Gustavo de Oliveira Zanetti

ADAPTAÇÕES MORFOLÓGICAS, METABÓLICAS E MOLECULARES DA MUSCULATURA ESQUELÉTICA DE CAMUNDONGOS AO TREINAMENTO FÍSICO AERÓBICO EM AMBIENTE QUENTE

Belo Horizonte

Escola de Educação Física, Fisioterapia e Terapia Ocupacional - UFMG

2023

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Linha de pesquisa: Aspectos psicobiológicos do desempenho humano

Orientador: Prof. Dr. Dawit Albieiro Pinheiro Gonçalves

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ATA DE DEFESA DE DISSERTAÇÃO

GUSTAVO DE OLIVEIRA ZANETTI

Às **14:00 horas** do dia **15 de maio de 2023**, a comissão examinadora, indicada pelo Colegiado do Programa de Pós-Graduação em Ciências do Esporte, reuniu-se no miniauditório e por videoconferência, para julgar, em exame final, a dissertação intitulada "**Adaptações morfológicas, metabólicas e moleculares da musculatura esquelética de camundongos ao treinamento físico aeróbico em ambiente quente**". Abrindo a sessão, o presidente da comissão, Prof. Dr. Dawit Albieiro Pinheiro Gonçalves (EEFFTO/UFMG), orientador, após dar a conhecer aos presentes o teor das Normas Regulamentares de Defesa do Trabalho Final, passou a palavra para o candidato, que realizou a apresentação da sua dissertação. Seguiu-se a arguição pelos examinadores, com a respectiva defesa do candidato. Logo após, a comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição do resultado.

Prof. Dr. Dawit Albieiro Pinheiro Gonçalves (UFMG - orientador)

Prof. Dr. Luiz Carlos Carvalho Navegantes (USP)

Profa. Dra. Danusa Dias Soares (UFMG)

Após as indicações, o candidato foi considerado **APROVADO**.

Nada mais havendo a tratar, eu, Prof. Dr. Dawit Albieiro Pinheiro Goncalves, presidente da comissão examinadora, dei por encerrada a reunião, da qual, para constar, lavrei a presente Ata, que, lida e aprovada, vai por todos assinada eletronicamente.

Belo Horizonte, 15 de maio de 2023.

Assinatura dos membros da banca examinadora:

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Aos meus pais, Mônica e William, minha Vó Ivone, minha irmã Isabella e ao meu namorado João.

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A todos vocês,

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Resumo

RESUMO

Apesar do desempenho físico aeróbico ser reduzido na exposição aguda ao ambiente quente (Q), a exposição crônica modula o metabolismo muscular e induz um fenótipo muscular mais oxidativo. Entretanto pouco se sabe sobre a exposição ao estresse térmico quente durante o treinamento físico aeróbico (TFA) de longo prazo (i.e., > 4 semanas) nos diferentes tipos de fibras musculares esqueléticas, em seu metabolismo e nas sinalizações intracelulares mediadoras desses efeitos. Objetivo: Avaliar as adaptações morfológicas, metabólicas e moleculares na musculatura esquelética induzidas pelo TFA de longo prazo em ambiente Q. Camundongos suíços machos e adultos foram divididos em quatro grupos: 1) Sedentários (SED) mantidos em ambiente temperado (T) (22 °C; SED/T), 2) SED mantidos em ambiente (Q; 32 ºC; SED/Q), 3) submetidos ao TFA [1h/dia, 5dias/semana, durante 8 semanas à 60% da velocidade máxima ($V_{máx}$) e 5° de inclinação] em T (TFA/T) e 4) submetidos ao TFA em Q (TFA/Q). Testes incrementais (TCI) na esteira em T e Q foram realizados antes do treinamento e após 4 semanas para a prescrição do TFA de TFA/T e TFA/Q, respectivamente. Após o período de treinamento, os animais foram eutanasiados e amostras de sangue e tecido muscular e hepático foram congeladas e armazenadas. O conteúdo de glicogênio hepático e muscular foi mensurado pelo método de Antrona e a atividade mitocondrial e área de secção transversa (AST) das fibras musculares foi mensurada por meio da técnica histológica de atividade da succinato desidrogenase (SDH). Os tipos de fibras musculares foram determinados pela expressão de miosinas de cadeia pesada (do inglês; *myosin heavy chain* – MyHC) por meio da técnica histológica de imunofluorescência. O conteúdo e atividade das proteínas musculares envolvidas na síntese/degradação proteica e metabolismo energético mitocondrial foram quantificados por meio de *western blot*. Com base no TCI pré-TFA, a velocidade de corrida de TFA/Q foi menor (~30%) do que TFA/T. Contudo, ambos os grupos TFA apresentaram melhora semelhante do desempenho físico (e.g., tempo de corrida) após 8 semanas. O conteúdo de glicogênio hepático também aumentou (~34%) de forma semelhante nos grupos TFA/T, TFA/Q e SED/Q quando comparado ao SED/T, sem alteração do glicogênio muscular. A atividade de SDH muscular aumentou (~9%) em ambos os grupos TFA quando comparado ao SED/T, e apenas o grupo SED/Q aumentou a CSA das fibras oxidativas em relação ao grupo SED/T. Apesar do conteúdo de MyHC *fast* e *slow* não ter alterado entre os grupos, observamos um aumento no percentual de fibras do tipo 2A nos grupos TFA (~16%) em relação ao grupo SED/T. A análise molecular revelou que o grupo TFA/Q apresentou uma redução no conteúdo das proteínas FoxO1 (~30%; marcador de degradação proteica e metabolismo energético). O conteúdo de proteínas dos complexos da fosforilação oxidativa mitocondrial (OxPhos) bem como da membrana mitocondrial (TOM20) e reguladores da qualidade dessa organela (AMPK/CAMKII/p38 - PGC1α e TFEB) não foram alterados em qualquer condição. Conclusão: A melhora do desempenho físico promovida por TFA em Q parece exigir uma menor intensidade de exercício para promover mudança no tipo de fibra, para um padrão mais oxidativo/lento e aumentar o conteúdo de glicogênio hepático, se comparado ao TFA em Q. Além disso, apenas o TFA em Q reduziu o conteúdo basal de FoxO1.

Palavras-chave: treinamento físico aeróbico; ambiente quente; metabolismo muscular; musculatura esquelética.

Abstract

ABSTRACT

Heat (H; ≥32°C) stress acutely worsens aerobic performance. However, H has emerged as a potential therapy to modulate muscle metabolism inducing aerobic phenotype and it is unknown the effects of training in this condition for long periods (> 4weeks) on muscle fiber type and trophism and intracellular pathways regulating muscle phenotype. To evaluate the morphological, metabolic and molecular adaptations in skeletal muscle induced by long-term endurance training (ET) in H. Adult, male Swiss mice (40g) were divided in: 1) Sedentary (SED) mice kept in the temperate (T) environment (22°C;SED/T), 2) SED kept in H (32°C;SED/H), 3) mice ET in treadmill (1h/day, 5days/week, 8weeks, 60% of maximum speed (S_{max})) in T (ET/T), and 4) ET in H (ET/H). All groups performed incremental load tests in T and H before (pre-training) and after 4 and 8 weeks of training. The liver and muscle glycogen content were measured by Anthrone method and mitochondrial activity. Muscle fiber types were determined by analyzing SDH activity and *myosin-heavy chain* (MyHC) isoforms by immunofluorescence techniques in histological slices. The content and activity of muscle proteins involved in protein synthesis/degradation (Akt/FoxO) and energy metabolism (AMPK-p38/PGC1α) were quantified by *western blot* (WB). In pre-training period, H impaired performance by reducing (~30%) S_{max}. After 8weeks, although ET/H exercised at a lower (26%) absolute intensity than ET/T, S_{max} were similarly increased (~22%) in both ET groups compared with SED/T. The liver glycogen content also increased by ~34% in the ET/T, ET/H and SED/H groups when compared to SED/T. The skeletal muscle SDH activity increased ~9% in both ET groups when compared with SED/T. SED/H group increased (~15%) cross sectional area (CSA) of oxidative fibers with no additional effects of ET. The protein content of slow (type 1) and fast (type 2) MyHC by WB did not change in any condition, but % of type 2A fibers was higher (~16%) in both ET groups than in SED/T group. The protein content of mitochondrial oxidative phosphorylation complexes (OxPhos) as well as mitochondrial membrane (TOM20) and the intracellular regulators of these organelles and oxidative fiber phenotype (i.e., AMPK/CAMKII/p38 - PGC1α and TFEB) were not altered in any group. Molecular analysis revealed that ET/H group reduced the content of FoxO1 (~30%; an inducer of protein degradation and energy metabolism and an inhibitor of angiogenesis). Although exercise in H is performed at a lower absolute intensity, training in both environments similarly improved performance and increased the proportion of type 2A fibers, the activity of the mitochondrial enzyme SDH, and the hepatic content of glycogen. Thus, the similar improvement in physical performance of ET in H and T appears to be due to a change in fiber type to a more oxidative phenotype. In summary, ET in H requires lower intensity to induce fast-to-slow fiber type shift and increase glycogen than ET in T. Furthermore, only ET in H reduce basal levels of FoxO1.

Keywords: mitochondria; heat stress; skeletal muscle metabolism; mitochondria; signaling pathways.

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Lista de abreviaturas

LISTA DE ABREVIATURAS

ACC	Aclimatação ao calor
Akt	Proteína quinase B
AMPK	Proteína Quinase Ativada por Monofosfato de Adenosina
ANGPT2	Angiopoietina-2
ANOVA	Análise de variância
ATP	Adenosina trifosfato
ATP5a	$ATP_{sintase}$ - Adenosima trifosfato sintase
AST	Área de secção transversa
Ca^{2+}	Íons cálcio
CAMKII	Proteína quinases dependentes de cálcio/calmodulina
DDT	Ditiotreitol
DNA	Ácido desoxirribonucléico
EDTA	Ácido etilenodiamino tetra-acético
EPM	Erro padrão da média
ERK1/2	Quinases regulas pelo sinal extracelular
FC	Frequência cardíaca
$FC_{máx}$	Frequência cardíaca máxima
FoxO	*Forkhead box class O*
Q	Quente
H&E	Hematoxilina e eosina
HSP72	*Heat-shock protein 72*
IAAF	*International Association of Athletics Federations*
KO	nocaute (*Knockout)*

KOH	Hidróxido de potássio
MAPK	Proteina quinase ativada por mitogeno
ml	Microlitro
mRNA	Ácido ribonucleico
MyHC	Miosina de cadeia pesada (*Myosin heavy chain*)
NaCl	Cloreto de sódio
NDUFB8	oxidoredutase NADH-ubiquinona
NFAT	Nuclear factor of activated T-cells
O_2	Molécula de Oxigênio
OxPhos	Complexos da fosforilação oxidativa mitocondrial
p38 MAPK	Proteina quinase ativada por mitogeno p38
PGC-1α	Coativador alfa do receptor gama ativado por proliferador de peroxissoma
rpS6	Proteína ribossomal S6
SDH	Succinato desidrogenase
SDS	Dodecil sulfato de sódio
SED	Sedentário
T	Temperado
T_a	Temperatura ambiental
T_{ABD}	Temperatura abdominal
T_C	Temperatura central
TCI	Teste de carga incremental
TFA	Treinamento físico aeróbico
TFEB	Fator de transcrição EB
T_m	Temperatura muscular

TOM20	Proteína de membrana externa mitocôndria
T_{pele}	Temperatura da pele
μm	Micrometro
UQCRB	Ubiquinol-citocromo c redutase
VEGF	Fator de crescimento endotelial vascular
VO_2	Consumo de oxigênio
$VO_{2máx}$	Consumo máximo de oxigênio
VO_{2pico}	Consumo pico de oxigênio
$V_{máx}$	Velocidade máxima

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INTRODUÇÃO

1 INTRODUÇÃO

O desempenho esportivo de atletas de elite envolvidos em competições de longa duração (i.e., *World Marathon Majors*, Circuito Mundial de maratona aquática e *Tour de France*) é produto da integração de fatores psicobiológicos, do regulamento da competição e das condições ambientais (Furrer; Hawley; Handschin, 2023). Nesse cenário, o principal desafio desses atletas é conseguir manter altas taxas de produção de energia a fim de manter elevada capacidade aeróbica por um longo período de tempo. Tal capacidade deriva do preciso controle do metabolismo energético a fim atrasar a fadiga (Furrer; Hawley; Handschin, 2023; Hawley, 2002). O treinamento físico aeróbico (TFA), constituído por repetidas sessões de exercício físico aeróbico, promove efeitos sistêmicos que melhoram a resistência à fadiga. Na musculatura esquelética o TFA promove a transição das fibras musculares para um padrão lento/oxidativo (aumentando fibras do tipo 1 e 2A), aumenta a síntese, densidade e função mitocondrial e, por consequência, melhorar a capacidade oxidativa (Egan; Zierath, 2013).

Por outro lado, a inadequada prescrição do TFA pode levar a comprometimentos físicos e mentais nos atletas (Schwellnus *et al.*, 2016). Jacobsson et al. (Jacobsson *et al.*, 2012), observaram que mais de 60% dos quadros de lesão física em atletas acontecem durante o treino. Segundo os autores, fatores como a sobrecarga na prescrição do treino podem explicar esse fenômeno, visto que boa parte desses eventos acontecem na pré-temporada, quando os atletas estão menos condicionados. Além dos danos à integridade individual dos atletas, lesões físicas impactam na permanência no programa de TFA. Corredores de elite apresentam uma prevalência anual de 43% para quadros de lesões físicas com duração mínima de três semanas (Jacobsson et al., 2012). Por um período coincidente, a interrupção do TFA (2 – 8 semanas) reduz a capacidade aeróbica em 5 a 10%. Dessa forma, ressalta-se a importância de estratégias que contribuam para atenuação da sobrecarga física imposta nas sessões de TFA e que mitiguem a perda das adaptações durante os processos de recuperação.

1.1 Respostas aguadas e fatores limitantes do exercício físico aeróbico

1.1.1 Consumo oxigênio

Os benefícios do TFA derivam de adaptações às repetidas repostas induzidas pelas sessões exercício físico de resistência aeróbica. Agudamente o exercício físico aeróbico, causa um desequilíbrio na homeostase sistêmica por aumentar a demanda do metabolismo energético e aumentar o consumo de oxigênio (VO_2) (Egan; Zierath, 2013). De acordo com a teoria do deslizamento dos filamentos (Podolsky e Schoenberg, 1983), a hidrolise do ATP é um processo fundamental para que ocorram as contrações musculares provenientes da formação de pontes cruzadas entre os filamentos das proteínas musculares actina e miosina. Por consequência, os produtos provenientes da quebra do ATP [i.e., Adenosina difosfato (ADP) e moléculas inorgânicas de fosfato (Pi)] estimulam sua ressíntese pelas organelas mitocondriais, via metabolismo dependente de O_2 (oxidativo). Portanto, a contrações musculares durante o exercício físico aumentam o VO_2.

Robisson et al., (1937) foram os primeiros a identificar que o consumo máximo de oxigênio ($VO_{2máx}$) é um dos fatores limitantes do desempenho aeróbico. O $VO_{2máx}$ é a capacidade máxima do organismo em captar e utilizar o O2, e por isso é limitado pela capacidade dos sistemas em captar (capacidade de difusão pulmonar e débito cardíaco máximo), transportar (conteúdo de proteínas carreadoras de O_2 e capilarização dos tecidos) e utilizar o O_2 (capacidade oxidativa do tecido muscular) (BASSETT; HOWLEY, 2000). Entretanto, simplesmente aumentar o fluxo sanguíneo para o músculo não é suficiente para aumentar o VO_2. Segundo modulações matemáticas (HONIG; CONNETT; GAYESKI, 1992) o espaço entre as células transportadoras de O_2 (hemácias) e a membrana muscular (sarcolema) é a região que apresenta maior resistência para a difusão do O_2. O aumento do consumo do O_2 pela atividade mitocondrial durante a contração muscular é uma etapa crucial para o transporte de O_2. Para atender a demanda de energia pelas contrações musculares, as mitocôndrias consomem mais O_2 e favorecem o gradiente para difusão do O_2 da hemácia para o músculo. De fato, Ivy et al. (1980) mostraram que o $VO_{2máx}$ está

significativamente relacionado ao conteúdo de mitocôndrias musculares. Embora indivíduos treinados apresentem maior $VO_{2máx}$ e capacidade oxidativa muscular (biopsia) se comparados a indivíduos sedentários, em ambos os grupos as variáveis apresentavam relação positiva.

A capacidade oxidativa e o conteúdo mitocondrial, varia de a cordo com o tipo de fibras. Os diferentes tipos de fibra podem ser classificados de acordo com sua atividade contrátil e atividade de enzimas relacionas ao metabolismo glicolítico e oxidativo. Mamíferos apresentam quatro principais tipos de fibras musculares cuja classificação coincide com a atividade da classe de enzimas que hidrolisam o ATP (ATP_{ase}) (Schiaffino; Reggiani, 2011). Segundo Schiaffino et al. (2011), as fibras do tipo 1 são fibras de contração lenta (baixa atividade da ATP_{ase}), ricas em proteínas musculares transportadoras de O_2 (mioglobinas) e alta atividade e conteúdo mitocondrial, o que lhes confere predomínio do metabolismo oxidativo e alta resistência à fadiga. Já as fibras do tipo 2A são de contração rápida, mas possuem metabolismo misto (oxidativo-glicolítico) e apresentam alta resistência à fadiga. Costil et al. (1976) compararam a capacidade oxidativa do músculo esquelético e resistência aeróbica de indivíduos não treinados, corredores de média distância e maratonistas de elite (42 km). Dos três grupos, os corredores de elite apresentavam menor tempo para realizar provas acima de 5 km, assim como maior capacidade oxidativa muscular. Segundo os autores os músculos dos corredores de elite (biopsia) apresentavam aumento na proporção das fibras do tipo 1 e na atividade de enzima relaciona ao metabolismo oxidativo (SDH). Portanto, a resistência aeróbica não depende apenas de fatores individuais, como a vascularização do tecido ou da capacidade oxidativa muscular, mas sim da interação entre esses fatores como um sistema.

1.1.2 Produção metabólica de calor

Outro subproduto da hidrolise do ATP é a produção metabólica de calor. Durante a contração muscular, uma significativa parte da energia produzida (~75 - 95%) é convertida em energia térmica e liberada na forma de calor (Edwards *et al.*, 1973; Wasserman, K.; Van Kessel; Burton, 1967). Dependendo da duração, a

produção metabólica de calor aumento a temperatura muscular (T_m) (Sawka, Michael N. *et al.*, 2011), uma resposta imprescindível para a função desse tecido durante o exercício (Cheuvront *et al.*, 2010). Isso porque, o aumento da T_m causado pelo exercício físico aumenta a capacidade fosforilação dos complexos mitocondriais (responsáveis pela produção do APT) mitocondrial (Brooks *et al.*, 1971).

A literatura destaca que, dependendo da duração e intensidade de exercício físico, à medida que a T_m aumenta, observa-se consequente aumento na temperatura central (T_c) do corpo. Em reposta, mecanismos termorregulatórios são ativados a fim de estabelecer um balanço entre a produção e perda de calor para tecidos periféricos à musculatura (condução) e o meio ambiente (convecção do sistema circulatório). Isso porque, o sangue que perfunde a musculatura atua como um transportador do calor para outros tecidos do corpo. Destaca-se a transferência de calor para a pele a fim de facilitar a troca de calor com o meio, evitando acumulo nas cavidades internas do corpo e retardando o aumento da T_c (Sawka et al., 2011). (Sawka, Michael N. *et al.*, 2011). Sabendo que a temperatura da pele (T_{pele}) aumenta em proporção direta a temperatura ambiente (T_a) (Gagge; Gonzalez, 2011). A elevação da T_a constitui um estressor adicional ao exercício físico, por reduzir o gradiente de temperatura entre a T_a e T_{pele} e dificultar a transferência de calor para o meio. O acumulo de calor aumenta da T_c durante o exercício físico e exacerba diversas respostas fisiológicas.

1.2 Resistência aeróbica no ambiente quente

1.2.1 Exercício físico aeróbico agudo no ambiente quente

Guy et al. (2015) analisaram as médias dos 10 melhores desempenhos em competições de atletismo ao longo de 7 consecutivos Campeonatos Mundiais da IAAF (*International Association of Athletics Federations*) ocorridos entre os anos de 1999 e 2011. Os autores observaram uma redução significativa do desempenho físico tanto em homens como em mulheres nas provas de longa distância (5, 10 e 42 km) realizadas em ambiente quente (Q, ≥ 25 ºC). A principal

resposta fisiológica que explica a redução de resistência aeróbica pelo estresse térmico Q é o aumento na demanda cardiovascular (Rowell, 1974). Nessas condições, há o aumento da concorrência do débito cardíaco para a pele (função termoregulatória) e para os tecidos/órgãos mais ativos, em especial o musculoesquelético (aporte de substratos energéticos). Assim, ao comprometer o aporte adequando de sangue para a musculatura ativa há uma diminuição do $VO_{2máx}$ associada ao desenvolvimento de hipertermia que limitam a realização de exercício prolongado em ambiente quente (Febbraio, 2000; Périard; Racinais, 2015).

Além da corrida, outros esportes de longa duração têm apresentado prejuízo na resistência aeróbica em ambiente Q. Nadadores competitivos expostos a uma única sessão de exercício em água quente (33 ºC) reduziram a distância de nado no teste de contra relógio de 20 min, comparado a avaliação em água mais fria (28 ºC) (Bradford *et al.*, 2015). Portanto, fica evidente que a exposição aguda ao ambiente Q prejudica a resistência a fadiga por aumentar a demanda fisiológica. Quanto a isso a literatura destaca algumas estratégias que mitigam os efeitos deletérios do ambiente Q (Ely *et al.*, 2007), é bem estabelecido que repetidas exposições ao ambiente Q combinadas ao exercício físico podem melhorar o desempenho físico nesse ambiente, processo conhecido como aclimatação ao calor (AAC).

1.2.2 Aclimatação ao calor

A exposição repetida ao ambiente Q com estresse de magnitude suficiente para elevar tanto T_c como T_{pele} e provocar transpiração profusa, induz ajustes biológicos que reduzem os efeitos negativos, fenômeno denominado AAC (Périard *et al.*, 2016; Racinais *et al.*, 2015). No geral, as principais melhorias induzidas pela AAC derivam de aprimoramentos na capacidade termorregulatória (Sawka, Michael N. *et al.*, 2011). Isso por que, a extensão do efeito da T_a sobre a capacidade de se exercitar aerobicamente depende da capacidade corporal de dissipar calor e manter o fluxo sanguíneo para os músculos ativos (Sawka, Michael N. *et al.*, 2011). De fato, indivíduos AAC

apresentam redução da T_{pele}, melhora da capacidade sudorípara e aumento do fluxo sanguíneo cutâneo o que favorece a termorregulação e reduz a T_c durante o exercício físico no ambiente Q (Eichna *et al.*, 1950; Lorenzo *et al.*, 2010; Nadel *et al.*, 1974; Roberts, M. F. *et al.*, 1977). Além disso, indivíduos ACC apresentam não só melhorias na capacidade de dissipar calor, mas também menor produção metabólica de calor pelos tecidos ativos (Sawka, M. N. *et al.*, 1983). Em conjunto, aprimoramentos na termodinâmica (i.e, balanço entre a produção e dissipação de calor) contribuem para o para aumento da resistência aeróbica durante o exercício em ambiente Q (Sawka, Michael N. *et al.*, 2011). Em geral, observa-se um aprimoramento significativo das respostas fisiológicas após 15 dias de exposição repetida ao ambiente Q (Karlsen et al., 2015).

1.2.3 Adaptações ao treinamento físico aeróbico no ambiente quente

Embora os múltiplos efeitos benéficos da AAC em curto e médio prazo sejam relativamente bem conhecidos, a intensidade dos protocolos utilizados é atenuada (Lorenzo *et al.*, 2010). Com isso, poucos estudos têm investigado os efeitos fisiológicos envolvidos no processo adaptativo ao TFA em ambiente Q por um longo período tanto. Resultando em uma escassez de informações quanto as demandas fisiológicas impostas sobre indivíduos que cronicamente se excitam sob altas temperaturas, como atletas que treinam ao ar livre. Isso porque, durante os meses de verão muitas regiões geográficas apresentam temperaturas acima de 29 ºC (Sawka, Michael N. *et al.*, 2011). O trabalho de Kodesh e Horowitz (2010) foi um dos poucos que de fato se propôs a investigar a somação de efeitos do TFA de corrida e do estresse térmico Q (34 ºC) em médio-longo prazo (i.e., 4 semanas) em ratos. Após 30 dias de TFA em ambientes Q, os ratos apresentam aumento da massa e da força muscular quando comparado com o TFA em ambiente T (24 ºC). Mas curiosamente os autores não avaliaram a resistência aeróbica

Em vista dos dados escassos na literatura, desenvolvemos um trabalho de iniciação científica (resultados ainda não publicados; apêndice 1) no qual a resistência aeróbia foi comparada entre camundongos submetidos ao TFA (60%

da velocidade máxima de corrida; $V_{máx}$) em ambiente Q (32 °C) e T (22 °C) ao longo de 8 semanas (1 h/dia, 5 dias/semana). Embora o ambiente Q tenha piorado a resistência aeróbica (~30%), os grupos não apresentaram diferenças no VO_{2pico}. Assim o ambiente Q poderia promover uma dissociação entre intensidade absoluta e relativa de exercício, ao demandar uma menor intensidade de corrida para se atingir o mesmo VO_{2pico}. O que indica uma possível equivalência entre a demanda fisiológica apesar da reduzida demanda a mecânica ($V_{máx}$). De fato, treinar sob essas condições fez com que ambos os grupos melhorassem de forma equivalente a resistência aeróbica no ambiente T e Q. Tais achados ressaltam a manipulação da T_a como uma ferramenta para reduzir a intensidade absoluta de exercício e manter a demanda fisiológica necessária para promover adaptações ao TFA em ambos ambientes T e Q.

1.2.4 Contribuições das adaptações ao treinamento físico aeróbico no ambiente quente para a resistência aeróbica em ambiente temperado

Em vista do potencial do ambiente Q em aumentar a intensidade relativa de exercício, Lorenzo et al. (Lorenzo *et al.*, 2010) propuseram o protocolo de AAC como uma ferramenta pra melhorar a resistência aeróbica em ambiente T. Ciclistas treinados foram submetidos a um protocolo de AAC de intensidade reduzida (50%$VO_{2máx}$). Após 10 dias de AAC, os autores observaram que além da AAC os ciclistas aumentaram o $VO_{2máx}$ em comparação aqueles treinados em ambiente T (13 ºC).

Por outro lado, no recente trabalho de Mikkelsen et al. (Mikkelsen *et al.*, 2019) os autores pretendiam também avaliar potencial do ambiente Q para melhora da resistência aeróbica em T, mas em condições de TFA. Por esse motivo, os voluntários formam orientados a realizar seu TFA habitual (i.e., prescrito pelos próprios treinadores) mas em ambiente quente (40 ºC) por 5½ semanas. O protocolo foi responsável por induzir a ACC nos atletas reduzindo a $FC_{máx}$ ao término do exercício, mas não promoveu aprimoramentos adicionais no $VO_{2máx}$ durante o teste em ambiente T (14 ºC). Segundo os autores, as adaptações promovidas pelo TFA em ambiente Q, são dependentes do nível de treinamento

e se mostram pouco proveitosas para indivíduos treinados se exercitando em ambiente T. Com isso, entender os mecanismos biológicos regulados pelo TFA em ambiente Q podem contribuir para o entendimento das incongruências na literatura.

1.3 Adaptações celulares do tecido muscular esquelético induzidas pelo treinamento físico aeróbico

A adaptação das fibras musculares ao treinamento é mediada pelos padrões de disparo dos neurônios motores (Chin *et al.*, 1998), como ocorre durante o exercício físico. Os padrões observados em fibras musculares do tipo 1 e do tipo 2A são responsáveis pela liberação frequente de pequenas quantidades de Ca^{2+} do retículo sarcoplasmático para o citosol (Chin, 2010). Por sua vez, essa liberação de Ca^{2+} ativa membros da família das proteínas cinases dependentes de cálcio/calmodulina (CAMKII), que juntas alteram o estado de fosforilação de múltiplos agentes envolvidos na regulação do fenótipo oxidativo muscular. Um desses alvos é PGC-1α, um coativador transcricional envolvido com a biogênese mitocondrial. O papel de PGC-1α foi confirmado por estudos em camundongos transgênicos que suprexpressam PGC-1α (Lin *et al.*, 2002). Nesses animais ocorreu mudança do tipo de fibra aumentando a proporção de fibras musculares do tipo 1 e do tipo 2A com alta densidade e função mitocondrial, metabolismo oxidativo e expressão de proteínas musculares características das fibras musculares tipo 1 e tipo 2A (i.e., troponina 1 e mioglobina) (Lin *et al.*, 2002).

Além disso, outro importante regulador do remodelamento mitocondrial é o fator de transcrição EB (TFEB) (Mansueto *et al.*, 2017). A localização nuclear e a função de TFEB são reguladas por sua fosforilação. Quando TFEB é fosforilado em resíduos de serina (Ser^{211}) pela proteína alvo da rapamicina de mamíferos (mTOR) e pelas cinase regulada pelo sinal extracelular (ERK2), esse fator transcricional é translocado para o citosol, mantendo-se inativo (Settembre *et al.*, 2011). Já, sob condições de estresse como o jejum, há uma inibição de mTOR e com isso TFEB é desfosforilado e translocado para o núcleo celular, onde aumenta a transcrição de genes relacionados a autofagia. Entretanto, estudos recentes indicam que TFEB atua também na biogênese mitocondrial

independentemente de PGC-1α (Mansueto *et al.*, 2017). Assim, PGC-1α e TFEB são importantes reguladores conteúdo mitocondrial o que promove a resistência à fadiga ao favorecer a maior capacidade do tecido muscular esquelético de oxidar substratos energéticos.

A disponibilização de substratos energéticos durante a sessões de TFA depende da mobilização dos substratos. Assim, em condições de déficit energético, como acontece durante as sessões de TFA, o aumento na razão entre ADP/ATP e de Ca^{2+} é responsável por ativar AMPK. AMPK é uma enzima cinase com papel fundamental na regulação do metabolismo energético celular (Hardie; Carling, 1997). Quando fosforilada em resíduos de treonina (Thr^{172}) fosforila um fator de transcrição Forkhead box O3 (FoxO3) em resíduos de serina (Ser^{413}) aumentando seu papel transcricional e ativa a degradação de substratos energéticos. De fato Slopack et al. (2014), observaram que uma única sessão de exercício físico aumentou os níveis de mRNA dos isótipos FoxO1 e FoxO3. Entretanto, o TFA promoveu uma atenuação nessa reposta de FoxO1 induzida pelo exercício. Além disso, a repressão do FoxO é crítica para a angiogênese induzida pelo TFA. Ao estudarem animais transgênicos nocautes (KO) para FoxO1 no tecido endotelial vascular os autores observaram uma resposta antecipada no tecido endotelial para promoção de angiogênese. Portanto, FoxO, atua não só no fornecimento de substratos, principalmente em exercícios de longa duração, mas também nas adaptações decorrentes do ET.

1.4 Adaptações celulares do músculo esquelético ao estresse térmico quente

O aumento da produção metabólica de calor é essencial para o aumento da atividade muscular durante o exercício físico. Segundo Brooks et al. (Brooks *et al.*, 1971), o aumento da T_m facilita a dissociação do O_2 da hemoglobina e aumenta a atividade mitocondrial. De forma passiva. Kuhlenhoelter et al. (Kuhlenhoelter *et al.*, 2016) demonstraram que o aumento da T_m dos membros inferiores em ~0,5 ºC ao longo de 90 min promoveu aumento da expressão de mRNA de VEGF (*vascular endothelial growth factor*) e de ANGPT2

(Angiopoietina-2), fatores que favorecem a angiogênese (i.e., aumento da capilarização). Sugerindo que o aumento da capilarização em reposta ao TFA, pode ser mediado pelo aumento da produção metabólica de calor.

A avaliação em larga escala de mRNA muscular por microarranjo de DNA em resposta ao estresse térmico local também identificou um aumento significativo da expressão da proteína ligante da ubiquinol-citocromo c redutase (UQCRB), um gene relacionado à fosforilação oxidativa, sugerindo que o estresse térmico também poderia estimular a ressíntese de ATP por vias oxidativas (Goto *et al.*, 2011). Em concordância, a exposição de corpo inteiro de ratos ao ambiente quente (40 ºC, 30 min/dia, 5 dias/semana, 3 semanas) após a sessão de exercício físico potencializou as adaptações induzidas pelo treinamento físico em diferentes enzimas mitocondriais (i.e., citrato sintase e complexos da OxPhos) e a aumentou a ativação da proteína cinase p38 MAPK e AMPK (TAMURA *et al.*, 2014). Ambas proteínas AMPK e p38 têm sido classicamente associada a estimulação da expressão e atividade de PGC-1α (Bartlett *et al.*, 2012; Puigserver *et al.*, 2001). Em especial, foi observado que a proteína cinase AMPK aumenta atividade de PGC-1α em resposta ao exercício físico (Bartlett *et al.*, 2012) e ambas contribuíram para a biogênese mitocondrial induzida por estresse térmico em miotubos C2C12. Dessa maneira, de forma isolada tanto o TFA quanto o ambiente Q são potencialmente capazes de gerar benefícios ao metabolismo aeróbico, ao promover a capacidade oxidativa do tecido musculoesquelético.

Em conjunto, as adaptações celulares induzidas pelo TFA em Q, podem elucidar as melhorias de resistência aeróbica a um menor custo mecânico, conforme observado em nosso estudo anterior. Destacando o potencial para manter a aptidão física ou atenuar a perda de capacidade aeróbica associada a um processo prolongado de reabilitação após uma lesão, especialmente naqueles incapazes de se exercitar em altas intensidades.

Objetivos

2 OBJETIVO

O presente trabalho avaliará as possíveis adaptações morfológicas, metabólicas e moleculares na musculatura esquelética induzidas pelo TFA de longo prazo em ambiente quente.

2.2 Objetivos específicos

Analisar o conteúdo/estado proteico, variáveis histológicas e morfológicas dos diferentes tecidos, em especial muscular esquelético de camundongos que foram submetidos ao TFA por 8 semanas em ambiente quente. Mais especificamente, propomos avaliar:

a. A massa, a morfometria e os tipos de fibras do músculo esquelético;
b. Os níveis hepáticos e musculares de glicogênio;
c. A localização nuclear e a integridade das miofibrilas, por meio de coloração com Hematoxilina e eosina (H&E);
d. Alterações na atividade mitocondrial por meio de marcação do complexo II da OxPhos, a Succinato desidrogenase (SDH);
e. Identificação de modificações do tipo de fibra muscular por meio de Imunofluorescência;
f. O conteúdo proteico de PGC-1α, TFEB, OxPhos, Miosinas musculares (MyHC lenta e rápida) e marcador mitocondrial (TOM20) e o estado de fosforilação de suas proteínas reguladoras (AMPK, p38, CAMKII e FoxO).

Materiais e métodos

3 MATERIAIS E MÉTODOS

3.1 Animais

Para realização do experimento, foram utilizados 32 Camundongos suíços (*Software G*Power* configurado da seguinte forma: *Test Family* = F *tests*; *Statistical test* = ANOVA: repeated measures, within-between interaction; *Type of power analysis* = A priori; *Effect size "f"* = 0,25; *α err prob* = 0,05; *Power (1-β err prob)* = 0,9 (90%); *Number of groups* = 4 *Number of measurements* = 3; *Corr among rep measures* = 0,7; e *Nonsphericity correction e* = 1), machos, 2-3 meses de idade, provenientes do Centro de Bioterismo (CEBIO) do ICB-UFMG e mantidos no Biotério do Laboratório de Fisiologia do Exercício (LAFISE) da Escola de Educação física, Fisioterapia e terapia ocupacional (EEFFTO) da UFMG. Todos os animais receberam dieta balanceada para roedores e água *ad libitum* em ambiente com ciclos luz-escuro de 12 horas e temperatura ambiente (T_a) controlada para 24 ± 2 ºC. Todas as avaliações feitas durante o período de TFA (descrito a seguir) foram realizadas no intervalo das 7 às 14h. Assim, após o termino da execução do protocolo de TFA, as amostras de tecidos foram armazenadas em freezer a -80 ºC para análises a serem executadas posteriormente no laboratório de controle do metabolismo da Faculdade de medicina de ribeirão preto (FMRP) da USP. Todos os procedimentos experimentais desenvolvidos neste projeto foram aprovados pela Comissão de Ética no Uso de Animais – CEUA da UFMG (220/2019).

3.2 Grupos experimentais

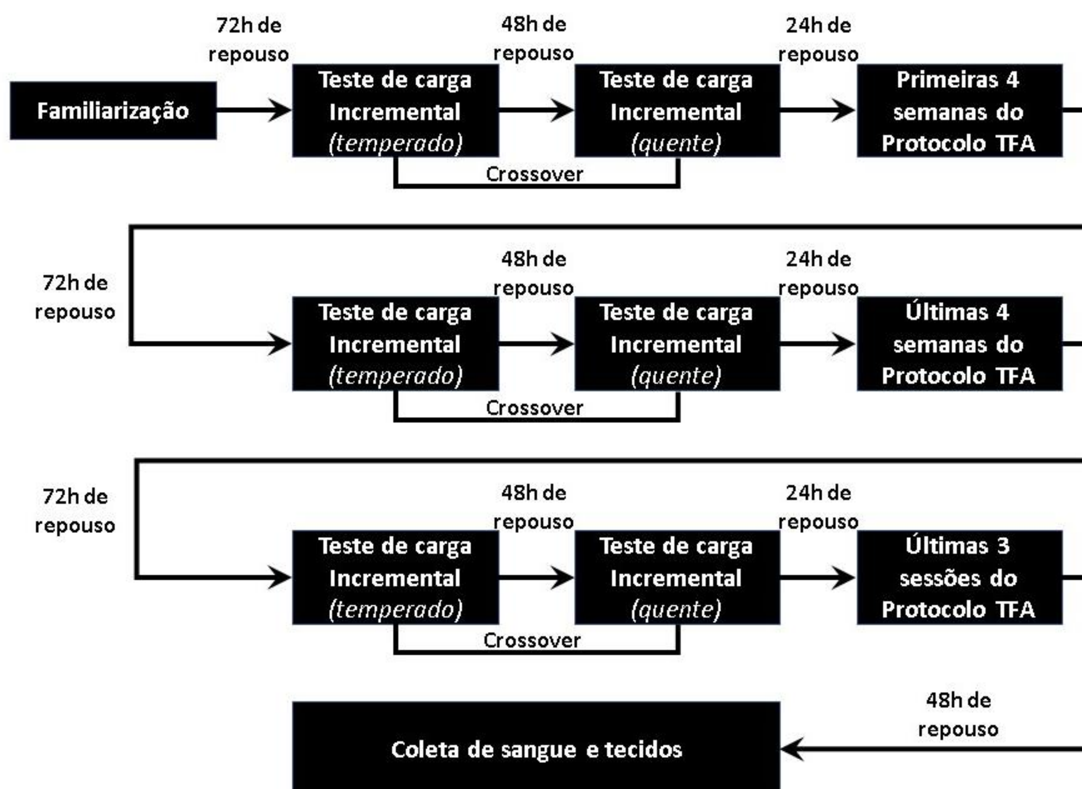
Com base no primeiro teste de carga incremental (TCI; descrito abaixo), os animais foram divididos de forma balanceada em 4 grupos experimentais: 1) animais Sedentários (SED) mantidos em ambiente temperado (T; 22 °C) (SED/T) durante o protocolo de treinamento físico aeróbico (TFA), 2) animais submetidos ao protocolo de TFA (descrito abaixo) em T (TFA/T), 3) animais SED mantidos em ambiente quente (Q; 32 ºC) (SED/Q) durante o protocolo de TFA e 4) animais submetidos ao protocolo de TFA em Q (TFA/Q). A intensidade de treinamento

dos grupos TFA foi prescrita com base no teste de carga incremental (TCI) em seu respectivo ambiente.

3.3 Delineamento experimental

Conforme a figura 1, todos animais foram submetidos a 5 sessões/dias de familiarização a corrida na esteira rolante e, dado 3 dias de repouso, iniciou-se a primeira rodada de TCI. Assim, todos os animais, independente do grupo, realizaram dois TCI em ambiente T e Q com intervalo de 48h de repouso. Como já mencionado, o desempenho obtido nesses dois primeiros TCI, foi utilizado para balancear os grupos experimentais. Após 1 a 2 dias de repouso, iniciou-se o protocolo de TFA por 4 semanas, prescrito com base no desempenho dos animais em seu respectivo ambiente. Assim, a intensidade do grupo TFA/T foi relativa ao teste em T e, da mesma forma, a intensidade do grupo TFA/Q foi relativa ao teste em Q. Dado três dias de repouso após o termino das primeiras 4 semanas de TFA, todos os animais foram submetidos novamente a segunda rodada de TCI, nas mesmas condições da primeira. Na sequência, 1 a 2 dias após o último TCI, as cargas de treinamento foram reajusta com base nos últimos testes e as sessões de TFA foram reiniciadas por mais 4 semanas, totalizando as 8 semanas de TFA. Novamente, ao termino da 8ª semana de TFA, os animais foram reavaliados na terceira e última rodada TCI, executada nas mesmas condições das anteriores. Vale mencionar que o estudo foi realizado de forma cruzada-balanceada (*crossover*), para metade da amostra a ordem dos TCI foi cronologicamente realizada em T e Q, enquanto que para segunda metade do número amostral realizou em ordem inversa (i.e., primeiro teste em Q e depois teste em T). Por fim, após 1 a 2 dias de repouso, foram realizadas mais 3 sessões do TFA e, após 2 dias de repouso, os animais foram decapitados com guilhotina para coleta dos tecidos de interesse.

Figura 1. Representação esquemática dos procedimentos experimentais em camundongos. TFA, treinamento físico aeróbico. Temperado, ambiente temperado (22 °C). Quente, ambiente quente (32 °C) (Fonte: elaboração própria).



3.4 Familiarização à corrida na esteira rolante

Realizado por todos animais em esteira rolante (Panlab*/Havard Apparatus,* Cornella, Espanha; Figura 2) mantida em inclinação de 5º e durante 5 sessões/dias consecutivos, como descrito na tabela 1. O protocolo consistiu em 3 estágios, sendo que sempre o primeiro deles era de repouso de 3 min dentro da baia da esteira desligada. Já no segundo estágio a duração foi fixada em 5 min e intensidade aumentou progressivamente de 5 – 6 m/min ao longo dos dias. Já no terceiro estágio, ao longo dos dias, houve aumento progressivo da duração e intensidade de 3 - 5 min e 6 - 8 m/min, respectivamente (adaptado de Wanner *et al.*, 2014). No entanto, no quinto dia os animais foram submetidos à familiarização ao teste de carga incremental com atenuado critério de

interrupção (5 s na zona de fadiga; Figura 2) (Dougherty; Springer; Gershengorn, 2016). A familiarização a esse teste é importante, uma vez que experimentos piloto do nosso laboratório, avaliando 3 testes incrementais sequenciais aplicados com intervalo de 48 h, foi demonstrado que os animais apresentam um aumento significativo no tempo de corrida e na $V_{máx}$ no 2º teste, quando comparado ao 1º teste, e não há alterações adicionais no 3º teste (dados ainda não publicados), sugerindo que o aprimoramento do desempenho físico entre o 1º e 2º teste deve depender da aprendizagem nesse curto período, o que inevitavelmente criaria um "artefato" na interpretação entre os nossos testes em ambientes T e Q.

Os camundongos foram encorajados a correr por meio de estimulação elétrica leve (0,5 mA) que foi fornecida por uma grade localizada no final da cinta da esteira. Durante as sessões de familiarização e todos os experimentos, um ventilador, que produz uma velocidade de fluxo aéreo de 2,0-2,5 m/min, foi posicionado na frente e atrás da esteira rolante. A T_a foi controlada em 24 ± 2 ºC durante a familiarização.

Tabela 1. Protocolo de familiarização planejado para ensinar o camundongo a correr na esteira rolante* (Fonte: elaboração própria).

Dia	Estágio 1	Estágio 2	Estágio 3
1	3 min de repouso	5 min à 5 m/min	3 min à 6 m/min
2	3 min de repouso	5 min à 6 m/min	3 min à 8 m/min
3 e 4	3 min de repouso	5 min à 6 m/min	5 min à 8 m/min
5	Familiarização ao teste de carga incremental (na mesma esteira do teste)		

*, a inclinação da esteira rolante foi sempre configurada em 5º

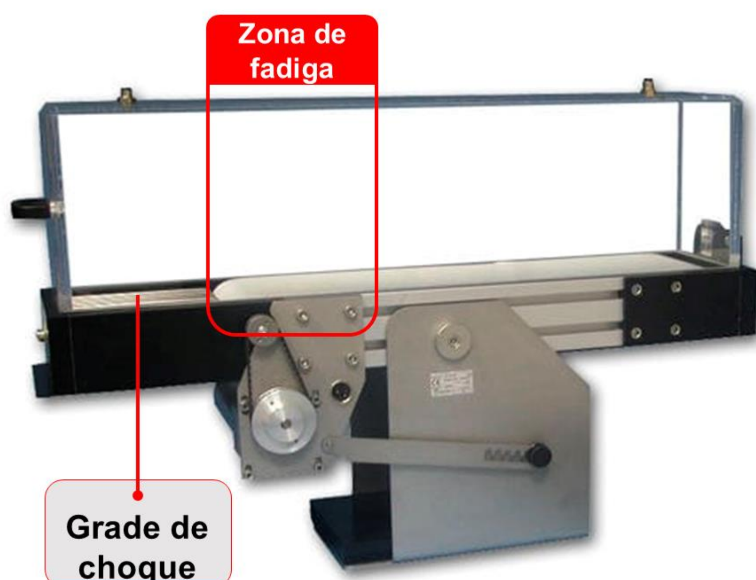
3.5 Protocolo do teste de carga incremental em esteira rolante

Utilizado como parâmetro do desempenho físico, o TCI em esteira rolante (Panlab/*Havard Apparatus*, Cornella, Espanha; Figura 2) foi executado por todos animais em ambas temperaturas (22 e 32 ºC) em 3 momentos, i.e., pré-TFA, após 4 e 8 semanas de TFA, a fim de determinar as variáveis de desempenho

físico [tempo até a fadiga e distância percorrida]. O teste foi iniciado na velocidade de 10 m/min, com aumento de 3 m/min a cada 3 min (inclinação em 5º). A fadiga foi determinada quando os animais eram incapazes de acompanhar o ritmo da esteira, permanecendo na grade de choque (Figura 2) por 5 s (Ayachi *et al.*, 2016).

Durante o teste em ambiente Q, para aquecer aparte interna da câmara de acrílico que contém a esteira rolante, um aquecedor elétrico (Britânia modelo AB 1100, Curitiba, Brasil) foi posicionado no mesmo nível de distância (~40 - 45 cm) do ventilador e foi ligado à 1.200 W (Wanner *et al.*, 2014). Os testes em ambiente T e Q foram separados por pelo menos 48 h e realizados entre 7:00 e 14:00 para minimizar as influências cronobiológicas.

Figura 2. Ilustração representativa da esteira utilizada no protocolo de familiarização à corrida e teste de carga incremental (fonte: adaptado de Panlab/Havard Apparatus, Cornella, Espanha).



3.6 Protocolo do teste de carga incremental em esteira rolante

A progressão do volume e intensidade relativa de cada sessão de TFA foi o mesmo para os grupos TFA. Em resumo, os animais iniciaram o TFA 1 a 2 dias após os primeiros TCI realizados no período pré-TFA. Apesar de todos animais realizarem TCI em ambos ambientes T e Q, a intensidade de treino prescrita foi relativa a $V_{máx}$ obtida no TCI no respectivo ambiente de cada grupo TFA.

Como mostra a tabela 2, os camundongos iniciaram o protocolo de TFA correndo em esteira rolante na velocidade equivalente à 50% da $V_{máx}$ (0º de inclinação) por 25 e 35 min na 1ª e 2ª sessão, respectivamente. A partir da 2ª semana, a duração das sessões continuou aumentando em 15 min por dia até atingir 60 min e, a partir da 4ª sessão, a intensidade do esforço aumentou para 60% da $V_{máx}$. Finalmente, a partir da 3ª semana até a conclusão do protocolo de TFA (8ª semana), as sessões de corrida foram realizadas à 60% da $V_{máx}$ por 60 min com a inclinação da esteira mantida em 5º. Destaca-se que a intensidade de TFA foi determinada novamente após a 4ª semana, ajustando assim a intensidade relativa dos grupos ao segundo TCI.

Vale destacar que, por serem condições ambientais diferentes, as sessões de TFA dos grupos TFA/T e TFA/Q não foram concomitantes. Desse modo, durante a sessões de TFA do grupo TFA/Q, a T_a foi mantida em 32 ºC pela combinação de aquecimento promovido tanto pelo ar-condicionado da sala (KOP4UQC, KOMECO™, Palhoça, Brasil) quanto por dois aquecedores elétrico (Britânia modelo AB 1100, Curitiba, Brasil) ligado à 1.200 W e posicionado a ~40 - 45 cm das baias da esteira de corrida.

Tabela 2. Protocolo de treinamento físico aeróbico (TFA) (baseado em Ferreira et al., 2007; Kodesh; Horowitz, 2010; Wanner et al., 2014) (Fonte: elaboração própria).

	Sessão #1	**Sessão #2**	**Sessão #3**	**Sessão #4**	**Sessão #5**
1ª Semana					
Inclinação (º)	0	0	--	--	--
Intensidade (% da $V_{máx}$)	50	50			
Duração *(min)*	20	35			
2ª Semana					
Inclinação (º)	0	0	0	0	0
Intensidade (% $V_{máx}$)	50	50	50	60	60
Duração *(min)*	45	60	60	60	60
3-8ª Semana					
Inclinação (º)			5		
Intensidade (% $V_{máx}$)			60		
Duração *(min)*			60		

$V_{máx}$, Velocidade máxima

3.7 Determinação do glicogênio hepático e muscular

As determinações do conteúdo de glicogênio foram realizadas a partir de amostras de tecido hepático e muscular colhidas imediatamente após a eutanásia, pesadas e armazenadas em freezer -80 ºC. No momento da quantificação, os tecidos foram colocados em tubos de centrífuga contendo 2 ml de KOH 30%. A extração foi realizada através do método de Sjögren et al. (1938) e a quantificação do glicogênio foi realizada pelo método da Antrona (Carroll; Longley; Roe, 1956).

3.8 Morfologia muscular e tipagem de fibras musculares

Para análise histológica foram utilizados cortes do músculo tríceps sural (TS). Após a eutanásia, o tecido foi cuidadosamente excisado da pata do animal,

estirado cuidadosamente sobre uma plataforma acrílica, congelados em nitrogênio líquido e armazenados em freezer -80 ºC. Para montagem das lâminas, os músculos foram cortados a -20 ºC em uma espessura de 10 µm em um criostato (*Criostato – Leica CM 1860*). Os cortes foram cuidadosamente posicionados em lâminas gelatinizadas para posteriores tratamentos e avaliações histológicas.

3.8.1 Coloração com hematoxilina e eosina (H&E)

Cortes histológicos de TS foram corados com Hematoxilina & eosina (H&E) para demonstração da integridade das fibras do músculo esquelético e visualização da localização do núcleo celular, um indicativo de processo de regeneração muscular (Clark, 1946).

A hematoxilina por possui característica acidófila marca em azul-púrpura os ácidos nucleicos presentes no núcleo miofibrilar. Já a eosina, como é basófila cora em rosa as proteínas não especificas no citoplasma e na matriz extracelular (Fischer *et al.*, 2008). Na sequência, as lâminas foram montadas com solução Entellan™ (Merck KGaA, Darmstadt, Alemanha) e as imagens foram coletadas com microscópio de fluorescência Olympus BX61VS e a proporção de núcleo centralizados foi quantificada com o software ImageJ (*Fiji is Just*) versão 1.51h.

3.8.2 Atividade mitocondrial

A SDH é uma importante enzima aderida a parede interna nas organelas mitocondriais e possui um papel importante no metabolismo energético, atuando tanto no ciclo do ácido tricarboxílico quanto na fosforilação oxidativa (complexo II da OxPhos). Dessa forma, a mensuração da atividade mitocondrial por meio SDH, ocorre pela capacidade da enzima em converter o sal tetrazolium no sal de cor azul, formazan. Dessa maneira, a interação com o sal indica visualmente fibras com maior (oxidativas) ou menor (glicolíticas) atividade da enzima.

Portanto, cortes histológicos de TS foram incubados em solução contendo sal de tetrazolium a 37 ºC por 30 min. Após lavagem com água destilada, as lâminas foram montadas com solução Entellan™ (Merck KGaA, Darmstadt, Alemanha) e as imagens foram coletadas com microscópio de fluorescência Olympus BX61VS. A intensidade da marcação e a área de secção transversa (AST) foram mensuradas no software ImageJ (*Fiji is Just*) versão 1.51h.

3.8.3 Ensaio de imunofluorescência para a marcação do tipo de fibra muscular

Imunomarcações específicas marcam os diferentes tipos de miosinas de cadeia pesada por meio de interação com anticorpo especifico (acoplado a marcador florescente), indicando os diferentes tipos de miosinas presentes e suas respectivas AST (Gonçalves *et al.*, 2019).

Cortes histológicos de TS foram lavados em PBS (*Phosphate buffer saline*) e incubados com reagente de bloqueio *Mouse on Mouse* (M.O.M™) em temperatura ambiente por 1 h. Após lavagem em PBS, os cortes foram incubados por aproximadamente 12h (*overnight*) a 4 ºC em solução (BSA 5%) contendo os anticorpos primários fornecidos pela *Developmental Studies Hybridoma Bank* (DSHB, *University of Iowa*): SC-71 (IgG1, 1:150) para MyHC2A, BF-F3 (IgM, 1:200) e MyHCX não recebeu marcação e por isso apareceram em preto. Para facilitar a visualização do perímetro das fibras, Distrofina (Dys; 1:100). No dia seguinte, os cortes foram novamente lavados em PBS e foram incubados por 1h a 37 ºC em solução (*goat sérum* (2%) e BSA (0,5%) em PBS) contendo os anticorpos secundários *Alexa Fluor* 488 *anti-mouse* (1:150) e *Alexa Fluor* 647 *anti-rabbit* (Life Technologies) (1:200). Após rápida lavagem em PBS as lâminas foram montadas com solução DAKO *Fluorescent Mouting Medium* e as imagens foram coletadas com microscópio de fluorescência Olympus BX61VS. A proporção entre os tipos de fibra e a AST foram mensuradas no software ImageJ (*Fiji is Just*) versão 1.51h.

3.9 Conteúdo e estado de fosforilação de proteínas

Os tecidos musculares (Gastrocnêmio) congelados foram homogeneizados no Politrom tampão Tris-HCl (50 mM; pH 7,4; 4°C) contendo 150 mM de NaCl, 1 mM de EDTA, 1% de Triton X-100, 1% de deoxicolato de sódio, 1% de dodecil sulfato de sódio (SDS), inibidores de proteases (1 mM PMSF, 5 µg/ml de aprotinina e 1 µg/ml de leupeptina) e inibidores de fosfatases (10 mM ortovanadato de sódio, 10 mM pirofosfato de sódio e 100 mM fluoreto de sódio). O homogenado foi centrifugado a 15.000 rpm, 4°C, durante 20 minutos. Parte do sobrenadante foi utilizada no método de Lowry e colaboradores (1951) para a quantificação proteica. Em seguida, na parte restante do sobrenadante, foi adicionado o tampão de Laemmli [solução contendo SDS 4%, 125 mM de Tris-HCl, glicerol 20%, 100 mM de DTT, azul de bromofenol 2% e pH 6,8 ajustado com 1M de HCl].

A corrida eletroforética foi realizada em gel de SDS-PAGE, segundo o método de Laemmli (1970). Após aquecimento (70 ºC, 10 min), as amostras foram aplicadas em sistema de mini-gel vertical (modelo Protean III e IV Cell BioRad, *Bio-Rad Laboratories*, CA, EUA) de acrilamida:bisacrilamida com 1,00 (para MyHC) ou 0,75 mm (para demais proteínas) de espessura, gel de separação variando de 06 a 16%. No primeiro poço foi aplicado o padrão de massa molecular PageRuler™ Prestained Protein Ladder (apenas MyHC, 10-250 kDa; Fermentas Life Sciences, EUA) ou Prestained Protein Ladder (10-170 kDa; Fermentas Life Sciences, EUA). A eletroforese foi realizada em cubas de acrílico preenchidas por tampão de corrida (25mM de Tris-HCl, pH 8,4,115mM de glicina, SDS 0,1%), sob voltagem de ~100 Volts, durante 1,5 - 2 horas.

Após a corrida eletroforética, o gel foi preparado para a transferência em sistema molhado (exclusivamente para MyHC, Mini trans-blot cell BioRad, Bio-Rad Laboratories, CA, EUA) ou em sistema semisseco (BioRad Trans-Blot SD Cell, EUA) (TOWBIN et al., 1979). Inicialmente, o gel e a membrana de nitrocelulose foram colocados na solução de transferência (48mM de Tris, 39mM de glicina, SDS 10% e 0,2M de metanol). Após a montagem dos sistemas de transferência, as proteínas presentes no gel de poliacrilamida foram transferidas para a membrana de nitrocelulose, sendo o processo de transferência molhada

realizado durante 1,5h sob a voltagem de 100 - 110 volts; ou semisseco por 30 minutos sob a amperagem de 400 mA e voltagem de no máximo 25 volts, à temperatura ambiente. Após o término da transferência, a membrana de nitrocelulose foi submetida à incubação por 1 hora, sob agitação, à temperatura ambiente com leite desnatado em pó 10%, diluído em TBS-T (0,02 M de Tris-HCl, 0,16M de NaCl e 0,1% Tween 20).

Após a eletro-transferência das proteínas do gel e o bloqueio, as membranas foram incubadas a 4 ºC com anticorpos primários específicos (Tabela 3). Posteriormente a remoção do excesso de anticorpo, por meio de lavagem (TBS-T), as membranas foram incubadas com anticorpo secundário conjugado com peroxidase (1:10000 para MyHC lenta, β-actin; 1:8000 para MyHC rápida; 1:6000 para OxPhos, TOM20; 1:5000 para α-tubulin; 1:3000 para p-ERK e pThr^{180}/Tyr^{182}-p38, TFEB; 1:2000 para pThr^{286}-CAMKII, CAMKII, p38; 1:1000 para p-Ser^{473}-Akt, Akt, ERK, pThr^{172}-AMPK, PGC-1α, pThr^{24}-FoxO1/pThr^{32}-FoxO3, pSer^{413}-FoxO3, pSer^{294}-FoxO3, FoxO3, FoxO1, Dynein). A revelação foi feita com reagente amplificador de quimiluminescência em fotodocumentador digital (ChemiDoc XRS+ BioRad, *Bio-Rad Laboratories*, CA, EUA). A análise da concentração das proteínas de interesse, indicada pela densidade das bandas (densitometria), foi realizada no software ImageJ (Fiji is Just) versão 1.51h e os resultados obtidos foram comparados aos do grupo controle (SED/T), o qual foi considerado como 100%.

Tabela 3. *Lista de anticorpos para Western blot (fornecidos por:* Cell Signaling Technology*, Massachusetts, EUA;* Santa Cruz Biotech*, Texas, EUA;* Merck KGaA*, Massachusetts, Alemanha;* AbCam*, Cambridge, Reino Unido) (Fonte: elaboração própria).*

Anticorpo primário	**Diluição**	**Número no catalogo**	**Fornecedor**
Anti-CAMKII	1:1000	sc-5606	Santa Cruz
Anti-FoxO1	1:1000	#9454S	Cell Signaling
Anti-FoxO3	1:500	#2497	Cell Signaling
Anti-MyHC fast	1:5000	M4276	Merck KGaA,
Anti-MyHC slow	1:5000	M8421	Merck KGaA
Anti-OxPhos	1:1000	ab110413	AbCam

Anti-PGC-1α	1:500	sc-13067	Santa Cruz
Anti-TFEB	1:500	sc-48784	Santa Cruz
Anti-TOM20	1:500	#4206	Cell Signaling
Anti-p38	1:1000	#9212	Cell Signaling
Anti-pThr^{286}-CAMKII	1:500	#12716S	Cell Signaling
Anti-pser^{294}-FoxO3	1:500	#5538S	Cell Signaling
Anti-pser^{413}-FoxO3	1:500	#8174S	Cell Signaling
Anti-pthr^{172}-AMPK	1:1000	sc-33524	Santa Cruz
Anti-pthr^{24}-foxo1/pthr^{32}-FoxO3	1:500	#9464L	Cell Signaling
Anti-pThr^{180}/Tyr^{182}-p38	1:1000	#9211S	Cell Signaling
Anti-pThr^{202}/Tyr^{204}-ERK1 / pThr^{185}/Tyr^{187}-ERK2	1:1000	#9102	Santa Cruz
Anti-pSer^{473}-Akt	1:1000	#9271S	Cell Signaling
Anti-Akt	1:1000	#9272S	Cell Signaling
Anti-dynein	1:1000	sc-13524	Santa Cruz
Anti-α-tubulin	1:1000	sc-32293	Santa Cruz
Anti-β-actin	1:2000	sc-81178	Santa Cruz
Anti-ERK1/2	1:1000	sc-292838	Santa Cruz

3.10 Análise estatística

Os resultados de desempenho físico foram expressos como % de alteração em relação ao grupo SED/T ± EPM (Erro Padrão da Média). A distribuição normal dos dados foi conferida pelo teste de normalidade de Shapiro-Wilk. Para os resultados de desempenho físico, comparações de duas dos resultados pré-TFA foram feitas por meio do Teste-t de *Student* e para múltiplas comparações dos resultados de pré e após 4 e 8 semanas de treinamento, foram realizadas análises de variância de duas vias e de medidas repetidas (*two-way ANOVA-RM*), seguido pelo pós-teste de Tukey. Para a análise do glicogênio hepático e muscular, morfologia muscular, Identificação do tipo de fibra muscular e conteúdo/estado de fosforilação de proteínas, múltiplas comparações foram

feitas também usando o ANOVA de duas vias ordinária (*two-way ANOVA*), seguido pelo pós-teste de Tukey. O nível de significância utilizado foi de 5%.

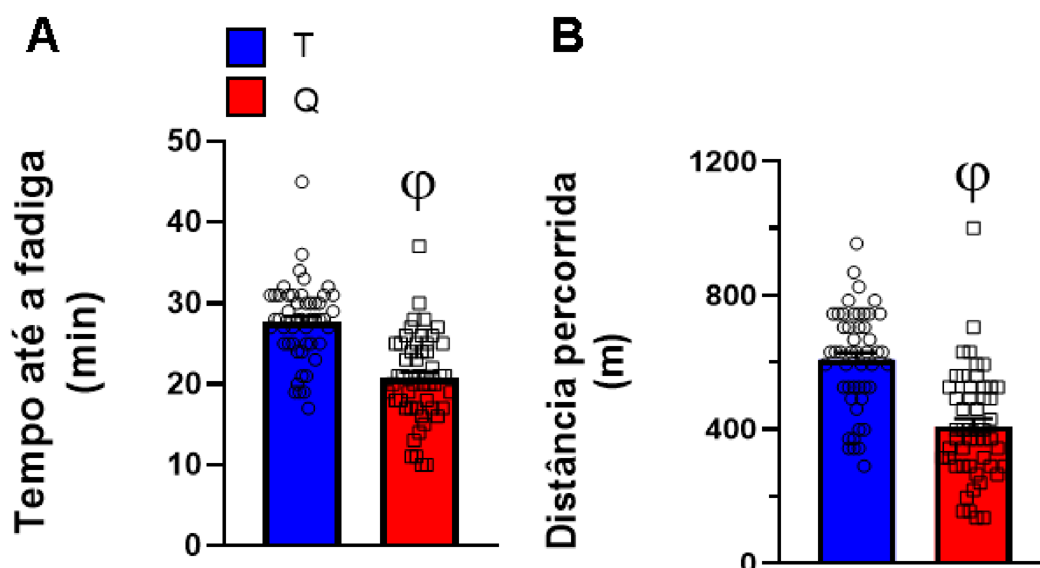
Resultados

4 RESULTADOS

4.1 Efeitos agudos e crônicos do ambiente quente no desempenho de resistência aeróbica

Primeiramente, o TCI foi realizado em todos os camundongos em ambientes tanto T como Q a fim de: 1) determinar o desempenho físico aeróbico e 2) balancear os grupos experimentais. Como esperado, o ambiente Q causou uma redução nos parâmetros relacionados à resistência a fadiga, como tempo até a fadiga (38%) e distância percorrida (35%), quando comparado com o teste em ambiente T (Figuras 4A e B). Esses achados estão de acordo com aqueles obtidos pelo nosso grupo anteriormente (apêndice 1, dados não publicados), o ambiente Q também reduziu a $V_{máx}$ no TCI realizado no período de pré-TFA. Por essa razão, a velocidade de corrida absoluta (m/min) correspondente a 60% de $V_{máx}$ prescrita para o grupo TFA/Q durante 8 semanas de TFA foi ~30% menor se comparada ao do grupo TFA/T.

Figura 3. Efeito agudo do ambiente quente (Q) no tempo até a fadiga (A) e distância percorrida (B) no teste de carga incremental (TCI) comparado ao ambiente temperado (T) no período pré-treinamento físico. Os valores são expressos como média ± erro padrão da média ± erro padrão da média. ϕ, P ≤ 0,05 vs. T (n = 12 – 13/grupo) (Fonte: elaboração própria).



Para além do TCI realizado no período pré-TFA, outros dois TCI em ambos ambientes T e Q foram realizados após 4 e 8 semanas de TFA a fim de avaliar a resistência aeróbica dos animais. Os dados referentes ao TFA mostram que, no ambiente T, os todos os grupos apresentaram semelhantes valores pré-TFA de tempo até a fadiga (~27 min) e distância percorrida (~630 m) (Figuras 5A e B). As Figuras 5A e B mostram que ambos os grupos TFA (i.e., TFA/T e. TFA/Q) aumentaram ~18% o tempo até a fadiga e a distância percorrida na 4ª e 8ª semana, em comparação com seu período de pré-TFA (semana 0) e com seus respectivos grupos SED no mesmo período (i.e., TFA/T *vs.* SED/T e TFA/Q *vs.* SED/Q). No ambiente Q, os valores pré-TFA do tempo até a fadiga (~21 min) e distância percorrida (385 m) também foram semelhantes entre os grupos (Figura 6A e B). Na semana 4, apenas o grupo TFA/Q aumentou significativamente o tempo até a fadiga e a distância percorrida (~20%) em comparação com seu período de pré-TFA (Figura 6A e B). Na semana 8, os grupos TFA/T e TFA/Q aumentaram (~20%) o tempo até a fadiga e a distância percorrida em comparação com seu respectivo período de pré-TFA. Esses achados estão de acordo com aqueles obtidos pelo nosso grupo anteriormente (apêndice 1, dados não publicados), quando observamos melhora na resistência aeróbica, indicada pela $V_{máx}$, acompanhada de aumento no VO_{2pico} após TFA. Em conjunto, esses achados mostram que embora o ambiente Q prejudique o desempenho físico aeróbico e a intensidade de exercício físico, TFA melhora a resistência aeróbica de forma semelhante em qualquer T_a avaliada**.**

Figura 4. Efeito do treinamento físico aeróbico (TFA) por 8 semanas em ambiente quente (Q) e temperado (T) no tempo até a fadiga (A) e na distância percorrida (B) em ambiente T. Os valores estão expressos como % do grupo SED/T no pré-TFA. *, P ≤ 0,05 vs. TFA/T no pré-TFA; &, P = 0,05 – 0,09 *vs.* TFA/Q no pré-TFA; @, $P \leq$ 0,05 *vs.* SED/T no mesmo período. SED/T, grupo de animais sedentário mantidos em ambiente T. TFA/T, grupo de animais submetido ao TFA em ambiente T. SED/Q, grupo de animais SED mantidos em ambiente Q. TFA/H, grupo de animais submetido ao TFA em ambiente Q. Pré-TFA representa a semana 0 do protocolo de TFA, ou seja, antes do início do

programa de TFA. 4 e 8 semanas, representam os testes de carga incremental realizados na 4ª e 8ª semana de protocolo experimental, respectivamente (n = 12 – 13/grupo).

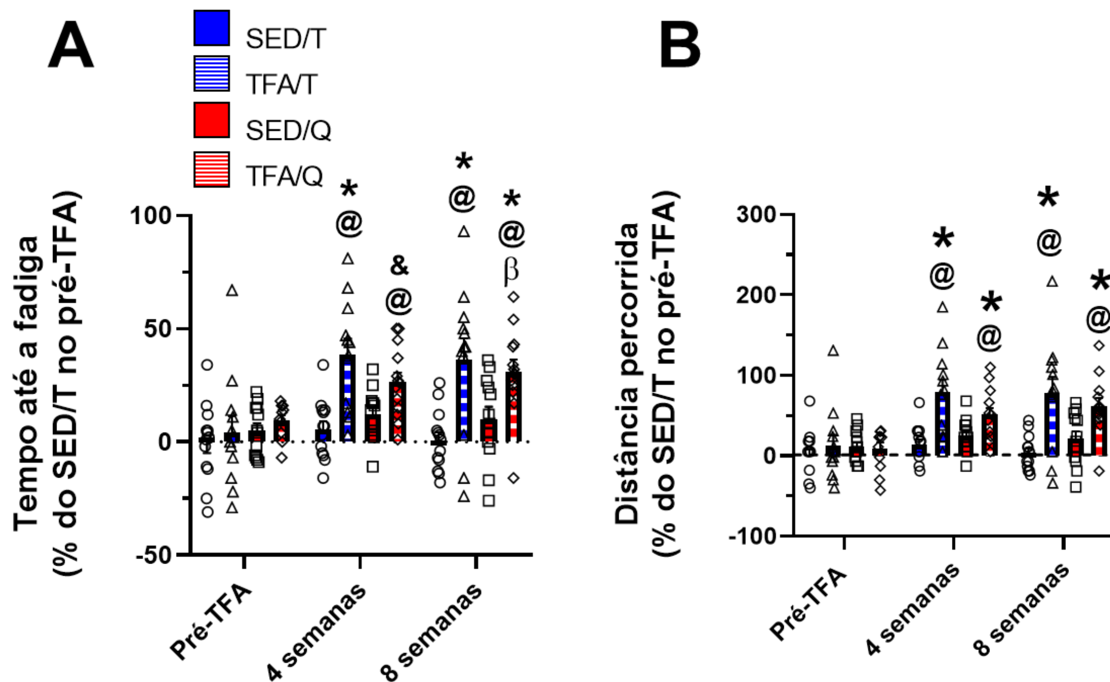
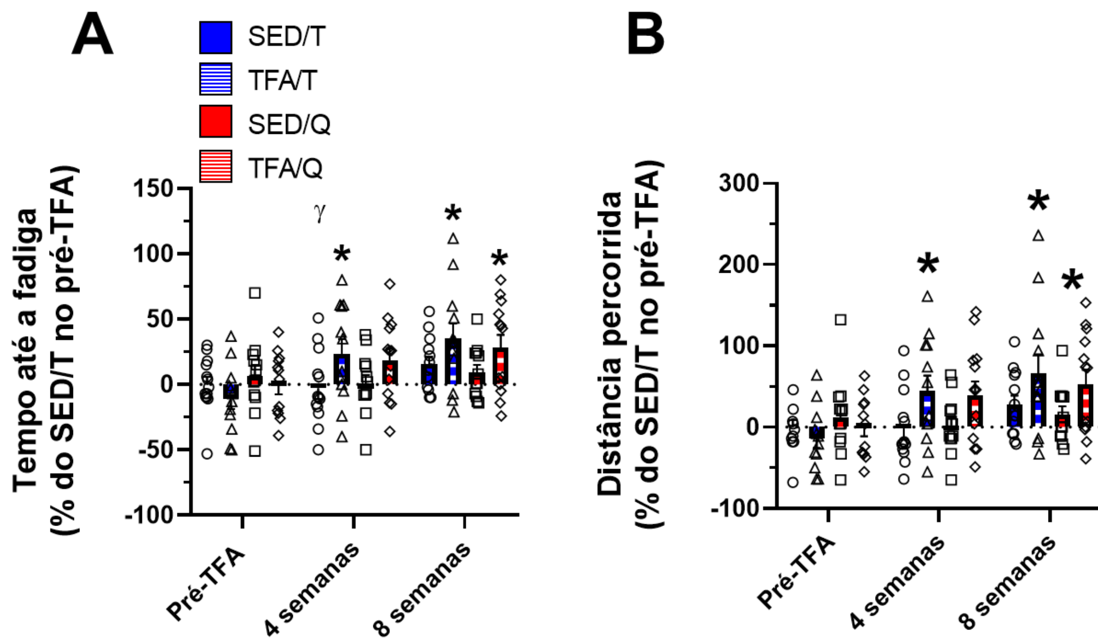


Figura 5. Efeito do treinamento físico aeróbico (TFA) por 8 semanas em ambiente quente (Q) e temperado (T) no tempo até a fadiga (A) e na distância percorrida (B) em ambiente Q. Os valores estão expressos como % do grupo SED/T no pré-TFA. *, P ≤ 0,05 vs. TFA/T no pré-TFA; γ, P = 0,05 – 0,09 vs. SED/T no mesmo período. SED/T, grupo de animais sedentário mantidos em ambiente T. TFA/T, grupo de animais submetido ao TFA em ambiente T. SED/Q, grupo de animais SED mantidos em ambiente Q. TFA/H, grupo de animais submetido ao TFA em ambiente Q. Pré-TFA representa a semana 0 do protocolo de TFA, ou seja, antes do início do programa de TFA. 4 e 8 semanas,

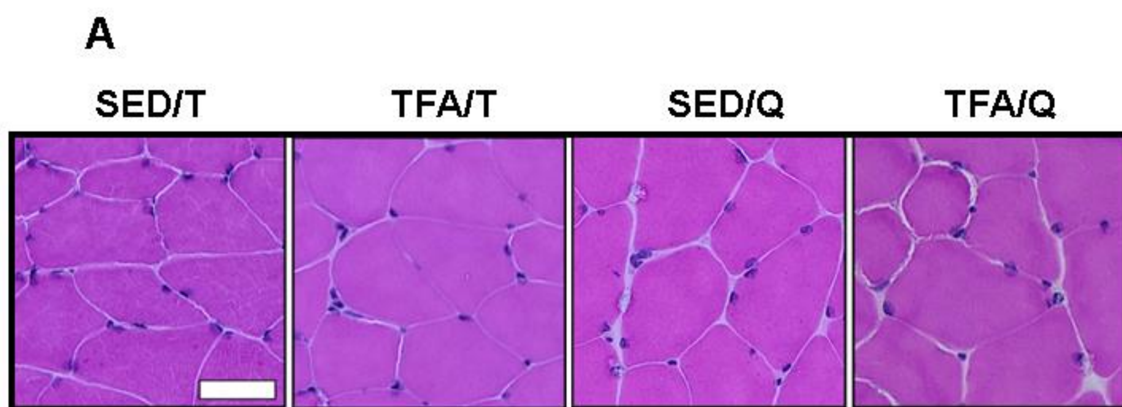
representam os testes de carga incremental realizados na 4ª e 8ª semana de protocolo experimental, respectivamente (n = 12 – 13/grupo).



4.2 A exposição crônica ao ambiente quente não causa danos musculares em animais sedentários ou treinados

O estresse térmico causa uma sobrecarga fisiológica que pode evoluir para complicações relacionadas ao exercício físico no calor, conhecido em inglês como *Exertional heat illness*, dentre elas destaca-se a lesão muscular (ROBERTS, WILLIAM O. *et al.*, 2021) que poderia prejudicar a saúde do animal e o desempenho de resistência aeróbica. A análise qualitativa de cortes musculares pela técnica de H&E revelou que todos os grupos estudados apresentam uma boa integridade histomorfológica do músculo TS, ou seja, não há alterações morfológicas características do processo de degeneração-regeneração e miopatias, como fibras atróficas pequenas, achatadas ou irregularmente formadas, fibras com núcleos centralizados, fibras alvo e fibras necróticas (Figura 7). Nossos resultados sugerem que a exposição crônica ao ambiente Q não causa danos musculares ou outras anormalidades histológicas em animais.

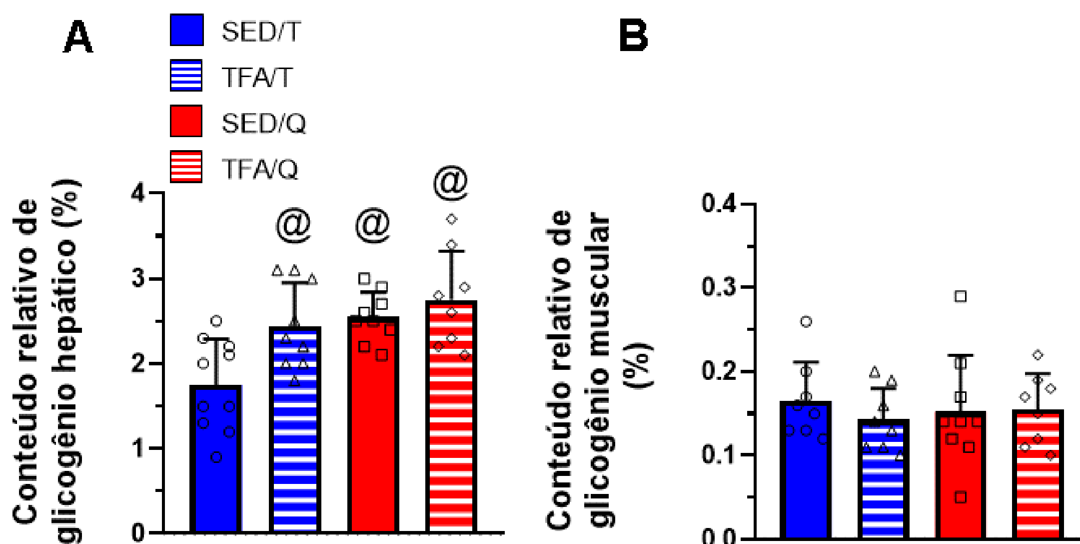
Figura 6. Imagens microscópicas de cortes histológicos transversais de fibras musculares coradas com hematoxilina e eosina (H&E) do músculo tríceps sural de camundongos. O azul-purpura é marcado pela hematoxilina e evidencia os ácidos nucleicos presentes no núcleo. A coloração rosa marcada pela eosina evidencia as proteínas não especificas no citoplasma e na matriz extracelular. SED/T, grupo de animais sedentário mantidos em ambiente T. TFA/T, grupo de animais submetido ao TFA em ambiente T. SED/Q, grupo de animais SED mantidos em ambiente Q. TFA/H, grupo de animais submetido ao TFA em ambiente Q. Barra de escala de 50 µm.



4.3 Efeitos do treinamento físico aeróbico no conteúdo de glicogênio hepático e muscular

Para avaliar se o glicogênio hepático e muscular poderia explicar a melhoria de desempenho de resistência aeróbica induzidas pelo TFA, o conteúdo de glicogênio nesses órgãos foi avaliado. O conteúdo de glicogênio no fígado aumentou aproximadamente 34% nos grupos TFA/T, SED/Q e TFA/Q em comparação com SED/T (Figura 8A). Por outro lado, o glicogênio no músculo gastrocnêmio não foi afetado em nenhum grupo (Figura 8B). Esses dados indicam que tanto a exposição ao ambiente Q quanto o TFA de corrida promovem acúmulo de glicogênio no fígado, mas não no músculo esquelético. Além disso, outras adaptações musculares deveriam explicar a melhora do desempunho.

Figura 7. Efeito do treinamento físico aeróbico (TFA) por 8 semanas em ambiente quente (Q) e temperado (T) no conteúdo relativo (%) de glicogênio hepático (A) e muscular (B). @, P ≤ 0,05 *vs.* SED/T. SED/T, grupo de animais sedentário mantidos em ambiente T. TFA/T, grupo de animais submetido ao TFA em ambiente T. SED/Q, grupo de animais SED mantidos em ambiente Q. TFA/H, grupo de animais submetido ao TFA em ambiente Q (n = 12 – 13/grupo).



4.4 Efeitos do treinamento físico aeróbico em ambiente quente na expressão de miosinas de cadeia pesada e nos tipos das fibras musculares

Considerando que os grupos TFA/T e TFA/Q aumentaram a resistência à fadiga e essa é uma das características funcionais das fibras musculares de contração lenta, analisamos por meio da técnica de WB o conteúdo das isotipos de MyHC *slow* e *fast* no músculo gastrocnêmio a fim de estimar se o TFA e/ou o ambiente Q causariam mudanças no tipo de fibra musculares, mas não foram observadas diferença entre os grupos (Figuras 9A e B). Entretanto, as fibras musculares que expressam MyHC *fast* podem ser subdivididas em fibras rápidas intermediárias de metabolismo oxidativo e glicolítico (MyHC2A) e fibras rápidas de metabolismo predominantemente glicolítico (MyHC2X e 2B) (SCHIAFFINO; REGGIANI, 2011). Por isso, imunomarcação para essas três isoformas de MyHC *fast* foram

realizadas em cortes transversais do músculo TS. A análise dos tipos de fibra reportou que ambos os grupos TFA/T (~ 30%) e TFA/Q (~ 30%, *P* = 0,072) apresentaram uma proporção maior de fibras do tipo 2A em comparação ao o grupo SED/T (Figuras 10A e B). Em contrapartida, a proporção de fibras do tipo MyHC 2X e 2B não foi alterada em nenhum grupo (Figuras 10A e B). O grupo SED/Q evidenciou que apenas a exposição ao ambiente Q ao longo de 8 semanas levou a uma tendência (16%, *P* = 0,087) de aumento da AST das fibras musculares em comparação com o grupo SED/T (Figuras 10A e C). Em resumo, esses resultados indicam que o TFA de intensidade absoluta reduzida no ambiente Q promove transição no tipo de fibra para um fenótipo possivelmente mais oxidativo de forma similar ao ambiente T.

Figura 8. Efeito do treinamento físico aeróbico (TFA) por 8 semanas em ambiente quente (Q) e temperado (T) no conteúdo de miosinas de cadeia pesada (do inglês, Myosin heavy chain; MyHC) slow (lenta) e fast (rápida). As bandas (*blots*) representativas (A) ilustram os valores expressos como Média ± EPM no histograma (B). A α-tubulin foi usada como controle da quantidade de proteína aplicada para a eletroforese. SED/T, grupo de animais sedentário mantidos em ambiente T. TFA/T, grupo de animais submetido ao TFA em ambiente T. SED/Q, grupo de animais SED mantidos em ambiente Q. TFA/H, grupo de animais submetido ao TFA em ambiente Q (N = 6 - 7/grupo).

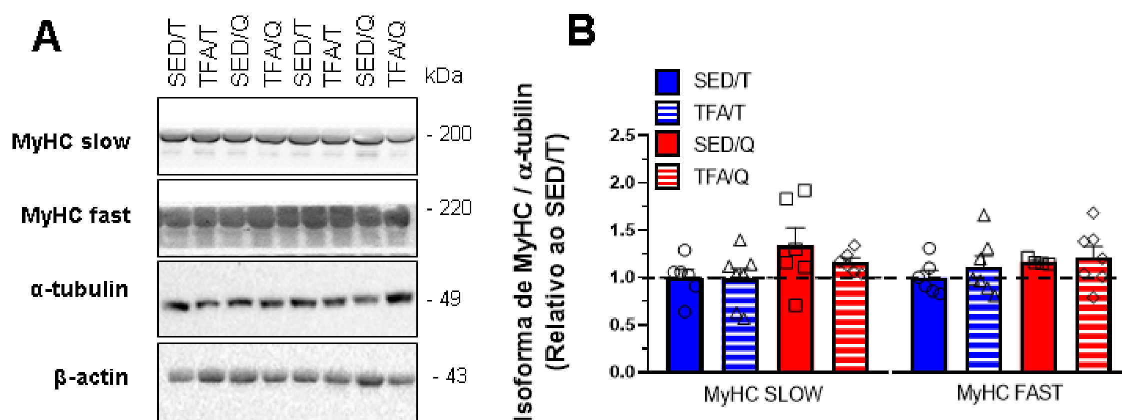
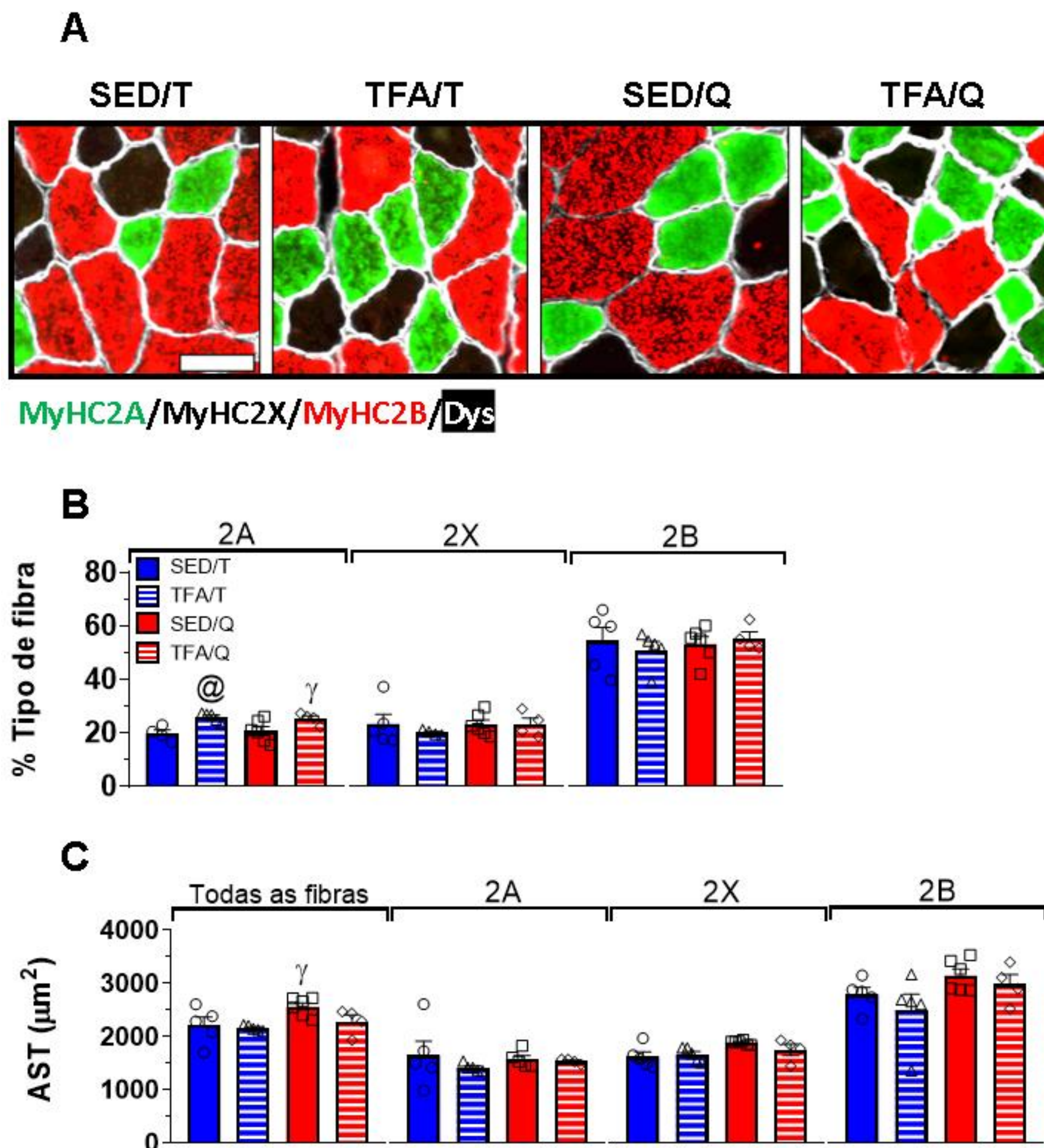


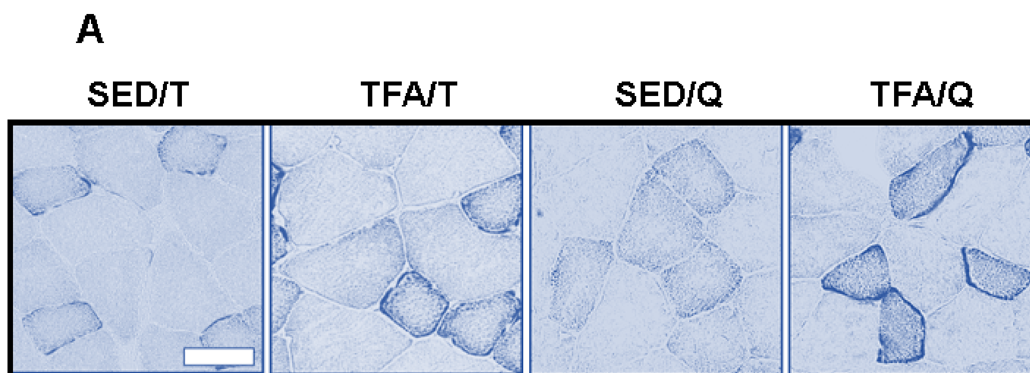
Figura 9. *Efeito do treinamento físico aeróbico (TFA) por 8 semanas em ambiente quente (Q) e temperado (T) nos isotipos (2A, 2X e 2B) da miosina de cadeia pesada (do inglês, Myosin heavy chain; MyHC) fast marcadas em ensaio de imunofluorescência. As imagens representativas de cortes transversais do músculo triceps sural (A) ilustram a proporção do tipo de fibra e a área de secção transversa (AST) expressos como Média ± EPM no histograma (B e C, respectivamente). @,* P ≤ *0,05* vs. *SED/T; γ,* P = *0,05 – 0,09* vs. *SED/T. SED/T, grupo de animais sedentário mantidos em ambiente T. TFA/T, grupo de animais submetido ao TFA em ambiente T. SED/Q, grupo de animais SED mantidos em ambiente Q. TFA/H, grupo de animais submetido ao TFA em ambiente Q (N = 5 - 8/grupo).*

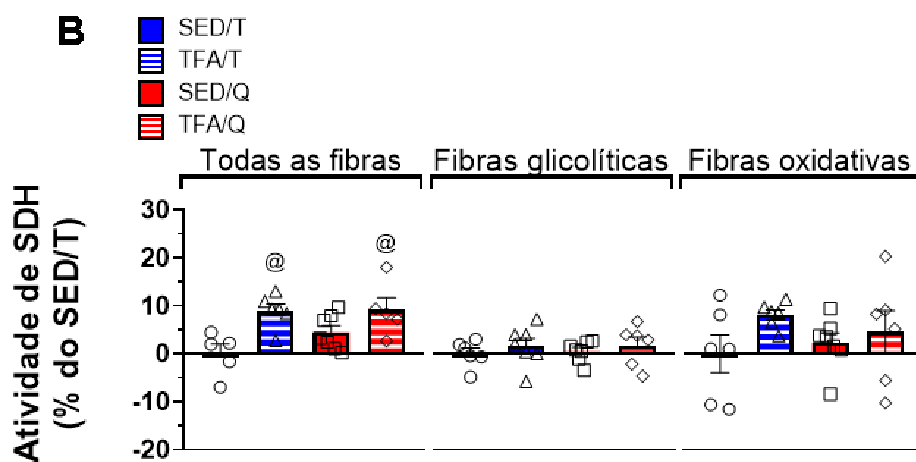


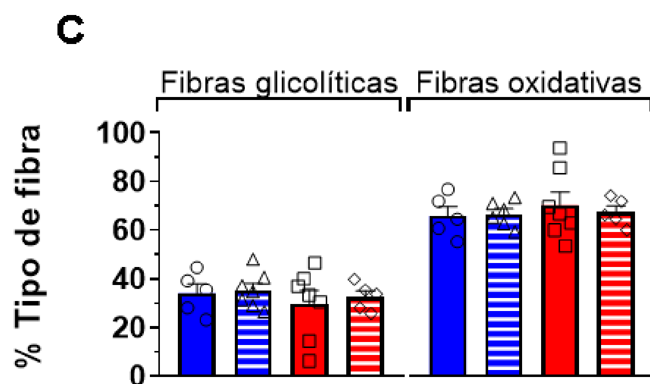
4.5 Efeitos do treinamento físico aeróbico em ambiente quente na atividade e conteúdo de enzimas mitocondriais

Além do tipo de fibra determinado pelo conteúdo das MyHC, o fenótipo muscular depende de diversas características celulares. Assim, caracterizamos ainda mais a identidade do tipo de fibra estimando a atividade metabólica oxidativa em cortes histológicos transversais corados para a atividade da enzima mitocondrial SDH. Como esperado, a atividade de SDH em todas as fibras, sem distingui-las, aumentou em cerca de 9% em ambos grupos TFA/T e TFA/Q (Figuras 11A e B). Entretanto, não observamos mais esse tipo de alteração quando analisamos a atividade de SDH em fibras pobres em mitocôndrias com predomínio do metabolismo glicolítico (fibras pálidas) e as fibras ricas em mitocôndrias com predomínio do metabolismo oxidativo (fibras escuras; Figuras 11A e C). Da mesma forma, a proporção de fibras oxidativas e glicolíticas não foi afetada por nenhuma condição (Figuras 11A e C). Como esperado, a análise da AST pela marcação da atividade da SDH confirmou os resultados obtidos na imunofluorescência para isoformas de MyHC, ou seja, as fibras oxidativas (15%) e a média de todas as fibras (~14%, $P = 0{,}065$) do grupo SED/Q apresentaram uma maior AST do que SED/T, (Figuras 11A e D). Os grupos TFA/T e TFA/Q não apresentaram qualquer alteração na AST. Em contraste com a atividade do SDH, a avaliação por Western blot do conteúdo dos cinco complexos da OxPhos, que inclui: Complexo I, NADH desidrogenase (NDUFB8), Complexo II, SDH (SDHB), Complexo III, oxidoredutase da ubiquinona-citocromo c (UQCRC2), Complexo IV, citocromo c oxidase I (MTCO1); Complexo V, adenosina trifosfato sintase ($ATP_{sintase}$; ATP5a); revelou que o TFA e o ambiente Q não foram capazes de alterar o conteúdo dessas proteínas mitocondriais (Figuras 12A - F). Além disso, nenhuma condição alterou o conteúdo da mitocondrial quantificado pela proteína de membrana mitocondrial externa (TOM20; Figura 12G). Assim, nossos dados sugerem que o TFA, tanto em T quanto em Q, induz aumento na atividade das mitocôndrias do músculo esquelético, estimada pela marcação da atividade da SDH em cortes histológicos, sem alterar o conteúdo da organela mitocondrial (TOM20) dos complexos da OxPhos.

Figura 10. Efeito do treinamento físico aeróbico (TFA) por 8 semanas em ambiente quente (Q) e temperado (T) na capacidade oxidativa do músculo esquelético mensurada por marcação histológica da atividade da enzima succinato desidrogenase (SDH). As imagens representativas de cortes transversais do músculo triceps sural (A) ilustram a atividade de SDH, proporção do tipo de fibra e a área de secção transversa (AST) expressos como Média ± EPM no histograma (B, C e D, respectivamente). @, $P \leq 0{,}05$ *vs.* SED/T; γ, $P = 0{,}05 - 0{,}09$ *vs.* SED/T. SED/T, grupo de animais sedentário mantidos em ambiente T. TFA/T, grupo de animais submetido ao TFA em ambiente T. SED/Q, grupo de animais SED mantidos em ambiente Q. TFA/H, grupo de animais submetido ao TFA em ambiente Q (N = 5 - 8/grupo).


A
SED/T
TFA/T
SED/Q
TFA/Q


B
SED/T
TFA/T
SED/Q
TFA/Q
Todas as fibras
Fibras glicolíticas
Fibras oxidativas
Atividade de SDH (% do SED/T)
30
20
10
0
-10
-20
@
@


C
Fibras glicolíticas
Fibras oxidativas
% Tipo de fibra
100
80
60
40
20
0

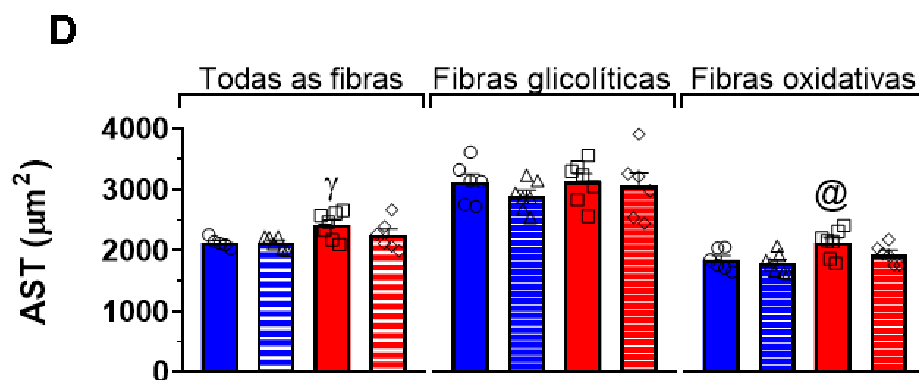
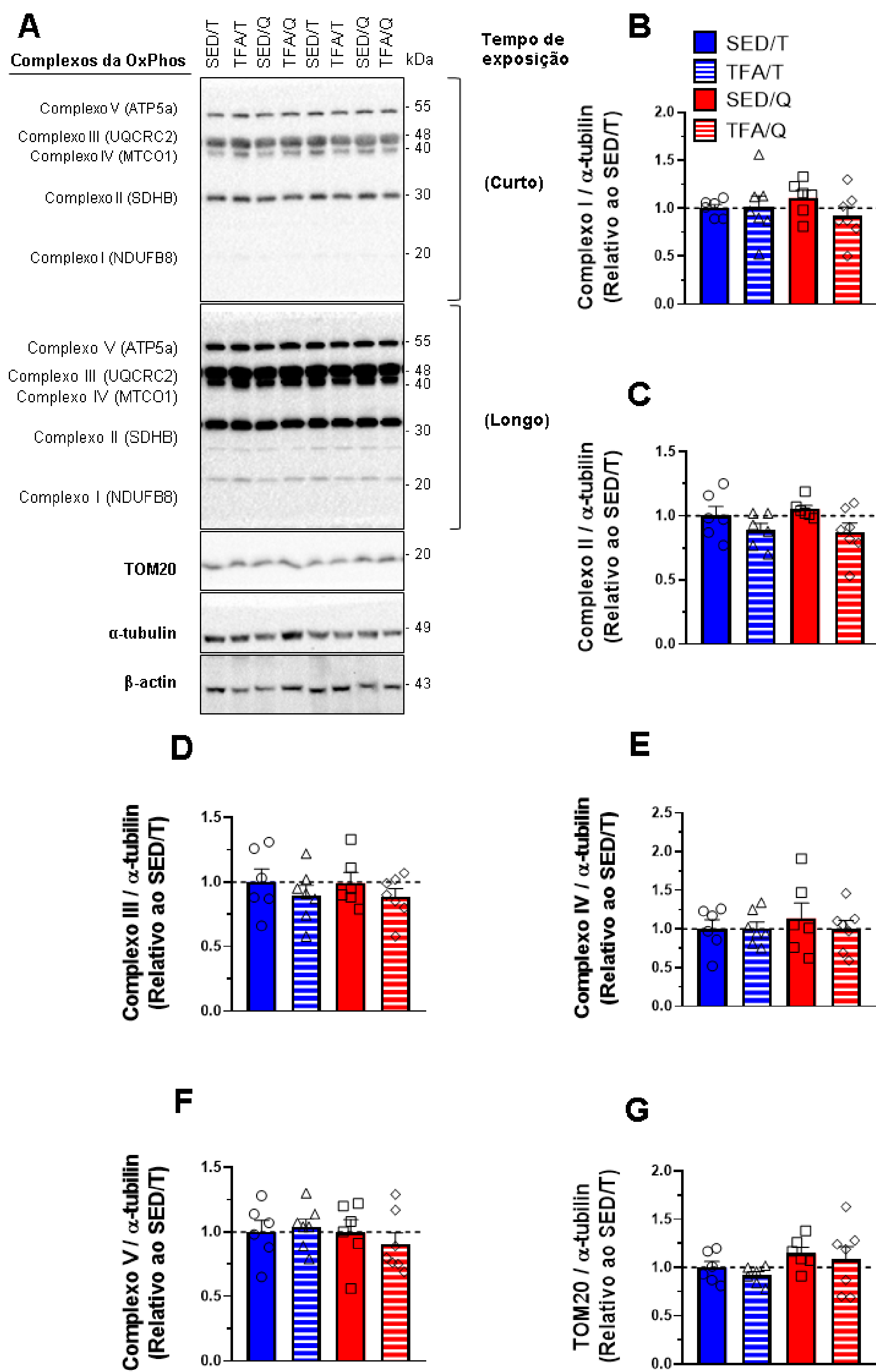

D
Todas as fibras
Fibras glicolíticas
Fibras oxidativas
AST (μm²)
4000
3000
2000
1000
0
γ
@

Figura 11. Efeito do treinamento físico aeróbico (TFA) por 8 semanas em ambiente quente (Q) e temperado (T) no conteúdo dos complexos (I – V) da cadeia transportadora de elétrons (OxPhos) e no conteúdo mitocondrial. As bandas (blots) representativas (A) ilustram os valores expressos como Média ± EPM nos histogramas (B - G). A α-tubulin foi usada como controle da quantidade de proteína aplicada para a eletroforese. SED/T, grupo de animais sedentário mantidos em ambiente T. TFA/T, grupo de animais submetido ao TFA em ambiente T. SED/Q, grupo de animais SED mantidos em ambiente Q. TFA/H, grupo de animais submetido ao TFA em ambiente Q (N = 6 - 7/grupo).

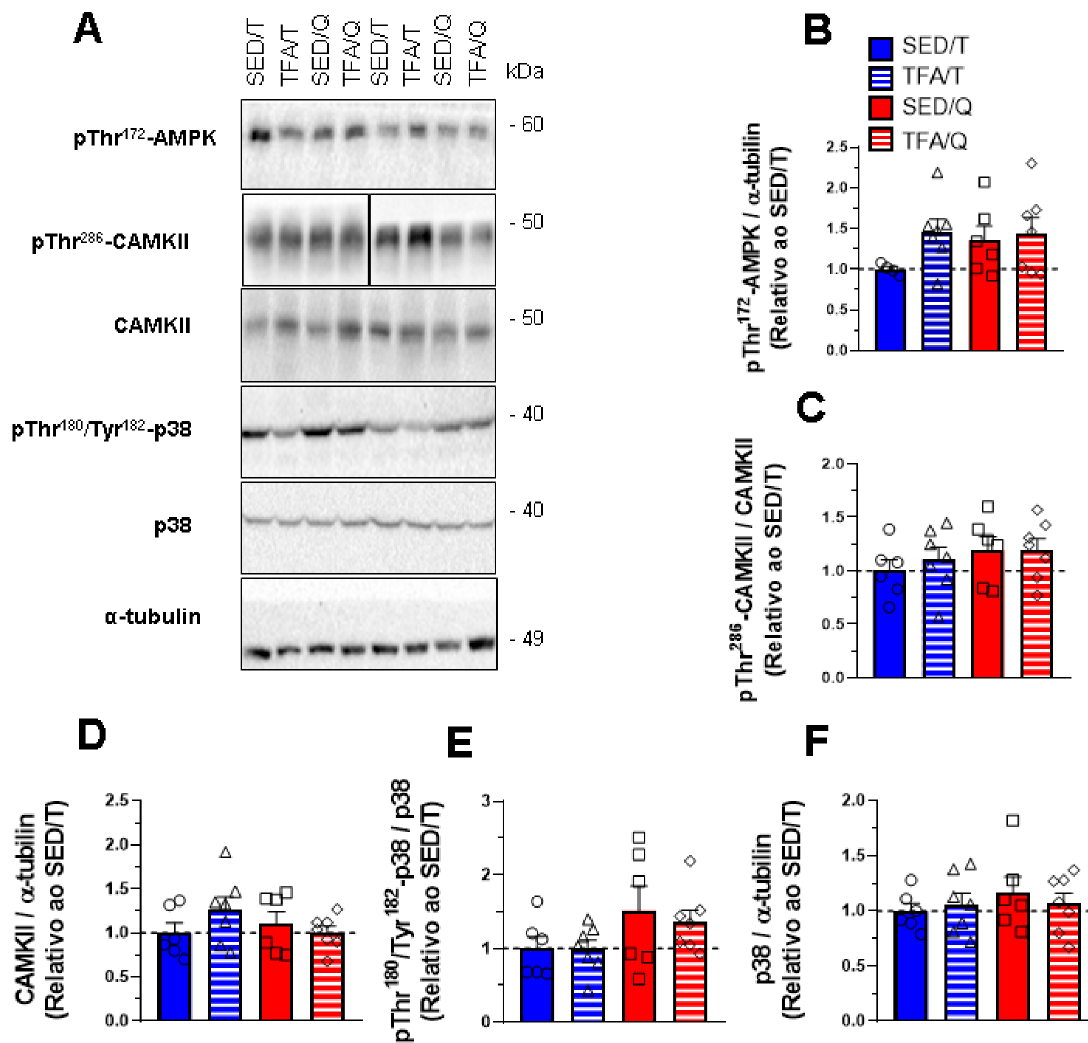


A
Complexos da OxPhos
SED/T
TFA/T
SED/Q
TFA/Q
kDa
Tempo de exposição
Complexo V (ATP5a)
Complexo III (UQCRC2)
Complexo IV (MTCO1)
Complexo II (SDHB)
Complexo I (NDUFB8)
(Curto)
(Longo)
TOM20
α-tubulin
β-actin
B
C
D
E
F
G
Complexo I / α-tubilin (Relativo ao SED/T)
Complexo II / α-tubilin (Relativo ao SED/T)
Complexo III / α-tubilin (Relativo ao SED/T)
Complexo IV / α-tubilin (Relativo ao SED/T)
Complexo V / α-tubilin (Relativo ao SED/T)
TOM20 / α-tubilin (Relativo ao SED/T)

4.6 Efeitos do treinamento físico aeróbico em ambiente quente nas vias de sinalização que regulam fenótipo muscular oxidativo

O fenótipo de fibras musculares oxidativas pode ser determinado por diferentes vias de sinalização intracelular, dentre elas as vias mediadas pelas proteínas cinases AMPK, CAMKII e p38 têm mostrado envolvimento no controle de diversos processos adaptativos em resposta ao TFA (Akimoto *et al.*, 2005; Bergeron *et al.*, 2001; Raney; Turcotte, 2008). As figuras 13A - F mostram que o conteúdo e o estado de fosforilação de AMPK, CAMKII e p38 não diferiu entre os grupos experimentais. Trabalhos mais recentes têm atribuído também um papel importante no controle do fenótipo oxidativo para as proteínas cinases Akt e ERK1/2 (Boyer *et al.*, 2019; Jaiswal *et al.*, 2022). Contudo, conteúdo dessas proteínas não foi alterado por nenhuma condição (Figuras 14A - E). Embora essas sinalizações não apresentem alterações no momento estudado (i.e., 2 dias após a última sessão de exercício) em nossas condições experimentais, é possível que elas tenham sido ativadas durante as sessões de exercício físico e isso tenha desencadeado a estimulação de fatores ou coativadores transcricionais envolvidos com o fenótipo oxidativo.

Figura 12. Efeito do treinamento físico aeróbico (TFA) por 8 semanas em ambiente quente (Q) e temperado (T) no conteúdo e estado de fosforilação de AMPK, CAMKII e p38. As bandas (blots) representativas (A) ilustram os valores expressos como Média ± EPM nos histogramas (B - F). A α-tubulin foi usada como controle da quantidade de proteína aplicada para a eletroforese. SED/T, grupo de animais sedentário mantidos em ambiente T. TFA/T, grupo de animais submetido ao TFA em ambiente T. SED/Q, grupo de animais SED mantidos em ambiente Q. TFA/H, grupo de animais submetido ao TFA em ambiente Q (N = 6 - 7/grupo).



Os alvos controladores da transcrição avaliados foramPGC1α e TFEB. Da mesma forma que as sinalizações, nenhuma mudança significativa nos níveis de PGC-1α e TFEB foi detectada em qualquer condição experimental (Figuras 15A e C). Para além dessas proteínas, FoxO1 e FoxO3 são fatores chave da homeostase energética muscular. As figuras 15A e I mostram que o conteúdo total de FoxO1 diminuiu (~32%) apenas em resposta ao TFA/Q em comparação ao SED/T. O estado de fosforilação de FoxO1 [em resíduos treonina (Thr^{24}) regulado por Akt] e o conteúdo total e estado de fosforilação de FoxO3 (em resíduos de treonina (Thr^{32}) regulado por Akt e resíduos de serina (Ser^{294} e Ser^{413}) regulados por ERK1/2 e AMPK) não apresentaram alterações significativas entre os grupos experimentais (Figuras 15A, D - H). Nossos resultados indicam que 8 semanas de TFA em ambiente Q promovem redução crônica no conteúdo FoxO1, mas não alteram os níveis basais de outras

proteínas envolvidas no fenótipo muscular oxidativo como PGC1a, TFEB e FoxO3.

Figura 13. Efeito do treinamento físico aeróbico (TFA) por 8 semanas em ambiente quente (Q) e temperado (T) no conteúdo e estado de fosforilação de Akt e ERK1/2. As bandas (*blots*) representativas (A) ilustram os valores expressos como Média ± EPM no histograma (B - E). A α-tubulin foi usada como controle da quantidade de proteína aplicada para a eletroforese. SED/T, grupo de animais sedentário mantidos em ambiente T. TFA/T, grupo de animais submetido ao TFA em ambiente T. SED/Q, grupo de animais SED mantidos em ambiente Q. TFA/H, grupo de animais submetido ao TFA em ambiente Q (N = 6 - 7/grupo).

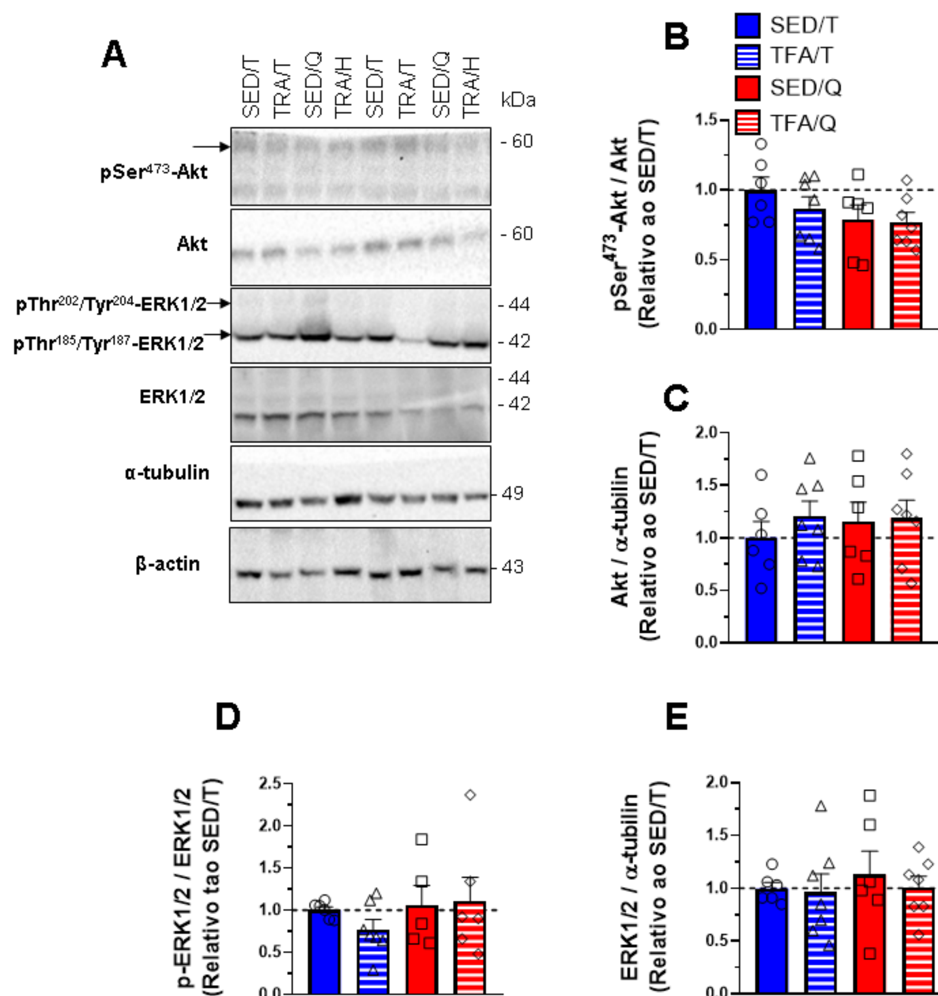
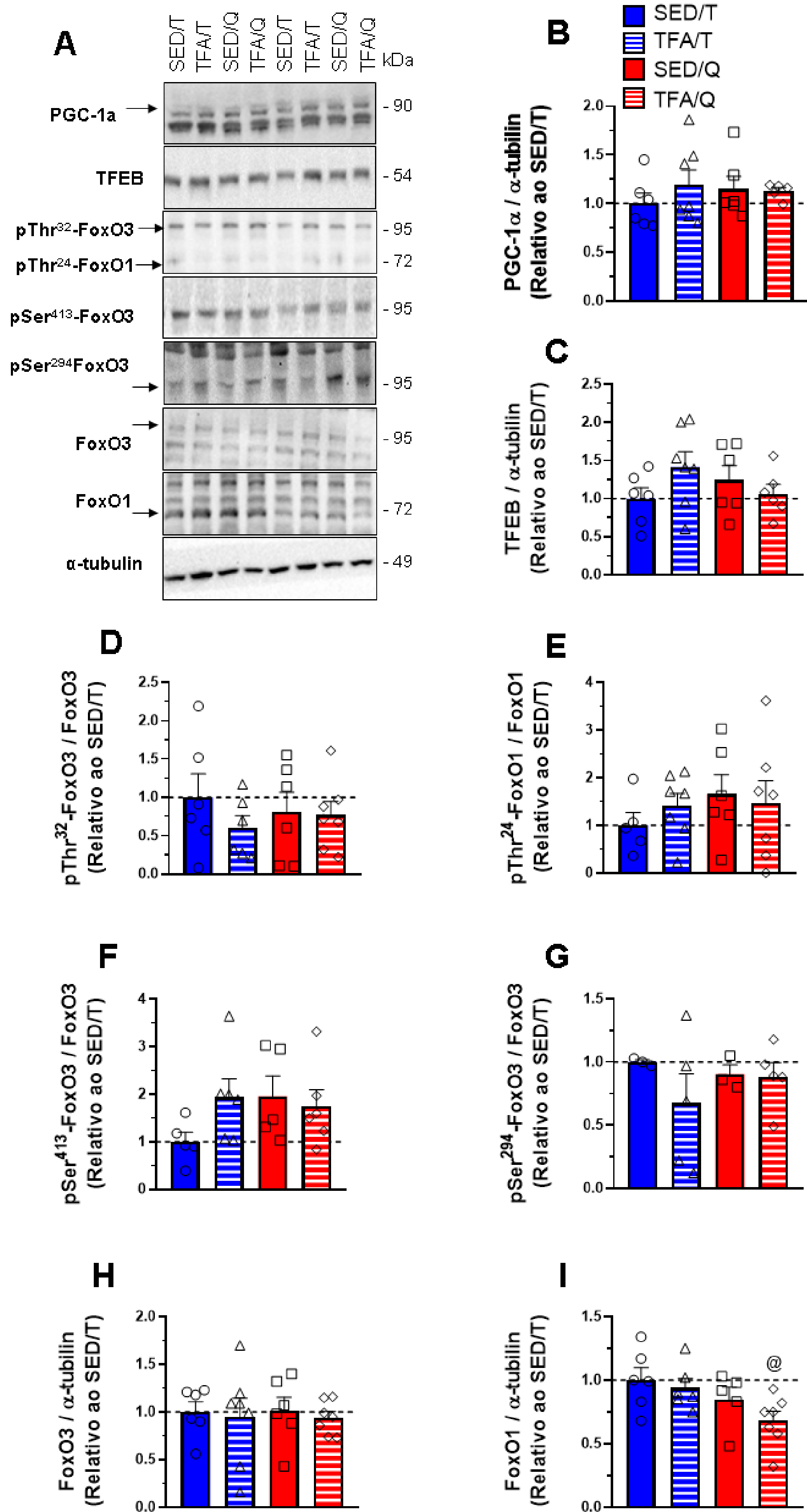


Figura 14. Efeito do treinamento físico aeróbico (TFA) por 8 semanas em ambiente quente (Q) e temperado (T) no conteúdo de PGC-1α e TFEB e conteúdo e estado de fosforilação de FoxO1 e FoxO3. As bandas (*blots*) representativas (A) ilustram os valores expressos como Média ± EPM nos histogramas (B - I). A α-tubulin foi usada como controle da quantidade de proteína aplicada para a eletroforese. @, $P \leq 0,05$ *vs.* SED/T. SED/T, grupo de animais sedentário mantidos em ambiente T. TFA/T, grupo de animais submetido ao TFA em ambiente T. SED/Q, grupo de animais SED mantidos em ambiente Q. TFA/H, grupo de animais submetido ao TFA em ambiente Q (N = 6 - 7/grupo).


A
SED/T
TFA/T
SED/Q
TFA/Q
kDa
PGC-1a
TFEB
pThr32-FoxO3
pThr24-FoxO1
pSer413-FoxO3
pSer294FoxO3
FoxO3
FoxO1
α-tubulin
B
C
D
E
F
G
H
I
PGC-1α / α-tubilin (Relativo ao SED/T)
TFEB / α-tubilin (Relativo ao SED/T)
pThr32-FoxO3 / FoxO3 (Relativo ao SED/T)
pThr24-FoxO1 / FoxO1 (Relativo ao SED/T)
pSer413-FoxO3 / FoxO3 (Relativo ao SED/T)
pSer294-FoxO3 / FoxO3 (Relativo ao SED/T)
FoxO3 / α-tubilin (Relativo ao SED/T)
FoxO1 / α-tubilin (Relativo ao SED/T)
@

Discussão

5 DISCUSSÃO

Embora seja bem conhecido que a AAC é capaz de mitigar os efeitos deletérios da exposição ao Q na resistência aeróbica, até o presente momento nenhum estudo havia avaliado o efeito do TFA em Q nas adaptações musculares esqueléticas e nas vias de sinalização intracelular, como PGC1α, TFEB, FoxO e seus reguladores, responsáveis por essas possíveis alterações fenotípicas. Apesar do ambiente Q reduzir significativamente a intensidade absoluta do exercício (i.e., velocidade de corrida) durante o TFA, as melhorias na resistência aeróbica dos animais treinados nessa condição foram semelhantes àquelas do grupo treinado em ambiente T com intensidade absoluta mais elevada. Esse efeito no desempenho de ambos os grupos treinados foi acompanhado de uma mudança no fenótipo das fibras musculares para um perfil rápido oxidativo-glicolítico, caracterizado pelo aumento na proporção de fibras do tipo 2A e na atividade da enzima relacionada ao metabolismo oxidativo, a SDH.

5.1 Efeito do ambiente quente no desempenho físico aeróbico

Inicialmente, observamos no TCI que a exposição aguda ao ambiente Q piorou a resistência aeróbica, levando a redução do tempo até a fadiga e da distância percorrida. Os nossos dados estão de acordo com a literatura em estudos com humanos e roedores (Galloway; Maughan, 1997; Guy *et al.*, 2015; Parkin *et al.*, 1999; Wanner *et al.*, 2014). Isso provavelmente ocorre porque o ambiente Q atua como um agente estressor adicional ao exercício físico, aumentando ainda mais a demanda do sistema cardiovascular (Périard et al., 2016). Essa resposta cardiovascular "exagerada" ocorre na tentativa de manter o fluxo sanguíneo para os tecidos ativos (i.e., musculo esquelético) e ainda redistribuir parte do débito cardíaco para a periferia do corpo, em especial a pele, a fim de facilitar a troca de calor com o ambiente. Assim, condições de altas T_a, a redistribuição do debito cardíaco pode ser inadequada, limitando a perda de calor e levando a um aumento exacerbado da T_c. Apesar de alguns achados na literatura questionarem sobre a existência de um valor critico de T_c responsável pela fadiga (Ely, Brett R. et al., 2009; Nybo, 2012), sabe-se que ocorre uma progressiva

inibição de áreas cerebrais responsáveis pela ativação motora à medida que a T_c e temperatura cerebral aumentam (Nybo; Nielsen, 2001; Thomas et al., 2006), o que poderia prejudicar a resistência aeróbica. Em camundongos, Wanner et al. (2014) reportaram que o aumento da T_a aumenta a magnitude da hipertermia induzida pelo exercício físico. De forma semelhante, observamos em estudos prévios (dados não publicados, apêndice 1) que o ambiente H foi capaz de aumentar a $T_{ABDmáx}$ no TCI em comparação ao ambiente T.

O VO_{2max} é um fator importante para resistência aeróbica (Bassett; Howley, 2000) e depende da capacidade máxima integrada dos sistemas respiratório, cardiovascular e musculoesquelético para captar, transportar e utilizar O_2, respectivamente. Por isso, González-Alonso & Calbet (2003) avaliaram o VO_{2max} durante o exercício físico em cicloergômetro (80% do pico de potência) em ambiente Q e observaram uma redução nesse parâmetro. Segundo esses autores, a redução do VO_{2max} no ambiente Q pode ser explicada pela sobrecarga no sistema cardiovascular que limita o fluxo sanguíneo muscular e, consequente, a entrega de O_2 para os músculos ativos. Em desacordo, em nosso estudo prévio (dados não publicados, apêndice 1) não observamos redução do VO_{2pico} em ambiente Q em comparação à avaliação ambiente T. essas diferenças devem ser explicas por diferenças entre os protocolos utilizados e/ou por diferenças na termorregulação entre as espécies.

Por outro lado, apesar de ser bem conhecido que a ACC promove adaptações fisiológicas importantes beneficiando a resistência aeróbica (Périard *et al.*, 2011; Sawka, Michael N. *et al.*, 2011), não fomos capazes de encontrar na literatura qualquer estudo investigando os efeitos fisiológicos envolvidos no processo adaptativo do TFA em ambiente Q por um longo período (i.e., superior a 6 semanas). Dessa forma, desenvolvemos em nosso estudo anterior (dados não publicados, apêndice 1) um modelo com camundongos submetidos ao protocolo de TFA por 8 semanas em ambiente Q e observamos que esses animais, juntamente com o grupo TFA em ambiente T, aumentaram o tempo até a fadiga e por consequência a distância percorrida após 4 semanas, essas alterações se mantiveram até a 8ª semana de TFA. A melhora semelhante de desempenho aeróbico em ambos os grupos TFA é surpreendente, já que o primeiro TCI em Q (i.e., pré-TFA) resultou em uma redução de 30% no desempenho físico do grupo

TFA/T comparado ao grupo TFA/Q. Portanto, embora a *intensidade relativa* tenha sido igual para os dois grupos submetidos ao TFA (i.e., 60% da $V_{máx}$ do TCI no respectivo ambiente de treinamento), a *intensidade absoluta*, ou seja,_a velocidade de corrida (m/min), foi 30% menor para o grupo TFA/Q em comparação ao grupo TFA/T. O desafio cardiovascular imposto aos indivíduos submetidos ao TFA em Q pode explicar essa dissociação entre intensidade absoluta e relativa. Arngrímsson et al. (2002) demonstraram que o aumento da frequência cardíaca observado durante o exercício físico submáximo no H está parcialmente relacionado a uma maior utilização da fração do $VO_{2máx}$ (%VO_{2max}) em menor intensidade. Embora não tenhamos observado uma redução do VO_{2pico}, o ambiente Q pode ter aumentado o %VO_{2pico} utilizado para uma mesma intensidade absoluta em comparação ambiente T. Com isso, apesar do exercício físico praticado no ambiente Q reduzir a intensidade absoluta de treino prescrita para TFA/Q, tal combinação também deve aumentar o %VO_{2peak} correspondente a prescrição. Treinar sob essas condições, pode fornecer um caminho alternativo para realizar exercício em alta intensidade relativa a um baixo custo mecânico.

O aumento da demanda fisiológica induzida pelo ambiente Q tem ressaltado sua utilização não só como um meio para promover AAC nos indivíduos, mas também como potencial ferramenta para induzir aprimoramentos exacerbados (i.e., efeito ergogênico) do desempenho físico aeróbico em T. Todavia, em especial no que diz respeito ao estado de treinamento, existem controvérsias sobre os benefícios do TFA em ambiente Q para a resistência aeróbica em T mensurada pelo $VO_{2máx}$. Está bem consolidado que a ACC aumenta o $VO_{2máx}$ de indivíduos não treinados durante avaliação em T (Takeno; Kamijo; Nose, 2001; Young *et al.*, 1985), contudo, os achados em indivíduos treinados são ambíguos. Lorenzo et al. (2010) relataram melhora do desempenho físico aeróbio (ciclismo) e do $VO_{2máx}$ avaliados em ambiente T (13 ºC) em atletas de elite submetidos ao protocolo de AAC (50%$VO_{2máx}$ a 40 ºC por 10 dias). Vale destacar que, segundo esses autores, a intensidade prescrita nesse protocolo não levaria a aumento de $VO_{2máx}$ dado o nível de treinamento dos voluntários. Por outro lado, Mikkelsen et al. (2019) observaram que o ET em ambiente H (40 ºC) por 5 ½ semanas ciclistas de sub-elite promoveu AAC, marcada pela redução da FC ao final da sessão, mas não promoveu aprimoramentos adicionais no $VO_{2máx}$ durante avaliação em

ambiente T (14 ºC). Entretanto, os pesquisadores não prescreveram o protocolo de TFA e orientaram aos atletas que realizassem o treino habitual, mas no ambiente Q. Sugerindo diferenças individuais na intensidade de TFA de cada atleta. Dessa forma, não há consenso na literatura sobre uma prescrição adequada e/ou controlada do TFA em ambiente Q. Nesse sentido, fomos os primeiros a observar que a TFA em Q é realizada em moderada intensidade relativa e menor intensidade absoluta, mas mesmo assim promove melhorias semelhantes no VO_{2pico} (dados não publicados, apêndice 1) e desempenho aeróbico, quando comparado ao ambiente T.

Portanto, os resultados dessa parte do trabalho sugerem que o TFA em Q é capaz de reduzir a intensidade absoluta de treino, mas promover as mesmas adaptações na resistência aeróbica em comparação ao TFA em ambiente T. Assim, indivíduos (i.e., atletas lesionados e indivíduos com sobrepeso ou obesidade) que por alguma razão não podem se exercitar em altas intensidade absolutas devido ao grande estresse mecânico poderiam se beneficiar da manipulação da T_a que, aparentemente, apresenta um papel importante no controle das respostas fisiológicas. Além disso, mesmo para indivíduos hígidos, o ambiente Q poderia ser considerado como uma variável no programa de TFA, dado seu papel não só na AAC, mas também na promoção do desempenho físico aeróbico com estresse mecânico reduzido.

5.2 Adaptações no tecido musculoesquelético e hepático induzidas pelo treinamento físico aeróbico em ambiente quente

A fim de entender as adaptações metabólicas que poderiam explicar a melhora da resistência aeróbica, avaliarmos o conteúdo de glicogênio muscular e hepático. Isso porque carboidratos são um dos principais substratos energéticos utilizados durante o exercício físico de resistência aeróbica (Van Loon *et al.*, 2001) e seu esgotamento é uma das principais causas da fadiga (Xirouchaki *et al.*, 2016). Em desacordo com a literatura (Bergström; Hultman, 1966), não observamos aumento no conteúdo de glicogênio muscular e, ao contrário, observamos aumentos no conteúdo de glicogênio hepático em todos os grupos.

Quanto a isso, é conhecido que a mobilização do glicogênio hepático contribui para o atendimento da demanda aumentada de glicose do músculo esquelético em contração (Petersen; Price; Bergeron, 2004; Wasserman, D. H. *et al.*, 1989). Em estudo realizado com camundongos noucute (*knockout*, KO) para glicogênio sintase (enzima responsável pela síntese do glicogênio), os autores observaram uma redução de 95% no conteúdo de glicogênio hepático acompanhada por uma redução da resistência aeróbica durante teste de corrida em esteira até a exaustão (Irimia *et al.*, 2010). Dessa forma, propomos que o TFA aumenta o conteúdo de glicogênio no fígado, o que pode contribuir para uma melhora na resistência aeróbica.

Considerando os efeitos promovidos pelo TFA em ambiente Q para a resistência aeróbica e a importância da capacidade oxidativa do músculo esquelético para o $VO_{2máx}$ (Korzeniewski; Zoladz, 2001), avaliamos as adaptações desse tecido em nosso modelo. O estudo de Kodesh e colaboradores (2010) foi o único que avaliou as adaptações do tecido muscular em resposta ao TFA realizado em ambiente Q por médio a longo prazo (i.e., 30 dias) e observou um aumento da massa e força muscular. Contudo, de forma surpreendente, apesar dos autores terem empregado um protocolo de TFA, os efeitos sobre o metabolismo aeróbico/oxidativo não foram avaliados neste estudo. Assim, fomos os primeiros a descrever os efeitos do TFA em ambiente Q sobre aspectos morfológicos e celulares do tecido musculoesquelético e a demonstrar que essa intervenção foi capaz de induzir um fenótipo rápido oxidativo-glicolítico muscular ao aumentar a proporção de fibras do tipo 2A, avaliadas pela marcação por imunofluorescência das diferentes MyHC. Os músculos dos animais do TFA em ambiente T exercitados em maior intensidade absoluta apresentaram a mesma adaptação. Embora não existam estudos que avaliaram as adaptações fenotípicas do músculo esquelético em reposta ao TFA em ambiente Q, o TFA em ambiente T é conhecido por promover a capacidade oxidativa de todos os tipos de fibras, em especial das fibras do tipo 2A (Howald et al., 1985). Aagaard et al. (2011) observaram uma melhora da resistência aeróbica, mensurada por um teste de contra relógio de 45 min (ciclismo), acompanhado por aumento na proporção das fibras do tipo 2A em biopsias do músculo vasto lateral. Destaca-se que ao comparar as propriedades contráteis e metabólicas das fibras musculares,

revelou que as fibras do tipo 2A são aquelas com menor velocidade de encurtamento (lentas) (Bottinelli; Schiaffino; Reggiani, 1991) e maior atividade e conteúdo mitocondrial avaliados pela enzima SDH (Bloemberg; Quadrilatero, 2012), por consequência, apresentam menor fatigabilidade se comparadas as fibras do tipo 2X (Bottinelli *et al.*, 1999). Esses fatores poderiam explicar a contribuição das fibras do tipo 2A para o aumento da resistência aeróbica. Além disso, a literatura tem apresentado correlação positiva entre o conteúdo de mitocôndrias musculares e o $VO_{2máx}$ (Hoppeler et al., 1973).

De fato, Sjöström et al. (1982), mensuraram a densidade mitocondrial intermiofibrilar pré e pós-TFA de 6 semanas nas fibras do músculo vasto lateral. Os autores observaram que as fibras do tipo 2A apresentam maior intervalo de aumento do volume mitocondrial entre o valor pré e pós-TFA (3,6 – 4,5%) se comparada as do tipo 1 (5,2 – 5,5%). Essas alterações mitocondriais foram acompanhadas por um aumento de 26% no $VO_{2máx}$. Em concordância, os nossos achados indicam que o aumento na proporção das fibras do tipo 2A foi acompanhado por aumento na atividade da enzima mitocondrial SDH. Contudo, o conteúdo proteico dos complexos da OxPhos, incluindo o complexo II que se refere à SDH, e da proteína TOM20, um marcador de conteúdo mitocondrial, não foram alterados em qualquer condição estudada. Isso sugere que a melhora da resistência aeróbica induzida pelo TFA nos ambientes Q e T foi resultado de um aumento da proporção de fibras rápidas oxidativas-glicolíticas do tipo 2A e da atividade mitocondrial, mas não de sua quantidade.

Para além das adaptações musculares do TFA associado ao estresse térmico Q, Horowitz et al. (1986) submeteram ratos ao protocolo de 3 semanas de ACC passiva (32 ºC, em repouso e ao longo de 2 meses) e observaram mudança na distribuição dos tipos de fibra do músculo cardíaco para um fenótipo mais lento, caracterizado pelo aumento da isoforma da enzima (isoenzima) ATP_{ase} lenta (V_3) em comparação a isoenzima rápida (V_1). Tais mudanças podem desempenhar um papel importante na adaptação do músculo cardíaco ao estresse térmico Q, pois, segundo os autores, das três isoenzimas avaliadas, a V_3 é a ATP_{ase} mais eficiente em termos de metabolismo energético o que garante a mesma atividade a um menor custo metabólico. Assim, o aumento na eficiência do músculo deve contribuir para a AAC, pois os efeitos deletérios da exposição aguda ao ambiente

Q estão relacionados ao aumento da intensidade relativa, conforme já discutido. No presente trabalho, observamos que ambos grupos TFA apresentaram aumento na proporção de fibras do tipo 2A e aumento da resistência fadiga no ambiente Q, um indicativo de AAC. Ressalta-se o grupo T, que mesmo sem o aumento da T_a apresentou equivalente AAC. Quanto a isso, um importante mecanismo de AAC é o aumento da atividade da proteína de choque térmico (Hsp) que respondem a diversas situações de estresse, incluído o exercício físico (Henstridge; Febbraio; Hargreaves, 2018). Ao serem ativadas, as Hsp atuam na proteção celular contra apoptose e protegem proteínas à desnaturação por estresse térmico quente (função chaperona). O'Neill et al. (O'neill *et al.*, 2006) sugeriram que a transição para do tipo de fibra para um padrão oxidativo parece ser um processo crítico para induzir mudanças crônicas na expressão constitutiva da proteína de choque térmico Hsp70, um membro da família das Hsp. Desse modo, o aumento da capacidade oxidativa do musculo esquelético, além de melhorar o desempenho físico aeróbico, pode ter contribuído para o processo de ACC.

Em vista da ausência de estudos que poderiam explicar a regulação molecular da alteração do fenótipo muscular, experimentos in vitro indicaram que a repetida exposição ao estresse térmico Q pode levar a um aumento na atividade mitocondrial, no conteúdo de MyHC do tipo 1 (Patton *et al.*, 2018) e na mudança do tipo de fibra para um padrão lento, atribuído à ativação de PGC-1α (Yamaguchi *et al.*, 2010). No entanto, em nosso estudo, o TFA em ambos os ambientes T e Q, não aumentou o conteúdo de PGC-1α e, tão pouco, o de TFEB. Dessa forma, como fizemos uma avaliação do conteúdo de PGC-1α, optamos por avaliar também as proteínas AMPK, CAMKII e MAPK p38, reconhecidas pela regulação na atividade de PGC-1α (Finck; Kelly, 2006). No entanto, não observamos alterações nos níveis dessas proteínas 48 horas após a última sessão de TFA. Vale ressaltar que o presente estudo se propôs a investigar os efeitos do TFA de 8 semanas nas adaptações crônicas dessas vias moleculares. Com isso, o período de descanso entre a última sessão e o momento da eutanásia, pode ter ocultado a participação de vias oscilatórias nas adaptações permanentes (i.e., tipo de fibra). Portanto, até o momento, o papel da mudança

de tipo de fibra e do aumento da atividade mitocondrial por meio da sinalização de quinases e PGC-1α permanece incerto.

Uma possível explicação para isso é o potencial de TFEB em regular a biogênese mitocondrial de forma independente de PGC-1α (Mansueto *et al.*, 2017). Para entender mais especificamente o papel de TFEB para o metabolismo mitocondrial, Mansueto et al. (2017) analisaram músculos de animais transgênicos que hiperespesavam TBEB e animais KO. Os autores observaram que a ausência de TFEB causou uma intolerância ao exercício físico, devido um acúmulo de mitocôndrias disfuncionais que apresentam comprometimento na atividade do complexo II da OxPhos. Por outro lado, o aumento na expressão de TFEB aumentou biogênese mitocondrial, resultando na melhoria da atividade dos complexos da cadeia respiratória e aumentos a produção de ATP. Entanto, o TFA nos ambientes Q e T não foi capaz de aumentar o conteúdo de TFEB sugerindo que outras vias estejam envolvidas na regulação do fenótipo oxidativo do músculo esquelético.

Visto que a qualidade mitocondrial não é regulada apenas pela indução do processo de biogênese dessa organela, mas sim por um equilíbrio homeostático com processos de degradação, como a mitofagia, os fatores de transcrição de FoxO1 e FoxO3 têm sido apontados como proteínas chave no remodelamento mitocondrial (Cheng, 2022). Além disso, uma das funções importantes de FoxO1 e FoxO3 é a regulação da degradação proteica por meio de diferentes sistemas proteolíticos, incluindo o sistema ubiquitina-proteassoma (UbP) e a autofagia (MILAN *et al.*, 2015). Em relação ao sistema ubiquitina-proteassoma, estudos mostraram que a ativação de FoxO leva a um aumento na expressão de genes que codificam proteínas envolvidas na marcação de proteínas para a degradação (ubiquitinação), como a atrogin-1 e MuRF-1 (Mammucari *et al.*, 2007). Já o sistema autofágico é uma das principais vias de degradação de componentes celulares danificados atuando na remoção de mitocôndrias disfuncionais pelo processo conhecido como mitofagia (Ivankovic *et al.*, 2016; Sandri *et al.*, 2004). Contudo, a ativação exacerbada desses sistemas pode levar a perda de mitocôndrias e a atrofia muscular (Sartori; Romanello; Sandri, 2021). Camundongos transgênicos que hiperexpressavam FoxO1 no músculo esquelético apresentam uma redução significativa na massa muscular

esquelética em comparação com os camundongos controle (Kamei *et al.*, 2004). Além disso, segundo os autores os camundongos transgênicos apresentaram uma redução dos genes associados às fibras musculares do tipo 1 (i.e., troponin C), o que sugere uma alteração na composição das fibras musculares do tipo 1. É interessante observar que os músculos dos animais do grupo TFA/Q apresentaram redução nos níveis de FoxO1 que foi associada a um aumento da proporção de fibras rápidas oxidativas-glicolíticas e da atividade mitocondrial, sugerindo que a redução de FoxO1 poderia ter contribuído para a mudança do tipo de fibra rápidas oxidativas-glicolíticas do tipo 2A

Além do controle mitocondrial, a capacidade oxidativa muscular e o $\dot{V}O_{2pico}$ são determinados pela vascularização, cujo aumento otimiza a entrega de oxigênio e nutrientes para os tecidos e a extração de CO_2 e produtos do metabolismo a partir deles (Bassett; Howley, 2000; Saltin, 1998) e consequentemente aumentar a resistência a fadiga. Hesketh et al. (2019) relataram que a terapia de calor passiva em humanos sedentários, aumentou a capilarização da musculatura esquelética e aumentou o VO_{2pico}. Entendendo o importante papel da vascularização muscular para o desempenho, Slopack et al. (2014) investigaram os mecanismos moleculares que controlam essas adaptações. Esses autores relataram que o exercício físico aumenta agudamente os níveis musculares, em especial das células endoteliais vasculares, de FoxO1 e FoxO3 que, por sua vez, estimulam a transcrição da proteína angiostática trombospondina-1 (THBS1), impedindo a angiogênese nos primeiros dias de TFA. Contudo, após 10 dias, o TFA atenua os níveis de FoxO1 e reduz a sua localização nuclear nos vasos sanguíneos, o que também resulta na redução da expressão de THBS1. Neste mesmo estudo, os autores geraram animais com deleção gênica (KO) de FoxO1 e FoxO3 exclusivamente nas células endoteliais e reportaram que esses animais aceleravam a resposta angiogênica de 14 dias em animais selvagens para 7 dias em animais KO para FoxO1 e FoxO3. Portanto, a redução de FoxO1 que observamos nos músculos dos animais TFA/Q pode ter contribuído para angiogênese nesses animais mais precoce nesses animais, comparado com o grupo TFA/T. Estudos futuros devem testar essa hipótese.

Conclusão

6 CONCLUSÃO

A respeito dos efeitos induzidos pelo ambiente quente na resistência aeróbica, conclui-se que, de forma aguda, o ambiente quente reduziu a resistência aeróbica, indicada pela redução do tempo até a fadiga e distância percorrida durante teste de carga incremental. Confirmando nossas hipóteses, o treinamento físico aeróbico em ambiente em ambiente Q, embora executado em menor intensidade absoluta, promoveu o aumento da resistência aeróbica da mesma forma que o treinamento físico aeróbico em ambiente T. Além disso, tanto o treinamento físico aeróbico quanto a exposição ao ambiente Q aumentaram similarmente o conteúdo de glicogênio hepático, sem alterações no conteúdo de glicogênio muscular;

Na tentativa de identificar os mecanismos histológicos e moleculares, observamos que o treinamento físico aeróbico em ambos ambientes T e Q aumentou a proporção de fibras rápidas glicolíticas-oxidativas do tipo 2A, a atividade mitocondrial e apenas o treinamento físico aeróbico no ambiente Q reduziu o conteúdo total de FoxO1.

Portanto, o treinamento em ambiente Q apresenta-se como uma potencial ferramenta que contribui para a prescrição do treinamento físico aeróbico. Uma vez que, parece compensar a redução de intensidade absoluta e pode contribuir para a prescrição e exercícios físico para população geral, apresentando-se com mais um possível componente da carga de treino, mas também como uma estratégia para grupos que não podem se exercitar em altas intensidades ou para aqueles em processo de recuperação/retorno a pratica esportiva.

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Apêndice 1

1 **TITLE:** LONG-TERM HEAT ACCLIMATION TRAINING IN MICE: SIMILAR
2 METABOLIC AND RUNNING PERFORMANCE ADAPTATIONS DESPITE A
3 LOWER ABSOLUTE INTENSITY THAN TRAINING AT TEMPERATE
4 CONDITIONS
5
6 **RUNNING HEAD:** HEAT REDUCES TRAINING INTENSITY BUT PROMOTES
7 SIMILAR ADAPTATIONS OF TEMPERATE
8
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39 **CONFLICT OF INTEREST**
40 None.

ABSTRACT

This study investigated the impact of long-term heat acclimation (HA) training on mouse thermoregulation, metabolism, and running performance in temperate (T) and hot (H) environments. Male Swiss mice were divided into 1) Sedentary (SED) mice kept in T (22 ºC; SED/T), 2) Endurance Trained mice (ET, 1 hour / day, 5 days / week, 8 weeks, 60% of maximum speed) in T (ET/T), 3) SED kept in H (32 ºC; SED/H), and 4) ET in H (ET/H). All groups performed incremental load tests (ILT) in both environments before (pre-ET) and after four and eight weeks of ET. In the pre-ET period, H impaired (~30%) performance variables (maximum speed and external work) and increased (1.3 ºC) maximum abdominal body temperature compared with T. In T, after four weeks, although ET/H exercised at a lower (~30%) absolute intensity than ET/T, performance variables and aerobic power (peak oxygen uptake, $\dot{V}O_{2peak}$) were similarly increased in both ET groups compared with SED/T. After eight weeks, the external work was higher in both ET groups compared with SED/T. Only ET/T significantly increased $\dot{V}O_{2peak}$ (~11%) relative to its pre-ET period. In H, only after eight weeks, both ET groups improved (~19%) maximum speed and reduced (~46%) post-ILT blood lactate concentrations compared with their respective pre-ET values. Liver glycogen content increased (34%) in both ET groups and SED/H compared with SED/T. Thus, ET/H was performed at a lower absolute intensity but promoted similar effects to ET/T on metabolism, aerobic power, and running performance. Our findings open perspectives for applying HA training as part of a training program or orthopedic and metabolic rehabilitation programs in injured or even obese animals, reducing mechanical load with equivalent or higher physiological demand.

Keywords: Aerobic training; Heat stress; Peak oxygen consumption; Thermoregulation.

65 **ABBREVIATIONS**

66 BAT - Interscapular brown adipose tissue;

67 EDL - Extensor digitorum longus;

68 EPI – Epididymal white adipose tissue;

69 ET - Endurance training;

70 ET/H - Endurance-trained mice in a hot environment;

71 ET/T - Endurance-trained mice in a temperate environment;

72 H - Hot environment;

73 HA - Heat acclimation;

74 HR - Heart rate;

75 ILT - Incremental load test;

76 MES – Mesenteric white adipose tissue;

77 SED/H - Sedentary mice kept in a hot environment;

78 SED/T - Sedentary mice kept in a temperate environment;

79 RETRO – Retroperitoneal white adipose tissue;

80 SUB – Subcutaneous white adipose tissue;

81 T - Temperate environment

82 T_a - Ambient temperature;

83 TA - Tibialis anterior;

84 T_{core} - Core body temperature;

85 TS - Triceps surae;

86 $\dot{V}O_2$ - Oxygen uptake;

87 $\dot{V}O_{2max}$ - Maximal oxygen uptake;

88 $\dot{V}O_{2peak}$ - Peak oxygen uptake;

89 **1. INTRODUCTION**

90 Exercise training promotes a variety of health and performance benefits that depend upon
91 the intensity, frequency, duration, and type of exercise along with the progression (Egan
92 and Zierath, 2013; Haugen et al., 2022; Powell et al., 2011; Smith, 2003). It is well known
93 that the higher the exercise intensity, the greater the challenge to physiological
94 homeostasis and adaptations (Egan and Zierath, 2013). However, despite the benefits of
95 exercise training, more intense exercise, like a faster running pace, increases peak vertical
96 and horizontal forces, thereby leading to overload injuries in the lower limb (Boullosa et
97 al., 2020). To decrease injury, improve the adaptive response to endurance training (ET),
98 and potentially enhance endurance performance, coaches and athletes have searched for
99 strategies to maximize metabolic and physiological strain at a lower level of mechanical
100 stress. Still, only a few of them have been scientifically tested.
101 Prolonged exercise in a hot (H) environment [ambient temperature (T_a) ≥ 25 ºC] increases
102 skin and core body (T_{core}) temperatures and induces extreme disturbances to homeostasis,
103 such as increases in heart rate (HR) and cardiac output to adequately perfuse active
104 skeletal muscle to support metabolism while simultaneously perfusing the skin to sustain
105 heat loss (SAWKA *et al.*, 2011). Therefore, environmental heat stress and muscle
106 metabolic heat production can interact synergistically to increase T_{core}, accelerate fatigue,
107 and degrade endurance performance (Ely et al., 2007; Tatterson et al., 2000). In contrast,
108 the most critical intervention to alleviate heat strain and optimize endurance performance
109 is heat acclimation (HA) training, which is developed following exposure to an H
110 environment during exercise for 1-2 weeks (Pryor et al., 2019; Racinais et al., 2015).
111 These physiological adaptations to HA training include, for example, reduced oxygen
112 uptake ($\dot{V}O_2$) at a given exercise intensity, muscle glycogen sparing (SAWKA, *et al.*,
113 1983), reduced blood lactate at a given exercise intensity (Young et al., 1985), and

114 improved myocardial efficiency (i.e., a performance measure calculated as the ratio
115 between the mechanical energy generated by the ventricle and the consumed chemical
116 energy from aerobic metabolism) (HOROWITZ *et al.,* 1993) in an H environment. While
117 these effects are well-described, it is uncertain if HA training enhances physiological
118 response and endurance performance in cool-temperate conditions.

119 On one hand, McCleave *et al.* (2017) addressed this issue and showed that nine sessions
120 of interval training at 33 °C [~24 to 45 min running from 65 to 100% of speed associated
121 with peak oxygen uptake ($\dot{V}O_{2peak}$; aerobic power) at 33 ºC] for three weeks increased 3-
122 km time-trial performance in cool-temperate conditions (14 ºC) in well-trained runners,
123 but there were no correlations between changes in performance, running economy and
124 other physiological parameters. On the other hand, KEISER *et al.* (2015) evaluated the
125 effects of HA training (90 min at 50% of maximal oxygen uptake ($\dot{V}O_{2max}$) at 38 ºC) for
126 ten consecutive days in well-trained cyclists. They observed classical HA adaptations
127 such as increased sweat rate and improved $\dot{V}O_{2max}$ and time trial performance in the heat,
128 but not in cool-temperate conditions (18 ºC). The difference in findings from these two
129 studies may be, to some extent, related to the HA duration.

130 It is recommended that the intervention be for at least two weeks to maximize all benefits,
131 specifically the endurance improvement in the heat (Racinais et al., 2014). Because most
132 previous studies have performed HA training for short- and medium-term (≤ 2 weeks), it
133 is reasonable to speculate that more extended periods should be necessary to translate the
134 enhancement of endurance performance in the heat to the cool-temperate conditions.
135 Animal models could be valuable in evaluating long-term HA training protocols'
136 physiological and performance adaptations before applying them to humans. KODESH
137 *et al.* (2010) assessed global genomic responses of ‘homeostatic genes’ and isometric
138 force generation in rats training on a treadmill in an H environment (34 ºC) for 30 days.

139 However, the authors did not analyze endurance performance. To our knowledge, no
140 study evaluated the effects of long-term HA training in laboratory rodents on running
141 performance in either temperate (T) or H environment. Based on these gaps in the
142 literature, we investigated the impact of HA training for eight weeks on thermoregulation,
143 metabolism, and running performance in both T and H environments in mice. Our data
144 show that long-term HA training reduces blood lactate and increases liver glycogen,
145 aerobic power, and endurance performance similar to ET in a T environment but requires
146 a lower absolute intensity (i.e., running speed).

147 # 2. METHODS

148 ## 2.1.Animals

149 Swiss mice (8-week-old male mice, 40g), provided by *Centro de Bioterismo (CEBIO)* of
150 the *Universidade Federal de Minas Gerais*, were used in all experiments. Mice were
151 housed in the vivarium of the Exercise Physiology Laboratory (LAFISE) in collective
152 cages under controlled light (7:00 a.m. to 7:00 p.m.) and T_a (24.0 ± 2.0 ºC) conditions
153 with access to water and chow *ad libitum* for at least one week before the beginning of
154 the experiments. All experimental procedures were approved by the local Ethics
155 Commission on the Use of Animals (*Comissão de Ética no Uso de Animais* - CEUA;
156 protocol number 220/2019).

157 A sample size of 53 animals (n = 13-14/group) was reported by G*Power software,
158 version 3.1.9.4 (Erdfelder et al., 2009), and four experiments (n = 3-4/group/experiment)
159 were performed to achieve this sample size. In all experiments, mice were divided into
160 four experimental groups: 1) Sedentary mice (SED) kept on a treadmill turned off in a
161 temperate environment (T; 22 ºC) (SED/T) during the same time as the endurance training
162 (ET) groups, 2) ET mice exercised on treadmill in T (ET/T), 3) SED mice kept on
163 treadmill turned off in a hot environment (H; 32 ºC) (SED/H) during the same time as the
164 ET groups, and 4) ET mice in H (ET/H). Mice were counterbalanced among groups based
165 on running performance immediately after the pre-ET incremental load test (pre-ILT) in
166 both environments. After completing eight weeks of ET (or SED), animals were
167 euthanized by decapitation using a guillotine to collect blood. Additionally, muscles were
168 promptly weighed, frozen in liquid nitrogen, and stored at -80 °C for subsequent analysis.
169 All experiments were performed between 7 a.m. and 1 p.m. to minimize chronobiological
170 influences.

171

172 **2.2.Experimental design**

173 Fig. 1 shows a schematic representation of the experimental procedures to which SED
174 and ET mice were submitted.

175

176 **2.3.Measurements of core body temperature (T_{core}) and ambient temperature**
177 **(T_a)**

178 For the analysis of T_{core}, a temperature telemetry transmitter (G2 E-Mitter series, Mini
179 Mitter, Bend, OR) was implanted in the mouse peritoneal cavity to register abdominal
180 temperature (ABT), and the maximum value (ABT_{max}) obtained during ILT was taken.
181 ABT was recorded as an indicator of T_{core}. Before starting the surgery, mice were
182 anesthetized with ketamine (84 mg/kg body mass, i.p.) and xylazine (8 mg/kg body mass,
183 i.p.). A small incision was made in the linea alba of the abdominal muscle, and the
184 peritoneal cavity was exposed. The sensor was affixed to the left lateral abdominal wall
185 using sutures. Finally, the abdominal muscle and skin were sutured. After seven days of
186 recovery, mice started the familiarization with treadmill running exercise. As previously
187 reported (Steiner et al., 2007; Wanner et al., 2014), this recovery period was prescribed
188 as sufficiently long for mice to recover.

189 For the analysis of T_a, a thermocouple (YSI-400A, Yellow Springs Instrument, Yellow
190 Springs, OH) was placed inside an acrylic chamber that contained the treadmill. The
191 thermocouple was positioned on the ceiling halfway between the fan and the electrical
192 grid. The thermocouple was connected to a real-time temperature reader displaying T_a
193 values.

194

195 **2.4.Regulating ambient temperature (T_a)**

196 To heat the environment inside the acrylic chamber that contains the treadmill, an electric
197 heater (Britânia model AB 1100, Curitiba, Brazil) was positioned at ~40-45 cm from the
198 front of the treadmill and turned on at 1,200 W (Wanner et al., 2014). To stabilize the
199 temperature, the test room air conditioning (KOP4UQC, KOMECOTM, Palhoça, Brazil)
200 was set at 22 (T) or 32 ºC (H). These T_a values were chosen based on previous studies
201 showing that 31 – 32 °C enhances body hyperthermia and accelerates fatigue in exercising
202 rats compared with 23 – 24 °C (Ribeiro Hudson et al., 2020; Teixeira-Coelho et al., 2021;
203 Zaretsky et al., 2018).
204

205 **2.5.Familiarization with treadmill running**

206 The familiarization protocol was modified from WANNER *et al.* (2014). The
207 familiarization protocol consisted of running on a treadmill over five consecutive days.
208 During the first four days, the protocol consisted of three stages: i) the animals rested for
209 3 min with the treadmill turned off; ii) mice ran for 5 min, maintaining a pace of 5 $m.min^{-}$
210 ^{1} on day 1 and 6 $m.min^{-1}$ on days 2 to 4; iii) on days 1 and 2, mice ran for additional 3 min
211 at speeds of 6 and 8 $m.min^{-1}$, respectively, and, on days 3 and 4, they ran for additional 5
212 min at 8 $m.min^{-1}$. The treadmill slope was always set at 5º. On the last day (i.e., 5th day),
213 mice were familiarized with a "lighter version" of ILT. The criterion for test completion
214 was modified, and mice had to spend five continuous seconds in the designated fatigue
215 zone (i.e., the rear of the treadmill, ranging from approximately 'one body length' from
216 the shock grid to, and including, the shock grid) as proposed by (DOUGHERTY *et al.,*
217 2016).
218 Mice were encouraged to run by light electrical stimulation (0.4 mA) provided by a grid
219 at the rear end of the treadmill belt. T_a was controlled at 24 ± 1 ºC during the
220 familiarization protocol.

221

222 **2.6.Measurement of endurance (aerobic) performance by incremental load test**

223 Mice rested for 72 h after familiarization protocol. Then, they were submitted to a
224 modified ILT (Ayachi et al., 2016) in both T and H environments with two days of rest
225 in between. The treadmill was set up in a metabolic chamber, and tests were started with
226 a speed of 10 $m.min^{-1}$, with increments of 3 $m.min^{-1}$ every 3 min (2° slope). Exercise
227 sessions consisted of running to fatigue, defined as the mouse's inability to maintain
228 speed despite being in contact with the shock grid for more than five consecutive seconds
229 (Ayachi et al., 2016; Mille-Hamard et al., 2012). Endurance performance {i.e., maximal
230 speed, and external work [external work = Σ_{stages} (Body mass (kg) × Stage duration (min)
231 × Stage speed ($m.min^{-1}$) × treadmill grade sin], metabolic [i.e., $\dot{V}O_{2peak}$, and blood lactate
232 and glucose], and thermoregulatory parameters [i.e., abdominal temperature]} were
233 measured. The $\dot{V}O_{2peak}$ was taken as the highest $\dot{V}O_2$ value recorded
234 during the ILT. Blood samples from the tail vein were collected to determine glycaemia
235 and lactatemia 30 min before starting (pre-ILT) and immediately after (post-ILT) the test.

236

237 **2.7.Measurements of glycaemia and lactatemia**

238 Blood glucose was measured using the glucometer Accu-chek® Guide (Roche). For
239 analyzing lactate, a blood samples (30 µL each) from each mouse were collected in a
240 heparinized capillary tube and transferred to a tube containing 50 µL sodium fluoride
241 (1%). Blood lactate concentration was determined using an electroenzymatic method with
242 a lactate analyzer (YSI 1500 Sport Lactate Analyzer; Yellow Springs Instrument, Yellow
243 Springs, OH, USA).

244

245 **2.8.Endurance training (ET) protocol**

246 Mice rested for 48 h after ILT and then were submitted to ET protocol modified from
247 (Ferreira et al., 2007; Kodesh and Horowitz, 2010). ET protocol was similar for both
248 trained groups (ET). However, T_a was distinct, i.e., ET/T and ET/H performed exercise
249 at 22 and 32 ºC, respectively. ET consisted of eight weeks of running on a treadmill
250 (Panlab, Harvard Apparatus, Cornellà, Spain), 5 days / week, and intensity and duration
251 gradually increased over the weeks, as described below. In the first week, mice ran at
252 50% of the maximum speed (no slope; 0º) for 20 and 35 min in the first and second
253 sessions, respectively. In the second week, the duration of the first session was 45 min
254 and increased to 60 min in the next one; exercise intensity increased to 60% of the
255 maximum speed from the fourth session. From the third week of ET, running sessions
256 were performed at 60% of the maximum speed, 60 min/day, and 5° slope. An ILT was
257 performed after four weeks of ET to determine the effects of training on running
258 performance, especially on maximum speed, thereby adjusting training intensity.
259

260 **2.9. Quantification of hepatic and muscular glycogen**

261 Hepatic and muscular glycogen were measured using the Antrone method (CARROLL
262 et al., 1956). Firstly, liver (~500 mg) or skeletal muscle (~70 mg) tissues are homogenized
263 and treated with alcohol to extract glycogen. The extracted glycogen was then hydrolyzed
264 with hydrochloric acid. The anthrone reagent (containing 2 g anthrone in 100 ml
265 concentrated sulfuric acid) was added to the solution, producing glucose derived from
266 glycogen. The color intensity was measured spectrophotometrically, and a glucose
267 standard curve calculates the glycogen concentration in the sample. This method, utilizing
268 hydrochloric acid in the hydrolysis step, provides a quantitative assessment of glycogen
269 levels in tissues based on color development, with the intensity directly proportional to
270 the glycogen concentration.

271

272 **2.10. Statistical analysis**

273 For ET experiments, each datum was relativized by the mean value of the SED/T group
274 in the pre-ET period. Thus, data are reported as % of SED/T in pre-ET ± SEM. Normality
275 was assessed by the Shapiro-Wilk test. To compare the means values of two
276 environmental conditions (T *vs.* H environment in the pre-ET period), paired Student's t-
277 tests were used. Multiple comparisons between four groups at different time points were
278 performed using mixed-model two-way analysis of variance with repeated measures
279 followed by post hoc Tukey's test. The significance criterion was set at $P<0.05$. Statistical
280 analysis was performed using Prism – GraphPad 8 software.
281

282 **3. RESULTS**

283 **3.1. Acute exposure to the H environment impairs endurance performance**
284 **without affecting $\dot{V}O_{2peak}$ and glucose metabolism**

285 ILT was performed in all mice in both T and H conditions to determine endurance
286 performance and counterbalance experimental groups before starting ET. As expected,
287 the H environment caused an increase of 1.3 ºC in ABT_{max} and a reduction in endurance
288 performance as determined by lower maximum speed (27%) and external work (34%)
289 when compared with T (Fig. 2A - C). Irrespective of T_a, ILT induced a continuous
290 increase in $\dot{V}O_2$ during exercise to fatigue (Fig. 3A). $\dot{V}O_{2peak}$ was similar in T and H
291 environments (Fig. 3B). Blood glucose concentrations were unaffected by H conditions
292 and exercise in ILT (Fig. 3C and D). Pre- and post-ILT blood lactate values were similar
293 between T and H environments (Fig. 3F). Post-ILT blood lactate values were elevated
294 (65%) compared with pre-ILT values in both T and H. Still, H did not cause any additional
295 effect (Fig. 3E). These findings indicate that acute exposure to H increased ABT_{max} and
296 reduced endurance performance. For this reason, absolute running speed ($m.min^{-1}$)
297 corresponding to 60% of the maximum speed was 20 and 40% lower in ET/H group than
298 in ET/T at weeks zero to four (i.e., 0 – 4 wk) and four to eight weeks (i.e., 4 – 8 wk),
299 respectively (18 $m.min^{-1}$ in ET/H *versus* 23 $m.min^{-1}$ in ET/T at weeks 0 – 4 and 20 $m.min^{-1}$
300 in ET/H *versus* 28 $m.min^{-1}$ in ET/T at weeks 4 – 8).

301

302 **3.2. ET/T and ET/H alters body composition**

303 Fig. 4A shows that from the 3rd to the 8th week, the ET/H group exhibited a food intake
304 57% higher than the SED/H group at the same time. Additionally, in the 4th week, the
305 food consumption of the ET/H group was 21% higher compared with the SED/T group.
306 In the 8th week, the food consumption of the SED/T and SED/H groups showed a

307 reduction (25% and 66%, respectively) compared with their respective values in the Pre-
308 ET week. In contrast, body mass gain tended to reduce (62.5%, $P = 0.065$) between weeks
309 4 and 8 in ET/H compared with SED/H (Fig. 4B). Intra-group analysis in ET/T revealed
310 that body mass gain during this period (4 - 8 wk) was significantly lower (45%) than
311 during the first four weeks (i.e., 0 - 4 wk) (Fig. 4B). Body mass gain was unaltered among
312 groups between weeks 0 and 4 and analyzing the whole period (i.e., 0 - 8 wk) (Fig. 4B).
313 Despite these data, the relative muscle masses of extensor digitorum longus (EDL; 11%)
314 and soleus (21%) were increased in ET/T compared with SED/T (Table 1). The relative
315 muscle masses of EDL (9%) and gastrocnemius (8%) were increased, and triceps surae
316 (TS; 7%, $P = 0.078$) tended to increase in SED/H compared with SED/T. The relative
317 muscle mass of the soleus (16%) and gastrocnemius (6%) was also significantly increased
318 in ET/H compared with SED/T. There is no difference among groups in the relative
319 masses of interscapular brown adipose tissue (BAT) and the and the epididymal (EPI),
320 retroperitoneal (RETRO), and mesenteric (MES) white adipose tissues (Table 1), as well
321 as the adiposity index (Oliveira et al., 2013) (24.67, 18.39, 24.89, and 22.31% in SED/T,
322 ET/T, SED/H, and ET/H, respectively). The relative mass of the adrenal gland, a stress-
323 responsive organ, was increased in ET/T (33%) and tended to increase in ET/H (14%, P
324 $= 0.080$) compared with SED/T, but it was unaffected in SED/H. The relative masses of
325 tibialis anterior (TA), plantaris muscle, heart, and liver did not alter in any condition.
326

327 **3.3.ET/T and ET/H similarly improve endurance performance in a T_a-**
328 **independent manner**

329 In the T environment, ABT_{max} (40.9, 39.8, 39.4, and 39.9 ºC in SED/T, ET/T, SED/H,
330 and ET/H, respectively) and endurance performance variables [i.e., maximum speed
331 (35.9, 36.3, 38.1, and 35.0 $m.min^{-1}$ in SED/T, ET/T, SED/H, and ET/H, respectively) and

332 external work (8.9, 7.9, 8.8, and 8.1 J in SED/T, ET/T, SED/H, and ET/H, respectively)]
333 obtained in ILT were similar among groups in the pre-ET period (Fig. 5A - C). At week
334 4, both the ET/T and ET/H groups exhibited elevated values in maximum speed (30%
335 and 19% of SED/T at pre-ET in ET/T and ET/H, respectively) and external work (83%
336 and 49% of SED/T at pre-ET in ET/T and ET/H, respectively) (Fig. 5B – C). Similarly,
337 by week 8, both ET/T and ET/H groups demonstrated increased maximum speed (25%
338 and 19% of SED/T at pre-ET in ET/T and ET/H, respectively) and external work (73%
339 and 63% of SED/T at pre-ET in ET/T and ET/H, respectively) (Fig. 5B – C). In some
340 instances, both ET/T and ET/H displayed higher values in both maximum speed groups
341 compared with their respective SED groups at the same time point (i.e., ET/T versus
342 SED/T and ET/H versus SED/H) (Fig. 5B and C). ABT_{max} was unaffected in any group
343 (Fig. 5A).
344 In the H environment, Fig. 6A - C shows that ABT_{max} (41.8, 41.4, 41.9, and 41.5 ^{o}C in
345 SED/T, ET/T, SED/H, and ET/H, respectively), maximum speed (30.3, 35.6, 34.1, and
346 30.3 m.min^{-1} in SED/T, ET/T, SED/H, and ET/H, respectively), and external work (6.3,
347 4.0, 7.1, and 5.9 J in SED/T, ET/T, SED/H, and ET/H, respectively) were also similar
348 among groups in the pre-ET period. Both ET/T and ET/H groups improved maximum
349 speed (26 and 22% of SED/T at pre-ET in ET/T in ET/T and ET/H) at week 8 compared
350 with their pre-ET period (i.e., week 0) (Fig. 6B). At week 8, the external work was
351 significantly higher (59% of SED/T at pre-ET) only in ET/H than in its pre-ET period
352 (Fig. 6C). In contrast, only ET/T showed significantly higher values of the maximum
353 speed (18% of SED/T at pre-ET) and external work (41% of SED/T at pre-ET) at week 4
354 compared with its pre-ET period (Fig. 6B and C). Again, ABT_{max} did not change in any
355 group (Fig. 6A). Together, these findings indicate that ET/T and ET/H for eight weeks
356 similarly improve running performance in a T_a-independent manner.

357

358 **3.4.ET/T and ET/H improve aerobic power and change glycaemia and**
359 **lactatemia during incremental load tests in a T_a-dependent manner**

360 In the T environment, aerobic power [i.e., $\dot{V}O_{2peak}$ (52.2, 49.0, 53.8, and 59.3 ml/min.kg^{-}
361 $^{0.75}$ in SED/T, ET/T, SED/H, and ET/H, respectively)] and delta values (post- minus pre-
362 ILT) of blood glucose (11.7, 10.8, -8.1, and -16.4 mmol/L in SED/T, ET/T, SED/H, and
363 ET/H, respectively) and lactate (8.0, 4.9, 6.4, and 4.7 mmol/L in SED/T, ET/T, SED/H,
364 and ET/H, respectively) concentrations were similar among groups in pre-ET period (Fig.
365 7A, D, and E, respectively). Both ET/T and ET/H groups had higher values (14 and 13%
366 of SED/T at pre-ET in ET/T and ET/H, respectively) of $\dot{V}O_{2peak}$ at week 4 compared with
367 SED/T at the same time-point and, for ET/T, with its pre-ET period (Fig. 7A). At week
368 8, $\dot{V}O_{2peak}$ was significantly higher (11% of SED/T at pre-ET) only in ET/T than in its
369 pre-ET period (Fig. 7A). No significant change was observed in post-ILT values of
370 glycaemia in any time point (Fig. 7B). However, delta values of blood glucose in ET/T at
371 week 4 were lower (105%) compared with its pre-ET period (Fig. 7D). At the same
372 period, delta values of blood glucose in ET/H were also lower (133%) compared with
373 SED/H (Fig. 7D). At week 8, delta values of blood glucose tended to be lower (27%,
374 *P*=0.094) only in ET/T, with no significant effects on other groups (Fig. 7D). At week 4,
375 post-ILT and delta values of blood lactate in ET/T were lower (46 and 72%, respectively)
376 compared with SED/T at pre-ET period (Fig. 7C and E). Post-ILT and delta values of
377 blood lactate were unaltered in the other groups at any point in time (Fig. 7B and D).

378 In the H environment, similar to the T environment, $\dot{V}O_{2peak}$ (50.5, 46.4, 47.7, and 47.4
379 ml/min.kg$^{-0.75}$ in SED/T, ET/T, SED/H, and ET/H, respectively) and delta values of blood
380 glucose (-13.5, -9.4, -4.3, and -15.0 mmol/L in SED/T, ET/T, SED/H, and ET/H,
381 respectively) and lactate (5.3, 5.0, 4.9, and 5.1 mmol/L in SED/T, ET/T, SED/H, and

382 ET/H, respectively) obtained in ILT were similar among groups in the pre-ET period (Fig.
383 8A, D, and E, respectively). No changes in $\dot{V}O_{2peak}$ (Fig. 8A) and pre-, post-ILT, and delta
384 values of glycaemia (Fig. 8B and C, respectively) were observed in any ET group. Post-
385 ILT concentrations of lactate in ET/T (47%) and ET/H (32%) groups at week 8 were
386 significantly lower compared with their values at the pre-ET period (Fig. 8C). Delta
387 values of lactatemia were unaffected in any group and time point (Fig. 8E). Altogether,
388 these results show that ET/T and ET/H improve $\dot{V}O_{2peak}$ and reduce post-ILT glycaemia
389 in T environment. Still, post-ILT lactatemia is decreased only in the H environment,
390 indicating that some metabolic effects depend on T_a.
391

392 **3.5.ET/T and ET/H increase liver glycogen storage**

393 To understand whether liver and muscle glycogen could explain the adaptive changes in
394 glycaemia and lactatemia in ET groups in response to ILT, glycogen content in these
395 organs was evaluated. Liver glycogen content was increased by 39, 45, and 58% in ET/T,
396 SED/H, and ET/H groups, respectively, compared with SED/T (Fig. 9A). On the other
397 hand, glycogen in gastrocnemius muscle was unaffected in any group (Fig. 9B). These
398 data indicate that chronic heat exposure *per se* and ET in any T_a promote glycogen
399 accumulation in the liver, but not in skeletal muscle. Moreover, lower glycaemia and
400 lactatemia in ET groups in response to ILT are unrelated to reductions in glycogen content
401 in the liver and muscle.

402 ## 4. DISCUSSION

403 To the best of our knowledge, this is the first study to systematically investigate the effect
404 of long-term HA training on thermoregulation, metabolism, and endurance performance
405 in mice and the possibility of translating its adaptive effects to the T environment. Our
406 main findings show that HA training (i.e., ET/H) for eight weeks reduces blood lactate
407 and increases liver glycogen, aerobic power, and endurance performance similar to ET/T
408 but requires a lower absolute intensity (running speed) of training.

409 It has been extensively shown that acute exposure to an H environment has an ergolytic
410 effect on endurance performance (Ely et al., 2007; Guy et al., 2015; Macaluso et al., 2011;
411 Maia-Lima et al., 2017). GUY *et al.* (2015) have shown that marathon running exhibited
412 the most considerable performance impairment in the heat, with a mean reduction of ~3%
413 for males and females. Similar findings were reported in a mouse model of exercise in
414 the heat. The maximum speed and time to fatigue were markedly decreased in mice
415 running at 34 ºC compared with those at 24 ºC (Wanner et al., 2014). In agreement, our
416 data show that the H environment acutely impaired endurance performance during ILT
417 in SED mice, as indicated by reduced maximum speed and external work.

418 The H environment acts as an additional stressor to exercise and may exacerbate the
419 physiological demand for physical activity. In fact, ABT_{max} in mice running in the H
420 environment was 1.2 ºC higher than in the T environment. WANNER *et al.* (2014)
421 reported similar findings in a different incremental exercise protocol in mice, i.e., ABT_{max}
422 in mice running at 34 °C was 1.3 °C higher than at 24 °C. These findings confirm the
423 prominent role played by T_a in determining T_{core} levels attained at fatigue in small rodents
424 (Andrade et al., 2023).

425 Another physiological response that has been related to degraded endurance performance
426 in humans performing an ILT in H environment [$T_a > 35$ ºC, 50% relative humidity (RH)]

427 is reduced $\dot{V}O_{2max}$ (Arngrímsson et al., 2004). Severe heat stress has been shown to
428 diminish $\dot{V}O_{2max}$ by accelerating the reductions in cardiac output and mean arterial blood
429 pressure that lead to impairments in muscle blood flow, O_2 delivery, and O_2 uptake
430 (González-alonso and Calbet, 2003). These alterations could also lead to lower aerobic
431 metabolic rates and higher muscle and plasma lactate, mainly produced by anaerobic
432 glycolysis, as described in healthy men cycling at submaximal intensity (70% of $\dot{V}O_{2max}$)
433 in H environment [49 ºC, 20% relative humidity (RH)] compared with the cool
434 environment (21 ºC, 30% RH) (Young et al., 1985). However, despite the ergolytic action
435 in our study, the H environment did not affect either $\dot{V}O_{2peak}$ or blood lactate
436 concentrations in mice performing an ILT, suggesting that endurance performance in
437 mice may be impaired in the heat independently of changes in such metabolic parameters.
438 These discrepancies may be explained by the differences in exercise protocols, T_a, and/or
439 the interspecies differences in thermoregulation and metabolism.
440 On the other hand, repeated exposure to exercise in the H environment, i.e., HA training,
441 mitigates the ergolytic action of heat stress and enhances endurance performance in the
442 heat (Périard et al., 2011; Sawka et al., 2011). LORENZO *et al.* (2010) showed that
443 medium-term HA training (10 days, ~50% $\dot{V}O_{2max}$ at 40 °C) increased power output at
444 the lactate threshold and improved time-trial performance in H environment (38 ºC) in
445 trained cyclists. As expected, our data show that ET/H for eight weeks increased the
446 endurance-related parameters maximum speed and external work during ILT in H
447 environment. Unfortunately, we cannot compare our findings with others in the literature
448 because no study investigated the effects of the HA training on these parameters in mice.
449 Moreover, the studies in rats (Horowitz, et al., 1993; Kodesh and Horowitz, 2010) have
450 not determined the efficacy of their HA protocols in improving endurance performance
451 in the H environment.

452 Interestingly, ET/T exhibited similar outcomes to ET/H in endurance-related parameters
453 in the heat, but the effects occurred earlier in ET/T (i.e., maximum speed and external
454 work already increased in the 4th week). These findings in mice strengthen the idea of
455 RACINAIS *et al.* (2015) that ET/T may elicit partial HA in humans. In agreement, a
456 previous study demonstrated that young women who performed a high-intensity interval
457 training in a 'cool' environment (22 ºC, named here as the T environment) for a total of
458 40 h (spread over 11 weeks; 30-60 min/day) showed improved endurance performance
459 (i.e., time to fatigue in walking test at 30–35% $\dot{V}O_{2max}$) in the heat (45 ºC) (Cohen and
460 Gisolfi, 1982). Similarly, rats trained for eight weeks at 23 ºC also had better tolerance to
461 an ILT at 32 ºC than control rats (Teixeira-Coelho et al., 2021). HA-like adaptation in
462 ET/T is expected because the augmented metabolic rate in the working muscles during
463 each exercise bout increases heat production and, consequently, T_{core} (Sawka et al., 2011;
464 Wanner et al., 2015). Indeed, ABT_{max} was about 40 ºC in our mice during ILT in the T
465 environment. Despite the improvement in ILT's performance in the H environment in
466 both ET/T or ET/H groups, no change was observed in $\dot{V}O_{2peak}$. It is reasonable to
467 speculate that severe hyperthermia during ILT in the T environment could induce blood
468 flow redistribution from skeletal muscles to thermoregulatory effectors, such as the tail
469 vasculature (Gordon, 2017; Wanner et al., 2015), compromising gains in $\dot{V}O_{2peak}$.
470 Intriguingly, blood lactate concentrations were lower post-ILT in the H environment in
471 both ET/T and ET/H groups at week 8 compared with respective groups in the pre-ET
472 period, which may be due to 1) lower motivation of mice to achieve their maximal effort,
473 2) lower pre-exercise (basal) concentrations of muscle glycogen, or 3) higher oxidative
474 muscle phenotype. Our findings show that both ET/T and ET/H presented a higher
475 maximum speed and external work at week 8, suggesting that the first possibility could
476 be ruled out. Regarding muscle glycogen, our data show that its content in resting mice

477 was unaffected in ET/T and ET/H. Moreover, it has been demonstrated that ET/T can
478 spare muscle glycogen content in humans during exercise in the heat (Young et al., 1985).
479 Although it is unknown whether the same adaptation occurs in mice, these pieces of
480 evidence suggest that the second possibility could also be ruled out. Finally, lower blood
481 lactate concentrations in both ET/T and ET/H might be explained by increased
482 mitochondrial enzyme activity in skeletal muscle, lowering the dependence on glucose
483 metabolism and the appearance of lactate and maximizing the use of lipid metabolism
484 and the disposal of lactate, as observed in endurance athletes (Brooks, 2018; Furrer et
485 al., 2023). Although this hypothesis may explain the lower blood lactate concentrations
486 in ET/T post-ILT in the H environment, no study has adequately investigated the effects
487 of long-term HA training on skeletal muscle mitochondrial adaptations. KODESH *et al.*
488 (2010) have added evidence about muscle oxidative adaptation, showing that ET/H (34
489 ºC) for 30 days in rats on a treadmill up-regulated the muscle expression of the gene
490 related to mitochondrial lipid oxidation, mitochondrial carnitine O-palmitoyltransferase
491 II precursor (*CPT2*), when compared with ET/T (24 ºC). Further experiments are
492 underway to evaluate whether and how long-term HA training may promote the oxidative
493 muscle phenotype in mice.
494 The most exciting finding is that the ET/H group had a similar improvement to ET/T in
495 aerobic power and endurance performance in ILT in the T environment. These data are
496 very impressive because the ET/H group performed each exercise bout at 20 to 40% lower
497 absolute intensity (running speed) than ET/T, causing a lower level of mechanical stress
498 but a similar adaptive enhancement in maximum speed and external work during ILT in
499 the T environment. Similarly, LORENZO and coworkers (2010) showed that medium-
500 term HA training at 40 °C in an exercise intensity insufficient to induce training
501 adaptations for highly trained cyclists, i.e., ~50% of $\dot{V}O_{2max}$, improved time-trial

502 performance in a cool (13 ºC) environment. In agreement, PHILP *et al.* (2022) observed
503 that HA training prescribed via absolute intensity in the heat (1 h/day, rowing or cycling
504 at 45-55% of average power output at 34 ºC) for 10 days caused a large (Cohen's d effect
505 size) non-significant decrease in the rate of perceived exertion, a large significant
506 decrease in HR_{peak}, and a large non-significant increase in power output in performance
507 tests in T condition in national-level rowers. According to the authors, these signs of HA
508 could potentially improve short-duration (4-min) rowing time-trial performance in the T
509 condition while minimizing additional mechanical stress to the athletes.
510 In partial contrast, MIKKELSEN *et al.* (2019) have shown that sub-elite cyclists
511 submitted to long-term HA training (28 ± 2 sessions lasting 1 h at 60% of $\dot{V}O_{2max}$ at 40
512 ºC for 5 weeks and a half) have some classical HA adaptations, e.g., reduced steady-state
513 HR and improved submaximal exercise endurance in the heat, but no additional effects
514 on mean power output and time trial performance in a cool condition (14 ºC), when
515 compared with the control group. Our findings in running mice also show that the effects
516 of ET/H on metabolism and performance are not superior to those of ET/T. However, any
517 strategy that elicits similar improvements of ET/T in physiology and performance with
518 lower mechanical stress may be advantageous as part of a training program. For example,
519 mdx mice, an animal model for Duchenne Muscular Dystrophy that lacks dystrophin
520 protein and produces a mechanically fragile sarcolemma, submitted to a single 30-min
521 treadmill exercise session at 'moderate to high' intensity (i.e., 12 $m.min^{-1}$) had increased
522 markers of muscle damage such as serum CK level, oxidative stress and myofiber
523 necrosis (Radley-Crabb et al., 2012). However, mdx mice subjected to ET for 6 months
524 (30 min three times a week) at low to moderate intensity (i.e., 8 $m.min^{-1}$) improved tetanic
525 and specific force (Zelikovich et al., 2019). Considering these studies and our findings, it
526 is tempting to speculate that HA training could maximize metabolic and physiological

527 strain at a lower level of mechanical stress in mdx mice, improving the adaptive response
528 to ET with reduced muscle injury. This concept could also be applied to obese mice whose
529 myofiber membranes are more susceptible to mechanical damage in normal cage activity
530 or strenuous eccentric exercise (Knoblauch et al., 2013) and exhibit impaired running
531 performance in ILT (Petrosino et al., 2016). Further experiments are needed to confirm
532 the potential benefits of HA training in such conditions.

533 **5. CONCLUSION**

534 In summary, ET/H required a lower absolute intensity than ET/T to promote similar
535 improvements in metabolism, aerobic power, and running performance, representing an
536 effective alternative to reduce mechanical stress (load). Our findings open perspectives
537 for applying HA training as part of a training program or orthopedic and metabolic
538 rehabilitation programs in injured or obese animals.
539

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719

720 **LEGENDS**

721 **Fig. 1:** Schematic representation of the experimental procedures to which mice from SED
722 and ET were submitted. Seven days post-implantation of the telemetry transmitter,
723 animals were familiarized with running on a treadmill for five consecutive days. Three
724 days later, the first incremental load test (ILT) was performed in a 'crossover design' (i.e.,
725 animals from experiments 1 and 3 performed the test in a temperate environment (T; 22
726 ºC) and, two days later, in a hot environment (H; 32 ºC); animals from experiments 2 and
727 4 performed the tests in the opposite T_a order). Two days later, two blocks of 4-week
728 endurance training (ET) were performed. The second (middle) and third (last) ILTs were
729 performed at the beginning of weeks 5 and 9, respectively. Animals performed the last
730 three ET (or SED) sessions and were euthanized after two days. ET, Endurance training.
731 ILT, incremental load test.
732

733 **Fig. 2:** Acute effect of hot (H) environment on maximal abdominal body temperature
734 (ABT_{max}; A), maximum speed (B), and external work (C) during the incremental load test
735 (ILT) compared with temperate (T) environment in the pre-ET period. ϕ, $P \leq 0.05$ vs. T;
736 θ, $P \leq 0.05$ vs. same environment (n = 13-14/group).
737

738 **Fig. 3:** Acute effect of hot (H) environment on oxygen consumption ($\dot{V}O_2$; A), peak
739 oxygen uptake ($\dot{V}O_{2peak}$; B), pre- and post-incremental load test (ILT) (C) and post-minus
740 pre-ILT values (D) of blood glucose and pre- and post-ILT (E) and post- minus pre-ILT
741 values (F) of blood lactate compared with the temperate (T) environment in the pre-ET
742 period. Λ $P \leq 0.05$ vs. same-group during pre-ILT (n = 13-14/group).
743

744 **Fig. 4:** Effect of endurance training (ET) on the treadmill for eight weeks in temperate
745 (T) and hot (H) environments on food intake (A) and body mass gain (B). Food intake
746 was analyzed one week prior to ET (week -1) and during the whole period (8 wk). Daily
747 body mass gain is shown as mean values of the first four weeks (0 – 4 wk), the last four
748 weeks (4 – 8 wk) and the whole period (0 – 8 wk). μ $P \leq 0.05$ SED/T vs. same group pre-
749 ET; T $P \leq 0.05$ ET/H vs. SED/T same time-point; A $P \leq 0.05$ ET/H vs. SED/H same time-
750 point; μ $P \leq 0.05$ SED/T vs. same group -1 week; π $P \leq 0.05$ SED/H vs. same group -1
751 week. SED/T, sedentary in T. ET/T, endurance-trained in T. SED/H, sedentary in H.
752 ET/H, endurance-trained in H. 4 wk and 8 wk represent four and eight weeks of the
753 experimental protocol, respectively (n = 13-14/group).
754
755 **Fig. 5:** Effect of endurance training (ET) on the treadmill for eight weeks in temperate
756 (T) and hot (H) environments on maximal abdominal body temperature (ABT_{max}; A),
757 maximum speed (B), and external work (C) evaluated during the incremental load test
758 (ILT) in T environment. Data are expressed as % change of SED/T in pre-ET period (0
759 week). *, $P \leq 0.05$ vs. same group - pre-ET; @, $P \leq 0.05$ vs. SED/T same time-point; β,
760 $P \leq 0.05$ vs. SED/H same time-point; γ, P = 005 – 0.09 vs. SED/T same time-point; χ, P
761 = 0.05 – 0.09 vs. SED/H same time-point. SED/T, sedentary in T. ET/T, endurance-
762 trained in T. SED/H, sedentary in H. ET/H, endurance-trained in H. 4 wk and 8 wk
763 represent four and eight weeks of the experimental protocol, respectively (n = 13-
764 14/group).
765
766 **Fig. 6:** Effect of endurance training (ET) on the treadmill for eight weeks in temperate
767 (T) and hot (H) environments on maximal abdominal body temperature (ABT_{max}; A),
768 maximum speed (B), and external work (C) evaluated during the incremental load test

769 (ILT) in H environment. Data are expressed as % change of SED/T in pre-ET period (0
770 week). *, $P \leq 0.05$ vs. same group - pre-ET; γ, $P = 0.05 - 0.09$ vs. SED/T same time-point;
771 κ, $P \leq 0.05$ vs. SED/T – 4 wk. SED/T, sedentary in T. ET/T, endurance-trained in T.
772 SED/H, sedentary in H. ET/H, endurance-trained in H. 4 wk and 8 wk represent four and
773 eight weeks of the experimental protocol, respectively ($n = 13\text{-}14$/group).
774

775 **Fig. 7:** Effect of endurance training (ET) on the treadmill for eight weeks in temperate
776 (T) and hot (H) environments on peak oxygen uptake ($\dot{V}O_{2peak}$; A), the pre- and post-
777 incremental load test (ILT) and post- minus pre-ILT values [delta (Δ) values] of blood
778 glucose (B and D) and lactate (C and E) in T environment. Data are reported as mean ±
779 SEM. * $P \leq 0.05$ vs. same group - pre-ET; β $P \leq 0.05$ vs. SED/H same time-point; & $P =$
780 $0.05 - 0.09$ vs. same group - pre-ET; ε, $P = 0.05 – 0.09$ vs. ET/T same time-point. SED/T,
781 sedentary in T. ET/T, endurance-trained in T. SED/H, sedentary in H. ET/H, endurance-
782 trained in H. 4 wk and 8 wk represent four and eight weeks of the experimental protocol,
783 respectively ($n = 13\text{-}14$/group).
784

785 **Fig. 8:** Effect of endurance training (ET) on the treadmill for eight weeks in temperate
786 (T) and hot (H) environments on peak oxygen uptake ($\dot{V}O_{2peak}$; A), the pre- and post-
787 incremental load test (ILT) and post- minus pre-ILT values [delta (Δ) values] of blood
788 glucose (B and D) and lactate (C and E) in H environment of animals submitted to running
789 training for eight weeks. Data are reported as mean ± SEM. * $P \leq 0.05$ vs. same group -
790 pre-ET; & $P = 0.05 - 0.09$ vs. same group - pre-ET. SED/T, sedentary in T. ET/T,
791 endurance-trained in T. SED/H, sedentary in H. ET/H, endurance-trained in H. 4 wk and
792 8 wk represent four and eight weeks of the experimental protocol, respectively ($n = 13\text{-}$
793 14/group).

794

795 **Fig. 9:** Effect of endurance training (ET) on the treadmill for eight weeks in temperate
796 (T) and hot (H) environments on the glycogen content of liver (A) and gastrocnemius
797 muscle (B). @, $P \leq 0.05$ vs. SED/T same time-point. SED/T, sedentary in T. ET/T,
798 endurance-trained in T. SED/H, sedentary in H. ET/H, endurance-trained in H (n = 10-
799 11/group).

800

801 **Table 1**: Effect of endurance training (ET) on the treadmill for eight weeks in temperate
802 (T) and hot (H) environments on visceral organs, muscles, and adipose tissue masses.
803 Values are mean ± SEM. TA, tibialis anterior; EDL, extensor digitorum longus; TS,
804 triceps surae; EPI, epididymal; RETRO, retroperitoneal; SUB, subcutaneous; MES,
805 mesenteric; BAT, interscapular brown adipose tissue. @ $P \leq 0.05$ vs. SED/T; γ. $P = 0.05$
806 - 0.09 vs. SED/T; ∑. $P \leq 0.05$ vs. ET/T. SED/T, sedentary in T. ET/T, endurance-trained
807 in T. SED/H, sedentary in H. ET/H, endurance-trained in H. 4 wk and 8 wk represent
808 four and eight weeks of the experimental protocol, respectively (n = 13-14/group).

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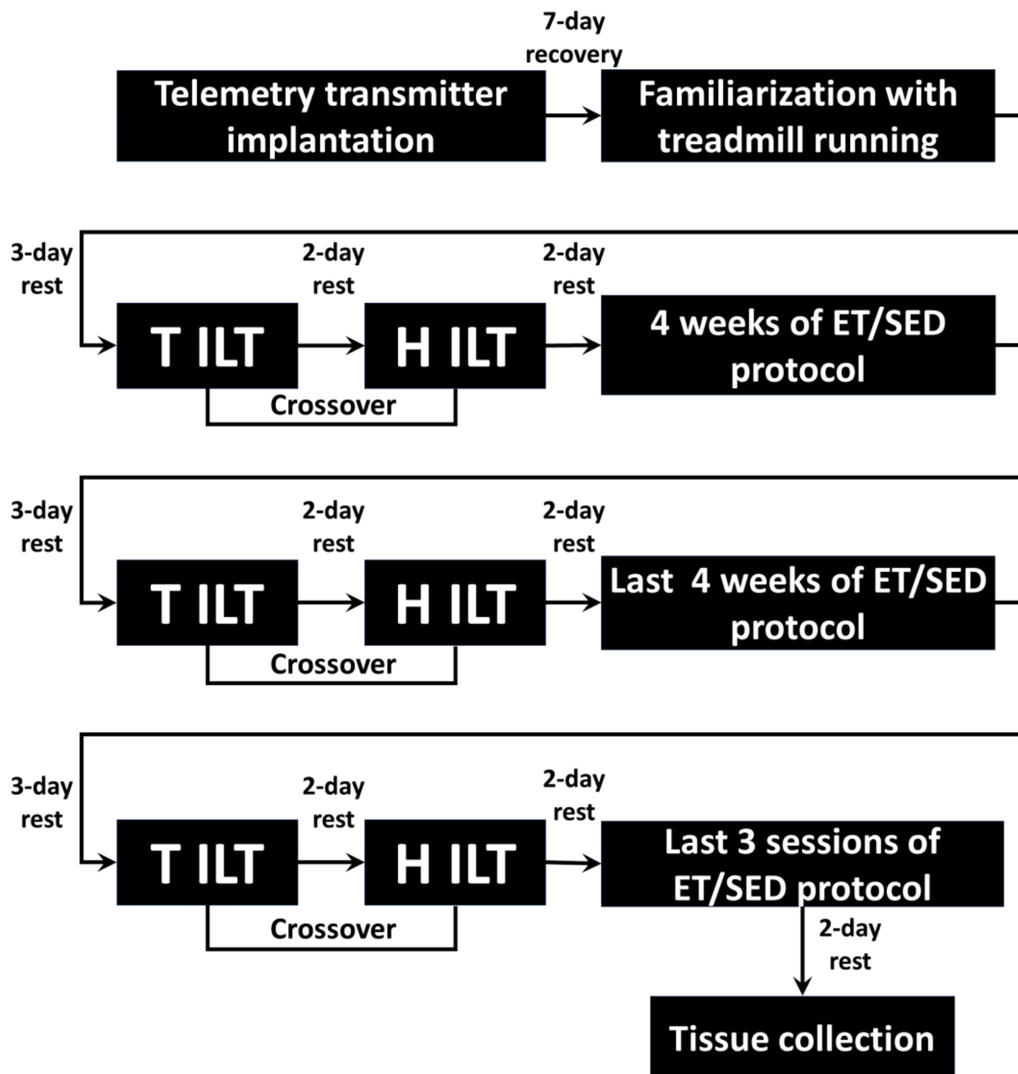

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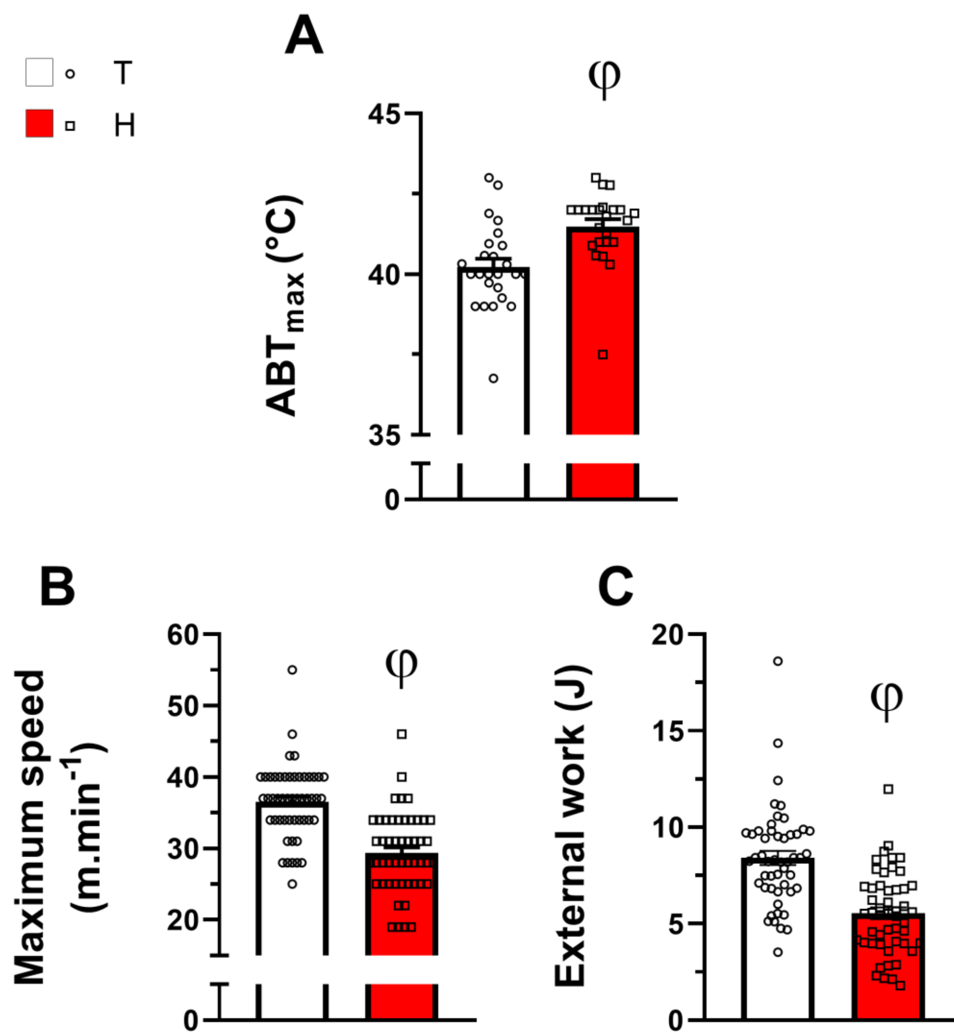
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ABTmax (°C)
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0
Maximum speed (m.min-1)
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External work (J)
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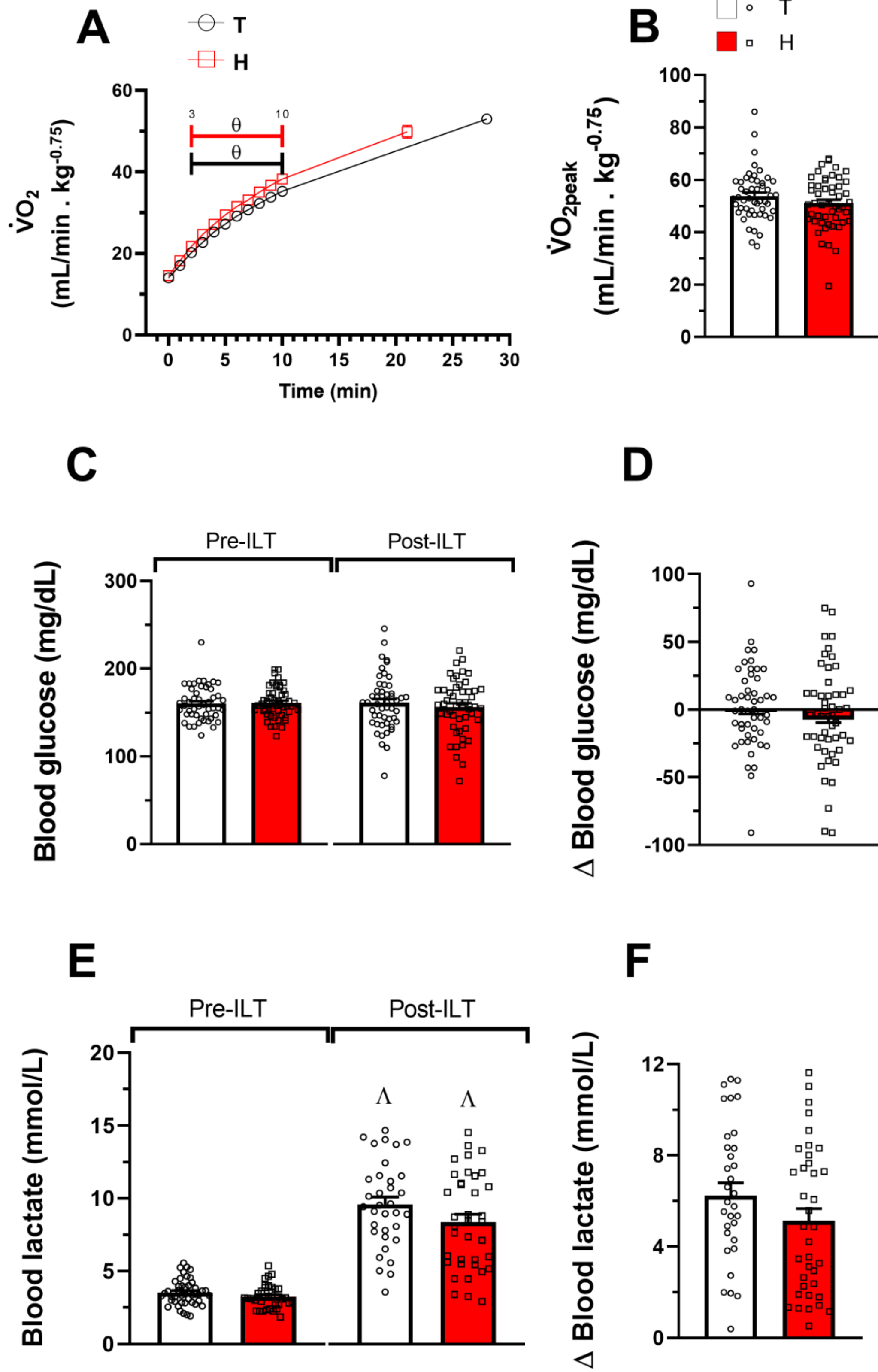

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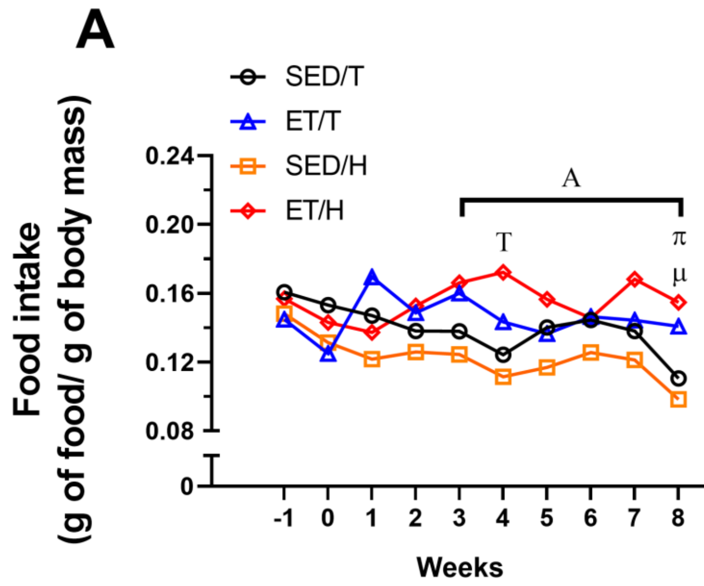
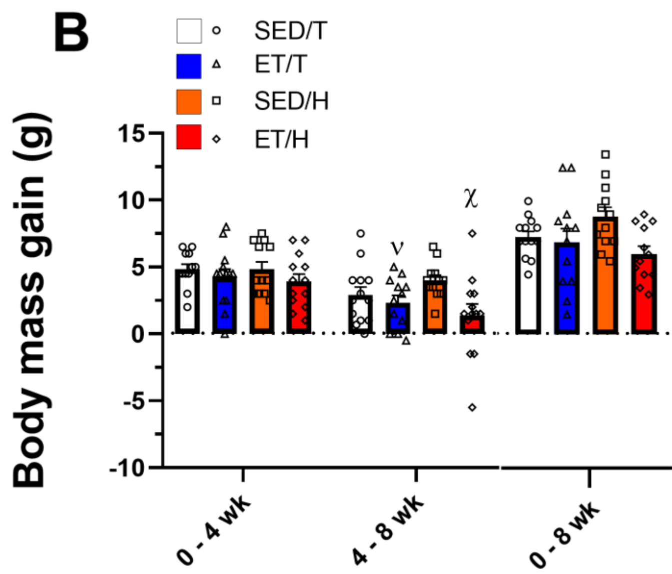
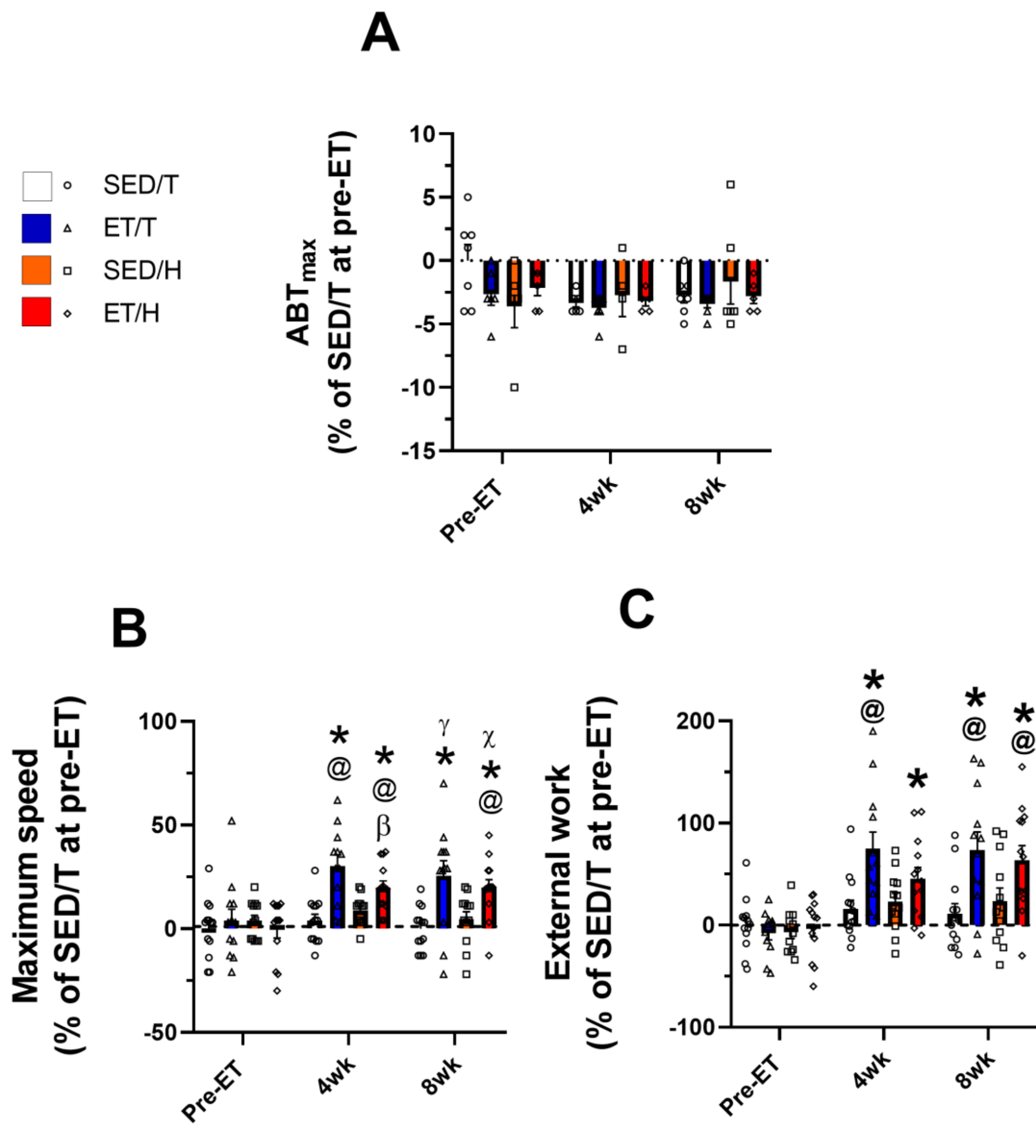
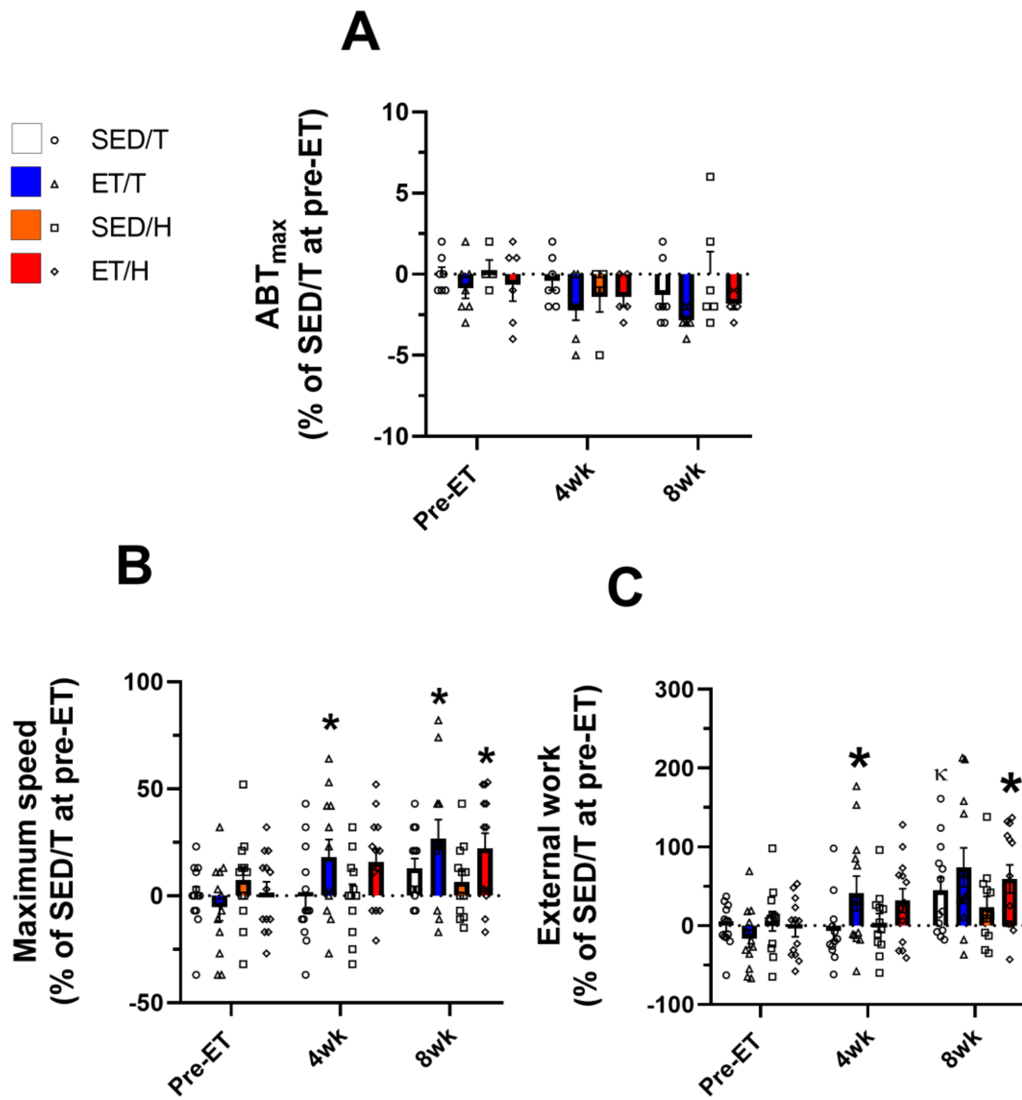
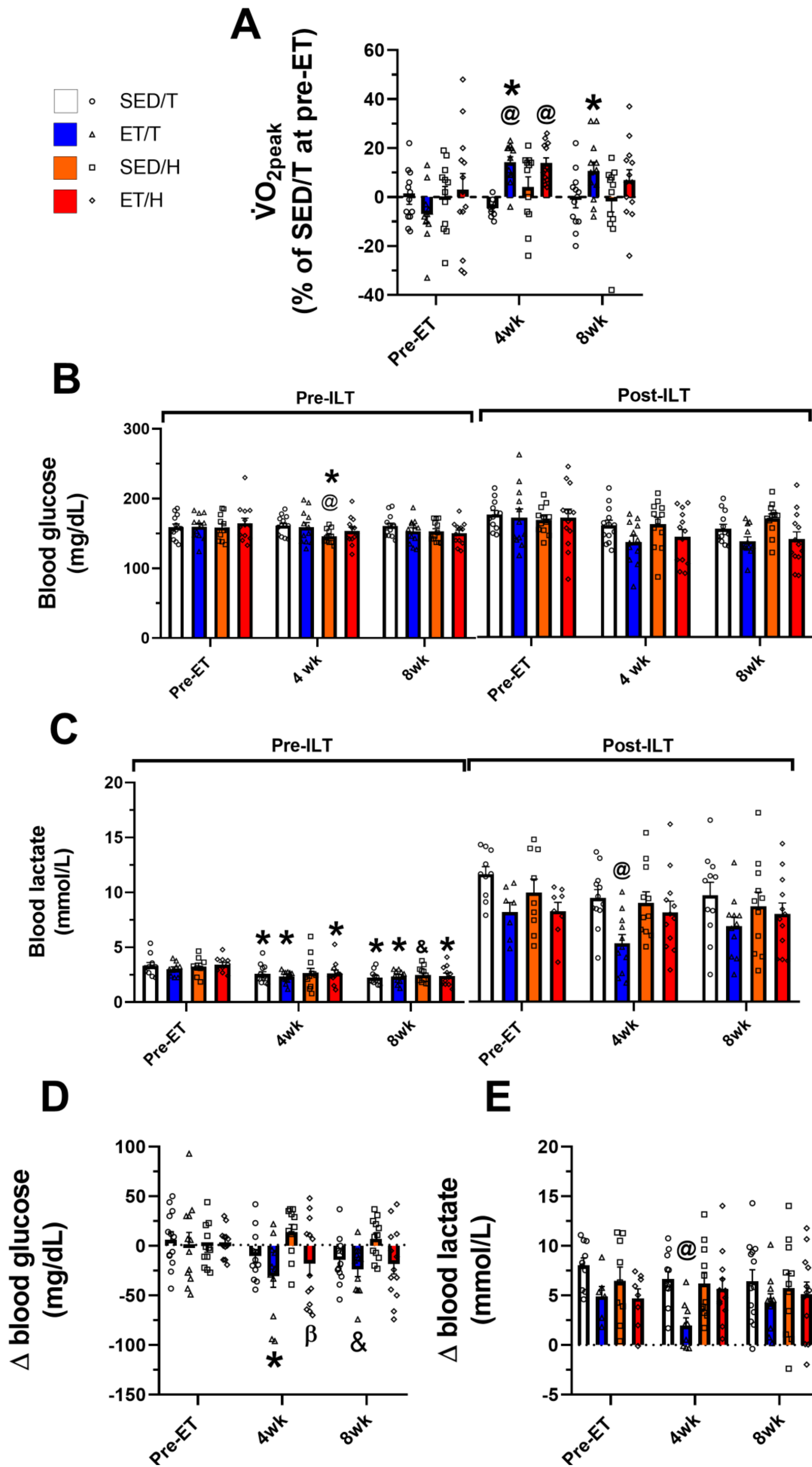
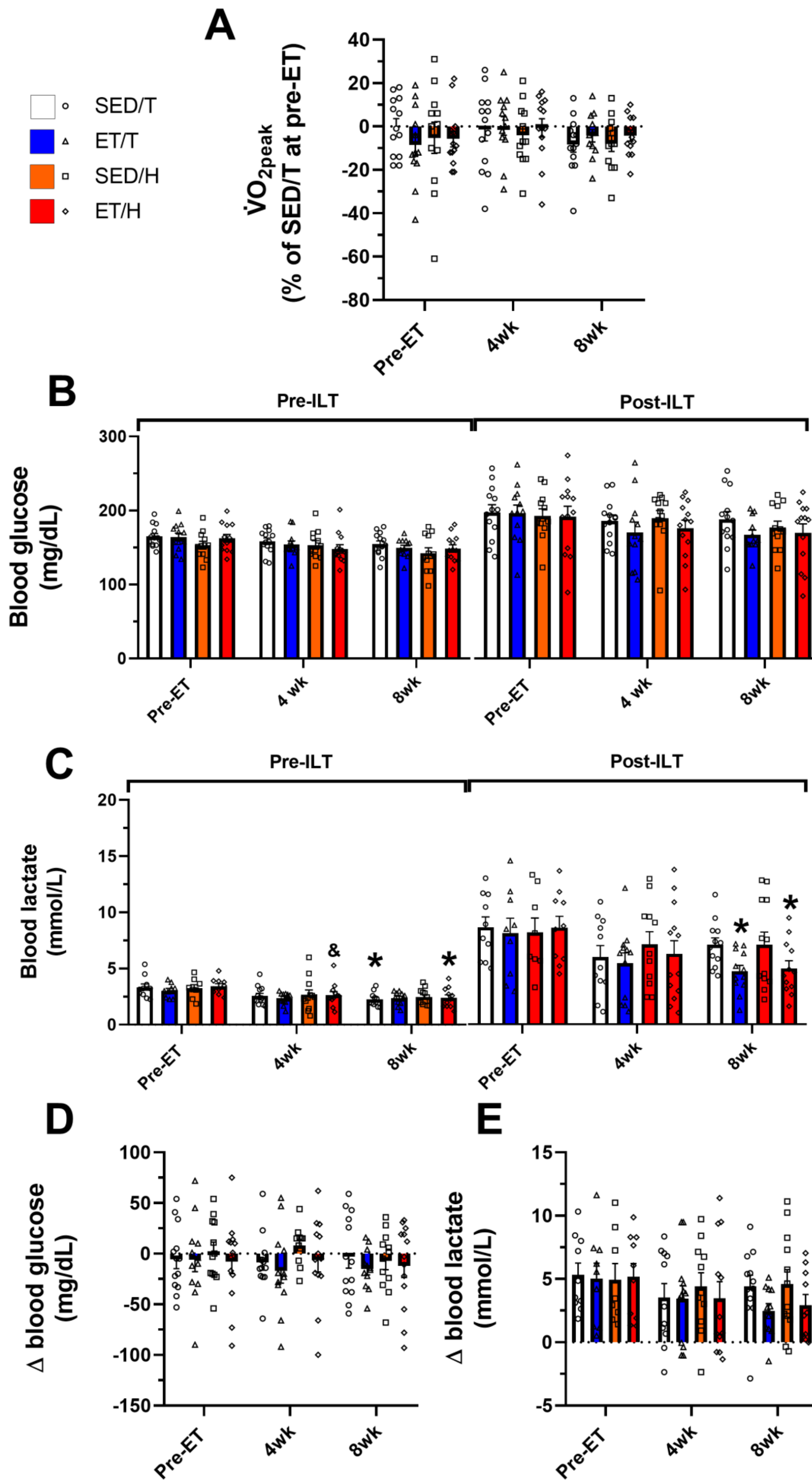

A
SED/T
ET/T
SED/H
ET/H
Food intake
(g of food/ g of body mass)
0.24
0.20
0.16
0.12
0.08
0
-1
0
1
2
3
4
5
6
7
8
Weeks
A
T
π
μ

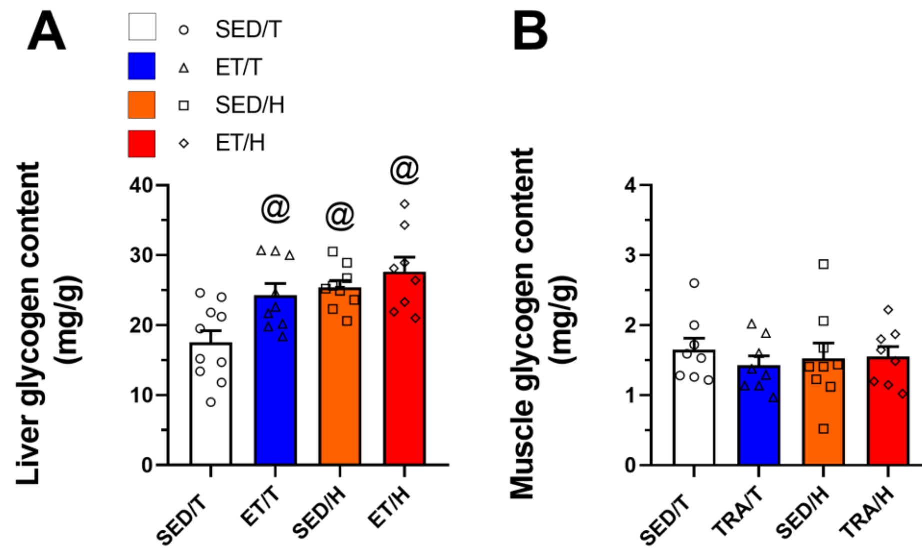

B
SED/T
ET/T
SED/H
ET/H
Body mass gain (g)
15
10
5
0
-5
-10
ν
χ
0 - 4 wk
4 - 8 wk
0 - 8 wk


A
SED/T
ET/T
SED/H
ET/H
ABT$_{max}$
(% of SED/T at pre-ET)
Pre-ET
4wk
8wk
B
Maximum speed
(% of SED/T at pre-ET)
C
External work
(% of SED/T at pre-ET)


A
B
C
SED/T
ET/T
SED/H
ET/H
ABT$_{max}$ (% of SED/T at pre-ET)
Maximum speed (% of SED/T at pre-ET)
External work (% of SED/T at pre-ET)
Pre-ET
4wk
8wk


A
SED/T
ET/T
SED/H
ET/H
V̇O2peak (% of SED/T at pre-ET)
Pre-ET
4wk
8wk
B
Pre-ILT
Post-ILT
Blood glucose (mg/dL)
Pre-ET
4 wk
8wk
C
Blood lactate (mmol/L)
D
Δ blood glucose (mg/dL)
E
Δ blood lactate (mmol/L)


A
SED/T
ET/T
SED/H
ET/H
V̇O2peak (% of SED/T at pre-ET)
Pre-ET
4wk
8wk
B
Pre-ILT
Post-ILT
Blood glucose (mg/dL)
Pre-ET
4 wk
8wk
C
Pre-ILT
Post-ILT
Blood lactate (mmol/L)
Pre-ET
4wk
8wk
D
Δ blood glucose (mg/dL)
E
Δ blood lactate (mmol/L)



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	Relative tissue mass to body mass (g/g)			
	SED/T	**ET/T**	**SED/H**	**ET/H**
Heart	3.82±0.1	4.07±0.08	3.77±0.1	3.87±0.1
Liver	48.45±0.51	48.97±0.98	46.32± 1.59	48.64±1.55
Adrenal	0.06±0.0	0.08±0.0$^{@}$	0.06±0.0	0.07±0.0$^{\gamma}$
Skeletal muscle tissue				
TA	1.43±0.02	1.53±0.04	1.53±0.04	1.51±0.04
EDL	0.3±0.0	0.33±0.01$^{@}$	0.33±0.01$^{@}$	0.31±0.01
TS	5.16±0.09	5.45±01	5.56±0.09$^{\gamma}$	5.32±0.15
Soleus	0.21±0.01	0.26±0.01$^{@}$	0.24±0.01	0.25±0.01$^{@}$
Gastrocnemius	4.48±0.06	4.72±0.05	4.87±0.1$^{@}$	4.75±0.08$^{\gamma}$
Plantar	0.46±0.02	0.49±0.02	0.5±0.03	0.48±0.02
Adipose tissue				
EPI	15.8±1.7	11.81±1.86	15.55±2.25	13.3±1.52
RETRO	3.96±0.39	2.81±0.4	3.04±0.29	2.98±0.31
SUB	11.37±0.71	8.6±0.63	11.65±1.57	9.23±0.41
MES	5.11±0.75	4.49±0.76	7.76±1.57	6.01±0.93
BAT	3.42±0.29	3.35±0.23	3.16±0.18	3.02±0.15
Adiposity index (%) ***	24.67±2.37	18.39±1.95	24.89±2.85	22.31±2.41

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Apêndice 2

1 **TITLE:** ENDURANCE TRAINING IN A HOT ENVIRONMENT REQUIRES A
2 LOWER ABSOLUTE INTENSITY TO INDUCE A SIMILAR SLOW MUSCLE
3 PHENOTYPE OF TEMPERATE
4
5 **RUNNING HEAD:** TRAINING IN THE HEAT REQUIRES LOWER INTENSITY TO
6 INDUCE A SLOW MUSCLE PHENOTYPE
7
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48 **CONFLICT OF INTEREST**

49 The authors have declared that no conflict of interest exists.

50 **ABSTRACT**

51 Although heat stress acutely worsens endurance (aerobic) performance, it has emerged as
52 a potential therapy to modulate muscle metabolism-inducing aerobic phenotype.
53 However, the effects of endurance training (ET) in this condition for long periods (> 4
54 weeks) on skeletal muscle phenotype and metabolism remain unclear. Therefore, we
55 investigate the role of 8-week ET in the hot environment (H) in the fiber type shift and
56 identify the intracellular mediators. Adult, male Swiss mice (40g) were divided into 1)
57 Sedentary (SED) mice kept in the temperate environment (T; 22 °C) (SED/T), 2) mice
58 trained on the treadmill (ET; 1h/day, 5 days/week, 8weeks, 60% of maximum speed) in
59 T (ET/T), 3) SED kept in H (32 ºC) (SED/H); and 4) ET in H (ET/H). In the pre-training
60 period, H impaired performance by reducing (~30%) time to fatigue (TTF). After 8
61 weeks, although ET/H exercised at a lower (30%) absolute intensity than ET/T, TTF, and
62 running distance were similarly increased (~22%) in both ET groups compared with
63 SED/T. The SDH activity increased ~9% in both ET groups compared with SED/T. The
64 SED/H group increased (~15%) the cross-sectional area of oxidative fibers with no
65 additional effects of ET. Although the western blot analyses did not indicate change in
66 protein content of slow and fast myosin heavy chain, immunofluorescence staining
67 indicated that % of type 2A fibers was higher (~30%) in both ET groups than in SED/T.
68 ET/H reduced the content of FoxO1 (~30%) compared with SED/T, however, the content
69 and activity of other intracellular regulators of oxidative muscle phenotype (i.e.,
70 mitochondrial oxidative phosphorylation complexes, TOM20, AMPK-p38/PGC1α and
71 TFEB) were not altered. Results from the present study indicate that ET in H requires a
72 lower absolute intensity to induce a similar fast-to-slow fiber type shift of T by increasing
73 the proportion of type 2A fibers, and the SDH activity in mice's skeletal muscle.

74 Keywords: Mitochondria, Heat stress, Skeletal muscle metabolism, Fiber type shift,

75 Aerobic training.

76 # 1. INTRODUCTION

77 Despite muscle contraction being utilized for work production, a significant proportion
78 (75 – 95%) is internally released as heat, leading to an elevation in core temperature (T_c)
79 (EDWARDS et al., 1973; GLADDEN; WELCH, 1978; WASSERMAN; VAN KESSEL;
80 BURTON, 1967). This rise in T_c not only triggers heat dissipation mechanisms but can
81 also be exacerbated by climatic conditions. Extensive evidence from studies (ELY et al.,
82 2007; GUY et al., 2015; MACALUSO et al., 2011; MAIA-LIMA et al., 2017) indicates
83 that brief exposure to a hot environment (H, ≥ 25 ºC) has an ergolytic impact on endurance
84 performance. Wanner et al. (2014) further illustrated that mice running at 34 °C
85 experienced markedly decreased maximum speed and time to fatigue (TFF) compared to
86 those at temperate (T; 24 °C) environment. While the longstanding understanding
87 suggests that repeated exposures over 10 – 15 days ablating the negative acute H effects
88 [i.e., heat acclimation (HA) (PÉRIARD et al., 2016; SAWKA, MICHAEL N. et al.,
89 2011)], recent research has highlighted that endurance HA-training in H can yield similar
90 metabolic and running performance adaptations, despite a lower absolute training
91 intensity compared to endurance training (ET) in T (Zanetti et al., 2024). However, the
92 physiological mechanisms driving these effects remain insufficiently elucidated.

93 Kodesh and Horowitz (2010) demonstrated that one month of HA at 34 ºC for four weeks
94 enhances force generation and leads to an up-regulation of genes related to lipid
95 metabolism in the rats' soleus muscles. While this adaptation could potentially contribute
96 to improved endurance performance, the study did not evaluate these aspects. In contrast,
97 in our recent work (Zanetti et al., 2024) we conducted the first assessment of the effects
98 of eight weeks of HA-training on endurance performance in mice. For this, mice ET in
99 both H (32 ºC) and T (22ºC) environments at the same relative intensity (i.e., 60% of
100 maximum speed) as the test conducted in the respective environment. Despite H reduced

101 endurance performance and the ET intensity in H, we observed similar increases in
102 maximum speed and peak oxygen consumption (VO_{2peak}) compared to ET in T.
103 Moreover, ET in H exhibited a comparable improvement to T in aerobic power and
104 endurance performance during an incremental load test (ILT) in T. The inclusion of heat
105 stress in training may, therefore, result in an ergolytic effect which reduced mechanical
106 load cost compared to ET in T conditions. However, our understanding of the molecular
107 aspects involved in HA-mediated adaptations remains limited.

108 Several lines of evidence indicate that local heat exposure promotes effects similar to
109 those classically associated with ET in skeletal muscle. Hafen et al. (2018) reported that
110 heat stress, leading to a 3.9 ºC increase in skeletal muscle temperature, enhances
111 phosphorylation of AMP-activated protein kinase (AMPK) and an increase in skeletal
112 muscle mitochondrial function. Traditionally, an increase in the ADP/ATP ratio and Ca^{2+}
113 levels, indicating an energy deficit, enhances AMPK phosphorylation ($pThr^{174}$-AMPK)
114 and activates members of the CAMKII family. This cascade leads to the activation of
115 PGC-1α and the nuclear translocation of TFEB, resulting in elevated expression of
116 mitochondrial electron transport protein complexes (OxPhos) I to V and enhanced
117 mitochondrial oxidative capacity. In accordance, the large-scale evaluation of muscular
118 mRNA in response to local heat stress identified a significant increase in the expression
119 of OxPhos III [ubiquinol-cytochrome c reductase binding protein (UQCRB)](Goto et al.,
120 2011). This finding suggests that the organism responds to heat stress by activating
121 metabolic pathways involved in cellular energy production by stimulating ATP
122 resynthesis through oxidative pathway, although the mechanism leading to this outcome
123 remains poorly elucidated. Given that, according to Zwaard et al. (2016), succinate
124 dehydrogenase (SDH, i.e., Oxphos II) activity (indicative of oxidative capacity) is
125 proportional to $\dot{V}O_{2max}$ in humans, the regulation of oxidative pathways by exposure to

126 heat stress could justify the ergolytic effect in VO_{2peak} observed in our previous study
127 (Zanetti et al., 2024). However, there is a gap in the literature regarding the molecular
128 mechanisms underlying this relationship.

129 The availability of energy substrates through energy metabolism and their delivery via
130 the vascular system during ET bouts represent additional factors regulating endurance
131 performance. pThr174-AMPK is also responsible to phosphorylates a transcription factor
132 Forkhead Box O3 (FoxO3) at serine residues (Ser^{413}), enhancing its transcriptional role
133 and activating pathways of energy substrate degradation. Indeed, Slopack et al. (2014)
134 observed that a single session of physical exercise increased mRNA levels of the FoxO1
135 and FoxO3 isoforms. However, ET attenuated this exercise-induced FoxO1 response.
136 Furthermore, transgenic knockout (KO) animals for FoxO1 in vascular endothelial tissue
137 exhibited an early angiogenic response. Underscoring the critical role of FoxO repression
138 in ET-induced angiogenesis. Therefore, FoxO not only acts in the provision of substrates,
139 especially in long-duration exercises but also in adaptations resulting from physical
140 exercise. Similarly to ET, Ihsan et al. (2020) demonstrated that whole-body heat treatment
141 (44 - 55 ºC, 50% humidity, 60 min) up-regulated mitochondrial biogenesis and FoxO1
142 and FoxO3 phosphorylation (i.e., reduction in transcriptional activity). This indicates that
143 multiple mechanisms may be involved in regulating the improved oxidative function
144 following heat exposure, but the studies did not investigate the effects of long-term HA
145 training on molecular pathways which regulates oxidative metabolism.

146 Given the role of heat and ET on muscle oxidative capacity, identifying mechanisms
147 underlying musculoskeletal-specific adaptations to training under heat stress is an
148 attractive target and worthy of investigation for the development of athletes' rehabilitation
149 programs and novel training strategy development. Thus, the purpose of the present work
150 was to investigate the role of nine-week ET in H in the in vivo skeletal muscle adaptation

151 and identify the intracellular mediators of these processes. We report that ET in H reduces
152 absolute training intensity compared to ET in T, but it induces similar increases in the
153 proportion of skeletal muscle 2A fiber type, enzyme succinate dehydrogenase (SDH)
154 activity, cross-section area (CSA), and FoxO1 downregulation.

155 # 2. METHODS

156 ## 2.1. Animals

157 Swiss mice (8-week-old male mice, ~40g) were housed in the vivarium of the Exercise
158 Physiology Laboratory (LAFISE) in collective cages under controlled light conditions
159 (7:00 a.m. to 7:00 p.m.) and a temperature (T_a) of 24.0 ± 2.0 ºC, with access to water and
160 chow *ad libitum*. All experiments were conducted in accordance with protocols approved
161 by the Ethics Commission on Animal Use of the Universidade Federal de Minas Gerais
162 (CEUA; 220/2019).

163 A sample size of 53 animals (n = 13-14/group) was determined using G*Power software,
164 version 3.1.9.4 (Erdfelder et al., 2009), and four experiments (n = 3-4/group/experiment)
165 were performed to achieve this number of sample size. Due to the consistency and clarity
166 of the results obtained with a representative sample, not all animals from the sample size
167 were used in the molecular biology and histological analyses. Mice were counterbalanced
168 based on running performance immediately after the first incremental load test (ILT),
169 with the following groups: 1) Sedentary mice (SED) kept on a treadmill turned off in the
170 temperate environment (T; 22 ºC; SED/T) during the same time as the endurance training
171 (ET), 2) ET mice on the treadmill in T (ET/T), 3) SED mice kept on a treadmill turned
172 off in the hot environment (H; 32 ºC; SED/H) during the same time as the ET groups, and
173 4) ET mice in H (ET/H). 48-hours after the nineth ET week, animals were euthanized
174 using a guillotine. Tissue and blood collection were quickly performed and storage in a -
175 80 ºC freezer. All experiments procedures were performed between 7 a.m. and 1 p.m. to
176 minimize chronobiological influences.

177

178 ## 2.2 Experiment design

179 Fig. 1 illustrates a schematic representation of the protocols to which the mice were
180 subjected.

181

182 **2.3 T_a control**

183 To monitor temperature changes, a thermocouple (YSI-400A, Yellow Springs
184 Instruments, Yellow Springs, OH) was situated inside an acrylic chamber housing the
185 treadmill. Positioned on the ceiling midway between the fan and the electrical grid. The
186 temperature detectors were connected to a data logging device (data logger, model AI-
187 24, Dianachart, Rockaway, NJ, USA). To regulate the temperature within the acrylic
188 chamber encompassing the treadmill, an electric heater (Britânia model AB 1100,
189 Curitiba, Brazil) was positioned approximately 40-45 cm from the front of the treadmill
190 and operated at 1,200 W, as per the method described by WANNER et al. (2014).
191 Additionally, room air conditioning was employed, set at 22 ºC (T test) and 32 ºC (H test).
192 The H ILT setup was previously verified to induce additional environmental heat stress,
193 resulting in a significant increase in core body temperature (T_{core}) by ~1.3 ºC when
194 compared to tests conducted in temperate conditions (Zanetti et al., 2024).

195

196 **2.4 Treadmill running familiarization protocol**

197 Modified from Wanner et al. (2014), the familiarization protocol consisted of running on
198 a treadmill over five consecutive days. During the initial four days, the protocol
199 comprised three stages: I - a three-min rest period with the treadmill turned off; II - mice
200 ran for five min, maintaining a speed of 5 $m.min^{-1}$ on the first day and 6 $m.min^{-1}$ by the
201 second to fourth day; III – on the first and second day, mice ran an additional three min
202 at speeds of 6 and 8 $m.min^{-1}$, respectively, and on the third and fourth day they ran an

203 additional five min at 8 $m.min^{-1}$. The treadmill slope was consistently set at 5°. On the
204 fifth day mice were acquainted with a lighter version of the ILT. As proposed by
205 Dougherty et al. (2016), the ILT completion criterion was determined when the animal
206 spent five continuous seconds in the designated fatigue zone (i.e., the rear of the treadmill,
207 ranging from approximately 'one body length' from the shock grid to, and including, the
208 shock grid).

209 Mice were encouraged to run by light electrical stimulation (0.4 mA) provided by a grid
210 at the rear end of the treadmill belt. Ta was controlled at 24 ± 1 ℃ during the
211 familiarization protocol.

212

213 **2.5 ILT protocol**

214 To evaluate physical performance, mice familiarized with running underwent a 72-hour
215 rest period and were then subjected to the ILT, modified from Ayachi et al. (2016) as
216 adapted by Zanetti et al. (2024). The ILT tests were conducted in both T and H
217 environments in a cross-balanced manner, with a 48-hour interval between sessions. The
218 ILT session commenced at 10 $m.min^{-1}$, with velocity increasing by 3 $m.min^{-1}$ every 3
219 min, lasting until exhaustion (Ayachi et al., 2016; Mille-Hamard et al., 2012). Physical
220 performance parameters, such as maximum speed and time to fatigue (TTF), were
221 measured during ILT. Maximum speed achieved during the first and fourth ILT sessions
222 were utilized to prescribe the intensity of training sessions in the subsequent weeks. Mice
223 were encouraged to run through light electrical stimulation (0.5 mA) provided by a grid
224 located at the rear end of the treadmill belt.

225

226 **2.6 ET protocol**

The mice commenced the ET protocol (Ferreira et al., 2007; Kodesh and Horowitz, 2010; Wanner et al., 2014) 48 hours after the first block of ILT sessions. In summary, the nine-week ET protocol involved treadmill running for 5 days per week, with a gradual increase in both intensity and duration over the ET period. In summary, during the first week, mice-initiated training by running on a treadmill at a speed equivalent to 50% of the maximum speed with a 0º slope. The first and second sessions lasted for 25 and 35 min, respectively. Starting from the second week, the duration of the sessions increased by 15 min per day until reaching a total of 60 min. After the fourth session, the intensity was adjusted to 60% of the maximum speed. Consequently, from the third week until the completion of the ET protocol, ET sessions were conducted at 60% of the maximum speed, lasting 60 min, and with a 5º slope. Maximum speed was reassessed after the fourth week of ET, and the relative intensity of the groups was adjusted accordingly to ensure the appropriate ET load.

2.7 Histology and microscopy

Gastrocnemius muscles (GAS) designated for histology were positioned to an acrylic surface and rapidly frozen in liquid nitrogen. Cryosections of the GAS were then prepared using a Leica CM1850 UV cryostat (Leica Microsystems, Wetzlar, Germany). Cryosections were stained for haematoxylin and eosin (H&E) and succinate dehydrogenase (SDH). H&E staining was performed by successively transferring the slides into staining jars with xylene, 100, 95, and 70% ethanol (for 3 min in each), thoroughly rinsing in tap water, immersing in hematoxylin (which stained nuclei dark blue) for 2 min, thoroughly rinsing in tap water to remove excess hematoxylin, dipping in eosin (which stained myoplasm pink) for 1 min, washing thoroughly in tap water to remove excess eosin, and successively transferring the slides into staining jars with 70,

252 90, 95, and 100, and finally xylene (for 3 min in each). SDH staining was performed by
253 incubating sections for 30 min at 37 °C in a 0.2 M sodium phosphate buffer containing
254 0.2 M sodium succinate and 4.4 mM nitro-blue tetrazolium. Sites of high SDH activity
255 were colored blue (Drury, 1981). Subsequent examination of H&E and SDH staining was
256 performed using a fluorescence microscope (Olympus BX61VS; ×20 magnification).
257 Skeletal muscle tissue integrity of more than 270 fibers stained with H&E per muscle was
258 evaluated. Cross-sectional area (CSA) and SDH activity were determined for over 270
259 fibers stained with SDH in each muscle.

260 CSA and fiber typing for the entire muscle were determined through indirect
261 immunofluorescence (IF) using a combination of monoclonal anti-myosin heavy chain
262 (MyHC) antibodies obtained from the Developmental Studies Hybridoma Bank (DSHB,
263 University of Iowa)(Gonçalves et al., 2019). The antibodies included BA-D5 (1/200 or
264 IgG2b) specific for MyHC1, SC-71 (1/150 for IgG1) specific for MyHC2A, and BF-F3
265 (1/200 for IgM) specific for MyHC2B. Type 2X fibers were purposely not labeled with
266 antibodies and were identified as black in the analysis. The skeletal muscle cell membrane
267 was stained for dystrophin (Dys; Abcam, reference ab15277; 1:100 dilution). Single
268 images were merged to create a complete muscle reconstruction using Adobe Photoshop
269 CC2019 (Adobe Systems Inc.).

270 Histological analyses were performed utilizing ImageJ software (Fiji is Just) version 2.3.0
271 (National Institutes of Health, USA). All histological analyses were carried out by a single
272 observer (GOZ), who was blinded to the identity of the mice.

273

274 **2.8 Western blotting analysis**

GAS was homogenized in RIPA buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM sodium orthovanadate, 5 µg/ml aprotinin, 1 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 50 µM MG132]. The homogenate was centrifuged at 21,000 g at 4 °C, and the protein content in the supernatant was determined using the Lowry method (Lowry et al., 1951) with bovine serum albumin as a standard.

The supernatant was mixed with sample buffer [20% glycerol, 125 mM Tris-HCl, 4% SDS, 100 mM dithiothreitol, 0.02% bromophenol blue, pH 6.8] and thermomixed at 70 °C for 10 min. Lysates (10-90 µg) were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6-12%). After electrophoresis, proteins were transferred to a nitrocellulose membrane and probed with primary antibodies [anti-CAMKII (1:1000), anti-FoxO1 (1:1000), anti-FoxO3 (1:500), anti-MyHC fast (1:5000), anti-MyHC slow (1:5000), anti-OxPhos (1:1000), anti-PGC-1α (1:500), anti-p38 (1:1000), anti-pThr^{286}-CAMkII (1:500), anti-pThr^{180}/Tyr^{182}-p38 (1:1000), anti-pSer^{294}-FoxO3 (1:500), anti-pSer^{413}-FoxO3 (1:500), anti-pThr^{172}-AMPK (1:1000), anti-pThr^{24}-FoxO1/pThr^{32}-FoxO3 (1:500), anti-TFEB (1:500), and anti-TOM20 (1:500)]. Detection of primary antibodies was done using peroxidase-conjugated secondary antibodies (1:10000 for MyHC slow; 1:8000 for MyHC fast; 1:6000 for OxPhos, TOM20; 1:3000 for p-p38, TFEB; 1:2000 for P-CAMKII, CAMKII, p38; 1:1000 for pThr172-AMPK, PGC-1α, pThr24-FoxO1/pThr32-FoxO3, pSer413-FoxO3, pSer294-FoxO3, FoxO3, FoxO1), and visualization was carried out using enhanced chemiluminescence reagents on a ChemiDoc XRS+ System (Bio-Rad). Band intensities were quantified using ImageJ software (Fiji is Just) version 2.3.0 (National Institutes of Health, USA).

2.9 Statistics

All individual physical performance data were normalized by the mean value of the SED/T group in the pre-ET period. Therefore, the data are presented as a mean ± SEM. Normality of data distribution was assessed using the Shapiro-Wilk normality test. For the comparison of means between two groups (T vs. H environments in the pre-ET period), paired Student's t-test was employed. For analyses involving four groups (SED/T, ET/T, SED/H, and ET/H) at the same time point (histology and western blotting analysis) were conducted using one-way analysis of variance (ANOVA one-way) followed by posthoc Tukey's test. For analyses involving four groups at different time points (ILT variables) were conducted using two-way ANOVA with repeated measures (ANOVA two-way RM) followed by posthoc Tukey's test. A significance level of $\alpha = 0.05$ was set for all analyses. Graphs and statistical analyses were performed using GraphPad Prism version 8.0.1.

313 # 3. RESULTS

314 ## 3.1 ET/H induce similar metabolic and running performance adaptations despite a
315 lower absolute intensity than ET/T

316 Firstly, ILT was performed in all mice in both T and H conditions to determine endurance
317 performance and counterbalance experimental groups. As expected, the H environment
318 caused a reduction in the endurance-related parameters TTF (38%) and running distance
319 (35%) when compared with the T environment (Fig. 2A and B). We have previously
320 shown that the H environment also reduced maximum speed and external work in ILT
321 (Zanetti et al., 2024). For this reason, the absolute running speed ($m.min^{-1}$) corresponding
322 at 60% of maximum speed in the ET/H group during ET period was 20 and 40% lower
323 than in the ET/T group at first (pre-ET – 4 wk) and least (4 – 8 wk) four weeks,
324 respectively (Zanetti et al., 2024).

325 In the T environment, pre-ET values of TTF (~27 min) and running distance (~630 m)
326 obtained in ILT were similar among groups (Fig. 2C and D). Fig. 2C and D show that
327 both ET groups increased by ~18% TTF and running distance after four and eight weeks
328 compared with their pre-ET period and with their respective SED groups (i.e., ET/T
329 *versus* SED/T and ET/H *versus* SED/H). In the H environment, pre-ET values of TTF
330 (~21 min) and running distance (385 m) were also similar among groups (Fig. 2E and F).
331 At week four, only ET/T significantly increased TTF and running distance (~20%)
332 compared with its pre-ET period (Fig. 2E and F). After eight weeks, ET/T and ET/H
333 groups increased (~20%) TTF and running distance compared with their pre-ET period.
334 Taken together, these findings show that acute exposure to H reduces endurance
335 performance, but ET/H improves performance in ILT similar to ET/T in any T_a, even
336 exercising at a lower absolute intensity than ET/T.

337 **3.2 ET/H at the same relative intensity as ET/T improves the expected muscle injury-**
338 **recovery phenotype after a two-day recovery period**

339 Similar to physical exercise inducing a skeletal muscle process of injury-recovery, heat
340 stress causes damage and dysfunction through physiological strain that may evolve into
341 exertional heat illness. We conducted a quantitative-qualitative histological examination
342 of H&E-stained transverse GAS muscle sections was performed to investigate
343 morphological characteristics of the degeneration-regeneration process and myopathies
344 such as small flattened or irregularly shaped atrophic fibers, target fibers, centrally
345 nucleated fibers (CNFs), and necrotic fibers (Fig. 3A). Quantitative analysis of the
346 proportion of CNFs revealed a significant increase of 106% in the ratio between cells
347 with centralized nuclei and intact ones only in ET/T, following two days of rest (Fig. 3B).
348 Qualitative analysis supported these findings and indicated sites with a high occurrence
349 of cells with centralized nuclei only in ET/T, while no other irregularities were discerned
350 (Fig. 3A). These results indicate that even after two days of rest, ET/T exhibited a
351 musculoskeletal injury-recovery process, but ET/H at the same relative intensity did not
352 show structural degeneration.

353

354 **3.3 ET/H induces a shift toward fast oxidative glycolytic fiber similar to ET/T but in**
355 **a lowered absolute intensity**

356 The expression of slow and fast MyHC isoforms was analyzed by western blot in the
357 GAS muscles after nine weeks of ET and there was no difference among groups (Fig. 4A
358 and B). Because the fast MyHC subtypes in limb muscles may be divided into one fast
359 oxidative glycolytic fiber (MyHC 2A) and two fast glycolytic fibers (MyHC 2X and 2B)
360 (Schiaffino and Reggiani, 2011), co-immunostaining for these MyHC isoforms was

361 performed in whole muscle cryosection (Fig. 4C). Both ET/T (~30%) and ET/H (~30%,
362 P = 0.072) groups presented a higher proportion of MyHC 2A fibers compared with the
363 SED/T group (Fig. 4D). The proportion of MyHC 2X and 2B fibers did not change in any
364 group (Fig. 4D). SED/H showed a tendency for higher values (16%, P = 0.087) of the
365 CSA of individual muscle fibers compared with the SED/T group (Fig. 4E). Altogether,
366 these results indicate that ET/H induces a shift toward fast oxidative glycolytic fiber
367 similar to ET/T, i.e., ET promotes a fiber-type transition in a T_a-independent manner.

368

369 **3.4 ET/H increases mitochondrial activity without affecting mitochondrial density**
370 **similar to ET/T but in a lowered absolute intensity**

371 Next, we further characterized fiber type identity by estimating mitochondria activity in
372 cryosections stained for SDH activity (Fig. 5A). The average SDH activity of all fibers
373 was increased by ~9% in both ET/T and ET/H, with no significant change in the oxidative
374 mitochondrial rich (dark fibers) and the glycolytic mitochondrial poor (pale fibers) fibers
375 (Fig. 5B). The proportion of oxidative and glycolytic fibers was unaffected by any
376 condition (Fig. 5C). Interestingly, the analysis of CSA in oxidative and glycolytic fibers
377 by SDH staining confirmed the results obtained in immunostaining for MyHC isoforms,
378 i.e., oxidative fibers from SED/H had higher (~15%) diameter than SED/T (Fig. 5D). In
379 contrast to SDH activity, the content of OxPhos, including complex II (SDH), was
380 unaltered in any condition (Fig. 6A - F). Neither ET nor the H environment changed the
381 mitochondrial content marker TOM20 (Fig. 6A and G). Thus, our data suggest that both
382 ET/H and ET/T increased muscle mitochondrial activity, estimated by SDH staining in
383 cryosections. However, these effects were not associated with a parallel up-regulation of
384 mitochondrial OxPhos and mitochondria content.

385

386 **3.5 ET/H regulates the intracellular signaling pathway involved in the oxidative**
387 **muscle phenotype**

388 Several studies have shown that AMPK, p38, and CAMKII proteins are involved in the
389 determination of muscle fiber type phenotypes (Akimoto et al., 2005; Bergeron et al.,
390 2001; Raney and Turcotte, 2008). However, neither ET nor the H changes the $pThr^{172}$-
391 AMPK, $pThr^{286}$-CAMKII, CAMKII, $pThr^{180}/Tyr^{182}$-p38, and p38 content among the
392 experimental groups (Fig. 7A – F). In addition, these kinases are three important signaling
393 cascades linked to PGC-1α (Combes et al., 2015), which is one of the factors regulating
394 the muscle fiber-type determination (Lin et al., 2002). However, no significant change in
395 the PGC-1α levels was detected after nine weeks of ET (Fig. B). Regardless of PGC-1α,
396 TFEB regulates mitochondrial biogenesis and function in the skeletal muscle (Grumati et
397 al., 2010; Mansueto et al., 2017) and could explain the improvement in oxidative
398 metabolism, however, protein its content also did not change in any condition (Fig. 8C).

399 In addition to these proteins, FoxO1 and FoxO3 are key factors of muscle energy
400 homeostasis through the control of mitochondrial metabolism. Fig. 8I shown that total
401 FoxO1 content decreases (~32%) only for ET/H compared with SED/T, but
402 phosphorylated (at Thr^{24}), as well as total and phosphorylated (at Thr^{32}, Ser^{413}, Ser^{294})
403 content of FoxO3 did not present significant alterations among the experimental groups
404 (Fig. 8A, D - H). These findings indicate that nine weeks of ET/H promotes chronic
405 FoxO1 downregulation, but did not change the baseline levels of others proteins involved
406 in oxidative muscle phenotype.

407 ## 4. DISCUSSION

408 This study investigated the effect of prolonged HA training for nine weeks on skeletal
409 muscles fiber type, muscle metabolism, and intracellular pathways adaptations in mice,
410 i.e., the cellular mechanism that underlining T_a as a ET intensity compensator. Our main
411 findings show that mice trained in either T and H environments had similar fast-to-slow
412 fiber type shift which exerts a crucial role in the regulation of tissue adaptation and
413 promotes endurance performance, whereas the lower absolute intensity (i.e., maximum
414 speed) and activation of alternative pathways in H environment.

415 The effects of acute heat stress on endurance have been extensively revised by literature.
416 Arngrísson et al. (ARNGRÍMSSON et al., 2002) measured VO_{2peak} during submaximal
417 exercise (walking for 20-min at ~33%VO_{2max}) in 25, 35, 40 and 45 ºC and consumption
418 was inversely proportional to test temperature. The analysis of the mean of the top ten
419 performances in marathon events (i.e., 42km) from seven consecutive International
420 Association of Athletics Federations (IAAF) World Championships (1999–2011)
421 revealed a significant impairment of endurance performance in hot environment (>25 ºC)
422 for both males and females (GUY et al., 2015). In accordance, we found that a single
423 session of exercise (i.e., pre-training ILT) in H caused a marked increase in T_c and
424 decrease in endurance performance (i.e., TTF and running distance) in non-HA mice.
425 Literature suggests that heat stress alone impairs endurance performance when
426 hyperthermia active a dissipation counter balance mechanism which shift brain and
427 skeletal muscle blood flow to the skin (Nybo et al., 2014; Périard et al., 2011; Rowell,
428 1974). Consequently, endurance athletes in the heat perform at a lower work rate than in
429 temperate environments (Ely et al., 2007; Racinais et al., 2015). These observations
430 shown that acute heat exposure decline endurance performance by enhanced T_C and acute
431 increase in physiological strain.

432 In our previous paper (Zanetti et al., 2024), we observed that mice aerobically trained in
433 either T and H environments had similar increases in aerobic power and improvements in
434 endurance performance, whereas the absolute intensity (i.e., running speed) was lower in
435 H environment. In accordance, in this study animals exercising in H had lower endurance
436 performance (i.e., lower TTF and running distance) and absolute training intensity
437 compared with those who trained in T, however, both ET groups had similar
438 improvements in endurance performance. As aforementioned, ET bouts in heat provides
439 a unique and progressively exacerbated challenge to the cardiovascular system compared
440 to that of temperate conditions (Périard et al., 2013). Increased physiological demand,
441 may act as exercise load component (i.e., volume, intensity, and bout), which
442 compensated the reduced absolute exercise intensity prescribed for ET/H group.
443 Accordingly, although additional environment heat stress acutely impairs the optimal
444 endurance performance, chronically it may provide an alternative pathway to reach
445 equivalent physiological strain without having high levels of mechanical workload.

446 The present study shows that the ET in both T and H has a shift fiber type outcome on
447 cellular skeletal muscle. For instance, our study is the first to report that ET in T and H
448 environment similarly enhances skeletal muscle oxidative metabolism by inducing a fast-
449 to-slow fiber type shift (increase in % 2A fibers), and increasing SDH activity, and 1h/day
450 heat exposure increase oxidative CSA. The distribution of cardiac myosin isoenzymes
451 was studied in rats during HA (34 ºC, 2-month), and it was demonstrated a fast-to-slow
452 myosin phenotype shift in conjunction with decreased thyroxin levels (Horowitz et al.,
453 1986). In accordance, O'Neill et al. (O'Neill et al., 2006) suggested that fast-to-slow
454 phenotypic remodeling appear to be a critical process to induce chronic changes in
455 constitutive expression of Hsp70 – a constitutively expressed protein which are
456 upregulated in response to protein-damaging stress. Furthermore, *In vitro* experiments

457 indicated that repeated hyperthermia may lead to increase in mitochondrial activity, slow
458 MHC content (Patton et al., 2018), and slow fiber-type shift attributed to activation of
459 PGC-1α (Yamaguchi et al., 2010). Heretofore, changes of MHC following long-term ET
460 under additional environment heat stress *in vivo* have not been reported, however,
461 increased skeletal muscle oxidative capacity may act as an important HA adaptive
462 mechanism.

463 Several signaling pathways regulate skeletal muscle fiber type shift. Although Lin et
464 al.(Lin et al., 2002) showed that overexpression of PGC-1α induced fast-to-slow fiber-
465 type transition in mice fast muscle (i.e., *plantaris*) in mice, Mansueto et al. (Mansueto et
466 al., 2017) reported that transcription factor EB (TFEB) regulates mitochondrial
467 biogenesis independently of PGC1α. However, in our study ET in both environments
468 does not increase the content of TFEB and PGC-1α. Besides AMPK, CAMKII, and
469 mitogen-activated protein kinases (MAPK) p38 pathways have been implicated in the
470 regulation of PGC-1α expression and activity (Finck and Kelly, 2006), other investigators
471 suggested that the MAPK pathway played an important role in the maintenance of fast-
472 twitch fiber phenotype. Ras-ERK pathway was required for reestablishment of the slow
473 fiber (Murgia et al., 2000). p38 MAPK has been reported to control MHC2X promoter
474 activity in myotubes (Meissner et al., 2007). AMPK activation during ET, is associated
475 with increases in PGC-1α expression (Baar, 2004). However, we did not observe changes
476 in protein levels 48h after the last exercise session of ET. It is worth to mention that the
477 present study investigated the effects of 8-week training on baseline protein levels, and
478 rest period may have hidden a transient pathway from a more lasting response. So far, the
479 role of fiber type shift and increased mitochondrial activity by kinases signaling remains
480 unclear.

481 Although regulators of protein breakdown, FoxO1 and FoxO3 are key factors of muscle
482 energy homeostasis through the mitochondrial metabolism. Yuan et al. (Yuan et al., 2011)
483 demonstrated that FoxO1 induces slow to fast-twitch fiber transition, and down-regulates
484 muscle oxidative capacity by inhibiting the calcineurin pathway and blocked resveratrol-
485 induced mRNA abundance of myoglobin and gene TnI slow, i.e., oxidative fiber markers.
486 In contrast, we observed that the fast-to-slow fiber type shift were accompanied by a
487 reduction in FoxO1 levels only in ET/H. Furthermore, Slopack et al. (Slopack et al., 2014)
488 reported that ET down-regulated the acute exercise-induced increase in endothelial
489 FoxO1 and FoxO3a, and reduction in FoxO1 results in an earlier angiogenic response.
490 The increase in muscle capillarization is an adaptation that contributes to the increased
491 $\dot{V}O_{2peak}$, and may optimize delivery and extraction of oxygen to the working tissues
492 (Bassett and Howley, 2000; Saltin, 1998) which may increase endurance. Hesketh et al.,
493 (Hesketh et al., 2019) related that passive heat therapy in sedentary humans increased
494 skeletal muscle capillarization, and in accordance with our previous study (Zanetti et al.,
495 2024) increased endurance in temperate conditions. Since our western blot analysis was
496 performed with whole muscle homogenate, including vessels, it remains unclear if this is
497 a vessel-specific adaptation or muscle also contribute to these reduced protein level.
498 Concerning FoxO3, its directly accumulated into mitochondria under low-glucose
499 condition (in an AMPK-dependent manner) increasing mitochondrial respiration by
500 binding to mitochondrial DNA (Peserico et al., 2013). However, 8-week ET did not
501 change the content of phosphorylated FoxO3 [at Ser^{413} (by AMPK), Ser^{294} (by MAPK
502 ERK1/2), and Thr^{32} (by Akt)] and its regulators [i.e., AMPK, Protein kinase B (Akt), and
503 MAPK ERK1/2; data not shown] (Wang et al., 2017). Thus, we hypothesized that training
504 in heat decrease FoxO1 content and improve endurance by an alternative pathway than
505 ET in temperate.

506 ## 5. CONCLUSION

507 Fast-to-slow fiber-type transition is a critical factor that plays an important role in skeletal
508 muscle to ET. In summary, the T_a-induced dissociation between relative and absolute
509 exercise intensity allow to train at lower absolute mechanical load, and by alternatives
510 pathways, i.e., selective increase in oxidative fibers CSA and decreased in FoxO1 content,
511 achieve the same adaptions. Here, we propose T_a as a novel ET prescription variable
512 which may assist individuals who are unable to undertake their usual mechanical loading
513 and aid both sporting and clinical evidence-based heat therapy.

514

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672

673 **LEGENDS**

674 **Fig. 1:** Schematic representation of the experimental procedures that animals were
675 submitted. ←→, Crossover; ET, Endurance training; FAM, familiarization protocol; H
676 ILT, Incremental load test in hot environment; TC, Tissue collection; T ILT, Incremental
677 load test in temperate environment.

678 **Fig. 2:** Effect of incremental load test on time to fatigue (TTF) and running distance (B)
679 in pre-endurance training (ET) period (A and B, respectively) and during nine week ET
680 on treadmill in temperate (C and D, respectively) and hot (E and F, respectively)
681 environment. ϕ, P ≤ 0,05 vs. T; *, $P \leq 0{,}05$ vs. same group - pre-training; @, $P \leq 0{,}05$ vs.
682 SED/T same time-point; β, $P \leq 0{,}05$ vs. SED/H same time-point; γ, $P = 0{,}05$ - 0,09 vs.
683 SED/T same time-point. SED/T, sedentary in T environment. ET/T, trained in T
684 environment. SED/H, sedentary in H environment. ET/H, trained in H environment. 4wk
685 and 8wk represent 4 and 8 weeks of experimental protocol, respectively (n = 13-
686 14/group).

687 **Fig. 3:** Effect of endurance training (ET) on treadmill for nine weeks in temperate (T)
688 and hot (H) environment on skeletal muscle integrity aspects (A) and centrally nucleated
689 fibers (CNFs; B) proportion. SED/T, sedentary in T environment. ET/T, trained in T
690 environment. SED/H, sedentary in H environment. ET/H, trained in H environment.

691 **Fig. 4:** Effect of endurance training (ET) on treadmill for nine weeks in temperate (T)
692 and hot (H) environment on total content of slow and fast myosin heavy chain (MyHC;
693 A and B) and MyHC (C) % fiber type (D), and cross-sectional area (E) by
694 immunofluorescence. Scale bar, 50µm. SED/T, sedentary in T environment. ET/T,
695 trained in T environment. SED/H, sedentary in H environment. ET/H, trained in H
696 environment (n = 6–7/group).

697 **Fig. 5:** Effect of endurance training (ET) on treadmill for nine weeks in temperate (T)
698 and hot (H) environment on staining for succinate dehydrogenase (SDH; A) activity (B),
699 % fiber type (C), and cross-sectional area (D). Scale bar, 50µm. @, $P \leq 0{,}05$ vs. SED/T
700 same time-point; γ, $P = 0{,}05 - 0{,}09$ vs. SED/T same time-point. SED/T, sedentary in T
701 environment. ET/T, trained in T environment. SED/H, sedentary in H environment.
702 ET/H, trained in H environment (n = 6–7/group).

703 **Fig. 6:** Effect of endurance training (ET) on treadmill for nine weeks in temperate (T)
704 and hot (H) environment on protein content of mitochondrial oxidative phosphorylation
705 complexes (OxPhos; A - F) as well as mitochondrial content (TOM20; A and G). SED/T,
706 sedentary in T environment. ET/T, trained in T environment. SED/H, sedentary in H
707 environment. ET/H, trained in H environment (n = 6–7/group).

708 **Fig. 7:** Effect of endurance training (ET) on treadmill for nine weeks in temperate (T)
709 and hot (H) environment on the content of the PGC-1α and TFEB intracellular regulators
710 (i.e., AMPK-p38-CAMKII; A - F). SED/T, sedentary in T environment. ET/T, trained in
711 T environment. SED/H, sedentary in H environment. ET/H, trained in H environment (n
712 = 6–7/group).

713 **Fig. 8:** Effect of endurance training (ET) on treadmill for nine weeks in temperate (T)
714 and hot (H) environment on the content of muscle proteins involved in energy metabolism
715 regulation (A – I). SED/T, sedentary in T environment. ET/T, trained in T environment.
716 SED/H, sedentary in H environment. ET/H, trained in H environment (n = 6–7/group).

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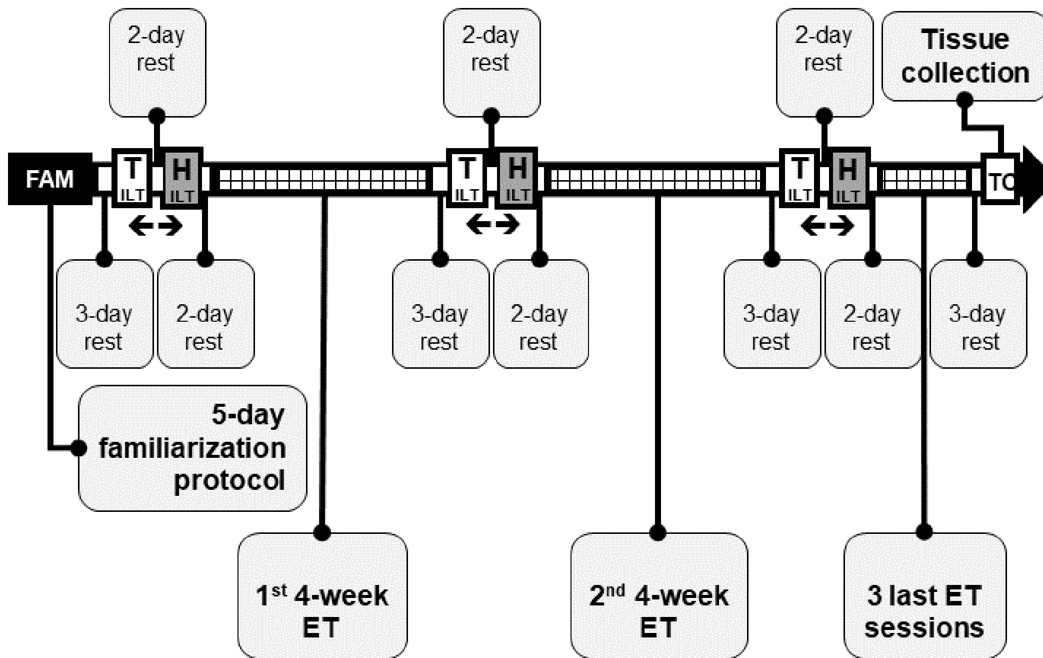
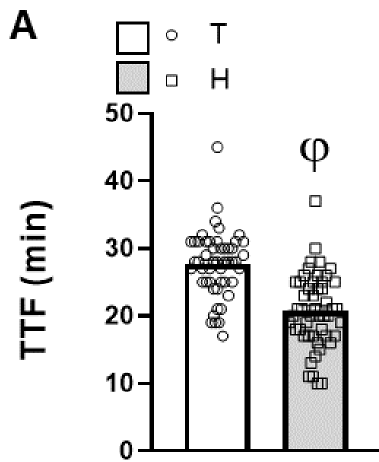
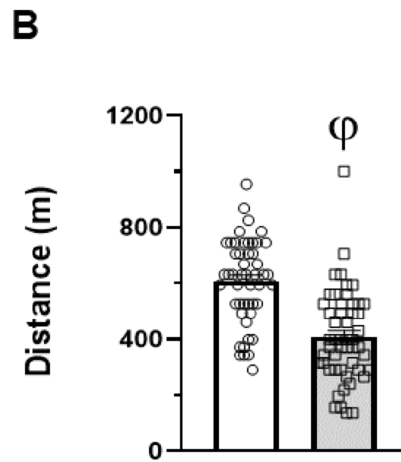
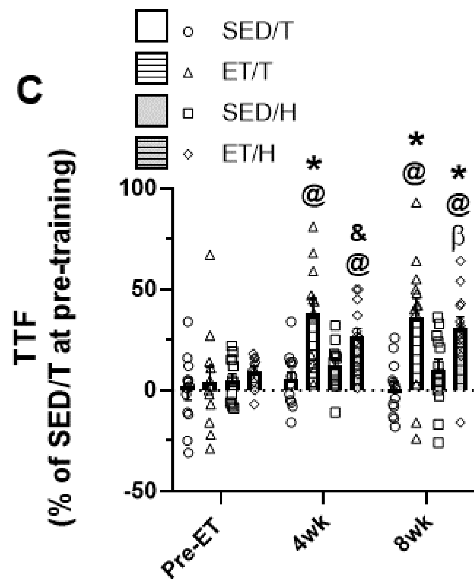
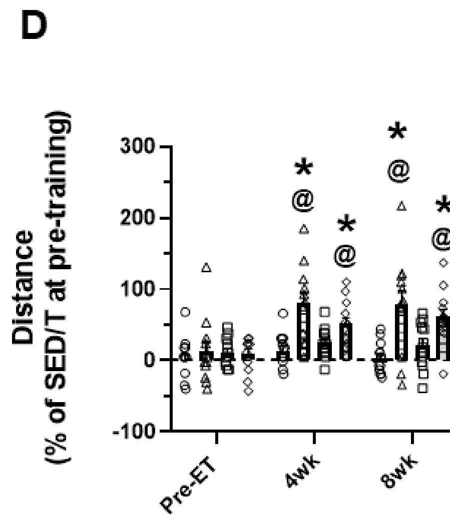


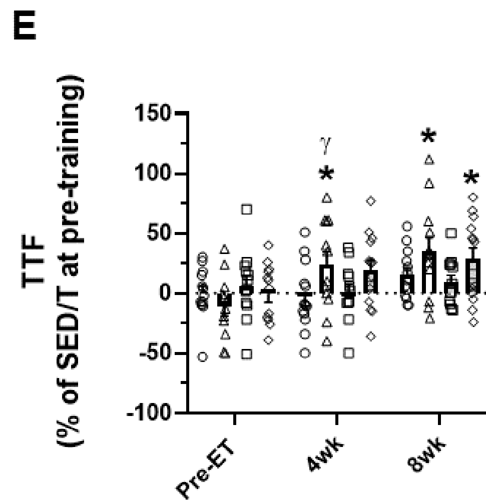
2-day rest
2-day rest
2-day rest
Tissue collection
FAM
T ILT
H ILT
TC
3-day rest
2-day rest
3-day rest
2-day rest
3-day rest
2-day rest
3-day rest
5-day familiarization protocol
1st 4-week ET
2nd 4-week ET
3 last ET sessions

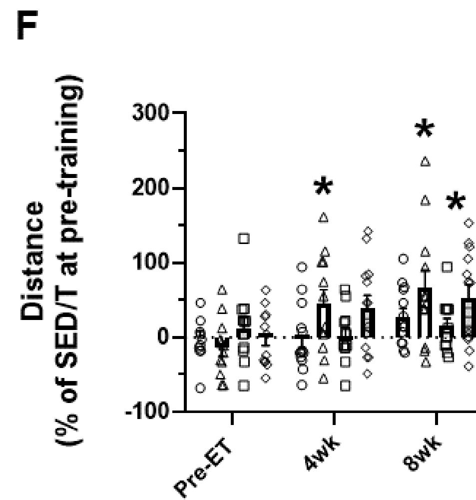

A
T
H
TTF (min)
φ
50
40
30
20
10
0

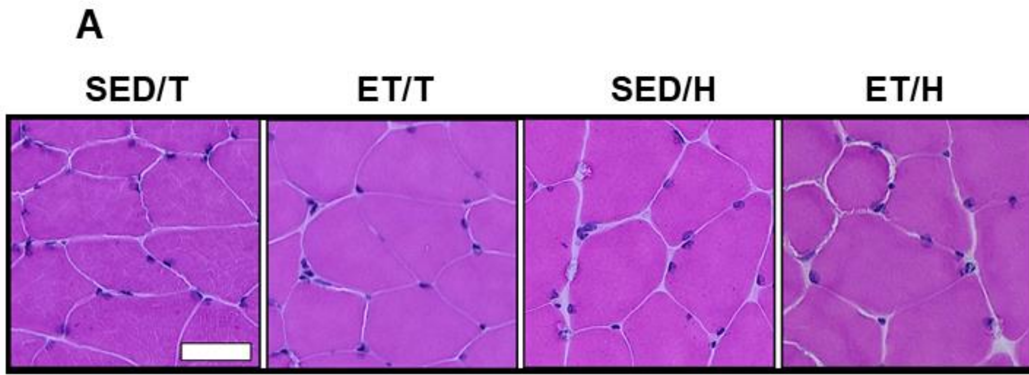

B
Distance (m)
φ
1200
800
400
0

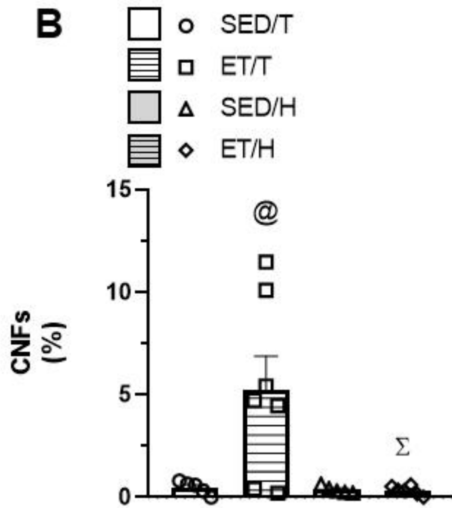

SED/T
ET/T
SED/H
ET/H
C
TTF
(% of SED/T at pre-training)
100
50
0
-50
Pre-ET
4wk
8wk

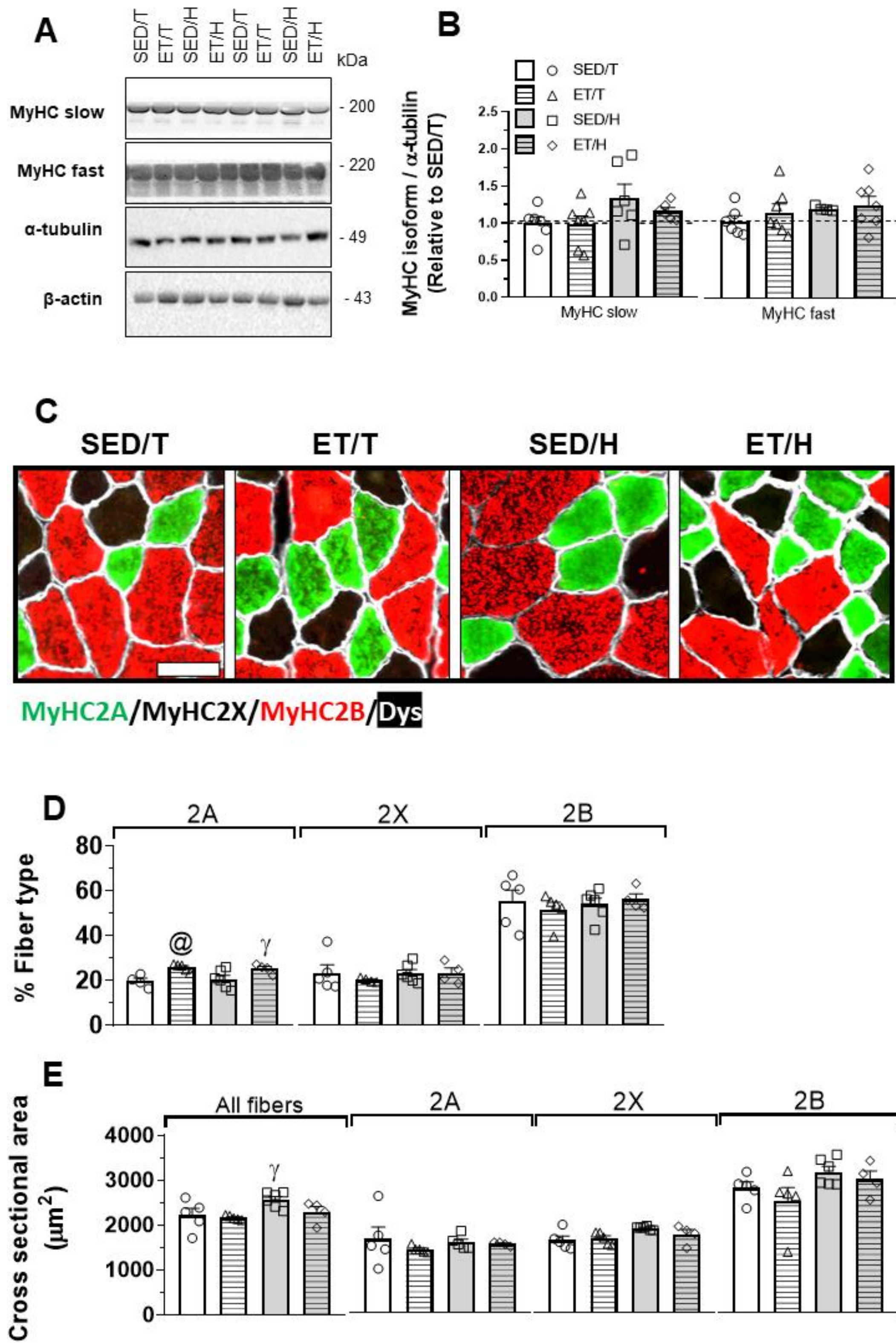

D
Distance
(% of SED/T at pre-training)
300
200
100
0
-100
Pre-ET
4wk
8wk

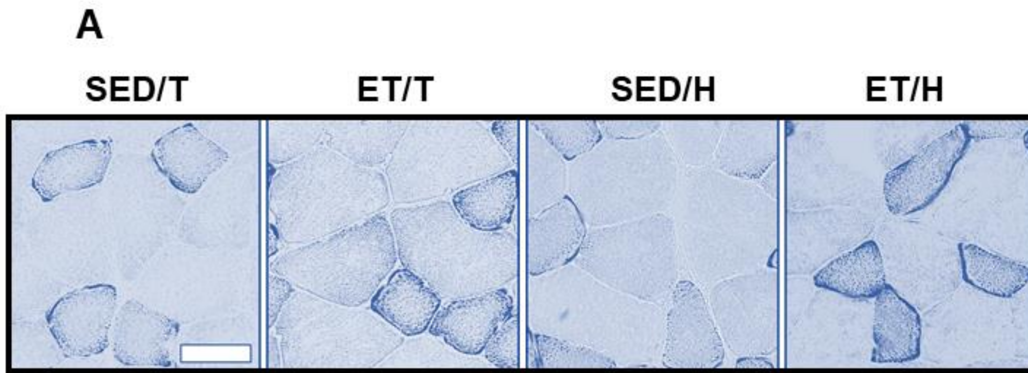

E
TTF
(% of SED/T at pre-training)
150
100
50
0
-50
-100
Pre-ET
4wk
8wk

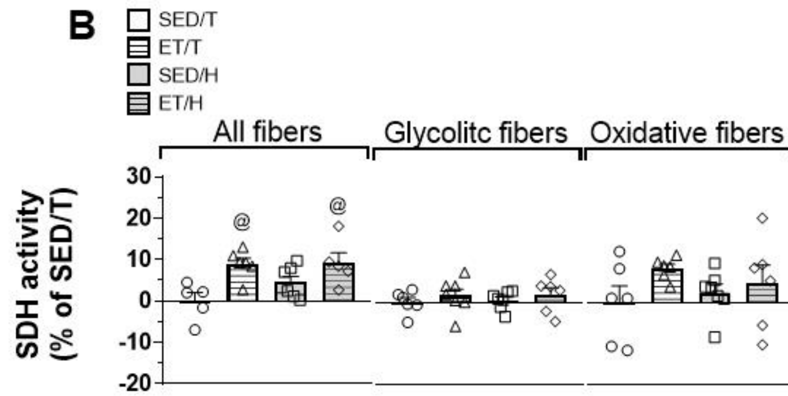

F
Distance
(% of SED/T at pre-training)
300
200
100
0
-100
Pre-ET
4wk
8wk

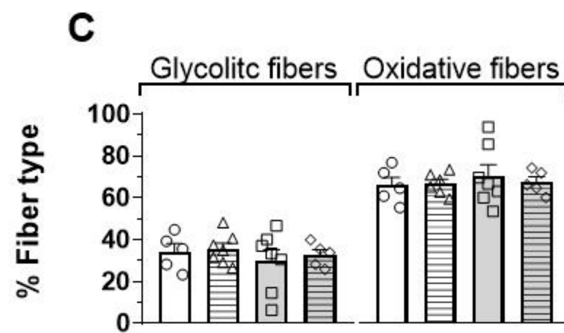

A
SED/T
ET/T
SED/H
ET/H

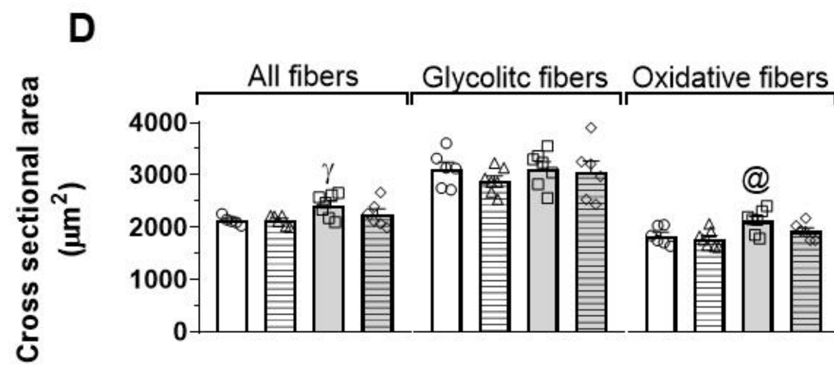

B
SED/T
ET/T
SED/H
ET/H
CNFs (%)
15
10
5
0
@
Σ

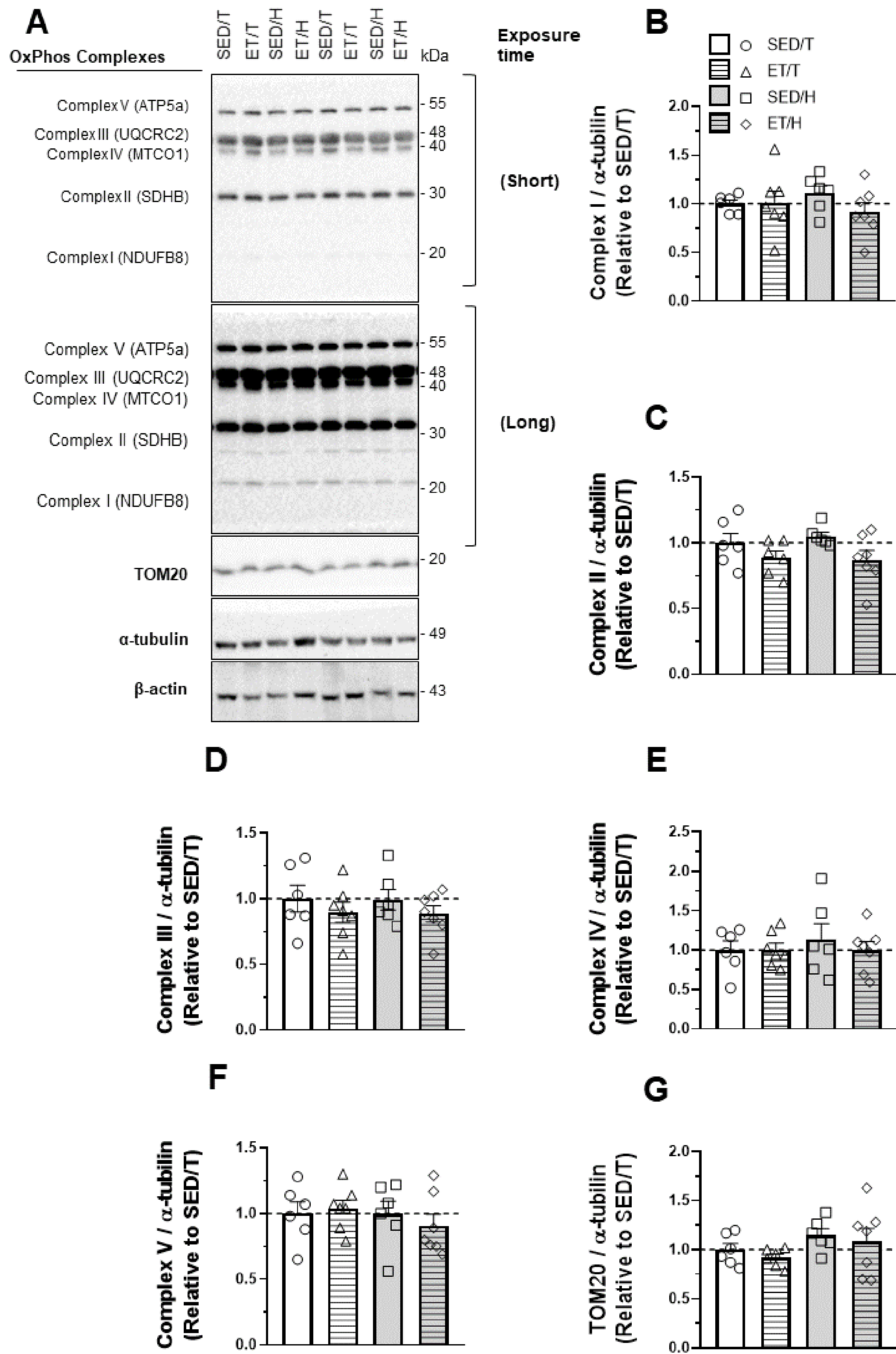

A
SED/T
ET/T
SED/H
ET/H
SED/T
ET/T
SED/H
ET/H
kDa
MyHC slow
- 200
MyHC fast
- 220
α-tubulin
- 49
β-actin
- 43
B
SED/T
ET/T
SED/H
ET/H
MyHC isoform / α-tubilin (Relative to SED/T)
MyHC slow
MyHC fast
C
SED/T
ET/T
SED/H
ET/H
MyHC2A/MyHC2X/MyHC2B/Dys
D
2A
2X
2B
% Fiber type
@
γ
E
All fibers
2A
2X
2B
Cross sectional area (μm²)
γ

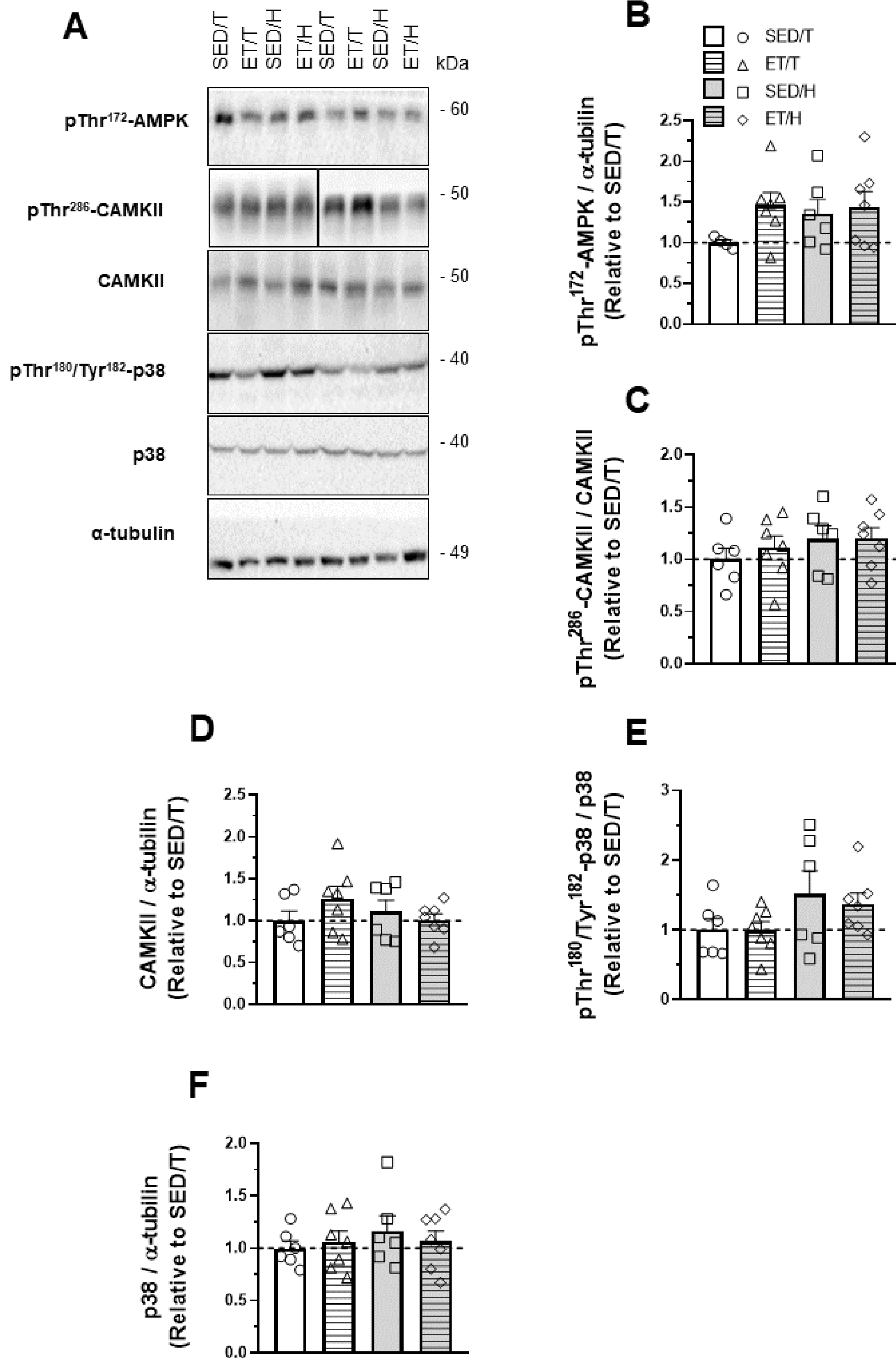

A
SED/T
ET/T
SED/H
ET/H

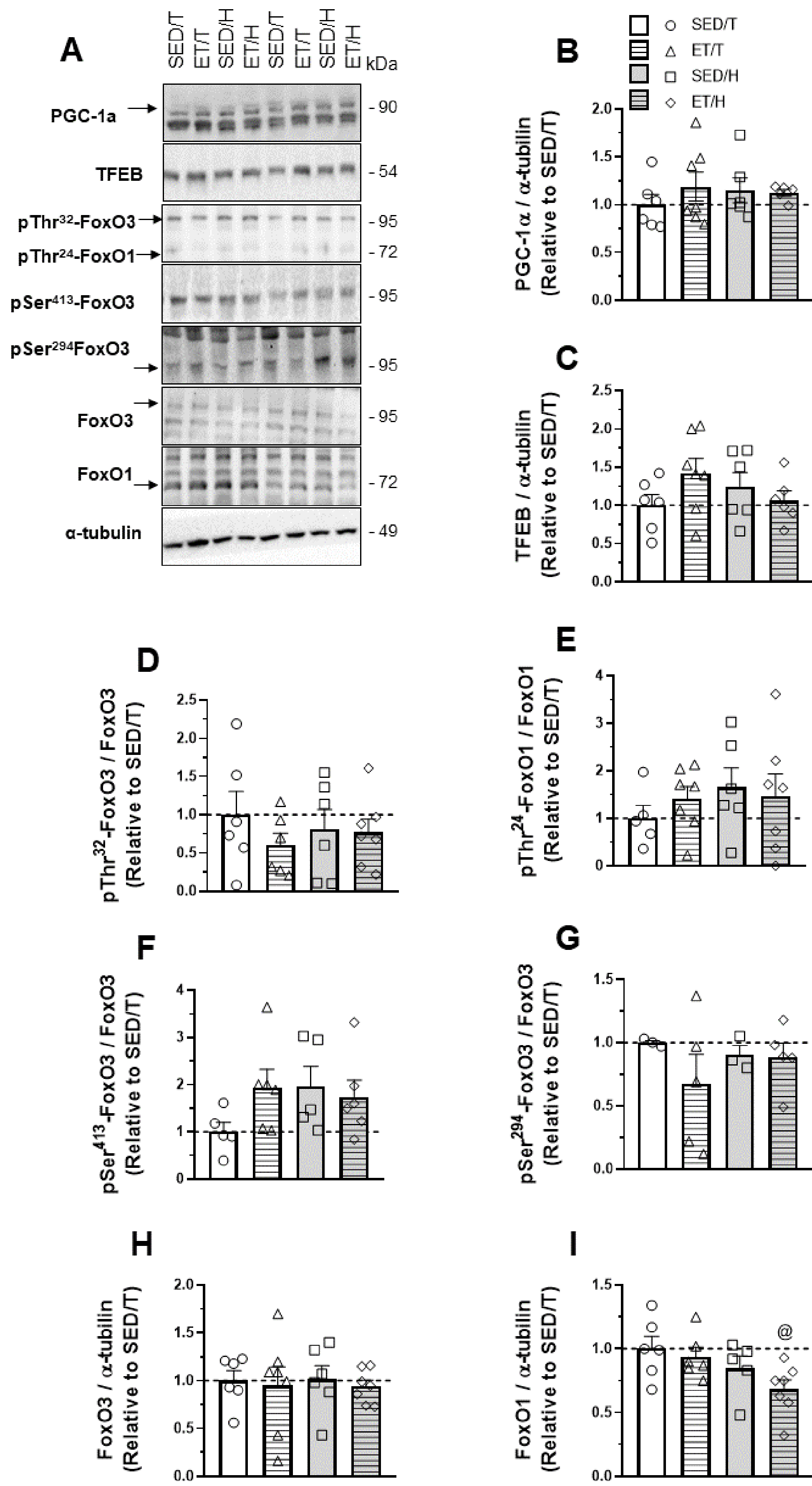

B
SED/T
ET/T
SED/H
ET/H
All fibers
Glycolitc fibers
Oxidative fibers
SDH activity
(% of SED/T)
30
20
10
0
-10
-20
@
@


C
Glycolitc fibers
Oxidative fibers
% Fiber type
100
80
60
40
20
0


D
All fibers
Glycolitc fibers
Oxidative fibers
Cross sectional area
(µm2)
4000
3000
2000
1000
0
γ
@


A
OxPhos Complexes
SED/T
ET/T
SED/H
ET/H
kDa
Exposure time
Complex V (ATP5a)
Complex III (UQCRC2)
Complex IV (MTCO1)
Complex II (SDHB)
Complex I (NDUFB8)
(Short)
(Long)
TOM20
α-tubulin
β-actin
B
C
D
E
F
G
Complex I / α-tubilin (Relative to SED/T)
Complex II / α-tubilin (Relative to SED/T)
Complex III / α-tubilin (Relative to SED/T)
Complex IV / α-tubilin (Relative to SED/T)
Complex V / α-tubilin (Relative to SED/T)
TOM20 / α-tubilin (Relative to SED/T)


A
SED/T
ET/T
SED/H
ET/H
SED/T
ET/T
SED/H
ET/H
kDa
pThr172-AMPK
- 60
pThr286-CAMKII
- 50
CAMKII
- 50
pThr180/Tyr182-p38
- 40
p38
- 40
α-tubulin
- 49
B
SED/T
ET/T
SED/H
ET/H
pThr172-AMPK / α-tubulin (Relative to SED/T)
C
pThr286-CAMKII / CAMKII (Relative to SED/T)
D
CAMKII / α-tubulin (Relative to SED/T)
E
pThr180/Tyr182-p38 / p38 (Relative to SED/T)
F
p38 / α-tubulin (Relative to SED/T)



727

Anexo

TITLE: Resveratrol directly suppresses proteolysis possibly via PKA/CREB signaling in denervated rat skeletal muscle

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Keywords: Intracellular Signaling Pathways; Muscle atrophy and hypertrophy; Sciatic nerve resection; Polyphenol; Protein Metabolism.

Running title: Resveratrol inhibits proteolysis via PKA pathway in denervated muscle

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50 **ABSTRACT**

51 Although there are reports that polyphenol resveratrol (Rsv) may cause muscle
52 hypertrophy in basal conditions and attenuate muscle wasting in catabolic situations, its
53 mechanism of action is still unclear. Our study evaluated the *ex vivo* effects of Rsv on
54 protein metabolism and intracellular signaling in innervated (sham-operated; Sham) and
55 3-day sciatic denervated (Den) rat skeletal muscles. Rsv (10^{-4} M) reduced total
56 proteolysis (40%) in sham muscles. Den increased total proteolysis (~40%) in muscle,
57 which was accompanied by an increase in the activities of ubiquitin-proteasome (~3-
58 fold) and lysosomal (100%) proteolytic systems. Rsv reduced total proteolysis (59%) in
59 Den muscles by inhibiting the hyperactivation of ubiquitin-proteasome (50%) and
60 lysosomal (~70%) systems. Neither Rsv nor Den altered calcium-dependent proteolysis
61 in muscles. Mechanistically, Rsv stimulated PKA/CREB signaling in Den muscles, and
62 PKA blockage by H89 (50µM) abolished the antiproteolytic action of the polyphenol.
63 Rsv reduced FoxO4 phosphorylation (~60%) in both Sham and Den muscles and Akt
64 phosphorylation (36%) in Den muscles. Rsv also caused a homeostatic effect in Den
65 muscles by returning their protein synthesis rates to levels similar to Sham muscles.
66 These data indicate that Rsv directly inhibits the proteolytic activity of lysosomal and
67 ubiquitin-proteasome systems, mainly in Den muscles through, at least in part, the
68 activation of PKA/CREB signaling.

69

INTRODUCTION

Skeletal muscle is the largest tissue in the body, accounts for 40-50% of body mass and plays a central role in whole-body metabolism, locomotion, thermoregulation, and other biological functions. Maintaining skeletal muscle mass and function is essential for human healthy. Muscle mass is regulated by dynamic protein turnover, which involves protein synthesis and breakdown. The imbalance between catabolic and anabolic processes may result in muscle hypertrophy (anabolic > catabolic) or atrophy (anabolic < catabolic), the latter being a common symptom of chronic diseases such as diabetes (Perry et al. 2016) and motor denervation (Den) (Furuno et al. 1990). Therefore, identifying therapeutic targets capable of inhibiting proteolysis is necessary for treating muscle loss and improve the disease prognosis.

Motor innervation is crucial for muscle physiology, including development, differentiation, metabolism, trophic status, and force production (Das et al. 2020). On the other hand, Den results in muscle inactivity and, consequently, atrophy during the early stages (Tang et al. 1984, Adhihetty et al. 2007). Den-induced muscle loss may be due to an increase in the rate of proteolysis exceeding the rate of protein synthesis (Furuno et al. 1990). Autophagy/lysosomal and ubiquitin-proteasome (UPS) systems have been considered the critical regulators for the bulk of muscle proteolysis (Sartori et al. 2021). However, these proteolytic systems cannot degrade myofibrils attached to the sarcomere. It has been suggested that calpains, the proteases of calcium-dependent proteolytic system, may initially cleave proteins leading to sarcomere disassembly and myofibrils degradation by UPS (Tidball & Spencer 2002, Kachaeva & Shenkman 2012). The blockade of calpain via the PD150606, a calpain inhibitor, attenuates UPS

94 activity and prevents unloading-induced skeletal muscle atrophy in rats (Shenkman et
95 al. 2015).

96 In addition to calpains, the transcription of a common set of atrophy-related
97 genes termed atrogenes is up- and down-regulated during muscle atrophy in different
98 catabolic states (Sacheck et al. 2007, Lecker et al. 2004). Muscle RING finger 1
99 (*MuRF1* or *Trim63*) and Atrogin-1 (also known as *MAFbx* or *Fbxo32*) are two muscle-
100 specific E3 ligases of UPS. These enzymes are up-regulated in several animal models of
101 atrophy, such as Den and fasting (Sacheck et al. 2007). Autophagy-related genes, such
102 as microtubule-associated protein 1 light chain 3 beta (*Map1lc3b*) and GABA type A
103 receptor-associated protein (*Gabarap*), are also up-regulated in muscles from Den or
104 fasted mice (Mammucari et al. 2007, Sartori et al. 2021). Most of these atrogenes are
105 under transcriptional control of Forkhead box O (FoxO) factors, which play a critical
106 role in muscle atrophy (Mammucari et al. 2007). FoxO, in turn, may be regulated by
107 several intracellular pathways, including insulin/insulin-like growth factor-1 (IGF-
108 1)/phosphatidylinositol 3-kinase (PI3K)/Akt signaling (Sandri et al. 2004, Milan et al.
109 2015, Mammucari et al. 2007). Both insulin and IGF-1 activate Akt that phosphorylates
110 FoxO proteins exporting them from the nucleus to the cytoplasm and reducing their
111 activity (Brunet et al. 1999, Sandri 2008) .

112 Resveratrol (Rsv; 3,5,4'-trihidroxistilbene), a natural antioxidant found in the
113 skins of red grapes as well as other plants such as peanuts, has shown to induce *in vivo*
114 both muscle hypertrophy in rest (Woodman et al. 2021) and exercise trained (Alway et
115 al. 2017) conditions and muscle antiatrophic effect in several experimental catabolic
116 situations, such as sarcopenic obesity (Huang et al. 2019) and Den (Asami et al. 2018).
117 However, the mechanism underlying Rsv action in muscle protein metabolism and
118 trophic status remains unclear. Moreover, whether Rsv acts directly or indirectly in

119 skeletal muscle promoting its protein anabolic and anticatabolic effects has been poorly
120 investigated. Rsv is a competitive and non-selective inhibitor of phosphodiesterases, a
121 superfamily of enzymes responsible for the intracellular degradation of cyclic adenosine
122 monophosphate (cAMP), thereby being able to elevate cAMP level as soon as 10 min
123 after the incubation with Rsv (Park et al. 2012). Upon binding of cAMP to regulatory
124 subunits of protein kinase A (PKA-R), their catalytic subunits (PKAcat) are activated
125 and detach from regulatory subunits phosphorylating several proteins, including CREB
126 transcription factor, the bona fide target of PKA (Silveira et al. 2020). Recently, it has
127 been shown that the genetic gain-of-fucntion of PKA mitigates FoxO activity and
128 atrogenes overexpression, and its genetic loss-of-function causes muscle loss in basal
129 condition (Silveira et al. 2020).

130 Based on these findings, we hypothesized that Rsv could directly stimulate
131 protein synthesis and inhibit protein degradation in innervated and denervated muscles
132 via activation of the cAMP/PKA pathway. Therefore, this study aimed to evaluate the
133 *ex vivo* effect of Rsv on protein metabolism and intracellular signaling pathways in
134 muscles innervated (sham-operated rats; sham) and undergoing atrophy due to sciatic
135 nerve resection (Den). Our data indicate that Rsv suppresses total proteolysis in both
136 innervated and Den muscles, especially the lysosomal and UPS proteolytic systems in
137 the latter condition. The pharmacological loss-of-function experiments suggest that
138 these effects of Rsv are mediated by the activation of PKA/CREB signaling. Rsv also
139 caused a homeostatic effect in Den muscles by returning their protein synthesis rates to
140 levels similar to Sham muscles.

141

142 **MATERIALS AND METHODS**

143 *Animals and sciatic denervation model*

144 Wistar male rats (±80 g body mass; 4 weeks old; *n* = 4-6/group) were used in all
145 experiments. Animals were housed in a room with a 12–12-h light-dark cycle and were
146 given free access to water and a normal laboratory chow diet for at least 2 days before
147 the beginning of the experiments, which were performed at 8:00 A.M. All animal
148 experiments were conducted following with the guidelines established by the Animal
149 Ethics Committee of Federal University of Pernambuco (CEUA/UFPE), which also
150 approved the protocols (Approval No. 23076.01234/2012-79), and are in accordance
151 with ethical principles in animal research.

152 The animals were anesthetized with ketamine hydrochloride solution (115 mg /
153 Kg, i.p.) and xylazine (10 mg / Kg, i.p.) and underwent bilateral motor denervation
154 (Den) caused by a surgical section of the sciatic nerve, with the removal of ~2 mm of
155 nerve. Den was chosen as an atrophy model because it induces a significant loss of
156 contractile proteins that is due primarily to the enhancement of protein breakdown
157 (Gonçalves et al. 2012). Sham (innervated) group was subjected to surgical stress,
158 which included nerve visualization, but not its transection.

159

160 *Isolated skeletal muscles and resveratrol incubation*

161 Three days after Den (or sham operation), animals were euthanized by cervical
162 dislocation, and *extensor digitorum longus* (EDL) muscles were carefully removed,
163 avoiding damage to the muscles. EDL muscles were chosen due to their high proportion
164 of fast glycolytic fibers that are more sensitive to the protein anabolic effects of cAMP
165 enhancers such as rolipram (Lira et al. 2011) and formoterol (Gonçalves et al. 2019).
166 The EDL muscle was rapidly macrodissected, weighed (innervated muscles: ~55g;
167 denervated muscles: ~40g), and maintained at approximately its resting length by
168 pinning their tendons on inert plastic supports. Tissues were incubated at 37°C in

169 Krebs-Ringer bicarbonate buffer (pH 7.4) containing 5 mM glucose equilibrated with
170 95% oxygen and 5% carbon dioxide. After 1h of preincubation in buffer medium,
171 muscle from one limb was incubated for 2 h in the presence of resveratrol (Rsv, 10^{-5} or
172 10^{-4} M), while muscle from the contralateral limb was incubated with vehicle
173 dimethylsulfoxide (DMSO, 1 %).
174
175 *Determination of total proteolysis and the activity of proteolytic systems*
176 Total proteolysis and the activity of proteolytic systems (ubiquitin-proteasome
177 (UPS), lysosomal, and calcium-dependent) were measured using a method as described
178 previously (Gonçalves et al. 2019). Briefly, proteolysis was measured by following the
179 tyrosine released into the medium in the presence of cycloheximide (0.5 mM),
180 preventing protein synthesis and reincorporation of tyrosine back into proteins. After 1
181 h preincubation, muscles were incubated for 2 h in a fresh medium with an identical
182 composition. For measurement of *UPS* activity, muscles from one limb were incubated
183 with a calcium-free medium containing 25 μM E64, 50 μM leupeptin, 10 mM
184 methylamine, 1 U/ml insulin, 170 μM leucine, 100 μM isoleucine, 200 μM valine to
185 prevent activation of calcium-dependent and lysosomal systems. Muscles from the
186 contralateral limb were incubated with the proteasome inhibitor MG132 (20 μM). For
187 measurement of *lysosomal* proteolysis, muscles from one limb were incubated in the
188 presence of the inhibitors of lysosomal proteolysis, i.e., insulin, leucine, isoleucine,
189 valine, and methylamine. Contralateral muscles were incubated in a "normal" buffer
190 medium without these inhibitors. For measurement of *calcium-dependent* proteolytic
191 activity, muscles from one limb were incubated in the presence of calcium and
192 inhibitors of the lysosomal system. Contralateral muscles were incubated in a calcium-
193 free medium containing lysosomal inhibitors and cysteine-protease inhibitors (E64 and

194 leupeptin). UPS, lysosomal and calcium-dependent proteolytic activities were calculated
195 from tyrosine release differences between the left and right muscles. Tyrosine release
196 was assayed using the fluorometric method (WAALKES & UDENFRIEND 1957).
197
198 *Determination of the rate of protein synthesis*
199 The protein synthesis rate was measured using a method as described previously
200 (Gonçalves et al. 2012). Briefly, EDL muscles were incubated in a buffer containing all
201 amino acids at concentrations similar to those of rat plasma. After a 1 h equilibration
202 period, L-[U-^{14}C]tyrosine (0.05 μCi/ml) was added to the replacement medium, in
203 which the muscles were incubated for the next 2 h. At the end of this period, the specific
204 activity of acid-soluble tyrosine (intracellular tyrosine pool) in each muscle was
205 estimated by measuring the radioactivity and the concentration of tyrosine in this pool,
206 which was determined by the method of (WAALKES & UDENFRIEND 1957). After
207 that, the rate of protein synthesis was calculated using the specific activity of the
208 intracellular pool of tyrosine in each muscle.
209
210 *Analysis of gene expression by real-time qPCR*
211 Real-time qPCR was used to analyze the expression levels of the atrogenes
212 *Fbxo32*, *Trim63,* and *Map1lc3b*. The analysis of qPCR was performed at the Laboratory
213 of Metabolism Control from Ribeirão Preto Medical School (University of São Paulo).
214 After the incubation procedure described above, EDL muscle was immediately frozen in
215 liquid nitrogen and stored at -80°C for less than one month. Total RNA was extracted
216 from muscle using TRIzol (50 mg of muscle was added to 0,5 ml of TRIzol,
217 Invitrogen®). Samples were homogenized in tubes using a TissueLyser II (Qiagen®)
218 with 5 mm stainless steel beads for 2 × 1 min cycles at 30 Hz, resting on ice in between.

219 Homogenates were cleared by centrifugation at 10,000 x g for 5 min at 4 °C. RNA
220 extraction was performed according to TRIzol manufacturer's instructions
221 (Invitrogen®). RNA was eluted in 50 µl of RNase-free water and stored at − 80 °C.

222 RNA samples were treated with DNase I, RNase-free (Thermo Fisher
223 Scientific®), to remove genomic DNA contamination. RNA samples were quantitated
224 using NanoDrop One spectrophotometer (Thermo Fisher Scientific®), following the
225 manufacturer's instructions. The same device was used to assess the purity of RNA by
226 measuring 260/280 and 260/230 ratios of absorbance values. Samples presenting a
227 260/280 ratio of ~2 and 260/230 ratio of 2 to 2.2 were accepted as "pure" for RNA.

228 According to the manufactures' protocols, one microgram of RNA was reverse
229 transcribed into cDNA using 0,5 µL of SuperScript IV First-Strand Synthesis System
230 (Invitrogen®). cDNA was diluted 25-fold with nuclease-free water. For qPCR, the total
231 volume per reaction was 10 µL containing 5 µL of cDNA (2 ng/µL), 4.8 µL of PowerUp
232 SYBR Green Master Mix (Thermo Fisher®), and 0.2 µL of primers (forward and reverse
233 mixture; 50 µmol/L stock). qPCR run on Applied Biosystems™ 7500 Real-Time PCR
234 System, using the recommended cycling conditions as follows: a pre-incubation of
235 2 min at 50 °C and 10 min at 95 °C, followed by a two-step amplification program of
236 40 cycles set at 95 °C for 15 s (denaturation) and 60 °C for 1 min (annealing +
237 extension) and, finally, a dissociation stage set at 95 °C for 15 s, 60 °C for 1 min and
238 95 °C for 15 s. The last stage was performed to evaluate the quality of qPCR reactions
239 regarding of nonspecific amplification and primer-dimer formation in a dissociation
240 curve for each gene. The amplification specificity for each primer was confirmed by
241 observing the single melt curve peak after the completion of qPCR.

242 Primers used were *Fbxo32* (forward 5'-CTT TCA ACA GAC TGG ACT TCT
243 CGA-3' and reverse 5'-CAG CTC CAA CAG CCT TAC TAC GT-3'), *Trim63*

244 (forward 5'-TCG ACA TCT ACA AGC AGG AA-3' and reverse 5'-CTG TCC TTG
245 GAA GAT GCT TT-3'), *Map1lc3b* (forward 5'-TTT GTA AGG GCG GTT CTG AC-
246 3' and reverse 5′-CAG GTA GCA GGA AGC AGA GG-3′) and *Rpl39* (forward 5'-
247 TCC TGG CAA AGA AAC AAA AGC-3' and reverse 5'-TAG ACC CAG CTT CGT
248 TCT CCT-3'). Primer sequences were designed utilizing Primer3Plus
249 (https://www.primer3plus.com/) in conjunction with OligoAnalyzer 3.1
250 (https://eu.idtdna.com/site) and cross-referenced using the Basic Local Alignment
251 Search Tool program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A six-point relative
252 standard curve was prepared for each gene by using five-fold serial dilutions of pooled
253 cDNA samples in duplicate. No threshold cycle quantification value for the no template
254 control was detected. The slope values and amplification efficiencies estimated from the
255 standard curve, respectively, were as follows: *Fbxo32* (-3,3 and 99%), *Trim63* (-3,1 and
256 112%), *Map1lc3b* (-3,8 and 83%) and *Rpl39* (-3,1 and 108%).

257 The relative expression levels of target genes were calculated using the $2^{-\Delta\Delta Ct}$
258 method (Schmittgen & Livak 2008). All reactions were carried out in four to five
259 biological replicates and two technical replicates. Data from the target genes were
260 normalized by the expression of *Rpl39*, which was used as a reference gene because it
261 has been shown to be the most stable gene in Den muscles compared with other
262 reference genes, such as *Gapdh*, *Actb* and, *Ppia* (unpublished data). Moreover, *Rpl39*
263 has been previously used as a reference gene in other studies in skeletal muscle
264 (Gonçalves et al. 2019, de O. Coelho et al. 2019).

265

266 *Analysis of protein expression by Western Blot*

267 After incubation, EDL muscle was quickly frozen in liquid nitrogen and stored
268 at -80 °C. These muscles were collected and homogenized in buffer RIPA (Tris-HCl 50

269 mM, pH 7.4), containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 % sodium
270 deoxycholate, 1% SDS, 10mM sodium pyrophosphate, 100mM sodium fluoride, 10mM
271 sodium orthovanadate, 5g/ml aprotinin, 1mg/ml leupeptin, 1mM phenylmethylsulfonyl
272 fluoride (PMSF), 50 μM MG132. The homogenate was centrifuged at 21,000 g at 4 °C.
273 The supernatant was collected, and the amount of protein was determined by the method
274 of (Lowry et al. 1951) using BSA (bovine serum albumin) as standard. An equal
275 volume of sample buffer [20 % glycerol, 125 mM Tris-HCl, 4 % SDS, 100 mM
276 dithiothreitol (DTT), 0.02 % bromophenol blue, pH 6.8] was added to the supernatant,
277 and the mixture was boiled at 70 °C for 10 min. 50 µg of total proteins were separated
278 by SDS-PAGE acrylamide gel at 10-12 %, and after transferred to a nitrocellulose
279 membrane~~,~~ and blotted with anti-phospho (p)-Ser^{473} Akt (1:750, #9271, Cell Signaling
280 Technology, Massachusetts, USA), anti-p-Ser$^{256/193}$ FoxO1/4 (1:750, #9461, Cell
281 Signaling), anti-p-Ser^{133} CREB (1:750, #9198, Cell Signaling), anti-p-Thr^{172} AMPK
282 (1:750, #2531, Cell Signaling), anti-p-Thr^{389} S6K1 (1:750, #9234, Cell Signaling) and
283 anti-β-actin (1:2000; sc-81178; Santa Cruz Biotech, Texas, USA). Primary antibodies
284 (Ab) were detected using peroxidase-conjugated secondary Ab (1:2000 for p-Ser$^{256/193}$-
285 FoxO1/4, p- Ser^{473}-Akt, p-Ser^{133} CREB, anti-p-Thr^{172} AMPK, anti-p-Thr^{389} S6K1 and
286 1:5000 for other primary antibodies) and visualized using ECL method using a
287 detection system ChemiDoc MP (Bio-Rad) and software ImageLab (version 5.2.1, Bio-
288 Rad). Band intensities were quantified using ImageJ2 (version 1.53c, National Institutes
289 of Health, USA).

290

291 *Statistical analysis*

292 The distribution and variance homogeneity were tested using Shapiro-Wilk test.
293 Data were expressed as mean ± S.E.M. The one-way analysis of variance (ANOVA)

294 followed by the Bonferroni test was employed to analyze total proteolysis in response to
295 different Rsv concentrations. Two-way analysis of variance (ANOVA) followed by the
296 Bonferroni test was employed to analyze total proteolysis (Rsv 10^{-4} M), proteolytic
297 systems, atrogenes mRNA expression and protein synthesis. Paired test-t was employed
298 to evaluate the phosphorylation levels of proteins because 1) the muscle from one limb
299 was incubated with Rsv while the muscle from the contralateral limb (control muscle)
300 was incubated with vehicle DMSO ("paired condition") in both Sham and Den
301 conditions, and 2) samples from these conditions (i.e., Sham and Den) ran in different
302 gels. $P < 0.05$ was considered statistically significant. GraphPad Prism 5.0 (San Diego,
303 CA, USA) was used to perform the statical analysis and create the graphs.

304

305 **RESULTS**

306 *Rsv reduces protein degradation in Sham and Den muscles*

307 Sham (innervated) EDL muscles from rats were incubated with either 10^{-5} or 10^{-}
308 ^{4} M. Figure 1A shows that total proteolysis was reduced by 25 % in muscles incubated
309 with 10^{-4} M of Rsv but not with 10^{-5} M. For this reason, the following experiments were
310 performed on Sham and 3-day Den EDL muscles incubated with the highest
311 concentration of Rsv (i.e., 10^{-4} M) for evaluation of total proteolysis and activity of
312 UPS, lysosomal and calcium-dependent proteolytic systems. Rsv reduced total
313 proteolysis (40 %) in Sham muscles (Fig. 1B) but did not cause a significant reduction
314 in the activity of UPS and lysosomal systems (Fig. 1C). As expected, Den up-regulated
315 total proteolysis (~40 %, Fig. 1B), which was accompanied by an elevation in the
316 activities of UPS (~3-fold) and lysosomal (100 %) systems (Fig. 1C). In contrast, Rsv
317 induced a significant reduction in total proteolysis (~60 %, Fig. 1B) that were paralleled
318 by an inhibition of UPS (50 %) and lysosomal (~70 %) proteolytic activities in Den

319 muscles (Fig. 1C). Neither Rsv nor Den altered calcium-dependent proteolysis in
320 muscles (Fig. 1C). To further investigate the expression of some essential components
321 of the proteolytic systems inhibited by Rsv, the expression of UPS-related genes
322 *Fbxo32* (Atrogin-1) *and Trim63* (MuRF1) and autophagy-related gene *Map1lc3b*
323 (LC3b) was determined. Rsv tended to down-regulate (~50%; $P > 0.05$) the expression
324 of *Fbxo32, Trim63, and Map1lc3b* in Sham and *Trim63 and Map1lc3b* in Den muscles
325 (Fig. 1D). Den *per se* did not change the expression of *Fbxo32*, *Trim63* and *Map1lc3b*.
326 These results demonstrate that Rsv mitigates total proteolysis in both Sham and Den
327 muscles, and this effect in atrophic muscles was due to the inhibition of the
328 hyperactivation of UPS and lysosomal proteolytic systems.
329

330 *Rsv reduces phosphorylation levels of Akt/FoxO4 in Den muscles*

331 To further explore the signaling pathways involved in the Rsv-induced
332 antiproteolytic effects, Akt/FoxO signaling was analyzed in Sham and Den muscles
333 incubated with the polyphenol Rsv. Analyzing the ratio of phosphoproteins to the
334 loading control β-actin, we observed that Rsv reduced the phosphorylation level of
335 FoxO4 (~60 %) in both Sham and Den muscles without altering FoxO1 phosphorylation
336 (Fig. 2B and D). This effect was associated with the down-regulation (36%) in Akt
337 phosphorylation only in Den muscles (Fig. 2D), suggesting that Akt/FoxO signaling
338 pathway does not mediate Rsv-induced inhibition of UPS and lysosomal systems and
339 other intracellular mediators may be recruited by Rsv.
340

341 *H89 abolishes the Rsv-induced antiproteolytic effects on Den muscles*

342 In order to determine whether the Rsv-induced antiproteolytic effects are
343 mediated by PKA/CREB pathway, we first evaluated the phosphorylation levels of

344 CREB in Sham and Den muscles. Rsv tended to increase (~50%; $P \geq 0.05$) the
345 phosphorylation levels of CREB in Sham muscles (Fig 3B) and this effect was
346 statistically significant (~40 %; $P < 0.05$) in Den muscles (Fig. 3D). These results
347 suggest that PKA/CREB signaling pathway could mediate the antiproteolytic effects of
348 Rsv.

349 In order to confirm the involvement of the PKA signaling in the negative
350 regulation of total proteolysis by Rsv, we analyzed this metabolic parameter in Sham
351 and Den muscles incubated in the absence or presence of Rsv and the PKA inhibitor,
352 H89. Again, Rsv reduced total proteolysis in Sham (~40 %, Fig. 3E). H89 *per se*
353 significantly reduced (73 %) total proteolysis in Sham muscles and the co-incubation of
354 H89, and Rsv did not cause an additional effect on total proteolysis (Fig 3E). In Den
355 muscles, as previously demonstrated, Rsv also reduced total proteolysis (~35 %, Fig.
356 3F). In contrast to Sham muscles, H89 did not affect total proteolysis in Den muscles
357 (Fig. 3F). However, the inhibition of proteolysis induced by Rsv was abolished in
358 muscles incubated with H89 (Fig. 3F), indicating that PKA mediates the antiproteolytic
359 effects of Rsv on Den muscles and this effect may depend on muscle innervation.

360

361 *Protein synthesis is reduced by Rsv in Den muscles*

362 As shown in Fig. 4A, the *ex vivo* rate of protein synthesis was unaltered by Rsv
363 in Sham muscles. Den *per se* tended to increase (~35 %, $P \geq 0.05$) muscle protein
364 synthesis. On the other hand, Rsv reduced (~40 %) rates of protein synthesis in Den
365 muscles to values similar to those in Sham muscles (Fig. 4A). We next analyzed the
366 phosphorylation levels of protein kinases S6K, a downstream target of the major
367 pathway (i.e., Akt/mTOR) that activates protein synthesis, and AMPK, a well-known
368 target of Rsv that inhibits mTOR and protein synthesis (Sartori et al. 2021). As shown

369 in Fig. 4C, analyzing the ratio of phosphoproteins to the loading control β-actin, Rsv did
370 not alter S6K phosphorylation in Sham muscles but tended to increase ($P \geq 0.05$)
371 AMPK phosphorylation. In Den muscles, S6K and AMPK phosphorylation was not
372 significantly affected by Rsv (Fig 4E). Our data demonstrate a possible homeostatic
373 effect of Rsv by returning protein synthesis in Den muscles to levels similar to Sham
374 muscles.

375

376 **DISCUSSION**

377

378 The present work sheds new light on intracellular mechanisms Rsv regulates
379 protein metabolism in Sham (innervated) and Den rat skeletal muscles. It shows that
380 Rsv directly inhibits lysosomal and UPS systems in Den muscles and suggests the
381 involvement of PKA/CREB signaling in such effects. Prior studies have examined the
382 effects of Rsv on muscle growth in cell cultures, animal models, and human beings
383 (Wilson et al. 2015, Montesano et al. 2013, Woodman et al. 2021, Alway et al. 2017).
384 Rsv *in vitro* has shown to induce cell cycle exit in C2C12 myoblasts, differentiation to
385 myotubes, and hypertrophy in the late phase of the myogenic process (Montesano et al.
386 2013). *In vivo* experiments have demonstrated that a “low” dose of 5 mg of Rsv·kg
387 body mass^{-1}·day^{-1} for 15 weeks promotes skeletal myofiber hypertrophy in sedentary
388 mice (Woodman et al. 2021). Similarly, southern flounder fish fed with a diet
389 supplemented with 600 μg Rsv·g of food^{-1} for 16 weeks had a greater length and body
390 mass than control fish (Wilson et al. 2015). In humans, it has been shown that 500 mg
391 of Rsv·day^{-1} for 12 weeks combined with aerobic and strength exercise programs in
392 older men and women increased myofiber cross-section area, total myonuclei, markers
393 of mitochondrial density, and muscle fatigue resistance (Alway et al. 2017). Altogether,

394 these findings indicate a positive effect of Rsv on skeletal muscle growth; however, it
395 has not been explored the underlying mechanism regulating protein metabolism and
396 whether this is a direct or indirect effect on skeletal muscle. As far as we know, this is
397 the first direct evidence showing that Rsv (10^{-4} M) *ex vivo* inhibits protein degradation
398 rates in innervated skeletal muscles without altering protein synthesis rates. Although a
399 previous study (Park et al. 2012) has shown that Rsv at low concentrations may activate
400 intracellular signaling pathways in C_2C_{12} myotubes by causing a significant increase in
401 cAMP levels that reaches a plateau at 40 μM, we may not rule out the possibility that
402 higher concentrations of Rsv (i.e., $> 10^{-4}$ M or > 100 μM) could stimulate rates of
403 protein synthesis in innervated muscles. More importantly, it is reasonable to speculate
404 that antiproteolytic effect of Rsv may be responsible for stimulating muscle growth *in*
405 *vivo* treatments previously reported above (Wilson et al. 2015, Woodman et al. 2021,
406 Montesano et al. 2013, Alway et al. 2017), because muscle hypertrophy occurs when
407 rates of protein synthesis exceed rates of degradation.

408 The protein metabolism imbalance may cause both muscle hypertrophy in
409 "normal" (innervated) conditions and muscle wasting in catabolic conditions. Since
410 protein synthesis in the Den muscle remains unchanged (Gonçalves et al. 2012, Furuno
411 et al. 1990) or even risen (Furuno et al. 1990), excessive protein degradation has shown
412 to essential for the development of skeletal muscle atrophy (Furuno et al. 1990,
413 Gonçalves et al. 2012, Sartori et al. 2021). Accordingly, we also observed a tendency to
414 increase protein synthesis and a significant increase in total proteolysis in 3-day Den
415 muscles. Although calcium-dependent proteolysis has been related to muscle atrophy
416 induced by unloading (Shenkman et al. 2015) and Den (Furuno et al. 1990), our data
417 show no change in its activity and are in agreement with data from Gonçalves et al.
418 (2012). UPS is responsible for degrading short- and long-lived proteins (e.g.,

419 myofibrillar proteins) and soluble misfolded proteins, whereas autophagy-lysosomal
420 degrades long-lived proteins, insoluble protein aggregates, and even whole organelles
421 (e.g., mitochondria). Excessive activation of the autophagy-lysosomal system and UPS
422 during pathophysiological conditions contributes to the degradation of sarcomeric
423 proteins and mitochondria loss resulting in energy imbalances, muscle wasting, strength
424 loss, and fatigue (Sartori et al. 2021). In agreement with previously reported data
425 (Gonçalves et al. 2012), our experiments demonstrated a dramatic activation of UPS
426 and lysosomal proteolytic systems in Den muscles and implied a role for these
427 processes in this atrophic model.

428 Rsv is a potential nutraceutical approach to counteract muscle wasting in a
429 plethora of conditions, including sarcopenic obesity and Den (Huang et al. 2019, Asami
430 et al. 2018). For example, Rsv supplementation (0.4 % in the diet) for 10 and 20 weeks
431 prevented muscle loss and myofiber size decrease and improved grip strength in aged
432 rats submitted to high-fat diet-induced sarcopenic obesity (Huang et al. 2019).
433 Moreover, mice feeding a diet containing 0.5% of Rsv were partially protected from
434 Den-induced muscle and myofiber atrophy (Asami et al. 2018). However, these studies
435 did not evaluate in Den muscles: 1) the direct action of Rsv in tissue and 2) rates of
436 protein metabolism, but only markers of protein degradation. Although the latter kind of
437 analysis may help to identify molecular mechanisms regulated by Rsv, it may
438 underestimate or overestimate the “real” changes in the rates of protein metabolism.
439 Indeed, we demonstrated that Rsv suppressed rates of UPS and lysosomal proteolysis in
440 rat Den muscles without any significant change in the gene expression of the Ub-ligases
441 *Fbxo32* and *Trim63* and the autophagy gene *Map1lc3b*. Similarly, Asami et al. (2018)
442 showed that Rsv did not alter the total amount of atrogin-1 protein in Den muscles,
443 whereas reducing atrogin-1-positive nuclei in muscle cryosections, suggesting that

444 subcellular localization and function of this Ub-ligase may be affected by Rsv rather
445 than its content or expression. It is noteworthy that there are several other atrophy-
446 related genes (i.e., atrogenes) belonging to UPS and autophagy-lysosomal systems, such
447 as the lysosomal protease cathepsin L (*Ctsl*) and the Ub-ligases *Musa1* and *Smart*
448 (Sartori et al. 2021) that were not investigated in our study. Further experiments are
449 needed to identify the atrogenes that Rsv may specifically regulate in Den muscles.

450 In skeletal muscle, FoxO proteins upregulate the transcription of several
451 atrogenes, such as *Map1lc3b, Ctsl*, *Fbxo32,* and *Trim63*, boosting the activity of UPS
452 and autophagy-lysosomal systems (Sartori et al. 2021). It is well known that Akt
453 phosphorylates FoxO and blunts its transcriptional function by inducing FoxO nuclear
454 export and keeping it away from its target genes (Sartori et al. 2021). Although FoxO1
455 phosphorylation was unaffected in any condition, unexpectedly, the Akt-induced
456 phosphorylation of FoxO4 was reduced by Rsv in both Sham and Den muscles and Akt
457 phosphorylation only in Den muscles, indicating that Rsv inactivates Akt/FoxO4
458 signaling. One limitation of our study was that the total protein levels of Akt and FoxOs
459 were not evaluated, and their phosphorylation levels were normalized to the loading
460 control β-actin. Therefore, we cannot determine whether the down-regulation of
461 Akt/FoxO4 phosphorylation induced by Rsv was due to changes in total protein levels
462 or modulation in the specific phosphorylation site. Independently of this limitation, the
463 results obtained in our study should hold significance to suggest the inactivation of
464 Akt/FoxO4 signaling by Rsv. These findings agree with the reduction in protein
465 synthesis rates caused by Rsv in Den muscles. In sharp contrast, Montesano et al.
466 (2013) reported that Rsv stimulates the IGF-1/Akt signaling pathway in C_2C_{12}
467 myotubes. However, to the best of our knowledge, no study has analyzed Akt and
468 FoxO4 phosphorylation in adult muscles incubated with Rsv *ex vivo*. One possible

469 explanation for Rsv-induced repression of Akt phosphorylation in our study is that the
470 antiproteolytic effect of Rsv could decrease the availability of free amino acids and,
471 consequently, the stimulus to activate PI3K/Akt signaling (Tato et al. 2011). Although
472 Akt does not seem to mediate the *ex vivo* effects of Rsv, we may not rule out the
473 involvement of FoxO factors in the antiproteolytic action of Rsv because many different
474 post-translational modifications, including phosphorylation, acetylation and
475 ubiquitination may regulate FoxO (Sartori et al. 2021). In fact, Alamdari et al. (2012)
476 have demonstrated that FoxO1 acetylation induced by the glucocorticoid
477 dexamethasone mediates the upregulation of Atrogin-1 and MuRF1 expression and
478 atrophy in cultured L6 myotubes and Rsv (10^{-4} M) is able to prevent these catabolic
479 effects. Further experiments should be performed to test this hypothesis in *ex vivo*
480 conditions of Den muscles.

481 The present study also shows that Rsv added to the incubation medium increased
482 CREB phosphorylation, especially in Den muscles. These data suggest that cAMP/PKA
483 signaling was stimulated by Rsv, because CREB is phosphorylated at Ser^{133} by PKA
484 (Silveira et al. 2020). Indeed, Park et al. (2012) demonstrated that Rsv acts as a non-
485 selective inhibitor of cAMP phosphodiesterases, particularly PDE 4, increasing cAMP
486 levels as soon as 10 min after incubation in C_2C_{12} myotubes. Another study reported
487 that bisphenols like curcumin also elevate cAMP and CREB phosphorylation (Ray
488 Hamidie et al. 2015). Several studies have shown that distinct cAMP inducers may
489 inhibit muscle proteolysis. For example, EDL muscles from diabetic rats incubated in
490 the presence of pentoxifylline, a non-selective PDE inhibitor, showed lower calcium
491 and UPS proteolytic activities than non-treated muscles (Baviera et al. 2007). Moreover,
492 rat muscles incubated with rolipram, a selective PDE 4 inhibitor, attenuated UPS
493 proteolytic activity and *Fbxo32* (Atrogin-1) expression induced by fasting (Lira et al.

494 2011). *In vivo* treatment with rolipram mitigated the loss of muscle mass and force in
495 Den muscles (Hinkle et al. 2005). Clenbuterol, a β_2-adrenergic agonist that elevates
496 intracellular cAMP levels, has shown similar effects of Rsv on proteolysis in Den soleus
497 muscles, i.e., clenbuterol ameliorated the hyperactivation of UPS and lysosomal
498 systems (Gonçalves et al. 2012). More importantly, the addition of 6-BNZ-cAMP, a
499 PKA activator, to the incubation medium of soleus muscle isolated from Sham and Den
500 rats reduced total proteolysis (Gonçalves et al. 2012). Considering all these findings, we
501 hypothesized that PKA could mediate Rsv-induced inhibition of muscle proteolysis. To
502 address this issue, Sham and Den muscles were co-incubated with Rsv and/or H89, and
503 total proteolysis was measured. Intriguingly, H89 alone decreased rates of proteolysis in
504 innervated muscles but not in Den ones. This *ex vivo* effect of H89 was previously
505 described and it was associated with a marked increase in Akt phosphorylation
506 (Gonçalves et al. 2012), supporting the idea that PKA may inhibit Akt activation (Mei
507 et al. 2002). In Den muscles, H89 *per se* did not cause any effect on proteolysis;
508 however, it blocked the suppressive action of Rsv in total proteolysis. These findings
509 indicates that PKA mediates, at least partially, the antiproteolytic effect of Rsv,
510 especially in Den muscles. Because FoxO1 phosphorylation at Ser^{256}, a target of both
511 Akt and PKA (Silveira et al. 2020), was unaffected by Rsv, it is possible to speculate
512 that PKA is inhibiting proteolysis by indirectly regulating FoxO via phosphorylation-
513 independent mechanisms or directly phosphorylating proteases or components of the
514 proteolytic systems. We have recently shown that genetic activation of PKA inhibits
515 FoxO transcriptional activity by multiple mechanisms *in vivo* (Silveira et al. 2020), such
516 as phosphorylation, acetylation, downregulation of its contente, and inhibition by PGC-
517 1α transcriptional coactivator, the latter has been considered a master regulator of
518 mitochondrial biogenesis (Lin et al. 2002). Future experiments addressing this

519 unresolved question will provide more insights into the signaling pathways and
520 molecular mechanisms regulated by Rsv in atrophic conditions.
521

522 **CONCLUSIONS**

523 In summary, our findings show that Rsv inhibits total proteolysis in both Sham
524 (innervated) and Den muscles and causes a marked suppression of the activities of UPS
525 and lysosomal proteolytic systems in Den muscles. The antiproteolytic effects of Rsv in
526 Den muscles seem to be mediated by PKA/CREB signaling. Moreover, Rsv reduces
527 protein synthesis rates in Den muscles to values similar to those in Sham muscles. Our
528 findings help identify novel mechanisms by which Rsv may attenuate muscle wasting in
529 atrophic conditions and develop new therapeutic approaches based on these findings.
530

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541

542 **AUTHOR CONTRIBUITIONS**

543 All authors contributed extensively to the work presented in
544 this paper and discussed the results. IISJ, DAPG and ECL designed the study,
545 researched data, and wrote the manuscript. TSV, FPA and GOZ researched data and
546 helped designing experiments and writing the manuscript. SPG, RRV and FAG
547 researched data. DAG, ICK and LCCN helped designing experiments and writing the
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549

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681 **FIGURES LEGEND**

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683 **Figure 1**. Effects of different concentrations (10^{-5} and 10^{-4} M) of Resveratrol (Rsv) on
684 total proteolysis (A) in EDL muscle of Sham rats. Effects of Rsv (10^{-4} M) on total
685 proteolysis (B), proteolytic activity of ubiquitin proteasome (UPS), lysosomal and
686 calcium-dependent systems (C) and mRNA expression of *Fbxo32*, *Trim63* and
687 *Map1lc3b* (D) in EDL muscle of Sham and Den rats. Values are presented as mean ±
688 SEM of 5-6 rats. Rsv: Resveratrol; Sham: sham-operated rats; Den: denervated rats.

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690 **Figure 2**. Effects of Rsv (10^{-4} M) on phosphorylation levels of p-Ser^{193} FoxO4, p-Ser^{256}
691 FoxO4 and p-Ser^{473}Akt and total content of β-actin in EDL muscle of Sham (A and B)

692 and Den (C and D) rats. Values are presented as mean ± SEM of 3 rats. Rsv:
693 Resveratrol; Sham: sham-operated rats; Den: denervated rats.
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695 **Figure 3**. Effects of Rsv (10^{-4} M) on phosphorylation levels of Ser^{133} CREB (A-D) and
696 total proteolysis (E and F) in EDL muscle of Sham (A, B and E) and Den (C, D and F)
697 rats. Representative blots (A and C) and densitometric analysis (B and D) are shown for
698 phosphorylation levels of Ser^{133} CREB. Values are presented as mean ± SEM of 3-6
699 rats. Rsv: Resveratrol; Sham: sham-operated rats; Den: denervated rats; H89: the PKA
700 inhibitor.
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702 **Figure 4**. Effects of Rsv (10^{-4} M) on protein synthesis (A), phosphorylation levels of
703 S6K and AMPK and total content of β-actin in EDL muscle of Sham (B and C) and Den
704 (D and E) rats. Values are presented as mean ± SEM of 3-6 rats. Resveratrol; Sham:
705 sham-operated rats; Den: denervated rats.
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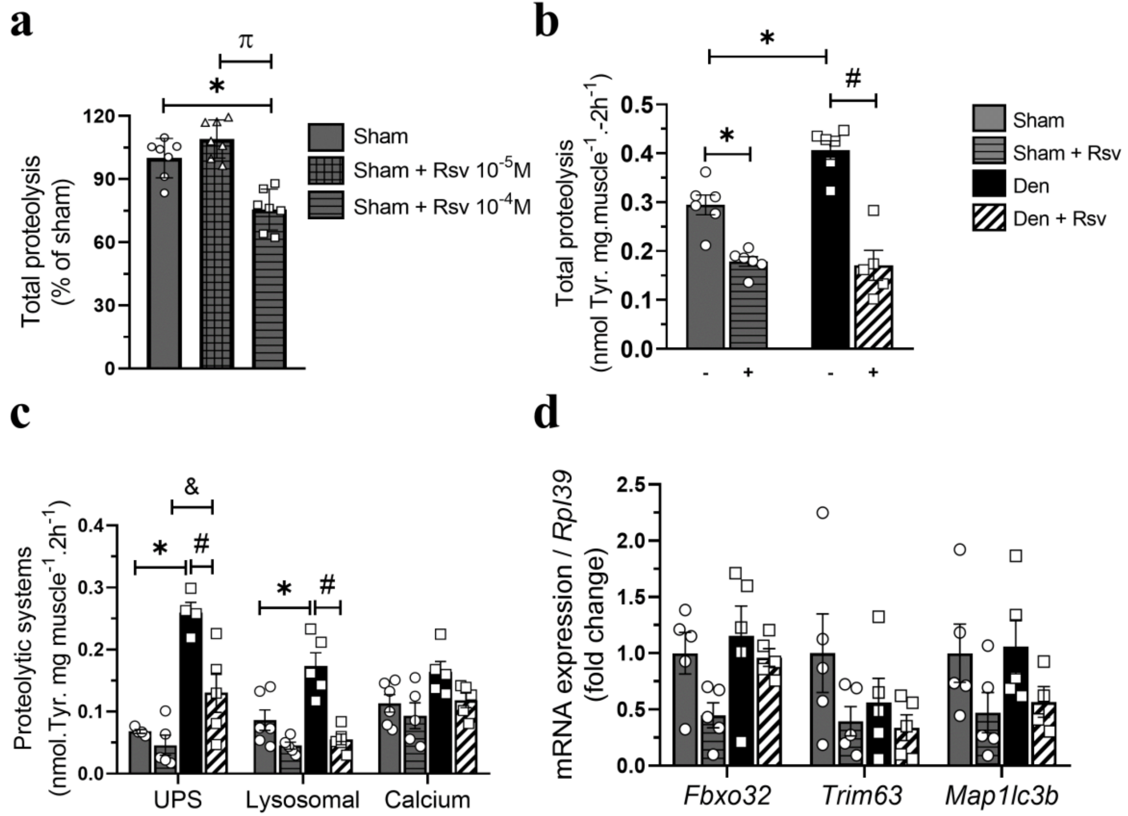

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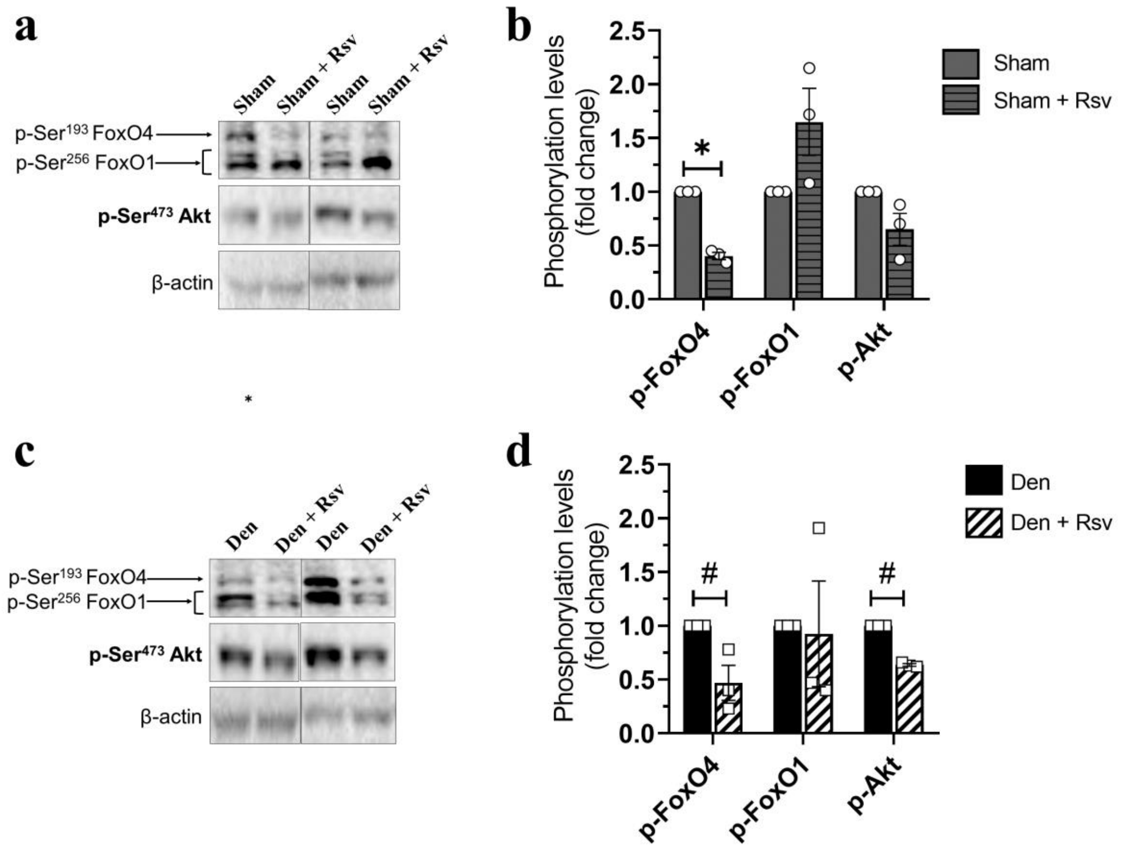

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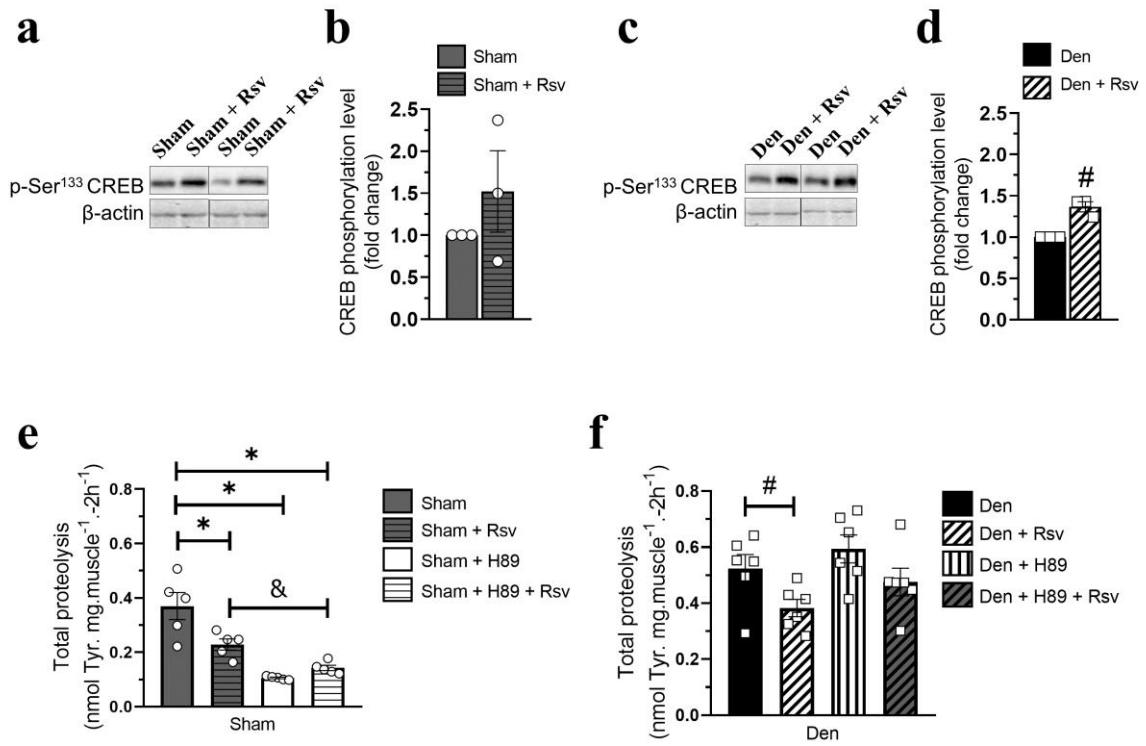

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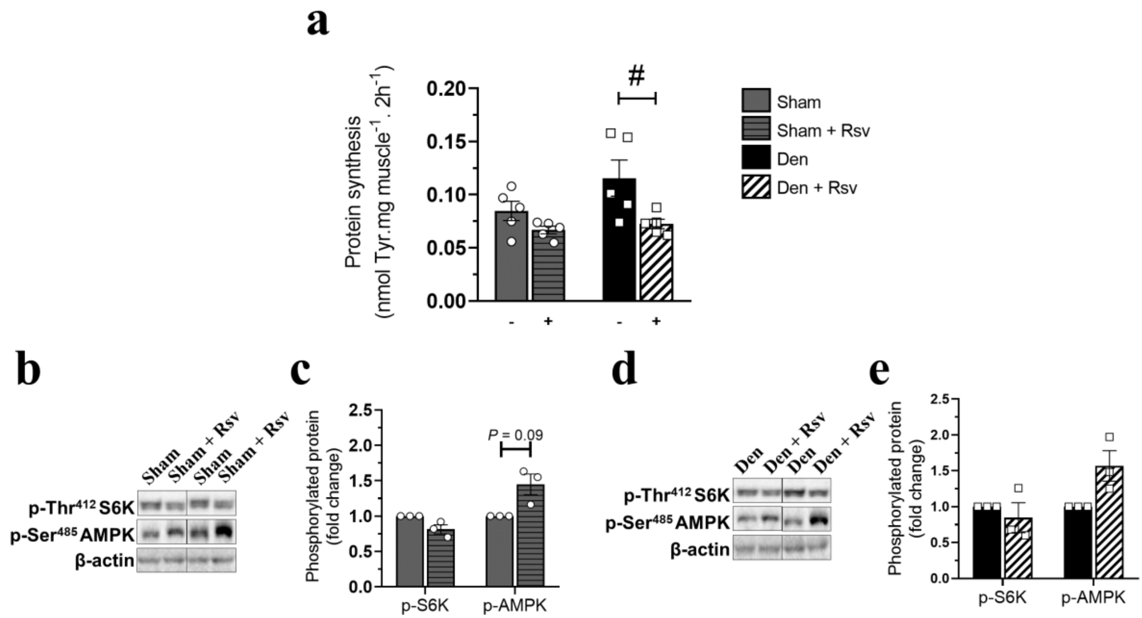

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