01 ± 0.19
					LMIE (swimming: 6 h in two 3-h sessions separated by 45 min of rest)			5h 3 ± 1.94↑	1.83 ± 0.44↑
								18h 1.56 ± 0.8	1.29 ± 0.37
Training program intervention	Saxena, S.(2016)(226)	8	Sprague-Dawley rats	2 wks	Control	Red gastrocnemius		809.08 ± 66.72	4145.5 ± 266.91
					LMIE (Swimming: 6 s/wk; 60min)		1 day	7089.89 ± 375.34↑	2577.38 ± 158.48↓
			Wistar rats	Wk 0				100.23 ± 84.11	101.41 ± 31.91

	Scariot, P.(2016)(2 27)	7 to 10		12 wks	Control	White gastrocnemiu s		25.93 ± 10.51↓*	169.5 ± 31.91↑*
					LMIE (Swimming: 7 s/wk; 40 min at 80% of the anaerobic threshold)		48h	34.34 ± 14.71↓*	185.1 ± 29.78↑*
				Wk 0		Soleus		99.74 ± 111.08	99.22 ± 23.25
				12 wks	Control			152.64 ± 34	71.31 ± 32.55
					LMIE (Swimming: 7 s/wk; 40 min at 80% of the anaerobic threshold)		48h	189.67 ± 34↑	113.17 ± 51.16
				LMIE: Low/Moderate-Intensity Exercise; wks: weeks; s/wks: sessions per week; ↑: Significant increase compared to control group; ↓: Significant decrease compared to control group; ↑*: Significant increase compared to baseline; ↓*: Significant decrease compared to baseline					

Table 7 Effect of low/moderate-intensity exercise on MCT1 and MCT4 protein content in humans.

	Study	N (women)	Duration	Exercise	Biopsy after exercise	MCT1 protein content		MCT4 protein content	
						PRE	POST	PRE	POST
Single-session intervention	Green, H.(2002)(218)	8	single-session	LMIE (Cycling: 300-360 min at 60%VO ₂ max)	2 days	100	121 ± 17.54↑	100	120 ± 23.48↑
					4 days		143 ± 31.11↑		137 ± 39.6↑
					6 days		114 ± 26.30↑		114 ± 27.72↑
Training program intervention	Bonen, A.(1998)(212)	7	1 wk	LMIE (Cycling: 7 s/wk; 120min at 65% VO ₂ max)	1 day	100	117.87 ± 23.17↑		
	Dubouchaud, H.(2000)(210)	9	9 wks	LMIE (Cycling: 6 s/wk; 50- 60 min at HR corresponding to 75 % VO ₂ peak	N.S.	6.86 ± 1.81	13.17 ± 2.36↑	5.12 ± 3.15	7.21 ± 5.43
	Neal, C.(2013)(213)	6	6 wks	LMIE (Cycling: 5-6 s/wk; 60 min at power output half-way between the LT and the LTP [3 s/wk]; ~240 min/wk < lactate threshold)	Week after training	100	110 ± 13	100	180 ± 41↑
LMIE: Low/Moderate-Intensity exercise; wk: week; s/wk: sessions per week; N.S.: Not Specified; ↑: Significant increase compared to PRE									

Table 8 Effect of low/moderate-intensity exercise on MCT1 and MCT4 protein content in animals.

	Study	N per group	Animal	Duration	Exercise	Muscle region	Biopsy after exercise	MCT1 protein content	MCT4 protein content
Single-session intervention	Coles, L. (2004)(25)	3 to 13	Sprague-Dawley rats	single-session	Baseline	Soleus		100	100
					LMIE (running: 30 min of exercise followed by 30 min rest until 2-h of exercise at 21m/min 15% grade)		0	122.48 ± 44.48	198.97 ± 68.09↑*
							5h	137.91 ± 35.92	332.53 ± 188.58↑*
							10h	178.18 ± 22.23↑*	422.94 ± 172.88↑*
							24h	154.69 ± 41.04↑*	325.34 ± 133.56↑*
					Baseline	Red gastrocnemius		100	100
					LMIE (running: 30 min of exercise followed by 30 min rest until 2-h of exercise at 21m/min 15% grade)		0	161.4 ± 58.17↑*	142.46↑*
							5h	250 ± 171.09↑*	198.97 ± 62.87↑*
							10h	258.05 ± 68.42↑*	231.84 ± 34.06↑*
							24h	118.45 ± 63.30	207.19 ± 62.84↑*
					Baseline	White gastrocnemius		100	100
					LMIE (running: 30 min of exercise followed by 30 min rest until 2-h of exercise at 21m/min 15% grade)		0	154.69 ± 42.78↑*	107.53
							5h	276.84 ± 41.07↑*	96.23
							10h	293.62 ± 87.26↑*	109.58
							24h	201 ± 53.05↑*	109.58
	Eydoux, N. (2000)(28)	10	Wistar rat	single-session	Baseline	Soleus		52.57 ± 7.63	
					LMIE (Running: 30 min at 25m/min with 8% grade)		N.S.	55.75 ± 10.04	
					Control	Red tibialis anterior		30.45 ± 6.02	
					LMIE (Running: 30 min at 25m/min with 8% grade)		N.S.	33.18 ± 6.41	
					Control	White gastrocnemius		11.96 ± 8.04	
					LMIE (Running: 30 min at 25m/min with 8% grade)		N.S.	10.6 ± 5.22	
		10			Control	Gastrocnemius		1	1

	Kim, T. (2019)(2 29)		Sprague- Dawley rats	single- session	LMIE (Swimming: 10% body weight until fatigue)		N.S.	1.82 ± 0.1↑	1.87 ± 0.18↑
Training program intervention	Baker, S. (1998)(2 32)	4 to 5	Sprague- Dawley rats	Control		Soleus		100	
				0 wks	LMIE (Running: 5 s/wk; 60min at 21m/min with 8% grade)		N.S.	100.55 ± 23.6	
				1 wks				101.11 ± 4.96	
				2 wks				98.33 ± 14.9	
				3 wks				102.77 ± 16.14	
				Control		Red gastrocnemius		100	
				0 wks	LMIE (Running: 5 s/wk; 60min at 21m/min with 8% grade)		N.S.	102.22 ± 26.08	
				1 wks				98.33 ± 9.93	
				2 wks				111.11 ± 13.66	
				3 wks				110.55 ± 14.90	
				Control		Extensor digitorum longus		100	
				0 wks	LMIE (Running: 5 s/wk; 60min at 21m/min with 8% grade)		N.S.	99.32 ± 17.32	
				1 wks				91.02 ± 22.27	
				2 wks				105.96 ± 16.08	
				3 wks				104.3 ± 9.89	
				Control		White gastrocnemius		100	
				0 wks	LMIE (Running: 5 s/wk; 60min at 21m/min with 8% grade)		N.S.	100.43 ± 16.08	
				1 wks				91.02 ± 24.74	
				2 wks				106.52 ± 13.61	
				3 wks				112.608↑	
				Control		Soleus		100	
				0 wks	LMIE (Running: 5 s/wk; 60min at 31m/min with 15% grade)		N.S.	100.55 ± 23.6	
				1 wks				130.55 ± 17.39↑	
				2 wks				147.22 ± 9.93↑	
				3 wks				171.66 ± 29.81↑	

				Control		Red gastrocnemius	N.S.	100	
				0 wks	LMIE (Running: 5 s/wk; 60min at 31m/min with 15% grade)			102.22 ± 26.08	
				1 wks				134.44 ± 18.63↑	
				2 wks				183.33 ± 19.87↑	
				3 wks				197.22 ± 23.6↑	
				Control		Extensor digitorum longus	N.S.	100	
				0 wks	LMIE (Running: 5 s/wk; 60min at 31m/min with 15% grade)			99.32 ± 17.32	
				1 wks				97.66 ± 12.37	
				2 wks				102.09 ± 12.37	
				3 wks				100.43 ± 12.37	
				Control		White gastrocnemius	N.S.	100	
				0 wks	LMIE (Running: 5 s/wk; 60min at 31m/min with 15% grade)			100.43 ± 16.08	
				1 wks				98.22 ± 55.68	
				2 wks				98.22 ± 37.12	
				3 wks				103.2 ± 35.88	
	Kim, S. (2011)(231)	8	Goto-Kakizaki rats (diabetes)	6 wks	Control	Soleus	N.S.	100.38	99.61
					LMIE (Running: 5 s/wk; 50 min at 21m/min)			137.93 ± 6.5↑	142.52 ± 21.67↑
					Control	Plantaris	N.S.	100.38	99.61
					LMIE (Running: 5 s/wk; 50 min at 21m/min)			131.55 ± 6.45↑	113.3 ± 4.3
	Saxena, S. (2016)(226)	8	Sprague-Dawley rats	2 wks	Control	Red gastrocnemius	1 day	925.61 ± 52.59	2424.49 ± 126.22
					LMIE (Swimming: 6 s/wk; 60min)			4675.43 ± 378.66↑	830.95 ± 47.33↓
	Takahashi., K.	7 to 9	ICR mice	3 wks	Control	Soleus	24h	0.65 ± 0.17	0.65 ± 0.19
					LMIE (running: 7 s/wk; 60 min at 20m/min)			0.68 ± 0.23	0.73 ± 0.23

	(2019)(230)				Control	Plantaris		0.64 ± 0.11	0.65 ± 0.1
					LMIE (running: 7 s/wk; 60 min at 20m/min)		24h	$0.74 \pm 0.12\uparrow$	0.62 ± 0.12
	Takahashi, K. (2022)(233)	10	Mice	5 wks	Control	Soleus		0.97 ± 0.27	0.97 ± 0.56
					LMIE (Running: 5 s/wk; 60 min at 15–20 m/min)		24h	0.95 ± 0.26	$1.36 \pm 0.6\uparrow$
					Control	Plantaris		0.98 ± 0.26	0.98 ± 0.12
					LMIE (Running: 5 s/wk; 60 min at 15–20 m/min)		24h	$1.38 \pm 4.75\uparrow$	0.97 ± 0.15
	Metz, L. (2005)(234)	8	male Wistar rats	10 wks	Control	Soleus		99.62 ± 27.75	98.8 ± 19.81
			male Zucker fa/fa rats (insuline resistance)				N.S.	89.66 ± 46.68	57.81 ± 39.63
			male Zucker fa/fa rats (insuline resistance)		LMIE (Running: 5 s/wk; 60min at 25m/min with 5% grade)			108.84 ± 22.29	72.87 ± 22.29
			male Wistar rats		Control	Red tibialis anterior		99.62 ± 39.11	99.32 ± 32.2
			male Zucker fa/fa rats (insuline resistance)				N.S.	$62 \pm 21.44\downarrow$	$56.23 \pm 22.79\downarrow$

			male Zucker fa/fa rats (insuline resistanc e)		LMIE (Running: 5 s/wk; 60min at 25m/min with 5% grade)			73.01 ± 24.81	65.51 ± 8.42↓		
			male Wistar rats		Control			White tibialis anterior	N.S.	99.77 ± 23.55	98.27 ± 16.84
			male Zucker fa/fa rats (insuline resistanc e)							34.2 ± 32.38↓	64.29 ± 11.89↓
			male Zucker fa/fa rats (insuline resistanc e)							47.43 ± 62.24	64.29 ± 8.42↓
	Takeda, R. (2022)(235)	7	C57BL6/J mice	6 wks	Control	Gastrocnemius	24h	0.98 ± 0.4	0.96 ± 0.16		
					LMIE (Running: 5 s/wk; 30 min at 20-25m/min)			1.01 ± 0.26	0.82 ± 0.19↓		
					Control	Soleus	24h	0.98 ± 0.27	0.97 ± 0.19		
					LMIE (Running: 5 s/wk; 30 min at 20-25m/min)			0.85 ± 0.22	0.79 ± 0.2↓		
					Control	Plantaris	24h	0.98 ± 0.45	0.93 ± 0.28		
					LMIE (Running: 5 s/wk; 30 min at 20-25m/min)			1.01 ± 0.38	0.83 ± 0.24		
	Suzuki, J. (2022)(236)	11 to 12	MCH(ICR)/jcl mice	4 wks	Control	Soleus		Percentile 25th:0.83/50th:0.99/75th:1.15	Percentile 25th:0.85/50th:0.89/75th:1.11		

					LMIE (Running: 6 s/wk; 75 min at 18m/min with 5 (π/180) incline increasing 1 m/min every 3 days)		48h	Percentile 25th:0.88/50th:1.08/75th:1.28	Percentile 25th:0.64/50th:0.75/75th:1.61	
					Control		Red gastrocnemius		Percentile 25th:0.56/50th:0.9/75th:1.35	Percentile 25th:0.98/50th:1.02/75th:1.78
					LMIE (Running: 6 s/wk; 75 min at 18m/min with 5 (π/180) incline increasing 1 m/min every 3 days)			48h	Percentile 25th:0.53/50th:0.69/75th:0.99	Percentile 25th:0.86/50th:0.87/75th:1.06
					Control		White gastrocnemius		Percentile 25th:1.09/50th:1.33/75th:1.54	Percentile 25th:0.5/50th:0.76/75th:1.45
					LMIE (Running: 6 s/wk; 75 min at 18m/min with 5 (π/180) incline increasing 1 m/min every 3 days)			48h	Percentile 25th:1.41/50th:1.61/75th:1.82	Percentile 25th:0.58/50th:0.85/75th:1.58
					Control		Plantaris		Percentile 25th:0.79/50th:0.98/75th:1.22	Percentile 25th:0.75/50th:0.8/75th:1.22
					LMIE (Running: 6 s/wk; 75 min at 18m/min with 5 (π/180) incline increasing 1 m/min every 3 days)			48h	Percentile 25th:1.11/50th:1.25/75th:1.35	Percentile 25th:0.83/50th:0.88/75th:1.08
					LMIE: Low/Moderate-Intensity Exercise; wk: week; s/wk: sessions per week; N.S.: Not Specified; ↑: Significant increase compared to control; ↓: Significant decrease compared to control; ↑*: Significant increase compared to baseline					

Table 9 Effect of other types of exercise on SLC16A1 and SLC16A3 (MCT1 and MCT4 genes) mRNA expression in humans.

	Study	N (women)	Duration	Exercise	Biopsy after exercise	SLC16A1 mRNA		SLC16A3 mRNA	
						PRE	POST	PRE	POST
Training program intervention	Millet, G.(2014)(206)	18	3 wk	HIE plus LMIE (Cycling: 5 s/wk; 60 min at 60% VO ₂ peak and 3 x (2 x 120s at 100% peak power); Rest: 120s)	Week after training	0.37 ± 0.22	0.72 ± 0.44	0.004 ± 0.007	0.007 ± 0.005
HIE: High-Intensity Exercise; LMIE: Low/Moderate-Intensity Exercise; wk: week; s/wk: sessions per week; N.S.: Not specified; ↓: Significant decrease compared to PRE									

Table 10 Effect of other types of exercise on SLC16A1 and SLC16A3 (MCT1 and MCT4 genes) mRNA expression in rodents.

	Study	N per group	Animal	Duration	Exercise	Muscle region	Biopsy after exercise	SLC16A1 mRNA	SLC16A3 mRNA
Training program intervention	Bonen, A. (2000)(238)	3 to 10	Sprague-Dawley rats		Control	Red tibialis anterior		163.2 ± 15.67	164.36 ± 10.68
				1 wks	Electrostimulation (24h, 10 Hz, 50 μs duration)		N.S.	161.6 ± 8.31	137.45 ± 11.33↓
				3 wks				196.8 ± 36.02↑	164.36 ± 6.29
					Control	White tibialis anterior		102.4 ± 15.67	165.09 ± 8.9
				1 wks	Electrostimulation (24h, 10 Hz, 50 μs duration)		N.S.	136.8 ± 13.85↑	143.27 ± 35.27↓
				3 wks				116 ± 15.24↑	164.36 ± 8.81
	Lima, T. (2020)(241)	18	male Fischer rats	4 wks	Control	Gastrocnemius		0.71 ± 0.16	
					VPE (Running: 8090,45 ± 2203,03 min/4 wks)		48h	1.29 ± 0.47↑	
			male Fischer rats (hipertension)		Control			0.89 ± 0.12	
					VPE (Running: 8090,45 ± 2203,03 min/4 wks)		48h	1.32 ± 0.5↑	
VPE: Voluntary physical exercise; wk: week; s/wk: sessions per week; N.S.: Not Specified; ↑: Significant increase compared to control group; ↓: Significant decrease compared to control group; ↑*: Significant increase compared to baseline; ↓*: Significant decrease compared to baseline									

Table 11 Effect of other types of exercise on MCT1 and MCT4 protein content in humans.

	Study	N (women)	Duration	Exercise	Biopsy after exercise	MCT1 protein content		MCT4 protein content	
						PRE	POST	PRE	POST
Training program intervention	Millet, G.(2014)(206)	18	3 wks	HIE plus LMIE (Cycling: 5 s/w; 60 min at 60% VO2peak and 3 x (2 x 120s at 100% peak power); Rest: 120s)	Week after training	2.28 ± 0.62	2.38 ± 0.63	1.55 ± 0.56	1.32 ± 0.61
	Neal, C.(2013)(213)	6	6 wks	HIE and LMIE (Cycling: HIE= 3s/wk; 6 x 240s at 5- 10% above lactate turnpoint; Rest: 120s; LMIE= ≈290 min/wk < lactate threshold)	Week after training	100	112 ± 13	100	233 ± 56↑
	Juel, C.(2004)(219)	10	6 wks	Control			100 ± 31.61		100 ± 34.98
		10		Strength (Leg press, knee extension, hamstring curl: 3 s/w; 3-4 sets of 10- 12 reps at 50-80% RM; Rest: 90s)	16h		149.16 ± 58.13↑*		132.20 ± 47.53↑*
		7 (Diabetes)		Control			65.52 ± 35.35		93.9 ± 60.03
		7 (Diabetes)		Strength (Leg press, knee extension, hamstring curl: 3 s/w; 3-4 sets of 10- 12 reps at 50-80% RM; Rest: 90s)	16h		114.09 ± 43.88↑*		99.66 ± 47.17
HIE: High-Intensity Exercise; LMIE: Low/Moderate-Intensity Exercise; wk: week; s/w: sessions/week; ↑: Significant increase compared to PRE; ↓: Significant decrease compared to PRE. ↑*: Significant increase compared to control group; ↓*: Significant decrease compared to control group.									

Table 12 Effect of other types of exercise on MCT1 and MCT4 protein content in animals.

	Study	N per group	Animal	Duration	Exercise	Muscle region	Biopsy after exercise	MCT1 protein content	MCT4 protein content
Single-session intervention	Tonouchi, M. (2002)(237)	13	Sprague-Dawley rats	single-session	Control	N.S. (muscle giant vesicle)		100	100
					Electroestimulation (2x 5 (50-60V, stimulation rate 100Hz, train delay<0,01ms, train duration 200ms, pulse duration 100µs) interrupted by a 1min rest)		N.S.	81.44 ± 22.3↓	60.05 ± 26.94↓
					Control	N.S. (muscle sarcolemma giant vesicle)		100	100
					Electroestimulation (2x 5 (50-60V, stimulation rate 100Hz, train delay<0,01ms, train duration 200ms, pulse duration 100µs) interrupted by a 1min rest)		N.S.	99.69 ± 8.16	79.75 ± 12.68↓
Training program intervention	Bonen, A.(2000)(238)	3 to 10	Sprague-Dawley rats	Control		Red tibialis anterior		460.64±73.44	549.19 ± 113.88
				1 wks	Electrostimulation (24h, 10 Hz, 50 µs duration)		N.S.	816.77 ± 174.32↑	522.18 ± 31.18
				3 wks		983.22 ± 441.44↑		535.69 ± 171.12	
				Control		White tibiales anterior		201.29 ± 61.2	571.7 ± 99.64
				1 wks	Electrostimulation (24h, 10 Hz, 50 µs duration)		N.S.	584.51 ± 216.39↑	495.17 ± 70.46
				3 wks				298.06 ± 51.93↑	531.18 ± 80.52
	McCullagh, K.(1997)(239)	6	Sprague-Dawley rats	1 wks	Control	Red tibialis anterior		44.82 ± 7.11	
					Electrostimulation (10Hz, 50micros, 24h/d during 7d)		N.S.	68.23 ± 10.22↑	

					Control	White tibialis anterior		19.23 ± 5.77	
					Electrostimulation (10Hz, 50micros, 24h/d during 7d)		N.S.	$55.89 \pm 7.55\uparrow$	
					Control	Extensor digitorum longus		42.1 ± 6.22	
					Electrostimulation (10Hz, 50micros, 24h/d during 7d)		N.S.	$81.85 \pm 12.44\uparrow$	
	Kitaoka, Y.(2015)(240)	5	Sprague-Dawley rats	4 wks	Control	Gastrocnemius		100.66 ± 20.07	100.22 ± 18.92
					Electrostimulation (5 sets of 10 x 3s (30V and 100Hz); Rest: 7s between contractions)		48h	109.57 ± 4.98	$124.72 \pm 16.93\uparrow$
	Yoshida, Y.(2004)(242)	6 to 7	ICR mice	1 wks	Control	Plantaris		100 ± 48.98	98.55 ± 49.69
				1 wks	VPE (Mice ran freely in the wheel)		N.S.	106.31 ± 41.25	121.73 ± 30.76
				3 wks	Control			100 ± 51.56	99.51 ± 54.43
				3 wks	VPE (Mice ran freely in the wheel)		N.S.	102.1 ± 41.25	68.59 ± 21.29
				6 wks	Control			100 ± 38.67	98.55 ± 16.56
				6 wks	VPE (Mice ran freely in the wheel)		N.S.	$148.42 \pm 20.62\uparrow$	101.44 ± 21.29
				1 wks	Control	Tibialis anterior		100 ± 54.14	96.61 ± 35.49
				1 wks	VPE (Mice ran freely in the wheel)		N.S.	128.42 ± 54.14	122.7 ± 30.76
				3 wks	Control			100 ± 46.41	98.55 ± 52.06
				3 wks	VPE (Mice ran freely in the wheel)		N.S.	116.84 ± 33.51	141.06 ± 82.83

				6 wks	Control			100 ± 41.25	96.61 ± 21.29	
				6 wks	VPE (Mice ran freely in the wheel)		N.S.	144.21 ± 51.56↑	113.04 ± 18.93	
				1 wks	Control		Soleus		100 ± 70.35	
				1 wks	VPE (Mice ran freely in the wheel)			N.S.	125.53 ± 45.6	
				3 wks	Control				100 ± 39.08	
				3 wks	VPE (Mice ran freely in the wheel)			N.S.	96.8 ± 13.02	
				6 wks	Control				100 ± 39.08	
				6 wks	VPE (Mice ran freely in the wheel)			N.S.	79.78 ± 15.63	
	Scariot, P.(2022)(243)	10	C57BL/6J mice	8 wks	Low physically active	Soleus		0.038 ± 0.033	0.043 ± 0.013	
					Low physically active+LMIE (5 s/w; 40 min at 80% critical velocity)		48h	0.049 ± 0.031	0.052 ± 0.02	
					High physically active			0.037 ± 0.024	0.048 ± 0.01	
					Low physically active+LMIE (5 s/w; 40 min at 80% critical velocity)		48h	0.044 ± 0.03	0.006 ± 0.006↓	

HIE: High-Intensity Exercise; LMIE: Low/Moderate-Intensity Exercise; VPE: Voluntary Physical Exercise; wk: week; s/w: sessions/week; N.S.: Not specified; ↑: Significant increase compared to control group; ↓: Significant decrease compared to control group.

DISCUSSION

This study systematically reviews the scientific literature on the impact of exercise on MCT1 and MCT4 protein content and mRNA expression of their respective genes in muscle tissue, to know: 1) the effect of exercise considering its intensity and duration of the intervention (single-session vs training program), 2) including human and rodent studies to observe whether both models provide aligned results, and 3) mentioning the methodological aspects that should be improved in futures studies. Our main results are that physical exercise is a stimulus that modifies protein levels in skeletal muscle in humans. Concretely, MCT1 protein content increases both after a single exercise session and after a training program in humans. Increases in MCT4 protein content can be observed after a training program in humans. Protein content responsiveness is higher for MCT1 than for MCT4, and most of the results indicate the same tendency regardless exercise characteristics. However, it still remains to be better defined what responses occur with each intensity and duration. On the other hand, the effect of exercise on mRNA results is less defined and more research is needed in this aspect in humans. In this line, the present review has pointed out that this new research should be developed with more robust designs and higher levels of clinical evidence since high-quality studies in this area are sparse. Moreover, results in animals only agree with humans results on the observed increase in MCT1 levels after a training program.

Effect of the methodological characteristics of human studies in the results and future methodological considerations

After performing the systematic review and reading the included studies in depth, we have observed methodological and design weaknesses, some of which have made impossible to perform a quantitative analysis (i.e. meta-analysis), as well as hindering the drawing of robust conclusions. Furthermore, practical comments and recommendations will be made in order to improve future research. Firstly, most of the selected studies did not include a control group (75%). Absence of a control group in most of the studies prevented to perform a quantitative analysis in this systematic

review. In this sense, the ideal study to test the effects of a single-session stimulus would be a randomized parallel design with two groups, one group performing exercise and the other group performing a control situation. Moreover, studies that want to observe the effect of an exercise program should perform a randomized control trial with an intervention group and a control group. Measurements should be performed in both groups, which did not occur in one of the included studies, in which only the experimental group was measured (203). Moreover, and contrary to some studies in which both the control and experimental groups performed an exercise intervention (205–207), we consider the control group should perform an intervention that does not provoke metabolic changes in the muscle, ideally not performing any type of physical activity. Other types of designs, like the one which opted to exercise one leg and take the other non-exercising leg as a control (204) are certainly not the best design. This is because the exercising leg causes systemic changes that likely affect the non-exercising leg. For example, the increase in blood lactate concentration caused by the exercising leg could affect the MCT1 response of the non-exercising leg (175).

Timing of the biopsies in the included studies was very heterogeneous, varying from 0 min to a week after the end of the exercise. As already pointed out, this is one of the key aspects (244), since the kinetics of *SLC16A1/SLC16A3* mRNA and MCT1/MCT4 protein levels seems to change over time after exercise. McGinley & Bishop reported that both *SLC16A1* and *SLC16A3* mRNA peaked 9h after exercise, whereas MCT proteins behaved differently from each other. More specifically, MCT1 protein levels was greater at 24-72h compared with the first 9h after exercise, contrary to MCT4 protein which levels did not change over the 72 h measurement period (216). Hence, to investigate the effect of a single exercise session on MCTs response we suggest extracting several biopsies after exercise from 0 min up to 72h after exercise, instead of performing only a single biopsy. The kinetics of the response of these two parameters (mRNA expression and protein level change) is a process that is not momentary but extends over time, so only one biopsy after exercise may proportionate a wrong picture of the whole process.

On the other hand, if the purpose is to investigate the effect of a training program, it is recommended to extract the biopsy far after the last exercise session, to avoid interferences from this last session. It has been previously suggested to measure at least 24h after exercise and at several time points (216). However, if the effects of the last exercise session can last up to 72h as shown above, we recommended to perform at least one measurement from 72h onwards.

Another important consideration is the way the results are reported, the most common being the presentation of post-exercise results relative to pre-exercise values (65% of the studies) (203,205,207–209,212–215,217,218). However, those studies expressing the response relative to the value before exercise did not report any measure of variability in the value before exercise, what leads to two problems. On the one hand, artificially reducing the dispersion of the pre-value increases the likelihood of finding significant differences between two means, what could misrepresent the actual results. On the other hand, the lack of a measure of variability in the pre-exercise value was another reason why it was impossible for us to carry out a quantitative analysis. We therefore propose to present the results relative to an internal control, as several studies have already done (206,211,216,219,220). In case of mRNA expression, we consider that the internal control should be a stable mRNA house-keeping expression. On the other hand, in case of protein level, we consider that the internal control should be an internal standard loaded in each gel, and each lane should be normalized to this value.

Last but not least, we consider imperative highlight the fact that less than 15% of the sample was composed by female participants. Such an excessive decompensation implies that the data available so far are only applicable to males. Sadly, this is in line with the publication trends in Sport Sciences, where females are still underrepresented (245,246). Thus, we encourage to follow the warnings made by some experts in various publications on the need to extend the studies to females (247,248), even more when we already have

data available indicating a possible dysmorphism in the response of monocarboxylate transporters, since sex hormones influence MCTs levels in rat skeletal muscle (249). Moreover, this consideration is especially important in the case of muscle MCTs, since they are dependent on the type of muscle fiber and the proportion of muscle fibers is different according to sex (250).

Therefore, although some methodological aspects have been properly carried out in the studies up to date, such as the homogeneity of the techniques used to measure protein levels and mRNA or the maintenance of the biopsy location, other should be considered to potentially alleviate the limitations described above. All of these recommendations have been already conducted by previous studies meaning that, although challenging, their implementation is feasible. In brief, these recommendations are: the inclusion of a control group with a non-effect intervention on mRNA or protein levels; the measurement at several time points (i.e. several biopsies), far from the last session when studying the effect of a training program, as in (216); reporting the values relative to an internal control instead of relative to pre values, as in (251); and the inclusion of more females as participants in future investigations, as in (211).

Effect of exercise on *SLC16A1/SLC16A3* mRNA and MCT1/MCT4 protein levels

Exercise is a complex stimulus, composed of several characteristics that model the final load of the intervention. Therefore, to thoroughly understand the effect of it, researchers should separately analyze the effect of each factor and then combining that knowledge to comprehend the responses that a particular intervention will elicit. From the existing literature reviewed here, the characteristics that we considered most relevant in order to determine the response were the intensity (low/moderate vs high intensity) and the duration of the intervention (single session vs. training program).

Influence of the exercise intensity

Since only two studies with high intensity (214,215) and no studies with low/moderate intensity have been performed in humans, it is complicated to

draw a pattern for mRNAs expression according to exercise intensity, regardless the duration of the intervention. However, data available to date suggest that high-intensity exercise in human males does not appear to affect mRNA expression in muscle MCT genes. The data in rodents are hardly comparable to human, since the vast majority have implemented low/moderate exercise intensity. Nonetheless, in rodent the behavior seems to be a more responsiveness of *SLC16A1* compared to *SLC16A3*, in addition to a fiber-specific response, which fits the distribution of the related proteins (i.e. MCT1 in more oxidative and MCT4 in more glycolytic fibers). Nevertheless, the discussion of the effect of intensity on mRNAs responses is conditioned by the fact already mentioned, that all the works in humans employed high-intensity exercise whereas in rodents the majority uses low/moderate-intensity exercise. Therefore, the present review reveals the lack of diversity in exercise protocols design among studies, which leads to a biased knowledge of the response to exercise of mRNA of muscle *SLC16As*. At this point, we strongly encourage the researchers to apply and compare a greater diversity of training and exercise characteristics in their future studies, in order to fulfil this gap in knowledge.

Regardless of intervention duration, the percentage of studies finding a significant increase in MCT1 protein content after high-intensity exercise is very similar to the percentage of low/moderate intensity exercise studies (around 70% in both cases). Therefore, the current data seem to indicate that, in the case of aiming to increase MCT1 protein levels, both low/moderate and high-intensity exercise could be a good strategy, even if only a single exercise session is applied. Regarding MCT4 protein content, the percentage of studies performing low/moderate-intensity exercise observing increases in MCT4 protein content is similar to the percentage of studies performing high-intensity exercise (around 50% in both cases). Even gathering interventions by exercise intensity, the responsiveness of the MCT4 protein content appears to remain lower than MCT1. Therefore, and contrary to what is generally believed (i.e., that high intensity is required to increase MCT4 protein content), changes in MCT4 protein content are similar between low/moderate

and high-intensity exercise. Traditionally, we have assumed that MCT1 protein levels increased with both low/moderate- and high-intensity exercise, while MCT4 protein levels increased only with high-intensity exercise. This assumption is probably because MCT1 is more present in type I fibres (presumably activated by both moderate- and high-intensity exercise), whereas MCT4 is more present in type II fibres (presumably activated only at high intensity). Our review shows results in line with the fact that MCT1 protein levels increase with both intensities. However, when we compiled all the studies in the literature we found that MCT4 protein levels do not necessarily increase with high-intensity exercise exclusively, since we found studies that observed an increase in MCT4 protein content after a low/moderate-intensity training program (210). Our main hypothesis is that MCT4-mediated lactate release occurs at both intensities and that an increase in MCT4 levels may be caused by this demand. Actually, in a previous excellent review aligned results were found (175).

The only two studies combining low/moderate and high intensity exercise (206,213) reported no differences. Although we cannot propose any explanation for their findings because they may be conditioned by the timing of the biopsy (week after training) or the training status of the participants (active/trained); we believe that this finding should be mentioned.

On the other hand, most of the studies in rodents involving low/moderate-intensity exercise reported a very heterogeneous response and only one study performed high-intensity exercise showed an increase (222). Hence, it is difficult to establish a pattern according to exercise intensity in rodents. With respect to the comparison between humans and rodents, as with studies investigating mRNA, most human studies use high-intensity exercise while most rodent studies use low/moderate-intensity exercise. So, it is important to take into account the knowledge bias that has been previously mentioned.

Influence of the exercise duration

Although based in scarce studies, single-session interventions with high-intensity do not appear to be sufficient stimulus to generate changes in

mRNA for any of the muscle *SLC16A1* or *SLC16A3* in male humans (no human study utilize low/moderate intensity exercise) (214,215). On the contrary, the four interventions in rodents with single-session interventions show increases at some point both in *SLC16A1* (221,223–225) and *SLC16A3* mRNA (221,224,225). The discrepancies between human and animals could be due to several reasons, like the training status of the sample (training status is not usually evaluated in rodents) or the setting of the exercise intensity (relative intensity in humans vs. absolute intensity in rodents). Furthermore, the way the muscle samples were taken could also affect, since in human were extracted by muscle biopsies while in rodents were extracted after sacrifice. In addition, biopsies in humans were always taken from the same muscle (vastus lateralis), while different muscles with different fiber composition were analyzed in rodents. Finally, an intra-subject study design was carried out in most of the studies in humans, while an inter-subject study design is used in rodents. With only 6 investigations conducted so far and with the substantial methodological differences, it is not prudent to draw conclusions. However, preliminary data suggest that the mRNA response to a training session is different between humans and rodents, the latter being more responsive.

The three studies in men reviewed in this section do not indicate a definite trend for either *SLC16A1* or *SLC16A3* mRNA after a training program, since only Bickham et al. (215) reported changes (i.e., decrease in expression) in both mRNAs after 6 weeks of training. Maybe the other two investigations (206,214) did not reach a sufficient stimulus to generates changes, but further research is needed anyway. In rodents, the data are also insufficient to establish a clear response. However, the analysis of muscles with different fibrillar composition hint at a possible different adaptation of mRNA depending on the abundance of fiber types: in more oxidative muscles (i.e. soleus and red gastrocnemius) an increase in *SLC16A1* and a decrease in *SLC16A3* mRNA are observed, while in more glycolytic muscles (i.e. white gastrocnemius) *SLC16A1* mRNA decreases and *SLC16A3* mRNA increases. Hence, the data available to date do not indicate a homogeneous response,

although it is true that the amount of knowledge in this regard is still scarce. It may be necessary for future studies to take into account other factors, such as the comparison of muscles with different fibrillar composition, the use of a greater training stimulus that generates measurable responses, or the selection of a homogeneous sample in terms of fitness status, since the latter is key to determining the response to a training program.

Regarding protein levels, only Green et al. investigated the effect of a single session at low/moderate intensity exercise in humans and reported increases in both proteins after 2, 4 and 6 days of a 5-6-h cycle at 60% VO₂peak. This suggests that low/moderate intensity exercise is a plausible strategy to increase MCTs protein levels after a single exercise session. On the other hand, it seems that the response of MCT1 and MCT4 protein abundance after a high-intensity session in humans is heterogeneous. We can highlight some interesting details. For instance, the only study with one session in women is the only one reporting decreases (217), implying a possible sexual dimorphism in the MCTs response. Moreover, the biopsy in this case was taken immediately post-exercise, as in the study by McGinley et al. (216). The difference between the study by Bishop et al (217) and the study by McGinley et al. (216) is not only the sex of participants (women and men, respectively), but also the exercise intensity, which was considerably higher in Bishop et al. investigation (i.e. 200% of the power output at VO₂peak) than in the other work extracting the biopsy just after exercise. Finally, when exercised until exhaustion, a higher protein content of MCT1 and no changes in the protein content of MCT4 were reported (215), suggesting that fatigue could affect differently to both transporters. In rodents, the effect of a single exercise session was studied only through low/moderate intensity exercise in three studies, where Eydoux et al. observed an increase in MCT1 protein content swimming until fatigue (228), Kim et al. found no change after running for 30 min (229) and Coles et al. observed an increased after 2 h of exercise composed by 30 min exercise and 30 min rest(225). The different type of exercise and duration of the exercise session are possibly influencing the different response between studies. Contrary, the only study performing electrostimulation

showed a reduced protein levels content of both transporters after a single session.

With multi-session programs in humans, most of the studies reported a significant increase in MCT1 protein content after a training intervention involving different types of training. Even the only study using a strength-training-based program found an increase in both proteins (219). These results are in line with the higher MCT1 protein content observed in well trained subjects compared to less trained subjects (201). It is important to mention that the lack of differences in MCT1 responses reported in some of the included studies could be due to methodological aspects already discussed, like the timing of biopsy. For example, two of the works which failed to find an increase in MCT1 protein content (206,213) are the only studies performing the biopsy a week after the end of the training. This may have implied a too-long period of time and therefore diluting the possible effects achieved with training. Similar results are found in rodents, since most of the studies observed a higher MCT1 protein content in the training groups compared to control groups. Thus, the existing data suggest an increase on muscle MCT1 protein content with regular and periodized exercise, which hints that the increase in this protein is an adaptation to training. This seems to be one of the common adaptations to training, as increased MCT1 protein levels have been linked to improved fatigue index and lactate clearance in humans (201). In addition, inhibition of MCT1 has recently been shown to reduce performance in rodents (252) suggesting this protein is clearly related to performance. Regarding MCT4 protein content, about half of the studies in humans included in the present review showed an increase in this protein with different training programs involving different exercise characteristics, suggesting a less pronounce response compared to MCT1. Results in rodents are elusive on MCT4 protein content because different response are found. This could indicate the MCT4 protein content response is more diverse in rodents compared to humans.

Although the pathways responsible for regulating MCT1 and MCT4 remain to be determined, the current review supports they respond to different stimuli, as previously suggested (211). In this sense, we observe that MCT1 seems to be more responder compared to MCT4 protein content after a training program independently of exercise characteristics. The higher frequency of increases observed in MCT1 protein content point out a greater plasticity of this carrier, which is consistent with its broader localization and greater number of functions (17). Since it is found in most of the tissues and transports a wide spectrum of monocarboxylates (193), a great variety of stimulus could trigger the increase of MCT1 protein content. In addition, the fact that MCT1 can transport lactate and H^+ in both directions (uptake and release) whereas MCT4 is specialized only in lactate/ H^+ release implies *per se* a situation of higher demand for MCT1 (193). In this sense, the different exercise characteristics represent different stimulus that increase MCT1 protein content supporting a putative greater plasticity of this carrier. In contrast, the MCT4 response is less frequent in the studies reviewed. This could be due to, among others, a greater specificity in the stimuli that trigger its change, responding only to more specific exercise characteristics; that the change occurs only in some muscle fibers, given that MCT4 is more present in glycolytic fibers (17); and/or that its changes are more genetically determined.

Regulatory mechanisms of mRNA expression and protein content that could be influenced by exercise

The changes reported by the articles included in this systematic review are driven by regulatory mechanisms that are likely to occur at all steps of the expression pathway, i.e., transcriptional, post-transcriptional, translational, and/or post-translational mechanisms. Nowadays, data regarding these regulation mechanisms are scarce, but we believe it helpful to summarize below the different steps where exercise could have an impact in order to have a general idea of how exercise influences these mechanisms of SLC16A1/SLC16A3 mRNA expression and MCT1/MCT4 protein levels.

Transcriptional Regulation: Exercise can stimulate the transcription of SLC16A1/SLC16A3 genes. Transcription factors activated during exercise may bind to specific regulatory regions in the DNA, promoting gene expression (253,254).

Post-Transcriptional Regulation: Exercise may influence the stability of SLC16A1/SLC16A3 mRNA. Certain RNA-binding proteins or non-coding RNAs can affect the degradation or stabilization of mRNA molecules (254).

Translational Regulation: We hypothesised exercise may modulate the initiation of translation, influencing the rate at which mRNA is translated into protein. Signalling pathways activated during exercise may impact the assembly of the translation initiation complex. To the best of our knowledge, we have not found any studies on this issue, opening the door for future studies to address this possibility.

Protein Synthesis: Acute exercise, especially resistance or high-intensity exercise, can stimulate protein synthesis (255), being MCTs one of the potential proteins synthesised by exercise.

Protein Degradation: Some studies suggest that acute exercise may influence protein degradation pathways (255), potentially preventing degradation of MCTs protein content.

Translocation to the Membrane: Translocation of MCT proteins to the membrane is a crucial step in their functional activation (256). Exercise may influence the translocation process, allowing MCTs to facilitate lactate transport more effectively.

Considerations for future studies

It is worthwhile to mention that the increase in MCT1 protein levels after a training program observed in most of the studies in humans is not preceded by an increase in SLC16A1 mRNA after a single exercise session or a training program, which would be expected as the most likely causal event (257). Therefore, it cannot be considered that an increase in SLC16A1 mRNA predicts an increase in MCT1 protein levels from the studies included in the

present review. Moreover, there is still controversy about whether changes in mRNA with exercise will affect protein abundance (244). In addition, mRNA response is usually a more transient increase over time compared to protein content, which is more stable (244) so results about protein content would be more consistent and should be the main outcome when analyzing training adaptation in humans. In this line, mRNA changes would be a desirable measurement when the focus of research is to unveil the mechanism behind these protein changes (258). The lack of relationship between changes in mRNA and protein seen in our results in humans suggests that post-transcriptional mechanisms are playing a role in the increase of protein content after a training program. A possible mechanism is that MCT1 is continuously demanded by the cell, since transports the greatest variety of monocarboxylates (193). Hence, *SLC16A1* might be expressed continuously and MCT1 protein is formed from the *SLC16A1* mRNA only when the environment is favorable. In this case, exercise could create a favorable situation for the generation of MCT1 protein (i.e., presence of facilitators for translation of mRNA to upregulate protein content). It is interesting to notice that our results in animals do support a greater match between mRNA and protein changes. The higher *SLC16A1* mRNA after exercise observed in most of the studies in animals (whether as a single session or as part of a training program) fits with the increase in MCT1 protein that we observed after most of the training programs. With these results, it is speculative but tempting to suggest different MCT1 regulation mechanisms between humans and rodents.

An important consideration when interpreting the effect of a training program is the suppressing phenomenon in the transcription responses induced by exercise of genes after training. The result of this phenomenon is a gradual decrease in the exercise-induced transcriptional response after a period of training, which has already been demonstrated in other studies (251). This phenomenon could have occurred in the studies of the present review investigating the effect of exercise at the same absolute intensity on mRNA and protein content before and after a training program. A possible

explanation is that adaptation to training cause a lower transcription-inducing signaling pathway (lower homeostatic perturbation, sensor activation, transcription factors and transcription coregulators) by the same exercise session reducing transcription and the subsequent protein content.

Also, futures studies could consider the protein extraction methods for western-blotting (whole lysate vs. membrane extraction). Whole lysate extraction may provide a snapshot of the total protein content, while membrane extraction specifically captures membrane-bound proteins. A previous study compared the effect of a training program (nine weeks of leg cycle endurance training at 75% $\text{VO}_{2\text{peak}}$) on MCT1 and MCT4 protein content in total muscle homogenates and sarcolemma-enriched fractions (210). Their results showed that MCT1 protein content increased after training in both protein extraction methods, while MCT4 protein content only increase in sarcolemma-enriched fractions but not total muscle homogenates. These results suggest that the choice of protein extraction method in western-blotting can significantly impact the results.

Finally, it is interesting to note that the only results coinciding between humans and rodents are when analysing the effect of a training program on MCT1 protein levels. In this case the response observed in most studies is an increase in both species, but in the other comparisons no common pattern is observed. Throughout this review we have noticed some aspects that might explain the differences found between human and rodent studies that are worth summarising. A first, training status is different between humans and rodents, in fact, training status of rodents is not usually reported. Exercise intensity setting is another aspect of controversy, since relative intensity is usually performed in humans and absolute intensity is used in rodents. Furthermore, muscles samples measurement is completely different, while a biopsy is extracted in humans, rodents are always sacrificed. In addition, biopsies in humans were always taken from the same muscle (vastus lateralis), while different muscles with different fiber composition were analyzed in rodents. Finally, an intra-subject study design was developed in

most of the studies in humans, while an inter-subject study design is used in rodents without reporting the sex and the litter.

CONCLUSION

We have provided an extensive review of the current knowledge regarding SLC16A1 and SLC16A3 mRNA and MCT1 and MCT4 protein content in response to single exercise session and training program of different intensities. The studies summarized in this systematic review demonstrate that exercise represents a powerful stimulus to increase MCT1 protein content in humans, regardless of whether a single session or a training program is used. In addition, MCT4 protein level increases can also be observed after a training program, although its responsiveness is lower compared to MCT1. This adaptation (i.e. increments) of both transporters is observed independently of the exercise intensity used, again with increases in MCT1 reported in a greater number of studies than for MCT4. Regarding mRNA changes, increases in MCT1 protein levels observed in humans are not preceded by increases in SLC16A1 mRNA indicating that a SLC16A1 mRNA increment may not predict an increase in MCT1 protein levels. This, together with the absence of changes in *SLC16A3* mRNA following interventions that do report changes in MCT4 levels, points to the existence of regulatory factors that we are not analyzing so far. At this point, and although it could sound cliché, we want to highlight the need for more studies that compare the effect of different intensities and types of exercise, considering all the methodological recommendations mentioned above.

When comparing results of human and rodent, they only coincide for the effect of a training program on MCT1 protein levels, reporting increments in both. Some aspects that may explain these differences could be training status, exercise intensity setting (relative vs. absolute), location of the biopsy, or the study design.

Finally, conducting the systematic review, we have noticed some important methodological aspects that we deem are worth considering to improve the

design of future studies. All of them have already been implemented in previous studies, which confirms their feasibility, and comprises the inclusion of a control group with a non-effect intervention, the measurement at several time points and far from the last session in case of a training program, reporting the values relative to an internal control instead of relative to pre-values and the inclusion of more female participants. It would be desirable that the future studies needed apply these considerations. Thus, the results and conclusions obtained will be more robust and a simple tool such as exercise will be safely used to modify MCTs, whose role is becoming increasingly crucial in clinical practice and in health in general.

4.2. Study II: Differences in plasma and RBC lactate concentration induced by intervallic exercise between genotypes for the T1470A SNP (rs1049434) on the MCT1 (SLC16A1) gene

APPROACH TO THE PROBLEM

Lactate has three main functions in the body: as an energy source, as a gluconeogenic precursor, and it is also considered a signaling molecule with autocrine-, paracrine- and endocrine-like effects, and has been called a “lactormone”(12,196).

For the signaling function it has been highlighted the role of the monocarboxylate transporters (MCTs) as responsible for the lactate exchange between loci of driver processes (i.e. glycolysis and glycogenolysis) and loci of lactate consumption (259). But, to accomplish this last function, lactate must not only be able to move across membranes (through MCTs) but also to reach tissues far away from the producing cells, being the vascular conductance (i.e. blood stream) the mean to achieve it (46,259). In this line, the blood is able to enhance this transport through two mechanisms: 1) the removal of lactate from interstitial fluids facilitates the net transport of lactate from intracellular to extracellular fluids and helps regulate pH in contracting skeletal muscle (260); and 2) during exercise red blood cells (RBC) have been suggested to function as a lactate sink, lowering plasma lactate concentration and increasing the gradient between muscle and plasma lactate concentrations (261). MCT1 (SLC16A1) is crucial in these two mechanisms, since is the major MCT in the RBC membrane (262).

Lactate dynamics between blood compartments have been studied with progressive submaximal exercise (35,44), progressive maximal tests (48,263), maximal exercise bouts (18,38,47,264) and steady state exercise (45) showing, in general, that lactate enters the RBC with some delay. Most of the studies reported lower lactate levels inside the RBC in comparison to plasma lactate concentration during exercise (35,38,44–48,263,264), being nearly half of it

(265). However, all these studies utilized the same estimation for erythrocyte lactate levels. The only study actually measuring erythrocyte lactate concentrations, observed higher values inside the erythrocyte compared to the plasma (18). These differences could be caused by the distinct exercise protocols used or the time moments of measurement (i.e. during exercise, immediately after exercise cessation or during recovery), being the type of exercise one of the most influential factors. In this line, to the best of our knowledge, this lactate distribution in blood compartments has not been described during high-intensity intervallic exercise with low-intensity active recovery in between, which is a frequent situation in many sports activities. Thus, it is unknown whether RBC/plasma lactate ratios would be maintained or change in this type of exercise, and whether RBCs would continue to act as a lactate sink. Describing these ratios, both during this type of intervallic exercise and during subsequent recovery may be important to understand the role that erythrocytes could have, both in the maintenance of the gradient between muscle and plasma lactate concentrations (near the producing muscles) and in the effect of lactate as a signaling agent to other distant organs (196).

Additionally, the data available so far indicate that the functionality of MCT1 may be different between sexes (16), in addition to the different energy metabolism between sexes, where females oxidize proportionally more lipid and less carbohydrate than males (266). These two factors could lead to different responses between males and females in lactate distribution. However, of the works reviewed that analyze blood lactate distribution, all but one has studied only male samples. The only one that includes females (263) investigated a smaller number of them and did not report a split analysis. This is contrary to numerous recent recommendations which encourage to include both male and female participants in similar numbers where possible and to always undertake sex disaggregation of their data, ensuring that research conducted is applicable to all and not just 50% of the population.

Finally, a single-nucleotide polymorphism (SNP), T1470A (rs1049434), has been described for the SLC16A1 gene, leading to an aspartic acid to glutamic acid change at codon 490 (Asp490Glu) (167). The impact of this SNP on females remains inconclusive (168,182,267) but numerous studies show that males carrying the Asp allele (i.e. the T allele) presented altered blood lactate levels during or after exercise, indicating impaired lactate transport by MCT1(168,170,171,268,269). This effect on lactate transport has been experimentally validated as well, with oocytes expressing the Asp (T) allele exhibiting reduced lactate uptake (270).

Therefore, the objective of the present study was to determine if the plasma and RBC lactate concentration changes induced by intervallic exercise differed between genotypes for the T1470A SNP (rs1049434) on the MCT1 (SLC16A1) gene in active males and females.

METHODS

Participants

Twenty males and twenty-one females moderately trained participated in the study (Table 13). Inclusion criteria were: 1) do not present any pathology that could impede the correct performance of the tests or could influence the main study variables; 2) non-smokers; 3) a BMI between 18 and 25 kg/m², 4) a self-reported physical activity of more than five hours per week and 5) answer negatively all the questions of the Physical Activity Readiness Questionnaire (PAR-Q) (271). Participants abstained from drinking alcohol, caffeine and intense exercise for at least 24 hours prior to the tests. Sleep hours were recorded prior to the test to ensure rest. Nutritional status was checked to ensure participants ate a similar diet prior to the tests. All the tests were carried out at the same time of the day to avoid the effect of circadian rhythms (149). Females performed all the tests in the early follicular phase. Prior to participating in the study, informed consent was obtained after a description of the study as well as detailing out any risks and/or benefits. This study was approved by the ethics committee of Technical University of Madrid (2019-044).

Table 13 Descriptive characteristics of the participants

	Males	Females
Age (years)	23.65±1.25	20.38±1.26
Body mass (kg)	71.61±1.35	56.9±1.35
Stature (cm)	177.02±1.2	163.3±1.44
Body mass index (kg/m ²)	22.86±0.39	21.36±0.5
Peak power output (W)	283.25±6.94	190±5.33
Peak power output (W/kg)	3.98±0.11	3.36±0.08
Data are shown as mean ± SE.		

Study design

The study consisted of three visits to the laboratory. On the first visit the volunteers signed the informed consent, height and weight were measured and a capillary blood sample was collected on filter paper for genetic analysis. On the second visit the level of physical activity was estimated through the IPAQ (International Physical Activity Questionnaire) and volunteers performed a maximal incremental test on a cycle-ergometer. On the third visit, an interval test was performed on a cycle-ergometer based on the thresholds determined in the previous maximal incremental test. During the protocol, venous blood was drawn to analyse lactate transport between plasma and erythrocytes. Clinical trials: NCT06104150.

Genotyping

Whole blood samples (5ml) were collected in ethylene-diaminetetraacetic acid (EDTA) from each participant, and DNA was extracted using the "QIAamp® DNA Blood Mini Kit" from QIAGEN (Hilden, Germany). Genotyping of the T1470A polymorphism of the SLC16A1 (MCT1) gene (rs1049434) was performed by polymerase chain reaction (PCR) amplification followed by direct sequencing for the overweight phase of the study (Cupeiro et al., 2010). For the obese phase, the genotyping was carried out using the TaqMan® SNP Genotyping Assays from Applied Biosystem in a StepOne™ Real Time PCR

System (Applied Biosystem, Foster City, CA, USA). Participants were grouped based on the MCT1 genotype following an additive model: Asp/Asp (T/T), Asp/Glu (T/A), and Glu/Glu (A/A), following previous studies. The subjects were classified into three groups according to the MCT1 genetic profile, following an additive model: AA genotype, TA genotype and TT genotype. Heterozygous subjects, those carrying the polymorphism in only one of the two alleles (AT group) were discarded to compare the two most genetically differentiated alleles. This discard was performed because we expected an additive effect of the polymorphism, based on previous studies (33,168,170,182,183). Therefore, the largest differences are expected to be found between the AA and TT genotypes.

Maximal incremental test

The test consisted of a maximal incremental test until exhaustion on a cycle-ergometer (Lode Excalibur Sport, Groningen, The Netherlands) which began with a warm-up of 5 min followed by increments of 35 W every 3 min. The cadence was maintained during the test between 70-90 rpm. The test finished when cadence was below 60 rpm. Capillary blood samples from the earlobe were analyzed at baseline, during the last 30 s of each step and immediately after exercise. Heart response was continuously monitored with the Polar RS800sd (Polar Electro Oy, Kempele, Finland). Lactate threshold 1 (LT1) and 2 (LT2) were evaluated as previously described by (31) and (272), respectively.

Interval test

A catheter was inserted in the antecubital vein before starting the test. The interval test was performed on the same cycle-ergometer. This test consisted of a 5min warm-up at the power output corresponding to 80% of the LT1. Subsequently, participants performed four intervals of 1 min and 30 s at the power output corresponding to 20% above the LT2 with a 5min rest between series at the power output corresponding to 80% of the LT1. The test ended with a final recovery of 20 min at the power output corresponding to 80% of the LT1. Venous blood samples were taken from the catheter in the antecubital vein at baseline, during the 30 s before and after the second, third

and fourth interval and 5 min, 10 min, 15 min and 20 min during the final recovery. Also, capillary blood samples were extracted from the earlobe at baseline, during the 30 s before and after the first, second, third and fourth interval and 10 min and 20 min during the final recovery. Heart rate was continuously monitored with the Polar RS800sd (Polar Electro Oy, Kempele, Finland).

Blood sample treatment

Capillary blood samples were analyzed with the Lactate Plus (Nova Biomedical, Cheshire, UK), calibrations were carried out according to the fabricant. All the venous samples were analyzed with the blood gas analyzer (ABL 80, Radiometer, Copenhagen, Denmark) which was routinely calibrated according to the fabricant. All the samples were processed as previously describe (44) with small modifications. Briefly, a total of 4 ml of blood was drawn in a heparinized tube in each extraction and a subsample was analyzed immediately for whole blood lactate. After this, each sample was immediately capped and submerged in a dry ice-ethanol solution for 5 s and then placed in ice water to minimize transport of lactate between the plasma and erythrocytes (273) for the short time before it was analyzed. After the test, the samples were centrifugated (Biosan LMC-3000 version V.5AD) at 3000 rpm during 15 min to separate plasma from erythrocytes. Just after centrifugation, the plasma was removed and analyzed for plasma lactate. After removing the plasma, the remaining erythrocytes were lysed through three freeze-thaw cycles in a dry ice-ethanol solution. Lysed erythrocytes were then centrifuged at 3000 rpm during 15 min. The lactate concentration of the supernatant was considered to represent erythrocyte lactate.

Data analysis

The lactate ratio was calculated dividing the concentration of lactate in erythrocytes by the lactate concentration in plasma (18,46). The lactate gradient was determined as the lactate concentration difference between plasma and erythrocytes (46).

Statistical analysis

Normal distribution and homogeneity of variance were confirmed using the Shapiro-Wilk test and the Levene's test, respectively. Because the lactate responses to exercise are different between sexes, the sample size estimation has been calculated independently based on data from a previous study (182) using an a priori sample size estimation with a repeated measures ANOVA. In case of males, the following inputs were introduced: Effect size $f=0.45$, $\alpha=0.05$, power=0.8; groups=3, repetitions=3, correlation=0.7, nonsphericity correction=1. Based on these inputs, at least 9 males were required. In case of females, the following inputs were introduced: Effect size $f=0.3$, $\alpha=0.05$, power=0.8; groups=2, repetitions=3, correlation=0.7, nonsphericity correction=1. Based on these inputs, at least 14 females were required. A repeated measures ANOVA (2x2x11) was used to compare lactate concentrations between types of blood analyzed (plasma and erythrocytes) and between genetic profiles (AA and TT genotype) at the different moments of measurement, in females and males separately. Moreover, A repeated measures ANOVA (2x11) was conducted to compared lactate ratio and lactate gradient between genetic profiles (AA and TT genotype) at the different moments of measurement, in females and males separately. ANOVA effect size was calculated by partial eta-squared (η^2_p) and the small, moderate, and large effects corresponded to values equal or greater than 0.01, 0.059, and 0.138, respectively (Cohen, 1988). Bonferroni post hoc tests were conducted where significant differences were found in any of the analyzed factors. Statistical analyses were performed using the statistical software Jamovi V1.6 (Jamovi, Sidney, Australia). Significant level was set at $p<0.05$.

RESULTS

Lactate concentration

In females, lactate concentration was higher in RBC than in plasma ($F(1,8)=16.38$; $p=0.004$; $\eta^2_p=0.672$). Moreover, lactate concentration changed at the different moments of measurement ($F(10,80)=9.73$; $p<0.001$; $\eta^2_p=0.549$). Additionally, similar lactate concentrations were found between genotypes ($F(1,8)=3.61$; $p=0.094$; $\eta^2_p=0.311$). Similar results were found in

males, lactate concentration was higher in RBC than in plasma ($F(1,15)=24.81$; $p<0.001$; $\eta^2_p=0.623$). Moreover, lactate concentration changed at the different moments of measurement ($F(10,150)=23.4$; $p<0.001$; $\eta^2_p=0.609$). Additionally, similar lactate concentrations were found between genotypes ($F(1,15)=1.22$; $p=0.286$; $\eta^2_p=0.075$). Finally, an interaction between type of blood and moment of measurement was found ($F(10,150)=5.21$; $p<0.001$; $\eta^2_p=0.258$). Specifically, lactate concentration was higher in RBC than in plasma at baseline, 10, 15 and 20 min after 4 interval (Figure 11).

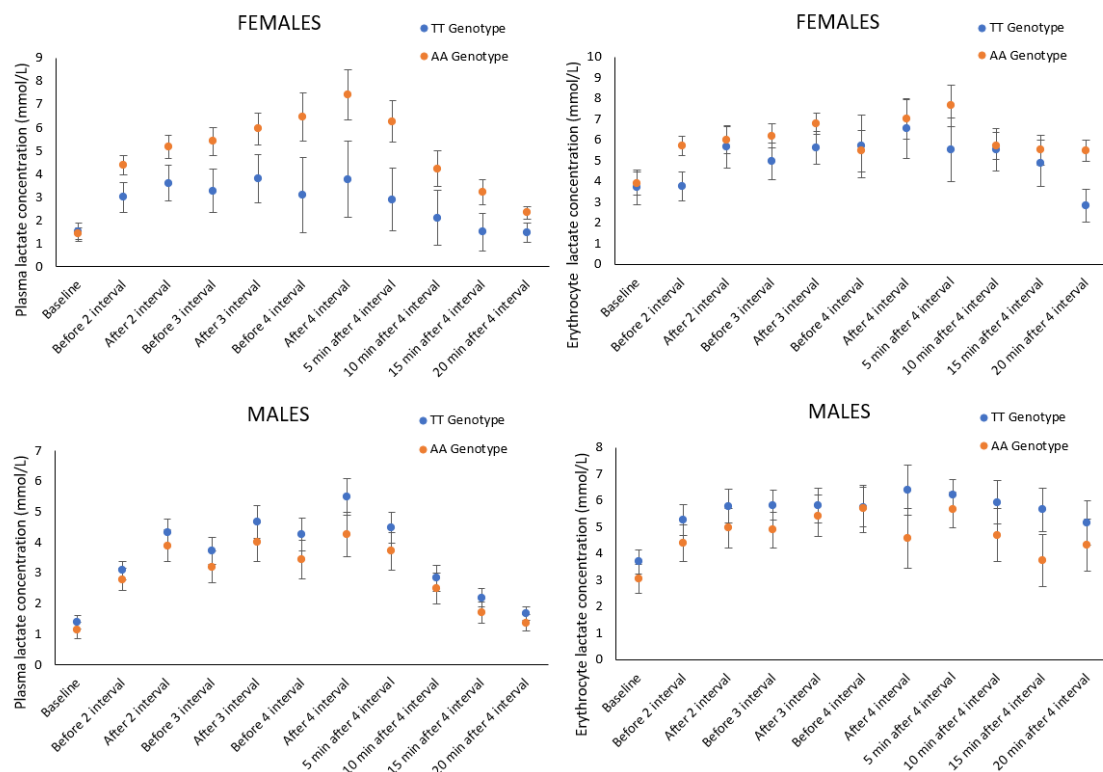


Figure 11 Lactate concentration between type of blood and genotypes at the different moments of measurement.

Lactate ratio

In females, lactate ratio was similar at the different moments of measurement ($F(10,80)=0.8$; $p=0.623$; $\eta^2_p=0.092$) and between genetic profiles ($F(1,8)=1.78$; $p=0.218$; $\eta^2_p=0.182$). Also, an interaction effect was not found ($F(10,80)=0.84$; $p=0.591$; $\eta^2_p=0.095$). In males, lactate ratio changed at the different moments of measurement ($F(10,150)=1.98$; $p=0.039$; $\eta^2_p=0.117$). In contrast, lactate ratio was similar between genetic profiles ($F(1,15)=0.136$;

$p=0.717$; $\eta^2_p=0.009$). Also, an interaction effect was not found ($F(10,150)=0.37$; $p=0.957$; $\eta^2_p=0.024$) (Figure 12).

Lactate gradient

In females, lactate gradient was similar at the different moments of measurement ($F(10,80)=1.01$; $p=0.442$; $\eta^2_p=0.112$) and between genetic profiles ($F(1,8)=1.55$; $p=0.248$; $\eta^2_p=0.162$). Also, an interaction effect was not found ($F(10,80)=1.36$; $p=0.216$; $\eta^2_p=0.145$). In males, lactate gradient changed at the different moments of measurement ($F(10,150)=5.21$; $p<0.001$; $\eta^2_p=0.258$). In contrast, lactate gradient was similar between genetic profiles ($F(1,15)=0.21$; $p=0.654$; $\eta^2_p=0.014$). Finally, an interaction effect was not found ($F(10,150)=0.825$; $p=0.605$; $\eta^2_p=0.052$) (Figure 12).

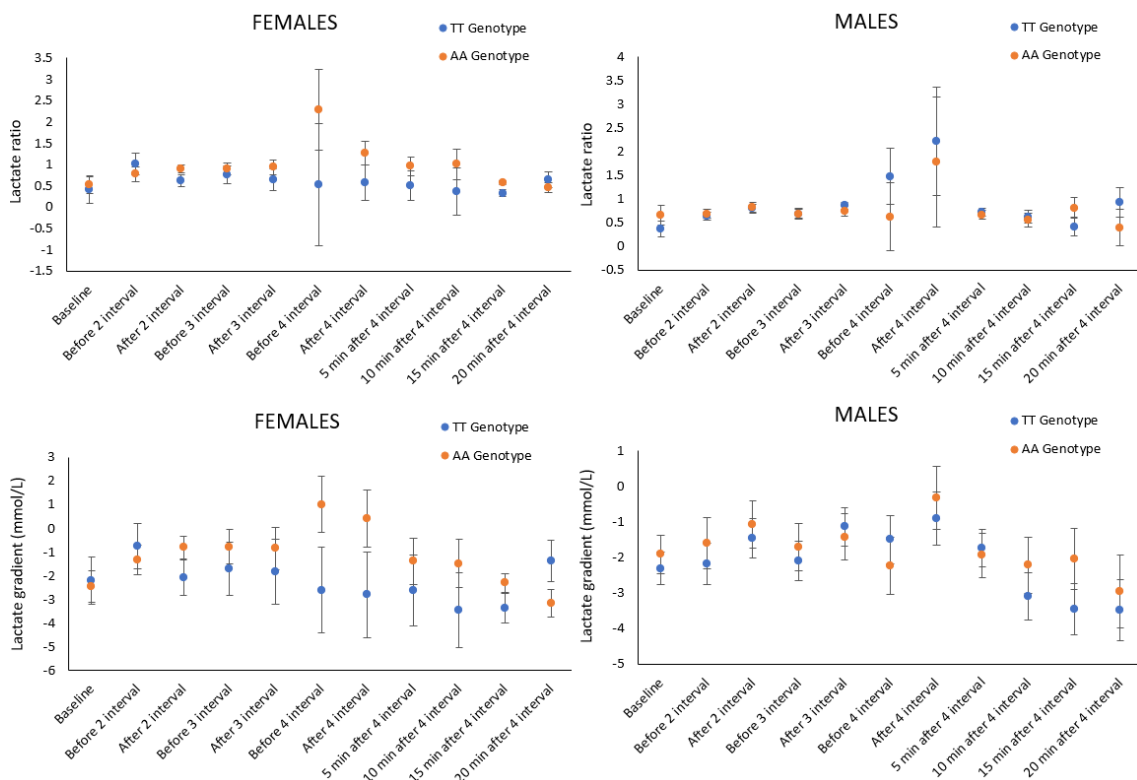


Figure 12 Lactate ratio between genotypes at the different moments of measurement.

DISCUSSION

The objective of this work was to describe if lactate levels in plasma and erythrocyte during an intervallic protocol and its recovery are influenced by

the genotype of the T1470A SNP (rs1049434) on the MCT1 (SLC16A1) gene, in males and females. The main results showed that 1) no significant difference in whole blood, plasma or RBC lactate concentration was found between genotypes at rest, during exercise or during the recovery and that 2) neither the RBC/plasma lactate ratio nor the lactate gradient differed between the genetic groups at rest or during exercise and recovery. Both results were found in males and females. These results indicate that it is not necessary to consider MCT1 genotype when analyzing blood lactate distribution and that the T1470A SNP have no influence regardless the sex of the participants with concentrations similar to the present work.

Our data show that lactate concentration significantly changed at the different moments of measurement in both sexes, indicating that the protocol was adequate to cause this lactate movement among compartments. (i.e. muscle, plasma and RBC). An interval protocol was carried out to cause a high movement of lactate between the muscles, the plasma and the erythrocytes due to the intensity changes throughout the different bouts and recoveries, and thus, trying to require the involvement of the MCT1 as the main pathway for lactate transport across the erythrocyte membrane, accounting for 80–90% of total lactate flux (274). The high-intensity segments were set at an intensity 20% above LT2 to maximize lactate production. Conversely, the recovery periods between sets and the final recovery phase were set at 80% of LT1, the optimal intensity for lactate clearance, as this is where lactate removal is most efficient (31). However, despite this requirement of MCT1 function, no differences were observed in lactate concentrations, ratios or gradients between genotypes. This may be due to a possible lower influence of the SNP in the MCT1 of the erythrocyte membrane, an over presence of the protein in the membrane to compensate the decreased functionality of the less-functional MCT1 variant, or to the already hypothesized possibility of the existence of a minimum lactate concentration threshold (approximately 10 mM/L), which would reveal the impact of the SNP (33,170,182).

Previous studies have documented lower lactate concentrations in erythrocytes compared to plasma (35,38,44–48). These studies, which utilized constant or incremental exercise protocols, resulted in continuous lactate production. When lactate production by active muscles exceeds the removal capacity of tissues, as is likely during constant or incremental exercise, lactate concentrations are higher in plasma than in erythrocytes. Conversely, during the recovery phases of intervallic protocols or during final recovery, where lactate removal from plasma exceeds production, plasma lactate concentration will be lower than that in erythrocytes. According to the lactate shuttle theory, lactate travels from producer cells to consumer cells (15). During exercise, active skeletal muscles release lactate into the plasma via MCT4 (17). In this line, it seems that, during intervallic exercise, the influx of lactate as an energy substrate occurs more rapidly from plasma to muscles and organs than from RBC to those body parts (14,46). This may explain why lactate concentrations are higher in RBC compared to plasma in venous blood samples during our exercise protocol and recovery (18). In this regard, the fact that lactate inside the erythrocytes is still elevated and the lactate ratios altered even at the end of the recovery, may reflect an enhanced role of erythrocyte in this type of exercise. As stated in the introduction, RBC have been considered a lactate sink (261), whereby lactate would be able to reach farther distances from the producing muscles to target locations (e.g., brain) where lactate could function as a signaling molecule. The barrier provided by the erythrocyte membrane (263) would prevent its uptake by muscles and consumer tissues, and could, to a certain extent, enhance the signaling function of lactate, particularly in this type of exercise.

The present work shows no difference between sexes in MCT1 genotype influence. Although very few studies have compared the role of SNP between sexes, it seems that the effect is more pronounced in male than in female athlete, since different blood lactate levels among genotypes has been described in exercising males but not in exercising females (168,182). Sex hormones play a role in the regulation of MCT1 protein expression and content in rodent liver and muscle (249,275), testosterone increasing MCT1

protein levels in rat muscle (249). This might be one of the reasons for the previous differences between sexes reported. However, no such difference was observed in our data, with an influence of the MCT1 SNP lacking for both males and females. This lack of difference could be due to the moderate lactate levels reported, which failed to show the SNP influence already reported, as mentioned above. Therefore, it seems that at moderate lactate concentrations, although there is movement across the erythrocyte membrane, this does not seem to be influenced by the SNP in a different way in each sex. In this sense, and in order to control in a much stricter way the hormonal variations that could condition the levels of MCT1 protein, all the female volunteers included were naturally menstruating, in addition to performing all the tests in their early follicular phase, which ensured a similar hormonal balance among all the females (276,277).

Finally, this study has some considerations that should be acknowledged. Firstly, we did not control for blood pH, which could have influenced lactate transport and distribution. Secondly, the amount of MCT1 in the erythrocyte membrane was not measured, which may have impacted our understanding of lactate dynamics across different individuals. Despite these limitations, our study possesses significant strengths. The sample was very homogeneous in terms of training level, a factor known to influence lactate dynamics (278). Additionally, the menstrual cycle phases of female participants were monitored and controlled, ensuring that hormonal variations did not confound the results. These strengths enhance the reliability and relevance of our findings within the context of trained populations.

CONCLUSION

The results of the present study observed no differences between genotypes for the T1470A SNP (rs1049434) on the MCT1 (SLC16A1) gene, for the different lactate variables studied (i.e. whole blood, plasma or RBC lactate concentrations, RBC/plasma lactate ratio, or lactate gradients) neither at rest, during exercise or during the recovery. This lack of differences was reported for males and females similarly. Consequently, the presence of the

T1470A SNP does not appear to alter the efficiency of lactate transport through the erythrocyte membrane during intervallic exercise, in either male or female athletes. This insight simplifies the analysis of blood lactate levels by removing the need to account for MCT1 genetic differences, thereby allowing for more straightforward and generalized interpretations of lactate measurements across diverse populations when lactate concentration are similar to the present work.

4.3.Study III: Greater relative first and second lactate threshold in females compared to males: Consideration for exercise prescription

APPROACH TO THE PROBLEM

Exercise prescription is often established as a percentage of a maximal value [maximal heart rate (HR_{max}), maximal oxygen consumption (VO₂_{max}), peak velocity, or peak workload] obtained during an incremental test to volitional exhaustion. Although this is a commonly accepted practice, this approach has two key limitations as described elsewhere (177): (a) there is a dissociation between the incremental test-derived workload and oxygen consumption (VO₂), in relation to what is observed during constant workload exercise (279,280). This dissociation is explained by the VO₂ mean response time and the rate of increase of the incremental test (280,281), and needs specific corrections for it to be accounted for (282,283); (b) exercising at a specific percentage of maximum values does not represent identical physiological stress for everyone, which leads to heterogeneous physiological responses in individuals who exercise at the same given percentage of VO₂_{max} or HR_{max} (284,285). Therefore, accurate exercise prescription that precisely quantifies the metabolic disturbance within the active tissues is needed to better control the acute responses (26,286,287) and chronic adaptations (288–290) to endurance exercise.

Based on the limitations of using percent of maximal values for accurate exercise prescription, an alternative solution would be to set the exercise intensity based on the metabolic stress given by the exercise intensity domains model (291). In fact, using this approach has been shown to be effective to produce more homogeneous responsiveness to endurance training interventions (290). Given the dissociations between VO₂ and workload during continuous exercise as compared to relatively fast incremental testing highlighted above, an alternative option to partition exercise intensity domains would be the use of the first lactate threshold (LT1) or the second

lactate threshold (LT2) as demarcation points. An advantage of this approach would be that by using longer steps that allow for a steady state (or quasi steady state) response during each step, the VO_2 mean response time would not be an issue to consider, and the VO_2 slow component that is not expressed during fast ramps or short steps incremental testing (280) would have the opportunity to be at least partly expressed. Indeed, a recent study demonstrated that LT2 determination from 3-min long steps offered accurate estimations of the workload associated with the maximal lactate steady state, provided that the expertise of the evaluator was used for the estimation of this threshold (292). Thus, using LT1 and LT2 as the demarcation points for exercise intensity estimations can be considered as a valid approach to separate exercise intensity domains, so that accurate exercise workload prescription can be assigned to participants.

Interestingly, some recent lines of research have indicated the percent of $\text{VO}_{2\text{max}}$ at which exercise thresholds (determined by gas-exchange and ventilatory response) occur is greater in females compared to males (177,285,293). This is critically important as females represent 50% of the population, but current exercise and physical activity guidelines use a heavily disproportionate volume of male data to inform recommendations (294). This is due to the dramatic sex bias found in the scientific literature (246,247,294). Due to this lack of research in females, scientists, coaches, and practitioners usually extrapolate studies in males to provide exercise intensity prescription to females. If the above-mentioned differences are true, females could be in a further disadvantage for getting accurate exercise prescription as, in addition to the known limitations of using the percent of maximal values for accurate exercise prescription, they would also experience a more inaccurate estimate of metabolic disturbance that the exercise bout generates. Since lactate thresholds-derived responses are one of the most common approaches to prescribe exercise intensity (28), understanding whether sex-related differences exist in the absolute and, most importantly, relative, heart rate (HR), and power output (PO) at which LT1 and LT2 occur is critically

important. However, no direct comparisons of these responses have been made between females and males.

Thus, the aim of this study was to compare the differences in absolute and relative HR and PO at LT1 and LT2 between females and males. Based on the available data, it was hypothesized that lactate thresholds would occur at a smaller absolute but a greater relative HR and PO in females compared to males.

METHODS

Twenty-four females and twenty males who were highly active participated in this study (Table 14). Inclusion criteria included: 1) absence of any pathology that could prevent the correct performance of the tests or that could influence the main study variables; 2) non-smokers; 3) a BMI between 18 and 25 kg/m²; 4) a self-reported physical exercise volume of more than five hours per week; and, 5) answer negatively all the questions in the Physical Activity Readiness Questionnaire (PAR-Q). Participants were asked to abstain from drinking alcohol, caffeine, and performing intense exercise for at least 24 hours prior to the tests. All tests were performed at the same time of the day to avoid the potential effect of circadian rhythms (146). Females completed all testing sessions in the early follicular phase (for natural menstruating females) or the placebo phase (for oral contraceptive users) (on the day 2.29±1.63 of bleeding) to avoid any possible modulation of sex hormones. Before participation, participants signed an informed consent form after being provided with detailed information about the study and its potential risks and/or benefits. This study was approved by the ethics committee of Universidad Politécnica de Madrid (2019-044). Clinical trials: NCT06104150.

Table 14 Descriptive characteristics of the participants.

	Females			Males			p
Age (years)	21	±	4	23	±	5	0.129
Body mass (kg)	56.9	±	6.6	71.6	±	6.0	<0.001
Stature (cm)	163.3	±	6.9	177.0	±	5.3	<0.001
Body mass index (kg/m ²)	21.3	±	2.4	22.8	±	1.7	0.027
Baseline HR (bpm)	73	±	17	76	±	12	0.455

Maximal HR (bpm)	184	±	10	187	±	8	0.374
Final lactate (mMol/L)	10.3	±	2.1	11.3	±	2.0	0.126
Absolute peak power (W)	175	±	23	266	±	32	<0.001
Relative peak power (W/kg)	3.0	±	0.3	3.7	±	0.4	<0.001
Data are shown as mean ± standard deviation.							

A maximal incremental test was performed on a cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). The ergometer was fitted to the ergonomics of each participant. HR was measured every 5 s with a Polar RS800sd device (Polar Electro Oy, Kempele, Finland) with a chest strap. The test started with a warm-up of 5 min at 75 W, followed by 35 W increments every 3 min until task failure, which was defined as the inability to maintain a cadence above 70 rpm for longer than ~5 s or the participant's voluntary withdrawal despite strong verbal encouragement. The cadence had to be kept stable between 70 and 90 rpm. Participants had to be seated during the whole test. Capillary blood samples were obtained from the earlobe and lactate concentrations were measured using a portable lactate analyser (Lactate Plus; Nova Biomedical, Cheshire, UK) at rest, in the last 30 seconds of each step, as well as immediately after the end of the test. Peak PO from the incremental test was calculated by the following equation (295):

$$\begin{aligned}
 \text{Peak power (w)} &= \text{Last step completed (W)} \\
 &+ \frac{\text{Last step time (s)}}{\text{Step time (s)}} \times \text{step increments (W)}
 \end{aligned}$$

In this equation, *last step completed* refers to the PO of the last completed step (Watts), *last step time* is the time spent in the last uncompleted step (seconds), *step time* is the duration of each step (seconds) and *step increments* is the increments of each step (watts).

After the incremental test, the log-log method for LT1 (296) and the ModDmax method for LT2 (23) was determined in each participant. Specifically, absolute PO, relative to peak PO, HR, and relative to maximal HR were calculated for LT1 and LT2.

Data are presented as mean $\pm$ standard deviation (SD). Normal distribution and homogeneity of variance were confirmed using the Shapiro-Wilk test and the Levene's test, respectively. A repeated measures ANOVA (Sex x LT) was performed to compare absolute and relative PO and HR according to sex at LT1 and LT2. Effect sizes for two means comparisons were estimated calculating Cohen's d , where 0.2 represents a small effect, 0.5 a moderate effect, and 0.8 a large effect(297). ANOVA effect size was calculated by partial eta-squared (η^2_p) and the small, moderate, and large effects corresponded to values equal or greater than 0.01, 0.059, and 0.138, respectively (297). Bonferroni post hoc tests were conducted where significant differences were found in any of the analysed factors. Statistical analyses were performed using the statistical software Jamovi V1.6 (Jamovi, Sidney, Australia). Significant level was set at $p < 0.05$. A post hoc power analysis was performed based on data from relative HR using statistical software (G*Power v. 3.1.9.2, Stuttgart, Germany), resulting in a statistical power > 0.95 .

RESULTS

A main effect of sex was found for PO ($F(1,36)=37.5$; $p < 0.001$; $\eta^2_p=0.51$), indicating smaller absolute PO in females compared to males. Also, an interaction was found between sex and LT ($F(1,36)=6.85$; $p=0.013$; $\eta^2_p=0.16$). Pairwise comparisons showed PO at LT1 tended to be lower in females ($p=0.077$) and PO at LT2 was lower in females compared to males ($p < 0.001$). Relative PO was greater in females than males ($F(1,38)=19$; $p < 0.001$; $\eta^2_p=0.333$), independently of the LT (interaction effect: $F(1,38)=19$; $p=0.445$; $\eta^2_p=0.015$) (Figure 13).

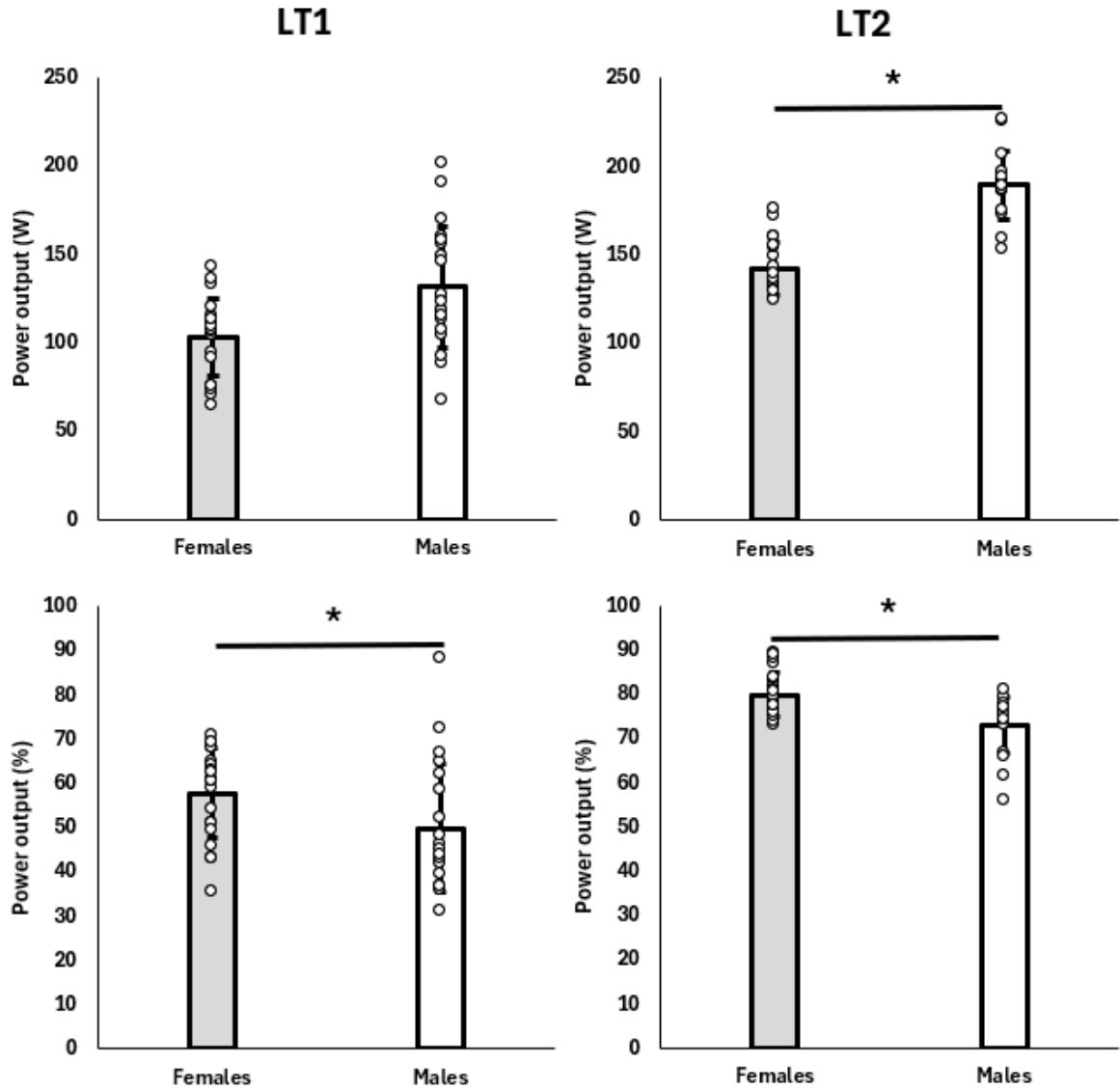


Figure 13 Absolute and relative peak power output in females and males at lactate threshold 1 (LT1) and 2 (LT2). *Significant main effect of sex.

A main effect of sex was found for HR at LTs ($F(1,29)=8.06$; $p=0.008$; $\eta^2_p=0.217$) indicating greater HR values in females compared to males, without significant interaction between sex and LT ($F(1,29)=2.18$; $p=0.151$; $\eta^2_p=0.07$). Finally, relative HR was greater in females compared to males ($F(1,29)=11.4$; $p=0.002$; $\eta^2_p=0.282$), regardless of the LT (interaction effect: ($F(1,29)=2$; $p=0.168$; $\eta^2_p=0.065$) (Figure 14).

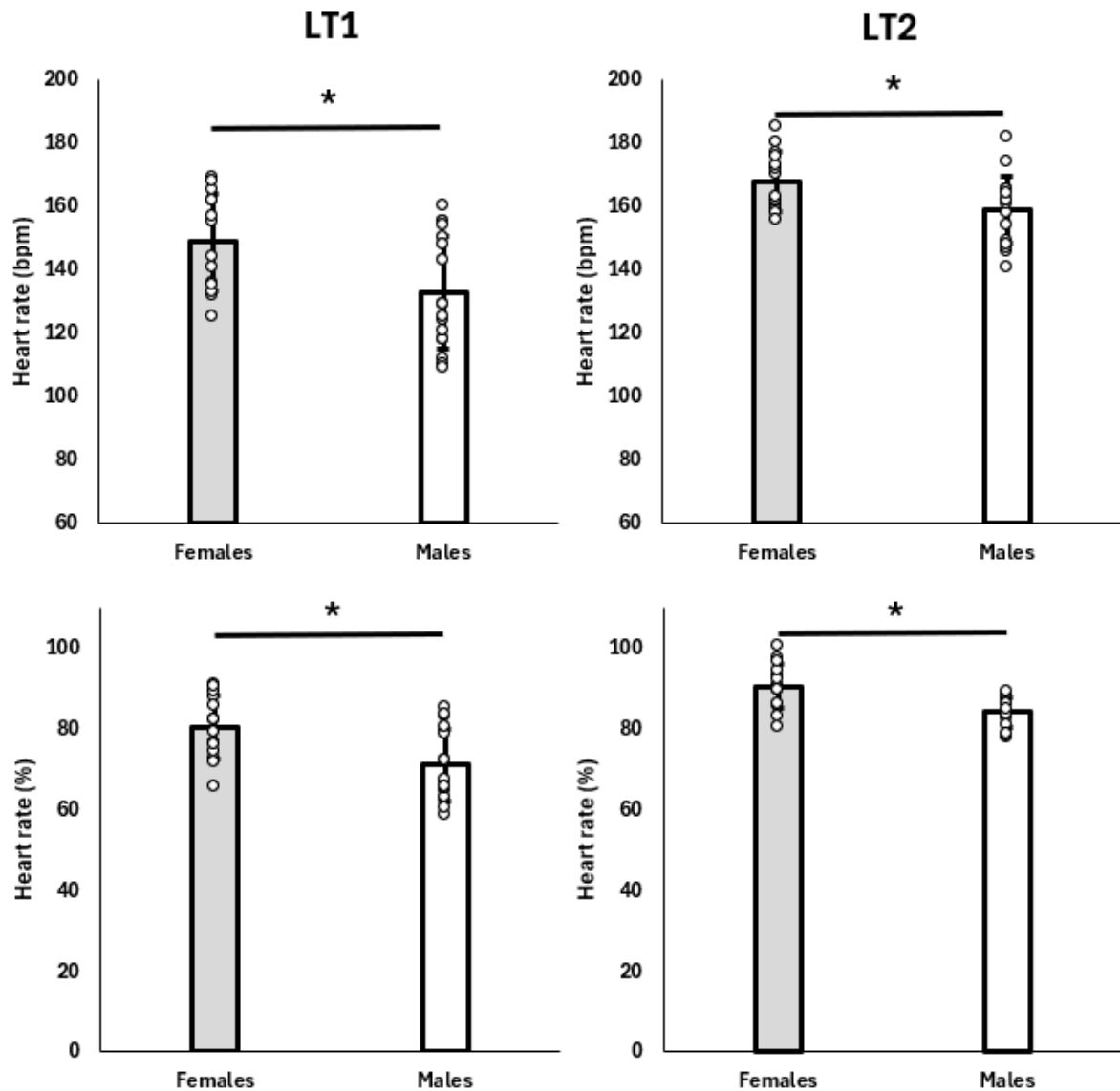


Figure 14 Absolute and relative to maximum heart rate in females and males at lactate threshold 1 (LT1) and 2 (LT2). * Significant main effect of sex.

DISCUSSION

To the best of our knowledge, this is the first study assessing the differences in absolute and relative HR and PO at LT1 and LT2 between females and males during an incremental cycle-ergometer test. The main findings from the present study were that: 1) despite the trend found in the absolute PO at LT1 and the higher absolute PO at LT2 in males compared to females, the relative PO at which these thresholds occurred was greater in females than in males; 2) females demonstrated a greater absolute and relative HR

response compared to males at the LT1 and LT2. These data have important implications as they support the idea that the sex of the participant needs to be taken into consideration to improve the accuracy of exercise prescription.

In recent years, more attention has been put into understanding the connection between the metabolic disruption that a given exercise intensity generates and the acute responses (26,286,287) and chronic adaptations (288–290) to endurance exercise. Data have indicated that using the exercise intensity domains model to better quantify the true metabolic stimulus elicited by the exercise results in more homogenous and predictable responses and adaptations (290). Although using lactate derived metabolic boundaries for exercise prescription is a common laboratory-based approach, subsequent exercise prescription relies on the identification of HR and/or PO zones to elicit a given metabolic rate. The present study provides novel information indicating that the relative HR and PO at which LT1 and LT2 occurred was greater in females compared to males, and highlights the importance of considering sex-related differences in the percent of maximal values at which these boundaries occur.

This study is the first to show a right shift in the percent of HRmax responses at which the lactate thresholds occur in females compared to males. This information agrees with Iannetta et al. (177) who demonstrated that females had their estimated lactate threshold (moderate to heavy intensity boundary) and their maximal lactate steady state (MLSS) (heavy to severe intensity boundary) at a greater percent of HRmax and $\text{VO}_{2\text{max}}$ during cycling exercise. Similarly, another study indicated in a large sample of 565 participants that the percent of $\text{VO}_{2\text{max}}$ at which the gas exchange threshold and the respiratory compensation point (proxies for metabolic rates at which the moderate-heavy, and heavy-severe boundaries occur, respectively) was significantly greater in females compared to males (285). In line with the findings of other studies measuring other types of demarcations of the moderate-heavy domain (LT estimated through gas exchange variables) and the heavy-severe domain (respiratory compensation point or MLSS), our results support the idea that there is a sex-related difference in the

percentage of maximal responses at which exercise intensity thresholds occur. Importantly, these differences remain independently of whether ramp incremental tests that rely on gas exchange and ventilatory data, or longer step incremental tests that rely on lactate evaluations are used to estimate the percent of HRmax at which these thresholds take place.

Another novel finding from our study is that relative PO at LT1 and LT2 was greater in females than males. This is in contrast with the study by Iannetta et al. (177), in which the estimated LT and the MLSS were not different between males and females when expressed relative to peak PO. One important difference between the studies was that in our study both sexes performed the same type of incremental test, whereas in Iannetta et al. (177), females performed ramp incremental tests with slower rate of increase in work rate compared to males. Previous studies have shown that exercise protocols with steeper slopes for the load increment result in greater values for peak workload (23,280), but not HR (298), at the different physiological boundaries as well as at maximal exercise. Another factor that could contribute to the difference between our study and that of Iannetta et al. (177) might be connected not only to the rate of increase in females and males, but also to the characteristics of the test. Whereas Iannetta et al. (177) used relatively fast ramp incremental testing that is known to exacerbate the differences in the relationship between constant load vs. incremental VO_2 and PO, the longer steps in the present study would allow for a greater expression of the VO_2 slow component and, thus, POs at the LT2 that are closer to those observed during maximal metabolic steady state (292). Future studies should further explore the relative PO at which the heavy-severe boundary established from different methods occurs in females compared to males.

The reasons for the right shift in the LTs in females compared with males cannot be elucidated from this study. However, some speculations can be made. The fact that LTs occur in females at a higher relative intensity indicates that the transition from the fully oxidative pathway to a progressive increase in glycolysis dependence is delayed in female athletes. Previous studies have shown a greater fat preference in females than in males

(158,299–302) at the same low/moderate relative intensity. Given the inverse relationship between lactate concentration and fat oxidation observed in previous studies (179), the rightward shift of LTs observed in females seems to indicate that this greater reliance on fat in females at low/moderate relative intensities also occurs at higher relative intensities.

Another potential factor that could contribute to explaining the sex differences is the different muscle fibre composition between females and males. Whereas males present a larger muscle cross-sectional area than females due to the overall greater muscle mass in males (250) that would explain the larger absolute PO in males, the percentage area occupied by the type I fibres is larger in females compared to males, and the percentage area occupied by the type IIa fibres is lower in females than in males, without significant differences in type IIx fibres (250). Since type I fibres are more oxidative (303), the greater proportion of type I fibres in females might explain, at least in part, the rightward shift in LT1 and LT2 in females compared to males.

Experimental considerations

It has to be considered that different approaches to estimate the lactate threshold might elicit different responses. Although the aim of this study was not to compare the responses between different models to estimate the lactate threshold, we examined the possibility that using different methods to determine LT1 and LT2 would change the interpretation of our results. Based on our analysis (see Supplementary material of the study III in Annexes for detailed information on the responses using different fitting approaches), the results remained the same independently of the selected model. This clarified, and given the information presented elsewhere indicating that not all models contribute to accurate determination of the maximal metabolic steady state (23), future studies should consider how the estimations obtained by different models might affect the exercise prescription.

In the present study, the incremental tests did not include evaluations of gas-exchange data. This would have been useful to identify the metabolic rates at

which the LTs occurred. However, what is novel and important from this study is that, for the first time, a sex-related difference in the relative intensity at which the LTs occurred was identified, which falls in line with previous data reporting similar differences during gas exchange and ventilatory responses analysis.

Practical applications

The present study provided novel information about a sex-related difference in LTs responses, where females showed a greater relative HR and PO associated to the LT1 and LT2 compared to males. This information has important implications for exercise prescription as it is becoming clear that exercise prescription that considers the metabolic boundaries is critical for better prediction of acute responses and chronic adaptations to endurance training. Thus, acknowledging these sex-related differences is key. We suggest that future guidelines should consider establishing exercise prescription recommendations according to sex as data from the present and other studies highlight the relevance of doing so.

CONCLUSION

Despite males tended to present a greater absolute PO at LT1 and presented a higher absolute PO at LT2 compared to females, the relative PO at which these thresholds occurred was greater in females than in males. On the other hand, females demonstrated a greater absolute and relative HR response compared to males at the LT1 and LT2.

4.4. Study IV: Females have better metabolic flexibility in different metabolically challenging stimuli

APPROACH TO THE PROBLEM

Metabolic flexibility can be broadly defined as the ability of an organism to maintain energy homeostasis by prioritizing a particular substrate to match fuel availability to energy demand in response to different stimuli, such as fasting, variation in meal composition, exercise or environmental fluctuation (304). Interestingly, assessment of metabolic flexibility is crucial, since metabolic inflexibility is associated with several pathological conditions, including metabolic syndrome, type 2 diabetes mellitus, and cancer (304). Therefore, understanding the different aspects that can affect metabolic flexibility seems key to improve patients' health (305). Specifically in the exercise field, one of the methodologies to determine metabolic flexibility is to perform an incremental intensity exercise test (stimulus) in a full substrate availability state measuring carbohydrate (CHox) and fat oxidation (FATox), as well as lactate concentration (56). In this context, to maintain energy homeostasis through adjustment of the energy demand caused by exercise intensity (stimulus), individuals whose metabolism prioritises FATox and maintain basal lactate concentration at low intensities, and begin to prioritise CHox and increase lactate concentrations at higher intensities present greater metabolic flexibility (56). This specific methodology has been implemented in males with different metabolic characteristics finding a greater metabolic flexibility (i.e. a higher FATox and a later increase in lactate concentration during the incremental test) the greater the fitness status (56). A similar methodology was implemented in females with different cardiorespiratory levels reporting significantly higher FATox in endurance-trained and recreationally active females compared to those who were overweight. In this case, lactate concentration failed to consistently differentiate between the three cardiorespiratory levels (178). Unfortunately, this specific methodology has not been previously used to explore metabolic flexibility differences between sexes.

A sex-based comparison on maximal fat oxidation normalized to fat-free mass (MFO/FFM) has been investigated, as previously recommended due to sex differences in FFM (302). In fact, some studies have reported greater MFO/FFM in females compared to males without overweight/obesity during an incremental intensity exercise test (299,300,306,307). Unfortunately, only one of these studies matched both sexes by training status and considered the reproductive profiles of females (306). These results lead us to suggest that females may have greater metabolic flexibility compared to males. Nevertheless, with the current knowledge we cannot confirm a different metabolic flexibility between sexes, since the aforementioned studies only compared MFO/FFM without comparing the effect of sex on different substrates throughout different exercise intensities. Therefore, sex-based differences in substrate oxidation to match fuel availability to energy demands to maintain energy homeostasis in response to exercise intensity remains unknown. Therefore, further studies are needed comparing FATox and CHox normalized to FFM (FATox/FFM and CHox/FFM, respectively) as well as lactate concentration throughout different exercise intensities (matching both sexes by training status and considering both the reproductive profile and the menstrual cycle phase of females (276)) to confirm whether a sexual dimorphism exists in metabolic flexibility.

Additionally, the only stimulus applied in the aforementioned studies was exercise intensity. However, multiple metabolically challenging situations can be applied during exercise as stimuli to the organism, apart from exercise intensity. For example, low carbohydrate availability which is a common nutritional strategy used by athletes with the intention to emphasize fat oxidation (oxidative pathway) (308), or the wide range of environmental temperatures at which exercise is performed affecting lactate concentration (glycolytic pathway). Hence, to investigate sex-based differences on metabolic flexibility in response to different metabolically challenging stimuli in addition to exercise intensity, such as glycogen depletion or heat, would allow to have a broader and more complete view of the potential different metabolic response between female and male. This is critically important as females

represent ~50% of the population. However, due to the dramatic sex bias found in the scientific literature (246,247,294), the physiological sex differences are poorly investigated. Therefore, the first the aim of the present work was to explore the difference in metabolic flexibility between sexes in response to the exercise intensity stimulus in a control situation. The second aim was to evaluate metabolic flexibility between sexes in response to exercise intensity adding two different metabolically challenging stimuli (glycogen depletion and heat). Important confounding aspects, not previously considered, were controlled (i.e. all trained individuals, naturally menstruating females measured in the early follicular phase and matching both sexes by VO_{2max}/FFM).

METHODS

Participants

Eleven males and nine females participated in this study (Table 15), all classified as trained individuals (309). The inclusion criteria were: a) being healthy, and between 18 and 40 years old; b) perform endurance training more than 3 hours per week; c) be a non-smoker; and for females: d) naturally menstruating; e) non-user of hormonal contraceptives ; f) not be pregnant (276) and; g) not abundant menstrual bleeding (<80 ml) (310). Participants were informed of the risks and benefits of the study before participating, and written informed consent was obtained from each subject before inclusion. Ethical authorization for this study was obtained from the Research Ethics Committee of the University where the research was conducted (ID: DATOS-20220524-RCC-LacFat). Subjects were informed of the risks and benefits of the study prior to participation and written informed consent was obtained from each subject before their inclusion.

Table 15 Descriptive characteristics of the participants.

	Males	Females
Age	22±3	22±2
Height	176.2±4.0	166.7±4.5*

Weight	68.4±4.9	61.9±2.9*
FFM (kg)	59.4±4.8	45.2±3.3*
Fat (%)	10.3±3.1	23.5±3.8*
VO _{2max} (ml/min)	3581±369	2898±282*
VO _{2max} /BW (ml/kg/min)	52.3±3.6	46.8±4.5*
VO _{2max} /FFM (ml/kg/min/kg FFM)	60.2±4.1	64.2±5.6
PPO (W/kg)	277.7±38.3	231.4±14.2*
PPO (W/kg)	4±0.3	3.7±0.2*
PPO (W/kg FFM)	4.6±0.4	5.1±0.4*
Data are shown as average ± SD. FFM: fat free mass; VO _{2max} : maximal oxygen uptake; VO _{2max} /BW: VO _{2max} normalised to body weight; VO _{2max} /FFM: VO _{2max} normalised to FFM; PPO: peak power output. *Significant differences between sexes (p<0.05).		

Study design

The study consisted of five visits to the laboratory (Figure 15). The first visit included informed consent, body composition assessment using whole-body dual-energy X-ray absorptiometry (DXA) and individualized nutritional plan. Subsequently, in the following appointments, the same maximal incremental tests were performed in three different experimental situations (control, glycogen depletion and heat). Tests were performed at the same time of day (±2 hours). For females, all tests were performed during the early-mid follicular phase of the menstrual cycle (day 3.6±1.6). The first test was in all cases a maximal incremental test in the standard situation (control situation). The order of the two remaining incremental tests (glycogen depletion or heat situations) was randomized. In order to deplete glycogen stores for the subsequent glycogen depletion test, 24 hours prior to this incremental test, an exercise protocol was performed to completely deplete muscle glycogen stores. Additionally, participants followed a low-carbohydrate diet from breakfast on the day of the glycogen depleting exercise day until the maximal incremental test. On the contrary, in control and heat situations, from breakfast the day before the test, participants followed a

high-carbohydrate diet. During both control and glycogen depletion situations, the ambient temperature was maintained between ~21-22 °C. Finally, during the heat situation, participants were placed in a heat chamber designed to maintain an ambient temperature between 36-38°C for 30 minutes before the incremental test, and the incremental test was performed at the same temperature (187). In all situations, body weight loss was less than 2%. Clinical trials:NCT05703100.

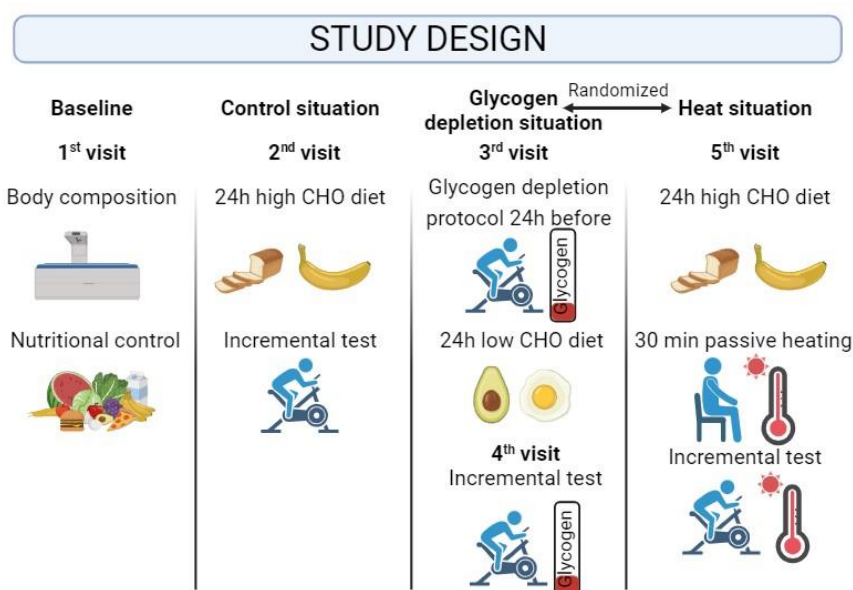


Figure 15 Overview of the study design. CHO: carbohydrate.

Body composition assessment

Body composition was measured by DXA scan (Version 6.10.029GE Encore 2002, GE Lunar Prodigy; GE Healthcare, Madison, WI, USA) to obtain fat mass and FFM. All participants performed the test wearing light sports clothing (e.g., top and shorts). In addition, they must be with their body and hands in a supine position and their feet joined by a strap. Participants were instructed not to move or talk while the scanner performed measurements. The scan was calibrated every two days using the phantom supplied by the manufacturer.

Nutritional intervention

High-energy, high-carbohydrate diet

Participants followed a specific diet at least 24 hours before the incremental test in the control and heat situations to ensure adequate energy and carbohydrate intake to avoid performance limitations during testing. Macronutrient distribution was standardized for all participants at an approximate ratio of 65% carbohydrate, 15% protein and 20% fat. Energy and carbohydrate intake were determined for all participants in relation to their FFM (energy: 60 kcal/kg FFM; carbohydrates: 9.8g/kg FFM) (188) (see example in Supplementary Material of the study IV in Annexes).

High-energy, low-carbohydrate and high-fat diet

This diet matched the energy intake of the high carbohydrate diet (60kcal/kg FFM/day). However, carbohydrate intake was severely restricted (<50 g/day) and protein and fat intake were 20% and 75-80%, respectively (188). The most challenging aspect was to achieve the previously established daily caloric intake without exceeding the carbohydrate limit. Therefore, supplementation with medium chain triglycerides was used to achieve this energy goal without contributing to increase carbohydrates (see example in Supplementary Material of the study IV in Annexes).

Exercise protocol

Incremental exercise test

The incremental test performed in the three different situations was performed on a cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). The ergometer was fitted to the ergonomics of each participant. The cadence throughout the test was constant, remaining between 70-90 rpm. Participants had to be seated during the whole test. The protocol started with 1 minute of rest followed by 5 minutes of warm-up at 30W. Subsequently, power was increased by 30 W every 3 minutes until task failure, which was defined as the inability to maintain a cadence above 70 rpm for longer than ~5 s or the participant's voluntary withdrawal despite strong verbal encouragement. Previous studies have already used 3 minutes steps to measure lactate concentration (24) and substrate oxidation (311).

Glycogen depletion protocol

The glycogen depletion protocol consisted of a 60-minute continuous cycling exercise at the power associated with 60% of $\text{VO}_{2\text{max}}$ calculated from the power- VO_2 ratio obtained during the maximal incremental cycling test performed in the control situation. The pedalling cadence was set at 70 rpm. After that, six 30-second all-out sprints were performed with a 7.5% body weight load with a 4-minute recovery between bouts. This protocol has been carried out in previous studies with the same objective, having already shown to be effective in the glycogen stores depletion (189).

Measurements

Heart rate (HR) was analysed beat by beat throughout the test via the Polar H10 (Polar Electro Oy, Kempele, Finland), although 12-lead ECG was continuously monitored to assess the presence of cardiovascular abnormalities. This band was connected via Bluetooth to a cell phone that received the signal and where the data were stored in the Polar Flow application. HR data averaged every 15 seconds were merged with those from the gas analyser. Maximal HR (HR_{max}) was considered as the highest value of HR obtained during the test.

Expired gas volume and composition were analysed breath-by-breath using the JAEGER® Vyntus CPX gas analyzer (Jaeger-CareFusion, Hoechberg, Germany). The equipment was previously calibrated according to the manufacturer instructions, whose validity and reliability have been demonstrated (coefficient of variation of the respiratory quotient less than 0.5%). VO_2 and CO_2 were averaged every 15 seconds from the gas analyser. $\text{VO}_{2\text{max}}$ was established as the highest value averaged every 15 seconds achieved during the incremental exercise test of each situation. For data analysis of the three different incremental tests, the average of the last 60 seconds of each step was taken (311). FATox and CHox were calculated from the respiratory quotients using standard indirect calorimetry equations (312).

$$\text{FATox (g/min)} = 1.67 \cdot \text{VO}_2(\text{L/min}) - 1.67 \cdot \text{VCO}_2(\text{L/min}) - 1.92 \cdot n$$

$$\text{CHox (g/min)} = 4.55 \cdot \text{VCO}_2(\text{L/min}) - 3.21 \cdot \text{VO}_2(\text{L/min}) - 2.87 \cdot n$$

n = negligible urinary nitrogen excretion

FATox/FFM and CHox/FFM were also determined by normalizing FATox and CHox to FFM, respectively. MFO was determined as the highest value of FATox during the tests. MFO/FFM was also determined by normalizing MFO to FFM. Additionally, VO_2 , percentage of $\text{VO}_{2\text{max}}$ ($\%\text{VO}_{2\text{max}}$), HR, percentage of HR_{max} ($\%\text{HR}_{\text{max}}$), power output (PO) and PO normalized to FFM (PO/FFM) were determined at the intensity corresponding to MFO. Energy expenditure normalized to FFM (EE/FFM) was determined from FATox/FFM and CHox/FFM (313).

$$\text{EE/FFM (cal/min/FFM)} = \text{FATox/FFM} \cdot 9.75 + \text{CHox/FFM} \cdot 4.07 \cdot 1000$$

Whole blood lactate concentration was measured during the test from capillary blood. These blood samples were drawn from the fingertip at rest, in the last 30 seconds of each step and immediately after the moment of maximum effort, and these samples were analysed with a lactate analyser (EKF Diagnostic GmbH, Barlebe, Germany). The first lactate threshold (LT1) was determined by the Log-log method (314). Lactate concentration, VO_2 , $\%\text{VO}_{2\text{max}}$, HR, $\%\text{HR}_{\text{max}}$, PO and PO/FFM were determined at the intensity corresponding to LT1.

At rest, in the last 30 seconds of each step and immediately after the moment of maximum effort, central temperature was monitored with a tympanic thermometer (Braun IRT6520, Switzerland), rate of perceived exertion was measured with the 0-10 and 6-20 scales, and ambient temperature and humidity (Dienmern, Guangdong, China) were determined.

Statistical analysis

Normal distribution and homogeneity of variance were confirmed by the Shapiro-Wilk test and Levene's test, respectively. A Student's t-test was used to compared descriptive characteristics between sexes. A three-way ANOVA (sex*intensity*situation) for repeated measures was used to compare FATox/FFM, CHox/FFM, EE/FFM and lactate concentrations between sexes throughout the different intensities across situations. Additionally, a two-way ANOVA (sex*situation) for repeated measures was used to compare MFO,

MFO/FFM, VO_2 , $\%\text{VO}_{2\text{max}}$, HR, $\%\text{HR}_{\text{max}}$, PO and PO/FFM at the intensity corresponding to MFO between sexes across situations. Furthermore, a two-way ANOVA (sex*situation) for repeated measures was used to compare lactate concentration, VO_2 , $\%\text{VO}_{2\text{max}}$, HR, $\%\text{HR}_{\text{max}}$, PO and PO/FFM at the intensity corresponding to LT1 between sexes across situations. When Mauchly's assumption of sphericity was not met, the Greenhouse–Geisser correction was used. ANOVA effect size was calculated by partial eta-squared (η^2_p) and small, moderate and large effect corresponded to values equal to or greater than 0.001, 0.059 and 0.138, respectively (315). Bonferroni's correction was applied for pairwise comparison. Statistical analyses were performed with the Jamovi V1.6 statistical software (Jamovi, Sidney, Australia). The significant level was set at $p < 0.05$. A post hoc power analysis was performed based on data from FATox/FFM, CHox/FFM and EE/FFM using statistical software (G*Power v. 3.1.9.2, Stuttgart, Germany), resulting in a statistical power close to 1 in all cases.

RESULTS

Fat oxidation

FATox/FFM was higher in females compared to males (sex: $F(1,18)=9.64$; $p=0.006$; $\eta^2_p=0.349$). Also, FATox/FFM was different across situations (situation: $F(2,35)=54.449$; $p < 0.001$; $\eta^2_p=0.752$). Specifically, it was greater in glycogen depletion than in control ($p < 0.001$) and in heat ($p < 0.001$). FATox/FFM was different throughout intensities (intensity: $F(1,25)=7.038$; $p=0.008$; $\eta^2_p=0.281$). Changes in FATox/FFM throughout the intensities were different across situations (situation*intensity: $F(4,76)=9.719$; $p < 0.001$; $\eta^2_p=0.351$), observing a greater FATox/FFM in glycogen depletion than in control and heat throughout the intensities ($p < 0.003$) (Figure 16).

MFO/FFM, VO_2 , $\%\text{VO}_{2\text{max}}$, HR and $\%\text{HR}_{\text{max}}$ at MFO were higher in females than in males. Moreover, MFO, MFO/FFM, VO_2 , $\%\text{VO}_{2\text{max}}$, HR, $\%\text{HR}_{\text{max}}$, PO and PO/FFM at MFO was significantly higher in glycogen depletion compared to control and heat (Table 16).

Carbohydrate oxidation

CHox/FFM was different across situations (situation: $F(2,35)=60.014$; $p<0.001$; $\eta^2_p=0.769$). Specifically, it was lower in glycogen depletion compared to control ($p<0.001$) and heat ($p<0.001$). CHox/FFM was different throughout the intensities (intensity: $F(1,21)=272.936$; $p<0.001$; $\eta^2_p=0.938$), observing a higher CHox/FFM the higher the intensity ($p<0.001$). Changes in CHox/FFM throughout the intensities were different between sexes (sex*intensity: $F(1,21)=8.269$; $p=0.007$; $\eta^2_p=0.315$), observing a greater increase in CHox/FFM as intensity increased in females compared to males, but pairwise comparisons showed no significant differences between sexes at any specific intensity. Changes in CHox/FFM throughout the intensities were different across situations (situation*intensity: $F(4,70)=15.146$; $p<0.001$; $\eta^2_p=0.457$), observing a lower CHox/FFM in glycogen depletion compared to control or heat throughout the intensities of the test ($p<0.001$) (Figure 16).

Energy expenditure

EE/FFM was higher in females than in males (sex: $F(1,18)=13.5$; $p=0.002$; $\eta^2_p=0.429$). Moreover, EE/FFM was different across situations ($F(1,21)=3.839$; $p=0.057$; $\eta^2_p=0.176$). Specifically, it was higher in heat than in glycogen depletion ($p<0.001$). EE/FFM was different throughout the intensities (intensity: $F(1,20)=385.16$; $p<0.001$; $\eta^2_p=0.955$), observing a greater EE/FFM the higher the intensity ($p<0.001$). Changes in EE/FFM throughout the intensities were different between sexes (sex*intensity: $F(1,21)=8.706$; $p=0.006$; $\eta^2_p=0.326$), observing a higher EE/FFM in females than in males at 120W ($p=0.044$). Changes in EE/FFM throughout the intensities were different across situations (situation*intensity: $F(3,56)=10.277$; $p<0.001$; $\eta^2_p=0.363$), observing a higher EE/FFM in heat than in the glycogen depletion at 180W ($p=0.003$) (Figure 17).

Lactate concentration

Lactate concentration was not different between sexes (sex: $F(1,18)=0.027$; $p=0.87$; $\eta^2_p=0.002$), but it was different across situations (situation: $F(2,33)=33.391$; $p<0.001$; $\eta^2_p=0.65$). Specifically, it was greater in heat compared to control ($p<0.001$) and glycogen depletion ($p<0.001$) and was

greater in control compared to glycogen depletion ($p=0.024$). Lactate concentration was different throughout the intensities (intensity: $F(2,29)=30.415$; $p<0.001$; $\eta^2_p=0.628$). Changes in lactate concentration throughout the intensities were different across situations (situation*intensity: $F(5,88)=5.852$; $p<0.001$; $\eta^2_p=0.245$) (Figure 17).

% $\text{VO}_{2\text{max}}$ at LT1 was higher in females than in males. Lactate at LT1 was lower in glycogen depletion compared to control and heat, VO_2 and % $\text{VO}_{2\text{max}}$ at LT1 was higher in glycogen depletion compared to heat, % HR_{max} at LT1 was lower in control than in glycogen depletion, PO at LT1 was higher in glycogen depletion than in heat (Table 17).

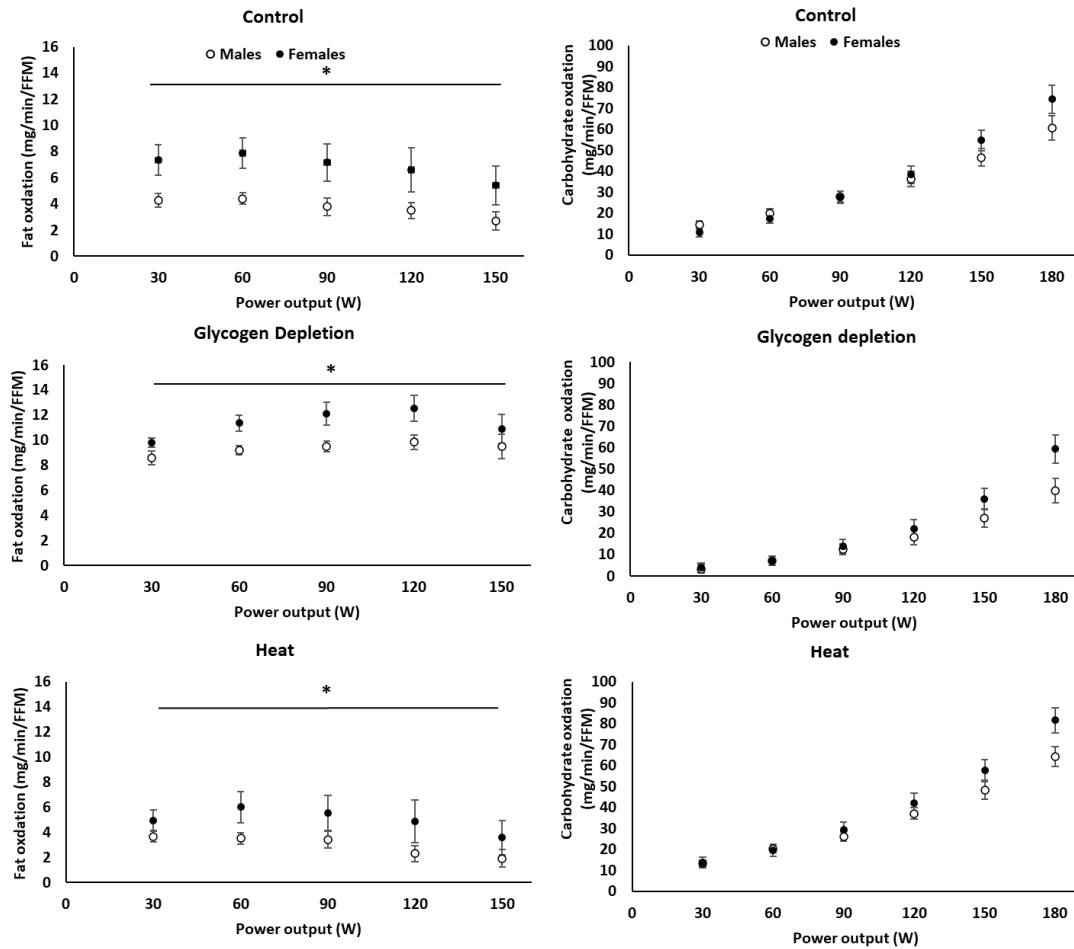


Figure 16 Fat oxidation and carbohydrate oxidation normalized to fat free mass throughout the intensities across situations. *: significant main effect of sex indicating a higher fat oxidation in females throughout the power outputs across situations. Data shown as mean $\pm$ standard error.

Table 16 Comparison between males and females at maximal fat oxidation.

		Control	Glycogen depletion	Heat	Sex main effect	Situation main effect	Interaction
MFO (mg/min)	Males	0.29±0.09*	0.65±0.15	0.25±0.11*	F(1,18)=0.201 p=0.66 η^2 p=0.011	F(2,36)=60.98 P<0.001 η^2p=0.772	F(2,36)=2.88 p=0.069 η^2 p=0.138
	Females	0.37±0.12*	0.58±0.08	0.3±0.15*			
MFO/FFM (mg/min/kg FFM)	Males	4.91±1.65*	10.98±2.38	4.26±1.63*	F(1,18)=10.7 p=0.004 η^2p=0.372	F(2,36)=53.479 p<0.001 η^2p=0.748	F(2,36)=0.565 p=0.573 η^2 p=0.03
	Females	8.32±2.99*	13±2.55	6.66±3.51*			
VO ₂ (ml/min/kg FFM)	Males	25.15±6.19*	32.37±7.27	22.38±6.28*	F(1,18)=7.43 p=0.014 η^2p=0.292	F(2,36)=23.101 p<0.001 η^2p=0.562	F(2,36)=0.5 p=0.61 η^2 p=0.027
	Females	29.82±8.21*	40.37±5.44	29.05±7.19*			
%VO _{2max} (%)	Males	41.45±8.68*	52.91±11.41	40.26±11.18*	F(1,18)=4.99 p=0.038 η^2p=0.217	F(2,36)=17.309 p<0.001 η^2p=0.49	F(2,36)=0.963 p=0.391 η^2 p=0.051
	Females	46.28±11.63*	65.63±13.01	46.14±10.75*			
Heart rate (bpm)	Males	105±12*	120±19	100±21*	F(1,18)=5.93 p=0.025 η^2p=0.248	F(2,36)=12.23 p<0.001 η^2p=0.404	F(2,36)=1.78 p=0.183 η^2 p=0.090
	Females	109±13*	134±14	120±11*			
%HR _{max} (%)	Males	54.52±9.28*	65.34±13.06	53.41±13.69	F(1,18)=11.5 p=0.003 η^2p=0.389	F(2,36)=8.372 p=0.001 η^2p=0.317	F(2,36)=0.782 p=0.465 η^2 p=0.042
	Females	59.55±7.63*	74.42±5.95	66.65±8.53			
PO (W)	Males	73.63±38.8*	98.18±52.11	62.72±39.01*	F(1,18)=0.006 p=0.936 η^2 p=	F(2,36)=13.51 p<0.001 η^2p=0.429	F(2,36)=0.489 p=0.617 η^2 p=0.026
	Females	66.66±36.05*	107.14±25.35	64.28±27.77*			
PO/FFM (W/kg FFM)	Males	1.21±0.59*	1.63±0.83	1.03±0.58*	F(1,18)=3.47 p=0.079 η^2 p=0.161	F(2,36)=14.82 p<0.001 η^2p=0.452	F(2,36)=1.24 p=0.302 η^2 p=0.064
	Females	1.5±0.88*	2.38±0.57	1.43±0.63*			

Data are shown as mean ± SD. MFO: maximal fat oxidation; FFM: fat free mass; VO₂: oxygen uptake at maximal fat oxidation; %VO_{2max}: percentage of maximal oxygen uptake at which maximal fat oxidation occurs; %HR_{max}: percentage of maximal heart rate at which maximal fat oxidation occurs; PO: power output; PO/FFM: power output normalized to fat free mass. *Pairwise comparisons of the situation effect showed a significant difference compared to glycogen depletion.

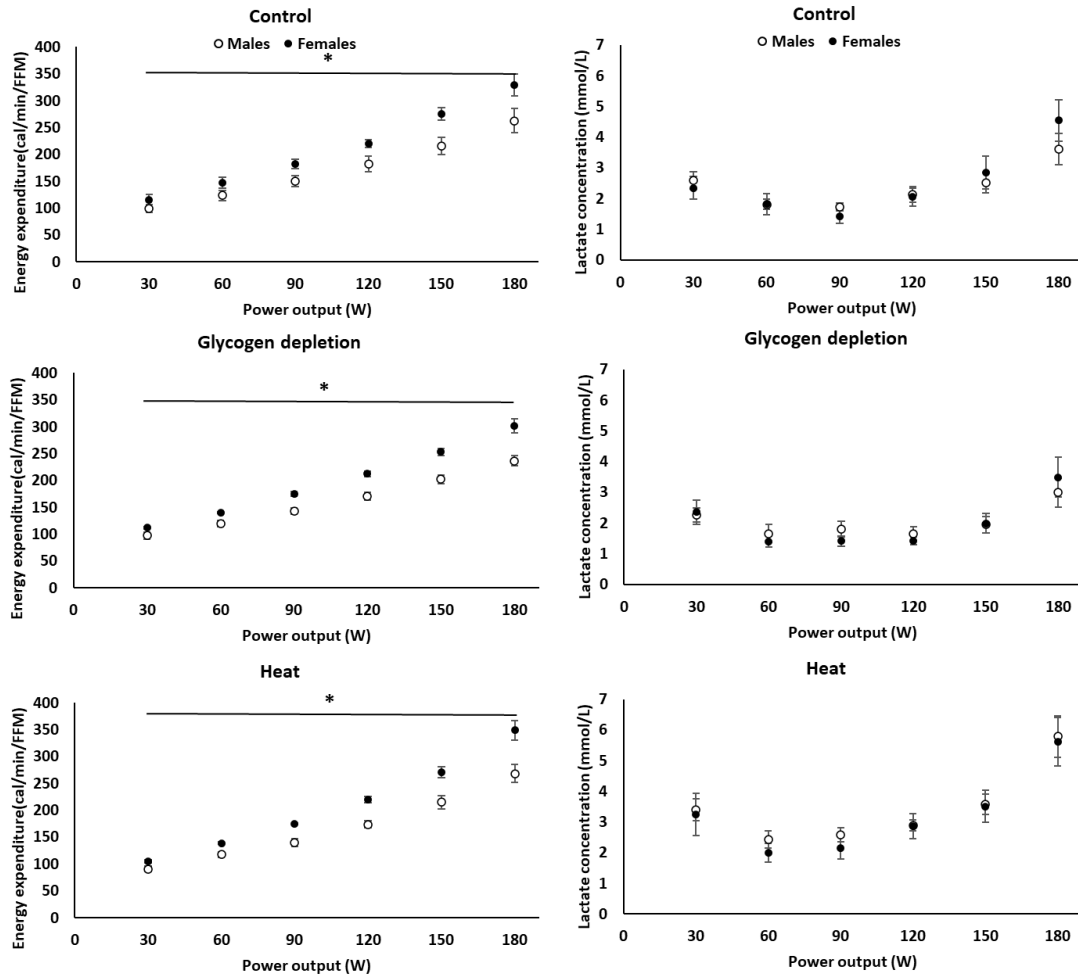


Figure 17 Energy expenditure normalized to fat free mass and lactate concentration throughout the intensities across situations. *: significant main effect of sex indicating a higher energy expenditure in females throughout the intensities across situations. Data shown as mean $\pm$ standard error.

Table 17 Comparison between males and females at lactate threshold.

		Control	Glycogen depletion	Heat	Sex main effect	Situation main effect	Interaction
Lactate (mmol/L)	Males	1.89±0.58*	1.51±0.73	2.45±0.77*	F(1,18)=1.93 p=0.181 η ² p=0.097	F(2,36)=8.946 p<0.001 η²p=0.332	F(2,36)=0.156 p=0.856 η ² p=0.009
	Females	1.8±0.71*	1.2±0.19	2.14±0.87*			
VO ₂ (ml/min/kg FFM)	Males	29.22±5.96	33.86±6.37	27.5±4.25*	F(1,18)=8.42 p=0.01 η ² p=0.319	F(2,36)=5.724 p=0.007 η²p=0.241	F(2,36)=0.734 p=0.487 η ² p=0.039
	Females	37.55±9.54	38.15±6.46	32.44±5.2*6			
%VO _{2max} (%)	Males	48.42±8.94	55.22±8.99	49.38±6.7*	F(1,18)=4.56 p=0.047 η²p=0.202	F(1,36)=4.946 p=0.013 η²p=0.216	F(1,36)=0.987 p=0.383 η ² p=0.052
	Females	57.92±10.36	62.41±14.87	51.38±5.54*			
Heart rate (bpm)	Males	119±16	126±13	120±13	F(1,18)=2.23 p=0.153 η ² p=0.11	F(2,36)=2.678 p=0.082 η ² p=0.13	F(2,36)=0.607 p=0.55 η ² p=0.033
	Females	124±11	131±10	131±11			
%HR _{max} (%)	Males	65.25±8.26*	69.02±6.09	67.38±7.8	F(1,18)=3.55 p=0.076 η ² p=0.165	F(2,36)=3.469 p=0.042 η²p=0.162	F(2,36)=0.233 p=0.793 η ² p=0.013
	Females	67.59±5.45*	73.33±6.07	72.07±3.47			
PO (W)	Males	106.07±29.3	119.10±43.48	93.29±30.93*	F(1,18)=0.527 p=0.477 η ² p=0.028	F(2,36)=4.088 p=0.025 η²p=0.185	F(2,36)=0.336 p=0.717 η ² p=0.018
	Females	105.07±29.37	104.66±29.68	85.04±23.94*			
PO/FFM (W/kg FFM)	Males	1.78±0.47	2.01±0.75	1.58±0.51	F(1,18)=3.95 p=0.062 η ² p=0.18	F(2,36)=3.944 p=0.028 η²p=0.18	F(2,36)=0.396 p=0.676 η ² p=0.022
	Females	2.33±0.62	2.3±0.6	1.88±0.53			
Data are shown as mean ± SD. VO ₂ : oxygen uptake at lactate threshold; FFM: fat free mass; %VO _{2max} : percentage of maximal oxygen uptake at which lactate threshold occurs; %HR _{max} : percentage of maximal heart rate at which lactate threshold occurs; PO: power output; PO/FFM: power output normalized to fat free mass. *Pairwise comparisons of the situation effect showed a significant difference compared to glycogen depletion.							

DISCUSSION

In the present study we explored the difference in metabolic flexibility between sexes in response to the exercise intensity stimulus in a control situation. Additionally, we evaluated metabolic flexibility between sexes in response to exercise intensity adding two different metabolically challenging stimuli (glycogen depletion and heat). Important confounding aspects, not previously considered, were controlled (i.e. all trained individuals, naturally menstruating females measured in the early follicular phase and matching both sexes by $\text{VO}_{2\text{max}}/\text{FFM}$). We found that FATox/FFM was higher in females throughout the different intensities across situations. In contrast, CHox/FFM was not significantly different between sexes at any specific intensity across situations. Consequently, EE/FFM was higher in females throughout the different intensities across situations. Finally, lactate concentration was not different between sexes at the same intensities across situation. The results of the present study reveal a sexual dimorphism in metabolic flexibility. Furthermore, it helps to elucidate physiological sex differences as well as to reduce the dramatic sex bias found in the scientific literature.

FATox/FFM throughout the different intensities and MFO/FFM was greater in females across situations. Also, it can be qualitatively observed that FATox/FFM seems to be higher in females at the same PO/FFM or $\% \text{VO}_{2\text{max}}$ (Supplementary material of the study IV in Annexes). Our results are aligned with previous studies performed in participants without overweight/obesity (299,300,306,307) reporting a higher MFO/FFM in females, with only one study matching both sexes by $\text{VO}_{2\text{max}}/\text{FFM}$ and considering the ovarian hormonal profiles of females (306). Altogether, females present a higher FATox/FFM throughout the different intensities, regardless of whether the test is performed in a situation emphasizing the oxidative pathway (glycogen depletion) or the glycolytic pathway (heat). In addition, we found that females presented the MFO at a higher $\% \text{VO}_{2\text{max}}/\% \text{HR}_{\text{max}}$. Also, a rightward shift in the $\text{FATox}/\text{FFM} \cdot \% \text{VO}_{2\text{max}}$ relationship can be qualitatively observed in females (Supplementary material of the study IV in

Annexes), which means that females can oxidize fat at a higher relative intensity. In line with the present study, three previous articles found that females tended to present the MFO at a higher %VO_{2max} (300,306,316) and two other studies reported a greater %VO_{2max} at MFO in females (299,307), with only one study failing to find significant differences (317). Collectively, MFO position (%VO_{2max}/%HR_{max}) is shifted to the right in females compared to their male counterparts. Based on the different physiological response between sexes at the same %VO_{2max}/%HR_{max}, males and females should train at a different %VO_{2max}/%HR_{max} to meet the intensity corresponding to MFO. According to the present results, future exercise intensity guidelines for maximizing fat oxidation should be stratified by sex in trained individuals.

CHox/FFM was not different between sexes across situations at any specific intensity. From a qualitative perspective, a lower CHox/FFM seems to be observed at the same PO/FFM or %VO_{2max} in females (Supplementary material of the study IV in Annexes). To the best of our knowledge, this is the first study comparing CHox/FFM between sexes at the same absolute intensities preventing to compare our results with the literature. Interestingly, our results seem to behave similarly regardless of whether the test is performed in a situation emphasizing the oxidative pathway (glycogen depletion) or the glycolytic pathway (heat).

It is worth mentioning that females oxidized more FATox/FFM throughout the intensities of the test across situations, while CHox/FFM was not different between sexes across situations at any specific intensity. That is, the greater difference in FATox/FFM than in CHox/FFM between sexes clearly represents the preference of females for oxidizing fats instead of carbohydrates for being able to cycle at the same intensity of males. Some of the main reasons reported in the literature for the preference of fats instead of carbohydrates of females compared to males are the difference in adrenergic activation (greater activation of β -receptors in females and the lower activation of α -receptors accompanied by a greater activation of β -receptors in males), sex hormones (the potential carbohydrate-sparing effect of oestrogen in females), muscle fibre distribution (the percentage area occupied by type I fibres is larger and the percentage area occupied

by type IIa fibres is lower in females), body composition (higher fat mass and lower FFM in females) and resting substrate content (higher intramyocellular lipid content in females) (301). Collectively, our results indicate that females maintain energy homeostasis by matching fuel availability to energy demand in response to exercise intensity by prioritising fat oxidation at lower intensities and shifting towards carbohydrate oxidation at higher intensities. Moreover, this is confirmed in a situation emphasizing the oxidative pathway (glycogen depletion) and other emphasizing the glycolytic pathway (heat).

EE/FFM was greater in females throughout the different intensities of the test across situations due to the greater FATox/FFM of females. Cycling at a specific intensity implies a higher PO/FFM in females than in males due to the lower FFM of the former. Consequently, females need more EE/FFM to cycle at the same intensity. This additional EE/FFM from females compared to males is obtained preferentially from fats, as mentioned above.

Lactate concentration was not different between sexes at the same intensities across situations. Thus, in contrast to FATox/FFM, lactate concentration was not able to detect differences in metabolic flexibility between sexes. Contrary, it can be qualitatively observed that lactate concentration seems to be lower in females at the same PO/FFM or %VO_{2max} (Supplementary material of the study IV in Annexes). Alternatively, females presented the LT1 at a higher %VO_{2max} and a tendency was found in %HR_{max}, indicating lactate concentration starts to increase at a higher relative intensity in females. Similar results have been found in the literature observing a higher %VO_{2max}/%HR_{max} at the estimated-LT1 in females (177,285). The inverse relationship between lactate concentration and fat oxidation (56,179) is in line with the concomitant right shift in the MFO position and the LT1 position observed in females compared to males. Hence, males and females should not train at the same %VO_{2max}/%HR_{max} with the intention to train at the LT1. In line with that, the physiological response could be different between sexes at the same %VO_{2max}/%HR_{max} because both sexes could be in a different training zone. Again, general guidelines should contemplate these differences and set exercise intensity stratified by sex in trained individuals.

Experimental considerations

Glycogen depletion was not directly measured in our study. However, the purpose of this situation was not to reach a state of complete glycogen depletion, but rather to shift the oxidation of different substrates compared to the control situation. In this sense, changes in blood lactate concentration and fat oxidation between control and glycogen depletion confirmed this shift in substrates oxidation and strongly suggest that participants had low glycogen levels. Additionally, the exercise protocol (189) and the low carbohydrate diet (188) used have already been effective in reducing glycogen levels.

CONCLUSIONS

Females need more EE/FFM than males to cycle at the same absolute intensity than males due to the lower FFM of the former. This additional energy from females is obtained preferably from fats. Hence, females maintain energy homeostasis by matching fuel availability to energy demand in response to exercise intensity by prioritising fat oxidation at lower intensities and shifting to carbohydrate oxidation at higher intensities than males. The results reveal a sexual dimorphism in metabolic flexibility in favour of females. In contrast, lactate concentration failed to detect differences in metabolic flexibility between sexes. This was confirmed under different metabolically challenging stimuli: a stimulus that emphasises the oxidative pathway (glycogen depletion situation) and a stimulus that emphasises the glycolytic pathway (heat situation). Importantly, this conclusion is achieved solving previous important limitations, such as considering naturally menstruating females evaluated at their early follicular phase and matching both sexes by $\text{VO}_{2\text{max}}/\text{FFM}$. Furthermore, it helps to elucidate physiological sex differences as well as to reduce the dramatic sex bias found in the scientific literature.

4.5. Study V: Changes in lactate concentration are accompanied by opposite changes in the pattern of fat oxidation: Dose-response relationship?

APPROACH TO THE PROBLEM

Lactate has different functions in the body: 1) it is an energy source, 2) it is the major gluconeogenic precursor and, 3) it is considered a signalling molecule with autocrine-, paracrine- and endocrine-like effects, being called “lactormone” (15). This last function has gained more attention in recent years, in areas of intermediary metabolism, redox biology, mitochondrial biogenesis, neurobiology, gut physiology, appetite regulation or nutrition (196).

One of the main signalling functions of lactate during exercise is the regulation of muscle metabolism in an autocrine and paracrine way (196). In 1962, Issekutz et al. were the first to describe that there was an inverse association between lactacidemia and free fatty acids in exercising dogs when infusing lactic acid (318). More than ten years later, the pioneer study in humans by Boyd et al., showed that infusion of D,L-sodium lactate during exercise decreased free fatty acid and glycerol levels in the blood. They suggested that a high lactate concentration during exercise produces a direct inhibition of lipolysis (319). Thereafter, at the beginning of this century, Achten et al. described a clear relationship between the increase of blood lactate concentration above baseline levels and the onset of a decrease in fatty acid oxidation (179). Specifically, they found that the intensity corresponding to the first rise in lactate concentration during incremental exercise was correlated with the intensity which elicited maximal fat oxidation rates (Fatmax). Moreover, they found that the intensity corresponding to the inflection point of the lactate curve during an incremental test was correlated with the intensity at which fat oxidation rates become negligible (Fatmin). Recently, San-Millán et al. went one step further observing that lactate concentration was positively correlated with carbohydrate oxidation and negatively correlated with fat oxidation across participants with different metabolic characteristics (elite endurance athletes, moderately active individuals, and patients with metabolic

syndrome). Interestingly, those subjects with a faster increase in blood lactate concentration presented a lower fat oxidation rate and vice versa (56).

Different situations can modify lactate concentration during exercise. For example, exercise in the heat in non-acclimatized individuals results in a greater reliance on carbohydrate metabolism (100,101). Since lactate is the end product of glycolysis (5), the greater reliance on glucose in a hot environment will lead to increased lactate production (100,101,104,108). In fact, a relationship between muscle temperature and muscle lactate has been observed (109). On the other hand, several studies have modified previous glycogen levels by combining diet and exercise, showing lower lactate concentrations with low glycogen levels (61–63). However, it has not yet been established whether these changes in lactate concentration produced by the different situations modify fat oxidation. If confirmed, we could determine a dose-response relationship between lactate concentration and fat oxidation that would help to elucidate the interaction between fat oxidation and blood lactate concentration during exercise. In addition, it would be possible to estimate fat oxidation from lactate concentration, and vice versa, regardless of the situation. This would avoid having to establish the blood lactate-fat oxidation relationship in each situation.

Therefore, the aim of this study was to assess whether changes in lactate concentration, due to glycogen depletion or heat, alter the pattern of fat oxidation during a maximal incremental cycle ergometer test in healthy young active females and males. It was hypothesized that blood lactate concentration was inversely correlated with fat oxidation in the different situations. Additionally, we hypothesized the lowest lactate concentrations observed in the glycogen depletion situation will be accompanied by increased fat oxidation. In contrast, the highest lactate concentrations in the heat situation will be accompanied by less fat oxidation.

MATERIAL AND METHODS

Participants

Eleven males and eight naturally menstruating females participated in this study. All of them were healthy and endurance trained (Table 18). Inclusion criteria required all subjects to: a) be healthy and between 18 and 40 years of age; b) undertake endurance training more than 3 hours per week; c) be a non-smoker; and for women: d) not be user of hormonal contraceptives; e) not be pregnant; f) have natural and regular menstrual cycles (276) and; g) not abundant menstrual bleeding (<80 ml) (310).

Table 18 Descriptive characteristics of the participants.

Age (years)	Males	22	±	3
	Females	22	±	2
Height (cm)	Males	176.2	±	4.0
	Females	165.1	±	5.3
Weight (kg)	Males	68.4	±	4.9
	Females	60.8	±	4.5
Fat (kg)	Males	6.80	±	2.14
	Females	14.06	±	2.51
Fat free mass (kg)	Males	59.42	±	4.86
	Females	43.82	±	5.37
Fat (%)	Males	10.3	±	3.1
	Females	24.4	±	4.7
Maximal oxygen uptake (ml/min)	Males	3581	±	369
	Females	2767	±	433
Maximal oxygen uptake (ml/min/kg)	Males	52.30	±	3.64
	Females	45.33	±	5.37
Data are shown as mean ± standard deviation				

Ethical authorization for this study was obtained from the Research Ethics Committee of the University where the research was conducted (ID: DATOS-20220524-RCC-LacFat). Subjects were informed of the risks and benefits of the study prior to participation and written informed consent was obtained from each subject before their inclusion.

Study design

This crossover study consisted of four situations carried out on five visits to the laboratory on five different days (Figure 18). The first visit included informed consent, an assessment of body composition by total body dual-energy X-ray

absorptiometry (DXA) and individualized nutritional plan. Afterwards, in the following appointments, the same maximal incremental tests were performed in three different experimental situations (control, glycogen depletion and heat). Tests were performed at the same time of day (± 2 hours). In the case of females, all tests occurred during the early-mid follicular phase of the menstrual cycle (day 4 ± 1.67). The first test was in all cases a maximal incremental test in the standard situation (control situation). The order of the two remaining incremental tests (glycogen depletion or heat situations) was randomized. In order to prepare for the glycogen depletion test, 24 hours before the incremental test, an exercise protocol was performed to completely deplete muscle glycogen stores. In addition, from breakfast on the same day of the glycogen depletion exercise protocol until the maximal incremental test, a low-carbohydrate diet was followed. On the contrary, in control and heat situations, from breakfast of the previous day of the tests, participants followed a high-carbohydrate diet. During both control and glycogen depletion situations, the ambient temperature was maintained at $\sim 21^{\circ}\text{C}$. Finally, during the heat situation participants were placed in a heat chamber designed to maintain an ambient temperature between $36\text{-}38^{\circ}\text{C}$ for 30 min prior the incremental test, and the incremental test was performed at the same temperature (187). In all the situations, body weight loss was less than 2%. Clinical trials: NCT05703100.

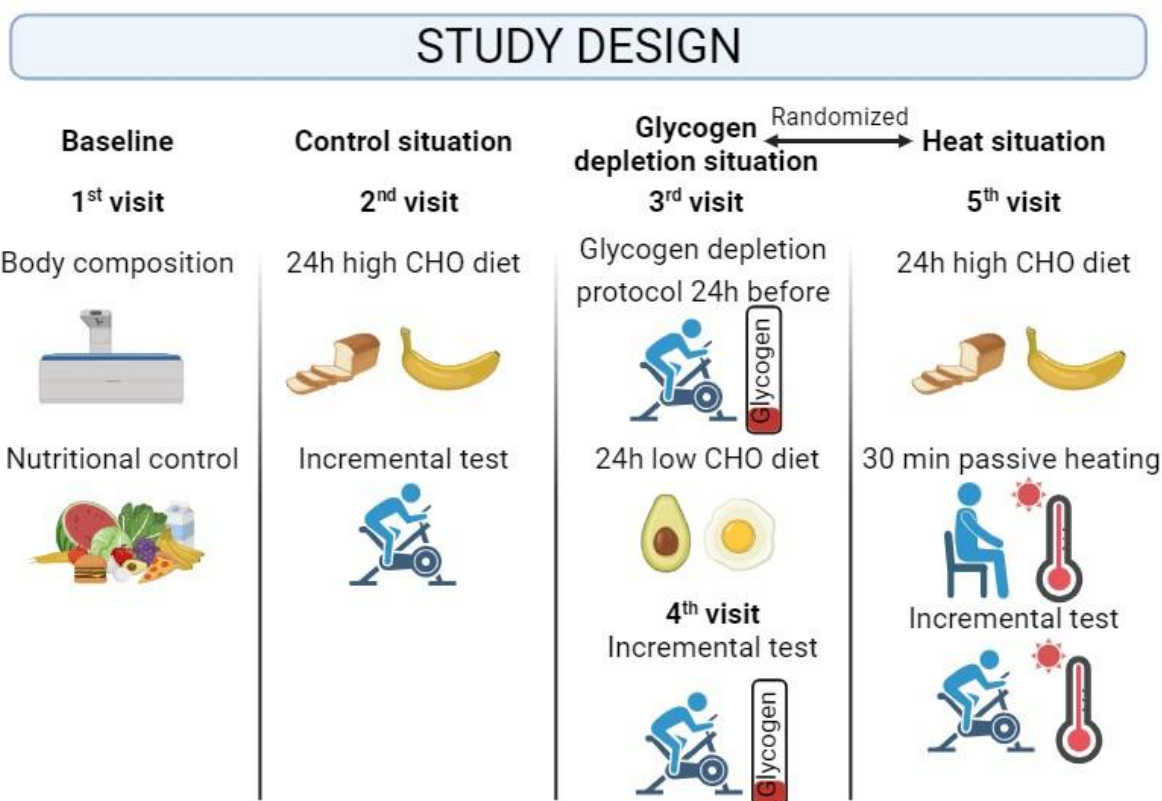


Figure 18 Overview of the study design. CHO: Carbohydrates.

Body composition assessment

Body composition was measured by a DXA scan (Version 6.10.029GE Encore 2002, GE Lunar Prodigy; GE Healthcare, Madison, WI, USA) to obtain the fat mass and fat-free mass (FFM). All participants performed the test wearing light sportswear (e.g., top and shorts). In addition, they must be with their body and hands in a supine position and their feet joined by a strap. Participants were instructed not to move or speak while the scanner performed the measurements. The scan was calibrated every other day using the phantom supplied by the manufacturer.

Nutritional intervention

High-energy, high-carbohydrate diet

Participants followed a diet at least 24 hours before the incremental test in the control and heat situations to ensure adequate energy and carbohydrate intake to avoid performance limitations during testing. The macronutrient distribution was standardized for all participants at an approximate ratio of 65% carbohydrate, 15%

protein and 20% fat. Energy and carbohydrate intake were determined for all participants in relation to their FFM (energy: 60 kcal·kg·FFM⁻¹; CHO: 9.8 g·kg·FFM⁻¹) (188) (see example in Supplementary Material of the study V in Annexes).

High-energy, low-carbohydrate and high-fat diet

This diet matched the energy intake of the high carbohydrate diet (60 kcal·kg⁻¹·FFM·day⁻¹). However, carbohydrate intake was severely restricted (<50 g·day⁻¹) and protein and fat intake were 20% and 75-80%, respectively (188). The most difficult nutritional objective to achieve was to reach the previously established daily caloric intake without exceeding the carbohydrate limit. Therefore, a medium-chain triglycerides supplementation was used to achieve this energy objective without contributing to increase carbohydrates (see example in Supplementary Material of the study V in Annexes).

Exercise protocol

Incremental exercise test

The incremental test performed in the three different situations was carried out on a cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). The cadence throughout the test was constant, remaining between 70-90 rpm. The protocol started with 1 min of rest followed by 5 min of warm-up at 30W. Subsequently, power was increased by 30 W every 3 min until maximum effort was reached, considering this when a reduction in cadence of 10 rpm was observed.

Glycogen depletion protocol

The glycogen depletion protocol consisted of a 60-minute continuous cycling exercise at the power associated with 60% of VO₂max calculated from the power-VO₂ ratio obtained during the maximal incremental cycling test performed in the control situation. The pedalling cadence was set at 70 rpm. After that, six 30-second sprints were performed at the maximum possible intensity with a 7.5% body weight load with a 4-minute recovery between bouts. This protocol has been carried out in previous studies with the same objective, having already demonstrated its efficacy in the depletion of glycogen stores (189).

Data analysis

The expired gas volume and composition were analyzed breath-by-breath and the data were averaged every 15 seconds using the JAEGER® Vyntus CPX gas analyzer (Jaeger-CareFusion, Hoechberg, Germany). The equipment was previously calibrated according to the manufacturer, whose validity and reliability has been demonstrated (coefficient of variation of the respiratory quotient lower than 0.5%). VO_2 max was established as the highest value averaged every 15 seconds reached during the test until exhaustion in the control situation. For data analysis of the three different incremental tests, the average of the last 60 seconds of each step was taken (Amaro-Gahete et al., 2019). Whole body fat oxidation was calculated from respiratory quotients using standard indirect calorimetry equations (312).

$$\text{Fat oxidation (g min}^{-1}\text{)} = 1.67 \cdot \text{VO}_2 - 1.67 \cdot \text{VCO}_2 - 1.92 \cdot n$$

n= negligible urinary nitrogen excretion

Heart rate was analyzed beat by beat throughout the three different incremental tests and averaged every 15 seconds through the Polar H10 (Polar Electro Oy, Kempele, Finland). This band was connected via Bluetooth to a cell phone that received the signal and where the data was stored. Moreover, the whole blood lactate concentration was measured during the three different incremental tests. Capillary blood was drawn from the fingertip at rest, in the last 30 seconds of each step and immediately after the moment of maximum effort, and these samples were analyzed with a lactate analyzer (EKF Diagnostic GmbH, Barlebe, Germany). Throughout the tests, central temperature with a tympanic thermometer (Braun IRT6520, Switzerland), rate of perceived exertion (RPE) with the 0-10 and 6-20 scales, and ambient temperature and humidity (Dienmern, Guangdong, China) was controlled.

Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD). Two sample size estimation were conducted using statistical software (G*Power v. 3.1.9.2, Stuttgart, Germany). Initially, to examine the feasibility of the project, sample size

estimation was performed based on data from a previous study (187) using an a priori paired student's t test with the following criteria: effect size $d_z=0.65$; $\alpha=0.05$; power=0.8. Based on these inputs, at least 17 participants were required. Subsequently, according to our data from a pilot study with 6 subjects, another sample size estimation was performed using an a priori One way ANOVA repeated measures within factors using power output at the lactate threshold (OBLA 4) in the three different situations of the study with the following criteria: effect size $F=2.015$; $\alpha=0.05$; power=0.95; number of groups=3; number of measurements=3; correlation among repeated measures=0.5; nonsphericity correction=1. Based on these inputs, at least 6 participants were required. The latter sample size estimation was taken as a reference. The normal distribution was confirmed by the Shapiro-Wilk test. A mixed linear model was used to compare the individual response (random effect) of lactate or fat oxidation between situations (fixed effect) at the different protocol intensities (fixed effect). Bonferroni correction was applied for post hoc analyses. Pairwise comparisons effect size was calculated by Cohen's d considering $d < 0.5$ as small, $d < 0.8$ as moderate and $d > 0.8$ as large (320). The association between lactate and fat oxidation was calculated using Pearson's correlation. Statistical analysis were carried out using Jamovi V1.6 statistical software (Jamovi, Sidney, Australia). The significant level was set at $p<0.05$.

RESULTS

Regarding participants, two males and two females were removed from the fat oxidation analysis because their fat oxidation during the control situation was negligible coinciding with a respiratory exchange ratio close to 1 from the beginning of the test.

In males and females, a very strong inverse association was found between lactate concentrations and fat oxidation in control, glycogen depletion and heat situation (Figure 19).

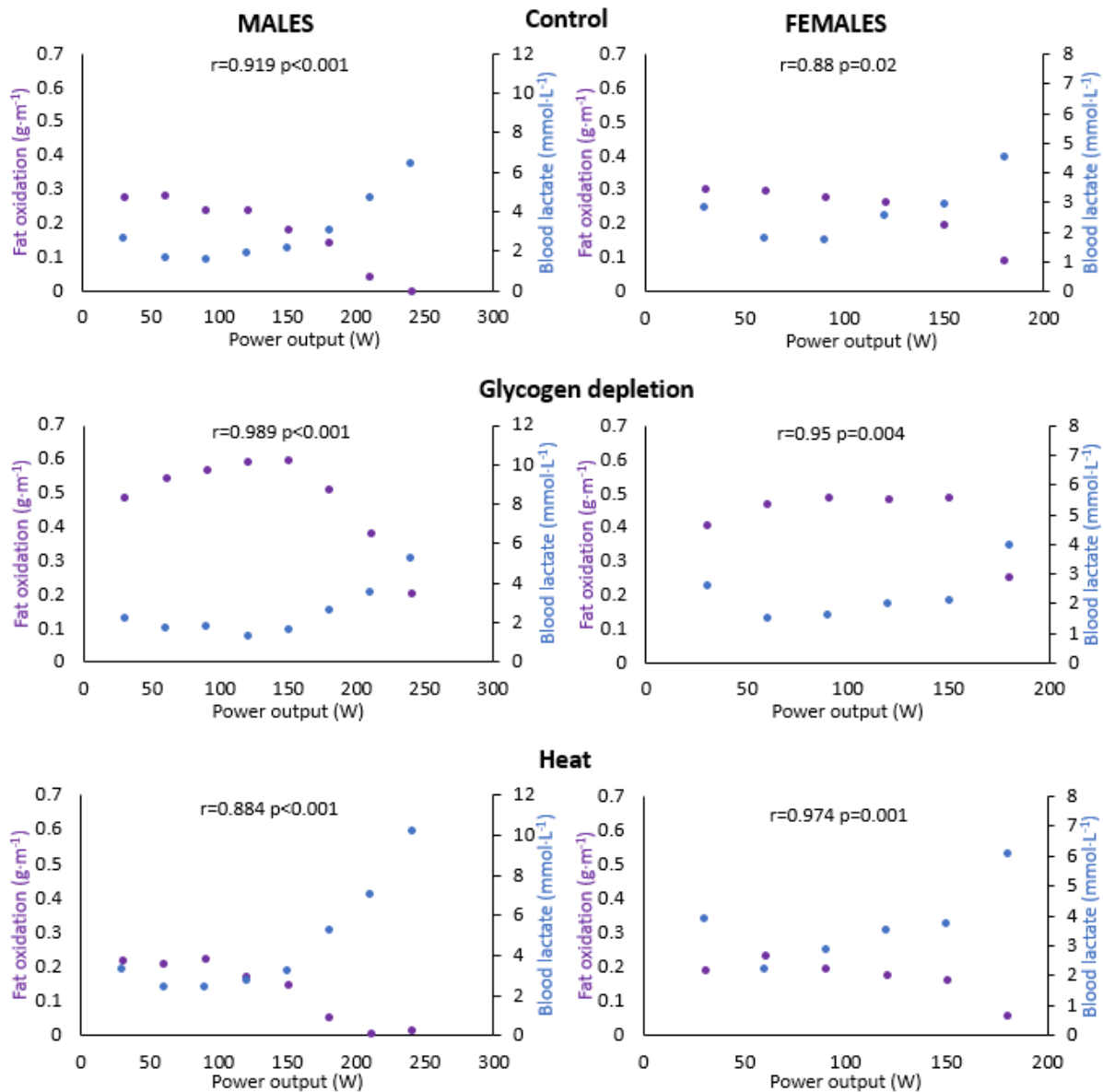


Figure 19 Relationships between the average blood lactate concentrations and fat oxidation as a function of exercise power output in control, glycogen depletion and heat situations in males and females.

In males, a main effect of the situation was found in blood lactate concentration ($F(2,220)=47.45$; $p<0.001$). Pairwise comparisons showed that blood lactate concentration was higher in heat compared to control ($p<0.001$; $d=0.473$) and glycogen depletion situations ($p<0.001$; $d=0.728$). In addition, blood lactate concentration was higher in control compared to glycogen depletion situation ($p=0.037$; $d=0.255$). On the other hand, fat oxidation was different between situations ($F(2,182)=216.24$; $p<0.001$). Pairwise comparisons showed that fat

oxidation was lower in heat compared to control ($p=0.047$; $d=-0.236$) and glycogen depletion situation ($p<0.001$; $d=-2.038$). Moreover, fat oxidation was lower in control compared to glycogen depletion situation ($p<0.001$; $d=-1.801$) (Table 19).

In females we observed similar results. Blood lactate concentration varied between situations ($F(2,112)=18.15$; $p<0.001$). Specifically, blood lactate concentration was higher in heat compared to control ($p=0.005$; $d=0.438$) and glycogen depletion situations ($p<0.001$; $d=0.809$). In addition, blood lactate concentration was higher in control compared to glycogen depletion situation ($p=0.020$; $d=0.371$). Regarding fat oxidation, it was significantly different between situations ($F(2,80)=48.87$; $p<0.001$). Post hoc test showed that fat oxidation was lower in heat compared to control ($p=0.019$; $d=-0.371$) and glycogen depletion situation ($p<0.001$; $d=-1.417$). Finally, fat oxidation was lower in control compared to glycogen depletion situation ($p<0.001$; $d=-1.045$) (Table 19).

Table 19 Blood lactate concentration and fat oxidation as a function of exercise power output in control, glycogen depletion and heat situations in males and females.

		Males						Females									
Intensity (W)	Situation	N	Lactate (mmol ·L ⁻¹)			N	Fat oxidation (g min ⁻¹)			N	Lactate (mmol ·L ⁻¹)			N	Fat oxidation (g min ⁻¹)		
30	Heat	11	3.40	±	1.18	9	0.221	±	0.099	8	3.48	±	2.073	6	0.192	±	0.136
	Control	11	2.59	±	0.94	9	0.277	±	0.144	8	2.79	±	0.809	6	0.305	±	0.141
	Depletion	11	2.27	±	0.74	9	0.486	±	0.096	8	2.41	±	1.124	6	0.410	±	0.091
60	Heat	11	2.44	±	0.93	9	0.212	±	0.099	8	2.16	±	0.981	6	0.236	±	0.198
	Control	11	1.81	±	0.57	9	0.285	±	0.130	8	1.96	±	0.915	6	0.299	±	0.189
	Depletion	11	1.66	±	0.96	9	0.544	±	0.082	8	1.52	±	0.602	6	0.472	±	0.144
90	Heat	11	2.58	±	0.78	9	0.228	±	0.154	8	2.59	±	1.620	6	0.199	±	0.208
	Control	11	1.73	±	0.40	9	0.238	±	0.173	8	1.84	±	1.153	6	0.279	±	0.206
	Depletion	11	1.81	±	0.82	9	0.569	±	0.094	8	1.61	±	0.728	6	0.489	±	0.227
120	Heat	11	2.88	±	0.58	9	0.172	±	0.130	8	3.44	±	2.002	6	0.181	±	0.243
	Control	11	2.13	±	0.81	9	0.238	±	0.175	8	2.65	±	1.577	6	0.265	±	0.214
	Depletion	11	1.65	±	0.78	9	0.596	±	0.134	8	1.85	±	1.277	6	0.488	±	0.260
150	Heat	11	3.57	±	1.08	9	0.148	±	0.159	7	3.51	±	1.629	5	0.165	±	0.200
	Control	11	2.52	±	1.10	9	0.182	±	0.190	7	2.89	±	1.306	6	0.200	±	0.163
	Depletion	11	1.94	±	0.91	9	0.598	±	0.221	7	1.98	±	0.978	5	0.490	±	0.162
180	Heat	11	5.78	±	2.21	9	0.054	±	0.100	7	5.61	±	2.410	5	0.061	±	0.138
	Control	11	3.61	±	1.68	9	0.142	±	0.167	7	4.39	±	1.270	5	0.091	±	0.089

	Depletion	11	3.00	± 1.57	9	0.513	± 0.251	7	3.50	± 1.971	5	0.254	± 0.184
210	Heat	10	7.25	± 3.31	9	0.007	± 0.023						
	Control	11	5.33	± 2.40	9	0.045	± 0.076						
	Depletion	11	4.37	± 2.45	9	0.383	± 0.269						
240	Heat	6	10.24	± 3.76	8	0.018	± 0.051						
	Control	10	6.75	± 3.22	9	0.000	± 0.000						
	Depletion	8	5.29	± 2.53	8	0.232	± 0.248						
Average	Heat		4.40	± 2.92		0.135	± 0.137		3.42	± 2.03		0.176	± 0.185
	Control		3.27	± 2.30 ^a		0.176	± 0.171 ^a		2.71	± 1.40 ^a		0.245	± 0.177 ^a
	Depletion		2.66	± 1.87 ^{ab}		0.494	± 0.213 ^{ab}		2.12	± 1.29 ^{ab}		0.438	± 0.192 ^{ab}
Data are shown as mean ± SD. ^a Significantly difference from heat; ^b Significantly difference from control.													

DISCUSSION

This study aimed to investigate whether changes in lactate concentration, due to glycogen depletion or heat, alter the pattern of fat oxidation during a maximal incremental cycle ergometer test in healthy young active females and males in a within-subject design. In agreement with our hypothesis, a very strong inverse association was found between lactatemia and fat oxidation in females and males in all three situations. Furthermore, the lowest lactate concentrations were accompanied by the highest fat oxidations in the glycogen depletion situation, while the highest lactate concentrations were accompanied by the lowest fat oxidations in the heat situation. This study helps to elucidate the interaction between lactate concentration and fat oxidation during exercise.

Our results show that the increase in blood lactate concentration coincides with the decrease in fat oxidation. This phenomenon agrees with previous studies observing an inverse association between lactacidemia/lactatemia and free fatty acids after infusing lactic acid/lactate in animals (318,321,322) or humans (319). In addition, our data are in concordance with the scientific literature showing an inverse relationship between lactatemia and fat oxidation (56,179). Hence, the very strong inverse association between blood lactate concentration and fat oxidation suggests that measuring blood lactate concentration is an effective way to indirectly assess changes in fat oxidation, as previously suggested (56), even across different situations. However, it is important to note that lactate-fat oxidation relationship is different between the three situations. This implies that

to determine fat oxidation from lactate, or vice versa, it is required to determine the lactate-fat oxidation relationship specific to each situation.

Due to the different lactate-fat oxidation relationship between situations, the same blood lactate concentration does not imply the same fat oxidation across the different situations. It was already known that a fixed blood lactate concentration does not reflect the same metabolic situation between different individuals (25). Now, with our study, we also know that a fixed blood lactate concentration may not reflect the same metabolic response (e.g., fat oxidation) even in the same individual exposed to different situations. To the best of our knowledge, this is the first study showing this within-subject comparison.

The highest lactate concentrations coincided with the lowest fat oxidations in heat, and the lowest lactate concentrations coincided with the highest fat oxidations in glycogen depletion. Interestingly, from a qualitative perspective, the lactate-fat oxidation crossover point was shifted to the left in heat and shifted to the right in the glycogen depletion both compared to control. This means that changes in lactate concentrations are accompanied by opposite changes in the pattern of fat oxidation. On the one hand, lactate oxidation inside mitochondria increases the production of acetyl-CoA and, subsequently, malonyl-CoA. In turn, malonyl-CoA inhibits the transport of fatty acids through the mitochondrial membrane transporter Carnitine Palmitoyl Transferase 1 (CPT1) (15). Moreover, chronic lactate exposure reduces fatty acid transport into mitochondria decreasing the activity of CPT1 (323). Additionally, an increase in blood lactate concentration decreases lipolysis in adipose tissue through activation of an orphan G protein-coupled receptor (GPR81) (324–326). All this evidence contributes to the assumption that lactate inhibits fat oxidation. Nevertheless, in our results, the greatest effect size in lactate concentration was found between the control and the heat situation; but on the contrary, the greatest effect size in fat oxidation was found between control and glycogen depletion. That is, the greatest change in blood lactate concentration does not coincide with the greatest change in fat oxidation. That means changes in lactate concentration are not proportional to changes in fat oxidation. Therefore, these results lead us to suggest that there may be other

mechanisms which play a major role than lactate regulating fat oxidation during exercise.

As any study, the present one is not exempt from limitations. Glycogen depletion was not directly measured in our study. Therefore, we cannot confirm a true glycogen depletion state of the individuals in the glycogen depletion situation. However, the changes in blood lactate concentration and fat oxidation between control and glycogen depletion strongly suggest that participants presented low glycogen levels. Furthermore, this exercise protocol (189) and this low carbohydrate diet (188) have already been effective reducing glycogen levels. On the other hand, to control the confounding effect of the menstrual cycle in females, they performed the different tests in the early-mid follicular phase. This decision was made because previous studies suggested that menstrual cycle could affect substrate utilization and blood lactate concentration (327). Since all tests could not be performed in the same menstrual cycle, most females performed all test at least one menstrual cycle apart (between two or three menstrual cycles). That means (de)training between menstrual cycles could be acting as a confounding variable in the females' results. This concern should be considered in future studies when deciding whether menstrual cycle should be controlled.

CONCLUSION

A very strong inverse association between lactatemia and fat oxidation was found in females and males in control, glycogen depletion and heat situations. Therefore, assessing blood lactate alone could be an effective way to indirectly assess fat oxidation even across different situations, or vice versa. However, lactate-fat oxidation relationship changes between situations. That means the same blood lactate concentration does not imply the same fat oxidation across the different situations. Therefore, a fixed blood lactate concentration may not reflect the same metabolic response even in the same individual exposed to different situations. This implies that to determine fat oxidation from lactate, or vice versa, it is required to determine the lactate-fat oxidation relationship specific to each situation. In addition, changes in lactate concentrations are accompanied by opposite changes in the pattern of fat oxidation in females and males in a within-

subject design; although, the greatest change in blood lactate concentration between situations does not coincide with the greatest change in fat oxidation. Hence, these results lead us to suggest that there are mechanisms apart from lactate that have a greater impact on changes in fat oxidation across different situations.

4.6. Study VI: A comparison between heart rate, RPE and power output to prescribe exercise intensity in control, low glycogen availability and heat

APPROACH TO THE PROBLEM

Exercise intensity prescription is usually set based on training zones (moderate, heavy, and severe) (177). These zones can be identified during an incremental test determining the first and second thresholds, respectively (177,288,328). The transition from the moderate to the heavy zone is indicated by the first threshold, determined through the first lactate or ventilatory threshold (LT1 and VT1, respectively). Subsequently, the transition from the heavy to the severe zone is indicated by the second lactate or ventilatory threshold (LT2 and VT2, respectively) (329).

When prescribing exercise intensity, these thresholds can be indicated by different variables of internal load (e.g., heart rate, rate of perceived exertion, or lactate concentration) or external load (e.g., power output or speed) (330). However, it remains unclear how these variables are affected by different situations (i.e., ambient temperature or nutritional status). Regarding ambient temperature, previous studies have investigated the stability of heart rate and power output under heat at different thresholds obtaining apparently contradictory results (187,331). Maunder et al. found a similar heart rate at VT1 and VT2 at 18°C and 35°C (187). On the contrary, power output was lower at these thresholds at 35 °C compared to 18°C (187). On the other hand, Bourgois et al. performed ramp incremental tests at 36°C and 18°C, determining VT1 and VT2, finding a higher heart rate and a similar power output at 36°C compared to 18°C (331). Due to these inconclusive results, it seems necessary to continue to investigate the effect of heat on different variables at the thresholds due to the wide range of environmental temperatures at which training sessions and competition are carried out. Regarding nutritional status, it is already known that muscle glycogen depletion affects the metabolic response during an incremental test (61,62,64). However, to the best of our knowledge, there is limited evidence comparing the effect of

glycogen depletion on the different variables typically used to determine ventilatory thresholds, such as power output or heart rate, in the same study. Only two studies explored the effect of glycogen depletion on heart rate at VT1 exclusively, observing a similar heart rate at VT1 under glycogen depletion compared to control (64,332). Another study reported a higher power output at the LT1 in glycogen depletion compared to control (66). Then, it is necessary to fill this gap in the literature to elucidate which is the best way to prescribe exercise intensity based on training zones under this nutritional strategy commonly used by athletes (308).

Therefore, the purpose of this study was to compare heart rate, rate of perceived exertion (RPE), and power output at VT1, VT2 and maximum in different situations: control, heat (36°C) and glycogen depletion. A secondary objective was to observe whether the physiological response (fat and carbohydrate oxidation, lactate, O₂ consumption, CO₂ production and ventilation) varied between situations at these physiological points.

MATERIAL AND METHODS

Participants

Eighteen participants (ten males and eight females) participated in this study (Table 20). The inclusion criteria required that all subjects met the following requirements: a) be healthy, and between 18 and 40 years old; b) perform endurance training more than 3 hours per week; c) be a non-smoker; and for females: d) non-user of hormonal contraceptives; e) not be pregnant; f) have natural and regular menstrual cycles (276) and; g) not abundant menstrual bleeding (<80 ml) (310).

Ethical authorization for this study was obtained from the Research Ethics Committee of the University of the authors (ID: DATOS-20220524-RCC-LacFat). Subjects were informed of the risks and benefits of the study before participating, and written informed consent was obtained from each subject before inclusion.

Table 20 Descriptive characteristics of the participants.

	Males			Females		
Age (years)	22	±	3	22	±	2
Height (cm)	176.6	±	3.9	165.1	±	5.3
Weight (kg)	69.0	±	4.7	60.8	±	4.5
Fat mass (kg)	6.59	±	2.13	14.05	±	2.50
Fat-free mass (kg)	60.04	±	4.64	43.82	±	5.36
Fat mass (%)	9.8	±	3.0	24.4	±	04.7
VO _{2max} (mL/min)	3604	±	380	27670	±	433
VO _{2max} (mL/min/kg)	52.18	±	3.82	45.32	±	5.37
Data are shown as mean ± standard deviation. VO _{2max} : maximal oxygen uptake.						

Study design

The study consisted of five visits to the laboratory (Figure 20). The first visit included informed consent, body composition assessment using whole-body dual-energy X-ray absorptiometry (DXA) and individualized nutritional plan. Subsequently, in the following appointments, the same maximal incremental tests were performed in three different experimental situations (control, glycogen depletion and heat). Tests were performed at the same time of day (± 2 hours). For females, all tests were performed during the early-mid follicular phase of the menstrual cycle (day 4 ± 1.67). The first test was in all cases a maximal incremental test in the standard situation (control situation). The order of the two remaining incremental tests (glycogen depletion or heat situations) was randomized. To prepare for the glycogen depletion test, 24 hours before the incremental test, an exercise protocol was performed to completely deplete muscle glycogen stores. Additionally, participants followed a high-carbohydrate diet from breakfast on glycogen depleting exercise day until the maximal incremental test. On the contrary, in control and heat situations, from breakfast the day before the test, participants followed a high-carbohydrate diet. During both control and glycogen depletion situations, the ambient temperature was maintained between 21-22 °C. Finally, during the heat situation, participants were placed in a heat chamber designed to maintain an ambient temperature between 36-38°C for 30 minutes

before the incremental test, and the incremental test was performed at the same temperature (187). In all situations, body weight loss was less than 2%.

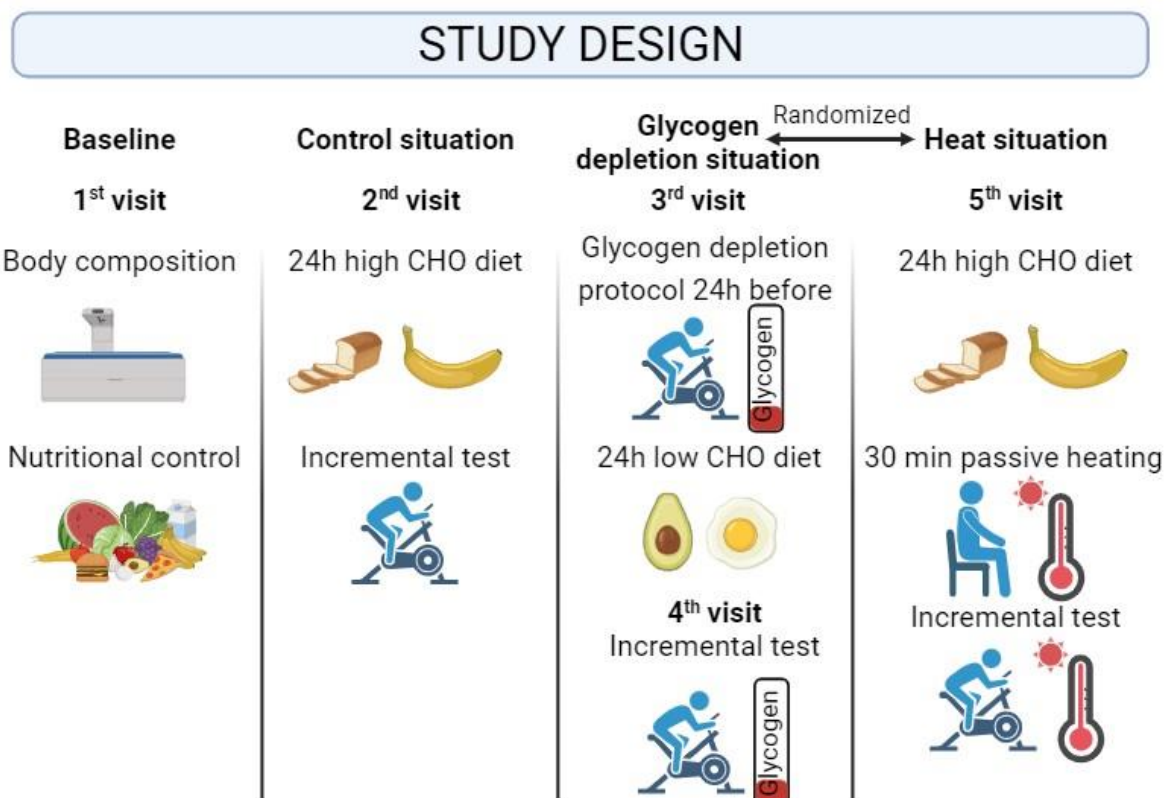


Figure 20 Overview of the study design. CHO: Carbohydrates.

Body composition assessment

Body composition was measured by DXA scan (Version 6.10.029GE Encore 2002, GE Lunar Prodigy; GE Healthcare, Madison, WI, USA) to obtain fat mass and fat-free mass (FFM). All participants performed the test wearing light sports clothing (e.g., top and shorts). In addition, they must be with their body and hands in a supine position and their feet joined by a strap. Participants were instructed not to move or talk while the scanner performed measurements. The scan was calibrated every two days using the phantom supplied by the manufacturer.

Nutritional intervention

High-energy, high-carbohydrate diet

Participants followed a diet at least 24 hours before the incremental test in the control and heat situations to ensure adequate energy and carbohydrate intake to avoid performance limitations during testing. Macronutrient distribution was standardized for all participants at an approximate ratio of 65% carbohydrate, 15% protein and 20% fat. Energy and carbohydrate intake were determined for all participants in relation to their FFM (energy: 60 kcal·kg FFM⁻¹; CHO: 9.8 g·kg FFM⁻¹) (188) (see Figure in Supplementary material of the study VI in Annexes).

High-energy, low-carbohydrate and high-fat diet

This diet matched the energy intake of the high carbohydrate diet (60 kcal·kg⁻¹ FFM·day⁻¹). However, carbohydrate intake was severely restricted (<50 g·day⁻¹) and protein and fat intake were 20% and 75-80%, respectively (188). The most challenging aspect was to achieve the previously established daily caloric intake without exceeding the carbohydrate limit. Therefore, supplementation with medium chain triglycerides was used to achieve this energy goal without contributing to increase carbohydrates (see Figure in Supplementary material of the study VI in Annexes).

Exercise protocol

Incremental exercise test

The incremental test performed in the three different situations was performed on a cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). The cadence throughout the test was constant, remaining between 70-90 rpm. The protocol started with 1 minute of rest followed by 5 minutes of warm-up at 30W. Subsequently, power was increased by 30 W every 3 minutes until maximum effort was reached, considering the end of the test when a reduction in cadence of 10 rpm was observed (24).

Glycogen depletion protocol

The glycogen depletion protocol consisted of a 60-minute continuous cycling exercise at the power associated with 60% of VO₂max calculated from the power-VO₂ ratio obtained during the maximal incremental cycling test performed in the control situation. The pedalling cadence was set at 70 rpm. After that, six 30-

second all-out sprints were performed with a 7.5% body weight load with a 4-minute recovery between bouts. This protocol has been carried out in previous studies with the same objective, having already shown to be effective in the glycogen stores depletion (189).

Data analysis

Expired gas volume and composition were analyzed breath-by-breath and data were averaged every 15 seconds using the JAEGER® Vyntus CPX gas analyzer (Jaeger-CareFusion, Hoechberg, Germany). The equipment was previously calibrated according to the manufacturer instructions, whose validity and reliability has been demonstrated (coefficient of variation of the respiratory quotient less than 0.5%). $\dot{V}O_{2\max}$ was established as the highest value averaged every 15 seconds achieved during the incremental exercise test of the control situation. The following respiratory and performance variables averaged every 15 seconds from the gas analyzer during the incremental exercise test were evaluated: ventilation (VE), oxygen consumption ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$), respiratory exchange ratio (RER) and power output. Whole body fat and carbohydrate oxidation were calculated from respiratory quotients using standard indirect calorimetry equations (312).

$$\text{Fat oxidation (g} \cdot \text{min}^{-1}) = 1.67 \cdot \dot{V}O_2 - 1.67 \cdot \dot{V}CO_2 - 1.92 \cdot n$$

$$\text{Carbohydrate oxidation (g} \cdot \text{min}^{-1}) = 4.55 \cdot \dot{V}CO_2 - 3.21 \cdot \dot{V}O_2 - 2.87 \cdot n$$

n= negligible urinary nitrogen excretion

Heart rate was analyzed beat by beat throughout the test via the Polar H10 (Polar Electro Oy, Kempele, Finland), although 12-lead ECG was continuously monitored to assess the presence of cardiovascular abnormalities. This band was connected via Bluetooth to a cell phone that received the signal and where the data was stored in the Polar Flow application. Heart rate data averaged every 15 seconds were merged with those from the gas analyzer. Whole blood lactate concentration was measured during the test from capillary blood. These blood samples were drawn from the fingertip at rest, in the last 30 seconds of each step and immediately after the moment of maximum effort, and these samples were analyzed with a lactate

analyzer (EKF Diagnostic GmbH, Barlebe, Germany). At rest, in the last 30 seconds of each step and immediately after the moment of maximum effort, central temperature was monitored with a tympanic thermometer (Braun IRT6520, Switzerland), rate of perceived exertion was measured with the 0-10 and 6-20 scales (RPE 0-10 and RPE 6-20, respectively), and ambient temperature and humidity (Dienmern, Guangdong, China) were determined.

The first and second ventilatory thresholds (VT1 and VT2, respectively) were set at the point of maximum agreement of the most common methods of assessment published previously (333). Briefly, VT1 was calculated: a) according to the V-slope method, whereby VT1 is the breaking point of the VCO_2 – VO_2 relationship; b) as the first exponential increment in VE; and c) as the first rise in the VE/VO_2 ratio without increments in the VE/VCO_2 ratio. VT2 was determined: a) as the second rise in ventilation; b) as the intensity that accompanied a second rise in the VE/VO_2 ratio with a simultaneous rise in the VE/VCO_2 ratio. All tests were evaluated by 2 researchers and a third researcher was asked in case of controversy.

Respiratory and performance variables at VT1, VT2 and maximum (end of the test) were extracted from the gas analyzer. Heart rate was also determined at VT1, VT2 and maximum from the results merged with those from the gas analyzer. Since lactate concentrations, RPE (0-10) and RPE (6-20) were evaluated at rest, in the last 30 seconds of each step and immediately after the moment of maximum effort, the values of these variables in VT1 and VT2 were calculated from the relation between power output and these variables by interpolation.

Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD). The initial sample size calculation was determined through a pilot study with 6 subjects, and a priori power analysis was conducted to determine the final sample size using statistical software (G*Power v. 3.1.9.2, Stuttgart, Germany). One-way ANOVA repeated measures within factors was selected using power output at the lactate threshold in the three different situations of the study with the following criteria: effect size $F=2.015$; $\alpha=0.05$; power=0.95; number of groups=3; number of measurements=3;

correlation among repeated measures=0.5; nonsphericity correction=1. Based on these inputs, at least 6 participants were required. Given the sample size and the variables distribution, non-parametric tests were used. The different study variables (heart rate, power output, RPE (0-10), RPE (6-20), fat oxidation, carbohydrate oxidation, lactate concentration, O₂ consumption, CO₂ production and ventilation) were compared between situations at VT1, VT2 and maximum using Friedman test. Pairwise comparisons were analyzed with Holm's correction. Subsequently, linear regression with the standard error of estimate (SEE), intraclass correlation coefficient (ICC) with 95% confidence intervals (CI) and Bland-Altman plots were performed to evaluate concordance in heart rate, RPE (0-10) and RPE (6-20) between situations at VT1, VT2 and maximum. Statistical analyses were carried out with the statistical software Jamovi V1.6 (Jamovi, Sidney, Australia). Significant level was set at $p < 0.05$.

RESULTS

Main effects of situation and pairwise comparisons of heart rate, power output, RPE 0-10 and RPE 6-20 are shown in Table 21. In males, power output at VT1 was different between situations. Specifically, it was higher in glycogen depletion compared to control ($p=0.021$), while it was lower in heat compared to control ($p=0.05$) and glycogen depletion ($p<0.001$). Moreover, power output at maximum was lower in glycogen depletion compared to control ($p=0.005$) and it was lower in heat compared to glycogen depletion ($p<0.001$) and control ($p<0.001$). RPE 6-20 at maximum was also different between situations. Specifically, it was lower in heat compared to glycogen depletion ($p=0.009$). In females, power output at VT1 was higher in glycogen depletion compared to control ($p<0.001$) and it was lower in heat compared to glycogen depletion ($p<0.001$). Also, power output at VT2 was lower in the heat compared to control ($p=0.002$) and glycogen depletion ($p<0.001$).

Table 21 Values of heart rate, power output, RPE 0-10 and RPE 6-20 at VT1, VT2 and Max in males and females.

		Control			Depletion			Heat			Situation effect
Males											
	VT1	121	±	13	126	±	12	120	±	17	χ ² (2)=2.8; p=0.247

Heart rate (bpm)	VT2	151	±	11	156	±	12	156	±	15	$\chi^2(2)=0.75$; $p=0.687$
	Max	181	±	5	182	±	4	180	±	6	$\chi^2(2)=1.14$; $p=0.565$
Power output (W)	VT1	109.5	±	31.73	121.5	±	23.98 ^a	91.7	±	41.45 ^{ab}	$\chi^2(2)=10.8$; $p=0.004$
	VT2	184.75	±	25.97	192.75	±	25.32	176.25	±	22.55	$\chi^2(2)=2.6$; $p=0.273$
	Max	284.25	±	33.38	270.25	±	30.06 ^a	249.75	±	24.96 ^{ab}	$\chi^2(2)=16.8$; $p<0.001$
RPE (0-10)	VT1	3.5	±	1.6	3.70	±	1.34	3.00	±	1.76	$\chi^2(2)=3.58$; $p=0.167$
	VT2	6.1	±	1.7	6.10	±	1.79	6.22	±	2.22	$\chi^2(2)=0.27$; $p=0.871$
	Max	9.8	±	0.4	9.80	±	0.42	9.30	±	0.82	$\chi^2(2)=5.33$; $p=0.069$
RPE (6-20)	VT1	11.10	±	2.13	11.60	±	2.01	10.00	±	2.83	$\chi^2(2)=4.71$; $p=0.095$
	VT2	15.00	±	2.16	15.20	±	2.30	15.33	±	2.45	$\chi^2(2)=0.58$; $p=0.748$
	Max	19.70	±	0.67	19.80	±	0.42	19.20	±	0.79 ^b	$\chi^2(2)=7$; $p=0.03$
Females											
Heart rate (bpm)	VT1	131	±	11	143	±	9	137	±	13	$\chi^2(2)=3.63$; $p=0.163$
	VT2	163	±	11	165	±	11	168	±	7	$\chi^2(2)=0.44$; $p=0.801$
	Max	179	±	14	176	±	13	182	±	11	$\chi^2(2)=0.26$; $p=0.878$
Power output (W)	VT1	96.88	±	29.54	124.69	±	34.45 ^a	91.56	±	31.93 ^b	$\chi^2(2)=12.1$; $p=0.002$
	VT2	169.06	±	40.82	171.56	±	35.30	155.50	±	26.83 ^{ab}	$\chi^2(2)=8.32$; $p=0.016$
	Max	218.13	±	38.42	209.69	±	36.68	199.69	±	28.89	$\chi^2(2)=4.71$; $p=0.095$
RPE (0-10)	VT1	3.3	±	0.9	3.3	±	0.7	3.3	±	1.2	$\chi^2(2)=0.23$; $p=0.891$
	VT2	6.4	±	1.4	6.1	±	1.4	6.6	±	1.5	$\chi^2(2)=1.08$; $p=0.584$
	Max	9.4	±	1.2	9.0	±	1.1	9.6	±	0.8	$\chi^2(2)=2$; $p=0.368$
RPE (6-20)	VT1	11.3	±	1.7	11.6	±	1.1	10.5	±	2.3	$\chi^2(2)=2$; $p=0.368$
	VT2	15.0	±	1.3	15.3	±	1.6	16.0	±	2.0	$\chi^2(2)=0.77$; $p=0.678$
	Max	18.9	±	1.1	19.0	±	0.9	19.3	±	1.1	$\chi^2(2)=0.8$; $p=0.67$
Data are shown as mean ± SD. RPE: rate of perceived exertion; VT1: ventilatory threshold 1; VT2: ventilatory threshold 2; Max: end of the test. ^a Significantly different from control. ^b Significantly different from glycogen depletion. $p<0.05$ highlighted in bold.											

Due to the lack of significant differences in heart rate, RPE (0-10) and RPE (6-20) between control and glycogen depletion or heat, a complementary concordance analysis was performed on these variables across VT1, VT2 and maximum in males and females. Overall, excellent concordance was found in these variables between control and glycogen depletion or heat as can be seen in Table 22. Additionally, Bland-Altman plots are shown in supplementary material of the study VI in Annexes.

Table 22 Concordance of heart rate, RPE (0-10) and RPE (6-20) at VT1, VT2 and Max between situations.

	Control vs Depletion				Control vs Heat			
	r	SEE	p	ICC (95% CI)	r	SEE	p	ICC (95% CI)
Males								

Heart rate	0.928	0.078	<0.001	0.914 (0.824-0.959)	0.921	0.076	<0.001	0.917 (0.822-0.963)
RPE (0-10)	0.952	0.06	<0.001	0.951 (0.9-0.976)	0.955	0.053	<0.001	0.944 (0.887-0.972)
RPE (6-20)	0.935	0.069	<0.001	0.932 (0.863-0.967)	0.951	0.052	<0.001	0.933 (0.869-0.966)
Females								
Heart rate	0.886	0.132	<0.001	0.846 (0.702-0.923)	0.962	0.067	<0.001	0.956 (0.902-0.980)
RPE (0-10)	0.907	0.096	<0.001	0.902 (0.790-0.956)	0.915	0.090	<0.001	0.912 (0.802-0.962)
RPE (6-20)	0.950	0.069	<0.001	0.947 (0.882-0.976)	0.903	0.082	<0.001	0.889 (0.764-0.950)
VT1: ventilatory threshold 1; VT2: ventilatory threshold 2; Max: end of the test; r: Pearson's correlation; SEE: standard error of estimate; ICC: intraclass correlation coefficient; 95% CI: confidence interval at 95%.								

As part of the secondary objective, table 23 shows the main effects of the situation and the pairwise comparisons of fat oxidation, carbohydrate oxidation, O₂ consumption, CO₂ production, ventilation and blood lactate at VT1, VT2 and maximum between situations. In general, in males, differences were found in all variables at some point between situations. In females, differences were found in fat oxidation, O₂ consumption and lactate at some point between situations.

Table 23 Fat oxidation, carbohydrate oxidation, O₂ consumption, CO₂ production, ventilation and lactate concentration at VT1, VT2 and Max in males and females.

		Control			Depletion			Heat			Situation effect
Males											
Fat oxidation (g/min)	VT1	0.309	±	0.198	0.631	±	0.165 ^a	0.264	±	0.097 ^b	χ²(2)=15.2; p<0.001
	VT2	0.102	±	0.170	0.470	±	0.290 ^a	0.083	±	0.119 ^b	χ²(2)=14.4; p<0.001
Carbohydrate oxidation (g/min)	VT1	1.730	±	0.539	0.981	±	0.491 ^a	1.518	±	0.476 ^b	χ²(2)=6.2; p=0.045
	VT2	3.536	±	0.856	2.560	±	0.966 ^a	3.449	±	0.781 ^b	χ²(2)=7.8; p=0.02
	Max	7.398	±	0.908	5.793	±	0.767 ^a	6.204	±	0.825 ^a	χ²(2)=10.4; p=0.006
Lactate (mmol/L)	VT1	1.86	±	0.50	1.72	±	0.66	2.75	±	0.74 ^b	χ²(2)=6.2; p=0.045
	VT2	3.45	±	1.38	2.98	±	1.62	5.00	±	1.60 ^{ab}	χ²(2)=11.4; p=0.003
	Max	12.45	±	2.05	10.95	±	3.03	11.52	±	2.69	χ ² (2)=2.4; p=0.301
O ₂ consumption (ml/min)	VT1	1921	±	535	2016	±	287	1669	±	421 ^{ab}	χ²(2)=10.4; p=0.006
	VT2	2608	±	445	2859	±	334	2614	±	326	χ ² (2)=2.4; p=0.301
	Max	3605	±	380	3644	±	289	3317	±	331 ^{ab}	χ²(2)=9.8; p=0.007
CO ₂ production (ml/min)	VT1	1735	±	465	1638	±	272	1511	±	392	χ ² (2)=3.8; p=0.15
	VT2	2617	±	448	2579	±	395	2602	±	380	χ ² (2)=2.6; p=0.273
	Max	4169	±	426	3844	±	275 ^a	3704	±	363 ^a	χ²(2)=9.8; p=0.007
Ventilation (L/min)	VT1	45.90	±	9.16	45.20	±	7.97	40.70	±	9.01	χ ² (2)=2.6; p=0.273
	VT2	70.50	±	11.16	74.30	±	10.45	72.20	±	7.76	χ ² (2)=1.8; p=0.407
	Max	160.20	±	16.95	150.80	±	19.74 ^a	136.60	±	19.63 ^{ab}	χ²(2)=12.8; p=0.002
Females											
Fat oxidation (g/m)	VT1	0.330	±	0.192	0.555	±	0.169 ^a	0.316	±	0.224 ^b	χ²(2)=8.32; p=0.016
	VT2	0.089	±	0.102	0.212	±	0.132	0.020	±	0.029	χ ² (2)=4; p=0.135
Carbohydrate oxidation (g/m)	VT1	1.245	±	0.660	1.098	±	0.274	1.259	±	0.515	χ ² (2)=0.58; p=0.748
	VT2	3.028	±	0.886	2.698	±	0.486	3.091	±	0.612	χ ² (2)=2.8; p=0.247
	Max	5.569	±	1.256	4.628	±	0.874	4.959	±	1.139	χ ² (2)=4.32; p=0.115
Lactate (mmol/L)	VT1	1.86	±	0.67	1.67	±	0.37	2.75	±	1.06	χ ² (2)=3; p=0.223
	VT2	5.00	±	1.49	3.66	±	0.98	4.04	±	0.99	χ ² (2)=5.2; p=0.074
	Max	11.49	±	1.34	8.66	±	2.28 ^a	9.90	±	2.61	χ²(2)=7.75; p=0.021
O ₂ consumption (ml/min)	VT1	1599	±	334	1948	±	355 ^a	1582	±	487 ^{ab}	χ²(2)=12.2; p=0.002
	VT2	2323	±	403	2444	±	407	2194	±	365 ^{ab}	χ²(2)=8.4; p=0.015
	Max	2768	±	434	2695	±	372	2736	±	387 ^{ab}	χ²(2)=8.4; p=0.015
CO ₂ production (ml/min)	VT1	1402	±	344	1616	±	277	1393	±	409	χ ² (2)=4.71; p=0.095
	VT2	2305	±	460	2317	±	375	2227	±	376	χ ² (2)=4.8; p=0.091
	Max	3177	±	551	2919	±	445	3020	±	478	χ ² (2)=1.23; p=0.542
Ventilation (L/min)	VT1	41.13	±	8.51	47.75	±	6.71	43.25	±	10.47	χ ² (2)=5.24; p=0.073
	VT2	66.88	±	9.76	70.88	±	7.30	63.60	±	8.26	χ ² (2)=2.8; p=0.247
	Max	118.00	±	19.99	109.38	±	14.58	114.75	±	20.21	χ ² (2)=0.58; p=0.748
Data are shown as mean ± SD. VT1: ventilatory threshold 1; VT2: ventilatory threshold 2; Max: end of the test.											
^a Significantly different from control. ^b Significantly different from glycogen depletion. p<0.05 highlighted in bold.											

DISCUSSION

The main objective of this study was to compare heart rate, RPE, and power output at VT1, VT2 and maximum under different situations: control, heat (36°C), and glycogen depletion. Accordingly, the main findings of the present study were that both heart rate and RPE were very similar at VT1 and VT2 between control, glycogen depletion and heat situations. On the contrary, power output showed differences at the same two points across these three situations. Interestingly, despite being at the same training zones, physiological differences were found (O_2 consumption, fat oxidation, carbohydrate oxidation or lactate concentration) between situations. This study helps to elucidate which variable is more accurate to prescribe exercise intensity according to training zones delimited by ventilatory thresholds when the nutritional state or the ambient temperature is different between the testing session and the training session. Furthermore, the results suggest that despite being at the same training zones, the physiological response can be conditioned by ambient temperature or nutritional status.

Heart rate was similar across situations at the ventilatory thresholds. Moreover, high concordance was observed in this variable between control and glycogen depletion or heat. However, the great inter-individual variability observed in the Bland-Altman plots (see Figure in Supplementary material of the study VI in Annexes) should not be overlooked. This shows that despite the average heart rate was not different between situations, some individual differences can be observed. With respect to the heat situation, our results are in line with those by Maunder et al. observing consistency of heart rate at VT1 and VT2 during step incremental assessments performed in 18°C and 35°C in males (187). Additionally, a similar heart rate was found by Tyka et al. at VT1 (334) or LT2 (335) (determined through the Exp-Dmax method) during a step incremental test at 23°C, 31°C and 37°C. However, the opposite results were found by Bourgois et al. finding a higher heart rate at 36°C compared to 18°C at VT1 and VT2 during a ramp incremental test (331). Furthermore, De Barros et al. found that heart rate was higher at VT2 at 40°C compared to 22°C, but they did not analyse VT1 (108). As can be observed, apparently contradictory results are reported in the literature. Some differences

between studies that could be affecting the results are the wide range of environmental conditions (temperature and humidity), different ways to monitor pre-hydration status (108,187,331), lack of dehydration measurement after the test (108,187,331,335), pre-acclimation protocol (such as lack of pre-acclimation protocol (108,335) or passive rest (187,331)), adaptation to heat (adapted vs non-adapted (331) individuals) or the incremental test (such as ramp (331) or step test (108,187,335)). Regarding glycogen depletion, previous investigations showed a similar heart rate at VT1 under glycogen depletion compared to control (64,332). Based on our results, since heart rate is very similar at ventilatory thresholds across situations, heart rate seems to be a good variable to prescribe training intensity based on training zones regardless ambient temperature or glycogen status. Future studies should continue to investigate this topic with more homogeneous study designs to ensure consistency among studies. Furthermore, a systematic review with exhaustive inclusion/exclusion criteria can reach a solid conclusion on this aspect.

Unlike heart rate, some differences were found between situations in power output at the ventilatory thresholds. Focusing on the heat situation, there seems to be more congruence in the literature, agreeing with our results. Most studies report a lower power output at ventilatory or lactate thresholds (108,187,334,335), except for one study observing a similar power output at 36°C and 18°C (331). On the other hand, as for glycogen depletion, a previous study already reported a higher power output at the LT1 in glycogen depletion compared to control (66). Altogether, the same power output may correspond to a different training zone in conditions of heat or glycogen depletion. Hence, differences found in power output at the different points would indicate that power output at ventilatory thresholds determined in the testing session will not accurately represent the desired intensity if ambient temperature or nutritional state is different during the training session.

According to the results in heart rate, RPE 0-10 and RPE 6-20 were similar at the ventilatory thresholds between situations. Furthermore, high concordance was found between control and glycogen depletion or heat. Concerning heat, our

findings are supported by a previous study reporting a similar RPE 6-20 at 40 °C compared to 22 °C at VT2 (108). Relative to glycogen depletion, a previous study is in line with our results reporting a similar RPE 6-20 at VT1 and LT1 in glycogen depletion compared to control (63). Therefore, as previously mentioned about heart rate, RPE seems to be a good option to prescribe exercise intensity based on training zones because a specific RPE value will correspond to the same training zone across different situations.

Regarding metabolic variables, fat oxidation was different at the different physiological points between situations in males and females, and carbohydrate oxidation was different between situations only in males. These results would indicate that despite being at the same training zone (demarcated by each ventilatory threshold), the substrate oxidation response is different across situations. That is, depending on each situation, the metabolic response could be dramatically different at the same training zone. Furthermore, these results also indicate that an absolute substrate oxidation does not delimit the training zones determined through ventilatory thresholds. Similar results were found in lactate, finding a different lactate concentration at the ventilatory thresholds in males, although only a trend was achieved in females. These results would indicate that, as occurs with substrate oxidations, a specific lactate concentration in the blood does not trigger a respiratory response defining the change from one training zone to another. This has already been suggested by other studies finding a dissociation between ventilatory and lactate thresholds (65,66,332). Previous studies have already shown that a fixed blood lactate concentration (e.g. the traditional concentrations of 2 and 4 mM/L) is not a good indicator of training zones between different individuals (20). Now with our study, it is shown that a fixed blood lactate concentration is not a good indicator of a specific training zone delimited by ventilatory thresholds even in the same individual exposed to different situations. Altogether, current knowledge indicates that absolute blood lactate concentrations measured in a control situation should not be used to assess exercise intensity according to training zones demarcated by ventilatory thresholds if ambient temperature or nutritional status change during the training session.

Regarding the respiratory variables, a different O_2 consumption was found between situations. That means that O_2 consumption at a specific training zone may change if the situation is different. Additionally, from the current results it can be assumed that ventilatory thresholds do not occur when a certain O_2 consumption is reached even in the same individual under different situations. However, CO_2 production and ventilation were very similar across situations at the ventilatory thresholds, while both variables were lower at maximum, both in glycogen depletion and heat compared to control. We know that one of the mechanisms to compensate metabolic acidosis is respiratory alkalosis. Specifically, the increase in H^+ produced by exercise is offset by HCO_3^- , resulting in an increase in CO_2 and H_2O . Finally, CO_2 is removed through ventilation (329). Thus, once a specific CO_2 production is reached, an increase in ventilation would be triggered. This compensatory mechanism demonstrates the strong relationship between CO_2 and ventilation. This strong relationship is consistent with the concomitant changes in CO_2 production and ventilation observed in our study. Therefore, from the current results it can be suggested that ventilatory thresholds occur once a specific CO_2 and ventilation are reached.

CONCLUSION

Heart rate, RPE 0-10 and RPE 6-20 showed high concordance at the ventilatory thresholds between control and glycogen depletion or heat. In contrast, some differences were observed in power output at these physiological points between the same situations. Therefore, we recommend heart rate, RPE 0-10 or RPE 6-20 as variables to set exercise intensity according to training zones delimited by ventilatory thresholds when ambient temperature or glycogen status is different during the training session compared to the situation in which the training zones were identified. On the other hand, it is important to highlight that despite being at the same training zone, the metabolic response (O_2 consumption, fat oxidation, carbohydrate oxidation or lactate concentration) can change depending on the ambient temperature or nutritional status. Therefore, it is crucial to know the effect of different situations to comprehend the acute response to exercise and future adaptations to training.

5. Conclusions

Specific conclusions of the influence of the main lactate transporters:

Study I

Exercise increases MCT1 protein content in human skeletal muscle, regardless of whether a single session or a training program is used. Skeletal muscle MCT4 protein levels can increase after a training program, although its responsiveness is lower compared to MCT1.

Study II

No differences were observed between genotypes of the polymorphism on the MCT1 (SLC16A1) gene, for the different lactate variables studied throughout the session.

Specific conclusions of the influence of sex:

Study III

Despite males presented a greater PO at LT1 and 2 to females, the relative PO at which these thresholds occurred was greater in females than in males. On the other hand, females demonstrated a greater absolute and relative HR compared to males at the LT1 and 2.

Study IV

Due to the lower fat free mass of females, to cycle at the same power output than males they produce a higher power output relative to fat free mass. This requires an additional energy expenditure relative to fat free mass, this additional energy is obtained preferably from fats.

Specific conclusions of the influence of temperature and nutritional status:

Study V

To determine fat oxidation from lactate, or viceversa, it is required to determine the lactate-fat oxidation relationship specific to each situation. Changes in lactate

concentrations are accompanied by opposite changes in the pattern of fat oxidation, although the changes in both variables are not proportional.

Study VI

We recommend heart rate or RPE as variables to set exercise intensity according to training zones when ambient temperature or glycogen status is different during the training session compared to the situation in which the training zones were identified.

6. Practical applications

The main following practical applications can be extracted from the present thesis:

- Exercise performed regularly is a powerful stimulus to increase MCT1 and MCT4 protein levels.
- Due to the sex differences in the relative heart rate and power output at lactate threshold 1 and 2, males and females should not train at the same percentage of maximal heart rate or power output to produce the same metabolic response.
- Blood lactate concentration can be used to indirectly determine fat oxidation. However, the relationship between both variables should be determined specifically in each specific situation (such as ambient temperature or nutritional status).
- Heart rate or RPE should be used to set exercise intensity according to training zones when ambient temperature or glycogen status during the training session are different compared to the situations in which the training zones were identified.

7. Strength and limitations

STRENGTHS

The strengths from the present thesis deserve to be highlighted:

- Preferred Reporting Items for Systematic review and Meta-Analyses (PRISMA) checklist was followed in the study II.
- Menstrual cycle phase and reproductive status of females were considered.
- Both sexes were matched by training status in the V study.
- The sample size meets the needs required to ensure statistical power.

LIMITATIONS

After carrying out the thesis, we present a critical view of our work, listing the main limitations below:

- A quantitative analysis was not performed in study II due to several methodological aspects of the studies included in the systematic review.
- Gas exchange was not analysed in the studies III and IV.
- Both sexes were not matched by training status in the IV study.
- Muscle glycogen content was not directly determined in the V, VI and VII studies.

8. Future research lines

In future studies, the following points could be taken into consideration:

- To determine which physiological factors explain the differences in energy substrate metabolism between sexes.
- To show the effect of training on the relationship between lactate concentration and fat oxidation.
- To investigate a cause-effect relationship between lactate concentration or fat oxidation and different mitochondrial characteristics.
- To investigate differences in fat oxidation and lactate concentration under different stimuli, such as low carbohydrate availability or heat, in individuals with different training statuses.

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Annexes

Supplementary material of the study I

Supplementary Table 1. Review authors' judgements on each risk of bias item for each included study.

Study	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Score*
McGinley, C. (2016)	High	High	High	Unclear	High	Low	4
Burgomaster, K. (2007)	High	High	High	Unclear	High	Low	4
Pilegaard, H. (1999)	Low	Unclear	High	Unclear	High	Low	2
Juel, C. (2004)	Low	Unclear	High	Unclear	High	High	3
Nordsborg, N. (2003)	Low	Unclear	High	Unclear	High	Low	2
Mohr, M. (2007)	Low	Unclear	High	Unclear	High	Low	2
Bishop, D. (2008)	High	High	High	Unclear	High	Low	4
Millet, G. (2014)	Low	Unclear	Unclear	Unclear	Low	Low	0
Carsten, J. (2004)	Low	Unclear	High	Unclear	Low	Low	1
Dubouchaud, H. (2000)	High	High	High	Unclear	Low	Low	3
Bishop, D. (2007)	High	High	High	Unclear	High	Low	4
Green, H. (2002)	High	High	High	Unclear	High	Low	4
McGinley, C. (2016)	Low	Unclear	High	Unclear	High	Low	2
Green, H. (2008a)	High	High	High	Unclear	High	Low	4
Green, H. (2008b)	High	High	High	Unclear	High	Low	4
Bangsbo, J. (2009)	Low	Unclear	High	Unclear	High	Low	2
McGinley, C. (2017)	Low	Unclear	High	Unclear	High	High	3
Bonen, A. (1998)	High	High	High	Unclear	High	Low	4
Neal, C. (2013)	Low	Unclear	High	Unclear	High	Low	2
Bickham, D. (2006)	High	High	High	Unclear	High	Low	4

*Each item evaluated as "High" was scored with 1 point.

Supplementary material of the study III

Table S1. Absolute power output and relative power output according to sex at the different LT methods.						
	Absolute power output (W)			Relative power output (%)		
	Females	Males*	Effect size (confidence interval)	Females	Males*	Effect size (confidence interval)
LT1 methods						
LTP1	103±21.9	131.2±34.3	-0.983 (-1.66, -0.29)	57.7±10.18	49.8±14.57	0.631 (-0.019, 1.266)
Log-log	70.2±15.5	93.9±34.3	-0.833 (-1.56, -0.183)	40.4±9.72	35.6±13.39	0.406 (-0.232, 1.034)
LT2 methods						
ModDmax	142.1±15.2	198.2±19.1	-2.758 (-3.81, -1.683)	79.8±5.06	72.9±6.27	1.212 (0.477, 1.925)
Dmax	139.2±12.4	184.3±20.5	-2.68 (-3.69, -1.64)	78.3±5.65	69.7±6.71	1.402 (0.641, 2.139)
Exp-Dmax	136±14.7	189.9±21.5	-2.958 (-4.05, -1.844)	76.2±2.67	71.6±2.56	1.761 (0.918, 2.579)
Log-Poly-ModDmax	139.9±14.8	189.8±23	-2.629 (-3.64, -1.591)	78.7±6.77	72.2±8.24	0.874 (0.189, 1.542)
Log-Exp-ModDmax	135.1±16.4	196.5±26.4	-2.813 (-3.86, -1.741)	75.7±3.89	74.1±8.09	0.252 (-0.369, 0.866)
LTP2	151.8±25.2	218.2±31.1	-2.335 (-3.27, -1.379)	84.7±8.07	82.4±11.04	0.243 (-0.377, 0.857)
Data are shown as mean ± standard deviation. Absolute power output: power output determined at the respective threshold; Relative power output: power output relative to peak power output determined at the respective threshold; Log-log: [1]; LTP1 [2]; Dmax [3]; ModDmax [4]; Exp-Dmax [5]; Log-Poly-ModDmax [4]; Log-Exp-ModDmax [4]; LTP2 [2]. Effect size determined by Cohen's d (95% confidence interval). A repeated measures ANOVA (Sex x LT1 methods) was performed to compare absolute and relative power output according to sex at LT1, and the same was performed for LT2. *Significantly different between females and males (p<0.05).						

Table S2. Heart rate and relative heart rate according to sex at the different LT methods.						
	Heart rate (bpm)			Relative heart rate (%)		
	Females	Males*	Effect size (confidence interval)	Females	Males*	Effect size (confidence interval)
LT1 methods						
LTP1	149±15	133±17	0.952 (0.143, 1.73)	80.3±7.84	71.1±8.82	1.094 (0.259, 1.9)
Log-log	133±16	116±8	1.335 (0.447, 2.19)	71.6±9.14	62±5.12	1.335 (0.447, 2.19)
LT2 methods						
ModDmax	168±9	159±10	0.885 (0.087, 1.65)	90.7±5.63	85.2±5.06	1.036 (0.211, 1.83)
Dmax	166±12	154±12	0.97 (0.157, 1.75)	89.4±6.96	82.2±5.37	1.178 (0.324, 2)
Exp-Dmax	164±11	156±7	0.857 (0.065, 1.62)	88.6±5.55	83.5±2.77	1.196 (0.338, 2.02)
Log-Poly- ModDmax	166±12	158±11	0.675 (-0.088, 1.42)	89.6±7.20	84.6±5.71	0.778 (-0.001, 1.53)
Log-Exp-ModDmax	164±11	158±6	0.677 (-0.087, 1.42)	88.3±5.57	85±1.87	0.828 (0.031, 1.60)
LTP2	173±13	167±13	0.952 (0.143, 1.73)	93.1±5.83	89.6±6.74	0.549 (-0.197, 1.28)
Data are shown as mean ± standard deviation. Heart rate: heart rate determined at the respective threshold; Relative heart rate: heart rate relative to maximal heart rate determined at the respective threshold; Log-log: [1]; LTP1 [2]; Dmax [3]; ModDMax [4]; Exp-Dmax [5]; Log-Poly-ModDmax [4]; Log-Expo-ModDmax [4]; :LTP2 [2]. Effect size determined by Cohen's d (95% confidence interval). A repeated measures ANOVA (Sex x LT1 methods) was performed to compare absolute and relative heart rate according to sex at LT1, and the same was performed for LT2. *Significantly different between females and males (p<0.05).						

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2. Hofmann P, Tschakert G. Intensity- and duration-based options to regulate endurance training. *Front Physiol.* 2017 May 24;8(MAY).
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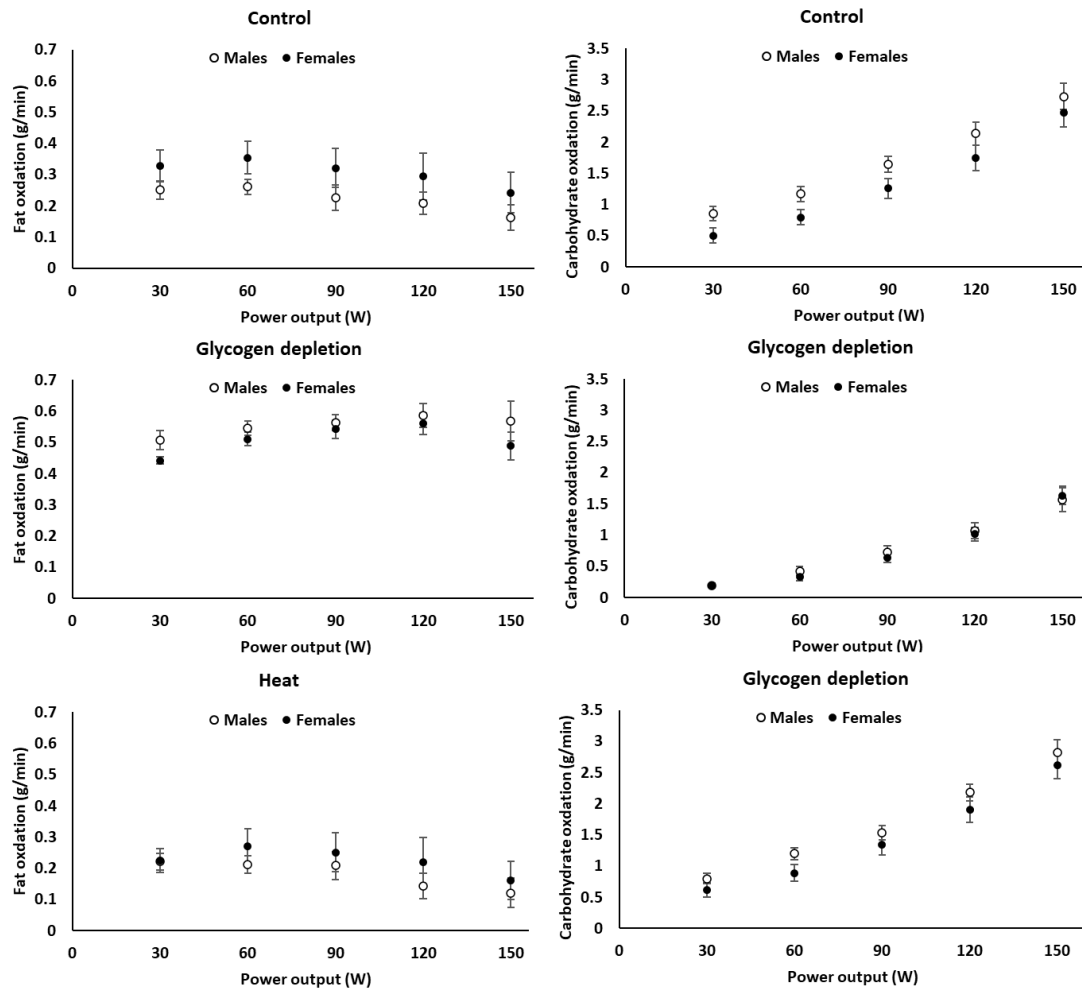


Figure S2: Fat oxidation and carbohydrate oxidation throughout the power outputs across situations. Data shown as mean $\pm$ standard error.

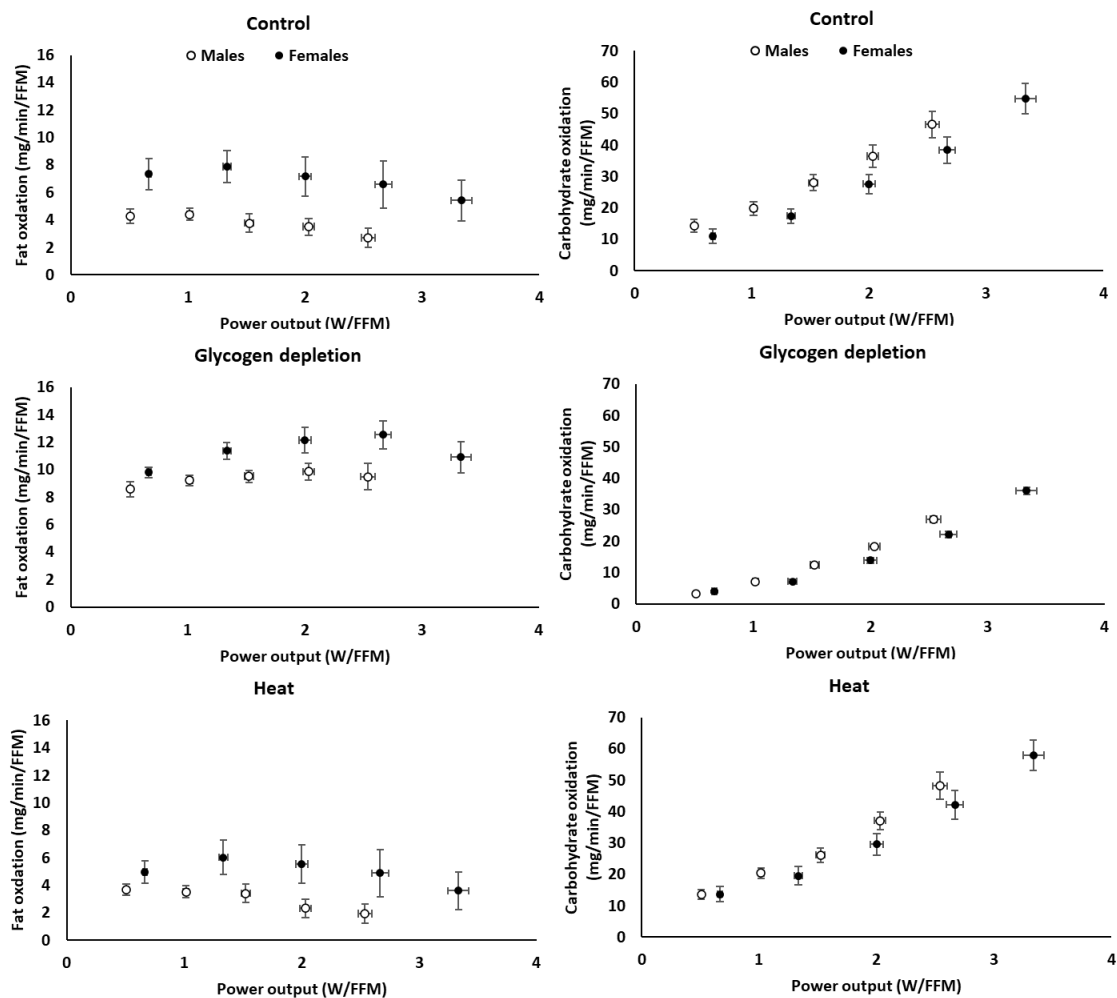


Figure S3: Fat oxidation and carbohydrate oxidation throughout the power outputs normalized to fat-free mass across situations. Data shown as mean $\pm$ standard error.

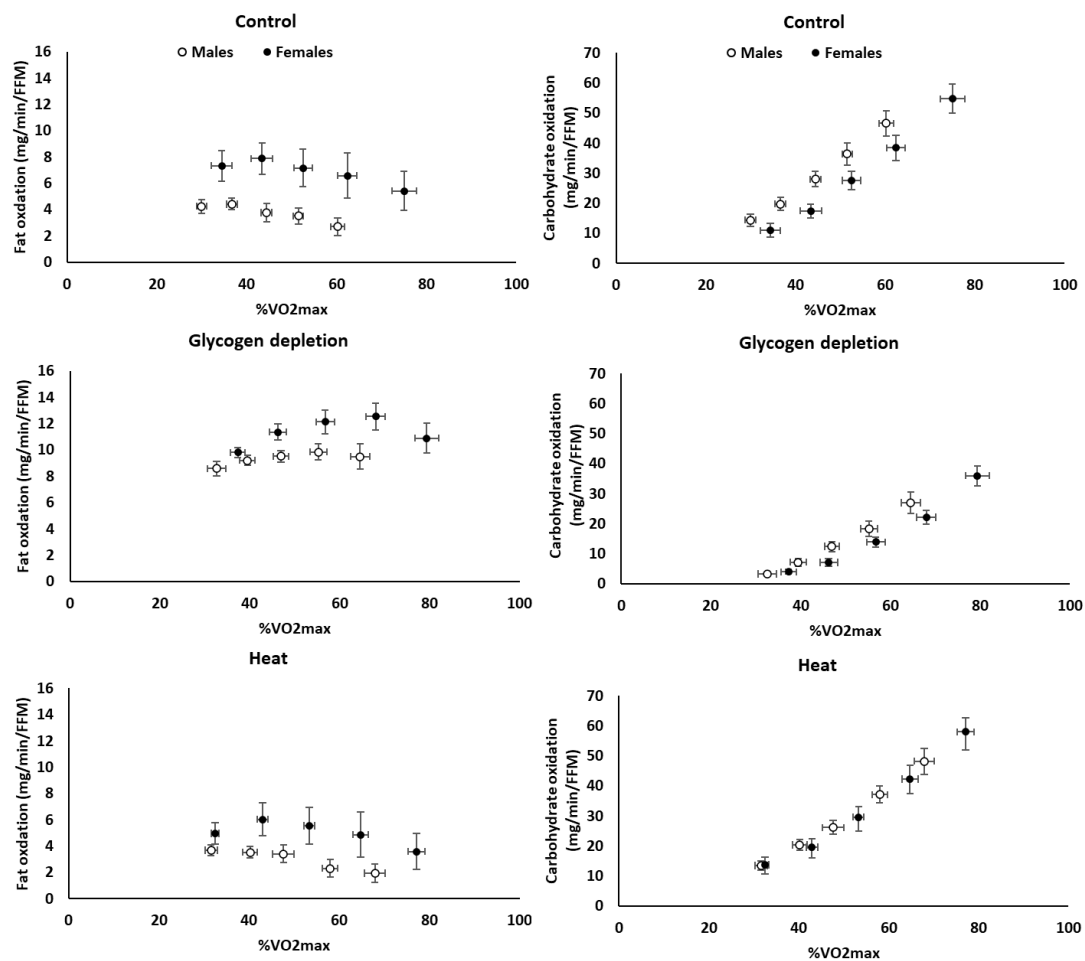


Figure S4: Fat oxidation and carbohydrate oxidation throughout the percentages of maximal oxygen uptake across situations. Data shown as mean $\pm$ standard error.

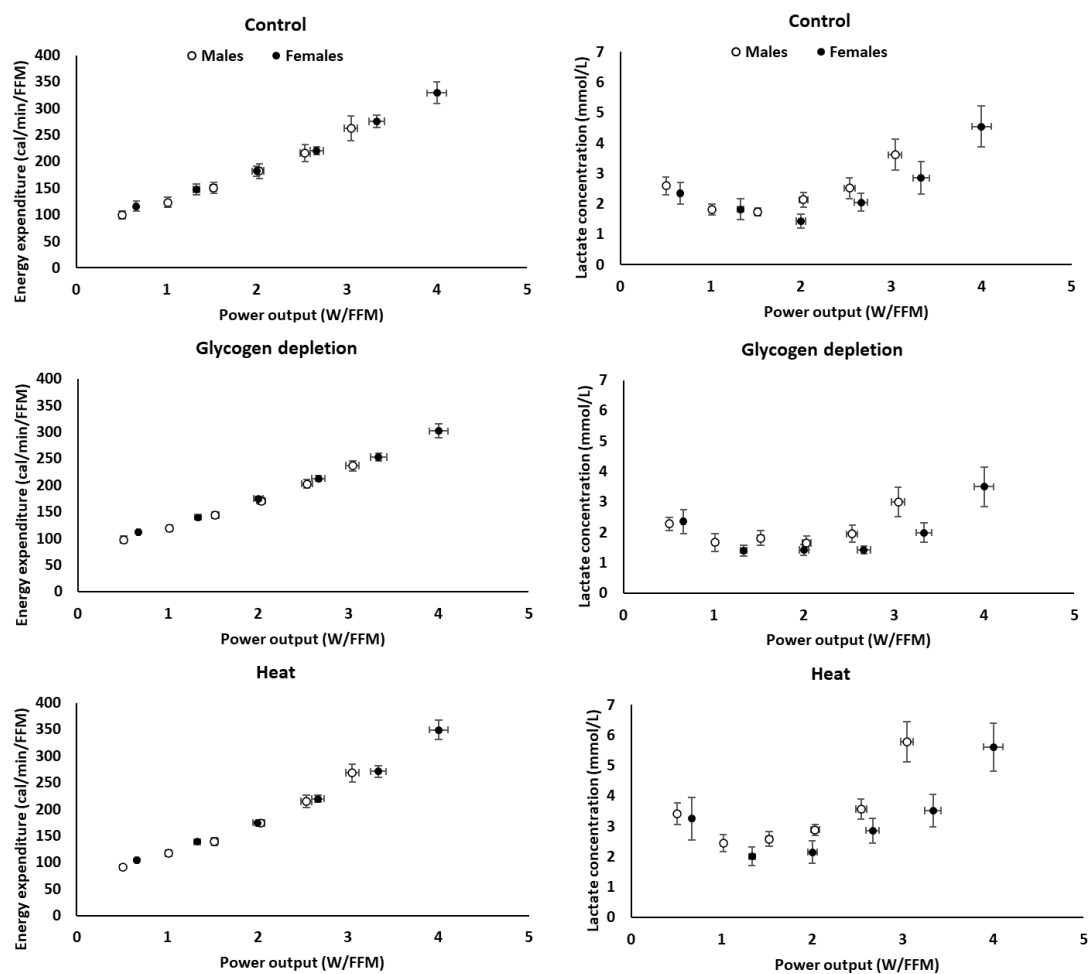


Figure S5: Energy expenditure and lactate concentration throughout the power outputs normalized to fat free mass across situations. Data shown as mean $\pm$ standard error.

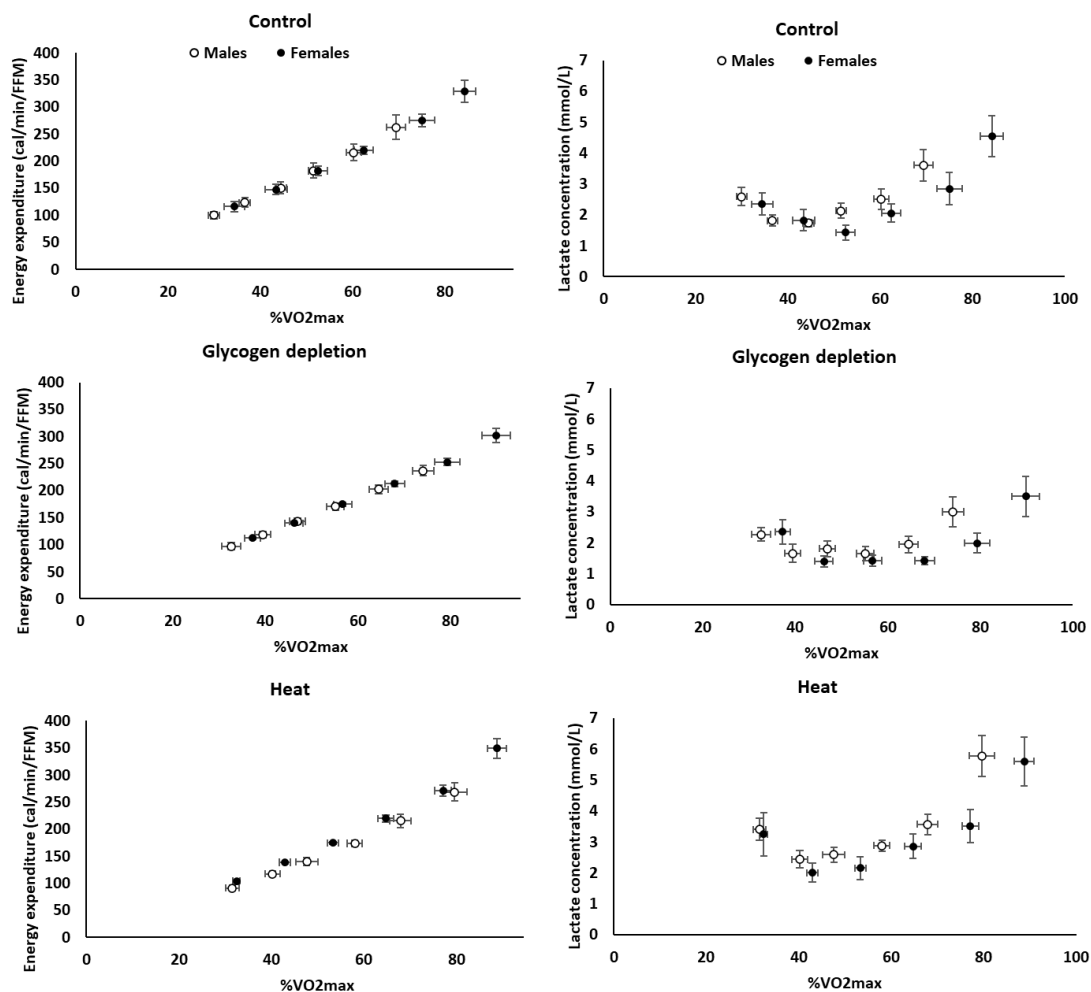


Figure S6: Energy expenditure and lactate concentration throughout the percentages of maximal oxygen uptake across situations. Data shown as mean $\pm$ standard error.

Fig. S1 Example of the High-energy, high-carbohydrate diet and the High-energy, low-carbohydrate and high-fat diet.

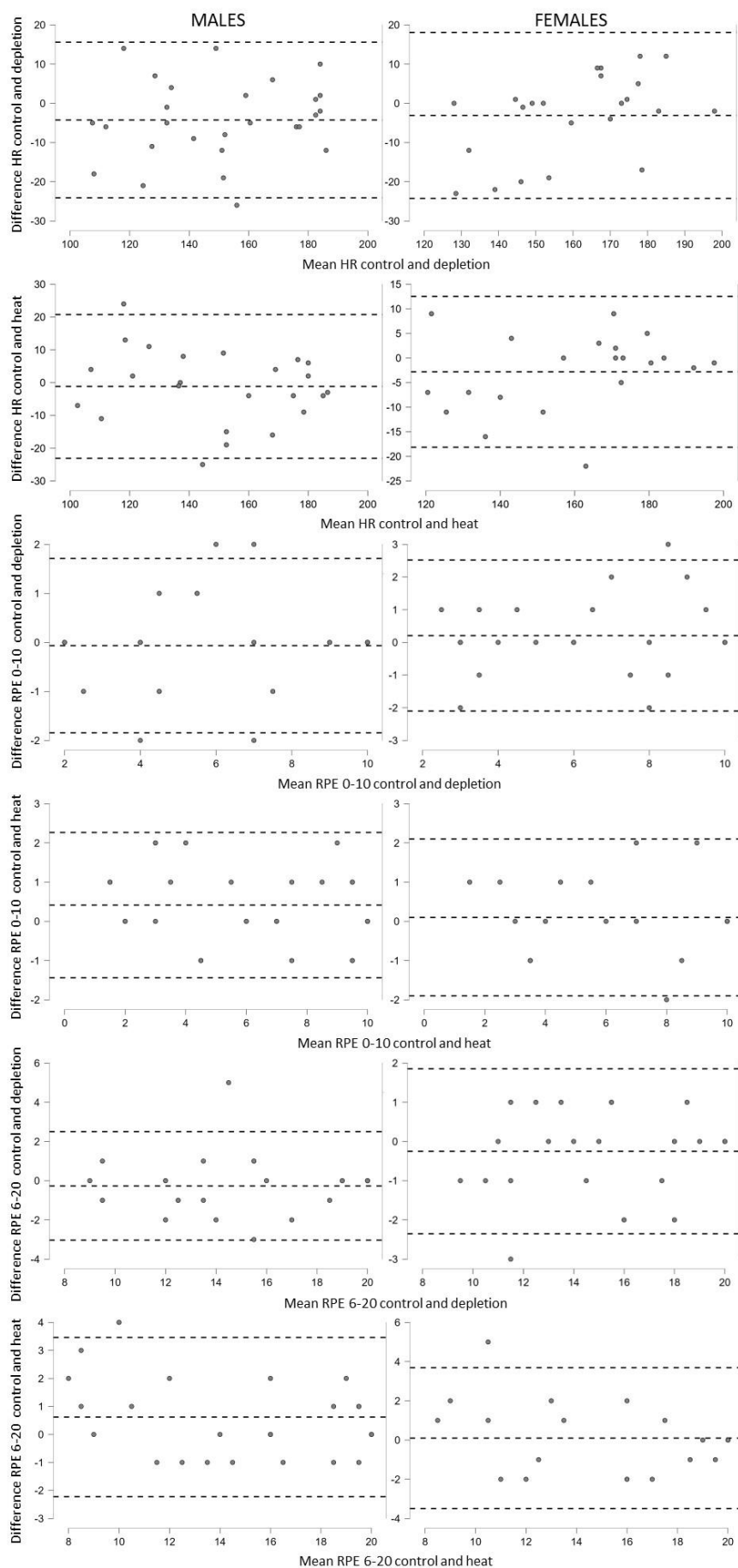


Fig. S2 Bland-Altman plots comparing heart rate, RPE 0-10 and 6-20 between control and glycogen depletion or heat.

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**EDUCATION**

2020-Current	Phd, Universidad Politécnica de Madrid
2019/2020	Master's degree in physical and Sporting Performance, Universidad Pablo de Olavide
2015/2019	Bachelor's degree in Sport Sciences, Universidad Politécnica de Madrid

WORK EXPERIENCE

2020-Current	Predoctoral scholar at Universidad Politécnica de Madrid, Faculty of Physical Activity and Sport Sciences, Department of Health and Human Performance.
2017-Current	Technical assistant in ergospirometry testing at the Laboratory of Exercise Physiology at the Universidad Politécnica de Madrid. Teaching in the subject "Bodybuilding, training methods in weight rooms" in the degree of Physical Activity and Sport Sciences at the Universidad Politécnica de Madrid (84h). Teaching in the subject "Systems Physiology" in the degree of Biotechnology at the Universidad Politécnica de Madrid (28h).
2021-Current	Teaching in the subject "kinesiology and systematics of physical activity and sport" in the degree of Physical Activity and Sport Sciences at the Universidad Politécnica de Madrid (72h). Teaching in the subject "Exercise physiology" in the degree of Physical Activity and Sport Sciences at the Universidad Politécnica de Madrid (12h).
2019-2022	Teaching at Master's degree in Personal Training, Universidad Politécnica de Madrid (10h).
2022	Teaching in the ATHENS programme at the Universidad Politécnica de Madrid (1.5h).

INTERNATIONAL STAY

2023 Institute for Health and Sport (iHeS), Victoria University, Melbourne, Australia 2023, David Bishop (3 months).

RESEARCH PROJECTS

2017-2019 IronFEMME project. Influence of menstrual cycle on iron metabolism and muscle damage in females' athletes. Funded by the Spanish Ministry of Economy, Industry and Competitiveness.

2021 MASCARILLAS TEXCON Y CALIDAD, S.L. (P2111600225), de la Universidad Politécnica de Madrid.

2023 Respuesta a la Actividad Física de la mujer con esclerosis múltiple a lo largo del ciclo menstrual (EMMA). Funded by the Ministerio de Cultura y Deporte de UE, Consejo Superior de Deportes (CSD).

2022-2023 LacFat. Universidad Politécnica de Madrid.

2019-2022 Transporte Eritrocitario de Lactato en Ejercicio (TELE). Funded by the Universidad Politécnica de Madrid.

2017-current Performance evaluation and monitoring. Universidad Politécnica de Madrid.

PUBLICATIONS

Armada-Cortés, E., Benítez-Muñoz, J. A., San Juan, A. F., & Sánchez-Sánchez, J. (2022). Evaluation of Neuromuscular Fatigue in a Repeat Sprint Ability, Countermovement Jump and Hamstring Test in Elite Female Soccer Players. *International Journal of Environmental Research and Public Health*, 19(22), 15069. <https://doi.org/10.3390/ijerph192215069>

Armada-Cortés, E., Benítez-Muñoz, J. A., Sánchez-Sánchez, J., & San Juan, A. F. (2022). Evaluation of Neuromuscular Fatigue According to Injury History in a Repeat Sprint Ability Test, Countermovement Jump, and Hamstring Test in Elite Female Soccer Players. *Applied Sciences*, 12(6), 2970. <https://doi.org/10.3390/app12062970>

Armada-Cortés, E., Peláez Barraón, J., Benítez-Muñoz, J. A., Navarro, E., & San Juan, A. F. (2020). Can We Rely on Flight Time to Measure Jumping Performance or

Neuromuscular Fatigue-Overload in Professional Female Soccer Players? *Applied Sciences*, 10(13), 4424. <https://doi.org/10.3390/app10134424>

Benítez-Muñoz, J. A., Cupeiro, R., Rubio-Arias, J. Á., Amigo, T., & González-Lamuño, D. (2024). Exercise influence on monocarboxylate transporter 1 (MCT1) and 4 (MCT4) in the skeletal muscle: a systematic review. *Acta Physiologica*.
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Ramos-Campo, D. J., Clemente-Suárez, V. J., Cupeiro, R., Benítez-Muñoz, J. A., Andreu-Caravaca, L., & Rubio-Arias, J. Á. (2023). The ergogenic effects of acute carbohydrate feeding on endurance performance: a systematic review, meta-analysis and meta-regression. *Critical Reviews in Food Science and Nutrition*, 52(11), 1–10.
<https://doi.org/10.1080/10408398.2023.2233633>

Rojo-Tirado, M. A., Benítez-Muñoz, J. A., Alcocer-Ayuga, M., Alfaro-Magallanes, V. M., Romero-Parra, N., Peinado, A. B., Rael, B., Castro, E. A., & Benito, P. J. (2021). Effect of Different Types of Face Masks on the Ventilatory and Cardiovascular Response to Maximal-Intensity Exercise. *Biology*, 10(10), 969.
<https://doi.org/10.3390/biology10100969>

Benítez-Muñoz, J.A., Rojo-Tirado, M.A., Benito, P.j., Murias, J.M., González-Lamuño, D., Cupeiro, R. Greater relative first and second lactate threshold in females compared to males: Consideration for exercise prescription. *International Journal of Sport Physiology and Performance*. In Press.

CONFERENCES PRESENTATIONS

A comparison between heart rate and power output to prescribe exercise intensity. International Symposium in Strength Training. Madrid, Spain. 15/12/2023. Universidad Politécnica de Madrid.

Body composition and cardiovascular fitness by sex according to bone mineral density level: a two-step cluster analysis. International Symposium in Strength Training. Madrid, Spain. 15/12/2023. Universidad Politécnica de Madrid.

Differences in the ventilatory threshold positions relative to $\text{vo}_{2\text{max}}$ according to vo_2 at each threshold. International Symposium in Strength Training. Madrid, Spain. 15/12/2023.

Universidad Politécnica de Madrid.

Concomitant changes in lactate and fat oxidation kinetics during exercise. Annual Congress of the European College of Sport Science. París, France. 05/07/2023. European College of Sport Science.

Restrictive effect of ventilation with the use of different types of masks in intense physical exercise. Annual Congress of the European College of Sport Science. París, France. 05/07/2023. European College of Sport Science.

A comparison between heart rate and power output to prescribe exercise intensity. . International Symposium in Strength Training. Madrid, Spain. 18/12/2022. Universidad Politécnica de Madrid.

Effect of the MCT1 T1471A Polymorphism on the lactate-load relationship: a pilot study. Annual Congress of the European College of Sport Science. Sevilla, Spain. 05/09/2022. European College of Sport Sciences.

Bone mineral density in resistance and endurance trained premenopausal females. International Symposium in strength training. Madrid, Spain. 2021. Universidad Politécnica de Madrid.

Different intragroup activation patterns in male and female professional soccer players: preliminary results. International Symposium in strength training. Madrid, Spain. 2021. Universidad Politécnica de Madrid.

Sex related differences in the second lactate threshold. International Symposium in strength training. Madrid, Spain. 2021. Universidad Politécnica de Madrid.

Testosterone profile throughout the menstrual and the oral contraceptive cycle. International Symposium in strength training. Madrid, Spain. 2021. Universidad Politécnica de Madrid.

Comparison of the different performance variables between force platform and MyJump application in female professional football players. Annual Congress of the European College of Sport Science. Online. 2020. European College of Sport Science.

Effect of MCT1 T1470A Polymorphism in maximal lactate production: a pilot study. Annual Congress of the European College of Sport Science. Online. 2020. European College of Sport Science.

30m-sprint differences in professional football players with and without an injury history: preliminary results. International Symposium in strength training. Madrid, Spain. 2019. Universidad Politécnica de Madrid.

Effects of oestrogen and progesterone on substrate oxidation and respiratory variables. Annual Congress of the European College of Sport Science. Praga, Czech Republic. 2019. European College of Sport Science.

Energy and nutrients intake analysis and performance of amateur master male athletes. Conferencia Científica de la Sociedad Española de Nutrición y la VI Reunión de jóvenes investigadores. Soria, Spain. 2019. Sociedad Española de Nutrición

Influence of menstrual cycle and oral contraceptives on rating of perceived exertion throughout an endurance exercise in female athletes. Annual Congress of the European College of Sport Science. Praga, Czech Republic. 2019. European College of Sport Science.

Low ferritin status and hepcidin response to exercise in female athletes. Annual Congress of the European College of Sport Science. Praga, Czech Republic. 2019. European College of Sport Science.

Menstrual cycle influence on oxygen consumption and ventilation in physically active women during an intervallic running protocol. Annual Congress of the European College of Sport Science. Praga, Czech Republic. 2019. European College of Sport Science.

Influence of menstrual cycle on muscle function and jump performance: ironfemme project. Annual Congress of the European College of Sport Science. Praga, Czech Republic. 2019. European College of Sport Science.

ORGANISING COMMITTEE

2017	International Symposium in strength training. Madrid, Spain. Universidad Politécnica de Madrid.
2018	International Symposium in strength training. Madrid, Spain. Universidad Politécnica de Madrid.
2021	International Symposium in strength training. Madrid, Spain. Universidad Politécnica de Madrid.
2022	International Symposium in strength training. Madrid, Spain. Universidad Politécnica de Madrid.
2023	International Symposium in strength training. Madrid, Spain. Universidad Politécnica de Madrid.

COMPLEMENTARY BACKGROUND

2020	Radiodiagnostic operator certificate. Stericycle.
2016	Lifeguard and first aid. Escuela de Socorrismo Profesional.

LANGUAGE

2018	English. B2. Aptis General.
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