UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CÂMPUS DE JABOTICABAL

RESPOSTAS METABÓLICAS E CONCORDÂNCIA ENTRE MÉTODOS PARA DETERMINAÇÃO DO LIMIAR DE LACTATO EM EQUINOS SUBMETIDOS A TREINAMENTO SUBMÁXIMO

Thayssa de Oliveira Littiere

Zootecnista

2024

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Discente: Thayssa de Oliveira Littiere

Orientador: Prof. Dr. Guilherme de Camargo Ferraz

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Ciência Animal

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IMPACTO POTENCIAL DESTA PESQUISA

A obesidade equina é preocupação na equideocultura, impactando não apenas a saúde e o bem-estar dos animais, mas também seu desempenho. Ao investigar as respostas metabólicas e a concordância entre diferentes métodos para predizer a máxima fase estável de lactato (MFEL), este estudo oferece uma base científica para desenvolver estratégias de combate à obesidade por meio do exercício físico. Além disso, a padronização de métodos para estimar a MFEL permite ajustar protocolos de forma individualizada para os cavalos, o que otimiza os programas de condicionamento, promovendo não apenas a perda de peso, mas também a melhora no desempenho físico dos animais. Deste modo, o presente estudo contribui para avanços no manejo e condicionamento físico dos cavalos, que estão diretamente relacionados ao bem-estar e saúde desses animais.

POTENCIAL IMPACT OF THIS RESEARCH

Equine obesity is a concern in horse breeding, impacting not only the animals' health and well-being but also their performance. By investigating metabolic responses and the agreement between different methods for predicting the maximum lactate steady state (MLSS), this study provides a scientific basis for developing strategies to combat obesity through physical exercise. Additionally, standardizing methods to estimate MLSS allows training protocols to be individually tailored to the horses, optimizing conditioning programs, promoting not only weight loss but also improved physical performance. Thus, this study contributes to advances in the management and physical conditioning of horses, which are directly related to their well-being and health.

UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Jaboticabal

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: RESPOSTAS METABÓLICAS E CONCORDÂNCIA ENTRE MÉTODOS PARA DETERMINAÇÃO DO LIMIAR DE LACTATO EM EQUINOS SUBMETIDOS A TREINAMENTO SUBMÁXIMO

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DADOS CURRICULARES DO AUTOR

Thayssa de Oliveira Littiere, nascida em 16 de maio de 1992, em Além Paraíba, Minas Gerais, filha de Irene de Oliveira Littiere e Marcos Netto Littiere. Graduou-se em Zootecnia pela Universidade Federal dos Vales do Jequitinhonha e Mucuri-UFVJM, Câmpus de Diamantina, em 2018. De setembro de 2013 a dezembro de 2014 realizou Graduação Sanduíche pelo “Ciências sem Fronteiras”, do governo federal, na Universidade Dalhousie, Campus de Agricultura de Truro, Nova Scotia, Canadá, sendo bolsista da Capes durante todo o período. Em 2020 obteve o título de mestre em Zootecnia, área de Produção Animal, pela Universidade Federal dos Vales do Jequitinhonha e Mucuri - UFVJM, Câmpus de Diamantina, sob orientação do Prof. Dr. Lucas Lima Verardo, com bolsa da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES. Atualmente é Doutoranda do Programa de Pós-Graduação em Ciência Animal, junto à Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista “Júlio de Mesquita Filho”, FCAV/UNESP, Câmpus de Jaboticabal, sob orientação do Prof. Dr. Guilherme de Camargo Ferraz. Durante o período de doutorado foi bolsista da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES.

“...Às vezes a felicidade demora a chegar

Aí é que a gente não pode deixar de sonhar

Guerreiro não foge da luta e não pode correr

Ninguém vai poder atrasar quem nasceu pra vencer

É dia de sol, mas o tempo pode fechar

A chuva só vem quanto tem que molhar

Na vida é preciso aprender, se colhe o bem que plantar

É Deus quem aponta a estrela que tem que brilhar”

Tá escrito – Xande de Pilates

Aos meus pais, Marcos e Irene, por todo o amor, por serem sempre meus pilares, e por me apoiarem em toda essa trajetória

Ao meu irmão, Thauan, que mesmo de longe nunca soltou minha mão

Aos cavalos, que são a razão de toda a minha dedicação, persistência e perseverança

AGRADECIMENTOS

À Faculdade de Ciências Agrárias e Veterinárias – Unesp campus de Jaboticabal, em especial ao Departamento de Morfologia e Fisiologia Animal, e ao Setor de Equinocultura, pelas oportunidades que me foram dadas, pelo empréstimo dos animais, equipamentos e instalações para realização deste trabalho.

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Aos meus amigos de Jaboticabal, em especial Dália, Dardânia e Stéphane, pela companhia em todos os momentos, apoio, risadas no Arouche e cervejinhas no Skina.

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Câmpus de Jaboticabal

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

C E R T I F I C A D O

Certificamos que o projeto de pesquisa intitulado **"Utilização da plataforma vibratória sobre respostas glicêmicas e insulinêmicas e glicogênio muscular de equinos"**, protocolo nº 2310/21, sob a responsabilidade do Prof. Dr. Guilherme de Camargo Ferraz, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 16 de setembro de 2021.

Vigência do Projeto	04/10/2021 a 31/07/2024
Espécie / Linhagem	*Equus ferus caballus*
Nº de animais	12 animais
Peso / Idade	± 450 kg / ± 15 anos
Sexo	Machos e fêmeas
Origem	FCAV Unesp Jaboticabal

Jaboticabal, 16 de setembro de 2021.

Fabiana Pilarski

Profa. Dra. Fabiana Pilarski
Coordenadora – CEUA

Faculdade de Ciências Agrárias e Veterinárias
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"JÚLIO DE MESQUITA FILHO"
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CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

DECLARAÇÃO

Declaramos que o trabalho de pesquisa intitulado "Utilização da plataforma vibratória sobre respostas glicêmicas e insulinêmicas e glicogênio muscular de equinos ", sob orientação do Prof. Dr. Guilherme de Camargo Ferraz e Certificado CEUA protocolo nº 002310/21, aprovado em reunião ordinária em 16 de setembro de 2021, teve o título alterado para "**Respostas glicêmicas e insulinêmicas de equinos com sobrepeso e concordância entre métodos para determinação do limiar de lactato**" com aprovação da alteração em reunião ordinária de 14 de junho de 2023.

Jaboticabal, 16 de junho de 2023.

Prof.ª Dr.ª Paola Castro Moraes
Vice-coordenadora – CEUA

Faculdade de Ciências Agrárias e Veterinárias
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RESPOSTAS METABÓLICAS E CONCORDÂNCIA ENTRE MÉTODOS PARA A DETERMINAÇÃO DO LIMIAR DE LACTATO EM EQUINOS SUBMETIDOS A TREINAMENTO SUBMÁXIMO

RESUMO

Objetivou-se: (1) avaliar as respostas metabólicas e mensurações de acúmulo de gordura subcutânea de cavalos submetidos a treinamento submáximo; (2) investigar métodos de predição da máxima fase estável de lactato (MFEL) em cavalos a partir do limiar de lactato. Exp. 1: 10 animais, avaliados em dois períodos: antes e após treinamento de sete semanas (PreTr e PostTr). Foram avaliados glicose, insulina, cortisol, LDL, VLDL, HDL, CHOL e FRU, assim como peso, ECC, ECP e circunferência do pescoço. Observou-se redução de glicose, insulina plasmática, pico de cortisol e CHOL em PostTr comparado ao PreTr. Houve redução na circunferência de pescoço em P25 e P50 no PostTr comparado ao PreTr. Exp.2: Os mesmos animais passaram por um único teste de esforço incremental (TEI) para determinar quatro métodos de predição para a MFEL. Os cavalos realizaram de 3-5 sessões de exercício de intensidade constante para obter a MFEL. As velocidades nas quais a concentração de lactato ($[La^-]$) atingiu 2 mM e 4 mM, V_2 e V_4, determinaram os limiares fixos. Um ensaio randomizado e cego utilizou análise visual (LT_V) e modelo de regressão linear bi-segmentado (LT_{BI}). A concordância entre as velocidades correspondentes a V_2, V_4, V_{LTV} e V_{LTBI} e a MFEL (V_{MFEL}) foi estabelecida usando a diferença média (MD), regressão ordinária dos mínimos produtos (OLP) e coeficiente de correlação (*r*). A $[La^-]$ média na MFEL foi 1,50 ± 0,37 mM, e a V_4 foi maior que a V_{MFEL} com MD de 2,12 ± 0,59 m/s entre elas. Observou-se menos viés médio de V_2 e V_{LTV} em comparação a V_{MFEL}. A V_{MFEL} apresentou correlação moderada com todos os métodos de predição. Conclui-se que o exercício pode ser uma ferramenta eficaz no combate à obesidade. Além disso, a utilização de métodos de predição da MFEL baseados no limiar individual de lactato pode ser mais precisa.

Palavras-chave: equino, exercício, limiar de lactato, parâmetros metabólicos, obesidade

METABOLIC RESPONSES AND AGREEMENT BETWEEN METHODS FOR DETERMINING LACTATE THRESHOLD IN HORSES UNDERGOING SUBMAXIMUM TRAINING

ABSTRACT

The objectives were: (1) to evaluate the metabolic responses and measurements of subcutaneous fat accumulation in horses subjected to submaximal training; (2) to investigate methods for predicting the maximal lactate steady state (MLSS) in horses based on the lactate threshold. Exp. 1: Ten animals were evaluated in two periods: before and after seven weeks of training (PreTr and PostTr). Glucose, insulin, cortisol, LDL, VLDL, HDL, CHOL, and FRU levels were measured, along with weight, BCS, CNS, and neck circumference. A reduction in glucose, plasma insulin, cortisol peak, and CHOL was observed in PostTr compared to PreTr. A decrease in neck circumference at P25 and P50 was observed in PostTr compared to PreTr. Exp. 2: The same animals underwent a single incremental exercise test (IET) to determine four prediction methods for MLSS. The horses performed 3-5 constant-intensity exercise sessions to establish the MLSS. The speeds at which lactate concentration ([La–]) reached 2 mM and 4 mM, V2 and V4, determined the fixed thresholds. A randomized, blinded trial used visual analysis (LTV) and a bi-segmented linear regression model (LTBI). The agreement between the speeds corresponding to V2, V4, VLTV, and VLTBI and the MLSS (VMLSS) was established using the mean difference (MD), ordinary least products regression (OLP), and correlation coefficient (r). The mean [La–] at MLSS was 1.50 ± 0.37 mM, and V4 was higher than VMLSS with an MD of 2.12 ± 0.59 m/s between them. Less average bias was observed for V2 and VLTV compared to VMLSS. VMLSS showed a moderate correlation with all prediction methods. It is concluded that exercise can be an effective tool in combating obesity. Furthermore, using prediction methods for MLSS based on the individual lactate threshold may be more accurate.

Keywords: equine, exercise, lactate threshold, metabolic parameters, obesity

LISTA DE ABREVIATURAS

ACTH – hormônio adrenocorticotrófico

ADF – acid detergent fiber

aNDF – neutral detergent fiber

ATP – adenosine trifosfato

AUC – area under the curve

BCS – body condition score

BM – body mass

bpm – beats per minute

°C – degree Celcius

Ca - calcium

CB – constant bias

CEUA – Ethics Committee of Animal Use

CF – crude fiber

CHOL – total cholesterol

CI – confidence interval

cm – centimetres

CNS – cresty neck score

CP – crude protein

CONCEA – National Council of Control in Animal Experimentation

DE – digestible energy

DM – dry matter

ECC – escore de condição corporal

ECP – escore de crista de pescoço

EDTA – ethylenediaminetetraacetic acid

EE – ether extract

ELISA – enzyme-linked immunosorbent assay

FCAV – Faculty of Agricultural and Veterinary Sciences

Fig – figure

FRU - fructosamine

g – grams

GLM – generalized linear model

GLUT4 – transportador de glicose tipo 4

ha – hectare

HDL – high-density lipoprotein

HPA – hipotálamo-pituitária-adrenal

HR – heart rate

HR_{max} – maximal heart rate

IET – incremental exercise test

IMC – índice de massa corpórea

IRS – insulin receptor substrate

IS – insulin sensitivity

kg – kilogram

LAFEQ – Laboratory of Pharmacology and Equine Exercise Physiology

$[La^{-}]$ – plasma L-lactate concentration / concentração de lactato

LCL – lower confidence limit

LDL- low-density lipoprotein

LL – limiar de lactato

LSM – least square means

LT – lactate threshold

LT_{BI} – bi-segmented linear regression model / modelo de regressão linear bissegmentado

LT_{V}– visual lactate threshold / limiar de lactato visual

LVC – lactate-velocity curve / curva velocidade-lactato

m^2 – square meters

Mcal/kg – megacalories per kilogram

MFEL – máxima fase estável de lactato

mg/dL – milligrams per deciliter

MLSS – maximal lactate steady state

mL – millilitre

mM – millimolar

mRNA – messenger RNA

m/s – meters per second

mU/L – mil unidades por litro

NFC – nonfiber carbohydrates

NIRS – near-infrared spectroscopy

No – number

$OBLA_{4mM}$ – onset of blood lactate accumulation / início do acúmulo de lactato sanguíneo

OLP – ordinary least products regression

p – p-value

P – phosphorus

PB – proportional bias

PCA – principal component analysis

PostTr – after seven weeks of conditioning program

PreTr – before seven weeks of conditioning program

P25 – proximal part of the neck

P50 – middle of the neck

P75 – distal part of the neck

r – Pearson's correlation coefficient

RI – resistência à insulina

SD – standard deviation

SE – standard error

TEI- teste de esforço incremental

TRI – triglycerides

ug/dL – micrograms per deciliter

UCL – upper confidence limit

UNESP – São Paulo University

VLDL – very-low-density lipoprotein

V_{LT} – velocity corresponding to LT

V_{LTBI} – velocity corresponding to LT_{BI} / velocidade no LT_{BI}

V_{LTV} – velocity corresponding to the LT_V / velocidade correspondente ao LT_V

V_{MFEL} – velocidade na MFEL

V_{MLSS} – velocity corresponding to MLSS

($\dot{V}O_2$) – oxygen uptake

V_2 – velocity at which the [La^-] reaches 2 mM / velocidade na qual a [La^-] atinge 2 mM

V_4 – velocity at which the [La^-] reaches 4 mM / velocidade na qual a [La^-] atinge 4 mM

µmol/L – micromoles per liter

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CAPÍTULO 1 – Considerações gerais

1. INTRODUÇÃO

A obesidade equina tem se tornado preocupação crescente na criação de cavalos, impactando não apenas a saúde e o bem-estar dos animais, mas também a eficiência de seu desempenho. A redução da obesidade em cavalos é crucial para melhorar a saúde geral e prevenir distúrbios metabólicos associados, como a resistência à insulina, síndrome metabólica equina e laminite endocrinopática (Hoffman et al., 2003; Carter et al., 2009; Coleman et al., 2018; Fitzgerald et al., 2019). Estratégias de perda de peso em equinos são mais eficazes quando envolvem combinações na restrição dietética e aumento da atividade física. Programas de perda de peso que incorporam balanço energético negativo, alcançado através de ajustes na dieta e aumento do nível de atividade física, podem promover a diminuição da massa corporal e melhorar os parâmetros metabólicos (Gordon et al., 2009; Bamford et al., 2019; Pratt-Phillips, 2024).

A implementação de um plano de condicionamento físico estruturado que permita aos cavalos atingir e manter a máxima fase estável de lactato (MFEL) otimiza os protocolos de treinamento. A MFEL é o padrão ouro para avaliar a capacidade aeróbica (Lindner, 2010; Miranda et al., 2014; Hering et al., 2018; Alves et al., 2020; De Maré et al., 2022; Ferraz et al., 2022), e seu alcance pode melhorar a eficiência do treinamento e a eficácia na redução de gordura corporal. No entanto, o protocolo é trabalhoso, pois requer pelo menos três sessões de exercício em velocidade constante, com duração de 30 minutos em dias alternados (Ferraz et al., 2022). Portanto, é necessário utilizar outros métodos para predizer a MFEL com única sessão de exercício. Estudos recentes têm explorado diferentes métodos para estimar a MFEL, incluindo testes de esforço incremental. Esses métodos ajudam a ajustar os protocolos de treinamento para atender às necessidades individuais dos cavalos, promovendo perda de massa corporal eficaz. Este estudo visa (1) avaliar as respostas metabólicas, escore de condição corporal, crista e circunferência de pescoço de equinos mantidos a pasto submetidos a treinamento submáximo. O protocolo de treinamento foi delineado para cavalos destreinados se tornarem capazes de realizar um protocolo para alcançar a máxima fase estável de lactato. (2) Este estudo verifica

a concordância da MFEL com quatro métodos de avaliação da capacidade aeróbia, baseados na concentração de lactato sanguíneo em cavalos.

2. REVISÃO DE LITERATURA

2.1 Obesidade

A obesidade pode ser definida como acúmulo anormal ou excessivo de tecido adiposo no corpo e tornou-se problema em ascensão, tanto em humanos quanto nas diversas espécies animais, incluindo os equinos (Geor, 2008; Nostell et al., 2021), principalmente por causa de mudanças no estilo de vida e alimentação (Ertelt et al., 2014). O fornecimento de concentrados ricos em grãos ou forrageiras com alto teor de carboidratos não estruturais, associado ao confinamento dos animais e baixa atividade física é um dos principais contribuintes para a indução da obesidade em cavalos (Frank et al., 2010; Daradics et al., 2021). Assim, estudos apontam que porcentagem significativa da população de equinos pode ser considerada com sobrepeso ou obesa (Wyse et al., 2008; Stephenson et al., 2011; Thatcher et al., 2012; Shepherd et al., 2021).

Em humanos, a obesidade está diretamente relacionada com doenças crônicas como diabetes tipo 2, doenças cardiovasculares e doença hepática gordurosa não alcoólica (Li et al., 2021). Já em equinos, o aumento nos índices de obesidade é preocupante porque está diretamente relacionada a distúrbios endocrinometabólicos como resistência à insulina, laminite endocrinopática, hiperlipedemia e síndrome metabólica equina (Hoffman et al., 2003; Carter et al., 2009; Coleman et al., 2018; Fitzgerald et al., 2019) e problemas de termorregulação (Goossens, 2008; Fitzgerald et al., 2019; Pratt-Phillips et al., 2023) que refletem na redução do desempenho reprodutivo e atlético dos animais. (Henneke et al., 1983; Frank et al., 2010; Morgan et al.,2014; Sheperd et al., 2021). Ademais, Jansson et al. (2021) relataram que o aumento do peso e do conteúdo de gordura corporal em cavalos reduz a aptidão fisiológica em termos de V_4 (velocidade na qual a concentração de lactato é igual a 4 mM), remoção de lactato plasmático, níveis de hematócrito, disponibilidade de glicose plasmática e, consequente, redução do desempenho dos animais. Pratt-Phillips e

Munjizun (2023) destacaram que a obesidade altera a biomecânica locomotora dos cavalos e também está relacionada com a redução da eficiência na utilização de oxigênio durante o exercício, diminuindo a capacidade cardiorrespiratória e, consequentemente, comprometendo negativamente o desempenho dos animais.

2.2 Avaliação do grau de obesidade

A avaliação do grau de obesidade dos animais é ferramenta fundamental para auxiliar nas mudanças, caso necessárias, do manejo, dieta, exercícios físicos e intervenção clínica, e principalmente, na prevenção do aumento de peso e gordura corporal dos cavalos. Uma ferramenta fácil de ser utilizada na rotina diária é a escala de escore de condição corporal (ECC) (Henneke et al., 1983), uma vez que não é invasiva e apresenta baixo custo. Além disso, possui correlação moderada ao índice de massa corpórea (IMC) utilizado para humanos (Donaldson et al., 2004). O ECC, segundo Henneke et al. (1983) classifica os equinos em escala de 1 a 9, onde o escore 1 é atribuído a animais em condições de extrema magreza e o escore 9 ao animal extremamente obeso. A avaliação é realizada visualmente e por meio de palpação das áreas de deposição de gordura corporal, sendo: borda dorsal do pescoço, cernelha, processos espinhosos lombares, inserção da cauda, costado e região caudal às espáduas.

Apesar da facilidade da avaliação do ECC, esse método não é eficiente na avaliação da deposição de gordura regional, que pode indicar obesidade e desenvolvimento de distúrbios metabólicos (Carter et al., 2009; de Laat et al., 2018; Fitzgerald et al., 2019). Além disso, o ECC é subjetivo e menos sensível em detectar variações na adiposidade ao longo do tempo (Geor, 2008; Thatcher et al., 2012; Potter et al., 2023). Deste modo, é possível associar o ECC com outros métodos, de maneira a tornar mais precisa a avaliação do grau de obesidade dos animais.

Escores e mensurações morfométricas de determinadas regiões do corpo características por depositar excesso de gordura subcutânea foram desenvolvidas ao longo dos últimos anos, como por exemplo o escore de crista de pescoço (ECP) e a avaliação da circunferência do pescoço. O ECP, desenvolvido por Carter et al. (2009) consiste em escala de 0 a 5, onde 0 é atribuído à crista não visível nem palpável e 5

ao acúmulo exagerado de gordura nesta mesma região, caracterizado pela crista aumentada e pendida para um dos lados do pescoço. Já a mensuração da circunferência do pescoço é realizada em três regiões do pescoço em relação ao seu comprimento total, correspondendo a 25, 50 e 75% (P25, P50 e P75) do comprimento do pescoço (Frank et al., 2006). O acúmulo de tecido adiposo no pescoço está associado a distúrbios metabólicos como obesidade, resistência à insulina, síndrome metabólica equina e aumento do risco de desenvolver laminite (Carter et al., 2009; Ragno et al., 2021), e por isso tem sido vastamente avaliado.

2.3 Respostas metabólicas em animais com sobrepeso ou obesos

O acúmulo de tecido adiposo compromete as respostas metabólicas de insulina e glicose, uma vez que a obesidade pode estar relacionada com resistência à insulina. A insulina é o principal hormônio anabólico do metabolismo energético em mamíferos, responsável também pela regulação do metabolismo de lipídeos (Duehlmeier et al., 2010). Este hormônio é secretado pelas células β das Ilhotas de Langerhans pancreáticas em resposta aos elevados níveis de glicose no sangue durante o período pós-prandial. Após sua liberação, a insulina se liga a ao receptor de insulina que está presente nas membranas celulares, o que gera cascata de sinalização e consequente ativação de várias proteínas e enzimas dentro da célula. Essa ativação em cascata resulta na mobilização de vesículas contento os transportadores de glicose tipo 4 (GLUT4) da região intracelular para a membrana plasmática, de maneira a permitir a captação de glicose para o interior dos tecidos (Sakamoto et al., 2008; Bird et al., 2017; Petersen et al., 2018; Pratt-Phillips, 2024), sendo estes o músculo esquelético, fígado e tecido adiposo. Assim, a insulina atua em várias etapas do metabolismo dos carboidratos, sendo responsável pela oxidação da glicose e o seu armazenamento na forma de glicogênio no fígado e tecido muscular (Wilcox, 2005). Além disso, a insulina também está relacionada com a redução da produção hepática de glicose por meio da inibição da glicogenólise e gliconeogênese e produção de ácido pirúvico, que será utilizado no ciclo de Krebs para síntese de adenosina trifosfato (ATP), através da ativação da glicólise (Wilcox, 2005). Ademais, este hormônio estimula a síntese de ácidos graxos no tecido adiposo, fígado e glândula mamária, o que resulta na

formação dos triglicerídeos que serão posteriormente armazenados no próprio tecido adiposo e fígado (Antu et al., 2016).

Cavalos obesos podem apresentar uma resposta reduzida dos tecidos à insulina, conhecida como resistência à insulina (RI). A RI está associada à diminuição na captação de glicose pelos tecidos, ao aumento da eficiência da gliconeogênese e ao aumento da lipólise (Treiber et al., 2006). A ligação entre obesidade e RI se dá pela produção elevada de adipocinas e citocinas, que interferem na sinalização da insulina para os tecidos. Além disso, em animais obesos, pode ocorrer acúmulo de gordura nas células musculares e em outros tecidos, o que reduz a sensibilidade à insulina nesses locais (Tadros e Frank, 2013). A hiperinsulinemia pode ocorrer como resposta do organismo para evitar períodos prolongados de elevada concentração de glicose no sangue, onde as células beta do pâncreas produzem mais insulina para compensar a RI. Segundo Frank et al. (2011), valores de glicose acima de 6.11 mM são considerados elevados. Em equinos, valores basais de insulina acima de 30 mU/L caracterizam a hiperinsulinemia (McGowen et al., 2008; Kaczmarek et al., 2016), que por sua vez está associada ao aumento da lipogênese nos tecidos muscular, subcutâneo e visceral e consequente redução da mobilização da gordura armazenada, o que favorece a obesidade. Apesar da obesidade estar intimamente relacionada à RI, é importante ressaltar que nem todos os cavalos obesos apresentam RI.

Alterações significativas nos níveis de lipídios sanguíneos também estão relacionadas com a obesidade, devido a mudanças no metabolismo lipídico e na regulação da insulina. O acúmulo excessivo de tecido adiposo é responsável pela liberação de ácidos graxos livres, citocinas pró-inflamatórias e adipocinas (Ferraz et al., 2008; Reynolds et al., 2019). Em equinos, foram relatadas funções mitocondriais comprometidas (Marycz et al., 2018), aumento do metabolismo de glicocorticóides (Morgan et al., 2017), aumento das concentrações de leptina plasmática (Buff et al., 2002) e lipidograma sanguíneo alterado (Elzinga et al., 2016). A dislipidemia associada à obesidade e à RI em humanos pode ser caracterizada por altas concentrações de triglicerídeos acompanhadas de concentrações reduzidas HDL, enquanto as concentrações de LDL podem se apresentar normais ou levemente alteradas (Otvos et al., 2011; Vekic et al., 2019). Em cavalos obesos com RI também

foi observada dislipidemia caracterizada pelas altas concentrações de VLDL, VLDL-triglicerídeo, e HDL-colesterol em equinos obesos (Frank et al., 2006).

A obesidade também está associada a desregulação na secreção de cortisol. O cortisol é um hormônio esteroide e um dos principais glicocorticoides sintetizados na zona fasciculada do córtex adrenal a partir do colesterol. Sua secreção é regulada pelo hormônio liberador de corticotrofina (CRH) e pelo hormônio adrenocorticotrófico (ACTH), liberado pela hipófise anterior. O ACTH aumenta o número de receptores de LDL e a atividade da enzima colesterol desmolase, enzima que converte colesterol em pregnenolona, o passo limitante na síntese de cortisol (Angelousi et al., 2020). Conhecido como hormônio do estresse, o cortisol é crucial para a resposta ao estresse físico e emocional. Além disso, ele desempenha papéis importantes na manutenção da homeostase, incluindo a regulação da pressão arterial, a modulação do sistema imunológico, a ação anti-inflamatória e o metabolismo de proteínas, carboidratos e gorduras (Thau et al., 2020; Katsu and Baker, 2021). Concentrações elevadas de cortisol e maior sensibilidade aos glicocorticoides foram observadas em homens com resistência à insulina e hipertensão, sendo relacionada a regulação anormal do eixo HPA (hipotálamo-pituitária-adrenal), além da exposição dos tecidos ao cortisol devido a modulações periféricas (Walker et al., 2000; Weinsberg et al., 2008). Elevadas concentrações totais de cortisol também foram observadas em equinos obesos (Glunk et al., 2015; Hart et al., 2016; Nostell et al., 2021). O cortisol é um hormônio antagonista da insulina e, em situações de estresse crônico, bloqueia a captação de glicose pelos tecidos dependentes de insulina, ocasionando hiperglicemia constante e consequente aumento da liberação de insulina, que estimula a lipogênese e o depósito de tecido adiposo. Além disso, níveis elevados de cortisol podem induzir resistência à leptina, que contribui para maior acúmulo de tecido adiposo (Peckett et al., 2011; Tsatsoulis et al., 2013; Kaczmarek et al., 2016).

2.4 Estratégias para redução da obesidade em equinos

A perda de massa corporal é o principal fator no combate à obesidade. A restrição calórica por meio da dieta tem sido amplamente estudada e apresenta

resultados satisfatórios em equinos (Van Weyenberg et al., 2008; Dugdale et al., 2010; Shepherd et al., 2021). Além de promover a perda de peso, a restrição dietética pode melhorar as respostas metabólicas, como a sensibilidade à insulina e a mobilização de triglicerídeos e ácidos graxos não esterificados (Van Weyenberg et al., 2008). Porém, é válido ressaltar que a restrição da dieta deve ser feita de maneira a atender as necessidades nutricionais da categoria em questão e que atenda à saciedade dos animais (Shepherd et al., 2021). Deste modo, Shepherd et al. (2021) indicam que um programa de perda de peso por meio da restrição dietética pode ser realizado de maneiras simples, por meio da redução da quantidade de concentrado fornecida ou alteração do tipo de concentrado e restrição da quantidade de volumoso fornecido, uma vez que o fornecimento de volumoso *ad libitum* pode não proporcionar restrição energética adequada para alguns indivíduos ou categorias, principalmente levando em consideração a qualidade do volumoso.

Além da restrição energética da dieta, programa de exercício regular pode auxiliar para a perda de massa corporal e ainda proporcionar melhorias nas respostas metabólicas dos animais, como na sensibilidade à insulina e metabolismo da glicose (Gordon et al., 2009; Bamford et al., 2019; Pratt-Phillips, 2024), aumento da porcentagem de massa magra, hipertrofia muscular e aumento da taxa metabólica basal (Pratt-Phillips, 2024). Desta forma, o exercício físico não só auxilia na perda de massa corporal, como também atua na prevenção de distúrbios metabólicos. (Powel et al., 2002; Stewart-Hunt et al., 2006; Gordon et al., 2009; Carter et al., 2010; Bamford et al., 2019).

2.5 Máxima fase estável de lactato e métodos para determinação do limiar de lactato

A máxima fase estável de lactato (MFEL) é considerada o método padrão ouro para avaliação da capacidade de fosforilação oxidativa e tem sido aplicado não apenas em humanos, mas também em equinos e cães (Urhausen et al., 1993; Faude et al., 2009; Lindner, 2010; Soares et al., 2014; Miranda et al., 2014; Ferraz et al., 2022). Apesar de sua origem incerta, a MFEL pode ser definida como a intensidade máxima de exercício onde a concentração de lactato ([La^-]) atinge condição dinâmica

de estado estacionário entre a produção, remoção e metabolismo de lactato (Heck et al., 1985; Brooks, 2020; Heck e Wackerhage, 2024), com variação de > 1mM durante os últimos 20 minutos da sessão de exercício de velocidade constante (Płoszczyca et al., 2020; Ferraz et al., 2022). No entanto, o teste da MFEL não é um protocolo prático de ser aplicado, uma vez que requer pelo menos três sessões de exercício em velocidade constante com duração de 30 minutos em dias alternados (Svedahl e MacIntosh 2003, Gondim et al., 2007). Por esse motivo, torna-se necessária a utilização de outras metodologias que possam predizer a MFEL com única sessão de exercício.

Assim, testes de esforço incremental (TEI) têm sido utilizados para determinar o limiar de lactato (LL), que é o ponto em que ocorre aumento exponencial na curva velocidade-lactato (LVC) e pode estimar a MFEL (Gondim et al., 2007; Ferraz et al., 2008; Lindner, 2010, Miranda et al., 2014; Ferraz et al., 2022). De acordo com a literatura disponível, foram identificados aproximadamente 25 métodos para determinar o LL (Faude et al., 2009; Poole et al., 2021). Nesse contexto, pesquisadores estudaram a concordância entre os métodos de predição e a MFEL com objetivo de destacar quais métodos podem ser considerados de fato válidos para predizer a MFEL para atletas humanos e modelos animais. Heck et al. (1985) realizaram estudo com atletas humanos para determinar a MFEL e afirmaram que ela ocorreu a velocidade de [La^-] de 4mM (V_4), também chamada de início do acúmulo de lactato sanguíneo (OBLA). Embora V_4 seja amplamente utilizado em estudos com cavalos, as evidências mostram que este método não é adequado, uma vez que não considera diferenças individuais na cinética do lactato (Svedahl e MacIntosh, 2003) e pode superestimar a MFEL (Gondim et al., 2007; Lindner, 2010; Miranda et al., 2014; Soares et al., 2014). Da mesma forma, os pesquisadores indicaram que V_2 também parece superestimar a MFEL em cavalos quando realizada em esteira (Lindner 2010, Soares et al., 2014).

Além dos métodos de lactatemia fixos, outros protocolos para determinação do LT foram aplicados para determinar a MFEL em humanos e animais. Uma delas é a inspeção visual do LT (LT_V) a partir das respostas [La^-] ao TEI. Cunha et al. (2009) mostraram que a velocidade correspondente ao LT_V (VLT_V) pode predizer a velocidade na MFEL (V_{MFEL}) em ratos idosos. Da mesma forma, resultados foram

obtidos para cães (Alves et al., 2020; Ferraz et al., 2022), sugerindo que o LT_V pode ser usado para predizer a MFEL. Além disso, ajustes matemáticos também são utilizados para determinar o LT. Zagatto et al. (2008) indicaram que modelo de regressão linear bissegmentado (LT_{BI}) aplicado a uma inspeção visual do aumento abrupto de [La^-] pode predizer a resistência aeróbica de jogadores de tênis de mesa. Ferraz et al. (2022) também sugeriram que a velocidade no LT_{BI} (V_{LTBI}) pode ser um método simples para predizer a MFEL em cães Beagle.

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CAPÍTULO 2 – A submaximal training as a preliminary strategy for maximal lactate steady state determination in horses on forage-only diet: some insights to lose weight

ABSTRACT

Equine obesity can be reduced through weight loss protocols that involve a negative energy balance. The objective of this study was to evaluate the metabolic responses and body measurements of ten teaching horses maintained on pasture and undergoing seven weeks submaximal training designed for untrained horses to become capable of performing a protocol to reach the maximal lactate steady state (MLSS). Metabolic responses and body measurements were evaluated at two-time points: before (PreTr) and after (PostTr) the conditioning program. Body measurements (BM, BCS, CNS, and neck circumference) and metabolic responses (glucose, insulin, cortisol, triglycerides, total cholesterol, LDL, VLDL, HDL, and fructosamine) were evaluated. The results showed that PostTr had a significant reduction in plasma glucose and insulin levels, as well as a decrease in cortisol peak compared to the PreTr. There was also a significant decrease in total cholesterol in PosTr compared to PreTr. Neck circumference measurements showed significant reduction, particularly in P25 and P50, in PosTr compared to PreTr. However, there were no significant differences in body condition scores and crest scores. In conclusion, training programs can assist in reducing equine overweight and improving the metabolic responses of horses.

Keywords: obesity, dyslipidemia, metabolic syndrome, laminitis, glucose, insulin

1. Introduction

Obesity has become a rising problem in horse breeding with a significant percentage of the equine population being overweight or obese (Wyse et al., 2008; Stephenson et al., 2011; Thatcher et al., 2012; Robin et al., 2015; Potter et al., 2016; Giles et al., 2020) and it can be characterized as a chronic inflammatory state which occurs due to a high accumulation of fat, leading to metabolic disorders such as insulin dysregulation, equine metabolic syndrome, endocrinopathy laminitis (Hoffman et al., 2003; Carter et al., 2009; Coleman et al., 2018; Fitzgerald et al., 2019) and thermoregulation disorders (Goossens, 2008; Fitzgerald et al., 2019; Shepherd, 2021). In addition, equine obesity can also affect reproductive efficiency and athlete performance (Frank et al., 2010; Morgan et al., 2014; Shepherd et al., 2021), directly affecting the well-being of horses. The main factors that are related to equine obesity are excess feed supply, especially diets rich in non-structural carbohydrates, confinement, and limited physical activity (Frank et al., 2010; Daradics et al., 2021), which are associated with an imbalance between energy intake and energy expenditure.

The key to reducing equine obesity is body mass loss. Usually, the weight loss protocols primarily include a negative energy balance achieved through dietary adjustments. In this way, some researchers have shown that dietary restriction effectively reduces body mass (Van Weyenberg et al., 2008; Dugdale et al., 2010; Shepherd et al., 2021). In addition to body mass loss, dietary restriction can improve metabolic responses, such as insulin sensitivity and mobilization of triglycerides and non-esterified fatty acids (Van Weyenberg et al., 2008). Gill et al. (2016) also suggested that 10 to 20% of energy intake restriction can improve insulin sensitivity and reduce body condition scores. It is important to highlight that energy restriction may be promoted by meeting essential nutrient requirements according to horse's categories and managing satiety (Shepherd et al., 2021).

Additionally, a combination of a caloric deficit from the diet with exercise can result in a successful body mass loss plan since the exercise can be effective in preventing and reducing obesity and improving glycemia and insulin sensitivity in horses (Gordon et al., 2009; Bamford et al., 2019; Pratt-Phillips, 2024). Regular exercise also alters lean mass percentage, contributes to muscle hypertrophy, and

makes skeletal muscle more active, increasing the basal metabolic rate (Pratt-Phillips, 2024). Few studies have demonstrated the benefits of physical exercise in reducing obesity and improving metabolic parameters in horses, which contribute not only to body mass loss but also to the prevention of metabolic diseases (Powel et al., 2002; Stewart-Hunt et al., 2006; Gordon et al., 2009; Carter et al., 2010; Bamford et al., 2019). We sought to contribute to the academic discussion on preventing and combating equine obesity by evaluating the metabolic responses and body measurements of overweight horses maintained on pasture and undergoing submaximal training. Notably, this training program was designed for untrained horses to become capable of performing a protocol to reach the maximal lactate steady state (MLSS). The MLSS is a well-known method for determining the aerobic fitness of athletic horses. It occurs at the highest exercise load with constant plasma lactate concentration. The gold standard protocol for determining the MLSS involves conducting repeated 30-minute exercise sessions near the suspected MLSS.

2. Material and methods

2.1 Ethical approval

The experiment was conducted at the Laboratory of Pharmacology and Equine Exercise Physiology (LAFEQ), located at the Faculty of Agricultural and Veterinary Sciences (FCAV), São Paulo State University (UNESP), Jaboticabal, São Paulo, Brazil. All procedures followed the Ethical Principles in Animal Experimentation as established by the National Council for Control in Animal Experimentation (CONCEA). The protocol underwent review and approval by the Ethics Committee for Animal Use (CEUA) – FCAV/UNESP, Jaboticabal, Brazil, under protocol No. 002310/21.

2.2 Horses

Ten teaching horses were used, seven Arabian purebred horses and three crossbreed (nine females and one gelding) aged ranging from 3 to 19 years old, with an average body mass of 428.6 ± 50.1 kg, body condition score (BCS) of 6.5 ± 0.84,

on a scale of 1-9 (Henneke et al., 1983), and cresty neck score (CNS) of 1.9 ± 0.46, on a scale of 0-5 (Carter et al., 2009). The horses belonged to the didact herd of the university. Since the study was conducted at the end of the reproductive cycle, the mares were possibly in anoestrus. The horses underwent a physical examination, as well as hematological and biochemical blood tests to determine their health status. They were also dewormed (Equijet, JA Saúde Animal, Brazil) and vaccinated against respiratory viruses and tetanus (Lexington Gold, Dechra Brazil, Brazil).

The horses had not participated in any conditioning program for four months (November 2022 to February 2023) leading up to the training program. They were kept in paddocks with Tanzânia grass (*Panicum maximum* cv. Tanzânia) and Massai grass (*Panicum maximum* cv. Massai) (Table 1), supplemented with 2 kg of concentrate meal formulated to supply the animal's requirements (NRC, 2007) (Table 1), mineral salt (GuabiTech Evolution 80, Guabi Nutrição e Saúde Animal S.A., Brazil) and water provided *ad libitum*. This management is routinely adopted for all horses at the institution during the vacation period when the horses are not training. The concentrate was removed from their diet 15 days before the training period. The horses continued to be kept in the same paddocks with mineral salt and water provided *ad libitum*.

Table 1. Nutrient composition by near-infrared spectroscopy (NIRS) of diet provided before the submaximal training program. All values are presented on dry matter basis.

Nutrient (%)	Concentrate meal	Pasture
DM	88.81	23.85
Ash	5.13	9.80
CP	17.17	11.29
EE	4.93	2.71
CF	4.77	-
aNDF	-	71.25
ADF	-	40.10
NFC[1]	-	10.34
Ca	1.75	0.35
P	0.74	0.36
DE[2]	-	1.84

DM, dry matter; CP, crude protein; EE, ether extract; CF, crude fiber; aNDF, neutral detergent fiber, NFC, nonfiber carbohudrates, Ca, calcium; P, phosphorus; DE, digestible energy.

[1]NFC, value provided in the bromatological analysis

[2]DE, estimated digestible energy NRC (2007) = 2,118 + 12.18 x (% CP) – 9.37 x (% ADF) – 3.83 x (% hemicellulose) + 47.18 x (% EE) + 20.35 x (% NFC) – 26.3 x (% ash)

2.3 Pasture management

The horses were kept in four paddocks (total area: 7.9 ha) in a rotational grazing system, with the paddocks fertilized (NPK 4-14-8, Heringer, Brazil) before the experimental period, according to a previously conducted soil analysis. Every seven days, always on Saturday mornings, the animals were relocated to a new paddock, respecting the grass height entry of 70 cm and exit of 30 cm of Tanzânia grass (Barbosa et al., 2007; Difante et al., 2009), which predominates in the four paddocks. Before relocating the animals, the forage height in each paddock was measured using equipment adapted with a tape measure. Once the paddock for relocation was determined, pasture sampling was carried out using a 50 x 50 cm plastic square, randomly thrown in a zigzag pattern across the paddock until the entire area was covered. Between 25-30 samples were standardized per paddock for each forage, with

all paddocks consisting of Tanzânia grass and Massai grass. Samples were collected every week for the eight weeks of the conditioning program, homogenized and weighed from the different sampling points, with a final sample of 700 g being taken, which was adequately stored in plastic bags and frozen at -20°C for later bromatological analysis.

2.4 Experimental design

A repeated measures study was conducted. All horses were included in the experimental training program, and the metabolic responses were evaluated at two-time points: before (PreTr) and after (PostTr) the seven-week conditioning program.

2.5 Conditioning program

The conditioning program lasted seven weeks and was composed of four training stages. Its goal was to prepare the horses for the gold-standard test to measure an individual's aerobic capacity, known as the maximal lactate steady state (MLSS). All information on MLSS protocol will be provided in the next chapter of this Tesis. All exercise sessions occurred on a high-performance treadmill (Galloper® 5500, Sahinco, Palmital—Brazil) in a climate-controlled room set at 22°C.

2.5.1 Stages 1 and 2: submaximal training at 130 and 160 bpm

The Stages 1 and 2 protocols were adapted from Carter et al. (2010). Stage 1 comprised of three weeks of submaximal training, during which each horse performed exercise sessions on the treadmill at a speed corresponding to 130 bpm for each individual or 59% of maximal heart rate (HR_{max}), considering the HR_{max} as 220 bpm (Gramkow and Evans, 2006). Horses were exercised three times per week enrolled into two training groups. The heart rate (HR) was measured during the exercise

session using a frequency meter Polar Pacer Pro receiver with a specific Polar H-10 transmitter for horses (Polar Electro®, Kempele, Finland). All horses performed a 2-minute warm-up exercise at 1.5 m/s with no surface inclination, followed by 10 minutes at a speed corresponding to 130 bpm (59% HR_{max}) for each individual with a 5% slope. Consequently, the speed during the 10 minutes was not constant, varying according to the HR of each animal. If the HR was below 125 bpm, the speed was increased in intervals of 0.1 m/s, and if the HR was above 140 bpm, the speed was reduced using the same interval. The exercise session ended when the horses reached an HR of 200 bpm or at the end of the 10 minutes. Additionally, stage 2 consisted of two weeks of submaximal training following the same protocol as stage 1. However, in this stage, each animal performed exercise sessions at a speed corresponding to 160 bpm (72% HR_{max}). Consequently, the speed during the 10 minutes was not constant, varying according to the HR of each animal. If the HR was below 155 bpm, the speed was increased in intervals of 0.1 m/s, and if the HR was above 170 bpm, the speed was reduced using the same interval. The exercise session also ended when the animals reached a HR of 200 bpm or at the end of the 10 minutes.

2.5.2 Stage 3: Incremental exercise test (IET) design

Horses underwent a single IET to determine the individual lactate threshold. The IET protocol was adapted from Lamprecht and Williams (2012). All horses performed a 3-minutes warm-up exercise at 1.5 m/s with no surface inclination, followed by 2 minutes at 3 m/s with 5% slope. Following the warm-up phase, the IET started at 3.5 m/s and the speed was increased by 0.5 m/s until reaching 10 m/s at 2 minutes intervals. Each speed step was interspersed with an active recovery period lasting 1 minute at 1.5 m/s. The treadmill slope was set at 5% throughout the IET. The IET was completed when the horses reached a HR of 200 bpm (93% HR_{max}) or at the end of the 10 m/s speed stage. At the end of each speed stage, blood samples were collected for subsequent determination of [La^-]. The IETs were always conducted between 06 and 11 AM. After completing the IET, horses actively recovered at 1.5 m/s for 5 minutes, with no treadmill slope.

2.5.3 Stages 4: Maximal lactate steady state (MLSS)

Once the individual lactate threshold and its corresponding velocity were obtained in the IET by visual inspection, horses underwent the MLSS test on the treadmill. The MLSS protocol was adapted from former agreement studies described for Arabian horses (Miranda et el., 2014) and Beagle dogs (Ferraz et al., 2022). In order to determine MLSS, the horses underwent 30-minutes submaximal bouts of constant-speed treadmill running (three to five sessions), every other day. The sessions began with a 5-minute warm-up phase at 2 m/s, followed by 25 minutes at a constant speed while maintaining a 5% slope. The speed of the first bout corresponded to the individual velocity of visual lactate threshold determined in the IET, while the speed of following sessions was adjusted up or down by 0.3, 0.5, or 1 m/s based on the [La^-] until a MLSS was reached. Blood samples were collected at 0, 5, 10, 15, 20, 25, and 30 minutes to assess the [La^-]. Thus, MLSS is the load at which the prescribed speed does not induce an increase in [La^-] by more than 1mM between 10 and 30 minutes of constant exercise (Carminatti et al., 2021; Ferraz et al., 2022).

2.6 Blood samples

All blood samples were collected through catheterization of the jugular vein using a 14G catheter (Safelet, NIPRO Medical Ltda, Brazil) coupled to a 120 cm extension tube (Embramed, Brazil). The region where the catheter was inserted was first shaved and prepared in an aseptic manner. After collected samples, 20 mL of 0.9% saline solution was flushed into the extension tube and catheter to maintain the system patency. Between the collections, saline remaining within the system was removed by withdrawing and discarding 20 mL before collecting the sample.

2.6.1 Lactate

To determine [La^-] during the IET and MLSS test, blood samples were stored in 4 mL tubes containing ethylenediaminetetraacetic acid (EDTA) and sodium fluoride (Diagnóstica Indústria e Comércio Ltda – Biocon, Belo Horizonte, Brazil). At the end of each session, the samples were centrifuged under refrigeration (10°C) during 10 minutes at 3,000 g (ALC – Multispeed refrigerated centrifuge PK121R, New Jersey, USA), the plasma separated, and kept refrigerated (4ºC) for analysis on the same day. An electro-enzymatic method with an automatic bioanalyzer (YSI 2300 Stat Plus®, Ohio, USA) was used to determinate the [La^-].

2.6.2 Glucose and insulin

To determine glucose and insulin curves, blood samples were obtained before and after the entire conditioning program. The horses were not subjected to a fasting period prior to blood collection; they were taken from the paddock, catheterized in the jugular vein, and housed in individual stalls (16 m^2) with water provided *ad libitum* and remained with no access to forage throughout the collection period. Basal sample was obtained one hour after catheterization of each individual's jugular vein, aiming to reduce potential high cortisol levels resulting from the stress caused by the procedure. After the basal collection of the last catheterized horse, they were fed with oats (Table 2) in order to raise the glucose and insulin peak. The amount of oats was provided individually, calculated based on each animal's body mass and respecting the limit of 1 g starch/kg body weight (Coenen and Vervuert, 2010). Subsequent sampling moments were conducted following the full consumption of oats, with samples to determine glucose being collected immediately after ingestion (0), 30, 60, 90, 120, 180, 240, and 300 minutes; and blood samples to determine insulin being collected 60, 90, 120, 180, and 240 minutes after ingestion.

Table 2. Nutrient composition by near-infrared spectroscopy (NIRS) of oats provided before sampling for the determination of plasmatic glucose and insulin. All values are presented on dry matter basis.

Nutrient (%)	Oat
DM	87.78
Ash	2.99
CP	14.88
EE	6.74
CF	5.43
aNDF	16.15
ADF	7.08
NFC[1]	61.53
Starch	54.19
Ca	0.14
P	0.51
DE[2]	3.69

DM, dry matter; CP, crude protein; EE, ether extract; CF, crude fiber; aNDF, neutral detergent fiber, NFC, nonfiber carbohudrates, Ca, calcium; P, phosphorus; DE, digestible energy.

[1]NFC, value provided in the bromatological analysis

[2]DE, estimated digestible energy NRC (2007) = 2,118 + 12.18 x (% CP) – 9.37 x (% ADF) – 3.83 x (% hemicellulose) + 47.18 x (% EE) + 20.35 x (% NFC) – 26.3 x (% ash)

Blood samples to determine plasma glucose were stored in 4 mL tubes containing EDTA and sodium fluoride (Diagnóstica Indústria e Comércio Ltda – Biocon, Belo Horizonte, Brazil). Blood samples to determine plasma insulin were stored in 4 mL tubes containing sodium heparin (Diagnóstica Indústria e Comércio Ltda – Biocon, Belo Horizonte, Brazil). At the end of each moment of collection, the samples were centrifuged under refrigeration (10°C) during 10 min at 3,000 g (ALC – Multispeed refrigerated centrifuge PK121R, New Jersey, USA), the plasma separated, and storage at freezer – 80º C until analysis. An electroenzymatic method with an automatic bioanalyzer (YSI 2300 Stat Plus®, Ohio, USA) was used to determinate the glucose parameters. The insulin was determinate using an enzyme-linked immunosorbent assay (ELISA) with a microplate photometer (Multiskan GO Thermo Fisher Scientific,

Waltham, MA, USA) with a commercial kit Mercodia Equine Insulin ELISA (Mercodia AB, Uppsala, Sweden).

2.6.3 Cortisol

Blood samples were collected before and after the entire conditioning program to determine the cortisol, at the same time as glucose and insulin, always in the morning. A baseline sample was obtained one hour after each individual's jugular vein catheterisation. Subsequent sampling moments were conducted following the completion of concentrated food intake, with samples to determine cortisol being the same as insulin: 60, 90, 120, 180, and 240 minutes. However, only the basal and peak times were evaluated. The peak time of each horse was determined from the individual glucose peak. Blood samples were stored in 4 mL tubes containing sodium heparin (Diagnóstica Indústria e Comércio Ltda – Biocon, Brazil), centrifuged, the plasma separated, and stored at freezer – 80º C until analysis. Plasma cortisol concentrations were measured using an ELISA kit (Accu Bind ELISA Microwells: Cortisol, Monobind Inc., Lake Forest, USA) and a microplate reader (Labsystems 352 Multiskan MS, Waltham, MA, USA).

2.6.4 Lipid profile and fructosamine

To determine the triglycerides (TRI), total cholesterol (CHOL), low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), high-density lipoprotein (HDL), and fructosamine (FRU), blood samples were collected before and after the entire conditioning program. The samples were collected one hour after catheterization of each individual's jugular vein, at the same time as the basal insulin and glucose samples, always conducted in the morning. Blood samples were stored in 9 mL tubes containing serum clot activator (Vacuette®, Greiner Bio-One, Americana, Brazil). At the end of blood collection, the samples were centrifuged at room temperature (25º C) for 10 min at 3,000 g (ALC – Multispeed refrigerated centrifuge PK121R, New Jersey, USA), the serum separated, and stored at -80ºC until analysis. An enzymatic

colorimetric method with a biochemistry analyser (Mindray BS-200E, Shenzhen, China) was used to determine the metabolic biomarkers.

2.6.5 Body mass, body condition score (BCS), cresty neck score (CNS), and neck circumference

The horses were weighed at the beginning of the first and last week of training, always on the first day of the training week, using a digital animal weighing scale (MGR-3000 Junior®, Toledo do Brasil Indústria de Balanças Ltda, São Bernardo do Campo, Brazil). Immediately after weighing, the body condition score (BCS) was assessed by three experienced evaluators independently, using a scale of 1 to 9 according to the methodology described by Henneke et al. (1983). Similarly, the cresty neck score (CNS) was also assessed by three experienced evaluators independently, using a scale of 0 to 5 as described by Carter et al. (2009). The average of the values provided by the specialists was used to establish the BCS and the NCS. Additionally, neck circumference was measured by a single experienced evaluator using a measuring tape. The total length of the neck from the poll to the cranial aspect of the withers was measured and neck circumference measurements were taken at three different heights relative to the total neck length (P25, P50, and P75) as described by Frank et al. (2006).

2.7 Statistical analysis

All the analysis were performed using the R software with the RStudio integrated development environment version 4.3.1 (Posit Software, PBC). The same statistical analysis was used to compare the mean ± standard deviation of glucose, insulin, and cortisol. For these three variables, the comparisons of PreTr vs. PostTr were performed by applying the repeated measures analysis of variance (ANOVA), followed by the paired t-test with Bonferroni correction for *post hoc* comparisons. The mean ± standard deviation of the body mass (BM), fructosamine (FRU), and the lipid profile (total

cholesterol, triglycerides, LDL, HDL, VLDL) were compared using F and Bartlett tests to evaluate the homogeneity of variances and the Shapiro-Wilk test to determine the normality of residues. The parametric variables were compared using the t-test, while the Wilcoxon test was applied to compare the non-parametric variables. The means of the neck circumference measurements were compared by applying the t-test. A significance level of 5% was considered for all tests used. For the analysis of body condition scores (BCS) and cresty neck score (CNS), a generalized linear model (GLM) adjusted by the binomial family was used. This adjustment was chosen due to the classification performed, namely: BCS < 6/9 were classified as 0 while BCS > 7/9 were classified as 1. For CN, values <1/5 were classified as 0, while values > 2/5 were classified as 1. Prior to the analyses, the quality of the model fit (goodness of fit [GOF]) was evaluated by calculating Nagelkerke's R^2 and the Hosmer-Lemeshow test. Once the model was established, *post hoc* comparisons between the different groups were calculated using Tukey's HSD test with Bonferroni correction. Regarding the data glucose and insulin values, the areas under the curve (AUC) were calculated for each individual, using spline interpolation. That is, the calculated values refer to the area under the natural cubic spline interpolation of the values obtained over time.

3. Results

3.1 Glucose and insulin

The plasma glucose levels were measured to identify the glycaemic curve of each group before and after the conditioning program (**Figure 1A**). It was observed that PostTr presented a higher baseline value of plasma glucose compared to PreTr (PostTr: 6.11 ± 0.98 mM vs. PreTr: 5.58 ± 0.73 mM, p = 0.046). Additionally, PostTr showed a significant reduction in glucose levels compared to PreTr at 60 minutes (PostTr: 7.32 ± 1.09 mM vs. PreTr: 8.78 ± 1.0 mM, p = 0.02), 90 minutes (PostTr: 7.50 ± 1.03 mM vs. PreTr: 9.51 ± 0.99 mM, p = 0.001), and 120 minutes (PostTr: 7.71 ± 1.10mM vs. PreTr: 10.00 ± 1.38 mM, p = 0.002). Similarly, the insulin curve was determined for each group by measuring plasma insulin levels (**Figure 1B**). There was

a significant reduction in insulin levels from PreTr to PostTr at 60 minutes (24.8 ± 12.5 mU/L to 19.4 ± 17.0 mU/L, p = 0.01), 90 minutes (30.1 ± 14.4 mU/L to 24.2 ± 28.7 mU/L, p = 0.001), 120 minutes (30.9 ± 14.7 mU/L to 28.3 ± 34.9 mU/L, p = 0.001), and 180 minutes (29.0 ± 14.4 mU/L to 18.1 ± 8.24 mU/L, p = 0.014).

The area under the curve (AUC) of glucose (**Figure 2A**) and insulin (**Figure 2B**) were also calculated. It was observed a significant decrease of AUC of glucose from PreTr to PostTr (2648.5 ± 352.79 vs. 2311.3 ± 314.22, p = 0.02). A reduction of the AUC of insulin from PreTr to PostTr was also observed (7632.4 ± 5211.20 vs. 5753.71 ± 6789. 25, p = 0.04).

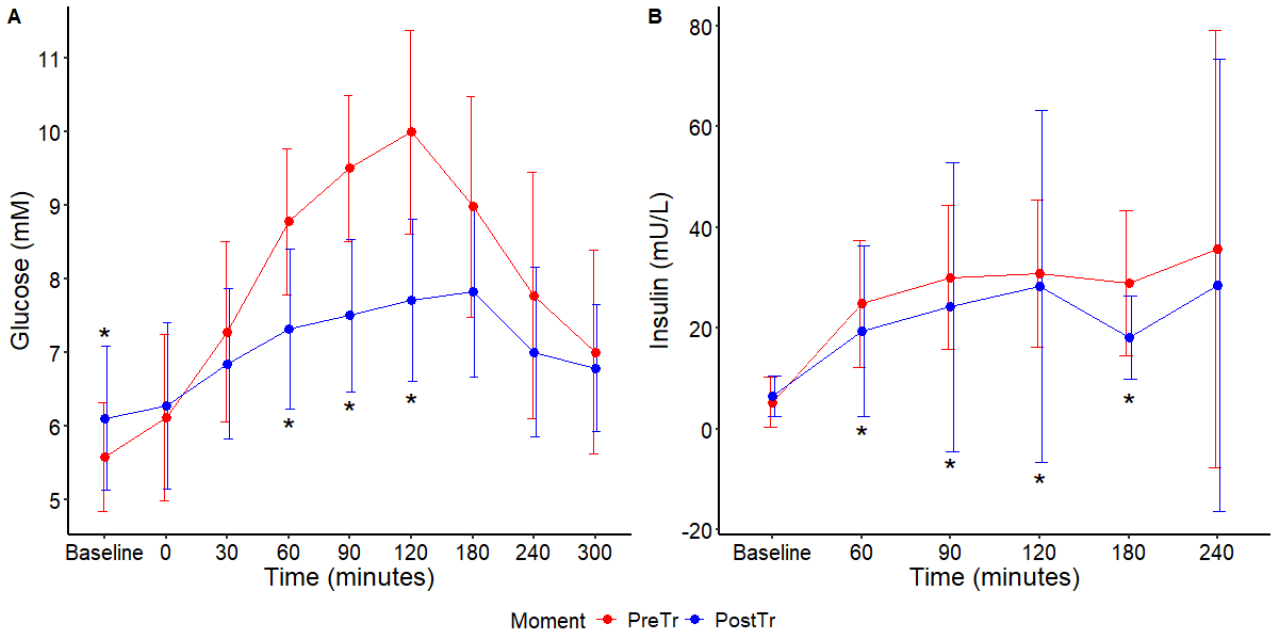

Figure 1. Means ± standard deviation of plasmatic glucose (A) and insulin (B) levels in horses subjected to a conditioning program at the beginning (PreTr) and at the end (PostTr) of the experimental period. Asterisks indicate statistical differences by paired t-test with Bonferroni correction, p < 0.05.

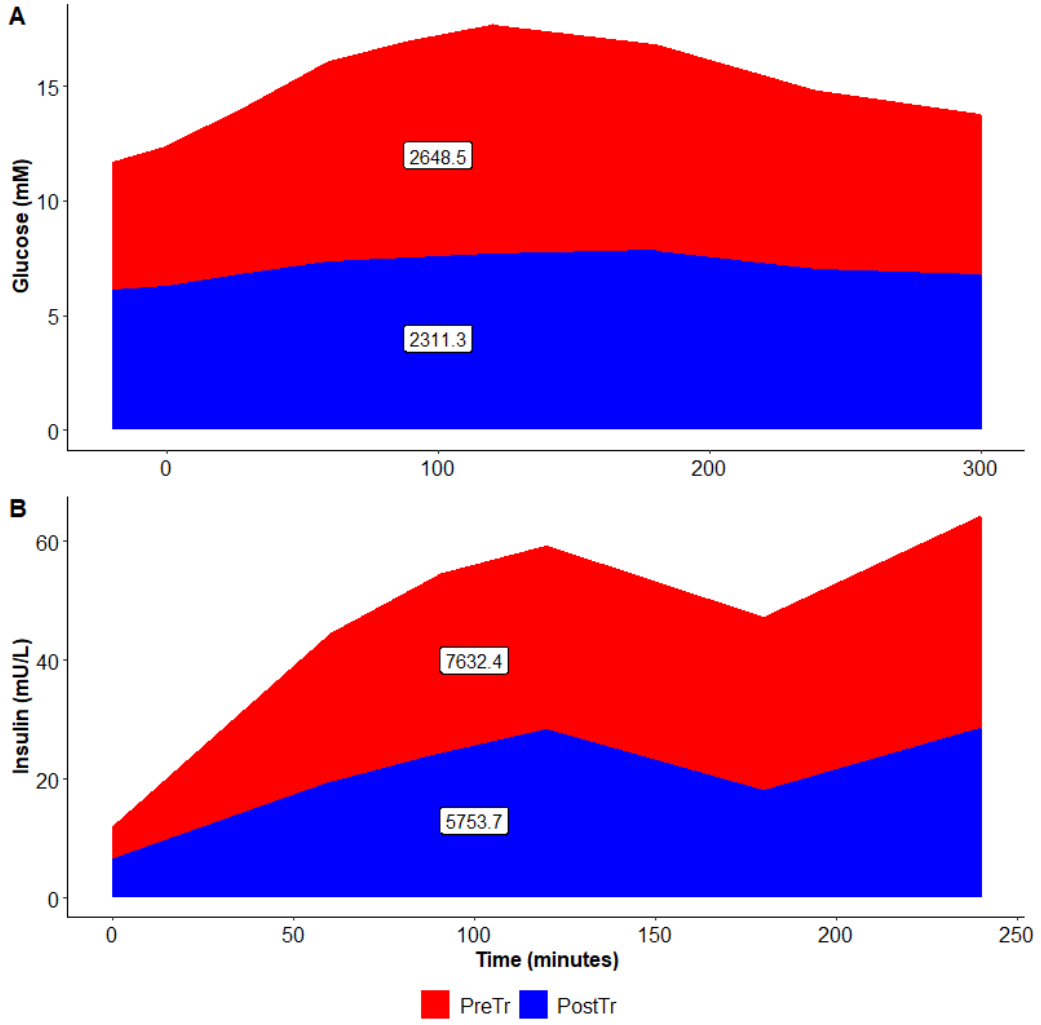

Figure 2. Area under the curve (AUC) plasmatic glucose (A) and insulin (B) of horses subjected to a conditioning program at the beginning (PreTr) and at the end (PostTr) of the experimental period. AUC presented was calculated by spline interpolation.

3.2 Cortisol

Figure 3 shows the cortisol levels of each group before and after the training period at the basal and peak timepoints. A significant reduction in peak cortisol concentrations was observed at PostTr compared to PreTr (PostTr: 7.84 ± 1.92 ug/dL vs. PreTr: 10.8 ± 3.04 ug/dL, p = 0.04).

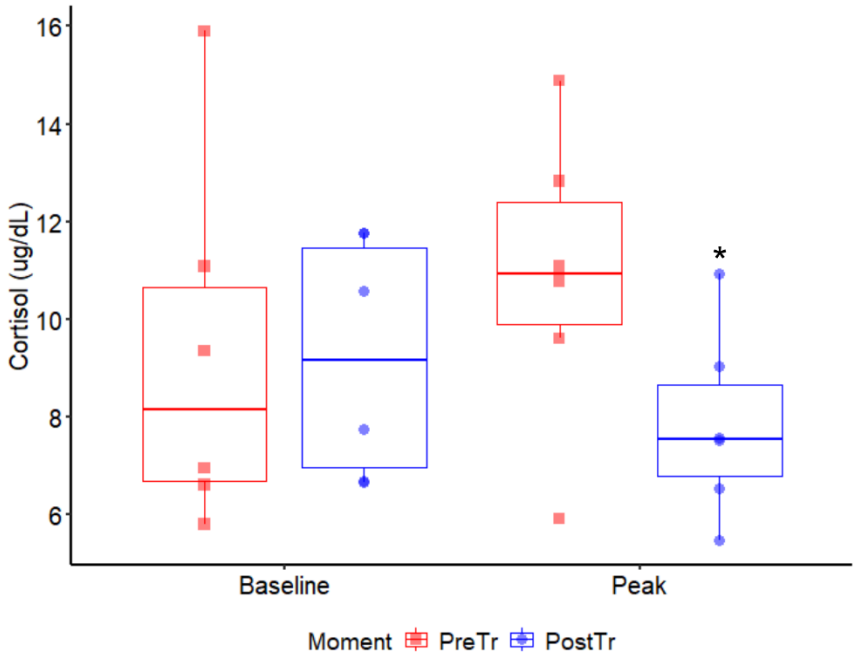

Figure 3. Boxplot indicating plasmatic cortisol levels in horses subjected to a conditioning program at the beginning (PreTr) and at the end (PostTr) of the experimental period. Asterisks indicate statistical difference by paired t-test with Bonferroni correction, $p < 0.05$.

3.3 Lipid profile and fructosamine

Table 3 presents the means ± standard deviations (SD) of serum concentrations for lipid profile and fructosamine. PostTr exhibited a significant reduction in cholesterol (CHOL) levels compared to PreTr ($p = 0.009$). No significant differences were observed between groups for triglycerides (TRI), low-density lipoprotein (LDL), high-density lipoprotein (HDL), very-low-density lipoprotein (VLDL), and fructosamine (FRU).

Table 3. Means and standard deviation (SD) of serum concentrations of lipid variables of fifteen horses before and after seven weeks of training

Groups	TRI (mg/dL)		LDL (mg/dL)		HDL (mg/dL)		VLDL (mg/dL)		CHOL (mg/dL)		FRU µmol/L	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PreTr	20.2	11.1	10.2	4.84	36.1	12.3	4.8	1.44	46.2 [a]	22.6	152	26.4
PosTr	11.2	3.88	9.12	4.77	32.0	7.97	4.4	1.67	25.4 [b]	5.50	163	28.4

TRI, triglyceride; LDL, Low-density lipoprotein; HDL, High-density lipoprotein; VLDL, Very-low-density lipoprotein; CHOL, Total cholesterol; FRU, fructosamine; PreTr, before seven weeks of conditioning program; PostTr, after seven weeks of conditioning program; SD, standard deviation.

Different letters in the same column represent a statistical difference by Wilcoxon test, $p < 0.05$.

3.4 Body mass, BCS, CNS, and neck circumference

PosTr presented a significantly reduction in body mass compared to PreTr ($p = 0.01$) (**Table 4**). The circumference of the proximal third of P25 significantly decreased from PreTr to PostTr ($p = 0.01$), as shown in Table 4. Similar results were found for P50, with PostTr presenting reduced values compared to PreTr ($p = 0.03$). No difference was observed for body condition score (BCS) and cresty neck score (CNS) (**Table 5**).

Table 4. Means and standard deviation (SD) of body mass and neck circumference measurements of horses before and after seven weeks of training

Moments	BM (kg)		NC (cm)					
			P25		P50		P75	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PreTr	404[a]	54.4	69.6[a]	3.42	87.2[a]	7.29	106.0	7.39
PostTr	389[b]	46.4	67.8[b]	2.92	84.0[b]	5.68	104.0	5.90

BM, body mass; NC, neck circumference; P25, proximal part of the neck; P50, middle of the neck; P75 distal part of the neck; PreTr, before seven weeks of conditioning program; PostTr, after seven weeks of conditioning program; SD, standard deviation.

For BM, different letters in the same column represent a statistical difference by Wilcoxon test, $p < 0.05$.

For NC, different letters in the same column represent a statistical difference by paired t-test, $p < 0.05$.

Table 5. Least square means and confidence limits of body condition score (BCS) and cresty neck score (CNS) of horses before (PreTr) and after (PostTr) seven weeks of training.

Moments	BCS				CNS			
	LSM	SE	LCL	UCL	LSM	SE	LCL	UCL
PreTr	0	0.63	-1.24	1.24	2.20	1.05	0.13	4.26
PostTr	-2.20	1.05	-4.26	-0.13	0.40	0.64	-0.86	1.67

BCS, body condition score; CNS, cresty neck score; PreTr, before seven weeks of conditioning program; PostTr, after seven weeks of conditioning program; LSM, least square means; SE, standard error; LCL, lower confidence limit; UCL, upper confidence limit

The crude protein (CP) and the digestible energy (DE) of the pasture (Tanzânia grass and Massai grass) provided to horses during the seven weeks of the conditioning program is illustrated in Figure 4. It was observed a mean CP of 11.84 ± 1.24 % and 1.91 ± 0.09 Mcal/kg of DE.

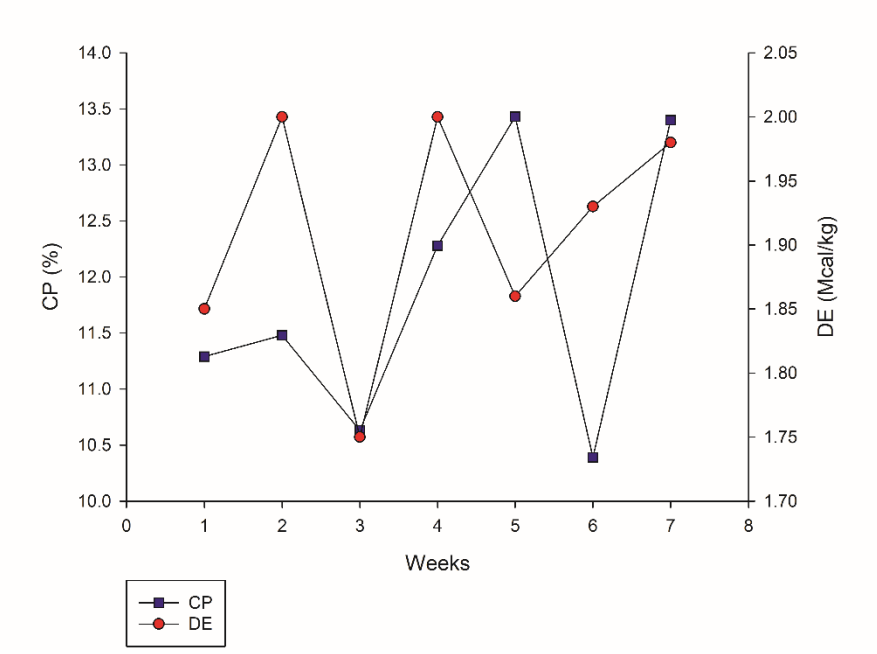

Figure 4. Crude protein (CP) (%) and digestible energy (DE) (Mcal/kg) of the pasture (Tanzânia grass and Massai grass) during the seven weeks of the conditioning program.

4. Discussion

This study evaluated the effect of a seven-week conditioning program on the metabolic responses and body measurements of horses maintained on pasture without concentrate supplementation. Throughout the experimental period, the horses had unrestricted access to forage and engaged in voluntary activity in the paddock. Pasture management was carried out to ensure high nutritional quality throughout the experimental period. The average digestible energy (DE) was estimated to be 15.28 Mcal, according to the DE prediction equation (Pagan, 1998), available in the NRC (2007). The DE requirements for this group of horses are 13.32 Mcal, determined based on the DE requirements for adult horses in maintenance with a weight of 400 kg, an alert temperament, and moderate voluntary activity, as indicated by the NRC (2007). Thus, the diet provided a caloric surplus of 1.96 Mcal.

The benefits of physical exercise in combating obesity and enhancing overall health and well-being are well-established. Weight loss is crucial in the fight against obesity and can be assessed by exercise programs. A reduction of 15 kg (approximately 3.7%) was observed after seven weeks of a conditioning program, though no difference in BCS and CNS was noted. Dugdale et al. (2019) observed a decrease in body mass of 12% in obese ponies after 12-week dietary restriction, however BCS and neck circumference were unchanged. Similar results were indicated by Carter et al. (2010) for overweight or obese horses with insulin resistance, showing a 4% decrease in body mass after eight weeks of moderate-exercise training, with no changes in subcutaneous fat thickness compared to pre-exercise values. These researchers suggested that these results can be explained by a possible reduction of other adipose depots, such as visceral or intramuscular, that can decrease with exercise training. Hallgreen and Hall (2008) indicated that in humans, it can be noticing a significant reduction in visceral adipose tissue when weight loss is observed. This visceral adipose tissue reduction was also associated with regular exercise without diet restriction in obese in patients with type II diabetes (Mourier et al., 1997). Besides, the intensity and duration of the exercise may have been sufficient to reduce body mass but not to cause a noticeable reduction in subcutaneous fat as evaluated by BCS and CNS.

Despite the lack of difference in the CNS, we observed a reduction in the neck circumference of P25 and P50 after the exercise training program. Measurements of circumference at different points along the neck can capture variations in fat distribution or changes in muscle mass that are not reflected in the cresty neck. The cresty neck may not have been significantly affected by the training, or changes in fat and muscle in other parts of the neck may not have been substantial enough to alter the size of the crest. Additionally, fat located in the cresty neck may be more resistant to mobilization and reduction in response to training compared to fat distributed along the neck.

Another notable benefit of exercise training is the enhancement of insulin and glucose metabolism. In our study, we observed a reduction in plasma glucose and insulin levels in horses following seven weeks of a submaximal exercise program. This improvement may be linked to metabolic adaptations resulting from physical conditioning, such as increased lean mass, enhanced capillarization of the muscles, and muscle hypertrophy, all of which contribute to a higher capacity for glucose uptake (Bird et al., 2017; Pratt-Phillips, 2024). These adaptations include elevated expression of mRNA and protein levels of GLUT4, the primary glucose transporter in horse skeletal muscle (Dela et al., 1994; Greiwe et al., 1999; Richter et al., 2013; Pratt-Phillips, 2024). Higher GLUT4 expression enhances insulin sensitivity (IS). Additionally, regular exercise increases glycogen synthase activity, which further supports glucose uptake (Greiwe et al., 1999; Pratt-Phillips, 2024). Exercise training also boosts the activation of insulin receptor substrate (IRS) (Bird et al., 2017; Pratt-Phillips, 2024). Bamford et al. (2019) observed that the association of 12 week low-intensity exercise program, with a dietary restriction led to an improvement in insulin sensitivity when compared with only dietary restriction, indicating that exercise can contribute to insulin metabolism in horses. In addition, Powell et al. (2002) indicated that a short-term light-moderate exercise training improved SI in obese mares. The potential improvement in insulin sensitivity of horses due to exercise may be associated with the decreased cortisol peak values observed after the conditioning program period.

No significant differences were observed in triglycerides, LDL, HDL, VLDL, and fructosamine levels, indicating that the lipid profiles of the horses remained consistent throughout the study, regardless of the training program. Similarly, Carter et al. (2010)

found that moderate exercise training did not affect triglyceride levels in overweight and obese horses. However, the exercise program led to a reduction in total cholesterol after the conditioning program, indicating an improvement in insulin sensitivity. This improvement can be associated with more efficient utilization of fat as an energy source.

It is important to acknowledge the limitations of the study. The results were derived from a small sample of horses, with considerable variation in age and breed. Therefore, additional studies with larger sample sizes and more homogeneous age and breed groups are needed. Besides, the energy expenditure of the conditioning program was not calculated. Without knowing the caloric deficit induced by this exercise program, we cannot accurately assess its effectiveness on the animal's energy balance.

5. Conclusion

It can be concluded that training programs can be implemented as allies in reducing obesity and should be included in weight loss programs, with improvements in the metabolic responses of horses. Although they contribute positively, better results may be achieved when combining physical exercise with dietary restriction. Besides, the training program applied in this study enable horses to perform a protocol to reach the maximal lactate steady state (MLSS).

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CAPÍTULO 3 – Evaluating the endurance hallmark indexes for predicting maximal lactate steady state in teaching horses[1]

ABSTRACT

There is an ongoing academic discussion about measuring plasma lactate concentration ([La^-]) during an incremental exercise test (IET) to establish thresholds associated with the maximal lactate steady state (MLSS), a precise indicator of endurance. Training studies on horses have utilized the onset of [La^-] ($OBLA_{4mM}$), also known as V_4, the velocity at which the [La^-] of 4 mM is reached. V_4 overlooks the individual differences in lactate kinetics, which may result in underestimation or overestimation of the MLSS in horses. This paper compares four methods with MLSS. Ten teaching horses underwent a single IET to determine four exercise intensity thresholds for predicting MLSS. The horses performed constant intensity running bouts to obtain the MLSS. The velocities at which the [La^-] reached 2 mM and 4 mM, V_2 and V_4 determined the thresholds. A randomized and blind trial used a visual analysis (LT_V) and a bi-segmented linear regression model (LT_{BI}). The agreement among the velocities corresponding to the V_2, V_4, V_{LTV}, and V_{LTBI} and the MLSS (V_{MLSS}) was established using mean difference (MD), ordinary least products (OLP), and correlation coefficient (*r*). The average [La^-] at MLSS was 1.50 ± 0.37 mM, and the V_4 was higher than V_{MLSS} with an MD of 2.12 ± 0.59 m/s between them. V_2 and V_{LTV} showed the lowest mean bias when compared to the V_{MLSS}. V_{MLSS} had a moderate correlation with all prediction methods. The V_4 threshold is unsuitable for estimating MLSS. Predicting the MLSS based on individual lactate thresholds may be more accurate.

Keywords*:* Exercise, anaerobic threshold, fatigue, exercise intensity domains, aerobic

[1]Este capítulo corresponde ao artigo científico submetido à revista *Journal of Equine Veterinary Science* e encontra-se em avaliação para publicação.

1. Introduction

The gold standard for assessing endurance performance is the maximal lactate steady state (MLSS), which indicates exercise intensity domains and has become a cornerstone of the aerobic training [1-9]. MLSS is the upper limit of plasma L-lactate concentration ([La^-]) that reaches a steady state during a constant external training loading [10,11]. However, it is a laborious protocol because it requires at least three sessions of constant-velocity exercise lasting 30 minutes on alternate days [4]. Using other curve-fitting procedures to predict MLSS with a single exercise session is necessary. An incremental exercise test (IET) is often conducted to determine the deflexion of the lactate-velocity curve (LVC) and establish the lactate threshold (LT) to predict MLSS. LT is the exercise intensity at which fast-twitch muscle fibers are mobilized, and muscular [La^-] production exceeds the body's ability to clear it, leading to an exponential increase in [La^-] [4,12].

The tipping points in LVC have been observed in horses, leading to the frequent use of a rigid cut-off unit of 4 mM [13]. In the 1970s, a group of German physicians and physiologists based in Cologne conducted studies on marathon runners, football players, and professional cyclists, leading to the development of a methodology for systematic lactate testing. It introduced a 4 mM-LT called the onset of blood lactate accumulation ($OBLA_{4mM}$) [10,11,14]. The origin of the V_4 approach in horses is not well-defined, but it is believed to have originated from studies of sport horses in the 1980s and 1990s [15-19]. The V_4-based training programs have influenced numerous studies on horse conditioning protocols [13]. Although V_4 is used in studies with horses, evidence shows that this method is unsuitable since it does not consider individual differences in [La^-] kinetics [2,20], and may diverge MLSS [2,6,7,8,13,20,21]. The human literature reviews identified approximately 25 methods to determine LT [3,22]. A large body of threshold concepts has evolved and has been the subject of a productive scientific debate for over 50 years [22,23]. Nevertheless,

few studies emphasize the agreement between LT prediction methods and MLSS in horses. This study checks the agreement of MLSS with four endurance hallmark indexes based on LVC in teaching horses. With a focus on the velocity corresponding to MLSS (V_{MLSS}), V_4 is likely higher than V_{MLSS}. Three other thresholds were also addressed using V_2, the velocity at which a [La^-] of 2 mM is reached, visual determination (LT_V) of the first sudden [La^-] increase, and a bi-segmented linear regression model (LT_{BI}).

2. Materials and methods

2.1 Ethical approval

All procedures for the study follow the Ethical Principles in Animal Experimentation as established by the National Council for Control in Animal Experimentation (CONCEA). The protocol underwent review and approval by the Ethics Committee for Animal Use (CEUA) – FCAV/UNESP, Jaboticabal, Brazil, under protocol No. 002310/21.

2.2 Research facility and horses

Ten teaching horses were used, seven Arabian purebred horses and three crossbreed (nine females and one gelding) aged ranging from 3 to 19 years old, with an average body mass of 406 ± 52 kg, belonging to the didact herd of the FCAV/UNESP – Jaboticabal Campus. The horses underwent a physical examination, as well as hematological and biochemical blood tests to determine their health status. They were also dewormed (Equijet, JA Saúde Animal, Brazil) and vaccinated against respiratory viruses and tetanus (Lexington Gold, Dechra Brazil, Brazil). The horses were kept in paddocks (total area: 7.6 ha) and fed solely Tanzânia grass (*Panicum maximum* cv. Tanzânia) and Massai grass (*Panicum maximum* cv. Massai). Water and mineral salt (GuabiTech Evolution 80, Guabi Nutrição e Saúde Animal S.A., Brazil) were provided *ad libitum*.

2.3 Incremental exercise test (IET) design

Horses underwent a single IET to determine the MLSS prediction methods, including V_2, V_4, LT_V, and LT_{BI}. IET protocol was adapted [24], and was conducted on a high-performance treadmill (Galloper® 5500, Sahinco, Palmital - Brazil), in a climate-controlled room, with a temperature of 22°C. All horses performed a 3-minutes warm-up exercise at 1.5 m/s with no surface inclination, followed by 2 minutes at 3 m/s with a 5% slope. Following the warm-up phase, the IET started at 3.5 m/s and the speed was increased by 0.5 m/s until reaching 10 m/s at 2 minutes intervals. Each speed step was interspersed with an active recovery period lasting 1 minute at 1.5 m/s. The treadmill slope was set at 5% throughout the IET. The IET was completed when the horses reached a heart rate of 200 bpm or at the end of the 10 m/s speed stage. At the end of each speed stage, blood samples were collected for subsequent determination of [La^-]. The IETs were always conducted between 06 and 11 AM. After completing the IET, horses actively recovered at 1.5 m/s for 5 minutes, with no treadmill slope.

2.4 MLSS prediction methods

Prediction methods provided by analysing [La^-] data include the identification of four lactate thresholds (V_2, V_4, LT_V, and LT_{BI}). The data-gathering process during the IET for each horse was transferred to a personal computer as an Excel file. An individual LVC was created by plotting each velocity step of the IET (m/s) on the *x*-axis against the corresponding [La^-] (mM) reached at each step on the *y*-axis [25]. The LT and LT corresponding to the velocity (V_{LT}) were determined using four prediction methods (lactate intensity thresholds). An example of each MLSS estimation method is illustrated in Fig 1. Firstly, V_2 and V_4 mean the fixed lactate concentrations values indicating the velocities at which the [La^-] reached 2 mM and 4 mM, respectively (Figs 1A and 1B) [8,11]. We applied the exponential growth function f = a*exp(b*x), where *y* represents [La^-] in mM and *x*

represents velocity in m/s, in order to validate the mathematical standard of the LVC and determine V_2 and V_4. In addition to these arbitrary values, LT was also determined by the visual lactate threshold (LT_V) (Fig. 1C). LT_V is the visual determination of the LVC's deflection point, which is the first sudden increase in [La^-], indicating an abrupt exponential rise in [La^-] [4,26,27]. In a randomized and blinded trial, six experienced researchers in exercise physiology independently identified the velocity at which the lactate threshold occurred. They did this by associating the inflection point with its corresponding to the velocity on the *x*-axis. The average of the values provided by the specialists was used to establish the V_{LTV}. Finally, the bi-segmented linear regression model (LT_{BI}) was used (Fig 1D). This model is a mathematical adjustment that involves two lines obtained from least products regression analysis to support the visual method. The velocity corresponding to the lactate threshold (V_{LTBI}) was determined by equating the *y* values of the two equations [4,28].

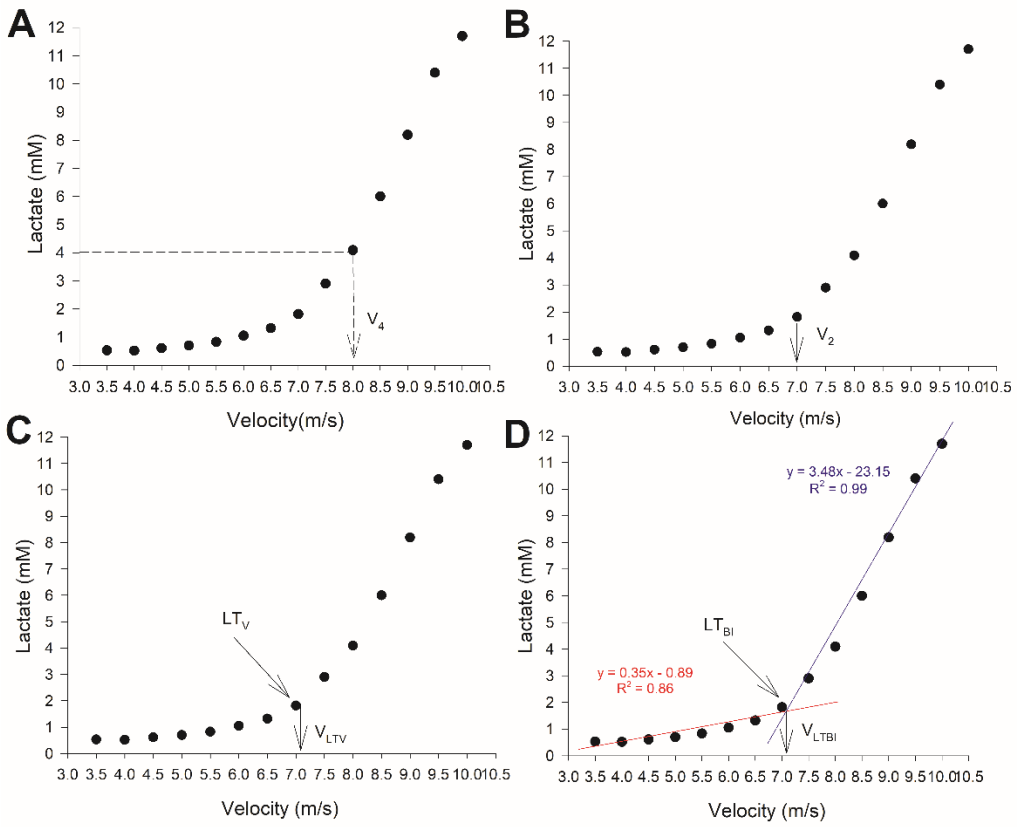

Figure 1. Determination of the lactate threshold in a single horse using four different methods to predict the maximal lactate steady state (MLSS). (A): V_4, the velocity at which the [La^-] is 4 mM); (B): V_2, the velocity at which the [La^-] is 2 mM; (C): visual determination (LT_V), V_{LTV}, the velocity at LT_V; (D): bi-segmented linear regression model (LT_{BI}), V_{LTBI}, the velocity at LT_{BI}.

2.5 Maximal lactate steady state (MLSS)

Once the individual V_{LTV} was obtained in the IET, horses underwent the MLSS test on the treadmill. The MLSS protocol was adapted from former agreement studies described for Arabian horses [7] and Beagle dogs [4]. For the determination of MLSS, the horses underwent 30-minute submaximal bouts of constant-velocity treadmill running (three to five sessions) every other day. The sessions began with a 5-minute warm-up phase at 2 m/s, followed by 25 minutes at a constant speed while maintaining a 5% slope. The speed of the

first bout matched the V_{LTV} determined in the previous test described in the 2.3. The speed of following sessions was adjusted up or down by 0.3, 0.5, or 1 m/s based on the [La^-] until a MLSS was reached. Blood samples were collected at 0, 5, 10, 15, 20, 25, and 30 minutes to assess the [La^-]. The MLSS is the load at which the prescribed velocity does not induce an increase in [La^-] by more than 1mM between 10 and 30 minutes of constant exercise [4,29]. Consequently, the velocity corresponding to the MLSS (V_{MLSS}) and the mean [La^-] of the last 20 minutes of the test ([La^-]$_{MLSS}$) were defined.

2.6 Blood samples and heart rate

To determine the [La^-] during the IET and MLSS test, blood samples were collected through catheterization of the jugular vein using a 14G catheter (Safelet, NIPRO Medical Ltda, Brazil) coupled to a 120 cm extension tube (Embramed, Brazil). The region where the catheter was inserted was first shaved and prepared in an aseptic manner. After collected the sample, 20 mL of 0.9% saline solution was flushed into the extension tube and catheter to maintain the system patency. Between the collections, saline remaining within the system was removed by withdrawing and discarding 20 mL before collecting the sample. Blood samples were stored in 4 mL tubes containing ethylenediaminetetraacetic acid (EDTA) and sodium fluoride (Diagnóstica Industria e Comércio Ltda - Biocon, Belo Horizonte, Brazil). At the end of each session, the samples were centrifuged under refrigeration (10°C) during 10 min at 3,000 g (ALC – Multispeed refrigerated centrifuge PK121R, New Jersey, USA), the plasma separated, and kept refrigerated (4ºC) for analysis on the same day. An electro-enzymatic method with an automatic bioanalyzer (YSI 2300 Stat Plus®, Ohio, USA) was used to determinate the [La^-]. The heart rate (HR) was measured during the MLSS tests using a frequency meter Polar, M430 receiver with a specific Polar H-10 transmitter, for horses (Polar Electro®, Kempele, Finland).

2.7 Statistical analysis

Data were presented as mean ± standard deviation. The [La^{-}] and the velocities correspondent to MLSS (V_{MLSS}) and prediction methods (V_2, V_4, V_{LTV}, V_{LTBI}) were compared by applying the Kruskal-Wallis one-way analysis of variance on ranks, followed by Tukey test for *post hoc* pairwise comparisons. Pearson's correlation coefficient (*r*) was carried out to indicate of the strength of the linear relationship between the velocities of all methods. The agreement between MLSS and exercise intensity thresholds (MLSS prediction methods) was assessed by comparing the mean difference (mean bias) between the methods with a 95% confidence interval and the 95% limits of agreement in the Bland-Altman method [30]. The analysis was performed using SigmaPlot software version 14.5 (Systat Software Inc.). Additionally, using the R software with the RStudio integrated development environment version 4.3.1 (Posit Software, PBC), an ordinary least products regression (OLP) was carried out to check the constant (CB) and proportional bias (PB). OLP allows us to identify CB and PB through the 95% CI of the *y*-intercept and the slope of the regression line, respectively. Thus, CB is not present if the CI of the *y*-intercept includes zero value, and PB is not present if the CI of the regression line slope includes a value of 1. OLP was applied instead of least squares regression considering presence of error in both methods [4,27,31,32]. Principal component analysis (PCA) of sample replicates was conducted using the prcomp() function from the 'stats' package in R, with the scale=TRUE argument enabled. The output was visualized using the 'factoextra' (v.1.0.7) and 'pca3d' (v.0.10.2) packages [4]. A significance level of $P < 0.05$ was considered to be statistically significant.

3. Results

To determine MLSS, 3-5 submaximal tests at a constant velocity lasting 30 minutes were conducted on different days. Five horses required three sessions, four needed four, and one

needed five. Notably, V_4 was found to be higher than V_{MLSS} ($P < 0.001$; Fig 2A and Table 1), with low agreement and a considerable mean difference between them (Fig 3A and Table 2). The limits of agreement and the confidence interval were wide indicating low V_4 agreement with MLSS despite correlation coefficient equal to 0.73. V_4 represented a higher load than V_{MLSS} by 39%. The [La^-] at V_4 was higher than V_{MLSS}, V_2, LT_V, and LT_{BI}, with corresponding *P* values of < 0.001, 0.024, 0.003, and < 0.001, respectively (Fig 2B). The Bland and Altman analysis revealed smaller mean differences between V_{MLSS} and V_2, V_{LTV}, and V_{LTBI} (Figs 3B, 3C, and 3D, respectively). In addition, the lowest limits of agreement with the MLSS were presented by V_2 and V_{LTV}.

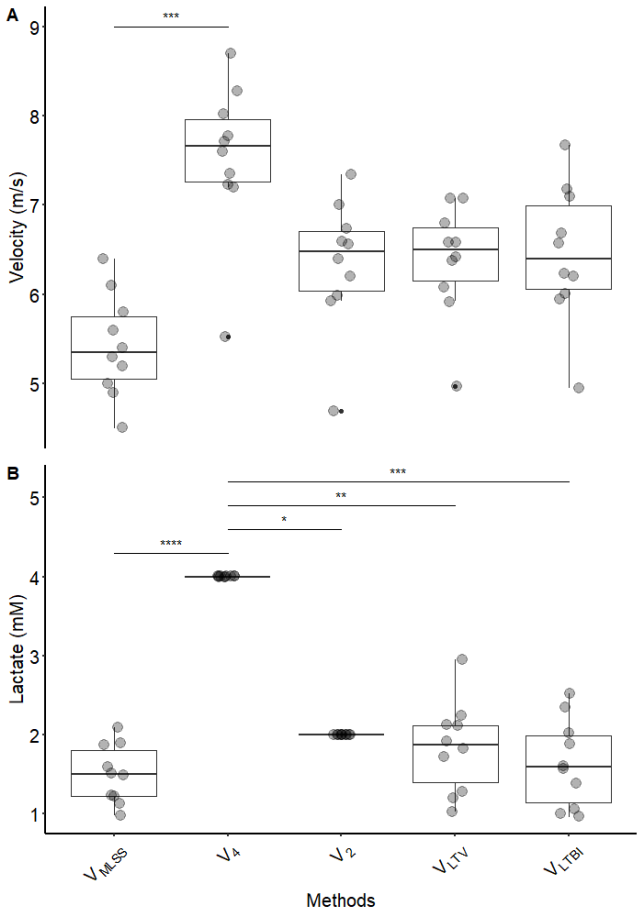

Figure 2. Boxplots indicating the velocities (A) and [La^-] (B) obtained by four different methods and the maximal lactate steady state in ten horses. V_{MLSS}, the velocity corresponding to MLSS; V_4, the velocity at which [La^-] is 4 mM; V_2, the velocity at which [La^-

] is 2 mM; V_{LTV}, the velocity at visual lactate threshold; V_{LTBI}, the velocity at lactate threshold by bi-segmented linear regression. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.

Table 1. Individual and average values of the velocities and lactatemia corresponding to the maximal lactate steady state (V_{MLSS} and $[La^-]_{MLSS}$), lactate threshold (LT) detected by different methods such as fixed lactatemia (V_4 and ($[La^-]_{V4}$); V_2 and $[La^-]_{V2}$), visual determination (V_{LTv} and $[La^-]_{VLTv}$), and visual determination of abrupt increase in $[La^-]$ using bi-segmented linear regression model (VLT_{BI} and $[La^-]_{VLTBI}$).

Horse (n=10)	V_{MLSS}	$[La^-]_{MLSS}$	V_4	$[La^-]_{V4}$	V_2	$[La^-]_{V2}$	V_{LTV}	$[La^-]_{VLTv}$	V_{LTBI}	$[La^-]_{VLTBI}$
1	6.40	1.89	8.70	4.00	7.34	2.00	7.08	1.38	7.67	2.35
2	5.00	1.59	8.28	4.00	7.00	2.00	6.58	1.02	6.69	0.96
3	5.60	1.13	7.60	4.00	6.39	2.00	6.80	2.95	7.18	2.52
4	6.10	2.09	7.78	4.00	6.59	2.00	6.08	1.20	5.94	1.00
5	5.40	1.87	7.23	4.00	5.92	2.00	5.92	1.73	6.01	1.61
6	4.90	1.22	7.35	4.00	6.21	2.00	6.38	2.11	6.20	1.39
7	4.50	1.49	5.52	4.00	4.69	2.00	4.97	2.12	4.95	1.88
8	5.80	1.23	8.02	4.00	6.74	2.00	7.08	1.82	7.10	1.57
9	5.20	1.52	7.20	4.00	5.98	2.00	6.42	2.24	6.57	2.02
10	5.30	0.98	7.72	4.00	6.56	2.00	6.58	1.92	6.23	1.06
Mean	5.42^{b}	1.50^{B}	7.54^{a}	4.00^{A}	6.34^{ab}	2.00^{B}	6.39^{ab}	1.84^{B}	6.45^{ab}	1.64^{B}
SD	0.57	0.37	0.85	0.00	0.73	0.00	0.63	0.57	0.77	0.55
CV (%)	0.11	0.24	0.11	0.00	0.11	0.00	0.10	0.31	0.12	0.34

SD, standard deviation and CV (%), coefficient of variation. Capital letters indicate statistical difference between lactatemia values of corresponding to the five methods. Lowercase letters indicate statistical difference between velocities values corresponding to the five methods. V_{MLSS}, V_4, V_2, V_{LTV}, V_{LTBI}, in m/s; $[La^-]_{MLSS}$, $[La^-]_{V4}$, $[La^-]_{V2}$, $[La^-]_{VLTV}$ and $[La^-]_{VLTBI}$, in mM.

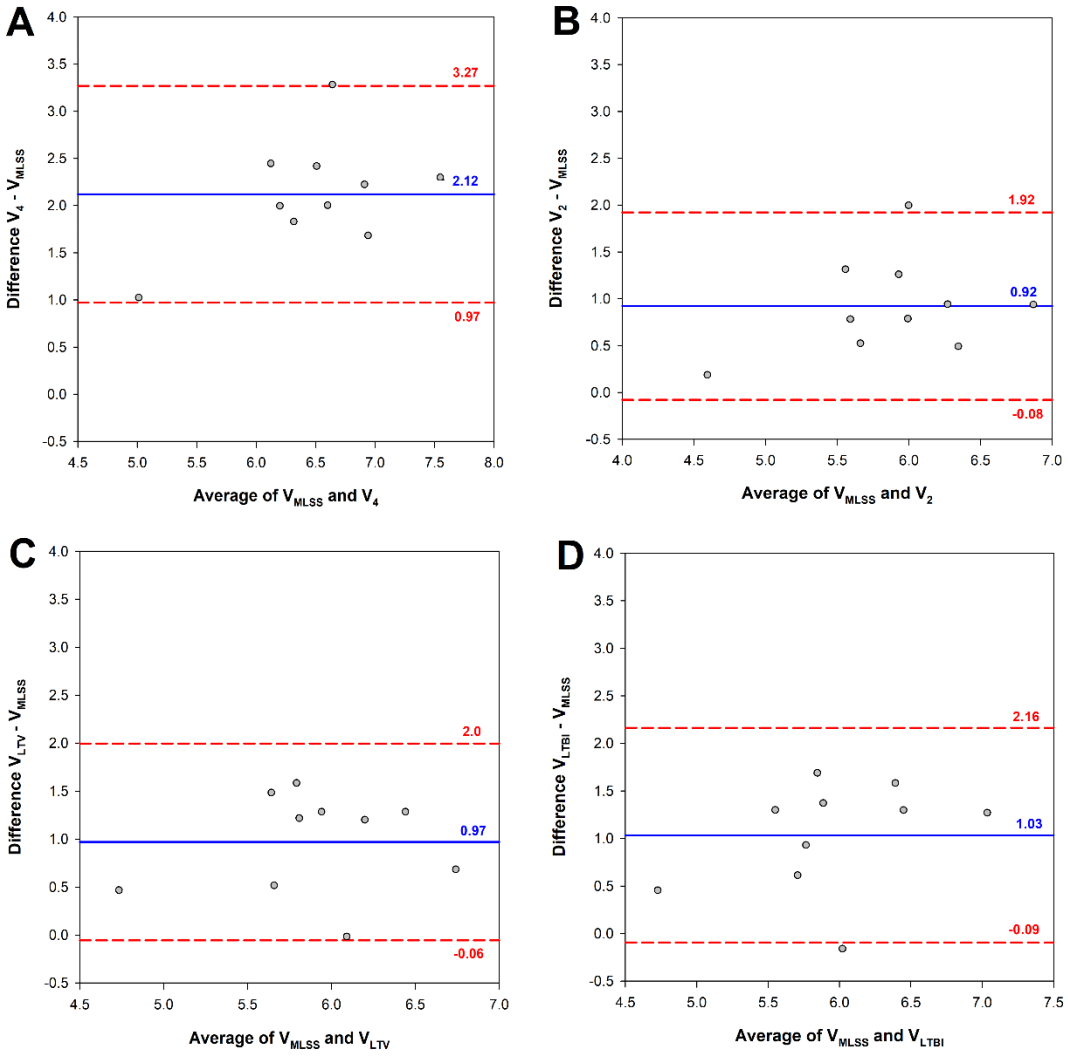

Figure 3. Bland-Altman plots to evaluate the agreement between predicted velocities through the four different methods and the maximal lactate steady state velocity (V_{MLSS}) in ten horses. The middle blue line corresponds to the estimated mean bias between the respective methods; the upper and lower dashed red lines represent the upper (mean + 1.96*SD) and lower (mean – 1.96*SD) limits of agreement (95% LoA). (A): V_{MLSS} and V_4 (velocity at which [La^-] is 4 mM); (B): V_{MLSS} and V_2 (velocity at which [La^-] is 2 mM); (C): V_{MLSS} and V_{LTV} (velocity at visual lactate threshold); (D): V_{MLSS} and V_{LTBI} (velocity at lactate threshold obtained by bi-segmented linear regression model).

Table 2. Analysis of the agreement between four lactate threshold prediction methods and maximal lactate steady state velocities using the Bland and Altman (1986) technique.

Methods		MD	SD	LoA	Bias (95% CI)	Lower LoA (95% CI)	Upper LoA (95% CI)
V_{MLSS}	V_4	2.12	0.59	0.97 to 3.27	1.69 to 2.55	0.23 to 1.71	2.53 to 4.01
V_{MLSS}	V_2	0.92	0.51	-0.08 to 1.92	0.55 to 1.29	-0.72 to 0.57	1.28 to 2.57
V_{MLSS}	V_{LTv}	0.97	0.52	-0.06 to 2.00	0.59 to 1.35	-0.72 to 0.61	1.33 to 2.66
V_{MLSS}	V_{LTBI}	1.03	0.58	-0.09 to 2.16	0.61 to 1.45	-0.82 to 0.63	1.44 to 2.89
V_4	V_{LTv}	-1.15	0.41	-1.96 to -0.34	-1.45 to -0.85	-2.48 to – 1.44	-0.86 to 0.18
V_4	V_{LTBI}	-1.09	0.47	-2.00 to –0.17	-1.43 to -0.75	-2.59 to -1.41	-0.76 to 0.42
V_2	V_4	1.20	0.15	0.91 to 1.48	1.09 to 1.30	0.73 to 1.10	1.30 to 1.67
V_2	V_{LTv}	0.05	0.34	-0.62 to 0.72	-0.20 to 0.30	-1.06 to -0.19	0.29 to 1.15
V_2	V_{LTBI}	0.11	0.45	-0.76 to 0.99	-0.21 to 0.44	-1.33 to -0.20	0.42 to 1.55
V_{LTv}	V_{LTBI}	0.06	0.27	-0.47 to 0.60	-0.13 to 0.26	-0.82 to -0.13	0.26 to 0.95

V_{MLSS}, velocity corresponding to maximal lactate steady state; V_4, velocity at which [La^-] is 4 mM; V_2, velocity at which [La^-] is 2 mM; V_{LTV}, velocity at visual lactate threshold; V_{LTBI}, velocity at lactate threshold by bi-segmented linear regression model. MD, mean difference (mean bias); SD, standard deviation; LoA (limit of agreement): 95% CI, confidence

The kinetic pattern of [La^-] used to establish MLSS in ten teaching horses is illustrated in Fig 4. The external loads led to variations in lactatemia, either higher or lower, aligned with MLSS. The mean ± SD [La^-] at MLSS was 1.50 ± 0.37 mM. All horses were able to finish the 30 minutes of continuous exercise.

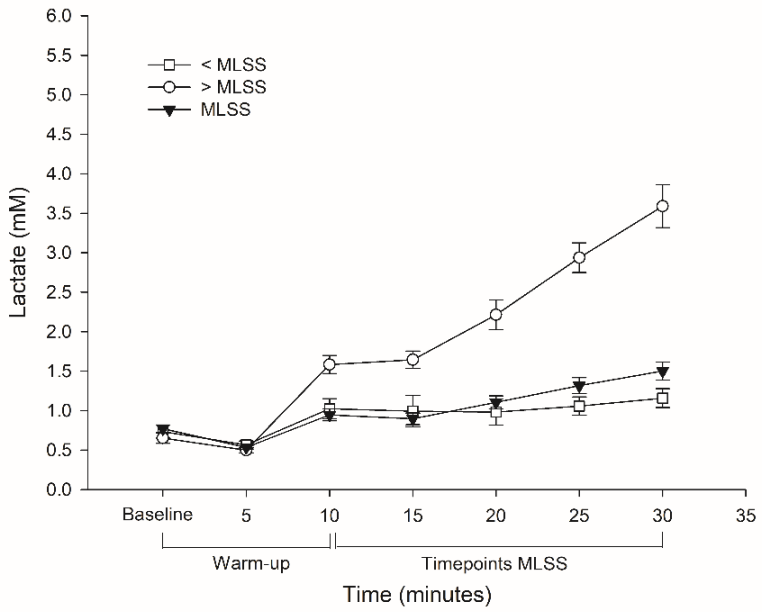

Figure 4. The [La⁻] response of ten horses during constant exercise session at the maximal lactate steady state (MLSS), below the MLSS, and above the MLSS. Blood samples were obtained every 5 minutes. WU: warm-up.

The OLP regression analysis is illustrated in Fig 5. PB and CB and also de Pearson's correlation coefficient can be found in Table 3. The confidence interval (CI) at 95% of *a* (*y*-interceptor) and *b* (slope) were used to determine constant and proportional bias, respectively. No PB was found between the methods and V_{MLSS}. CB was observed between V_{MLSS} vs. V_2, and V_{MLSS} vs. V_{LTBI}. CB was also observed when evaluating agreements between V_4 vs. V_{LTV}, V_2 vs. V_{LTV}, and V_{LTV} vs. V_{LTBI} despite moderate correlation coefficients between V_{MLSS} and all the prediction methods ($0.62 < r < 0.73$).

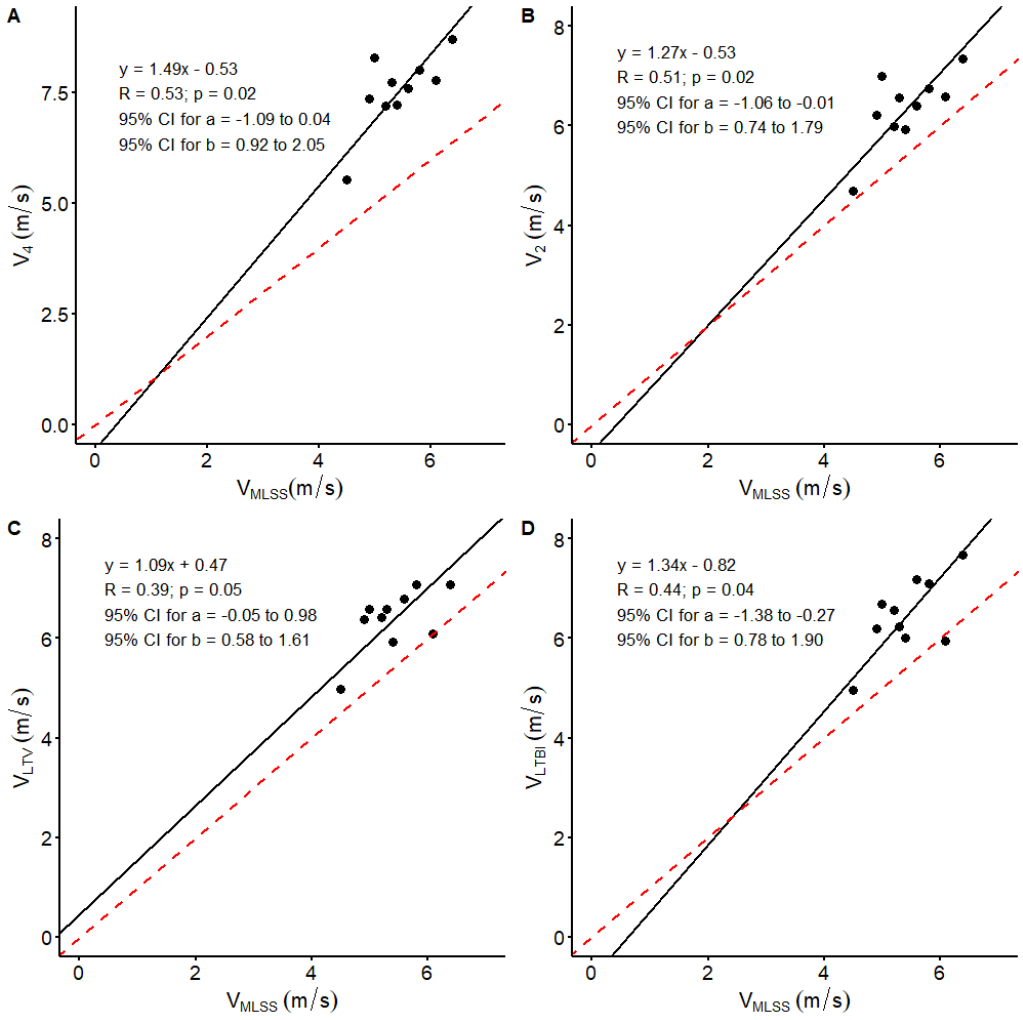

Figure 5. Ordinary least products regression plots to illustrate the agreement between predicted velocities through the four different methods and the maximal lactate steady state velocity (V_{MLSS}) in ten horses. The regression equation is presented as also the coefficient of determination (R), p-value (p) and the confidence interval (CI) at 95% of a (y-interceptor) and b (slope). The red dashed line corresponds to the line of identity (a = 0; b = 1). (A): V_{MLSS} and V_4 (velocity at which [La^-] is 4 mM); (B): V_{MLSS} and V_2 (velocity at which [La^-] is 2 mM); (C): V_{MLSS} and V_{LTV} (velocity at visual lactate threshold); (D): V_{MLSS} and V_{LTBI} (velocity at lactate threshold obtained by bi-segmented linear regression model).

Table 3. Analysis of the agreement between four lactate threshold prediction methods and maximal lactate steady state velocities using the ordinary least products regression analysis (OLP) to review proportional and constant bias and Pearson's correlation coefficient.

Methods		*a* (95% CI)	*b* (95% CI)	***r***	PB	CB
V_{MLSS}	V_4	-0.53 (-1.09 to 0.04)	1.49 (0.92 to 2.05)	0.73	No	No
V_{MLSS}	V_2	-0.53 (-1.06 to -0.01)	1.27 (0.74 to 1.79)	0.72	No	Yes
V_{MLSS}	V_{LTv}	0.47 (-0.05 to 0.98)	1.09 (0.58 to 1.61)	0.62	No	No
V_{MLSS}	V_{LTBI}	-0.82 (-1.38 to -0.27)	1.34 (0.78 to 1.90)	0.67	No	Yes
V_4	V_{LTv}	0.85 (0.46 to 1.25)	0.73 (0.34 to 1.13)	0.89	No	Yes
V_4	V_{LTBI}	-0.35 (-0.83 to 0.13)	0.90 (0.43 to 1.38)	0.89	No	No
V_2	V_4	0.10 (-0.11 to 0.31)	1.17 (0.96 to 1.38)	0.99	No	No
V_2	V_{LTv}	0.93 (0.53 to 1.33)	0.86 (0.46 to 1.26)	0.88	No	Yes
V_2	V_{LTBI}	-0.26 (-0.75 to 0.23)	1.06 (0.57 to 1.55)	0.82	No	No
V_{LTv}	V_{LTBI}	-1.40 (-1.77 to -1.03)	1.23 (0.86 to 1.60)	0.94	No	Yes

V_{MLSS}, velocity corresponding to maximal lactate steady state; V_4, velocity at which [La^-] is 4 mM; V_2, velocity at which [La^-] is 2 mM; V_{LTV}, velocity at visual lactate threshold; V_{LTBI}, velocity at lactate threshold by bi-segmented linear regression model. *a*, *b*, coefficients in ordinary least products regression model; *a*, *y*-axis intercept; b, slope; proportional bias, if 95% confidence interval (CI) for *b* does not include 1; constant bias, if 95% CI for *a* does not include 0. *r,* Pearson's correlation coefficient; PB, proportional bias; CB, constant bias.

The PCA analysis aims to reduce the dimensionality of the V_{MLSS}, V_2, V_4, V_{LTV}, and V_{LTBI} datasets into two components, accounting for 94.2% of the total variance (Fig 6). A biplot was used to assess the variability and similarities of the prediction methods, revealing a correlation between all the prediction methods and the V_{MLSS}.

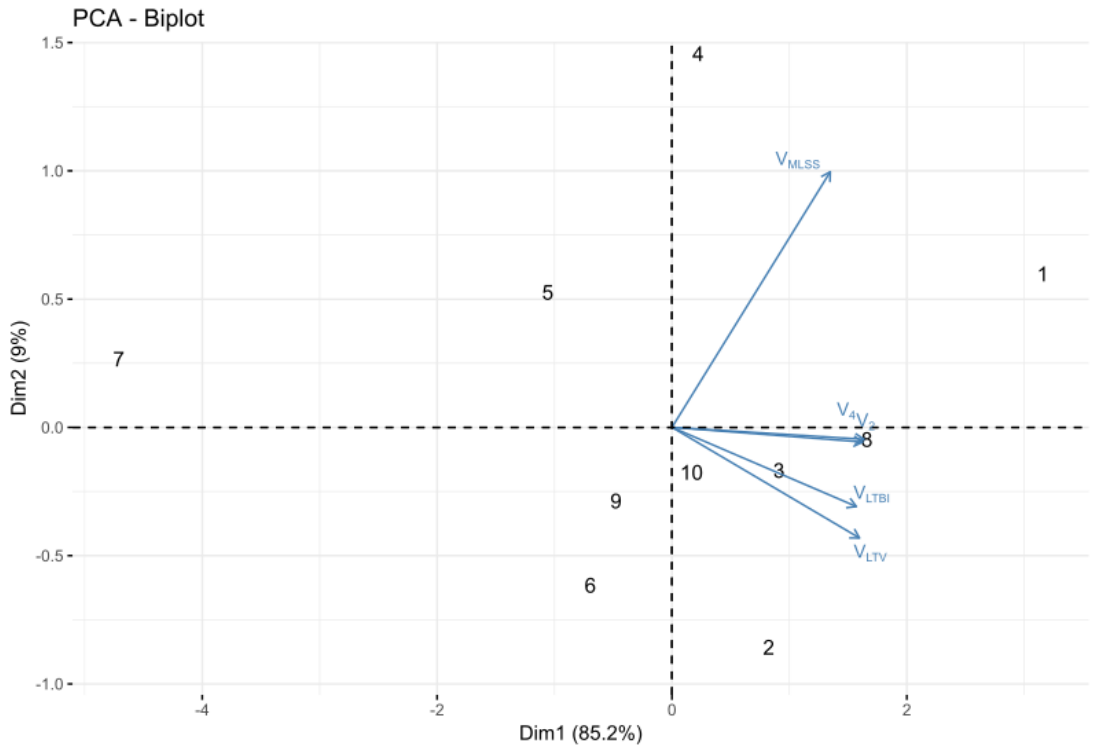

Figure 6. Principal component analysis (PCA) of ten horses undergoing an incremental exercise test, for the prediction of maximal lactate steady state (MLSS). Vectors are loadings on PC1 (x-axis) and PC2 (y-axis) for the four indices of MLSS prediction. V_{MLSS}, the velocity corresponding to MLSS; V_4, the velocity at which [La^-] is 4 mM; V_2, the velocity at which [La^-] is 2 mM; V_{LTV}, the velocity at visual lactate threshold; V_{LTBI}, the velocity at lactate threshold by bi-segmented linear regression.

The average heart rate at MLSS was 155 ± 2 beats/min (Fig 7), ranging from a minimum of 154 to a peak of 156 beats/min, corresponding to 72% of maximal heart rate for horses [33].

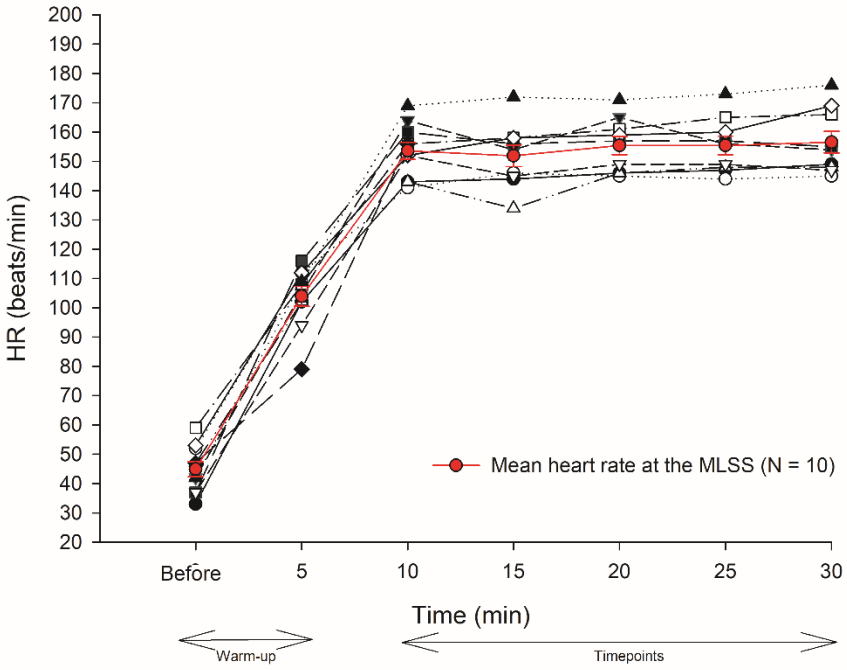

Figure 7. Heart rate (HR) corresponding to the maximal lactate steady state (MLSS) of ten horses performing a continuous exercise test on a treadmill. HR corresponding to MLSS was 155 ± 2 beats/min.

4. Discussion

The MLSS is closely linked to endurance performance. It has significant applications in sports science. This biomarker has been the focus of an intense debate and has occurred not only in humans [11] but also in other species, such as horses [6,7,8,13], and more recently in dogs [4]. It is crucial to acknowledge that the interconnectedness of several methods of MLSS prediction should discourage rigid and potentially arbitrary favouritism towards a specific definition of the LT over others. The main findings of the current study are as follows. The results shed new light on new methods for evaluating aerobic fitness in horses. V_{LTV}, V_{LTBI}, and V_2 yielded more favourable estimations of the MLSS than the $OBLA_{4mM}$. This discovery could lead to an alternative way of predicting MLSS in horses.

These indices can distinguish aerobic fitness and demarcate the domains of exercise that can be used to prescribe exercise and optimize training stimuli.

A moderate correlation (*r*) was indicated between MLSS and the other methods. Comparing agreement between methods is common in all fields using the *r*. It is inadequate because the *r* value only indicates only the strength of the linear relationship between two variables [34,35]. The PCA showed that the prediction methods were correlated with the MLSS. V_2, V_{LTV}, and V_{LTBI} methods exhibited a closer relationship with the V_{MLSS}, V_4 appears to overestimate the MLSS. Although the mean bias arises from the interaction of CB and PB, it fails to capture entirely the CB inherent in the compared methods [4,36]. This analysis may be complemented with the OLP regression, since it allows precise determination of CB and PB [4,31,32,36].

The OLP regression did not indicate PB between the V_{MLSS} and the prediction methods, suggesting a good degree of linearity among velocities [4,36]. A CB was not observed between V_{MLSS} and V_4, despite the mean bias overestimating the V_{MLSS} in almost 7.6 km/h (2.12 ± 0.59 m/s) with the large limits of agreement. The [La^-] associated with the MLSS and V_2, LT_V, and LT_{BI} were well below 4 mM, suggesting that V_4 is insufficient for predicting the MLSS and, as a result, the LT in this horse cohort. Overall, these findings align with former MLSS studies in horses [2,6,7,8], and human [37]. The leading cause for these inconsistencies is that the $OBLA_{4mM}$ approach is based on the association of threshold load values with a fixed constant [La^-] without considering individual variability and dynamics of changes in circulating [La^-] during IETs. Also, there needs to be more consensus on an appropriate IET protocol design to establish the lactate intensity thresholds, including duration, stage length, or number of stages.

A CB was also not observed between V_{MLSS} vs V_{LTV}. Unlike what was observed for V_4, the mean bias does not indicate that LT_V overestimate MLSS and can be used as prediction

method for this cohort of horses. To the best of our knowledge, the agreement between visual method and MLSS is unprecedented in horses. Studies indicated that this method could predict the MLSS in rats [26] humans [38] and dogs [4]. Although CB was observed between V_{MLSS} vs V_2 and V_{MLSS} vs V_{LTBI}, we considered the mean bias between V_2/V_{LTBI} and V_{MLSS} acceptable, since it may be possible to adjust one method to another simply by subtracting or adding this constant difference. Thus, these methods can be used for prescribing and evaluating horse training programs. As indicated to the visual method, to date no studies have been carried out to verify the agreement between LT_{BI} and the MLSS in horses.

In practical analysis, it was observed that the V_{MLSS} of each horse was lower than V_2. This suggests that no horse could complete the MLSS test at V_2 without a 1 mM increase. Similar findings were reported [8], indicating that horses undergoing a constant speed test on the treadmill at speeds predicted by V_2 were unable to maintain [La^-] without a 1 mM increment, despite moderate correlation between V_2 and V_{MLSS}. The average [La^-] at the MLSS was comparable to the results of a study involving horses from various disciplines such as trot and gallop races, eventing, jumping, dressage, and endurance [6]. This study illustrated that $V_{1.5}$ represented the intensity that best aligned with the definition of MLSS. It is suggested that $V_{1.5}$ could serve as a reliable predictor for MLSS in this group of horses and might be an alternative for determining the external training load for training horses. The $[La^-]_{MLSS}$ observed in this study was lower than the values reported for humans [10,39] which may be due to the overall volume of skeletal muscles engaged during physical exertion [6,40].

This paper reports the HR corresponding to the MLSS. The HR is a good parameter to indirectly predict the oxygen uptake ($\dot{V}O_2$). Our results showed that the mean HR corresponding to MLSS was 155 ± 2 bpm (roughly 72% of maximal HR) [33]. HR at the MLSS has emerged to appraisal the upper boundary of the moderate domain and the

intensity, indicating the upper limit of [La^-] steady state (i.e. MLSS). This value depends on factors such as the horse´s breed, predominant energy metabolism, and the type of exercise.

The study's limitations need to be recognized. The results were obtained by investigating a limited number of horses with wide variations in age and breed. Thus, further studies with larger sample sizes and using horses of the same age and breed should be conducted.

5. Conclusion

In summary, we can use single-exercise incremental tests to design and evaluate training programs and assess horses' aerobic fitness. This approach can help predict the MLSS using practical methods based on lactate thresholds (LT), such as LT_V, LT_{BI}, and V_2. V_4 is unsuitable for this group of horses, raising issues about its application for horses and emphasizing the need to use individual parameters to prescribe and evaluate aerobic fitness.

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