UNIVERSIDADE FEDERAL DE JUIZ DE FORA

INSTITUTO DE CIÊNCIAS BIOLÓGICAS

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

Maria Guilhermina Marçal Pedroza

Sustainability and Efficiency in Dairy Farming: an association between temperament, physiology, enteric methane emission and productive performance of dairy cattle of zebu origin

Juiz de Fora

2024

Maria Guilhermina Marçal Pedroza

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas – Biodiversidade e Conservação da Natureza da Universidade Federal de Juiz de Fora como requisito parcial à obtenção do título de Doutor em Ciências Biológicas. Área de concentração: Biodiversidade e Conservação da Natureza.

Orientadora: Aline Cristina Sant'Anna

Coorientadora: Mariana Magalhães Campos

Juiz de Fora

2024

Ficha catalográfica elaborada através do programa de geração
automática da Biblioteca Universitária da UFJF,
com os dados fornecidos pelo(a) autor(a)

Marçal-Pedroza, Maria Guilhermina.
Sustainability and Efficiency in Dairy Farming: An association between temperament, physiology, enteric methane emission and productive performance of dairy cattle of zebu origin : / Maria Guilhermina Marçal-Pedroza. -- 2024.
183 p.

Orientadora: Aline Cristina Sant'Anna
Coorientadora: Mariana Magalhães Campos
Tese (doutorado) - Universidade Federal de Juiz de Fora, Instituto de Ciências Biológicas. Programa de Pós-Graduação em Biodiversidade e Conservação da Natureza, 2024.

1. Comportamento animal. 2. Gado Leiteiro. 3. Desempenho produtivo. 4. Hormônios da lactação. 5. Emissão de metano entérico . I. Sant'Anna, Aline Cristina, orient. II. Campos, Mariana Magalhães, coorient. III. Título.

Maria Guilhermina Marçal Pedroza

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Aprovada em 27 de fevereiro de 2024.

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Juiz de Fora, 29/01/2024.

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Dedico esta tese a minha família,

meu bem mais precioso.

AGRADECIMENTOS

Agradeço primeiramente a Deus por ter preparado para mim a oportunidade de fazer doutorado e de escrever essa tese. Por de ter me dado forças para não desistir, me mostrando que tudo acontece no tempo e da maneira que Ele determina em nossas vidas. Por cuidar de mim e da minha família o tempo todo com amor e carinho, suportando nossos erros e nos ensinando com amor.

Agradeço ao meu esposo, Luiz Carlos, por estar sempre ao meu lado, pelas palavras de apoio e incentivo nos momentos difíceis, sempre me incentivando em seguir em frente, me fazendo acreditar que Deus sempre cuida de nós e prepara o que é melhor para os que Nele confiam. Te amo muito!

Agradeço ao meu filho amado, Daniel, para estar sempre ao meu lado, por aguentar a minha ausência nos momentos das coletas e da escrita dessa tese. Pelas nossas conversas, você ainda tão jovem e me ensinando e motivando a não desistir, você é o melhor presente que Deus me deu. Sou muito feliz e grata por ser sua mãe e ter a oportunidade de cuidar de você, meu amor!

Agradeço também à minha mãe, Aparecida, por me ajudar a cuidar do meu filho e da nossa casa nos momentos mais apertados dessa jornada. Que apesar de não entender o que significa do doutorado para mim, sempre esteve ao meu lado.

Agradeço às minhas primas-irmãs, Helena, Luzia, Ruth e Judith, pelas conversas e risadas, pelas palavras de incentivo, me mostrando como uma família unida e amorosa tem o poder de nos motivar a sermos pessoas melhores, que a vida acontece de fato nos bastidores.

Agradeço à minha orientadora, Aline Sant’Anna, por todo o apoio, paciência e ensinamentos. Você é um exemplo de profissional e inspiração para todos.

Agradeço à minha coorientadora, Mariana Campos, pela disponibilidade dos animais e da infraestrutura para realização dos experimentos que fazem parte dessa tese. Pela oportunidade de aprendizagem que me concedeu deste o mestrado, sem sua parceria e apoio, nada disso seria possível.

Agradeço às estagiárias da Embrapa gado de Leite que me ajudaram nas coletas e no cuidado com os animais.

Agradeço aos amigos do Núcleo de Estudos em Etologia e Bem-estar Animal – Nebea - pelas conversas e risadas ao longo da nossa jornada.

Agradeço ao Programa de Pós-graduação em Biodiversidade e Conservação da Natureza pelas oportunidades e atenção durante todo o período do Doutorado. À Coordenação e secretárias (Marlú, Priscila, Dayane e Rosi) pela ajuda, educação e carinho aos responder as minhas dúvidas e questionamentos.

Agradeço ao prof. Dr. Fábio Prezoto, de maneira especial, por todo apoio e incentivo desde quando voltei a UFJF para fazer mestrado. Professor, você é uma pessoa muito especial, generosa, alguém que queremos sempre ter por perto!

Agradeço aos demais membros da banca, Prof. Dra. Dionéia Cezar, Prof. Dr. Marcelo Rosa e Dra. Jessica Tatiana Morales por aceitarem o convite, estou certa de que vocês irão dar grandes contribuições, não apenas no texto, mas para a vida profissional.

Agradeço à Fundação de Amparo à Pesquisa do Estado de Minas Gerais – Brasil (Fapemig) pela bolsa de doutorado, apoio financeiro e oportunidade de desenvolver e executar o projeto. E em parte ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) Proc. nº. 409059/2016-1; # 311794/2020-3) pelo apoio financeiro e oportunidade de desenvolver e executar o projeto.

SUMMARY

Sustentabilidade e Eficiência na Pecuária Leiteira: uma associação entre temperamento, fisiologia, emissão de metano entérico e desempenho de bovinos leiteiros de origem zebuína

RESUMO - Os objetivos gerais com esta tese foram avaliar as implicações do temperamento de bovinos leiteiros cruzados de origem zebuína sobre o desempenho produtivo e a fisiologia da lactação, e sobre o desenvolvimento de bezerras na fase de aleitamento. Para isso, foram realizados uma revisão sistemática (RS) e uma metanálise (MA) e três estudos empíricos. A RS e MA teve como objetivo avaliar o efeito do temperamento de vacas leiteiras sobre a produção de leite. As buscas foram realizadas em quatro bases de dados (CABI Abstracts, Web of Science, PubMed, and Scopus), dentre os estudos que retornaram das buscas, apenas nove foram habilitados para entrarem na metanálise. Mais oito estudos foram avaliados de forma qualitativa, pois não apresentaram dados numéricos suficientes para serem inseridos na metanálise. Segundo os artigos incluídos na síntese qualitativa, vacas mais calmas produziram mais leite, porém, essa tendência não foi confirmada pelos resultados da síntese quantitativa e MA. O estudo 1 foi intitulado 'Vacas leiteiras com temperamento mais reativo são menos eficientes no metabolismo energético e produzem mais metano entérico?' e teve como objetivos: a) avaliar a relação entre o temperamento de vacas leiteiras cruzadas, o metabolismo energético e as emissões entéricas de CH_4; b) avaliar como a agitação das vacas nas câmaras respirométricas afeta o metabolismo energético e as emissões entéricas de CH_4. O temperamento de 28 vacas cruzadas F1 (Holandês-Gir) foi avaliado durante a ordenha e no curral de manejo, além da avaliação comportamental dentro das câmaras. Foram realizadas medições das emissões de metano entérico pelas vacas com o uso de câmaras respirométricas e avaliações metabólicas. Os resultados demonstraram que as vacas de temperamento mais calmo emitiram menos metano por litro de leite e alocaram mais energia para a lactação. Concluímos que o temperamento de vacas leiteiras esteve associado com a produção de leite e as emissões de metano entérico. O estudo 2 'O temperamento de vacas leiteiras cruzadas está relacionado com as concentrações de cortisol e ocitocina no leite, produção e qualidade do leite?' teve como objetivo investigar a relação entre temperamento e as concentrações de cortisol e ocitocina no leite, produção, ordenhabilidade e qualidade do leite de vacas leiteiras Holandês-Gir. O temperamento de 76 vacas cruzadas foi avaliado na sala de ordenha e no curral de manejo, além disso, foram realizadas coletas de leite para dosagem dos hormônios e qualidade do leite, e a medição dos parâmetros de ordenhabilidade. Nossos resultados indicaram que vacas mais reativas na sala de ordenha produziram leite com maior concentração de cortisol e ocitocina, e com menor teor de proteína e gordura. Além disso, vacas reativas apresentaram menor fluxo de leite e maior tempo de ordenha que as intermediárias. Vacas calmas e intermediárias durante as avaliações no curral de manejo produziram mais leite e apresentaram menor tempo de ordenha e maior fluxo médio de leite. Concluímos que a reatividade comportamental das vacas pode estar relacionada à intensidade de sua resposta ao estresse durante o manejo. Por sua vez, com o Estudo 3 'O temperamento de bezerras leiteiras cruzadas afeta o ganho de peso e o consumo médios diários?' buscamos: a) caracterizar o temperamento de bezerras leiteiras de origem zebuína; b) avaliar os efeitos do temperamento sobre o ganho de peso e o consumo médios diários durante a fase de aleitamento. Foram realizados três testes comportamentais (novo ambiente, novo objeto e aproximação voluntária) com 60 bezerras cruzadas (Holandês-Gir) e mensurado o ganho de peso médio diário e consumo de alimento dos animais durante 63 dias. Bezerras mais ativas e que interagiam menos com a pessoa desconhecida ganharam mais peso durante a fase de aleitamento, indicando que existe uma possível ligação entre temperamento e o desempenho de bezerras cruzadas leiteiras. Com essa tese esperamos ter gerado informações relevantes que possam ser úteis para os sistemas de produção leiteira com animais de origem zebuína.

Palavras-chave: comportamento, desempenho produtivo, hormônios da lactação, ordenhabilidade, qualidade do leite.

Sustainability and Efficiency in Dairy Farming: an association between temperament, physiology, enteric methane emission and productive performance of dairy cattle of zebu origin

ABSTRACT - The general aims of this thesis were to evaluate the implications of the temperament of crossbred dairy cattle of zebu origin on the productive performance, lactation physiology, and the development of calves during the pre-weaning phase. To this end, a systematic review (SR) and a meta-analysis (MA), and three empirical studies were carried out. The SR and MA aimed to evaluate the effects of dairy cows' temperament on milk yield. The searches were carried out in four electronic databases (CABI Abstracts, Web of Science, PubMed, and Scopus). Among the studies that returned from the searches, only nine were qualified to enter the meta-analysis. Eight studies were evaluated qualitatively as they did not present sufficient numerical data to be included in the meta-analysis. The articles included in the qualitative analysis indicated that calmer cows produced more milk, what was not confirmed by the quantitative synthesis and MA. Study 1 was entitled 'Are dairy cows with a more reactive temperament less efficient in energetic metabolism and do they produce more enteric methane?' and aimed a) to evaluate the relationship between cattle temperament assessed by traditionally used tests with energetic metabolism and enteric CH_4 emissions by crossbred dairy cows; b) to assess how cows' restlessness in respiration chambers affects energetic metabolism and enteric CH_4 emissions. The temperament of 28 primiparous F1 Holstein-Gyr cows was evaluated during milking and in the handling corral, in addition to behavioral evaluation inside the chambers. Measurements of enteric methane emissions by cows were carried out using respiration chambers and metabolic assessments. The results showed that cows with a calmer temperament emitted less methane per liter of milk and allocated more energy to lactation. We concluded that the cows' temperament was associated with milk production and enteric methane emissions. Study 2 'Is the temperament of crossbred dairy cows related to milk cortisol and oxytocin concentrations, milk yield, and quality?' aimed to investigate the relationships between temperament traits and concentration of milk cortisol and oxytocin, milk yield, milkability, and milk quality in Holstein-Gyr cows. The temperament of 76 crossbred cows was evaluated in the milking parlor and in the handling corral. In addition, milk collections were carried out to measure the hormones cortisol and oxytocin, and the measurement of milkability parameters. Our results indicated that cows that were more reactive in the milking parlor produced milk with a higher concentration of cortisol and oxytocin, and a lower protein and fat content, in addition to having lower milk flow and longer milking time than intermediate ones. Calm and intermediate cows during the evaluations in the handling corral produced more milk and had shorter milking time and higher average milk flow. We conclude that the behavioral reactivity of cows may be related to the intensity of their response to stress during handling. In its turn, with the study 3 'Does the temperament of crossbred female dairy calves affect weight gain and average daily starter feed consumption?' we intended a) to characterize temperament traits in dairy calves of zebu origin; b) to assess the effects of temperament on weight gain and on the average daily starter feed consumption during their pre-weaning stage. Three behavioral tests (novel object, novel environment, and voluntary approach) were carried out with 60 crossbred calves (Holstein-Gyr) and the animals' average daily weight gain and starter feed consumption were measured over 63 days. The results showed that calves that were more active and interacted less with the unknown person gained more weight, indicating that there is a possible link between temperament and the performance of crossbred dairy calves. With this thesis, we hope to have generated relevant information that could be useful for production systems formed by animals of zebu origin.

Keywords: behavior, productive performance, lactation hormones, milkability, milk quality.

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ABBREVIATIONS

SR	Systematic review
MA	Meta-analysis
NOS	Newcastle-Ottawa Scale
MD	Mean difference
EM	Effect measurement
95% CI	Confidence interval of 95%
GHG	Greenhouse gas
CH_4	Enteric methane
KOFF	Kick the milking cluster off
STEPS	Number of Steps
KICKS	Number of Kicks
GEI	Gross energy intake
Fecal-E	Daily fecal energy
Urine-E	Daily urinary energy
DMI	Dry matter intake
DM	Dry matter
DEI	Digestible energy intake
MEI	Metabolizable energy intake
CH_4-E	Enteric methane energy
HP	Heat production
O_2	Oxygen gas
CO_2	Carbon dioxide
NEL	Net energy of lactation
EB	Energy balance
ET	Entrance time
FS	Flight speed
SC	Squeeze chute

SCC	Somatic cell count
RSprep	Reactivity pre-milking udder preparation
RStca	Reactivity fitting the milking cluster
HG	Holstein-Gyr
ADG	Daily weight gain
ADC	Daily consumption
TC	Total consumption
CP	Crude protein
CC	Conventional concentrate
CD	Concentrate decreasing
PCA	Principal Component Analysis
PC	Principal Component
NOT	Novel object test
NET	Novel environment test
VAT	Voluntary approach test

x

Empresa Brasileira de Pesquisa Agropecuária

Ministério da Agricultura, Pecuária e Abastecimento

CERTIFICADO

Certificamos que o projeto intitulado "**Estudo metabólico de fêmeas Holandês-Gir com fenótipos divergentes para Eficiência Alimentar**", Protocolo Nº **05/2015**, sob a responsabilidade de **Fernanda Samarini Machado** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **APROVADO** pela Comissão de Ética no Uso de Animais (CEUA) da Embrapa Gado de Leite, em reunião de **19/06/2015**.

Vigência do Projeto	De 01/08/2015 a 31/12/2015
Espécie/Linhagem	*Bos taurus*/Bovino
Nº de Animais	36 Bovinos
Peso/Idade	8 - 15 meses e aprox. 250Kg
Sexo	Machos ( ) Fêmeas (X)
Origem	Biotérios da Embrapa Gado de Leite (x) Frigorífico ( )

Virginia S. C. Barbosa

Virginia de Souza Columbiano Barbosa
Secretária Administrativo da CEUA/EGL 2016

Embrapa Gado de Leite
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Embrapa Gado de Leite

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "TEMPERAMENTO DE BOVINOS DA RAÇA GIROLANDO: MÉTODOS DE AVALIAÇÃO E RELAÇÕES COM DESEMPENHO E EFICIÊNCIA DO MANEJO", protocolada sob o CEUA nº 5201240417 (ID 000139), sob a responsabilidade de **Aline Cristina Sant'Anna** *e equipe; Marcos Vinícius Gualberto Barbosa da Silva; Marta Fonseca Martins; Maria Guilhermina Marçal Pedroza* - 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 - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **APROVADA** pela Comissão de Ética no Uso de Animais da Embrapa Gado de Leite (CEUA/EGL) na reunião de 24/05/2017.

We certify that the proposal "Temperament of Girolando dairy cattle: Methods of assessment and relationship with performance and handling efficiency", utilizing 160 Bovines (160 females), protocol number CEUA 5201240417 (ID 000139), under the responsibility of **Aline Cristina Sant'Anna** *and team; Marcos Vinícius Gualberto Barbosa da Silva; Marta Fonseca Martins; Maria Guilhermina Marçal Pedroza* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **APPROVED** by the Ethic Committee on Animal Use of the Embrapa Gado de Leite Corporate (CEUA/EGL) in the meeting of 05/24/2017.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 07/2017 a 08/2018 Área: Núcleo Produção E Bem Estar Animal

Origem: Campo Experimental José Henrique Bruschi			
Espécie: Bovinos	sexo: Fêmeas	idade: 3 a 10 anos	Quantidade: 160
Linhagem: Cruzados Holandês x Gir		Peso: 500 a 650 kg	

Juiz de Fora, 20 de novembro de 2023

Dr. Rui da Silva Verneque
Coordenador da Comissão de Ética no Uso de Animais
Embrapa Gado de Leite

Maria Izabel Carneiro Ferreira
Vice-Coordenadora da Comissão de Ética no Uso de Animais
Embrapa Gado de Leite

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Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "EFEITO DA REDUÇÃO DO TEOR DE PROTEÍNA BRUTA DO CONCENTRADO SOBRE O DESEMPENHO DE BEZERRAS LACTENTES E AVALIAÇÃO DO EFEITO RESIDUAL DURANTE A FASE DE RECRIA", protocolada sob o CEUA nº 4422240120, sob a responsabilidade de **Mariana Magalhães Campos** *e equipe; Ana Caroline Ramos Teles da Silva; Alex Lopes da Silva; Luiz Gustavo Bruno Siqueira; Polyana Pizzi Rotta* - 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 - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Embrapa Gado de Leite (CEUA/EGL) na reunião de 04/03/2020.

We certify that the proposal "EFFECT OF REDUCING THE CONCENTRATE CRUDE PROTEIN CONTENT ON THE PERFORMANCE IN PREWEANING DAIRY CALVES AND EVALUATION OF THE RESIDUAL EFFECT DURING THE POSTWEANING DAIRY HEIFERS ", utilizing 120 Bovines (120 females), protocol number CEUA 4422240120, under the responsibility of **Mariana Magalhães Campos** *and team; Ana Caroline Ramos Teles da Silva; Alex Lopes da Silva; Luiz Gustavo Bruno Siqueira; Polyana Pizzi Rotta* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Embrapa Gado de Leite Corporate (CEUA/EGL) in the meeting of 03/04/2020.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 03/2020 a 08/2021 Área: Núcleo Produção E Bem Estar Animal

Origem:	Campo Experimental José Henrique Bruschi						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	3 a 60 dias	N:	60
Linhagem:	Holandês x Gir			Peso:	30 a 100 kg		
Origem:	Campo Experimental José Henrique Bruschi						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	60 a 365 dias	N:	60
Linhagem:	Holandês x Gir			Peso:	70 a 370 kg		

Local do experimento: Biotério Campo Experimental José Henrique Bruschi, Coronel Pacheco - MG - Retiro da Genizinha

Juiz de Fora, 18 de abril de 2022

Dra. Naiara Zoccal Saraiva
Coordenadora da Comissão de Ética no Uso de Animais
Embrapa Gado de Leite

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Certificados da CEUA referentes aos estudos que fazem parte da tese de Maria Guilhermina Marçal Pedroza

Ao Colegiado do Curso de Biodiversidade e Conservação da Natureza,

Prezado(a)s,

Envio abaixo os esclarecimentos da comissão de homologação de bancas acerca dos protocolos de aprovação no Comitê de Ética em Pesquisas com Animais (CEUA) da Embrapa Gado de Leite, local no qual foram realizados os estudos práticos da tese da aluna Maria Guilhermina Marçal Pedroza. As pesquisas foram realizadas no Campo Experimental José Henrique Bruschi do CNPGL vinculadas a projetos de pesquisas mais amplos, já em curso, realizados por aquela instituição.

- O Certificado da CEUA do projeto intitulado "**Estudo metabólicos de fêmeas Holandês – Gir com fenótipos divergentes para Eficiência Alimentar"** Protocolo nº 05/2015 está associado ao capítulo 3 da tese, intitulado "***Are dairy cows with a more reactive temperament less efficient in energetic metabolism and do they produce more enteric methane?***" no qual os mesmos animais foram avaliados em relação ao seu comportamento e sua associação com as metabolismo energético e as emissões de metano entérico.

- O certificado da CEUA do projeto intitulado" **Temperamento de bovinos da raça Girolando: métodos de avaliação e relações com desempenho e eficiência de manejo"**. Protocolo nº 5201240417 está relacionado ao capítulo 4 da tese, intitulado "***Is the temperament of crossbred dairy cows related to milk cortisol and oxytocin concentrations, milk yield, and quality*?** por se tratar dos mesmos animais.

- O certificado da CEUA do projeto intitulado **"Efeito da redução de teor de proteína bruta do concentrado sobre o desempenho de bezerras lactantes e avaliação do efeito residual durante a fase de cria"** Protocolo º 44222400120 está associado ao capítulo 5 intitulado **"*Does the temperament of crossbred female dairy calves affect weight gain and average daily consumption?*"** no qual os animais foram avaliados também em relação ao seu comportamento.

Juiz de Fora, 26 de janeiro de 2024.

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1. GENERAL INTRODUCTION

Animal production systems, both in Brazilian and international contexts, have increasingly attracted interest from public opinion due to how the animals are farmed and treated (Risius et al., 2017). In addition to this concern for the welfare of the animals, the improvement of productive efficiency is also receiving attention (Silva et al., 2015; Neikert et al., 2021), as well as the environmental impacts which are caused by the productive chain (Richardson et al., 2021). Thus, we must highlight the importance of keeping up with new demands from the consumer market and new animal production models, which must be based on animal welfare and on actions that guarantee the sustainability and efficiency of production systems.

In this context, research aiming to investigate the relationship between the individual behavioral differences in dairy cattle and the efficiency of production systems, including health, welfare, and productivity aspects, has been on the rise (Hedlund; Løvlie, 2015; Neja et al., 2015, 2017). Behavioral variation among individuals has been called personality, temperament, or coping style (Gosling; John, 1999; Koolhaas; Reenen, 2016). Such interindividual variations are considered consistent over time and different situations (Reále et al., 2007), being expressed as a combination of behaviors and physiological responses as an adaptation strategy to stressful environmental situations (Koolhaas et al., 2010).

Differently from psychology studies, most animal research does not differentiate the terms ‘temperament’ and ‘personality’, using them as synonyms (Gosling, 2001). However, in studies on farm animals, the most frequently used term is ‘temperament’. According to Reále et al. (2007), there are five main dimensions of temperament for animals, namely: activity, exploration, boldness, sociability, and aggressiveness. For production animals, especially cattle, reactivity during handling routines is one of the most investigated temperament traits, demonstrating the reaction of the animals when faced with the handling practices employed (Fordyce et al., 1982; Burrow, 1997). For dairy cows, reactivity to handling is usually measured in the milking parlor (Breuer, 2000; Rousing et al., 2004; Bertenshaw et al., 2008; Szentléleki et al., 2015), as this behavioral trait may be related to the productive performance of the animals. The extraction of one or more temperament traits depends on context and/or on the test used to classify the temperament of the animals.

In an attempt to expand knowledge about the influence of dairy cattle temperament on elements related to the animals' productive performance, we carried out theoretical and empirical studies that are part of this thesis. Which was divided into four studies that evaluate the temperament of dairy cattle and their relationship with aspects associated with animal productivity, milk yield and quality, lactation hormones, enteric emission and weight gain. The thesis is divided into four chapters. Chapter I- a systematic review and meta-analysis that investigated the effect of dairy cow temperament on milk yield. Chapter II – describes the results of the study of the relationship between temperament of crossbred dairy cows (Holstein x Gyr) F1 and its implications on energetic metabolism and enteric methane emission. Chapter III – presents the results of the investigation into the relationship between the temperament of crossbred dairy cows with the milk yield and quality, and lactation hormones. Chapter IV – evaluated the relationship between the temperament of crossbred female dairy calves and weight gain and daily consumption during the pre-weaning stage. And finally, brings the final considerations and implications generated by the empirical studies that are part of this thesis.

1.1 Theorical Contextualization

Animal temperament is a complex characteristic formed by several aspects (Gosling; John, 1999), which hinders its mensuration. Generally, the evaluation of temperament traits is carried out with the aid of behavioral indicators, which access one or a few aspects of each assessment. In practice the temperament indicators evaluate behavioral tendencies, for instance, an animal with a temperament considered docile and tame is easier to handle than the opposite, when the animal expresses more excitable behavior, making it difficult to handle (Paranhos da Costa, 2002, Sutherland; Huddart, 2012). Therefore, to assess temperament, some behavioral patterns are measured in frequency and duration, in addition to external manifestations of the animals' reactions when faced with certain stimuli (Manteca; Deag, 1993; Burrow et al., 1997).

1.2 Methods for assessing the temperament of dairy cows

Among the employed behavioral indicators, there is a variety of methodologies, appropriate for each species and animal category. For dairy cattle, particularly lactating cows, the behavioral assessments may occur within the milking parlor, especially during the milking process, by evaluating the cows' behavioral reactivity via the numbers of steps and kicks when their udders are cleaned and when the teat cups are attached (Wenzel et al., 2003; Rousing et al., 2004, 2006; Cerqueira et al., 2017; Marçal-Pedroza et al., 2020). Another measurement that can be obtained during milking is the temperament visual scores, that is, reactivity scores assigned according to their degree of agitation, such as the level of movement of hind legs, kicks, steps, and body movement when the cows are in contact with the milkers (Sutherland; Huddart, 2012; Sutherland et al., 2012; Szentléleki et al., 2015).

The evaluation of cows' behaviors in the milking environment may reveal aspects linked to their comfort during the procedures in the milking parlor (Chapinal et al., 2011; Szentléleki et al., 2015), also associated with their emotional states, e.g. fear of milkers, and even to the animals' health (Breuer et al., 2000; Rousing et al., 2004). It is also a good tool to assess the welfare of dairy cows (Rousing et al., 2004; Cerqueira et al., 2017).

In addition to the assessments in the milking parlor, the temperament of dairy cows has been evaluated in the handling corral, to enable the evaluations of consistency in different handling situations (Neave et al., 2020). In the corral, the animals are assessed by observing their reaction when faced with novel and challenging situations, provided through standardized tests, such as the novel object test (Ruiz-Miranda; Callard, 1992, Gibbons et al., 2009), in which the animal is exposed to an unknown object, which may be a ball, a bucket, or an umbrella. During the period of exposition, latency to touch and reaction to the unknown object are recorded, as well as time of exploration, activity levels, and other factors (Gibbons et al., 2009).

A second test is the open field one, used to evaluate general levels of locomotion and fearfulness of the animals. In this test, the individual is placed alone in an unknown environment, in the absence of objects or other animals, and its reactions, as well as the occurrences of defecation and urination, agitation level, and frequency of vocalizations, are recorded (Kilgour, 1975; Manteca; Deag, 1993; Neave et al., 2018, 2019). A third commonly used test is flight distance, in which the shortest distance an animal allows for the approach of an unknown observer is measured before expressing any signs of distancing itself or attacking said observer (Fordyce et al. 1982; Sutherland; Huddart, 2012; Sutherland et al., 2012). In addition to assessing temperament differences, this test is also known for assessing the quality of the human-animal relationship (Breuer et al., 2000; Waiblinger et al., 2006), thus being employed in studies with the goal of evaluating the effects of different handling practices used in the production environment (Boissy; Bouissou, 1988, Ceballos et al., 2018).

A fourth test that can be done is flight speed, when the speed with which the animal leaves the squeeze chute or the weighing scale toward an open area is measured, and the fastest animals are considered to have a reactive temperament (Burrow et al., 1988;

Curley et al., 2006; Marçal-Pedroza et al., 2020). Flight speed is an objective and easily obtained measure, which may be done during routine handling with the use of an electronic device. This device is comprised of a pair of photoelectric cells and a chronometer, which register the presence of the animal and the time it takes to exit the chute. Using these data and the distance covered, it is possible to calculate the speed in which the animal left the restraint area.

Lastly, it is also possible to measure agitation and movement levels within the squeeze chute, through the chute score test. In it, the animals receive a score between 1 (the lowest) and 4 (the highest), according to their degree of disturbance in the restraint area. The scores attributed to the animals are applied for intensity and frequency of movements, audible breathing, kicks, and attempts at jumping, lowering the body, and laying down within the squeeze chute (Tulloh et al., 1961; Fordyce et al., 1982; Sant'Anna; Paranhos da Costa, 2013; Ceballos et al., 2016, Marçal-Pedroza et al., 2020). The animals with the highest scores are considered those with the worst temperament in this test.

The tests in the handling corral were usually carried out mainly with beef cattle, however, they have recently started to be used to assess the temperament of dairy cows (Gibbons et al., 2011; Sutherland; Huddart, 2012; Marçal-Pedroza et al., 2020, 2021). Specifically for dairy calves, individualized temperament tests are used, including social isolation (open field) (Kilgour et al., 1975; De Passillé et al., 1995; Forkman et al., 2007, Lecorps et al., 2018), a test in which the calf is guided to a place far from other animals and is kept alone for a few minutes, with its behavior (locomotion, exploration, defecation, and urination) being recorded during that isolation time. Another test that can be used is the novel object one (Ruiz-Miranda e Callard, 1992; Forkman et al., 2007; Neave et al., 2018), as described for dairy cows. There is also the novel environment test (Le Neindre et al., 1989; Forkman et al., 2007; Neave et al., 2019), in which the calf is placed alone in an unknown environment and its behavior is recorded, similar to the open field test. Finally, there is the voluntary approach test (Lauber et al., 2006; Forkman et al., 2007; Neave et al., 2019), in which an unknown observer is placed in the center of the area without fixating their gaze on the calf and waits for the animal to voluntarily approach. These tests evaluate the same temperament traits found in adult bovines.

In addition to behavioral indicators, some authors have proposed the use of physiological parameters for the evaluation, such as heart rate (Kovacs et al., 2015), rectal temperature (Sanchez-Rodriguez et al., 2013; Lees et al., 2020), plasma cortisol levels

(Sutherland; Huddart, 2012; Sutherland et al., 2012), or milk cortisol levels (Wenzel et al., 2003; Gygax et al., 2006) in dairy cows. These are parameters which aid in defining temperament traits for cattle.

As it is a complex characteristic, the sum of different indicators contributes to a more complete assessment of the temperament of dairy cattle. Such an approach is supported by Koolhaas and Reneen (2016), who define a multidimensional model to evaluate animal personality (temperament), based on qualitative (how the animal reacts) and quantitative (the intensity of the reaction) dimensions, based on behavioral and physiological responses, including stress hormone levels and behavioral expression of emotions, such as the state of fear.

1.3 Implications of dairy cattle temperament on welfare, milk yield, performance, and livestock sustainability

Animal welfare is defined as the state of an individual throughout their attempts to cope to their environment (Broom, 1986). Based on this definition, we understand that there are variations between individuals in adjusting to an environment, and the temperament of the animals may explain part of this variation (Curley et al., 2008; Burdick et al., 2010). Additionally, evaluating the effects of temperament on productive performance may contribute to the improvement of animal welfare, as it helps identify new welfare indicators (Neja et al., 2015).

In that sense, dairy cows which are more reactive in the milking parlor may face welfare issues and be more susceptible to presenting signs of stress. Exposition to different stressors before and after milking (Bruckmaier et al., 1997; Wicks et al., 2004; Sutherland; Huddart, 2012) alters the behavior of the animals. It also increases heart and breathing rates, cortisol release, and retention of residual milk (Rushen et al., 1999; Van Reenen et al., 2002; Eicher et al., 2007), with a negative impact on animal welfare and productivity (Negrão et al., 2010; Hedlund; L¢vlie et al., 2015).

The high plasma cortisol and noradrenaline concentrations were associated with stress in the milking environment (Negrão; Marnet, 2003), as cortisol is one of the main hormones associated with stress in mammals (Cockrem, 2013). A greater increase in the glucocorticoids (cortisol) occurs due to a more intense activation of the hypothalamic-pituitary-adrenal (HPA) axis in response to some stressing agent, of a physical or emotional nature (Cockrem, 2013). According to Curley et al. (2008), the variation in function of the HPA axis is linked to the temperament of the animals, which has been demonstrated through challenges with CRH and ACTH in Brahman breed heifers. In the study, the authors found that animals with an excitable temperament had stronger activation of the pituitary and adrenal glands in response to the challenges. The authors also report that the temperamental heifers had higher basal plasma cortisol concentrations.

These results were similar to those reported by Cafe et al. (2011), who found that animals with excitable temperament (more reactive in the exit speed and chute reactivity score tests) also presented higher basal plasma cortisol, glucose, and lactate, even before carrying out the ACTH challenge. Additionally, in response to the challenge, glucose levels remained higher for longer in the reactive animals. This indicates that the individual differences in response to environmental stimuli and the variation in the concentration of glucocorticoids are associated with the differences in temperament in cattle (Cafe et al., 2011).

Previous studies indicate that dairy cows which are more reactive in the milking environment present higher concentrations of cortisol in their plasma (Van Reenen et al., 2002; Sutherland et al., 2012; Sutherland; Huddart, 2012), milk (Wenzel et al., 2003; Gygax et al., 2006), and saliva (Kovács et al., 2016). Some previous studies suggested that reactive dairy cows, which had a higher number of steps and kicks during the milking procedure, had greater concentrations of cortisol in their milk when compared to the calmer cows (Wenzel et al., 2003; Gygax et al., 2006). The concentrations of plasma and saliva cortisol were higher in cows which were previously classified as reactive, and these animals showed a greater cardiac autonomic response capacity to transrectal examination (Kovács et al., 2016).

1.3.1 Implications of temperament on the productivity of dairy cows

Regarding the productivity of dairy cows, there is evidence indicating that reactivity and susceptibility to stress are associated with animal productivity, as shown by some previous studies (Van Reenen et al., 2002; Rousing et al., 2004; Hedlund; L¢vlie et al., 2015; Cerqueira et al., 2017; Marçal-Pedroza et al., 2020). Regarding the greater cortisol concentration, a decrease is expected in the plasma concentration of oxytocin (Bruckmaier; Blum, 1998), which is the hormone responsible for ejecting milk and maintaining lactation (Bruckmaier, 2005). As reported in the study by Van Reenen et al. (2002), the inhibition of oxytocin release due to stress has increased the amount of residual milk and consequently decreased the amount of milk produced by primiparous animals in their first milkings. These results were corroborated by Bruckmaier (2005), who found that milk ejection may change due to reduced release or absence of oxytocin in the hypophysis. This phenomenon may occur with primiparous cows in their first milkings, or when the animals are milked in unknown locations, as the concentrations of cortisol and beta-endorphin were higher under these conditions. Bruckmaier (2005) also claims that the release of oxytocin and the milk ejection may be assessed via ACTH challenges. Thus, a comfortable environment without stressors could promote the ideal conditions for the adequate release of oxytocin, reduction of cortisol and residual milk levels, and, consequently, guarantee good productivity for the animals.

The relationship between productivity and temperament in dairy cows of different breeds has been investigated by several studies (Breuer et al., 2000 (Holstein-Friesian); Rousing et al., 2004 (Holstein); Praxedes et al., 2009 (Gyr); Gergovska et al., 2014 (Black and white); Hedlund; L¢vlie et al., 2015 (Swedish Red and White and Holstein); Cerqueira et al., 2017 (Holstein-Friesian); Sawa et al., 2017 (Holstein-Friesian); Marçal-Pedroza et al., 2020 (Holstein x Gyr). Some of these studies (Hedlund; L¢vlie et al., 2015; Cerqueira et al., 2017; Marçal-Pedroza et al., 2020) have shown that cows which are more reactive in the milking parlor (more steps and kicks) are less productive.

There are some proposed ideas to explain the mechanisms underlying the association of reactivity with milk yield. For instance, Abdel-Hamid et al. (2017) suggested that reactive cows possibly spend more energy in motor activities, such as walking and standing, and therefore have less energy left for milk yield. Another explanation was proposed by Langbein and Raasch (2000), who suggested that calmer cows would produce more because they spend longer feeding and ruminating, and, thus, their higher consumption would result in more energy intake and, consequently, more energy available for milk yield.

However, other studies have found different results, in which the reactive cows produced more milk (Rousing et al., 2004, Praxedes et al., 2009; Gergovska et al., 2012, 2014; Sawa et al., 2017). For Rousing et al. (2004), who assessed cow reactivity based on the number of steps taken within the milking parlor, the higher number of steps is indicative of discomfort during the milking process, especially in younger animals, and does not necessarily indicate a reactive temperament, which could explain why the reactive cows in their study were more productive.

In its turn, Gergovska et al. (2012), working with Black and White cows, and Sawa et al. (2017), working with Holstein cows, report that the reactive animals could achieve a higher social position in the herd hierarchy, leading to greater access to resources (food, water, and more comfortable resting areas). Thus, these cows would be more aggressive during feeding times and ingest more food, which results in greater milk yield, especially in systems where collective feeding takes place. Additionally, Praxedes et al. (2009), while evaluating the temperament of zebu cows, report that more reactive animals are kept in the herd due to their high productivity compared to calmer, but less productive, ones.

There are also studies in which no link was found between milking temperament and productivity for dairy cows, such as in the works of Orbán et al. (2011), who studied the temperament of Holstein and Jersey cows in the milking parlor using reactivity scores. The same results were found by Sutherland and Huddart (2012) and by Sutherland et al. (2012), who also evaluated the temperament of crossbred Holstein cows with reactivity scores during milking.

There is an obvious lack of consensus among studies evaluating temperament during milking and milk yield in dairy cows. Thus, we still cannot reach a conclusion regarding which temperament type is indeed associated with higher productivity in these animals. It could possibly be related to specific aspects of each herd, such as breed, age, birth order, and handling style employed, as highlighted by Sawa et al. (2017). Additionally, the difference in methodology to assess the temperament of the animals throughout the studies also limits the comparison of results. The association between temperament and milk yield has proven to be a complex matter, and further research is required.

Another factor associated with the high reactivity of dairy cows is the risk that these animals represent for the welfare of the stockpeople since handling reactive animals takes longer and is more dangerous (Sutherland; Huddart, 2012), which may lead to workplace accidents and damage to the facilities (Paranhos da Costa et al., 2000). Thus, it is extremely important that workers and handlers remain alert to signs of aggression and reactivity from the animals, and that they always be concerned with the improvement of handling done in the farms and look for ways to identify and remove the most reactive individuals.

1.3.2 Implications of temperament on milk quality and milkability parameters of dairy cows

Other factors which have also been associated with the temperament of dairy cows are milk quality (fat and protein content, and somatic cell count) and milkability parameters. Regarding quality, studies carried out so far have again shown a discrepancy in results on its association with temperament. According to the work of Kruszynski et al. (2013), who worked with Holstein cows; Radu et al. (2022), with Simental cows; Antanaitis et al. (2021), with Holstein cows; and Agravat et al. (2023), with Gyr cows; it was shown that individuals of a calmer and more docile temperament produced milk with higher fat and protein contents.

However, in the studies by Cziszter et al. (2016) with Simental animals and by Tamboli et al. (2018) with Sahiwal breed cows, they both report that the reactive individuals produced milk with higher fat contents when compared to calm ones. Morales Pineyrúa et al. (2022) (Holstein) found that the cows which were calmer during milking produced milk with lower levels of fat and protein. As for Gergorvka et al. (2014), while evaluating Black and White breed cows, the intermediate temperament animals were those whose milk had higher fat content. Finally, there are studies such as the one by Orbán et al. (2011) (Holstein and Jersey) where the authors failed to find any relation between temperament and milk quality.

The underlying mechanisms of this association are not deeply explored in the studies, but the connection between temperament and milk quality may occur due to the difference in which each animal reacts to stressful situations (Murray et al., 2009). As mentioned before, more reactive bovines respond more intensely to stressful events (Cafe et al., 2011). Thus, it is possible that reactive dairy cows, which are more susceptible to stress, produce less and with less quality due to metabolic changes (Rushen et al., 1999; Szentléleki et al., 2015). According to Etim et al. (2013), under chronic stress conditions with an increase in glucocorticoid concentrations, changes occur in the energetic metabolism of the animals, which may cause negative impacts on milk yield and quality.

In relation to somatic cell count, in general, milk produced by reactive cows presents greater values (Orbán et al., 2011; Gergosvka et al., 2014). This may be the result of a higher basal plasma cortisol concentration, which, in addition to affecting energetic metabolism, also alters the functioning of the immune system and the HPA axis in reactive animals (Yotova et al., 2004). The greater frequency of kicks over teat cups by reactive cows may also incur contamination of the milk, harming both milk quality and mammary gland health, as proposed by Paranhos da Costa et al. (2015).

Regarding the link between temperament and milkability parameters, such as milking flow, milking time and speed, previous studies have shown that calmer animals have a shorter milking time and greater flow (Shehar et al.; 2015a,b; Agravat et al., 2023).

All three of these studies were carried out with zebu cows. However, Radu et al. (2023), who worked with cows of Simental breed, demonstrated that calmer cows need longer to be milked, whereas Sutherland et al. (2012), working with Holstein cows, found that animals of an intermediate temperament had the lowest flow. In turn, Tamboli et al. (2018), who investigated the temperament of Sahiwal cows, found no association between temperament, milk yield, and milkability parameters.

Reactive Gyr cows are agitated and nervous during milking, which affects oxytocin release, leading to longer milking time. Consequently, that decreases milking speed and flow, and increases time spent on milking (Agravat et al., 2023). The same authors also found that, due to high reactivity, these cows are not fully milked, which increases the amount of residual milk and decreases milk yield.

For Visscher and Goddard (1995), who worked with Holstein-Friesian and Jersey cows, what increases the milking time for more reactive animals is the fact that handlers spend longer preparing the animals for milking. Handling is also more frequently interrupted when these cows kick and knock over the teat cup set more often, which may even cause the udder to be injured. Lastly, Tamboli et al. (2018), who worked with Sahiwal cows, concluded that the shorter milking time in cows of a calmer temperament indicates that these animals are comfortable in the milking environment and may therefore express their full productive potential.

Milking speed has been highlighted as one of the causes for the culling of cows, as both speed extremes are undesirable in milk yield. Slow speeds are bad as the handling time of the animal increases, and high speeds increase the risk of udder infections, and there is also an increase of milk somatic cell count (Govignon et al., 2016; Marete et al., 2018). According to Sewalen et al. (2011), milkability parameters are hereditary, as well as reactivity. Thus, the selection of calmer cows would contribute both to a better productivity and to reaching a satisfactory, safe, and efficient milking speed.

As we can see, most studies which have investigated the association between temperament, milk yield, quality, and milkability parameters have focused on animals of European breeds. Additionally, these studies showed different results, and few of them clarified the possible underlying mechanisms of this association. Therefore, more research is needed to clarify the underlying behavioral and physiological factors which affect the link between temperament and the productive performance of dairy cows, mainly for animals of zebu origin, which are the majority in herds in tropical countries. Considering these divergent previous results, other questions arise that need to be evaluated and rethought. For instance, which indicators should we use to classify the temperament of dairy cattle? When and where should tests be carried out? A methodological standardization of behavioral assessments should also be desirable, so that the results may be better understood.

1.3.3 Implications of temperament on enteric methane emissions of dairy cows

Methane gas, originated from enteric fermentation, and nitrous oxide, found in bovine feces, are considered two of the greenhouse effect gases (GEG), which contribute to the increase in the average temperature on the planet. Specifically, according to Machado et al. (2011), methane gas can contribute to the warming of the planet 25 times more than carbon dioxide, in addition to its lifespan ranging from 9 to 15 years. Thus, it is estimated to be responsible for 15% of global warming (Machado et al., 2011). The production of methane gas (CH_4) occurs due to the action of anaerobic microorganisms which colonize the rumen of cattle, through the fermentation of vegetable carbohydrates. Therefore, enteric methane is a natural product of the digestive process of ruminant animals (Beauchemin et al., 2008).

Enteric CH_4 emissions lead not only to environmental concerns but also economic losses, as the emissions represent a decrease in the energy efficiency of the animals, due to the loss of gross energy in the form of CH_4 (Johnson; Johnson, 1995). The energy released as CH_4 gas could be used for weight gain (in beef cattle) or milk yield (in dairy cattle). This energy loss may vary between 2 and 12% of the energy intake of the animals, depending on diet type (Johnson; Johnson, 1995). Thus, strategies to mitigate enteric emissions should result in environmental and economic gains, optimizing nutrient use.

The main emission mitigation strategies for cattle have been based on nutritional management, an intensive farming system, and environmental temperature (Cottle et al., 2011; de Vries et al., 2015; Yadav et al., 2016; Haque, 2018; Dini et al., 2019). However, there is evidence to suggest that physiological and behavioral responses to stress may be associated with greater enteric CH_4 emission (Yadav et al., 2016; Llonch et al., 2016; Llonch et al., 2018). In the work by Llonch et al. (2016), they investigated the link between temperament and methane gas emissions in beef cattle. The temperament of the animals was classified via flight speed and squeeze chute reactivity tests. The effect of stress after transportation was measured with the use of blood samples, by checking plasma cortisol levels, in addition to measuring CH_4 emissions. They found no association between temperament (with the tests used in their study) and enteric methane emissions, but they did find a positive association between cortisol and emissions. Thus, their results indicate that more stress-prone bovines may emit more enteric methane. It is important to highlight that the studies which have investigated this topic have been recent and few.

There are some propositions to explain the relationship between a reactive temperament and enteric methane emissions. For instance, as mentioned before, reactive animals had a more prolonged and intense activation of the HPA axis and the sympathetic-adrenomedullary receptors in response to stress (Cafe et al., 2011). And, as described by Cafe et al. (2011), both axes are involved in the control of catabolism, energy homeostasis, energy balance, and body energy storage. Thus, it is likely that reactivity and susceptibility to stress negatively affect enteric methane emissions per kilogram of product from reactive animals.

Additionally, another important point is that the production of enteric methane is done by rumen microorganisms, such as ciliate protozoa and methanogenic archaea, which can be adapted to the biological parameters of the host (Min et al., 2022). Therefore, the species and number of individuals colonizing the rumen of reactive animals may be different from those that inhabit the rumen in calmer ones, according to Kim et al. (2020), in response to thermal stress, Jersey and Holstein dairy cows exhibited changes in their rumen bacterial composition.

The mitigation of CH_4 emissions goes through many approaches, including changing the diet and improving rumen fermentation, which together contribute to improving food conversion Efficiency (Min et al., 2022). The same authors claim that it is possible to interfere positively with rumen fermentation and the structure of the microbiota community, in favor of enteric emission reduction.

1.3.4 Implications of temperament on the development of dairy calves

As a final topic, we will talk about the association between the temperament of dairy calves and their performance, as well as weight gain. In the study by Voisinet et al. (1997), the authors found a significant effect of temperament on average daily weight gain in crossbred *Bos indicus* and *Bos taurus* calves, indicating that calmer animals had greater daily gains than reactive ones under routine handling. A possible explanation for these findings is that more agitated/reactive calves spend more energy on motor activities, which leads to less weight gain.

More active and exploratory dairy calves in the novel environment test had gained more weight when compared to the less active animals (Neave et al., 2018, 2019) Similar results were found who evaluated the temperament of dairy calves with an isolation crate, similar to the novelty tests (environment and unknown person) (Costa et al., 2022). The animals which moved more (activity/exploration) in the crate gained more weight. According to Neave et al. (2018), the more active animals consumed greater quantities of starter feed, which resulted in greater weight gain. Additionally, they claim that, despite the greater energy expenditure caused by the motor activities, the more active calves had better feed efficiency.

The feeding rates and meal frequency were used as measures of temperament for Holstein calves. The results indicate that calves which ingested milk faster and more frequently were also more agile, and they considered this as a temperament trait. Thus, the calves classified as faster gained more weight (Carslake et al., 2022). A possible explanation for these findings could be provided by the "Life Syndrome" theory, which suggests that more active and exploratory animals tend to have more favorable development, as they are found within the "slow/fast" axis. On this axis, the more active/exploratory animals are classified as faster and, therefore, would have a greater weight gain (Reále et al., 2010; Dammhahn et al., 2018).

We must highlight that weight gain in young cattle is not only associated with nutrition, but also with several factors such as breed (Coffey et al., 2006), passive immunity transference (Elsohaby et al., 2019), diseases (Buczinski et al., 2021), handling (Silva et al., 2017), farming/housing system (Costa et al., 2016; Johnson et al., 2018), and environmental temperature (Shivley et al., 2018). These elements together interfere in the development and weight gain of calves in their initial life stage. Greater weight gain in calves contributes positively to the development of the mammary gland (Albino et al., 2019), decreases the age at first calving (Geiger et al., 2016), and increases milk yield in the first lactation (Carslake et al., 2022).

In summary, a reactive temperament, stress, and welfare issues may generate additional energy expenditure for the animals, when trying to face such circumstances. In addition to economic loss caused by the inefficient use of food resources and the reduction of milk yield and quality, a reactive temperament in cattle may be associated with enteric emissions and the weight gain of young animals. Moreover, it clearly is of concern when it comes to the risk of accidents and deteriorating work conditions in dairy farms (Hemsworth, 2003; Sutherland; Huddart, 2012).

Temperament assessment in dairy cows and calves of zebu origin, as well as understanding the mechanisms underlying the association of reactivity and stress with milk yield and quality, enteric methane emissions, and weight gain in young animals, is of great importance. That is because crossbred animals are known for expressing a more excitable temperament, reacting more intensely and with greater agitation to handling procedures (Paranhos da Costa, et al., 2015; Cerqueira et al., 2017). Among them, we highlight Gyr dairy cows (Negrão, 2008), which are widely used for crossbreeding in tropical countries like Brazil, where around 80% of the dairy herd is composed of crossbred Holstein x Gyr cows (Canaza-Caio et al., 2016). Under those conditions, it is expected that animals with greater zebu composition be more reactive to milking handling, which may result in negative effects on the welfare of both cows and stockpeople, as well as losses in milk yield and quality.

In this context and given the themes presented and discussed throughout this introductory chapter, we hope that this thesis may contribute to answering some questions regarding the temperament of crossbred (Holstein x Gyr) dairy cows and calves. We hope that our results will provide useful information for the Brazilian production systems, contributing to increasing productive performance, improving the welfare levels of animals and workers, and reducing enteric methane emissions from livestock activity.

To reach our goals, this thesis has been divided into four chapters, with the first; a systematic review and meta-analysis; the second, third, and fourth chapters being results of empirical studies.

4. GENERAL HYPOTHESES

Dairy cows that are more reactive during milking present behavioral and physiological indicators of stress, in addition, they produce less and low-quality milk, and emit more enteric methane. Furthermore, reactive dairy calves gain less weight during the pre-weaning phase.

SPECIFIC HYPOTHESIZE

Chapter 1. We hypothesize that calmer cows would produce more milk.

Chapter 2. We hypothesize that individuals with a more reactive temperament and restlessness in a situation of physical restraint would be metabolically and bioenergetically less efficient than the calmer ones, showing higher enteric CH_4 emissions.

Chapter 3. We hypothesized that more reactive cows in the milking parlor (with higher reactivity scores, more steps, and kicks) and in the handling corral (entered and exited the squeeze chute faster) would have higher concentrations of milk cortisol, oxytocin, and produce less milk with lower quality.

Chapter 4. We hypothesized that behavioral tests are capable of extracting temperament traits of crossbred dairy calves, and the less active animals during testing would consume more starter feed and gain more weight.

5. GENERAL OBJECTIVE

To evaluate the implications of dairy cow temperament on enteric methane emissions, energy metabolism, production performance, to milk quality and physiological indicators of stress during milking. In addition to evaluate the effect of dairy calves temperament on weight gain and starter feed consumption.

SPECIFIC OBJECTIVES

Chapter 1. The aim of this study was to evaluate the scientific evidence available in the literature using SR-MA to identify the effect of dairy cows' temperament on milk yield.

Chapter 2. The aims of this study were: a) to evaluate the relationship between cattle temperament assessed by traditionally used tests with energetic metabolism and enteric CH_4 emissions by crossbred dairy cows; b) to assess how cows' restlessness in respiration chambers affects energetic metabolism and enteric CH_4 emissions.

Chapter 3. The aim of this study was to investigate the relationships between temperament traits and concentration of milk cortisol and oxytocin, milk yield, milkability, and milk quality in Holstein x Gyr cows.

Chapter 4. The aims of this study were to: a) characterize the temperament of crossbred female dairy calves (Holstein x Gir), via standardized testing and; b) assess the effects of temperament on weight gain and starter feed consumption for female dairy calves of zebu origin during their m stage when subjected to different diets.

6. References

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Chapter 1- Effects of dairy cow temperament on milk yield: a systematic review and meta-analysis[1]

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Abstract

The temperament of dairy cows interferes in milk yield and quality, but there is a lack of consensus throughout the literature. Thus, systematic review (SR) and meta-analysis (MA) methodologies were used to assess the effects of dairy cow temperament on milk yield. Our literature search included four electronic databases (CABI Abstracts, Web of Science, PubMed, and Scopus) and bibliographies of the publications included on MA. As inclusion criteria, we considered publications about the temperament of lactating cows and its effect on daily milk yield and total milk yield (whole lactation). A random effect-MA was carried out separately for daily milk yield and total milk yield related to each class of cows' temperament, 'low' (low reactivity, calm animals), 'intermediate' (intermediate reactivity), and 'high' (high reactivity, reactive animals). A total of eight publications reporting 75 trials were included in the analyses for daily milk yield, and three publications reporting nine trials for total milk yield. For daily and total milk yield the heterogeneity between publications was high ($I^2 = 99.9\%$). Cows of European breeds with intermediate temperament produced less milk daily than the calm ($p = 0.020$) and reactive ones ($p < 0.001$). In the case of primiparous cows, those with intermediate temperament produced less milk daily ($p < 0.001$) than the reactive ones, while for multiparous, the intermediate produced less than calm ($p = 0.032$) and reactive cows (p <

0.001). Regarding the stage of lactation, cows evaluated throughout lactation with a calm temperament tended (p = 0.081) to produce more milk than the intermediate ones, but less than the reactive ones (p < 0.001). For total milk yield, reactive cows tended to produce more than the calm (p = 0.082) and intermediate (p = 0.001) ones. Among European and primiparous cows, reactive cows produced more than the intermediate (p = 0.001). According to our results, we can not confirm what we expected, that calmer cows would be the most productive for both daily and total yield.

Keywords: behavior, dairy cattle, performance, personality, reactivity

[1] Artigo publicado na revista Journal of Animal Science, v. 101, p. 1–12, 2023 (anexo 1)
https://doi.org/10.1093/jas/skad099

1. Introduction

Animal temperament is a complex trait that encompasses several behavioral aspects. According to Réale et al. (2007), temperament may be understood as the individual differences in the behavior of animals, in response to their environmental circumstances, given that those differences are relatively consistent over time and in distinct situations. In production animals, this trait may be assessed by observing the behavior of the animals during routine handlings, for example in the milking parlor (milking temperament) (Sawa et al., 2017), or through standardized tests, such as flight speed, reactivity in the handling corral, and flight distance (handling temperament) (Sutherland; Huddart, 2012). For dairy cows, the temperament is usually measured based on the cows' reactivity during milking, considering the intensity of reactions to milking procedure, such as leg movements and kicks (Breuer et al., 2000; Rousing et al., 2004).

In dairy cows, temperament has been associated with productivity (milk yield, quality and milkability), however, this is still a controversial topic. Contradictory results are reported in the scientific literature. Some studies report that calmer cows produce more milk (Sutherland; Dowling, 2014; Hedlund; L¢vlie, 2015; Cerqueira et al., 2017), with higher fat and protein contents (Kruszyński et al., 2013; Antanaitis et al., 2021). Others show that the reactive ones are more productive, with higher milk yield (Rousing et al., 2004; Sawa et al., 2017), milk fat and protein contents (Cziszter et al., 2016) than the calm ones. In addition, there are still studies that do not find association between temperament and productive parameters (Orbán et al, 2011; Sutherland et al., 2012; Szentléleki et al., 2008, 2015). Furthermore, there is a lack of standardization regarding the measurement used to assess the temperament of the animals throughout the studies, which may hinder the comparison of findings.

The behavior of dairy cows and its relationship with milk yield and quality are topics that interest both consumers and producers, due to their relationships with animal welfare, production efficiency, and sustainability of the livestock industry (Risius; Hamm, 2017; van Dijk et al., 2019; Marçal-Pedroza et al., 2021). Moreover, assessing the effects of temperament on performance may contribute to the improvement of animal welfare, as it aids in the identification of new welfare indicators (Neja et al., 2015).

Thus, in this study, we used systematic review (SR) and meta-analysis (MA) methodologies to explore the influence of dairy cattle temperament on milk yield and quality. We hypothesize that calmer cows would produce more milk. The aim of this study was to evaluate the scientific evidence available in the literature using SR-MA to identify the effect of the dairy cows' temperament on milk yield.

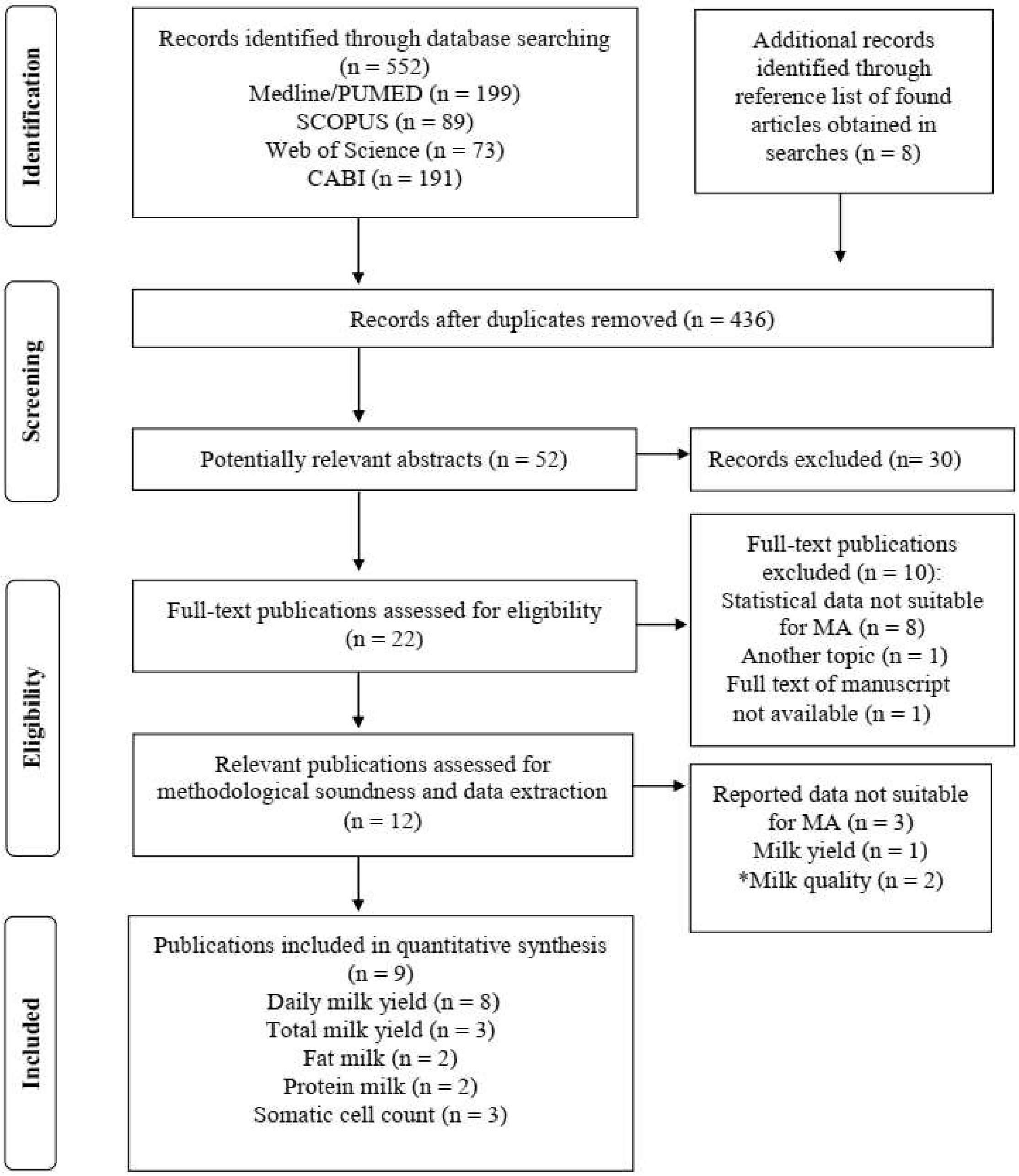
2. Materials and methods

2.1 Research question and protocol

This is a theoretical study and therefore did not need to be evaluated by an ethics committee. The systematic review followed the PRISMA guidelines (Page et al., 2021). The search strategy was defined based on PICO terms: population, intervention, comparison, and outcome (Brown et al., 2006). For population, we used the terms "lactating cow" or "dairy cow" or "dairy cattle"; for intervention, "temperament" or "reactivity" or "personality"; and for outcome, "milk production" or "milk yield" or "somatic cell count" or "protein" or "fat".

Dairy cow was the population of interest. The interventions were the different temperament types. As comparison, we considered groups of cows classified as different temperaments in 'Low' (lower reactivity class, also referred to as calm animals), 'Inter' (intermediate reactivity class, also referred to as normal animals), and 'High' (higher reactivity class, also referred to as reactive or nervous or excitable or aggressive animals in the publications reviewed). The outcomes of interest were daily milk yield, total milk yield (whole lactation), and milk quality, but the present study will report only the results regarding yield, despite our database search having included all these measures (Figure 1).

Figure 1. Flow diagram indicating the number of citations and publications included and excluded in each level of the systematic review on temperament of dairy cows and milk yield and milk quality, adapted from PRISMA guidelines (Page et al. 2021). All search results are included in the diagram to allow a better understanding of the total number of records found. *Data from both procedures (milk yield and milk quality) are presented in the flow diagram to allow the researchers to update the same systematic review



In order to be included in our SR, the publications had to assess at least one of the response variables of interest in association with dairy cows' temperament.

A search protocol was previously developed, and screening tools were adapted from forms used in previous studies (Canozzi et al., 2017; 2019) and tested prior to their application.

2.2 Search methods for the identification of publications

The systematic literature search was conducted from September to December 2020 in four electronic databases - CABI Abstracts (Thomson Reuters, 1910-2020), ISI Web of Science (Thomson Reuters, 1900-2020), PubMed (MEDLINE, 1940-2020), and Scopus (Elsevier, 1960-2020). Additional searches were carried out using the literature cited from the publications included in the MA to include peer-reviewed publications not identified by the literature search as well as abstracts published in conference proceedings that were relevant to the subject. All references were exported to EndNote Web software (Clarivate Analytics, Jersey, England) to organize and manually remove duplicate references.

2.3 Publications selection criteria and relevance screening

We applied the screening in all citations identified by the literature search using three stages. Before starting the screening, four reviewers were previously trained using 30 publications.

In the first stage, we aimed to identify possible citations of interest among those selected by the search. Each citation was evaluated by reading only the title and applying five simple questions (Table 1).

Table 1. Five questions used to identify possible citations of interest by reading the paper titles

1. Does this title investigate primary research?	2. Does this title investigate temperament / personality / reactivity in dairy cows?	3. Does this title investigate productive performance in dairy cows? (check all that apply)	4. Does this title investigate any milk quality indicators? (check all that apply)	5. Does this title investigate phenotypic aspects of the relationship between temperament and milk yield or milk quality?
a) Yes (include)	a) Yes (include)	a) Daily milk yield (include)	a) Somatic cell count (SCC) (include)	a) Phenotypic (include)
b) Literature Review (exclude)	b) No (exclude)	b) Monthly milk yield (include)	b) Fat content (include)	b) Phenotypis and genetic (include)
c) Other (exclude)	c) Cannot tell, but likely yes (neutral)	c) Milk yield throughout lactation (include)	c) Protein content (include)	c) Genetic (exclude)
d) Cannot tell, but likely yes (neutral)		d) Milk yield at peak lactation (include)	d) Other (please specify) (include)	d) Cannot tell, but likely yes (neutral)
		e) Other (please specify) (include)	e) Cannot tell, but likely yes (neutral)	
		f) Cannot tell, but likely yes (neutral)	f) None of the above (exclude)	
		g) None of the above (exclude)		

This stage was carried out by two researchers independently. In the next step, the remaining citations were evaluated by the same two reviewers, assessing the title, keywords, and abstract, based on eight questions (Table 2).

Table 2. Eight questions used to identify possible citations of interest by reading the title, keywords, and abstract

Question	Yes	No
1. Is this paper published in English, Portuguese or Spanish?		
2. Is the full paper available?		
3. Does this study investigate phenotypic aspects of the relationship between temperament and milk yield or milk quality?		
4. Does this study uses groups of cows with divergent temperaments?		
5. Are sufficient raw or unadjusted data provided for assesses the temperament association with milk yield and / or quality and / or content and / or milkability?		
6. Are the measure of dispersion for the raw or unadjusted mean data provided for assesses the temperament association with milk yield and / or quality and / or content and / or milkability?		
7. Are correlations or regressions coefficients provided for assesses the temperament association with milk yield and / or milk quality and / or content and / or milkability?		
8. If the paper was excluded, why?	(brief description of reason)	

When both evaluators answered "no" to one or more questions, the citation was excluded, and, in case of conflicting answers, both evaluators would consensually make the decision. A citation was considered relevant when it was peer-reviewed or conference proceedings assessing dairy cows' temperament, and its relationships with milk yield. In this last stage, we did not apply any restrictions to language or year of publication. The Microsoft Excel software was used throughout all screening stages.

2.4 Methodological assessment and data collection process

The first and last authors were responsible for the extraction of data from the selected publications. The relevance of the previously selected publications was confirmed by reading them in full.

The evaluated publications were restricted to the languages in which the research team was fluent (English, Spanish, and Portuguese). Data extracted from each publication was divided into characteristics related to population, intervention, measures, and outcome data, in addition to journal name, author(s), year of publication, and original language. The data extraction forms were adapted from previous studies (Canozzi et al., 2017; 2019).

We need to highlight the diversity of methods found within the selected publications, with different ways to assess temperament and data analyses, hindering the summarization of results. Furthermore, some of these papers allowed for only a qualitative analysis of data (Breuer et al., 2000; Rousing et al., 2004, Bertenshaw et al., 2008; Szentléleki et al., 2008; Dodzi; Muchenje, 2011; Sutherland; Dowling, 2014; Hedlund; L¢vlie, 2015; Cerqueira et al., 2017), as they presented results as correlations and / or regressions, making their inclusion in the MA impossible. Therefore, the included publications were divided into two groups: one for meta-analytical evaluation, and the other for qualitative evaluation.

2.5 Considerations for data collection and manipulation

A table with the data was created for each of the results of interest, including mean, standard deviation of mean or another dispersion measure, *P*-value, and the number of evaluated cows in each comparison: (Low vs. Inter), (Low vs. High), and (Inter vs. High), with each comparison for a temperament indicator (measure) being regarded as a 'trial'. For daily yield results, the obtained values refer to the average daily milk yield (in kg/day); and total milk yield (sum of milk yield throughout the whole lactating period, in kg). Some publications presented a greater number of scores and distinct classifications for temperament (Orbán et al., 2011; Gergosvka et al., 2014; Neja et al., 2017), so we standardized them to consider only three temperament types (Low, Inter, High). With these three temperaments, we formed three comparison groups for the analysis of subgroups: group 1 (Low x Inter), group 2 (Low x High), and group 3 (Inter x High).

For two publications that reported only the means values and *P*-values for means comparisons, without a measure of dispersion (Neja et al., 2015; Sawa et al., 2017), an estimate of common standard deviation was calculated using t-statistics and assuming the data was normally distributed, based on the following equation (Ceballos et al., 2009; Mederos et al., 2012):

$$S_P = \frac{(x_2 - x_1)}{t(\alpha df E)\sqrt{(1/n_2)+(1/n_1)}}$$

were $\chi_2 - \chi_1$ represents the means difference; t(α*df*E) is the percentile of the reference distribution, and n is the sample size of each group.

2.6 Quality assessment

The risk of publication bias in the publications was assessed using Newcastle-Ottawa Scale (NOS) (Wells et al., 2014). This is an appropriate tool to assess the quality of observational and not experimental randomized trials, based on three main criteria: ‘Selection’, ‘Comparability’, and ‘Outcome’. The publications receive one ‘star’ for each quality item included in the criteria of selection and outcome and a maximum of two ‘stars’ for comparability. In the end, the quality of the publications is expressed on a 9-point scale (Wells et al., 2014).

2.7 Meta-analysis

The publications which presented qualitative data that allowed us to estimate the mean difference (MD) between the evaluated temperament types and a confidence interval of 95% (95% CI) were included in this MA. The statistical analyses were carried out using the Stata V 16.0 software (StataCorp., Texas, EUA).

In subgroup analysis, we carried out an MA separately with data sets consisting of, at least, two individual publications which investigated the same comparative group and the same outcome of interest. The MA results were shown considering MD and 95% CI. Cochran’s Q (chi-square test for heterogeneity) and I^2 (percentage of total variation between publications due to heterogeneity and not by chance) were obtained based on the evaluated temperament type (groups 1, 2, and 3) and the outcome variable. The magnitude of I^2 was interpreted in the orders of 25%, 50%, and 75%, and considered as low, moderate, or high heterogeneity, respectively (Higgins et al., 2003).

2.8 Publication bias

Publication bias was assessed through a funnel plot and the statistical tests of Begg's correlation and Egger's linear regression. Bias was considered as present based on the visual analysis of the plot and if at least one of the statistical methods was significant ($P < 0.10$). In case there was any indication of the presence of bias, we used the "trim-and-fill" method to estimate its extension (Duval; Tweedie, 2000), which allows us to estimate the number of publications that should be included in the analysis in order for the graph to become symmetrical.

2.9 Meta-regression analysis

Univariate meta-regression was performed to identify possible sources of heterogeneity that could influence the results. The variables explored were: year of publication; geographic regions (North America, South America, Europe, Africa, Asia, Oceania); experiment time (days); sample size; racial group (European or Zebu); parity (primiparous or multiparous); lactation stage (beginning = first weeks of lactation or throughout lactation = over the whole lactation); observer effect (unfamiliar person, familiar person or milker); blinding (no, yes, not reported, or not applicable); clustering (no, yes, or not applicable); and identified and controlled confounders (no, yes, or not applicable). The results were reported only for variables that were significant.

2.10 Cumulative meta-analysis and influential publications

The cumulative MA was carried out to estimate the effect of the different temperament types on daily and total milk yield each time a new publication was published, to demonstrate the pattern of evidence over time (Borenstein et al., 2009). A sensibility analysis was carried out to check if a certain publication had influenced the effect measurement (MD), by successively removing manually one publication at a time and assessing if MD varied ± 30% after re-inserting the publication and removing the next one.

3. RESULTS

3.1 Publication selection

Our database search identified 552 citations. From that total, 52 were potentially relevant abstracts and 22 were selected for eligibility. Finally, 12 publications were fully read, and among those, nine had their data extracted (Figure 1) and included in this MA, with a total of 84 trials. For daily milk yield, a total of eight publications reporting 75 trials were included, and for total milk yield, it was considered three publications reporting nine trials.

The main characteristics of the included publications are shown in Tables 3 and 4. Three publications were excluded for presenting insufficient data for quantitative analysis (Table 5). We contacted the authors, but no numerical data were obtained, and, since we could not extract them manually, the publication was excluded.

Table 3. A descriptive summary of each relevant study included in the meta-analysis (n = 9) for daily milk yield and total milk yield

Reference	Country	Study population (breed / sample size)	Temperament indicator	*Comparison groups	Outcome parameter
Praxedes et al. (2009)	Brazil	Zebu (Gyr) / 2.507	Other	Group 1 Group 2 Group 3	Total milk yield
Orbán et al. (2011)	Hungry	Holstein Friesian / 69 Jersey / 283	Crush score (reactivity in score in the squeeze chute)	Group 1 Group 2 Group 3	Daily milk yield
Sutherland and Huddart (2012)	New Zealand	Holstein Friesian / 40	Flight speed (in m/s)	Group 1 Group 2 Group 3	Daily milk yield
Sutherland et al. (2012)	New Zealand	Holstein Friesian / 30	Flight speed (in m/s)	Group 1 Group 2 Group 3	Daily milk yield
Gergovska et al. (2014)	Bulgaria	Black and White / 143	Reactivity in scores in the milking parlor	Group 1 Group 2 Group 3	Daily milk yield
Neja et al. (2015)	Poland	Holstein Friesian / 11.629	Reactivity in scores in the milking parlor	Group 1 Group 2 Group 3	Daily milk yield / Total milk yield
Neja et al. (2017)	Poland	Holstein Friesian / 158	Reactivity in scores in the milking parlor	Group 1 Group 2 Group 3	Daily milk yield
Marçal-Pedroza et al. (2020)	Brazil	Zebu-crosses (Girolando) / 31	Reactivity in scores in the milking parlor/ Steps or kicks/ FSK[1] (or MOV)/ Entrance time / Crush score / Flight speed / Flight distance / Novel object test	Group 1 Group 2 Group 3	Daily milk yield
Sawa et al. (2017)	Poland	Holstein Friesian / 12.028	Reactivity in scores in the milking parlor	Group 1 Group 2 Group 3	Daily milk yield/ Total milk yield

* Comparison groups between temperament types, with group 1: low vs inter; group 2: low vs high; group 3: inter vs high. [1]FSK or MOV: Score based on the performance of flinching, stepping, and kicking or sum of the number of kicks and steps during milking.

Table 4. Descriptive characteristics of nine publications included in the meta-analyses (MA)

Variable	Categories	Number of publications
Study design	Observational study	7
	Controlled trial	2
Publication type	Peer-reviewed	8
	Conference proceedings	1
Indicator temperament	Reactivity in scores in the milking parlor	5
	Steps or kicks	1
	FSK (or MOV)[1]	1
	Entrance time (in s)	1
	Crush score	2
	Flight speed (in m/s)	3
	Flight distance (in m)	1
	Novel object test	1
	Other	2
Treatment (type of temperament)	Low	9
	Intermediate	9
	High	9
Year of publication	2009-2014	5
	2014-2020	4
Breed	Not reported	0
	Europen	2
	Zebu / Zebu-crosses	7
Calving order	Primiparous	3
	Multiparous	4
	Primiparous and multiparous	1
Lactation stage	Not reported	3
	Beginning of lactation	2
	Throughout lactation	4
Housing system	Not reported	3
	Free-stall or tie stall	3
	Loose housing / open yard	1
	Pastures / paddock	2
Milking system	Not reported	6
	Herringbone-milking parlor	2
	Parallel-milking parlor	0
	Tandem_milking parlor	0
	Rotary (Carousel) parlor	1
	Robotic milking parlor	0
Who performed the procedure	Not reported	5
	Unfamiliar person, technician, or researcher (authors)	4
	Familiar person or milker other	0
Outcome assessed	Daily milk yield	8
	Total milk yield	3
Continent	South America	2
	Oceania	2
	Europe	5
Sample size	N < 100	3
	n ≥ 100 and n < 1000	3
	n ≥1000	3

[1]FSK or MOV: Score based on the performance of flinching, stepping, and kicking or sum of the number of kicks and steps during milking.

Table 5. List of relevant publications excluded from the final dataset in the meta-analyses (MA)

Reference	Country	Indicator temperament	Temperament type	Outcome parameter	Reason for exclusion
Szentléleki et al. (2015)	Hungry	Reactivity in scores in the milking parlor	Low / High	Total milk yield	Insufficient numerical data
Kalińska and Slósarz (2016)	Poland	Reactivity in scores in the milking parlour	Low / Inter / High	Fat milk / Protein milk	Insufficient numerical data
El. Abdel et al. (2017)	Egypt	Reactivity in scores in the milking parlor	Low / Inter / High	Daily milk yield / Total milk yield / Fat milk / Protein milk	Insufficient numerical data

Eight publications evaluated daily milk yield, and three, total yield. The relationship of temperament with daily milk yield was assessed in 26.614 cows, and total milk yield in 23.885 cows.

3.2 Risk of Bias

The NOS tool was used to analyze the risk of bias, considering the type of publications used in this MA (observational) (Table 6). Of the nine publications included, four (Sutherland and Huddart, 2012; Sutherland et al., 2012; Neja et al., 2015; Sawa et al., 2017) were considered of moderate quality (score between 5 and 7), and the other seven were scored as high quality (scores 8 or 9). This result indicates a moderate to high quality and moderate to low risk of bias in the publications included.

Table 6. Risk of bias assessment in the nine studies included in the final dataset of the meta-analyses (MA)

Reference	SELECTION				COMPARABILITY	OUTCOME			Total
	Adequate definition of temperament groups	Representativeness of the cows used	Selection of divergent temperament groups	Control for disease or incidents that affected the outcome	Adjustment for confounders	Assessment of outcome	Enough time of outcome recording	Adequacy of outcome recording	
Praxedes et al (2009)	☆	☆	☆		☆☆	☆	☆	☆	8
Orbán et al. (2011)	☆	☆	☆		☆☆	☆	☆	☆	8
Sutherland and Huddart (2012)	☆	☆	☆	☆		☆	☆	☆	7
Sutherland et al. (2012)	☆	☆	☆			☆	☆	☆	6
Gergovska et al. (2014)	☆	☆	☆		☆☆	☆	☆	☆	8
Neja et al. (2015)	☆	☆	☆	☆		☆		☆	6
Neja et al. (2017)	☆	☆	☆		☆☆	☆	☆	☆	8
Sawa et al. (2017)	☆	☆	☆			☆		☆	5
Marçal-Pedroza et al. (2020)	☆	☆	☆	☆	☆☆	☆	☆	☆	9

3.3 Meta-analysis

In our MA, nine publications were included, six of which evaluated only daily yield and three, daily and total milk yield. The number of publications and types of outcome measures are shown in Table 3. For the analyses, in addition to temperament, the influence of breed, parity, and stage of lactation on milk yield were also evaluated.

3.3.1 Effect of temperament on daily milk yield

The daily yield was the most frequently studied outcome and was shown in eight of the nine publications included in the MA ($I^2 = 99.9\%$). Mean difference (MD) in daily yield (n = 8 publications, 75 trials) among Group 3 (i.e. Inter vs. High cows) was -0.82 kg of milk/day (95% CI: -1.01, -0.63; $p < 0.001$), suggesting that Inter cows produced less milk than the High ones, with high heterogeneity among publications ($I^2 = 99.4\%$).

3.3.1.2 Effect of temperament on daily milk yield considering breed, parity, and lactation stage

For the effect of breed on temperament, only studies with European breed (n = 7 publications, 35 trials) were evaluated, since only one publication assessed Zebu cows. The comparison among Group 1 (n = 6 publications, 35 trials) resulted in an MD of 0.67 kg/milk (95% CI: 0.10, 1.24; $p = 0.020$), indicating that daily milk yield was lower for Inter than for Low cows, with high heterogeneity between publications ($I^2 = 99.9\%$). In the comparison among Group 3 (n = 6 publications, 35 trials), MD was -1.18 kg/milk (95% CI: -1.41, -0.95; $p < 0.001$), with Inter cows producing less milk than High. In summary, for studies with European breeds, cows with intermediate temperament produced less milk than the calm and reactive ones.

Among primiparous animals (n = 4 publications, 50 trials) in Group 3, Inter cows produced less milk (MD = -0.74 kg/milk; 95% CI: -0.93, -0.56; $p < 0.001$) than High ones, with high heterogeneity among publications ($I^2 = 96.4\%$). Among multiparous (n = 6 publications, 25 trials) in Group 1 (n = 4 publications, 25 trials), Inter cows produced less milk (MD = 0.70 kg/milk; 95% CI: 0.07, 1.35; $p = 0.032$) than Low ones, with high heterogeneity among publications ($I^2 = 99.7\%$). In Group 3 (n = 5 publications, 25 trials), Inter individuals produced less than High ones (MD = -1.08; 95% CI: -1.54, -0.61, $p < 0.001$), with a 99.8% heterogeneity. So, intermediate cows produced less than the calm and reactive ones, without difference between the last ones.

When assessing the influence of the lactation stage (n = 3 publications, 50 trials) on daily milk yield, we only found significance for experiments carried out throughout lactation, but not at the beginning of lactation. In Group 1 (n = 3 publications, 13 trials), MD was 0.73 kg/milk (95% CI: -0.09, 1.55; $p = 0.081$), that is, Low cows tended to have a greater daily milk yield than Inter ones, with high heterogeneity among publications (I^2 = 99.7%). In Group 2 (n = 3 publications, 13 trials), MD was -1.01 kg/milk (95% CI: -1.34, -0.68; $p < 0.001$), Low cows produced less milk than High, with high heterogeneity among publications (I^2 = 97.5%). In Group 3 (n = 3 publications, 13 trials), Inter cows were less productive (MD = -1.24 kg/milk; 95% CI: -1.99, -0.49; $p = 0.001$) than the High ones, with high heterogeneity among publications (I^2 = 98.2%). In summary, the daily milk yield was higher for reactive, followed by calm and intermediate cows, which had the lowest milk yield.

3.3.2 Effect of temperament on total milk yield

Results for total milk yield were found in three publications (n = 9 trials), with high heterogeneity among publications (I^2 = 99.9%). In Group 2 (n = 3 publications, 9 trials), we obtained an MD of -1,217.57 kg/milk (95% CI: -2,589.08, 153.94), indicating that Low cows tended ($p = 0.082$) to produce less milk than the High ones, with high heterogeneity among publications (I^2 = 99.9%). In Group 3 (n = 3 publications, 9 trials), Inter animals had a yield -1,062.45 kg/milk (95% CI: -1,288.35, -836.54; $p < 0.001$) lower when compared to High ones, with high heterogeneity among publications (I^2 = 99.9%). It indicates that reactive cows produced more milk than the calm and intermediate ones.

3.3.2.1 Effect of temperament on total milk yield considering breed, parity, and lactation stage

For breed effect, subgroup analysis was carried out only with European breeds (n = 2 publications, 6 trials), since only one publication evaluated Zebu animals. In Group 3 (n = 2 publications, 6 trials), cows of Inter temperament yielded less milk (MD = -414.97 kg/milk; 95% CI: -656.05, -173.90; $p = 0.001$) than High ones, with high heterogeneity among publications (I^2 = 99.9%).

For primiparous cows (n = 2 publications, 6 trials), we observed difference only for Group 3 (n = 2 publications, 6 trials). High cows produced 414.97 kg (98% CI: -656.05, 173.90; $p = 0.001$) more milk than Inter ones, with high heterogeneity between publications

(I^2 = 99.9%). Among the three publications included, none assessed total milk yield in multiparous cows.

Regarding the lactation stage, only one of the three publications described it, which made such a comparison impossible.

3.4 Publication bias

The data included in this MA is quite heterogenous, therefore, results must be interpreted carefully. Both for daily and total milk yield, the asymmetry found in the funnel plot was confirmed by Egger's statistical test ($p < 0.001$ for both tests), and Begg's test was not significant ($p = 0.14$; $p = 0.75$, respectively), with no insertion of new publications by the "trim-and-fill" test.

3.5 Meta-regression analysis

Meta-regression results on daily milk yield: eight publications (n = 75 trials) were inserted in this analysis. Results showed that 99.9% of the variation among publications was due to chance. None of the eight variables were significantly associated with daily yield, and only three contributed to explaining the variation among publications: sample size (4.6%), lactation stage (4.2%), and identified and controlled confounders (5.5%).

***Meta-regression results on total milk yield*:** three publications (n = 9 trials) were considered in the meta-regression, and it was evidenced that 99.9% of the variation among publications was due to chance. Meta-regression indicated that with the increase of one year in the year of publication, there was an increase of 233.83 kg in the predicted value ($p = 0.050$). Publications carried out in Europe showed a 1,905.75 kg ($p = 0.019$) increase in the predicted value for total milk yield when compared to publications conducted in South America. The number of evaluated animals showed a significant effect, and the increase of one experimental unit rose the predicted value of 0.20 kg of milk ($p = 0.022$). Publications with animals of Zebu breeds showed a decrease of 1.90 kg in the predicted value ($p = 0.019$) when compared to those carried out with European cattle. When clustering factors were considered, the predicted value increased by 1.90 kg ($p = 0.019$) (Table 7).

Table 7. Univariate meta-regression results showing significant (P < 0.05) and marginally significant (0.05 ≤ P < 0.10) covariates investigated as potential sources of study heterogeneity for total milk yield.

No. of studies [A] (trials) [B]	Covariate (trials)	Estimate [C]	95% CI [D]	P-value	I^2 (%)	Adj-R^2 (%)
Total milk yield 3 (9)	Null model	-796.10	-1,765.62, -173.41	0.095	99.9	NA
	Publicarion year (9)	233.83	-0.52, -468.18	0.050	99.9	0
	Continent	-	-	0.019	99.9	0
	South America (9)	Reference				
	Europe (9)	1,905.75	413.93, 3,397.57	0.019		
	Sample size (9)	-2,563.72	0.04, 0.36	0.022	99.9	0
	Cattle group (9)	-	-	0.019	99.9	0
	Zebu (9)	Reference				
	Europe (9)	1,905.75	-3,397.57, -413.93	0.019	-	-
	Clustering (9)	-	-	0.019	99.9	0
	No (9)	Reference				
	Yes (9)	1,905.75	413.93, 3,397.57	0.019		

I^2 between-study residual variation; Adj-R^2 percentage of the residual variation;
[A] Number of studies included in the meta-regression.
[B] Number of trials included in the meta-regression.
[C] Standard mean difference of the effect size.
[D] These values represent 95% confidence intervals (CI) for the effect size.

3.6 Cumulative MA and sensitivity analysis

Daily milk yield: In the cumulative MA (2011-2020) for daily yield, there was clear evidence of a change in the estimated yield between temperament groups, going from a positive (MD = 0.16 kg/milk) to a negative value (MD = -0.54 kg/milk). Sensibility analysis showed that removing two publications (Orbán et al., 2011; Sutherland et al., 2012) reduced MD from -0.24 kg to -0.34 and -0.31 kg/milk, respectively. Removing the publication by Neja et al. (2017) increased MD from -0.23 to -0.09 kg/milk.

Total milk yield: In the cumulative MA (2009-2017) for total yield, there was any evidence of changes through the years. Removing the publication by Neja et al. (2015) decreased MD from -796.10 kg to -1,291.86 kg/milk, while removing the publication by Praxedes et al. (2009) increased MD from -796.10 to -171.43 kg/milk.

3.7 Qualitative analysis

Some publications assessed the influence of temperament on milk yield using correlation and regression analyses, thus, they were not included in the MA. Due to their relevance, they were considered and analyzed in a qualitative way (Table 8).

Table 8. A descriptive summary of each relevant study (n = 8) that was included in the qualitative synthesis (could not be included in the MA) for daily and total milk yield

Reference	Country	Study population (breed / sample size)	Temperament indicator	Outcome parameter
Breuer et al. (2000)	Australia	Holstein Friesian / 100-200	Reactivity in scores in the milking parlor, steps, and other	Total milk yield
Rousing et al. (2004)	Denmark	Holstein Friesian / 1.196	Steps, kicks, and other	Daily milk yield /
Bertenshaw et al. (2008)	United Kingdom	Holstein Friesian / 148	Steps and kicks	Daily milk yield
Szentléleki et al. (2008)	Hungary	Holstein Friesian / 17	Reactivity in scores in the milking parlour	Daily milk yield
Dodzi and Muchenje (2011)	South Africa	Holstein Friesian / 7, Jersey / 7, and crossbred / 7	Steps, kicks, FD, and FS[1]	Total milk yield
Sutherland and Dowling (2014)	New Zealand	Holstein Friesian / 150	FSK, FD[1]	Total milk yield
Hedlund and L¢vlie (2015)	Sweden	Holstein Friesian / 29, and Swedish Red and White cattle / 27	Steps, kicks, and NOT	Daily milk yield
Cerqueira et al. (2017)	Portugal	Holstein Friesian / 2.903	Steps, and kicks	Total milk yield

[1]FD: Flight distance; FS: Flight speed; FSK: Score based on the performance of flinching, stepping, or kicking during milking; NOT: Novel object test

All eight publications were carried out with European breeds and evidenced different patterns of relationship between temperament and milk yield. In one of them, milk yield was greater for reactive animals (Rousing et al., 2004), where cows that took more steps in the milking parlor yielded more milk (in kg/day), with Odds Ratios of 1.5 (20-to-30-liter production) and 2.2 (production of over 30 liters). In its turn, Szentleleki et al. (2008) did not find an association between temperament and milk yield using milking reactivity scores as temperament indicators.

Most of the publications (n = 6) reported a negative relationship between temperament and yield, that is, calmer cows produced more milk, as reported by Breuer et al. (2000) ($r = -0.38$; $p < 0.05$ for milking reactivity scores); Bertenshaw et al. (2008) ($r = -0.25$; $p = 0.01$ for steps); Dodzi and Muchenje (2011) ($r = -0.17$; $p < 0.05$ for kicks); Sutherland and Dowling (2014) ($r = -0.23$; $p < 0.05$ for milking reactivity scores); Hedlund and L¢vlie (2015) ($R^2 = -0.32$; $p < 0.02$ for steps); and Cerqueira et al. (2017) ($r = -0.10$; $p = 0.00$ for steps). Bertenshaw et al. (2008) report in the regression analysis, a 7.1% of the variation in productivity occurred due to the number of steps and kicks in the presence of humans ($R^2 = 0.07$; $p < 0.001$), which did not occur in the absence of humans ($R^2 = 0.002$; NS).

4. DISCUSSION

An SR followed by MA was carried out to quantitatively assess the effects of dairy cows' temperament on milk yield. According to our MA results, calmer cows were not the most productive for both daily and total milk yield, against our initial hypothesis. Despite the significant number of publications, only nine had enough information to be included in the quantitative synthesis (MA).

4.1 Effect of temperament on daily milk yield

In general, our MA results for daily milk yield evinced those cows classified as reactive (High) produced more than intermediates, and even more than the calm ones (Low), which differed from what we expected. According to Abdel-Hamid et al. (2017), reactive cows, possibly, spend more energy on motor activities, such as walking and standing. Additionally, reactive cows in the milking parlor drop teat cups more often and direct less liquid energy to lactating, which leads to a lower yield (Marçal-Pedroza et al., 2021). However, there are authors who argue that reactive cows are more aggressive during feeding and ingest greater amounts of food, resulting in greater productivity (Sawa et al., 2017). Despite our sensibility analysis not identifying it, the study by Marçal-Pedroza et al. (2020) could be influencing these results, since rumination frequency during milking was used as a temperament measurement. In this study, a significant relationship between temperament and milk yield was reported for the behavioral indicator of rumination in the milking parlor. In this specific case, cows classified as High ruminated more during milking, therefore being calmer and more relaxed, and reaching greater milk yield than the Low ones who spent less time ruminating. This classification was different from the other publications included in this MA, in which the High category animals were the most reactive.

The high variability found for the eight analyzed publications may be due to the different methods used to measure reactivity as an indicator of the cows' temperament. This makes it difficult to compare the data in published literature, since some methods may be more sensitive to recording the intensity of the behavioral responses of the animals than others (Sutherland; Huddart, 2012).

The effect of temperament on daily milk yield was assessed considering the subgroups of breed, parity, and lactation stage. Among the evaluated publications, only Marçal-Pedroza et al. (2020) studied Zebu cows. In the European cows, Inter animals produced less than the Low and High ones. For two (Orbán et al., 2011; Sutherland; Huddart, 2012) of the seven publications evaluated in the MA for European breeds, there was no evidence of any effect of temperament on daily milk yield, with only five publications leading to these results. Thus, it is evident that we need to be careful when interpreting results, mainly due to the low number of publications available.

Regarding the effect of parity, primiparous cows of Inter temperament yielded less than those of High temperament. Again, we highlight the work of Marçal-Pedroza et al. (2000), which, by using the frequency of rumination as temperament measurement, primiparous in the High category were the ones with the most rumination and higher milk yield. According to Sawa et al. (2017), the selection of animals to increase productivity may also increase the risk of selecting animals with undesirable temperaments, which might remain in the herd due to their greater milk yield (Praxedes et al., 2009).

Regarding multiparous cows, productivity was lower for Inter than for Low and High cows. In general, multiparous individuals are more used to the milking process, and their reaction to handling may be smaller, which possibly results in better productive performance for the calmer and for reactive ones compared to the intermediates (Sutherland; Huddart, 2012).

When considering lactation stage, the temperament classes differed only throughout the lactation, with a higher daily milk yield for reactive, followed by calm and intermediate cows that had the lowest milk yield. Among the four publications analyzed, two failed to find an influence of temperament on productivity (Orbán et al., 2011; Sutherland; Huddart, 2012), while the other two (Gergovska et al., 2014; Sawa et al., 2017) found greater productivity in High cows, in a total of 12.068 evaluated cows, and argued that High animals could have yielded more due to greater consumption. Whereas Gergovska et al. (2014) reported that High cows, despite their greater production, had an irregular lactation curve, which does not occur for Low cows.

4.2 Effect of temperament on total milk yield

Only three of the publications included in the MA evaluated the effect of animal temperament on total milk yield (over the whole lactation), which may compromise the interpretation of these findings. In general, High cows were more productive than Low and Inter ones. Regarding the breed effect, only two publications with European breeds were considered. In that case, High cows had greater productivity than Inter ones. Moreover, among the primiparous animals, also the High yielded more than Inter ones, possibly due to the previously mentioned relationship between greater feed intake and high milk yield in reactive animals.

Frequently used reactivity indicators for dairy cows have been the number of steps and kicks in the milking parlor (Rousing et al., 2004; Cerqueira et al. 2017; Marçal-Pedroza et al., 2020), but there is no consensus among authors regarding the real interpretation of these movements. Steps may represent a stress indicator, mainly for animals classified as aggressive (Wenzel et al., 2003), or have another meaning, e.g., younger animals with a high parasitic rate (ticks) may take more steps than those with a lower rate, signaling discomfort rather than a more excitable (or reactive) temperament (Rousing et al., 2004). This divergence of interpretation of the animals' temperament may lead to an incorrect association between temperament type and productivity variables. As highlighted by Sawa et al. (2017), the relationship between temperament and milk yield depends on several factors, such as the temperament indicator used, studied breed, age of the animals, and parity.

4.3 Meta-regression analysis

Of the eight covariables analyzed (year of publication, geographic region - continent, experiment duration, sample size, breed, parity, lactation stage, and controlled confounders), only three contributed to explaining the variation between publications: sample size, lactation stage, and controlled confounders have shown a direct correlation with the daily milk yield of cows. As for the total milk production, some variables showed an association with milk production, but none of them contributed to explaining the variability found between the publications.

Meta-regression indicated that with every one-year increase in the year of publication, there was an increase in MD, which is possibly related to the period of publication of the selected papers since all nine publications were published starting from the 2000's, a period of growing interest in issues related to behavior, productive performance, and welfare of farm animals (Hemsworth et al., 2000; Rousing et al., 2004; Broom, 2010; van Dijk et al., 2019). Another element we need to highlight is that most studies carried out in Europe showed an increase in MD for total milk yield when compared to studies conducted in South America (Praxedes et al., 2009; Marçal-Pedroza et al., 2020). It could be attributed to the longer period of selection for high productivity in the European breeds, resulting in higher productivity for these animals compared to the Zebu breeds and local crossbreeds used in Latin America (Brito et al., 2021). In spite of the lower milk production, the use of Zebu breeds and their crosses (such as Girolando), more adaptable to warm climates, would result in higher sustainability of dairy production in tropical regions (Canaza-Caio et al., 2016; Brito et al., 2021). The number of evaluated animals had a significant effect, which is probably because the publications had a great variation in sample size (from 30 to 12.028 animals).

For daily milk yield, there was clear evidence of change in the estimated MD, going from a positive value to a negative one, indicating that milk yield increases for the higher temperament classes (Inter and High). The exclusion of the publications by Orbán et al. (2011) and Sutherland et al. (2012) lead to a reduction in MD, but the daily yield of the reactive animals continues to be higher than that of calm and intermediate cows. Both publications together evaluated only 382 dairy cows, all of European breeds. In turn, the exclusion of Neja et al. (2017) resulted in increased MD, also maintaining greater production for reactive cows, and in their study, only 158 animals of European breed were evaluated.

Differently from daily yield, no tendencies were evidenced for total milk yield. The removal of Neja et al. (2015) decreased MD, and it was conducted with 11.629 cows of European breeds, but the total yield of the reactive cows remained higher than the intermediate and calm ones. The opposite happened when we excluded Praxedes et al. (2009), leading to an increase in MD, but the milk yield of reactive cows remained higher. Praxedes et al. (2009) investigated the production of 2.507 animals of Zebu breed, with a lower sample size when compared with the publications by Neja et al. (2015). The last one, published by Sawa et al. (2017), evaluated 12.028 cows. Neja et al. (2015) and Sawa et al. (2017) used European animals, which has possibly led to this variation alongside the fact that Zebu cows, in general, have lower milk yield than European breeds.

4.4 Qualitative analysis

The publication of Rousing et al. (2004), which evaluated the cows' temperament based on the number of steps in the milking pen, was the only one to find that High cows yielded more milk, in agreement with our results from MA. For these authors, the occurrence of steps is an indication of discomfort during the milking process, mainly in younger animals, and does not necessarily indicate reactive temperament, which could explain why High cows were more productive. In turn, Bertenshaw et al. (2008) and Dodzi and Muchenje (2011) reported that primiparous individuals which took more steps and kicks while milking were less productive. Hedlund and L¢vlie (2015) found the same pattern of association with nervous cows producing less milk, which was seen only in the first lactations. Cerqueira et al. (2017), who evaluated multiparous and primiparous cows, observed that the relationship between reactivity and production is associated with parity: cows with a greater number of calvings, i.e., the oldest of the herd, which took more steps, had a lower yield.

The quality of the human-animal relationship during the milking routine is possibly mediating the relationships between temperament and milk yield, as reported by Breuer et al. (2000) and Hemsworth (2003). Therefore, with high-quality handling, based on application of good practices, even the cows with the reactive temperament (more susceptible to stress) might express their best productive potential under adequate environmental conditions (Praxedes et al., 2009; Marçal-Pedroza et al., 2020).

Our SR/MA has some limitations that must be considered. Firstly, the low number of publications found on the subject. Secondly, some publications which could have been included did not present the data in a format that allowed it to be extracted for a MA. Even after trying to contact the authors to obtain details, as suggested by Lean et al. (2009), we were not successful to reach the numerical data. Additionally, some publications were analyzed separately from the MA in a qualitative manner, due to the relevance of their results. Also, the lack of standardization of the methods of temperament assessment in dairy cows associated with the large variation in productive performance of the animals made the analysis and interpretation of the results a challenging task. Putting it all together, the results obtained in this MA, reporting the greater production by High cows, may be due to how the behavior is interpreted in these studies (reactivity considering the leg movement levels). It is important to highlight the fact that the animals being less agitated, or even still, during the milking procedures does not necessarily mean a calmer temperament, but a fear state (Munksgaard et al., 2001). Understanding animal reactivity as an indicator of temperament type requires, aside from objective measurements, an interpretation of the intrinsic traits of animals, what could be achieved based on the inclusion of physiological measures.

5. Conclusion

This is the first SR-MA that assessed results published in the scientific literature on the effect of dairy cows' temperament on productivity. Our results of the MA did not support the original hypothesis, as we obtained that reactive cows generally produce greater milk yield than those of calm and intermediate temperament. On the other hand, correlation and regression data support our hypothesis of calm cows being more productive. This contrast leads us to further questions: which indicators should we use to classify animal temperament? And when should this classification be applied? In addition to the need for standardization of protocols for behavioral assessments, in order to allow for a better understanding of the results, and the need for more studies reporting this type of assessment for cows of Zebu breeds.

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Chapter 2 - Are dairy cows with a more reactive temperament less efficient in energetic metabolism and do they produce more enteric methane?[1]

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Abstract

The objectives of this study were: a) to evaluate the relationship between cattle temperament assessed by traditionally used tests with energetic metabolism and enteric CH_4 emissions by crossbred dairy cows; b) to assess how cows' restlessness in respiration chambers affects energetic metabolism and enteric CH_4 emissions. Temperament indicators were evaluated for 28 primiparous F1 Holstein x Gyr cows tested singly in the handling corral (entrance time, crush score, flight speed, and flight distance) and during milking (steps, kicks, defecation, rumination, and kick the milking cluster off). Cows' behaviors within respiration chambers were also recorded for each individual kept singly. Digestibility and calorimetry trials were performed to obtain energy partitioning and CH_4 measures. Cows with more reactive temperament in milking (the ones that kicked the milking cluster off more frequently) spent 25.24% less net energy on lactation ($p = 0.04$)

and emitted 36.77% more enteric CH_4/kg of milk ($p = 0.03$). Furthermore, cows that showed a higher frequency of rumination at milking parlor allocated 57.93% more net energy for milk production ($p < 0.01$), spent 50.00% more metabolizable energy for milk production ($p < 0.01$) and 37.10% less CH_4/kg of milk ($p = 0.04$). Regarding the handling temperament, most reactive cows according to flight speed, lost 29.16% less energy as urine ($p = 0.05$) and tended to have 14.30% more enteric CH_4 production ($p = 0.08$), as well as cows with a lower entrance time (most reactive), that also lost 13.29% more energy as enteric CH_4 ($p = 0.04$). Temperament and restless behavior of Holstein x Gyr cows were related to metabolic efficiency and enteric CH_4 emissions. Cows' reactivity and rumination in the milking parlor, in addition to flight speed and entrance time in the squeeze chute during handling in the corral, could be useful measures to predict animals more prone to metabolic inefficiency, which could negatively affect the sustainability of dairy systems.

Keywords: behavioral reactivity, greenhouse gas-GHG, Holstein x Gyr crossed cows, one welfare, sustainable dairy production

[1] Artigo publicado na revista Animal, v. 15, p. 100224, 2021 (anexo 2)
https://doi:10.1016/j.animal.2021.100224

1. Introduction

Sustainable livestock production has been a theme of debates in the international scene, raising new challenges for the stakeholders of farm animal production chains (van Dijk et al., 2019). Public opinion has shown an increasing interest in the acquisition of high-quality animal products. It includes the requirement of information about the products' origin and the productive processes, comprising issues related to their impacts on animal welfare and environment (Risius; Hamm, 2017). This is related to a growing global demand for an ethical and sustainable way to develop the economic activities, including the livestock production. The concept of "One Welfare" seems to be a useful guide to achieve this since it proposes that the activities that affect (positively or negatively) animal welfare, human wellbeing, biodiversity, and environmental conservation are closely connected and are mutually dependent of each other (García et al., 2016; Tarazona et al., 2019).

In this context, one of the challenges is the efficient use of resources and the mitigation of greenhouse gas (GHG) emissions by livestock (Herrero et al., 2016). Enteric methane (CH_4) is one of the GHG produced during the digestive process of ruminants by the action of anaerobic microorganisms that colonize the rumen, through fermentation of plant carbohydrate (Beauchemin et al., 2008). In Brazil, estimates pointed out that ruminants' enteric fermentation was responsible for 11 352 (t) of methane produced in 2017, and the dairy industry contributed with 0.33 L of methane/kg of milk in the country (SEEG, 2018).

There is a variation in the amount of CH_4 emission by ruminants; thus, it is important to understand which factors affect the enteric CH_4 production by these animals. For example, quality of the diet (Cottle et al., 2011), level of dry matter intake (Dini et al., 2019), environmental temperature (Yadav et al., 2016) were reported to be associated with CH_4 emissions. Thus, some possible alternatives for CH_4 mitigation have been investigated, most of them including nutritional strategies (Haque, 2018), besides other alternatives, such as intensification of productive system (de Vries et al., 2015). Despite considerable recent progress in the nutritional field, several other factors related to animal physiology may contribute to their bioenergetic efficiency and reduction of greenhouse gas emissions (Ornelas et al., 2019), which still deserve to be better understood.

There is some evidence showing that physiological and behavioral responses to stress might be associated with a higher enteric CH_4 production (Yadav et al., 2016; Llonch et al., 2018) and lower productivity in dairy cows (Hedlund; Løvlie, 2015). The emissions of enteric CH_4 represent an environmental concern and a source of energetic efficiency reduction due to the loss of gross energy as CH_4 (Johnson; Johnson, 1995). The energy released as CH_4 gas could be allocated for weight gain (in beef cattle) and milk yield (in dairy cattle), ranging from 2% to 12% of the animals' energy intake, depending on the type of diet (Johnson; Johnson, 1995). Thus, strategies for enteric CH_4 mitigation should result in environmental and economic gains, optimizing the use of nutrients.

Temperament had been defined as individual differences in animals' behavioral responses to stressors (Fordyce et al., 1982; Koolhaas et al., 2010). Previous studies have shown that 'nervous' and restless cows produce less milk (Sutherland; Dowling, 2014; Hedlund; Løvlie, 2015); however, the metabolic mechanisms underlying this relationship are poorly understood. One could expect that animals with divergent temperaments would differ in their efficiency to convert the feed energy into milk, i.e, reactive cows could be less efficient than the reactive ones. Thus, cattle temperament could affect the energetic partition, decreasing the energy to milk yield. If reactive cows, in fact, lose a higher percentage of energy through feces, urine, heat production, and CH_4, the temperamental animals may show a more significant impact on the sustainability of the dairy industry. However, these hypotheses still lack empirical support for dairy animals, remaining unknown whether animals with more reactive temperament and restless behavior produce more CH_4 (Llonch et al., 2016) and are less bioenergetically efficient than the calmer ones.

Therefore, the aims of this study were a) to evaluate the relationships between cattle temperament assessed by traditionally used tests with energetic metabolism and enteric CH_4 emission by Holstein x Gyr dairy cows; b) to assess how cows' restlessness in the respiration chambers affects energetic metabolism and enteric CH_4 emissions. We hypothesize that individuals with a more reactive temperament and restless in a situation of physical restraint would be metabolically and bioenergetically less efficient than the calmer ones, showing higher enteric CH_4 emission.

2. Material and methods

2.1 Animals and housing conditions

Data was collected from April to November 2017, at the Multiuse Livestock Complex of Bioefficiency and Sustainability of the Brazilian Agricultural Research Corporation, Embrapa Dairy Cattle (Coronel Pacheco, Minas Gerais, Brazil), with 28 primiparous F1 Holstein x Gyr lactating cows, aging 30 ± 1.04 years (mean ± SD) and weighing 568 ± 41.50 kg. Cows were kept in a free stall barn equipped with electronic feeding system (AF-1000 Master Gate, Intergado Ltd., Contagem, MG, Brasil) and water troughs (WD-1000, Intergado Ltd., Contagem, Minas Gerais, Brazil). Twice a day cows were milked in a fishbone milking parlor (2×4) (DeLaval, Tumba, Sweden), always by the same two stockpearsons. More details about the animals and facilities were previously published in Marçal-Pedroza et al. (2020), that it is part of the same study. Individual daily milk yield data was recorded automatically on the days of the behavioral observations.

2.2 Temperament assessment

The cows' temperament was measured based on the cows' behavioral responses to being handled by humans, assessed during milking (i.e., milking temperament) and during handling in the corral (handling temperament). The temperament data used come from data collected in a previous study (Marçal-Pedroza et al., 2020). The milking temperament of the lactating cows was evaluated 45 days after calving, and the subsequent sessions with an average interval of 45 days, performing three sessions along the early lactation period. In each session, data collection was made on three consecutive days, always in the morning milking (a total of nine days of assessment). The following behavioral indicators of cattle temperament were recorded by a previously trained observer, as described in Marçal-Pedroza et al. (2020): number of Steps (STEPS), number of Kicks (KICKS) and the occurrences of behaviors defecation, rumination, and kick the milking cluster off (KOFF), from the time that the milking cluster was attached until its extraction when milking was finished.

The handling temperament was assessed on the last day of each milking evaluation session, in a total of three evaluations in the corral. The following measures were used: Entrance Time (in s), Crush Score, Flight Speed (in m/s), Flight Distance (in m). For the full description of the temperament methods used please see Marçal-Pedroza et al. (2020).

2.3 Whole tract digestibility and Energy partitioning

The digestibility assays took place every 45 days throughout all lactation, for a total of six sampling periods. For the digestibility assays, groups of 9 cows were transferred to a tie-stall system with individual feeders and water troughs. Individual samplings of feces were collected for five days per group. Total urine was collected on the first two days of the fecal collection. Aliquots of silage, concentrate, and orts were daily collected along the five consecutive days and stored at -20 °C.

For the calculation of the energy partition, the gross energy intake (GEI), daily fecal (Fecal-E, Mcal/d) and urinary (Urine-E, Mcal/d) energy outputs were obtained by multiplying dry matter intake (DMI) and fecal and urinary dry matter (DM) excretion with their respective energy contents. Digestible energy intake (DEI, Mcal/d) was calculated as the difference between GEI and fecal energy excretion. Metabolizable energy intake (MEI, Mcal/d) was derived as the difference between DEI and the sum of urine energy and CH_4 energy (CH_4-E, Mcal/d), which was assumed to be 45 Kcal/L (Brower, 1965). Energy retention was calculated as the difference between MEI and heat production (HP). Heat production (Kcal/d) was determined based on measurements of O_2 consumption (L/d), CO_2, and CH_4 production (L/d), using the equation of Brower (1965). The net energy of lactation (NEL) was also obtained based on the feed energy available for milk production after digestive and metabolic losses (in Mcal/kg). The additional measures were also used in the analyses: metabolizable energy/digestible energy (MEI/DEI), metabolizable energy/gross energy (MEI/GEI), energy balance (EB), and milk energy/metabolizable energy (Milk-energy/MEI). These methods were described in Ornelas et al. (2019), carried out under the same conditions and installations of our study.

2.4 Respiration measurements

The open-circuit respiration chambers (n = 4) were used to measure gas exchanges. The full description of the chambers system used, and its validation was previously published in Machado et al. (2016). Briefly, the net volume of each chamber is 21.10 m^3, containing a 2.26 × 1.26 m pen. The chambers have large, double-glazed windows (150 cm high, 150 cm wide) to guarantee visual communication between the animals. Each chamber is fitted with one large back door for animal access and a smaller front door for operator access and feeding. The common gas analysis and data acquisition system were shared by the four chambers (Sable Systems International, Las Vegas, USA). Infrared technology was used to analyze CO_2 and CH_4 concentrations, whereas fuel cell technology was used for O_2. The injection of known volumes of CO_2 and CH_4 in each chamber was used to perform the recovery test of the whole system, using a mass flowmeter (MC-50SLPM-D, Alicat Scientific Inc., Tucson, AZ). The average recovery of the four chambers for CO_2 (mean ± SD) was 87.87 ± 0.04% and for CH_4 was 84.75 ± 0.07% (Figure 1).

Figure 1: (A) Overview of the respiration chambers; (B) view from inside the chamber with the food and water boxes; (C) animal inside the respiration chamber (Machado et al., 2016)

The animals were halter-trained, adapted to handling and to respiration chambers before the trial began. Six sessions of two-days of respiration measurements in chambers were done, performing a total of 12 days of evaluation per cow. The respiration chambers evaluation began on the 45th day after calving with a 45-days interval between sessions, for each four cows at a time, as there were only four respiration chambers available. The sessions started immediately after morning feeding at 9:00 a.m. The respiration indirect calorimetry reading was initiated, and gas exchanges were measured during 21 to 23 hours, with an extrapolation of 24 hours. The animals were randomly allocated to each chamber where they remained singly and then confined for 48 hours, leaving only for milking (morning and afternoon).

Data acquisition and analysis software (Expedata Data Analysis Software 1.8.5, version PRO, Sable Systems International) was used to calculate the consumption of O_2, CO_2, and CH_4 production (L/day). Individual enteric CH_4 production (g/day), CH_4 yield (g/kg DMI), and CH_4 intensity (g/kg milk) were calculated. Inside the chambers, there was a feeding and watering trough, and a video camera that recorded the behaviors of the animals throughout the experimental period.

2.5 Behavior within the respiration chambers

For the record of behavior, the videos (seven hours per cow, on average, performing a total of 196 h of video footages) captured by video cameras (VM 310 IR, an infrared camera from Intelbras S / A - Brazilian Electronic Telecommunications Industry, Manaus

/ AM, Brazil) between the two daily milking procedures at the first day of respiration chamber confinement were used. The videos of each one of the 28 cows were observed using focal-animal sampling and instantaneous sampling, with one-minute intervals to register. The following behavioral categories were used as measures of cows' restlessness in the respiration chambers: lying, feeding, ruminating in the chamber, shaking ears, shaking the head, moving and being inactive, considering the time spent in each behavior, expressed in relative frequencies (%). A continuous recording was used to register the occurrences of steps, vocalization, and turning the head, expressed as number of occurrences. The behaviors are described in the ethogram (Table 1).

Table 1. Description of behaviors recorded inside the respiration chambers

Behavior	Description
Lying	Prone / lateral decubitus leaning on the floor or supported on the paws.
Feeding	Eating feed from trough or head over trough and exhibiting chewing behavior.
Ruminating	Animal far from the trough, standing or lying, regurgitating, chewing or swallowing food.
Shaking ears	Movement of the ears when the animal is not eating or chewing the cud.
Shaking the head	Head movement without the animal feeding or ruminating.
Moving	Animal standing, moving its paws.
Inactive	Animal standing still, not even moving its head and ears.
Steps	The animal removes one of the front or hind legs from the ground.
Vocalization	The animal moos, demonstrated by the lowering of the head and neck and opening of the mouth, together with the apparent contraction of the abdomen.
Turning the head	The animal tries to turn the head and neck towards the abdomen, with an angle equal to or greater than 90º, to one side of the body.

2.6 Statistical analysis

First, to analyze the temperament indicators and energetic metabolism variables, a single individual measurement was obtained for each indicator, through the average of the sessions carried out throughout the study.

To assess the effects of temperament and behaviors in the chambers on the energetic metabolism and CH_4 emission measures, linear mixed models for longitudinal data were fitted by using PROC MIXED of SAS (version 9.2, SAS Institute Inc., Cary, NC). Models included the dependent variables of energetic metabolism (Fecal-E, Urine-E, CH_4-E, Heat-E, MEI/DEI, MEI/GEI, Milk-energy/MEI, NEL, EB) and CH_4 emissions measures (production, yield, and intensity). Fixed effects of temperament and behavioral measures (one measure at a time), evaluation session, and their interactions, in addition to milking group were included. The random effect of animal (subject) was considered as a repeated measure within the evaluation session. In all analyses, means were compared using post-hoc Tukey Test, and *P*-values were assumed as significant when < 0.05 and as a trend when < 0.10.

For inclusion in the mixed models as fixed effects, the handling temperament, milking temperament indicators, and behavioral measures were categorized into three scores (low, average, and high). Most of the variables were classified based on the terciles of distribution (low = fist tercile, intermediate = second tercile, and high = third tercile), except by Entrance Time and Flight Distance, which were classified based on threshold values, as follows: Entrance Time ('low' = 0 to 9.9 s; 'intermediate' = 10 to 20 s; 'high' = over 20 s); Flight Distance ('low' = 0 cm; 'intermediate' = 0.1 to 0.99 cm; and 'high' = over 1 m). Finally, the behaviors Defecation, Rumination, KOFF that were binomial variables (occurs or not) were classified based on the number of occurrences across the 3-days session: 'low' = 0 occurrence; 'intermediate' = 1 occurrence; and 'high' = 2 or 3 occurrences. Behavioral measures in the respiration chambers (steps in the chamber, turning the head, lying, feeding, ruminating in the chamber, ear shaking, head shaking, vocation, and being inactive) were also categorized in terciles.

3. Results

3.1 Effects of temperament indicators on energetic metabolism and CH_4 emissions

Regarding the effects of the milking temperament indicators, the number of STEPS showed a significant effect on Urine-E ($p = 0.02$), MEI/DEI ($p = 0.03$) and a tendency on DMI ($p = 0.06$) and GEI ($p = 0.07$) (Table 3). Similarly, a tendency for number of KICKS was found on CH_4-E ($p = 0.07$), CH_4 production ($p = 0.09$) and Heat-E ($p = 0.09$) (Table 2). Cows classified as intermediate for STEPS-$_{Inter}$ had 26.96% lower loss of energy as urine, 2.35% higher MEI/DEI rate, and 8.98% higher gross energy intake than those classified as STEPS-$_{Low}$. Either the cows defined as intermediate for KICKS-$_{Inter}$ tended to show reduced losses of energy as CH_4-E, as Heat-E, and lower CH_4 production (differences of 9.19%, 7.24%, and 9.93%, respectively) than those defined as KICKS-$_{Low}$ (Table 2).

Table 2. Adjusted means (± SE) of energetic metabolism and CH4 emissions measures for each temperament indicators (n = 28)

Dependent Variables[1]	Low	Intermediate	High	$F_{2,23}$	*P-value*
Handling Temperament Indicators					
		FS (m/s)			
Urine-E (Mcal/d)	5.04 ± 0.38[a]	4.27 ± 0.27 [ab]	3.57 ± 0.40[b]	3.52	0.05
CH_4 Production (g/d)	229.31 ± 11.40[b]	261.43 ± 8.28 [a]	262.10 ± 12.04[a]	2.88	0.08
		ET (s)			
Urine-E (Mcal/d)	3.95 ± 0.32[b]	4.27 ± 0.30[b]	5.34 ± 0.49[a]	2.86	0.08
CH_4-E (Mcal/d)	5.34 ± 0.14[a]	5.08 ± 0.13[a]	4.63 ± 0.22[b]	3.73	0.04
Milking Temperament Indicators					
		KOFF			
NEL (Mcal/d)	12.68 ± 0.77[a]	14.37 ± 1.27[a]	9.48 ± 1.33[b]	3.67	0.04
CH_4 Intensity (g/Kg milk)	19.17 ± 1.63[b]	15.49 ± 2.69[b]	26.22 ± 2.83[a]	3.92	0.03
		RUMI			
NEL (Mcal/d)	9.51 ± 1.07[c]	12.41 ± 0.78[b]	15.02 ± 0.99[a]	7.19	< 0.01
Milk-energy/MEI	0.14 ± 0.01[c]	0.17 ± 0.01[b]	0.21 ± 0.01[a]	8.17	< 0.01
CH_4 Intensity (g/kg milk)	25.39 ± 2.54[a]	19.07 ± 1.83[b]	15.97 ± 2.35[b]	3.83	0.04
		KICKS			
CH_4-E (Mcal/d)	5.33 ± 0.15[a]	4.84 ± 0.15[b]	5.30 ± 0.21[ab]	2.98	0.07
Heat-E (Mcal/d)	34.11 ± 0.83[a]	31.64 ± 0.80[b]	32.00 ± 1.16 [ab]	2.65	0.09
CH_4 Production (g/d)	261.54 ± 9.93[a]	235.57 ± 9.49 [b]	268.68 ± 13.58[a]	2.68	0.09
		STEPS			
DMI (Kg/d)	14.93 ± 0.39[b]	16.29 ± 0.41[a]	15.97 ± 0.52[ab]	3.09	0.06
GEI (Mcal/d)	66.24 ± 1.71[b]	72.19 ± 1.83[a]	70.78 ± 2.28[ab]	3.04	0.07
Urine-E (Mcal/d)	4.97 ± 0.30[a]	3.63 ± 0.32[b]	4.29 ± 0.40[ab]	4.47	0.02
MEI/DEI	0.85 ± 0.01[b]	0.87 ± 0.01[a]	0.86 ± 0.01[ab]	3.94	0.03

[1] FS = Flight Speed (m/s), ET = Entrance time (s), KOFF = kick off the milking cluster, RUMI = rumination, KICKS = number of Kicks, STEPS = number of Steps, Urine-E = % urine energy, CH_4-E = % methane energy, NEL = Net energy of lactation, Milkenergy/MEI = milk energy/EM intake, CH_4 intensity = methane emission, Heat-E = % heat energy, DMI = dry matter intake, GEI = gross energy intake, MEI/DEI = metabolizable energy/digestible energy.
[a - c] Adjusted means without a common letter differ statistically from each other (Tukey test. P < 0.10).

The milking behaviors of rumination and kicking the milking cluster off affected NEL ($p < 0.01$, $p = 0.04$, respectively) and CH_4 intensity ($p = 0.04$, $p = 0.03$), in addition to a significant effect of rumination on Milk-energy/MEI ($p < 0.01$) (Table 2). Cows that kicked the milking cluster off more frequently (KOFF-$_{\text{High}}$) and ruminated less frequently (RUMINATION-$_{\text{Low}}$) allocated less net energy on lactation (differences of 25.24%, 57.93%, respectively) and more CH_4 intensity (36.77%, 37.10%, respectively) per liter of milk than cow classified as KOFF-$_{\text{Low}}$ and RUMINATION-$_{\text{High}}$, respectively. The animals classified RUMINATION-$_{\text{High}}$ had 50.00% greater Milk-energy/MEI than cow classified as RUMINATION-$_{\text{Low}}$.

Concerning to cows' temperament in the handling corral, Flight Speed showed a significant effect on Urine-E (p = 0.05) and a tendency on CH_4 production (p = 0.08) (Table 3). Additionally, Entrance Time affected CH_4-E (p = 0.04) and also showed a tendency on Urine-E (p = 0.08). Cows classified as Flight Speed-$_{High}$ tended to lose 29.16% less energy as Urine-E and 14.29% more CH_4 production than Flight Speed-$_{Low}$. Cows with Entrance Time-$_{High}$ showed 35.18% more energy loss as Urine-E and 13.29% less energy loss as CH_4 -E than cows with Entrance Time-$_{Low.}$

3.2 Effects of behaviors in the respiration chambers on the energetic metabolism and CH_4 emissions

The cows' behavior within the respiration chambers during the respiration assay affected some measures of energetic metabolism (Table 3). Cows that spent less time being inactive showed 2.35% less MEI/DEI (p = 0.04), and higher frequency of vocalizations was related to 6.61% more of energy loss as CH_4 (lower CH_4-E) (p = 0.03). Finally, cows that took more steps in the chamber showed a tendency of reduction of 5.65% in NEL (p = 0.10) and an increase of 12.95% in CH_4 intensity (p = 0.09) (Table 3).

Table 3. Adjusted means (± SE) of energetic metabolism and CH4 emissions measures for each behavior within the respiration chambers (n = 28)

Dependent Variables[1]	**Low**	**Intermediate**	**High**	$F_{2,50}$	***P-value***
		Steps			
NEL (Mcal/d)	12.74 ± 0.66[a]	12.39 ± 0.68[ab]	12.02 ± 0.67[b]	2.42	0.10
CH_4 Intensity (g/Kg milk)	18.37 ± 1.53[b]	20.50 ± 1.58[a]	20.75 ± 1.53[a]	2.60	0.09
		Vocalization			
CH_4-E (Mcal/d)	4.84 ± 0.14[b]	5.27 ± 0.12[a]	5.16 ± 0.14[a]	3.83	0.03
		Inactive			
MEI/DEI	0.85 ± 0.006[b]	0.86 ± 0.005[a]	0.87 ± 0.006[a]	3.38	0.04

[1] NEL= net energy of lactation, CH_4-E= % methane energy, MEI/DEI= metabolizable energy intake/digestible energy intake.
[a - b] Adjusted means without a common letter differ statistically from each other (Tukey test. P < 0.10).

4. Discussion

The objectives of the present study were to evaluate the effects of temperament and behavior in respiration chambers of dairy cows on energy metabolism and enteric methane emissions. Cows' temperament and behaviors in the chambers influenced energy metabolism and methane emissions, with more reactive cows allocating less energy for lactation and emitting more methane per liter of milk produced compared to calmer animals. In addition, cows with an intermediate temperament measured by steps and kicks in the milking parlor lost less energy as urine, heat and CH_4 and also produced less methane per day, compared to reactive cows.

4.1 Effects of temperament indicators on energetic metabolism and CH_4 emissions

Animals with temperament categorized as ‘intermediate’ for STEPS and KICKS lost less energy in the form of urine and had higher rates of MEI / DE, besides presenting a tendency to produce less CH_4 and lower loss of energy as heat and CH_4. The number of leg movements has been considered a valid indicator of cows' reactivity in the milking parlor, with less reactive cows taking lower numbers of steps (Hemsworth, 2003). Nevertheless, Munksgaard et al. (2001) have observed that when some cows are kept under situations of tension and stress, they might have an opposite reaction, remaining immobile during milking. Under such perspective, it would be plausible that cows that took a few steps (as for cows in the 'intermediate' score) could be more relaxed than those that remained immobile (cows in 'low' score). Cows classified as intermediate for numbers of STEPS and KICKS showed higher DMI and could be considered more efficient as well, given the reduced losses of energy as Urine-E and CH_4-E, and lower CH_4 production. In a previous study conducted with the same animals of the present during the raising period, Ornelas et al. (2019) found a negative correlation between DMI and CH_4 production. Cows with a higher feed intake are more efficient if the metabolizable energy that exceeds maintenance are retained, associated with reduced losses of energy as urine, heat, and CH_4 (Chaokaur et al., 2014). It could explain the higher DMI in addition to lower loss of energy as urine, heat, CH_4, and higher MEI / DEI rate in cows classifies as 'intermediate' for STEPS and KICKS, that could be considered more efficient.

Cows that were more reactive in the milking ($KOFF\text{-}_{High}$) and ruminated less in the milking parlor ($RUMINATION\text{-}_{Low}$) were less efficient, allocating less net energy to milk production. Kicking the milking cluster off indicates cows' reactivity related to discomfort and emotional state of agitation (Marçal–Pedroza et al., 2020). Similarly, rumination was related to emotional states of relaxation, while its reduction could reflect tension and stress (Manteca et al., 2013). A previous study of our research group has shown that cows ruminating more frequently in the milking parlor produced 17.26% more milk than those with a lower frequency of rumination (17.59 vs. 15.00 kg/day) (Marçal–Pedroza et al., 2020). Based on the results of the present study, it is possible to infer that the increased production for more ruminating cows derives, in parts, from their better performance in allocating energy for milk production associated with lower losses as methane. This result might reveal the implications of cows milking behaviors for the sustainability of milk production.

Cows' reactive temperament in the handling had also influenced the energy metabolism and methane emissions, with cows exiting the squeeze faster (Fligth Speed-$_{High}$) showed less energy in the urine and more CH_4 production, while the animals that entered faster (Entrance Time-$_{Low}$) lost less energy as urine and produced more CH_4-E. It is worth to remember that the most reactive cows showed Flight Speed-$_{High}$ (in m/s) and Entrance Time-$_{Low}$ (in s), since they spent less time to enter into the squeeze and exit faster (high speed); thus, these measures were inversely correlated. Cows that entered and exited the squeeze chute faster (characterizing states of fear and agitation) tended to show higher losses of energy as CH_4-E and enteric CH_4 production. The flight speed and entrance time reflect an innate tendency of general fearfulness and high behavioral reactivity, revealing a susceptibility to stress in temperamental cows (the faster ones) (Cafe et al., 2011). The emotional state of fear has implications on the physiological control of metabolism, being a potential psychological stressor that leads to a higher activation of the hypothalamic-pituitary-adrenal (HPA) axis, resulting in the release of glucocorticoids (Hemsworth, 2003). A relationship between reactive temperament (measured by flight speed) and susceptibility to stress was previously shown in several studies (Cafe et al., 2011). Reactive temperaments in cattle (high flight speed and crush score) were related to a more prolonged and more intense activation of HPA axis and sympatho-adrenomedullary system in responses to stress (Cafe et al., 2011). Both axes are involved in the control of catabolism, energetic homeostasis, energy balance, and storage of energy in the body. At the best of our knowledge, the present study is the first to assess the relationships between temperament, energy partitioning, and CH_4 emissions in cattle. In the study by Llonch et al. (2016), the authors investigated the relationships between beef cattle temperament (measured by flight speed and crush score), cortisol levels following transportation and methane emissions. In spite of those authors did not find a relationship of flight speed and crush score with methane emissions, they reported a positive association between cortisol following transport and CH_4 yield (g / kg CMS).Thus, the present study contributes to the scarce evidence that characteristics intrinsic to the behavior of ruminants, such as temperament, emotional states, and intensity of behavioral and physiological responses to stressors, should be taken into account in the development of alternatives to mitigate enteric CH_4 by cattle (Llonch et al., 2016, present study).

4.2 Effects of behaviors in the respiration chambers on the energetic metabolism and CH_4 emissions

The behavior of cows in respiration chambers affected energy metabolism and methane emissions. Cows expressing behaviors indicative of restlessness (less time inactive, vocalized more and took more steps) had lower rates of MEI / DEI and lost more energy as CH_4, and tended to allocate less NEL and more CH_4 intensity. For confined beef cattle, Llonch et al. (2018) showed that higher level of activity in the home pens (measured as number of steps per day) was related to lower feed efficiency (poorer residual feed intake), what the authors attributed to the higher energy expenditure for muscle activity in more active individuals. Additionally, in beef cattle, efficient animals show lower maintenance requirements as well as better usage of metabolizable energy for growth (Cantalapiedra-Hijar et al., 2018). These results might explain the lower MEI / DEI and lower NEL in cows that took more steps, that probably were less efficient.

Vocalizations and steps in situations involving physical restraint can be used as indicators of cows' restlessness since confinement and social isolation are stressors for social animals (Llonch et al., 2018). Restless cows might lose more energy as CH_4-E, allocating less energy for milk yield, in parts, due to more intense physiological responses to stress in these animals. Stress responses are detrimental for efficiency in energy use, leading to reduced productivity and the rise of enteric CH_4 emissions (Hedlund and Løvlie, 2015; Llonch et al., 2018). On the other hand, calmer and relaxed cows might have the potential to be more productive and efficient in energy partitioning and use, along with CH_4 intensity reduction per unity of product (Yan et al., 2010).

Our study has some limitations that have to be taken into account. First, the measures of metabolism and methane emissions were taken in potentially stressful situations. The tie stall and respiratory chambers involve physical restraint and reduced social interactions, in spite of the visual contacts were maintained. All the cows were exposed to the same experimental conditions when they were heifers (Ornelas et al., 2019) and were previously habituated to the experimental settings prior to our trials during lactation period of this study. The feed intake was monitored to do not exceed 5% compared to feed intake in the free stall, as a measure of behavioral changes in tie stall and chambers. Thus, we expect that all the cows were adapted to the conditions of this study, leading us to consider our results valid, even so, caution is required when extrapolating our findings to non-experimental or commercial conditions. A second limitation was the lack of ruminal microbiome community assessment in our study. It is known that the ruminal microbiome composition plays an important role in cows' feed efficiency, cows' energy utilization and methane emissions (Difford et al., 2018; Schären et al., 2018) and have could affect our results.

In summary, reactive temperament, stress, and welfare problems potentially cause additional energy expenditure for animals to cope with such situations. Beyond the economic losses caused by the inefficient use of feeding resources and reduced milk yield, the reactive temperaments of cattle might cause concerns related to the risks of accidents and deteriorate the labor conditions in dairy farms (Hemsworth et al., 2003; Sutherland; Huddart, 2012). Finally, this study has shown that environmental consequences might arise from the increasing CH_4 emissions for temperamental cattle. All these factors are integrated within the perspective of 'One Welfare' (García et al., 2016; Tarazona et l., 2019). Thus, we recommend the improvement of temperament throughout animal breeding and good practices of cattle handling as viable strategies for attaining a more sustainable dairy production.

5. Conclusion

Cattle temperament assessed during milking and in the handling corral, in addition to cows' behaviors within the respiration chambers, were related to energy partitioning and CH_4 emissions by crossbred dairy cows under the experimental conditions of the present study. Animals classified as more reactive allocated less energy for lactation and emitted more enteric CH_4 per unity of product. All those impacts of reactive temperaments are undesirable for an efficient and sustainable livestock activity. A selection of calmer cows and the adoption of good practices of cattle handling could favor the welfare of cows, stockpeople, and the environment.

Acknowledgements

This study is part of the doctoral thesis of the first author prepared for the Graduate Program in Biodiversity and Nature Conservation of the Federal University of Juiz de Fora (UFJF), Brazil.

Financial support statement

This work was supported by in part by the Fundação de Amparo à Pesquisa do Estado de Minas Gerais – Brasil (Fapemig) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Proc. # 409059/2016-1).

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Chapter 3 - Is the temperament of crossbred dairy cows related to milk cortisol and oxytocin concentrations, milk yield, and quality?[1]

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Abstract

Reactive dairy cows are more susceptible to stress, and this may result in negative effects on milk yield and quality. The aims of this study were to investigate the relationships between temperament traits and concentration of milk cortisol and oxytocin, milk yield, milkability, and milk quality in Holstein x Gyr cows. Temperament traits were assessed in 76 Holstein x Gyr cows in the milking parlor (by scoring milking reactivity and recording the numbers of steps and kicks during pre-milking udder preparation and when fitting the milking cluster) and during handling in the corral (by measuring the time to enter in the squeeze chute, ET and flight speed, FS). Milk samples were collected for milk quality (% fat, % protein, % lactose, and somatic cell count, SCC), and milk cortisol and oxytocin. Milk yield, milking time, and average flow were also measured. The calmer cows during milking management (class 'low') produced milk with higher protein (p = 0.028) content and tendencies for lower fat (p = 0.056) and higher lactose (p = 0.055) contents. Regarding the hormones, the most reactive cows (class 'high') in the milking and handling corral produced milk with higher concentrations of cortisol (p < 0.001) and oxytocin (p = 0.023). In addition, the temperament of the animals affected some of the productive measures evaluated. Cows with reactive temperament had lower milk flow and longer milking time than the intermediate ones and had higher fat and a tendency for lower protein percentage in milk compared to cows with intermediate temperaments. Calm and intermediate cows in the handling corral produced more milk and presented better milkability parameters, such as a shorter milking time and greater average milk flow. Our results suggest that the cows' behavioral reactivity can be related to the intensity of their response to stress during handling.

Keywords: Holstein x Gyr, Lactation hormones, Milkability, Milk quality, Personality

[1] Artigo publicado na revista **PLOS ONE**, v. 18, p. e0286466, 2023 (anexo 3).
https://doi.org/10.1371/journal.pone.0286466

1. Introduction

Bovines, like other animals, present individual differences in behavior when exposed to challenging situations, and these behavioral differences are often described as temperament (Reále et al., 2007). Temperament is expressed through a set of behavioral and physiological responses as a strategy to adapt to stressful situations in the environment (Koolhaas et al., 2010). However, most studies recognize that the characterization of temperament is complex since it can consider various traits, such as coping style, emotionality, and sociability (Reále et al., 2007; Koolhaas; Reenen, 2016).

Studies have shown the importance of cattle temperament in livestock husbandry. Some studies have reported that calmer and more docile dairy cows in the milking parlor (milking temperament) produced greater milk yield (Breuer et al., 2000; Hedlund; L¢vlie, 2015; Marçal-Pedroza et al., 2020; Neave et al., 2022) , while others have found opposite results (Gergovska et al., 2012; Sawa et al., 2017) or did not find any association between milking temperament and milk yield (Sutherland et al. 2012; Sutherland; Huddart, 2012), showing a lack of consistency among results. It is important to highlight that these articles used different methods to assess milking temperament. Hedlund and Løvlie (2015); Marçal-Pedroza et al. (2020); and Neave et al. (2022) used the number of steps and kicks as measures of reactivity during milking procedure. Breuer et al. (2000); Sutherland et al. (2012); Sutherland and Huddart (2012) measured reactivity based on the intensity of leg movements, whereas Gergovska et al. (2012, 2014) and Sawa et al. (2017) assigned subjective temperament scores.

Additionally, there is a lack of studies assessing the relationship between cows' temperament, milk quality (Gergavska et al., 2014), and milkability parameters (Shehar et al., 2015a, 2015b). Some of these studies have indicated that calmer animals produced milk with greater contents of fat and protein (Antanaitis et al., 2021; Kruszyński et al., 2013), while others showed contrasting results, with the most reactive cows showing higher percentages of fat in the milk (Cziszter et al., 2016). It has also been reported that calmer cows had better milkability parameters, such as greater milk flow and lower milking time (Shehar et al., 2015a, 2015b). Considering the small number of studies addressing these issues and the divergent results, more research is needed to clarify the underlying behavioral and physiological factors affecting the relationship between temperament and productivity of dairy cows. All these cited studies used reactivity scores in the milking parlor to measure the milking temperament.

It is of particular interest to assess the temperament of dairy cattle breeds known for expressing a more reactive temperament, reacting more intensely and with greater agitation to the handling procedures (Cerqueira et al., 2017). Among them, we highlight the dairy Gyr cattle (Negrão, 2008) which are widely used for crossbreeding in tropical countries, like Brazil, where around 80% of the dairy herd are Holstein x Gyr crossbred cows (Canaza-Caio et al., 2016). Under such conditions, it is expected that the crossed dairy cows with a greater Zebu breed composition will be more reactive to milking management, which may result in negative effects on milk yield and quality. Along with a higher cortisol concentration, a reduction in plasma oxytocin concentration is also expected (Bruckmaier; Blum, 1998), which is responsible for milk ejection and maintenance of lactation (Bruckmaier, 2005). Few studies have investigated the relationship between oxytocin concentration and the temperament of dairy cows and they have found contradictory results. Sutherland and Tops (2014) showed that Zebu crossbred cows displaying higher levels of agitation (measured by a reactivity score during the milking cluster attachment) in a new milking environment tended to present a greater concentration of blood oxytocin, but Sutherland et al. (2012) did not find any association between reactivity in the milking parlor and the concentration of plasmatic oxytocin.

Thus, this study aimed to investigate the relationships between temperament traits and concentration of milk cortisol and oxytocin, milk yield, milkability, and milk quality in Holstein x Gyr cows. We hypothesized that more reactive cows in the milking parlor (with higher reactivity scores, more steps, and kicks) and in the handling corral (entered and exited the squeeze chute faster) would have higher concentrations of milk cortisol, oxytocin, and produce less milk with lower quality.

2. Material and methods

This study is in accordance with the ethical principles of animal experimentation and was approved by the Embrapa Dairy Cattle Animal Care and Use Committee, Juiz de Fora, MG, Brazil (Protocol n. 5201240417).

2.1 Animals and handling

The study was carried out in the Campo Experimental da Embrapa Dairy Cattle 'José Henrique Bruschi' (Coronel Pacheco, MG), by evaluating 76 Holstein (H) x Gyr (G) primiparous and multiparous cows with 2.75 ± 1.35 lactations (mean ± SD), average daily milk yield 19.90 ± 6.30 kg, and days in lactation 138.56 ± 91.91 at the beginning of the study. The animals were classified in four breed compositions: $^{3}/_{8}$HG (n = 8); ½HG = F1 HG (n = 25); ¾HG (n = 35) and $^{7}/_{8}$HG (n = 8). Cows were kept on pasture and were milked twice a day in a herringbone milking parlor (2 × 6), beginning at 07.30 a.m. and 03.00 p.m., always by the same milker, who was previously trained in good handling practices.

2.2 *Temperament assessment*

The behavioral responses of all 76 animals were assessed during the handling routines in the milking parlor (milking temperament) and the corral (handling temperament). The milking temperament was assessed during the morning milking for three consecutive days per month from June to August 2018, resulting in nine repeated measurements per cow. Only one milker and one observer were present during the behavioral recordings. The milker prepared each cow individually to be milked, so the observer could record the behavior of each cow in a direct and individualized manner. The reactivity measurements were taken by only one previously trained observer, considering the movement of the hind legs based on the following criteria: a) Reactivity score which is a behavioural-based score of the type and intensity of leg movement, assessed during pre-milking udder preparation (RSprep, from the first contact of the milker with the cow's teats, pre-dipping, evaluation of forestripping milk until the drying of teats) and when fitting the milking cluster (RStca, from the beginning of attachment of the first until the attachment of the last teat cup), by attributing one of the following scores: 1 = hind legs remained immobile throughout the procedure; 2 = one or two slow and gentle movements (hoof elevated at less than 15 cm from the ground) performed with one or both hind legs; 3 = three or more inconstant slow and gentle movements; 4 = constant (most of the observation time) slow and gentle movements; 5 = vigorous (elevating hooves above 15 cm from the ground), but inconstant movements; 6 = constant (most of the observation time) and vigorous movement of the hind limbs; 7 = the cow kick (elevating the hind hoof above hock line and directing it laterally towards the stockperson) and 8 = had to have one or both hind legs tied to be milked; b) Number of STEPS (elevations of the hooves below the hock line): corresponds to the sum of steps the animals took during pre-milking udder preparation and when fitting the milking cluster; c) Number of KICKS (defined as elevations of the hind hoof above hock line and directing it laterally towards the stockperson): corresponds to the sum of kicks during pre-milking udder preparation and during when fitting the milking cluster.

The handling temperament was assessed one day after assessing milking temperament, totalling three recordings throughout the study (one per month). The behavioral recordings were performed by individual observations for each animal by another observer who was unfamiliar with the animals and had experience with handling temperament assessment. Briefly, after the morning milking, the farm workers took the cows to a handling corral close to the milking parlor in a calm manner, according to the good management practices used on the farm. The following measurements were taken: a) Entrance time (ET), by measuring the time (in seconds) that each animal takes to go through the single-file race until entering the squeeze chute. The cow was allowed to move alone for ten seconds, without using any mechanism to encourage it to move. After this interval, those cows who stopped and refused to move forward were encouraged to move using voice command and, if necessary, were gently touched until they entered the squeeze chute (Pajor et al., 2000); and b) Flight speed (FS), by measuring the speed that each cow left the squeeze chute. It was done using equipment (Duboi®, Campo Grande, Brazil) comprised of two pairs of photoelectric cells and a chronometer, one of them fixed just after the exit gate of the squeeze chute and the other 2 m away. When the cow went through, the first pair of cells and the chronometer were activated and were stopped when she went through the second pair. The time interval displayed on the equipment was used to calculate the speed of each cow, in m/s (faster animals were considered the most reactive ones).

2.3 Milk cortisol and oxytocin

The samples used to measure the concentrations of oxytocin and cortisol were collected during the morning milking, simultaneously with the milk collections for milk quality assessment, and on the last day of each milking temperament session (the third day of each monthly assessment). For the cortisol and oxytocin analyses, only ½HG and ¾HG cows were included to reduce the variation due to genetic composition. Among the 60 cows available (½HG, n = 25; ¾HG, n = 35), some had more than 6 lactations, or more than 180 days in lactation, or had clinical signs of mastitis on the days of milking sampling, and therefore were excluded. Thus, a subsample of 38 cows (½HG, n = 19 and ¾HG, n = 19) were assessed for these analyses. Hormones were measured in milk by immunoassay analysis (EIA) using commercial kits according to the manufacturer's instructions (cortisol: Monobind, Lake Forest, CA, EUA; oxytocin: Mybiosource, San Diego, CA, EUA). As hormone concentrations in milk were substantially lower than those measured in plasma, we extracted milk samples. Briefly, we centrifuged the milk sample to separate the fatty and aqueous fractions. Each fraction was lyophilized, and the milk samples were 10-fold less diluted than the plasma samples. Regarding the milk, the intra-assay CVs were 4.8 and 6.5, and the inter-assay CV was 6.0 and 9.0% for cortisol and oxytocin, respectively.

2.4 Productive performance and milkability parameters

The individual daily milk production (kg/day), daily milking time (average of morning and afternoon milkings, in seconds), average milk flow (average of morning and afternoon milkings, in kg/s), and lactation days were manually recorded by the same observer who performed the behavioral observations, one day after performing the milking temperament assessment.

2.5 Milk quality indicators

To assess milk quality (percentage of fat, protein, lactose, and somatic cell count), individual milk samples were collected from all 76 cows, always on the last day of each of the three-monthly data collections in the milking parlor. The milk samples were kept in plastic containers of 50 mL each. The Centesimal Composition Analysis and Somatic Cell Count in Raw Milk Samples tests were performed at Embrapa Dairy Cattle (Juiz de Fora, MG, Brazil). The analyses of fat, protein, and lactose content (% = g/100 g of raw milk) were carried out via absorption spectrometry in a mid-range infrared sensor (ISO 9622 | IDF 141) (Bentley Instruments, Bentley FTS, Id.: 85015); whereas the somatic cell count was performed via Flow cytometry (ISO 13366-2 | IDF 148-2); (Bentley Instruments, SomaCount FCM, Id.: 82015).

2.6 Data analysis

First, a descriptive statistical analysis of the data from each evaluation month was carried out using the UNIVARIATE process of the SAS statistical package (SAS Inst. Inc. Cary. NC, version 9.3). Then, we used the Kolmogorov–Smirnov test to assess whether the distribution of milking temperament measures (RSprep, RStca, STEPS, and KICKS) and handling temperament measures (ET and FS), production and physiology variables met normality. We also checked if the temperament measures differed across the months and between the breed compositions, using linear mixed models for repeated measures, via PROC MIXED of SAS, including each temperament measurement as a dependent variable, and the fixed effects of breed composition ($^3/_8$HG, ½HG, ¾HG, and $^7/_8$HG), month (1 to 3), parity (1, 2, 3, and 4 or more calvings) and the random effect of animal. The temperament measures did not differ between the months of evaluation (P > 0.05 for all). Regarding the breed composition, we found a significant effect for RSprep (p=0.031) and FS (p=0.002), with $^3/_8$HG and $^1/_2$HG cows being more reactive (higher averages for both traits) than the other breed compositions. Parity did not affect any of the temperament measures evaluated (P > 0.05 for all).

To assess the relations of milking temperament with cortisol and oxytocin concentrations, milk yield, milkability parameters, and milk quality parameters, first, we calculated the individual monthly averages of milking temperament measures (RSprep, RStca, numbers of STEPS and KICKS), milk yield, and milkability to eliminate the 'day' effect and obtain a single monthly measure for all of the measures studied (3 repetitions, from June to August). Then we categorize the temperament to include them as fixed effects in the models (classes low, intermediate, and high). The categorization was done based on the tertiles of distribution for the 76 cows within each month (the first tertile was categorized as 'low', the second as 'intermediate', and the third tertile as 'high' for each temperament measure). Considering the low occurrence of KICKS its distribution was considered binomial, so this variable was categorized as "low" = no occurrence of kicks and "high" = 1 or more occurrence of kicks. We did a chi-square test in contingency table to determine if there were differences in the temperament categories distribution between the three months. Non-significant results (P > 0.05) were obtained for all of the temperament measures, showing that the temperament categories distributions did not change across the months.

Finally, linear mixed models were fitted using PROC MIXED of SAS when the residuals attained normality and generalized linear models using PROC GLIMMIX for somatic cell count, adopting lognormal distribution of dependent variable. The models included as dependent variables the concentration of cortisol and oxytocin, average daily milk production (in kg/day), milkability parameters (milking time and milk flow), milk quality (percentages of fat, protein, and lactose, and somatic cell count), and the fixed effects of temperament measurements (one trait included at a time), assessment month (1 to 3), breed composition, parity and days in lactation as covariates with linear effect. In all models, the random animal effect (*SUBJECT*) was considered as a repeated measurement within the evaluation month (1 to 3). In all of the analyses *P*-values ≤ 0.05 were considered as significant and ≤ 0.10 were discussed as trends.

3. Results

3.1 Relationships between temperament and concentrations of milk cortisol and oxytocin

Milk cortisol was related to the milking temperament, assessed by RSprep ($p < 0.001$), RStca ($p < 0.001$), STEPS ($p < 0.001$), and a tendency for KICKS ($p = 0.087$) (Table 1). Cows with a greater reactivity during pre-milking udder preparation (RSprep-$_{High}$) had 95.05% more cortisol in their milk than calmer cows (RSprep-$_{Low}$). Animals classified in the RStca-$_{High}$ had a cortisol concentration 100.09% greater than the cows classified as RStca-$_{Low}$. Cows that took more steps during the milking (STEPS-$_{High}$) had 81.43% more cortisol in their milk than cows with a calm temperament (STEPS-$_{Low}$). Finally, animals that kicked during milking tended to have 28.40% more cortisol in their milk when compared to cows that did not kick. Regarding handling temperament, cows in the FS-$_{Inter}$ category tended to have 36.96% more cortisol than FS-$_{low}$ individuals ($p = 0.088$). These results indicate that reactive cows had a higher concentration of cortisol in milk.

The milking temperament was also related to oxytocin concentration, with significant effects for RStca ($p = 0.023$) and tendencies for the RSprep ($p = 0.083$) and FS ($p = 0.095$) measurements. The RSprep-$_{High}$ cows had 49.5% more oxytocin in milk than RSprep-$_{Low}$ cows (Table 1). The RStca-$_{High}$ cows had 46.9% more oxytocin in milk than RStca-$_{Inter}$ ones. Finally, milk from the animals in the FS-$_{High}$ category had 36.83% more oxytocin than milk from cows in the FS-$_{Low}$ category (Table 1). The ET was not related to milk cortisol and oxytocin concentrations ($P > 0.05$).

Table 1. Least-square means (± SE) of concentration of cortisol and oxytocin as a function of classes of temperament indicators (n = 38)

Dependent variables[1]	Temperament classes: Low	Intermediate	High	$F_{2,104}$	*P*-value
		RSprep			
Cortisol, ng/ml	6.23 ± 0.56 [b]	7.35 ± 0.54 [b]	12.15 ± 1.12 [a]	10.87	<0.001
Oxytocin, pg/ml	5.29 ± 0.49 [b]	5.75 ± 0.47 [b]	7.82 ± 0.99 [a]	2.54	0.083
		RStca			
Cortisol, ng/ml	5.44 ± 0.60 [b]	6.89 ± 0.54 [b]	10.88 ± 0.71 [a]	17.56	<0.001
Oxytocin, pg/ml	5.82 ± 0.55 [ab]	4.91 ± 0.49 [b]	7.21 ± 0.65 [a]	3.91	0.023
		STEPS			
Cortisol, ng/ml	6.03 ± 0.53 [b]	7.23 ± 0.63 [b]	10.93 ± 0.88 [a]	11.36	<0.001
Oxytocin, pg/ml	5.50 ± 0.50	6.56 ± 0.56	5.01 ± 0.79	1.52	0.225
		KICKS		$F_{1,105}$	
Cortisol, ng/ml	7.06 ± 0.44 [b]	-	9.06 ± 1.05 [a]	2.99	0.087
Oxytocin, pg/ml	5.76 ± 0.36	-	5.87 ± 0.87	0.01	0.910
		FS (m/s)			
Cortisol, ng/ml	6.19 ± 0.69 [b]	8.48 ± 0.70 [a]	7.88 ± 0.85 [ab]	2.49	0.088
Oxytocin, pg/ml	4.74 ± 0.57 [b]	6.49 ± 0.60 [a]	6.50 ± 0.70 [ab]	2.41	0.095
		ET (s)			
Cortisol, ng/ml	7.22 ± 0.83	7.16 ± 0.55	8.05 ± 0.85	0.40	0.673
Oxytocin, pg/ml	5.39 ± 0.68	5.74 ± 0.45	6.23 ± 0.70	0.36	0.699

[1]RSprep= reactivity scores pre-milking udder preparation, RStca = reactivity scores when fitting the milking cluster, STEPS = number of steps, KICKS = number of kicks, ET= entrance time, FS = flight

[a – b] Means followed by the same letters in the row are not statistically different (P < 0.05).

3.2 Relationships of temperament with milk yield and milkability

The milking temperament was not related to milk yield, or the milkability parameters (Table 2). Regarding handling temperament, ET had a significant relationship with milk yield (p = 0.004). Cows classified in the ET_{Inter} category produced 27.62% more milk than ET_{High} cows (Table 2). Among the milkability parameters, milking time was influenced by ET (p < 0.000) and FS (p = 0.000). Cows with both extreme temperaments (high and low) for ET and FS were more difficult to milk and took more time to be milked than the intermediate ones. Cows classified as ET_{High} spent 20.22% longer time being milked than ET_{Low} cows. The same happened for animals who left the squeeze chute more slowly (FS_{Low}), which spent 19.91% longer being milked than FS_{High} cows (Table 2). ET had also a significant relationship (p = 0.046) with milking flow. The ET_{Inter} cows had a flow rate 14.80% faster than the ET_{Low} cows, which did not significantly differ from ET_{High}.

Table 2. Least-square means (± SE) of milk yield and milkability traits as a function of the temperament indicators (n = 76)

Dependent variables[1]	Temperament classes: Low	Intermediate	High	$F_{2,211}$	P-value
		RSprep			
Milk yield, kg/d	20.10 ± 1.23	18.67 ± 1.39	19.25 ± 1.50	0.57	0.565
Milking time, s	420.81 ± 12.83	435.80 ± 14.45	465.14 ± 18.15	2.22	0.111
Flow, g/s	20.45 ± 1.27	18.80 ± 1.46	21.67 ± 1.56	1.36	0.259
		RStca			
Milk yield, kg/d	19.62 ± 1.24	19.19 ± 1.36	19.56 ± 1.39	0.05	0.951
Milking time, s	421.16 ± 14.08	439.44 ± 14.04	450.36 ± 16.22	1.09	0.337
Flow, g/s	20.87 ± 1.29	19.67 ± 1.41	20.43 ± 1.45	0.33	0.718
		STEPS			
Milk yield, kg/d	20.55 ± 1.20	18.69 ± 1.44	18.43 ± 1.35	1.31	0.273
Milking time, s	435.72 ± 13.33	439.37 ± 15.53	431.19 ± 15.65	0.08	0.921
Flow, g/s	21.21 ± 1.25	18.88 ± 1.49	20.31 ± 1.41	1.20	0.303
		KICKS			
Milk yield, kg/d	19.08 ± 1.06	-	20.90 ± 1.61	$F_{1,211}$=1.25	0.264
Milking time, s	432.91 ± 10.38	-	446.85 ± 19.50	$F_{1,210}$=0.46	0.497
Flow, g/s	19.95 ± 1.10	-	22.15 ± 1.71	1.63	0.203
		FS (m/s)			
Milk yield, kg/d	21.05 ± 1.52	18.79 ± 1.12	19.69 ± 1.65	1.03	0.360
Milking time, s	516.44 ± 19.42 [a]	435.89 ± 14.22 [b]	430.68 ± 21.07 [b]	8.77	0.0002
Flow, g/s	20.78 ± 1.61	20.00 ± 1.16	21.75 ± 1.74	0.58	0.562
		ET (s)			
Milk yield, kg/d	18.49 ± 1.18 [b]	21.77 ± 1.25 [a]	17.06 ± 1.71 [b]	5.78	0.004
Milking time, s	416.38 ± 15.30 [b]	494.35 ± 16.20 [a]	500.60 ± 21.92 [a]	10.34	<0.001
Flow, g/s	19.31 ± 1.24 [b]	22.18 ± 1.31 [a]	18.86 ± 1.79 [ab]	3.13	0.046

[1] RSprep = reactivity score during pre-milking udder preparation, RStca = reactivity score when fitting the milking cluster, STEPS = number of steps, KICKS = number of kicks, ET= entrance time, FS = flight speed.
[a-b] Means followed by the same letters in the row are not statistically different (P < 0.05).

3.3 Relationship between milk temperament and milk quality

The milking temperament measured by RStca showed a tendency in the percentage of fat (p = 0.056). The milk from cows categorized as RStca-$_{\text{Inter}}$ had 11.83% higher fat content than the milk from cows with lower reactivity (RStca-$_{\text{Low}}$) (Table 3).

Regarding protein, cows with lower reactivity scores (RSprep-$_{\text{Low}}$) produced milk with 5.21% higher protein content (p = 0.028) than the milk produced by cows of a more reactive temperament (RSprep-$_{\text{High}}$). The cows classified as STEPS-$_{\text{Inter}}$ tended (p = 0.088) to produce milk with 3.45% lower protein content when compared to cows classified as STEPS-$_{\text{Low}}$ (Table 3). Protein content was also influenced by handling temperament, as the milk from cows with ET-$_{\text{Low}}$ tended (p = 0.073) to have 5.24% greater protein content than the milk from cows with ET-$_{\text{High}}$.

Lactose content tended to be related with ET (p = 0.055), as the milk from cows classified in the ET-$_{\text{Inter}}$ category had 3.17% more lactose than cows with ET-$_{\text{Low}}$ (Table

3). Finally, the SCC was not related to any of the temperament traits, either during milking or in the handling in the corral (Table 3).

Table 3. Least-square means (± SE) of milk quality traits as a function of the temperament indicators (n = 76).

Dependent variables[1]	Temperament classes: Low	Intermediate	High	$F_{2,203}$	*P-value*
		RSprep			
Fat, %	1.12 ± 0.05	1.15 ± 0.05	1.26 ± 0.06	2.07	0.129
Protein, %	3.33 ± 0.05 [a]	3.33 ± 0.05 [a]	3.17 ± 0.06 [b]	3.63	0.028
Lactose, %	4.49 ± 0.06	4.47 ± 0.06	4.44 ± 0.07	0.20	0.817
SCC, log cel/ml	5.53 ± 0.20	5.16 ± 0.23	5.30 ± 0.25	1.40	0.249
		RStca			
Fat, %	1.12 ± 0.05 [b]	1.25 ± 0.05 [a]	1.19 ± 0.05 [ab]	2.92	0.056
Protein, %	3.27 ± 0.05	3.30 ± 0.05	3.27 ± 0.05	0.19	0.825
Lactose, %	4.48 ± 0.06	4.43 ± 0.06	4.49 ± 0.06	0.53	0.588
SCC, log cel/ml	5.38 ± 0.20	5.52 ± 0.23	5.22 ± 0.23	0.74	0.478
		STEPS			
Fat, %	1.13 ± 0.05	1.24 ± 0.05	1.18 ± 0.05	1.99	0.140
Protein, %	3.31 ± 0.05 [a]	3.19 ± 0.05 [b]	3.30 ± 0.05 [a]	2.46	0.088
Lactose, %	4.47 ± 0.05	4.42 ± 0.06	4.50 ± 0.06	0.70	0.498
SCC, log cel/ml	5.46 ± 0.20	5.44 ± 0.24	5.18 ± 0.23	0.73	0.481
		KICKS			
Fat, %	1.18 ± 0.04	-	1.14 ± 0.06	$F_{1,211}$=0.33	0.568
Protein, %	3.26 ± 0.04	-	3.35 ± 0.06	$F_{1,211}$=1.80	0.181
Lactose, %	4.46 ± 0.05	-	4.50 ± 0.07	$F_{1,208}$=0.33	0.565
SCC, log cel/ml	5.42 ± 0.18	-	5.20 ± 0.27	$F_{2,213}$=0.68	0.409
		FS (m/s)			
Fat, %	1.25 ± 0.06	1.14 ± 0.04	1.19 ± 0.06	1.86	0.158
Protein, %	3.23 ± 0.06	3.27 ± 0.04	3.32 ± 0.06	0.35	0.701
Lactose, %	4.56 ± 0.07	4.44 ± 0.05	4.43 ± 0.07	1.69	0.187
SCC, log cel/ml	5.21 ± 0.25	5.42 ± 0.19	5.45 ± 0.28	0.37	0.691
		ET (s)			
Fat, %	1.19 ± 0.05	1.12 ± 0.05	1.23 ± 0.07	1.98	0.140
Protein, %	3.33 ± 0.05 [a]	3.25 ± 0.05 [ab]	3.16 ± 0.07 [b]	2.66	0.073
Lactose, %	4.41 ± 0.05 [b]	4.55 ± 0.06 [a]	4.44 ± 0.08 [ab]	2.93	0.055
SCC, log cel/ml	5.45 ± 0.20	5.27 ± 0.21	5.33 ± 0.29	0.30	0.741

[1]RSprep = reactivity score during preparation for milking, RStca = reactivity score during milking cluster attachment, STEPS = number of steps, KICKS = number of kicks, ET = entrance time, FS = flight speed, SCC, somatic cell count.

[a-b] Means followed by the same letters in the row are not statistically different ($P < 0.05$).

4. Discussion

4.1 Relationships between temperament and concentrations of milk cortisol and oxytocin

The concentration of milk cortisol was greater for cows with a more reactive temperament during milking, as measured by our high reactivity scores during preparation and teat cup attachment, and by the high number of steps and tended to kick more during milking. It should indicate that these cows presented behavioral and physiological signs of stress during milking, suggesting that reactive cows are more susceptible to stress during routine handlings. This is similar to the findings by Wenzel et al. (2003) and Gygax et al. (2006) in which cows that kicked more or took more steps in the milking parlor produced milk with higher concentrations of cortisol when compared to their calmer counterparts. However, this differed from the results by Sutherland et al. (2012) and Sutherland and Huddart (2012), who evaluated the reactivity of the animals using reactivity scores similar to ours and did not find an association between the agitation of the cows in the milking parlor and the concentration of plasmatic cortisol. The same was reported by Van Reenen et al. (2002), who did not find an association between the number of steps and kicks in milking and the concentration of plasmatic cortisol. These different results could be due to the cortisol sampling methods. In our study, we assessed the concentration of cortisol in the milk, as it is a less invasive method that does not cause additional stress during sampling collection. Van Reenen et al. (2002); Sutherland et al. [(2012) and Sutherland and Huddart (2012) used blood sampling, which could increase the levels of plasmatic cortisol even in less reactive cows.

Blood cortisol is widely used to assess the neuroendocrine stress response (Rushen et al., 2001; Van Reenen et al., 2002; Sutherland et al., 2012; Sutherland and Huddart, 2012), but it is an invasive technique that could activate the HPA axis and cause an increase in plasma cortisol levels in cows (Rushen et al., 2008). A non-invasive alternative has been to measure cortisol in the milk. Cortisol, like other steroid hormones, can permeate and cross the epithelial layer between blood vessels and the alveoli of the mammary gland (Rushen et al., 2008), resulting in a positive correlation between the concentration of cortisol in the blood and milk in response to different milking techniques (Bremel and Gangwer, 1978; Gygax et al., 2006; Thinh et al., 2011). Milk cortisol may be used as a biomarker to assess stress response to short- medium-term (12 h) environmental challenges in dairy cow (Pošcic et al., 2017).

Studies using ACTH challenge to investigate the changes in milk cortisol concentration found that the cortisol in milk might remain elevated until 8-10 h after receiving the stimulus, depending on the ACTH dosage (Fox et al. 1981; Bremel; Gangwer, 1978; Thinh et al., 2011). In the study of Sgorlon et al. (2015), the animals were milked twice a day (12 h intervals), as in the present study. In these situations, the cortisol concentration in the milk possibly reflects the variation of the plasma concentration in the interval of 10 to 14 h before the milk sampling, *i.e.* the previous milking (Sgorlon et al., 2015).

Our results confirm the hypothesis that cows that are more reactive during milking are also more susceptible to physiological stress during handling and show a higher concentration of cortisol in milk. The high concentrations of cortisol and noradrenaline in the blood are associated with stress in the milking environment (Negrão; Marnet, 2003) as cortisol is one of the main hormones associated with physiological stress response in mammals (Cockrem, 2013). A greater increase of this glucocorticoid occurs due to a stronger activation of the hypothalamic-pituitary-adrenal (HPA) axis in response to a stressing agent, that might be physical or emotional (Cockrem, 2013). Individual differences in response to environmental stimuli are expected, and the variation in the glucocorticoid concentration has been associated with differences in temperament in beef cattle measured by the flight speed test (Cafe et al., 2011).

The concentration of oxytocin was also higher in cows that presented greater reactivity scores during milking, as measured by high reactivity scores during teat cup attachment. Our results corroborate those of Sutherland and Tops [2014], where cows with greater levels of RStca agitation in a new milking environment (psychological stressor) tended to present a greater concentration of blood oxytocin, suggesting that oxytocin may be related to the behavioral stress response in dairy cows. According to the authors, cows that present a heightened response to a psychological stressor and have higher concentrations of oxytocin could have greater stress coping mechanisms. In turn, Sutherland et al. (2012) did not report any association between reactivity in a familiar milking parlor and concentrations of plasmatic oxytocin.

Oxytocin is the hormone responsible for milk ejection and maintenance of lactation (Bruckmaier; Blum, 1998) but has also been pointed to as a physiological reaction to stressing agents (Sutherland et al., 2012; Sutherland; Tops, 2014). In our study, the milk from reactive cows had higher cortisol and oxytocin concentrations, suggesting that a higher concentration of oxytocin might be part of the stress response in these cows, likely as a stress coping mechanism. That may occur as an attempt to mitigate the effects of stress during the milking process, as oxytocin has anti-stress (Chen; Sato, 2017) and anxiolytic effects (Amico et al., 2004), both associated with the HPA axis (Cafe et al., 2011; Chen; Sato, 2017). However, some studies report that a high oxytocin concentration in female rodents leads to a decrease in cortisol concentration (Amico et al., 2004). The same happens in dairy cows habituating to a new milking environment, where there is an increase in oxytocin release as the cows get used to the new environment (Sutherland; Tops, 2014), accompanied by a decrease in cortisol concentration. Sutherland et al. (2012) found that in a new milking environment (psychological stressor), the blood cortisol concentration was greater before milking, and the oxytocin concentration was greater after milking. These results suggest that the level of cortisol before milking attenuated the oxytocin response to the new situation.

However, other studies have indicated that high levels of cortisol do not suppress the secretion of oxytocin (Bruckmaier; Blum, 1998; Negrão; Marnet, 2006), similar to what occurred with the concentration of both hormones in the milk of our cows. Therefore, our results show that Holstein x Gyr crossbred cows with high reactivity had behavioral and physiological signs of stress during milking, even if they were milked in a familiar environment and by milkers using good handling practices, but the stress experienced by the cows seems not to affect the milk production. Reactive cows during milking had lower milk flow and longer milking time. They also showed an increase in oxytocin concentration during milking. Thus, a higher concentration of oxytocin does not necessarily mean a good milk ejection. That is, cows could release oxytocin and retain milk. Therefore, to analyze milking quality as a function of cows' temperament, it is necessary to gather data from oxytocin release, milk flow, milking time, and milk yield.

Unlike milking temperament, the cows with intermediate handling temperament measured by FS tended to have higher concentrations of milk cortisol and oxytocin compared to those with extreme temperaments (low and high). These results differ from those of Sutherland et al. (2012), who found that the more reactive cows (with high FS) had a higher basal cortisol concentration in a familiar milking environment (*i.e.* a rotary milking parlor where the cows were usually milked), but there was no variation in the cortisol concentration between cows of different FS categories exposed to an exogenous ACTH challenge. When exposed to a novel milking environment (a herringbone parlor within the same farm), these cows did not show variation in the concentration of plasmatic cortisol in relation to FS. In the same study, Sutherland et al. (2012), working with multiparous cows, found that the concentration of blood oxytocin was higher for cows in the novel environment, regardless of FS category. However, in primiparous cows, the concentration of plasmatic cortisol was higher in cows with high FS during the first milking sessions (Sutherland; Huddart, 2012). In general, the authors found that the heifers previously trained to be milked reached lower plasmatic cortisol concentration. Flight speed is commonly used to assess differences in temperament for beef cattle (cafe et al., 2011, Sant'Anna et al., 2013), but fewer studies have used this indicator for dairy cattle (Gibbons et al., 2011; Sutherland et al., 2012; Marçal-Pedroza et al., 2020). Since the concentration of cortisol and oxytocin had a positive and linear relationship with the reactivity measures during milking (but non-linear relation with the reactivity to handling in the corral), we might infer that the cows had different perceptions of the stimuli in the two distinct handling locations and reacted distinctively, resulting in different patterns of relationships between behavioral and physiological responses.

4.2 Relationships between temperament, milk yield, and milkability

We hypothesized that milking temperament would be related to milk yield based on previous studies reporting that cows who are more reactive to milking (measured by the number of steps and kicks) produced less milk (Breuer et al., 2000; Hedlund; L¢vlie, 2015; Neave et al., 2022). Nevertheless, none of the milking temperament measures assessed in the present study were related to milk yield. The lack of association between milking temperament and milk yield was previously reported by Van Reenen et al. (2002); Orbán et al. (2011); and Sutherland and Huddart (2012).

In contrast to the results reported by Sutherland and Dowling [2014], Sutherland and Huddart [2012], we did not find any association between FS and milk yield. Regarding milkability parameters, FS was associated with milking time and average milk flow. The cows which exited the squeeze chute slowly, considered to have a calmer temperament, spent more time being milked than more reactive cows, contrary to what we expected, but similar to what was reported by Sutherland and Huddart (2012).

Among the handling temperament measures assessed in this study, only ET was related to milk yield, with cows classified as intermediate producing more milk than those classified as low and high for ET. It is possible that among the cows with the highest values for ET, some refused to walk and need to be stimulated with voice commands and / or touch to go into the squeeze chute. In its turn, those with the lowest ET values should include cows that entered running (i.e., more reactive ones). In this specific case, the

Intermediate class should include animals with a better temperament that entered walking the single-file race and did not need to be stimulated to walk. Both extremes (low and high) for this measure, could be regarded as undesirable behaviors in the production environment. The ET was also related to milkability parameters since the intermediate cows showed greater average flow than the low and high classes. Furthermore, cows that took longer to enter the squeeze chute (possibly including cows that refused to walk as a response to fear), were the ones that took longer to be milked. Contrasting results were reported by Sutherland et al. (2012), who found that dairy cows of intermediate temperament (average exit time – i.e., between 2 and 4s) reached a lower average flow when compared to those of calmer (exit time > 4s) and more reactive (exit time < 2s) temperaments, revealing a lack of consensus, that is probably related to the different types of temperament measures used.

It is interesting to highlight that few studies (Sutherland; Huddart, 2012; Sutherland; Dowling, 2014; the present] evaluated the relationships between handling temperament with productive parameters for lactating dairy cows. Most of the studies with dairy cows limited the temperament assessment to the milking reactivity. In future studies, assessing the temperament of dairy cows should include indicators from different handling situations (beyond the milking parlor) to evaluate if the temperament in a broader sense could be related to productive parameters.

4.3 Relationship between temperament and milk quality

Calmer cows, measured by reactivity score during preparation, produced milk with a higher protein content and calmer cows during teat cup attachment tended to produce lower fat content. Similar results were found by Morales Pineyrúa et al. (2002) for Holstein cows, in which calmer cows based on a milking reactivity score similar to ours, had lower protein and fat content. The handling temperament also influenced the milk quality. Cows that entered the squeeze chute faster (i.e., low class for ET) tended to have higher protein content while cows that entered the chute calmly (intermediate ET) tended to produced milk with higher contents of lactose than the faster cows. Kruszynski et al. (2013) found that calmer cows produced milk with higher protein and fat contents. In turn, Cziszter et al. (2016) reported that the milk produced by more agitated cows in the milking parlor had greater fat percentages than the milk from cows of intermediate temperament, which had a lower content of protein than the calmer and more agitated ones. In contrast, Gergovska et al. (2014) found that both more agitated and calmer cows produced milk with a higher fat content than those of intermediate temperament. Finally, Orbán et al. (2011) failed to find a significant effect of temperament on the protein and fat contents in the milk of Jersey and Holstein cows. All of these studies assessed temperament based on the cows' reactivity during milking. The lack of consensus on the effect of dairy cows' temperament on fat and protein milk contents is likely due to differences in temperament assessment methods, breed, or handling conditions. In the present study, animals with a calmer temperament in the milking parlor produced milk that could be regarded as more desirable by consumers of fluid milk, that is, with higher protein content and lower fat content (Mccarthy et al., 2017). The relationship between temperament and milk quality should be further investigated in future research since there are few studies published on this topic.

Finally, the present study had some limitations that must be discussed. The research was conducted on an experimental farm where the animals are handled more frequently, which would make them more habituated to handling (being regarded as 'calmer') than the average Zebu cows in Brazilian commercial herds. Additionally, our sample varied in days in lactation, parity, and genetic group. To standardize these sources of variation we would have to exclude animals from our sample, leading to an even lower sample size. Therefore, we decided to include all of the cows available in the herd and control for these factors in the statistical analyses. Finally, we expected to find a genetic group effect in the temperament measures, but we were not able to investigate this relationship because of the low sample of animals within each genetic group. Future studies on this topic should include larger samples of crossed Zebu cows to allow for the assessment of genetic group effects on temperament and hormone concentration. It would also be of interest to integrate physiological and temperament indicators assessed in different handling situations (corral and milking parlor) (Koolhaas; Reenen, 2016). The inclusion of other tests traditionally used to assess temperament in cattle should also be investigated in future studies, such as novel object, novel human, avoidance distance, and restraint tests (Neave et al., 2022). It would allow for a broader view of the cows' temperament, including traits that go beyond milking reactivity. The integration of various temperament tests should be assessed using statistical methods for data dimensionality reduction, such as principal component analyses or factor analysis, which would help identify key components or factors that provide a better overall understanding of Zebu cows' temperament.

5. Conclusions

We conclude that handling temperament is related to milk yield and milkability, since calm and intermediate cows in the handling corral produced more milk and presented better milkability parameters, such as a shorter milking time and greater average milk flow. Additionally, the cows with better temperament in the milking parlor (calm and intermediate cows) produced milk with lower fat content and higher protein content. More reactive cows during milking produced milk with higher concentrations of cortisol and oxytocin, showing that behavioral reactivity could be related to the intensity of the physiological stress response. Future studies should investigate measures that lead to the improvement of temperament of crossbred Zebu cows, such as genetic selection and the use of good practices of handling, with the aim of reducing the cows' reactivity to handling and improving the welfare of the cows, the workers, and the productive indices, making the dairy industry more sustainable and efficient.

Acknowledgments

This study is part of the first author's doctoral thesis prepared for the Graduate Program in Biodiversity and Nature Conservation of the Federal University of Juiz de Fora (UFJF), Brazil. The authors thank EMBRAPA Dairy Cattle for kindly providing the animals and infrastructure necessary for the study, and the NEBEA students and Embrapa staff who collaborated with the study. We are grateful to the International Society for Applied Ethology for the English editing assistance as part of the English Language Help Service.

Financial support statement

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Grant # 409059/2016-1; # 311794/2020-3) and by the Fundação de Amparo à Pesquisa do Estado de Minas Gerais – Brasil (Fapemig).

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Chapter 4 - Does the temperament of crossbred female dairy calves affect weight gain and average daily starter feed consumption?

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Abstract

Previous studies suggest that the temperament of dairy bovines might be related to performance since bird. The aims of this study were to characterize temperament traits in dairy calves of crossed and to assess the effects of temperament on weight gain and on the average daily starter feed consumption during their pre-weaning stage. Three behavioral tests (novel object, novel environment, and voluntary approach) were carried out with 60 crossbred Holstein x Gyr female calves over two periods during their pre-weaning stage. The animals were divided into six distinct feeding regimens according to milk allowances (4, 6, or 8 L/day) and starter feed supplying strategies (control – 24%, or treatment with decreasing crude protein content – 24%, 18%, 14%). Body weight was measured weekly up to 63 days of age, and weight gain (g/d) was calculated for: (ADG1 ($_{1-28}$) period between days 1-28; (ADG2 ($_{28-63}$) period between days 28-63; and (ADG3 ($_{1-63}$) period, between days 1-63. The obtained starter feed consumption measurements were divided into average daily consumption (ADC, g/d/calf) and total consumption for 63 days (TC, g). A principal component analysis (PCA) was carried out with the behaviors recorded throughout the tests to determine temperament traits. We later adjusted a generalized linear model (GLM – ANOVA) to evaluate the effects of temperament, feeding regimens, and the interaction of both on weight gain starter feed and consumption. The PCA generated four principal components which explained 51.98% of the total data variance, interpreted as: 'activity'; 'fearfulness'; 'neophilia'; and 'exploration'. The 'activity' trait tended to be positively associated with ADG2 $_{(28-63)}$ (p=0.086) and ADG3 $_{(1-63)}$ (p = 0.069), whereas 'exploration' was positively associated with ADG1$_{(1-28)}$ (p = 0.002) and ADG3 $_{(1-63)}$ (p = 0.018). None of the starter feed consumption measures were linked to temperament (P > 0.05). There was no interaction of diet with temperament, but the milk allowance offered did interfere in weight gain and consumption, as the animals fed with 8 L gained more weight than the animals receiving 6 and 4 L. In turn, the animals which received 4 L consumed more starter feed. We found that animals with a more active/exploratory behavior and which interacted less with the unknown person gained more weight during their pre-weaning stage, indicating that there is a possible link between temperament and milk allowances with the performance of crossbred female dairy calves.

Keywords: dairy livestock, novel tests, personality, productive efficiency.

1. Introduction

Stable individual differences in the behavior or temperament of farm animals have been found to be responsible for causing variations in the efficiency of productive systems (Hedlund; Løvlie, 2015; Cerqueira et al., 2017). Such interindividual differences, according to Reále et. (2007), repeat over time and on various occasions throughout the life of the animals (Neave et al., 2020). Temperament can be characterized via traits such as activity, reactivity, sociability, aggressiveness, exploration, fearfulness, and boldness (Gibbons et al., 2011; Lecorps et al., 2019; Costa et al., 2020). These traits may be accessed through direct observation of the animals' behavior of the during handling at the farm (Marçal-Pedroza et al., 2020) or through the application of temperament tests, in which the animal is individually evaluated when exposed to novel or challenging situations (Gibbons et al., 2011).

Cattle temperament has important effects on production, both for beef (Cafe et al., 2011) and dairy cattle (Hedlund; Løvlie, 2015; Neja et al., 2017) in all breeding stages. Previous studies show that animals with a more excitable and reactive temperament present lower rates of weight gain (Del Campo et al., 2009; Sebastian et al., 2011) and lower meat quality (Sant'Anna et al., 2013). In addition to a lower milk yield (Hedlund; Løvlie, 2015) and lower solid content in the milk, such as lower protein content (Marçal-Pedroza et al., 2023), they also have lower milkability levels (Neja et al., 2015) and increased enteric methane emissions (Marçal-Pedroza et al., 2021) when compared to animals of a calmer and less reactive temperament.

However, especially during the calfhood of female dairy calves, there are few studies which investigated the presence of stable interindividual differences in calves, as well as their effects on the behavior and performance (Neave et al., 2018, 2019; Costa et al., 2014, 2020). The weight gain of the animals is positively associated with important performance parameters, including milk yield in the first lactation (Carsake et al., 2022), and the development of the mammary gland (Albino et al., 2015). Additionally, age when giving birth (Geiger et al., 2016) and weight gain (Van Stroet et al., 2016) are also influenced by the development and feeding of bovines since their birth.

Nevertheless, the few studies carried out so far on the relationship between temperament, starter feed consumption, and weight gain were done only with calves of European breeds, namely Holstein (Neave et al., 2018, 2019; Costa et al., 2020) and Norwegian Red (Whalin et al., 2022). There are still no studies investigating such associations for dairy calves of zebu origin. Zebu animals tend to have a high temperament when compared to animals of European origin (Paranhos da Costa et al., 2015). Thus, our study aims were to: a) characterize the temperament of crossbred female dairy calves (Holstein x Gyr), via standardized testing and b) assess the effects of temperament on weight gain and starter feed consumption during their pre-weaning stage when subjected to different diets. The following hypotheses were tested: novel tests are capable of extracting temperament traits of crossbred dairy calves, and the more active animals during testing consume more starter feed and gain more weight.

2. Material and methods

2.1 Animals and housing conditions

The study was conducted from March to August 2021, at the Multiuser Laboratory for Livestock Bioefficiency and Sustainability of the Brazilian Agricultural Research Corporation, Embrapa Dairy Cattle (Coronel Pacheco, Minas Gerais, Brazil), with 60 dairy calves, whose genetic grouping ranged from 5/8 (n =26) to 3/4 (n = 34) Holstein x Gyr. Immediately after birth, the calves were separated from their dams, dried, and their navels were treated with 10% iodine to prevent infections. Navel healing was carried out twice a day until the umbilical stump fell off. Additionally, the calves were weighed (body weight at birth = 32.8 ± 5.25 kg) and fed with colostrum (10% of their body weight at birth), standardized at 25% Brix. On the second and third days of life, they received 4 L/day of transition milk from their dams. Around 48 hours after being fed colostrum, the animals were subjected to a blood sample collection to assess the efficiency of passive immunity transfer. Only animals with a serum Brix of 8.1% or above were included in the experiment (Lombard et al., 2020).

The calves were housed in individual stalls with wood-shaving bedding and rubber flooring throughout their first three days of life. On the fourth day, the wood shavings were removed from the stalls and the experiment started, with the animals being randomly distributed into the six treatments described further below. From days 4 to 63 of life, the calves remained housed in individual pens (1.25 × 1.75 m) tethered with 1.2 m chains, equipped with a rubber mat (WingFlex, Kraiburg TPE GmbH & Co., Waldkraiburg, Germany), and allocated in an open-sided barn provided with feeding and drinking troughs. The divisions of the stalls allowed for limited visual and physical contact with the animals in neighboring stalls (Figure 1). Milk was provided twice a day, at 8 a.m. and 2 p.m., whereas the starter feed and water were provided ad libitum.

Figure 1: Calves housed in individual stalls

The health score was assessed every morning, by evaluating rectal temperature and fecal score. Rectal temperature was considered as within normality standards when it was below 39.5º C, and the feces were evaluated according to a scale of 1 to 4, where 1 represented normal feces and 4 represented liquid feces (Slanzon et al., 2022). The animals were evaluated only on days when they had good health indicators. At 46 days of age, the calves were dehorned with hot iron. The animals received local anesthesia prior to the procedure, followed by anti-inflammatory medication to control pain levels (AVMA, 2014).

2.2 Diet strategy and performance

Parallelly to the present study, an experiment was carried to assess effect of reducing starter feed crude protein content with different milk allowances on the performance of calves. This experiment was carried out in a completely randomized design in a 3×2 factorial scheme, with 3 different milk allowances (4, 6 or 8 L/d) and 2 starter feed supply strategies (fixed or decreasing CP content). The first strategy fixed starter feed supply was based on 18% CP (from 4 to 73 days, starter feed control). Meanwhile, the animals subjected to the second starter feed supplying strategy, animals received starter feed with 24% CP from days 4 to 24 of age; 18% CP from days 25 to 45; and 14% CP from days 46 to 73 of age (starter feed treatment). Thus, 6 groups were formed: 4 L/d of milk and starter feed control (4 L + SFC); 6 L/d of milk and starter feed control (6 L + SFC); 8 L/d of milk and starter feed control (8 L + SFC); 4 L/d of milk and starter feed treatment (4 L + SFT); 6 L/day of milk and starter feed treatment (6 L + SFT); 8 L/d of milk and starter feed treatment (8 L + SFT).

Milk consumption was recorded every meal, whereas starter feed consumption was measured daily. With the starter feed consumption data, we were able to generate two measurements: average daily consumption (ADC, in g/d) and total consumption (TC, g/calf). The performance of the animals was assessed by weighing them with the use of a mechanical scale. Such measurements were taken on their first day of life (at birth), and then weekly until their 63rd day of age, thus generating three measures for weight gain for the calves, namely ADG1 $_{(1-28)}$, which stands for the average daily weight gain from 1 to 28 days of age; ADG2 $_{(28-63)}$, which is the average daily weight gain from 28 to 63 days of age; and ADG3 $_{(1-63)}$, which is the average total weight gain, from 1 to 63 days of age.

2.3 Temperament assessment

Temperament tests were carried out over two periods during the pre-weaning stage, first between 30 and 40 days of age and then between 55 and 63 days of age. The tests consisted of evaluating the behavior of the animals when facing novel situations, such as the novel environment test, novel object test, and voluntary approach by an unknown person test, as performed in the works of Neave et al. (2018; 2019) and Whalin et al. (2022). The tests were carried out individually in an experimental pen (16 m^2) with concrete flooring, divided into 1m^2 squares, delineated in white crayon (Figure 2a). The walls were solid, to prevent the animals from having visual contact with anything outside the experimental pen. The three tests were video recorded for 10 minutes each, beginning as soon as the corral gate was closed. The calves were always guided gently and by the same handler, respecting the good handling practices implemented on the farm. The video recordings were always carried out by the same observer, using a video camera (Canon VIXIA HF R800) placed atop one of the corral walls, at a height of about 1.80 m from the floor.

The tests in the first testing period were carried out in the following order: first, the novel environment test (NET), followed by the voluntary approach test (VAT) and, two days later, the novel object test (NOT). On the second battery of testing, the order was inverted, first with the novel object test and then two days later with the novel environment test followed by the voluntary approach test.

During the novel environment test each calf was placed alone in the experimental pen and, after closing the gate, the animal was kept there for 10 minutes (Figure 2a). In the voluntary approach test, an unknown observer was placed in the middle of the pen and remained still throughout the duration of the test, with their gaze directed to the floor (Figure 2b). For the novel object test, we first placed a colorful ball with 120 cm of diameter in the pen, and then a colorful umbrella in the second round, also put in the middle of the pen (Figure 2c and 2d).

Figure 2: Behavioral tests performed: A) novel environment test, B) voluntary approach test, C) novel object test 1 and D) novel object test 2

Later, three observers recorded the behaviors, according to the ethogram, adapted from Neave et al. (2018) (Table 1). Behaviors were recorded as duration and reported as percentage of obervation time (10 min of test). We used the Kendall test to verify intra- and interobserver reliability, and we obtained coefficients over 0.8 for all recorded behaviors.

Table 1: Ethogram of behaviors during each of the 3 tests of temperament when calves (n = 60) were tested individually in novel environment, human approach, and novel object tests

Test and behavior	Description
All tests	
Vocalizations	All types of vocalizations, sounds emitted from the mouth (nº. of events)
Locomotory	Jumping: both forelegs off the ground and extended forward (nº. of events) Bucking: Both hind legs off the ground and extended backward (nº. of events)
Running	Running: calf trotting (2 beats) or galloping (3 beats) across or around the enclosure
Defecation	Defecations occurrences (nº. of events)
Urination	Urination occurrences (nº. of events)
Novel environment test	
Active	Total number of squares crossed with both forelegs (test arena divided into 16 equal squares)
Inactive	Time spent standing still without sniffing or licking walls or floor
Walking	Time spent walking around the arena
Exploration	Time spent with muzzle or tongue in contact with either walls or flooring substrate while moving or stationary
Voluntary approach and novel object tests	
Latency to touch	Time until moment calf touches human or object
Attentive	Time spent with head oriented toward human or object, excluding touching and object play behaviors (close: within 1 body length away; far: more than 1 body length away)
Touching	Time spent with muzzle in contact with human or object (muzzle within 5 cm)
Object/Human play	Butting (head in contact with) human or object, or mock butt where head is oriented downward and toward but not in contact with human or object
Inactive	Time spent standing still without sniffing or licking walls or floor

Ethogram adapted from Neave et al. (2018)

2.4 Statistical analysis

First, we carried out descriptive analyses on the behavioral data, consumption measurements, and the three weight gain measurements, using the Jamovi software (version 2.3.26). Then, we obtained a unique individual measure for each behavior recorded during testing, through the average of the values obtained in both testing periods.

Multivariate statistics was employed to extract temperament dimensions, through Principal Component Analysis (PCA), from all recorded behaviors. The analysis was run on Statistica. PCA gathers variables in a P data matrix and finds combinations between them to generate indices (principal components) which describe data variation (Manly, 2008). Therefore, the PCA included behavioral variables recorded in the novel environment test (active, vocalizations, locomotor, and running; time spent inactive, walking, and exploring), in the novel object and in the voluntary approach tests (latency to touch novel object/human, duration of touch with novel object/human, duration of object/human play, duration of state of attentive to novel object/human; duration of inactive number of vocalizations, locomotor play and running). The behaviors included in the PCA could be combined and represented by four principal components (PC), which were interpreted as principal traits animals' temperament. With this analysis, we could obtain an interpretable combination of correlations between the behavioral variables, generating a description based on a correlation, keeping the variables with values over 0.5. The number of jumping and the number of bucking were added together and transformed into locomotor play numbers. The behaviors of inactive, resting, defecation and urination rarely occurred and were excluded from the analyses.

To evaluate the link between temperament and weight gain and starter feed consumption, generalized mixed models were adjusted with the use of the GLM (ANOVA) JAMOVI software (version 2.3.26). The models included average daily weight gain ($ADG1_{(1\text{-}28)}$, $ADG2_{(28\text{-}63)}$ or $ADG3_{(1\text{-}63)}$) or starter feed consumption (ADC or TC) as dependent variables. As independent variables, the principal components (one at a time) and fixed effects of milk allowance, starter feed supply strategies and their interactions. In all analyses, means were compared using the post-hoc Bonferroni test; *P*-values < 0.05 were considered as significant and < 0.10 were discussed as trends.

3. Results

3.1 Descriptive results

The descriptive statistics of the behaviors recorded throughout the three temperament tests (novel environment, novel object, and voluntary human approach) are in Table 2. The behavioral measurements which showed more variation were: active, ranging from 61.00 to 335.00 seconds, inactive, from 92.50 to 4011 seconds, latency to touch the novel object, ranging from 1.00 to 600.00 seconds; latency to touch an unknown person, ranging from 2.00 to 477.5 seconds, and duration of touch with an observer, ranging from 1.00 to 569.5 seconds.

Table 2: Behavioral responses (mean ± SD) of calves (n = 60) when tested individually in novel environment, voluntary approach, and novel object tests

Behaviours	Mean	SD	Minimum	Maximum
Novel environment test				
Active	164.87	66.60	61.00	335.00
Inactive	200.53	67.76	92.50	401.00
Resting	0.00	0.00	0.00	0.00
Walking	175.52	45.26	77.00	279.50
Exploration	224.62	65.60	98.50	372.50
Vocalizations	10.47	11.22	0.00	51.50
Locomotor play	8.16	7.24	0.00	25.00
Defecação	0.00	0.00	0.00	0.00
Micção	0.00	0.00	0.00	0.00
Running	12.96	9.20	0.00	45.00
Novel object test				
Latency to touch	148.90	133.86	1.00	600.00
Attentive	29.97	35.10	3.00	259.00
Touching	36.50	40.19	0.00	163.00
Object play	4.85	17.01	0.00	120.00
Vocalizations	9.61	7.72	0.00	32.00
Locomotor play	7.64	6.15	0.00	30.00
Running	7.64	7.49	0.00	22.00
Resting	0.00	0.00	0.00	0.00
Voluntary approach test				
Latency to touch	86.79	122.37	2.00	477.50
Attentive	43.19	29.21	11.00	136.50
Touching	173.47	171.31	1.00	569.50
Object play	11.66	28.20	0.00	153.00
Inactive	0.00	0.00	0.00	0.00
Vocalizations	2.14	3.28	0.00	14.50
Locomotor play	4.57	5.55	0.00	24.50
Running	4.26	4.18	0.00	22.00
Resting	0.00	0.00	0.00	0.00

The descriptive statistics for the weight gain and starter feed consumption values are in Table 3. The values for the variables $ADG1_{(1\text{-}28)}$, $ADG2_{(28\text{-}63)}$, and $ADG3_{(1\text{-}63)}$ were between 170 and 1.150 g/d/calf), 170 and 1.170 (g/d/calf), and 230 and 1.050 (g/d/calf) respectively. For the starter feed consumption measurements, ADC had values between 50 and 640 (g/d/calf) and TC ranged between 3120 and 40520 (g/calf).

Table 3. Mean (± SD), minimum, maximum values of average daily gain (ADG) and total starter feed consumption (TC) over a 63-day experimental

	Mean	**SD**	**Minimum**	**Maximum**
	Average daily gain (g/d/calf)			
ADG1 (1-28)	540	220	170	1.150
ADG2 (28-63)	680	180	170	1.170
ADG3 (1-63)	610	170	230	1.050
	Average daily starter feed consumption (g/d/calf)			
ADC	210	130	50	640
	Total starter feed consumption (g/calf)			
TC	13.410	7.970	3.120	40.520

Total starter feed consumption in relation to the milk allowances was between 3.820 and 32.760 g/calf for the 4 L allowance, between 3.320 and 40.520 g/calf for the 6 L, and between 3.120 and 19.080 g/calf for the 8 L allowance. Finally, total starter feed consumption in relation to the fixed percentage of crude protein varied from 3.320 to 40.520 g/calf, and in relation to the decreasing crude protein percentage, it ranged from 3.820 to 35.120 g/calf (Table 4).

Table 4: Mean (± SD), minimum, maximum values of total starter feed consumption (TCC) over a 63-day experimental as a function milk diet and fixed starter feed and feed decreasing crude protein content

	Mean	**SD**	**Minimum**	**Maximum**
	Total starter feed consumption (g/calf)			
4 (L/d)	15.930	7.250	3.820	32.760
6 (L/d)	14.610	10.330	3.320	40.520
8 (L/d)	9.80	3.970	3.120	19.080
	Total starter feed consumption (g/calf)			
% fixed protein	15.530	8.900	3.320	40.520
% decreasing protein	11.200	6.310	3.820	35.120

3.2 Principal Component Analysis

The PCA was used to assess the correlation between the measurements of behaviors recorded throughout the temperament tests with crossbred (Holstein x Gyr) female dairy calves. The PCA generated four principal components which together explained 51.98% of the total data variance (Table 5).

The first component (PC1) explained 19.20% of data variance, showing greater positive values for the active, exploration, and running instances variables during the novel environment test, and negative values for inactive and vocalizations for the novel environment and novel object tests. Thus, PC1 ranged from active animals (greater PC1 scores), which ran more, crossed more quadrants and spent longer exploring the new environment to. Those which were less vocal and inactive longer (lower scores in PC). Therefore, PC1 may be interpreted as a general 'activity' level axis for the temperament of the calves.

The PC2 expressed 12.59% of variance and had only positive values for variables, namely walking and running in the novel environment test, latency to approach and number of vocalizations during the voluntary approach test. Thus, animals that spent more time locomotor play and running during the novel environment test vocalized more and took longer to approach (or failed to approach) the unknown person. Therefore, higher scores in PC2 reflect the 'fearfulness' trait.

For PC3 (11.41%), time spent in a state of attentive and time spent touching the novel object obtained the greatest positive values, whereas the latency to touch the novel object had the greatest negative value, distinguishing the calves which spent longer in a state of attentive and touching the novel object from those which took longer to interact with the object (Table 5). Thus, PC3 expressed the neophobia (animals with lower scores in PC3) – neophilia (animals with greater scores) axis, which was denominated 'neophilia' trait.

In turn, the variables with the greatest positive values shown in PC4 (8.76%) were activity (locomotor) and running, and the one with the greatest negative value was duration of touch in the voluntary approach test (Table 5). This dimension distinguished animals which moved the most around the test location (positive values) from those which spent less time interacting with the person (negative values). Therefore, PC4 reflected the 'exploration' trait.

Table 5. Principal component analysis of measures of behaviors recorded during temperament tests in bold loads represent the highest values (over 0.5) for each major component (PC) (N=60)

Behaviours	**PC1 "Activity"**	**PC2 "Fearfulness"**	**PC3 "Neophilia"**	**PC4 "Exploration"**
		Novel environment test		
Active	**0.602**	0.475	-0.433	-0.225
Inactive	**-0.739**	-0.054	0.341	0.077
Walking	0.392	**0.556**	-0.125	-0.042
Exploration	**0.500**	-0.290	-0.312	-0.203
Vocalizations	**-0.691**	0.031	-0.058	-0.140
Running	**0.611**	**0.519**	-0.354	-0.202
Locomotor play	0.432	0.469	0.038	0.096
		Novel object test		
Latency to touch	-0.358	0.171	**-0.504**	-0.071
Attentive	0.410	0.193	**0.609**	0.058
Touching	0.457	-0.212	**0.516**	-0.117
Object play	0.264	0.058	0.428	-0.348
Vocalizations	**-0.601**	0.298	-0.130	-0.185
Locomotor play	0.178	0.171	0.449	0.236
Running	0.360	0.144	0.400	0.164
		Voluntary approach test		
Latency to touch	-0.385	**0.560**	0.257	0.088
Attentive	0.005	0.477	0.293	0.078
Touching	0.174	-0.390	-0.076	**-0.675**
Human play	0.244	-0.307	-0.204	0.404
Vocalizations	-0.485	**0.633**	-0.174	0.114
Locomotor play	0.232	-0.233	-0.183	**0.660**
Running	0.234	-0.034	-0.364	**0.571**
Eigenvalue	4.033	2.645	2.398	1.832
Total variance	19.21%	12.59%	11.42%	8.76%

3.3 Link between temperament, consumption and weight gain throughout the experiment

A tendency was found between 'activity' and ADG2 $_{(28\text{-}63)}$ (p = 0.086) and ADG3$_{(1\text{-}63)}$ (p = 0.069), which shows that ADG tended to be greater for the more active animals (Table 6). We also found a linear and positive relation of 'exploration' with ADG1$_{(1\text{-}28)}$ (p = 0.002) and ADG3$_{(1\text{-}63)}$ (p = 0.018) (Table 6), suggesting that the animals which interacted less with the unknown person were those which gained the most weight during the periods of 1-28 and 1-63 days in the experiment. The dimensions of 'fearfulness' and 'neophilia' were not associated with ADG and none of the four traits were associated with ADC and TC. The temperament traits extracted in the principal component analysis (p > 0.10).

Table 6. Estimate means (± e.p.) of average daily gain (ADG) and starter feed consumption (ADC and TC) as a function of the four factors obtained principal component analysis performed with the behaviors recorded during the temperament tests.

Item	**ACP1 - "activity"**				**APC2 – "fearfulness"**				**APC3 – "neophilia'"**				**APC4 – "exploration"**			
	Estimate	s.e	p	**β**	Estimate	s.e	p	**β**	Estimate	s.e	p	**β**	Estimate	s.e	p	**β**
ADG1$_{(1-28)}$	0.01	0.01	0.435	0.091	-0.006	0.01	0.685	-0.046	0.00	0.02	0.865	0.02	**0.05**	**0.02**	**0.002**	**0.320**
ADG2$_{(28-63)}$	**0.01**	**0.00**	**0.086**	**0.168**	0.00	0.01	0.894	0.012	0.00	0.01	0.391	0.08	4.93-4	0.01	0.968	0.003
ADG3$_{(4-63)}$	**0.01**	**0.00**	**0.069**	**0.154**	-0.00	0.00	0.825	-0.019	0.00	0.00	0.463	0.06	**0.03**	**0.00**	**0.018**	**0.188**
ADC	0.004	0.00	0.596	0.074	-0.00	0.00	0.479	-0.097	-0.00	0.01	0.610	-0.00	-0.05	0.06	0.398	-0.116
TC $_{(1-63)}$	0.022	0.49	0.640	0.065	-0.43	0.60	0.466	-0.100	-0.35	0.62	0.576	-0.076	-0.73	0.71	0.306	-0.14

ADG1 ($_{1–28}$), average daily gain from Day 1 to 28 of the feeding period; ADG2 ($_{28–63}$), average daily gain from Day 28 to 63; ADG3 ($_{1–63}$), average daily gain from Day 1 to 63;

3.4 Effect of milk allowances on to the four temperament traits of calves

There was a tendency observed for the starter feed supplying strategies on the weight gain of the animals, for the ADG2 (28-63) (p = 0.083) and ADG3 (1-63) (p = 0.098) measurements, with greater gain for the animals which received a fixed crude protein content (18% CP) (Figures 3 and 4). In addition, we found that the calves which received 4 L of milk daily consumed greater quantities of starter feed when compared to the calves of the 6 and 8 L treatments (Table 4).

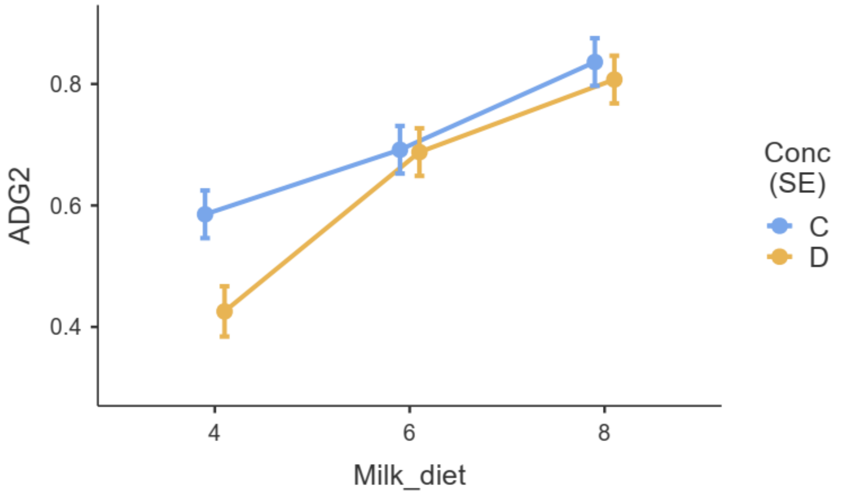

Figure 3: ADG2 weight gain (28-63) of calves depending on different milk allowances and starter feed concentrations (C- starter feed18% crude protein and D – starter feed 24%, 18% and 14% crude protein)

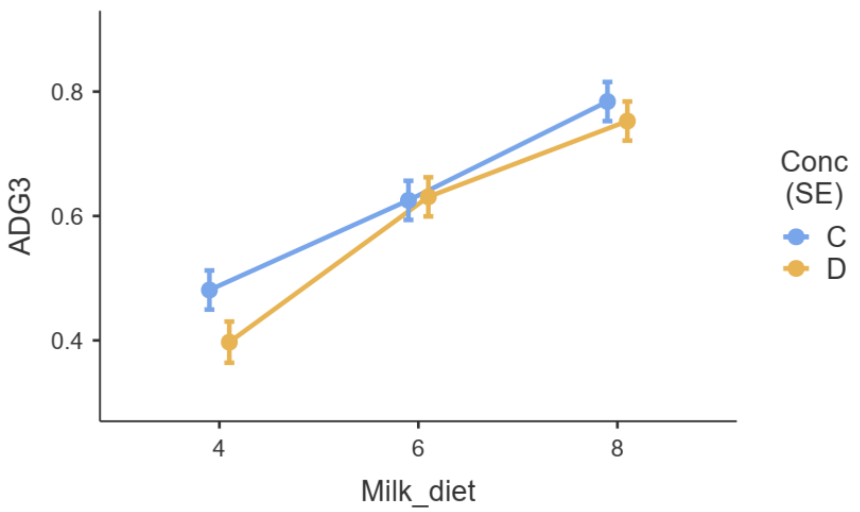

Figure 4: ADG3 weight gain (1-63) of calves depending on different milk allowances and starter feed concentrations (C- starter feed 18% crude protein and D – starter feed 24%, 18% and 14% crude protein).

The milk allowance offered had a significant effect ($p < 0.001$) on weight gain. In the ADG1 $_{(1-28)}$, ADG2 $_{(28-63)}$ and ADG3 $_{(1-63)}$ periods, the calves that received 8 L of milk gained more weight when compared to those that received 6 and 4 L (Figures 5-12).

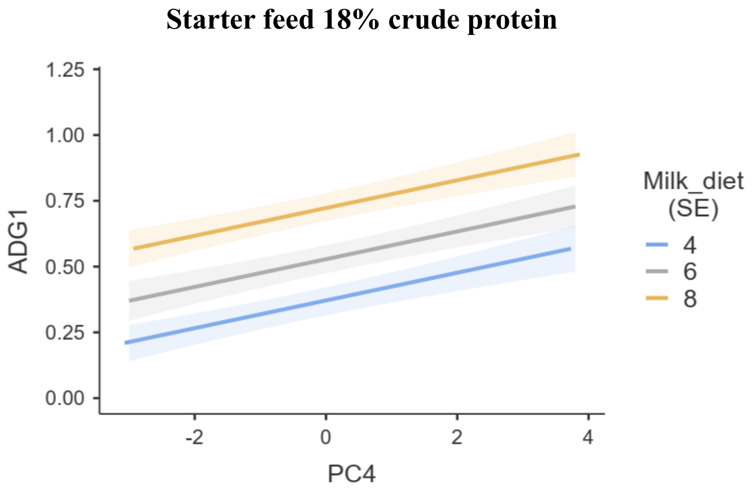

Figura 5: Weight gain ADG1 (1-28) with different milk allowances, for calves classified in PC4 “exploration”

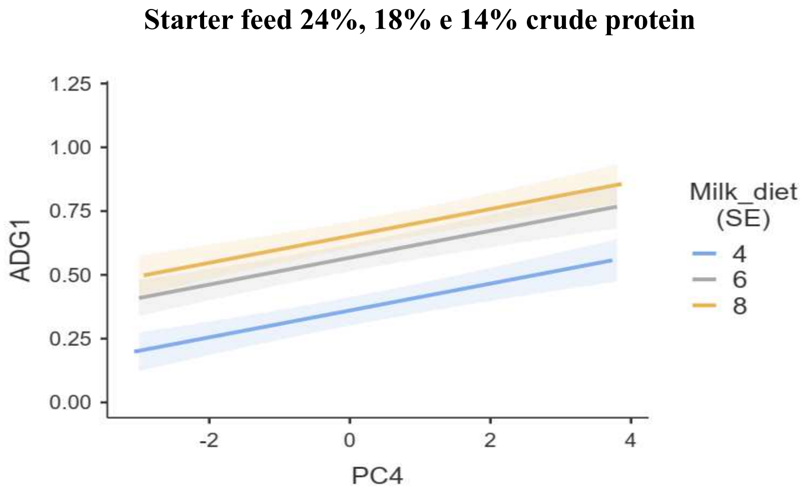

Figura 6: Weight gain ADG1 (1-28) with different milk allowances, for calves classified in PC4 “exploration”

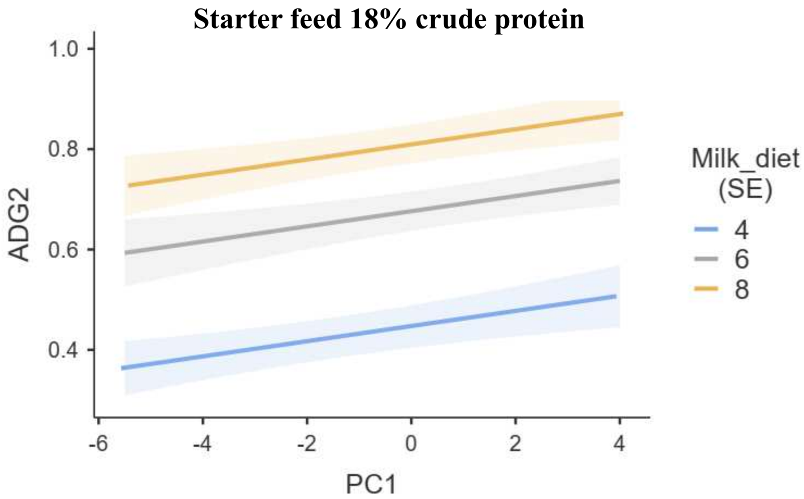

Figura 7: Weight gain ADG2 (28-63) with different milk allowances, for calves classified in PC1 “activity”

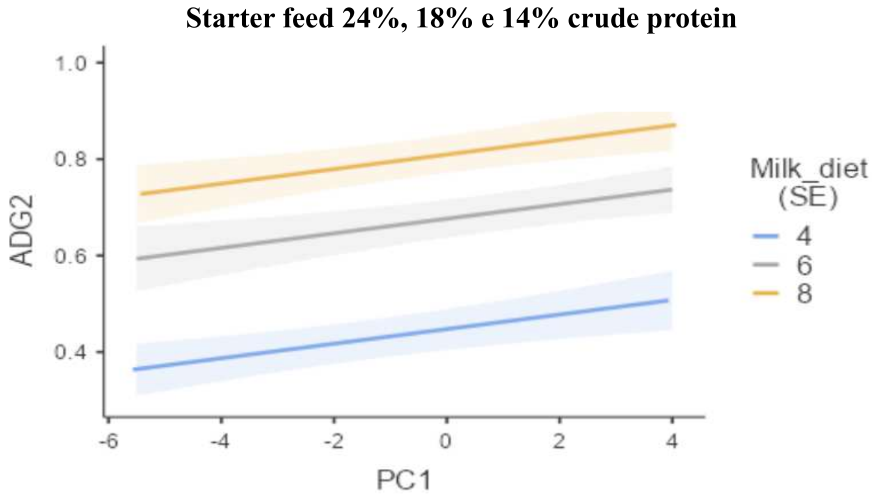

Figura 8: Weight gain ADG2 (28-63) with different milk allowances, for calves classified in PC1 “activity”

Starter feed 18% crude protein

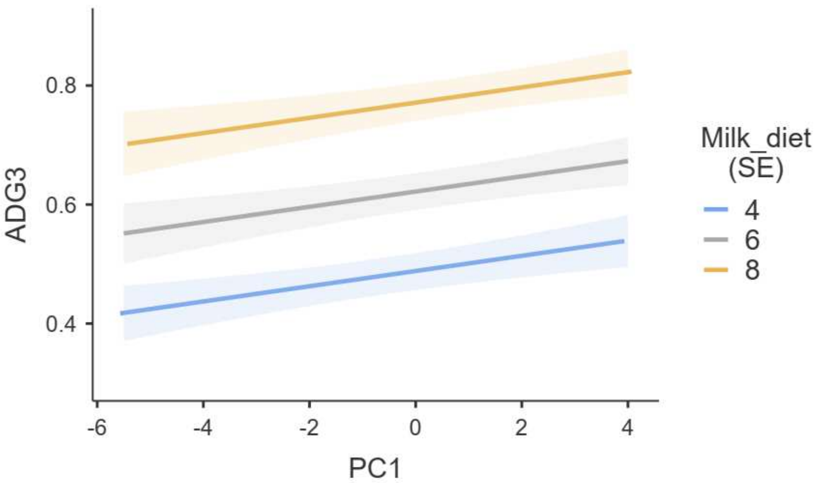

Figura 9: Weight gain ADG3 (1-63) with different milk allowances, for calves classified in PC1 "activity"

Starter feed 24%, 18% e 14% crude protein

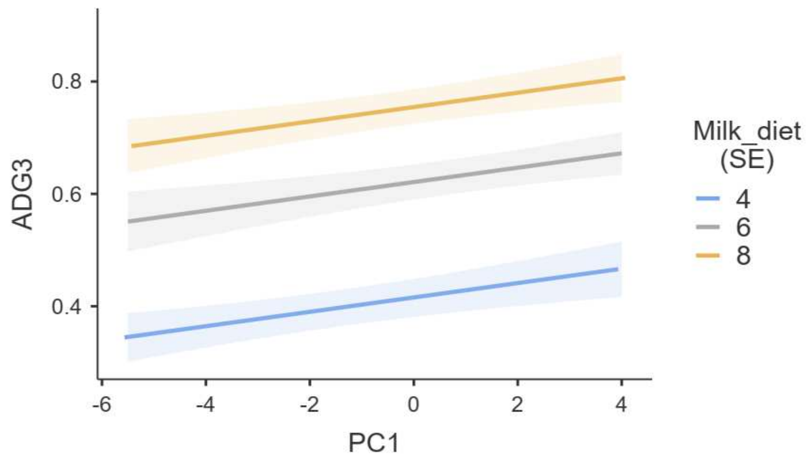

Figura 10: Weight gain ADG3 (1-63) with different milk allowances, for calves classified in PC1 "activity"

Starter feed 18% crude protein

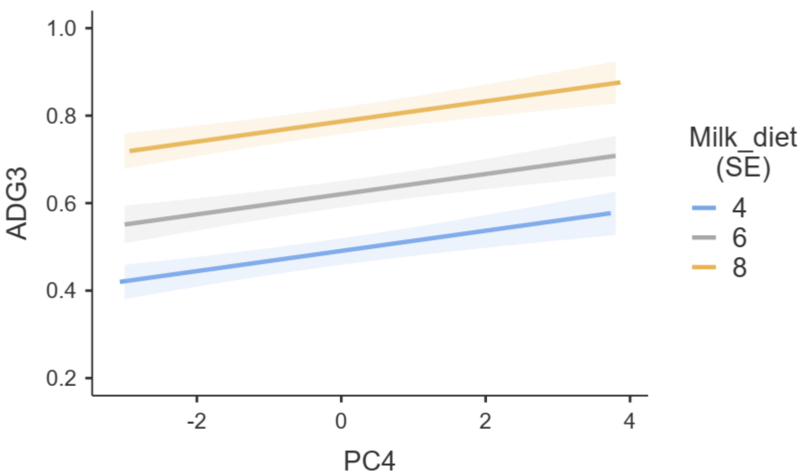

Figura 11: Weight gain ADG3 (1-63) with different milk allowances, for calves classified in PC4 "exploration"

Starter feed 24%, 18% e 14% crude protein

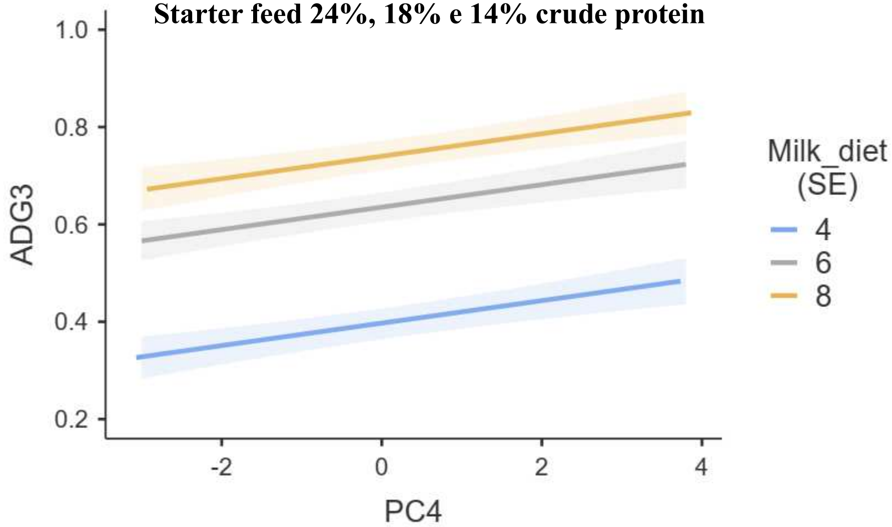

Figura 12: Weight gain ADG3 (1-63) with different milk allownaces, for calves classified in PC4 "exploration"

4. Discussion

The present study aimed to extract temperament traits of crossbred female dairy calves using standardized tests, such as novel environment, novel object, and voluntary approach, and to evaluate the link between the temperament dimensions, weight gain and starter feed consumption of the animals. The tests used were able to extract four temperament dimensions, defined as 'activity', 'fearfulness', 'neophilia' and 'exploration'. The 'activity' and 'exploration' traits were positively associated with the weight gain of the calves, that is, calves which were more active and interacted less with the unknown person gained more weight throughout the experiment. Calves fed 8 L of milk gained more weight when compared to the animals fed 6 and 4 L. And, lastly, the temperament of the animals did not influence starter feed consumption rates during the pre-weaning stage.

The animals classified as more active throughout the temperament tests, that is, calves which crossed more quadrants, spent longer exploring and running around the test area. In addition to the animals which spent less time staying still and vocalized less, there were those which gained the most weight during the pre-weaning stage.

Our results are similar to those of Neave et al. (2018, 2019), which evidenced that more active and exploratory animals in temperament tests tended to gain more weight. We must highlight that the animals in the study of Neave et al. (2018) gained more weight due to the increase in starter feed consumption recorded in the experimentation period (pre-weaning), but our analyses failed to find a link between starter feed consumption and temperament, since we could not identify variations in consumption due to temperament. According to the authors, the fact that the animals gained more weight despite the greater energy expenditure caused by the motor activities would have occurred due to the more active calves having a better feed efficiency.

In our study, the animals were not housed in collective stalls, but rather in individual pens, with their movement restricted to that area. However, they could have visual and even physical contact with the neighboring calves. Therefore, our results indicate that a more active and exploratory behavior by our animals during testing represents the motivation of confined animals to explore the open space when they have the opportunity. Additionally, these animals vocalized less or did not even vocalize during the tests, suggesting that they were not in an emotional state of fear, as excessive vocalization may be an indicator of fear and pessimism in this kind of test (Lecorps et al., 2018). This likely happened due to the housing environment of the animals being individual stalls with visual contact with other animals. Thus, the place provided them with an environment that mixed isolation and social contact, which has certainly contributed to the expression of the ‘activity’ trait, but not ‘fearfulness’, during testing. This indicates that, similarly to what is suggested by Neave et al. (2018), animals which are less reactive (more active and exploratory) to the novel environment show a better performance than animals with a more excitable temperament (which vocalized more and stood still for longer) (Neave et al., 2018).

Another possible explanation for our findings could be provided by the “Life Syndrome”, which predicts that more active and exploratory animals would tend to have better development, as they are found on the “slow/fast” axis, similar to that proposed in the study of Carsake et al. (2022). On this axis, more active/exploratory animals are classified as faster animals, and thus would have greater weight gain (Reále et al., 2010; Dammhahn et al., 2018). Additionally, being kept in the stalls was also a limiting factor for all individuals, both more and less active ones, to spend their energy on motor activities such as running, jumping and playing inside the stalls, which has possibly contributed to the more active ones to still gain more weight. Such a link could not be extrapolated for situations in which there are more exercise opportunities and greater levels of activity, such as pastures and paddocks. In such conditions, the relationship between a more active temperament and weight gain in dairy calves is yet to be investigated.

Lastly, we should also highlight that other previous studies have found different link patterns between activity levels and weight gain for calves. For instance, Woodrum Sestser et al. (2022) reported a negative relation between animal activity during novel interaction tests (NOT, VAT) and weight gain in Holstein calves. However, this same study by Woodrum Sestser et al. (2022) showed a positive relation between weight gain and activity levels recorded in an isolation crate with movement meters. In turn, Whalin et al. (2022) failed to find a link between weight gain and the activity trait in NOT and TNE for Norwegian Red calves. Thus, we see that the association between activity level and weight gain might be context-specific, varying greatly among studies.

Animals with greater ‘exploration’ PC4 scores, that is, those which spent a more time running and locomotor play, and less time in contact with the unknown person, were the ones which gained the most weight in the $ADG1_{(1-28)}$ and $ADG3_{(1-63)}$ periods. These results were similar to those of Woodrum Setser et al. (2022), in which the calves that took longer to interact with the unknown person and spent longer in a state of attentive in the tests had a greater average weight gain before weaning. However, Neave et al. (2018) found that animals which remained longer in contact with the unknown person tended to gain less weight. However, that was not confirmed in subsequent studies by the same authors, as they could not find a link between human presence and weight gain in calves in the pre-weaning stage (Neave et al., 2019).

Our findings were likely a result of the running and jumping behaviors being performed due to the same motivation of the PC1 'activity' behaviors when the animals were more motivated to explore the novel environment than to interact with an unknown person. We should highlight that our study was carried out in an experimental farm, where the animals are in constant contact with people. Therefore, human presence was likely not perceived as novel or as a threat by the calves (Forkman et al., 2007). Thus, the animals dedicated more time exploring the environment and less time interacting with the unknown person. As such, the movement of our animals during the voluntary approach test seems to be more related to activity than to fear of human presence (Boissy; Bouissou, 1995), since movement may reflect a motivation of exploratory behavior and not only fearfulness (Forkman et al., 2007).

The temperament of the animals was not linked to starter feed consumption, despite the animals presented an increase in it during our experiment, with emphasis on the animals of the 4 L treatment, which consumed more starter feed when compared to those which received 6 and 8 L. These results were like those of the study by Neave et al. (2019), but different from those described by Neave et al. (2018) and Whalin et al. (2022), who found a link between temperament and starter feed consumption. In those studies, the more active/exploratory animals consumed more that the inactive one.

The lack of association between temperament and starter feed consumption in our study may have occurred due to our animals not undergoing a decrease in milk allowance, as milk supply was maintained throughout the experiment. This opposes what happened in the study by Neave et al. (2018), where the animals underwent a reduction in the milk allowances, receiving 12 L up to 21 days of age and then suffering a 25% reduction until the end of the experiment. In the study by Whalin et al. (2022), there also was a gradual decrease; the animals were fed 12 L up to 30 days of age, then there was a first reduction of supplied milk by 25%, followed by a second reduction after 42 days, based on starter feed consumption.

Among the milk allowances, the animals fed 8 L daily in our trial gained more weight than the calves receiving 6 and 4 L. These were similar results to several previous studies which investigated weight gain in Holstein calves (Rosenberg et al. 2017; Jafari et al., 2021; Suarez-Mena et al., 2021; Kazemi-Bonchenari et al., 2022; Parsons et al., 2022). Weight gain in young bovines depends on several factors, such as genetic potential and breed (Coffey et al., 2006), passive immunity transfer (Elsohaby et al., 2019), occurrence of illnesses (Buczinski et al., 2021), handling (Silva et al., 2017), breeding/housing system (Johnson et al., 2018; Costa et al., 2016), and environmental temperature (Shivley et al., 2018). Together, all these factors affect performance and weight gain of animals in their first life stage.

Our study has some limitations, as it was carried out in an experimental farm where calves are in constant contact with people, which has possibly impacted their temperament positively, since good handling practices are employed in the experimental environment. Therefore, it would be interesting to assess the temperament of the animals in future studies by carrying out research in commercial farms, thus increasing sample size and broadening representation of the temperament of crossbred animals.

We conclude that the novel tests we used were able to show individual differences in the behavior of Holstein x Gyr female dairy calves during their pre-weaning stage. The 'activity' and 'exploration' temperament dimensions were positively associated to the weight gain in the animals. Our study is the first that we know of which has assessed the link between temperament traits, weight gain and starter feed consumption in crossed dairy calves in their pre-weaning stage. Thus, we must highlight that this is a starting point for future studies which seek to broaden our understanding about the temperament of young animals of zebu and its impact on development and efficiency of dairy livestock.

Acknowledgments

We would like to express our gratitude to Maria Eduarda Caçador Branco (UFJF, Brazil)**,** Victor Nascimento Costa e Silva (UFJF, Brazil), Carla Loures (Universidade Federal de Viçosa and Anaclara Resende Schmitz Loures (Universidade Salgado Filho, Brazil) for contributing to the data collect. This work was supported by the Fundação de Amparo à Pesquisa do Estado de Minas Gerais – Brasil (Fapemig) The study was part of the doctoral thesis of the lead author of the Graduate Program in Biodiversity and Nature Conservation at the Federal University of Juiz de Fora (UFJF), Juiz de Fora, Campus, Brazil.

Conflict of Interest Statement

The authors have declared no conflicts of interests.

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6. FINAL CONSIDERATIONS AND IMPLICATIONS

Currently, the national dairy livestock is characterized by farming crossbred Holstein and Gyr cows, which comprise about 80% of the national herd. These crossbred animals have the rusticity of the Gyr breed and the high productivity of the Holstein breed, thus contributing to greater efficiency and sustainability of Brazilian production systems. However, cattle of zebuine origin have a more excitable temperament, being more agitated and reactive during routine handlings, when compared to European breeds. As we can see, the temperament of the animals is directly associated with welfare, milk yield, and efficiency in animal production systems. Thus, studies that aim to investigate these associations are relevant when it comes to crossbred cows, for which research is scarce in this field.

Consequently, the present thesis was developed to deepen the knowledge about the temperament of crossbred dairy cattle and its implications on production systems. Our results show that there are interindividual behavioral differences among crossbred dairy cows and calves when assessed with the use of standardized temperament tests. The behavioral and physiological indicators used have proven to be efficient for classifying animal temperament and, therefore, we highlight the importance of employing different indicators for a more integrated analysis of bovine temperament.

For dairy cows, the findings of the systematic review and meta-analysis did not confirm our initial hypothesis that reactive cows would produce less milk. However, the results of the qualitative analysis revealed that the calmer animals produced more than the reactive ones, which we predicted. In turn, the empirical studies in the present thesis corroborate our hypotheses that animals of a calmer temperament have greater milk yield and quality, in addition to being more energetically efficient, as more net energy is allocated for lactation. Calmer cows were also more efficient in the environmental aspect, since they emitted less methane per liter of milk, and wasted less energy as methane when compared to reactive cows.

Regarding the hormonal assessment, the results also confirmed our hypothesis that cows of a reactive temperament would have higher levels of cortisol in their milk than calmer ones. In fact, our findings show that cows which are more reactive in the milking parlor produced milk with greater concentrations of cortisol and oxytocin, indicating that both hormones are associated with high behavioral reactivity. Despite the greater reactivity of the animals, there was no reduction in milk yield due to their reactive temperament. This may have happened due to the conditions of the experiment, in which the stress of the animals could have been insufficient to cause alterations in the productivity of the cows.

For dairy calves, the three behavioral tests used were able to reveal the temperament traits of the animals while still young. Our results show that more active calves, which explored the environment longer, and those which interacted less with the unknown person, had a greater average daily weight gain in the pre-weaning stage, with the animals fed 8 liters of milk gaining more weight. Despite the differences in weight gain, animals of distinct temperaments did not differ in starter feed consumption.

Therefore, based on the knowledge generated by our studies, we recommend that dairy cattle have their temperament assessed, which can be done during routine handling, taking little time or effort. The assessment may begin in calfhood, as temperament is directly associated with development since birth. Observing temperament in their juvenile stage contributes to predicting animal reactivity and the productivity of dairy cows when they reach their reproductive period. In addition, assessing the temperament of dairy cattle would help in formulating recommendations for good handling practices, aiming not only for the improvement of production indices, but also the improvement of the welfare of both animals and handlers and, also, the potential reduction of environmental impacts. Thus, it will be possible to create conditions to meet the demands of society and the consumer market, which has become increasingly demanding when it comes to animal production.

APPENDIX OF THESIS

APPENDIX 1. Artigo publicado no *Journal of Animal Science*: **MARÇAL-PEDROZA, M. G.,** CANOZZI, M. E. A., CAMPOS, M. M., & SANT'ANNA, A. C. Effects of dairy cow temperament on milk yield: A systematic review and meta-analysis. **Journal of Animal Science**, v. 101, p. skad099, 2023. https://doi.org/10.1093/jas/skad099

APPENDIX 2. Artigo publicado na *Animal* - The international journal of animal biosciences: **MARÇAL-PEDROZA, M. G.,** M. M. CAMPOS, J. P. SACRAMENTO, L. G. R. PEREIRA, F. S. MACHADO, T. R. TOMICH, AND A. C. SANT'ANNA. Are dairy cows with a more reactive temperament less efficient in energetic metabolism and do they produce more enteric methane? **Animal.** v. 15, p.100224, 2021. https://doi:10.1016/j.animal.2021.100224

APPENDIX 3. Artigo publicado na PLOS ONE: **MARÇAL-PEDROZA, M. G.,** CAMPOS, M. M., MARTINS, M. F., SILVA, M. V. B., PARANHOS DA COSTA, M. J. R., NEGRÃO, J. A., SANT'ANNA, A. C. Is the temperament of crossbred dairy cows related to milk cortisol and oxytocin concentrations, milk yield, and quality?. **Plos one,** v. 18, n. 6, p. e0286466, 2023. https://doi.org/10.1371/journal.pone.0286466

APPENDIX 4. Artigo de divulgação científica publicado na revista AG-revista do produtor: **MARÇAL-PEDROZA, M. G.,** CAMPOS, M. M., SANT'ANNA, A. C. 2022. O risco das arriscas. **AG-revista do produtor**. Abril de 2022, ano 25, n. 255, p. 51-54

APPENDIX 5. Artigo de divulgação científica publicado na Revista digital Leite Integral: **MARÇAL-PEDROZA, M. G.,** CAMPOS, M. M., SANT'ANNA, A. C. 2022. Vacas mais calmas emitem menos metano. **Revista Leite Integral**. Outubro de 2022, ano 25, n. 255, p.1-8 www.revistaleiteintegral.com.br

APPENDIX 6. Artigo de divulgação publicado na Folha de São Paulo, na seção Mercado. **MARÇAL-PEDROZA, M. G.,** CAMPOS, M. M., SANT'ANNA, A. C. 2022. Vaca brava emite 40% mais gás metano e produz menos leite, afirma Embrapa. 14 de dezembro de 2021. https://acervo.folha.uol.com.br/digital/

APPENDIX 7. Artigo de divulgação publicado no site da Embrapa Gado de Leite. **MARÇAL-PEDROZA, M. G.,** CAMPOS, M. M., SANT'ANNA, A. C. 2022. Vacas "reativas" emitem mais metano e produzem mais leite. 14 de dezembro de 2021. https://www.embrapa.br/gado-de-leite

APPENDIX 8. Coorientação no trabalho de conclusão do curso em Ciências Biológicas, intitulado "*Relações entre o comportamento e crescimento em bezerras leiteiras na fase de aleitamento mantidas em baias individuais",* do aluno Victor Nascimento Cerqueira Silva.

APPENDIX 9. Participação como membro avaliador no trabalho de conclusão de curso em Ciências Biológicas, intitulado, intitulado "*A Relação entre Temperamento e Comportamentos em Cativeiro de Papagaios do Gênero Amazona",* da aluna Ana Luíza de Almeida Cândido Vargas.

APPENDIX 10. Participação como membro avaliador no trabalho de conclusão de curso em Ciências Biológicas, intitulado, intitulado "*A capacidade de voo e a aversão ao ser humano podem interferir na rotina comportamental de papagaios do gênero Amazona em cativeiro?",* da aluna Maria Eduarda Caçador Branco.

Journal of Animal Science, 2023, **101**, 1–12
https://doi.org/10.1093/jas/skad099
Advance access publication 2 April 2023
Animal Behavior and Cognition

OXFORD

AMERICAN SOCIETY OF ANIMAL SCIENCE

Effects of dairy cow temperament on milk yield: a systematic review and meta-analysis

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Abstract

The temperament of dairy cows interferes in milk yield and quality, but there is a lack of consensus throughout the literature. Thus, systematic review (**SR**) and meta-analysis (**MA**) methodologies were used to assess the effects of dairy cow temperament on milk yield. Our literature search included four electronic databases (CABI Abstracts, Web of Science, PubMed, and Scopus) and bibliographies of the publications included on MA. As inclusion criteria, we considered publications about the temperament of lactating cows and its effect on daily milk yield and total milk yield (whole lactation). A random effect-MA was carried out separately for daily milk yield and total milk yield related to each class of cows' temperament, 'low' (low reactivity, calm animals), 'intermediate' (intermediate reactivity), and 'high' (high reactivity, reactive animals). A total of eight publications reporting 75 trials were included in the analyses for daily milk yield, and three publications reporting nine trials for total milk yield. For daily and total milk yield the heterogeneity between publications was high (I^2 = 99.9%). Cows of European breeds with intermediate temperament produced less milk daily than the calm (P = 0.020) and reactive ones (P < 0.001). In the case of primiparous cows, those with intermediate temperament produced less milk daily (P < 0.001) than the reactive ones, while for multiparous, the intermediate produced less than calm (P = 0.032) and reactive cows (P < 0.001). Regarding the stage of lactation, cows evaluated throughout lactation with a calm temperament tended (P = 0.081) to produce more milk than the intermediate ones, but less than the reactive ones (P < 0.001). For total milk yield, reactive cows tended to produce more than the calm (P = 0.082) and intermediate (P = 0.001) ones. Among European and primiparous cows, reactive cows produced more than the intermediate (P = 0.001). According to our results, we cannot confirm what we expected, that calmer cows would be the most productive for both daily and total yield.

Lay Summary

Individual differences in the behavior of dairy cows can affect their productive performance. In an attempt to summarize the scientific information available, we conducted a systematic review and meta-analysis to identify the effects of dairy cows' temperament on milk yield. We hypothesize that calmer cows would produce more milk. We found nine publications with quantitative data available to be included in a meta-analysis. Eight additional publications that addressed the topic of interest but did not present data enough to be included in the meta-analysis (i.e., evaluated the relationships between temperament and milk yield using correlations or regressions) were used to perform a qualitative synthesis. The results of our meta-analysis indicated that the reactive cows were more productive than the calm or intermediate ones, contradicting our initial hypothesis. According to the results of the qualitative synthesis, most of the publications reported a negative association between reactive temperament and milk yield, indicating that calmer cows would produce more milk. We concluded that there are divergences in the information available about the temperament and production of dairy cows. We highlight the need for greater methodological and analytical standardization to allow a broader quantitative synthesis of the temperament effects on milk yield.

Key words: behavior, dairy cattle, performance, personality, reactivity

Abbreviations: SR, systematic review; MA, meta-analysis; NOS, Newcastle-Ottawa Scale; MD, mean difference; 95% CI, confidence interval of 95%

Introduction

Animal temperament is a complex trait that encompasses several behavioral aspects. According to Réale et al. (2007), temperament may be understood as the individual differences in the behavior of animals, in response to their environmental circumstances, given that those differences are relatively consistent over time and in distinct situations. In production animals, this trait may be assessed by observing the behavior of the animals during routine handlings, for example in the milking parlor (milking temperament) (Sawa et al., 2017), or through standardized tests, such as flight speed, reactivity in the handling corral, and flight distance (handling temperament) (Sutherland and Huddart, 2012). For dairy cows, the temperament is usually measured based on the cows' reactivity during milking, considering the intensity of reactions to

Received November 12, 2022 Accepted March 29, 2023.

milking procedure, such as leg movements and kicks (Breuer et al., 2000; Rousing et al., 2004).

In dairy cows, temperament has been associated with productivity (milk yield, quality, and milkability); however, this is still a controversial topic. Contradictory results are reported in the scientific literature. Some studies report that calmer cows produce more milk (Sutherland and Dowling, 2014; Hedlund and L¢vlie, 2015; Cerqueira et al., 2017), with higher fat and protein contents (Kruszyński et al., 2013; Antanaitis et al., 2021). Others show that the reactive ones are more productive, with higher milk yield (Rousing et al., 2004; Sawa et al., 2017), milk fat and protein contents (Czisz-ter et al., 2016) than the calm ones. In addition, there are still studies that do not find association between temperament and productive parameters (Szentléleki et al., 2008, 2015; Orbán et al, 2011; Sutherland et al., 2012). Furthermore, there is a lack of standardization regarding the measurement used to assess the temperament of the animals throughout the studies, which may hinder the comparison of findings.

The behavior of dairy cows and its relationship with milk yield and quality are topics that interest both consumers and producers, due to their relationships with animal welfare, production efficiency, and sustainability of the livestock industry (Risius and Hamm, 2017; van Dijk et al., 2019; Marçal-Pedroza et al., 2021). Moreover, assessing the effects of temperament on performance may contribute to the improvement of animal welfare, as it aids in the identification of new welfare indicators (Neja et al., 2015).

Thus, in this study, we used systematic review (**SR**) and meta-analysis (**MA**) methodologies to explore the influence of dairy cattle temperament on milk yield and quality. We hypothesize that calmer cows would produce more milk. The aim of this study was to evaluate the scientific evidence available in the literature using SR–MA to identify the effect of the dairy cows' temperament on milk yield.

Materials and Methods

Research question and protocol

This is a theoretical study and therefore did not need to be evaluated by an ethics committee. The systematic review followed the PRISMA guidelines (Page et al., 2021). The search strategy was defined based on PICO terms: population, intervention, comparison, and outcome (Brown et al., 2006). For population, we used the terms "lactating cow" or "dairy cow" or "dairy cattle"; for intervention, "temperament" or "reactivity" or "personality"; and for outcome, "milk production" or "milk yield" or "somatic cell count" or "protein" or "fat".

Dairy cow was the population of interest. The interventions were the different temperament types. As comparison, we considered groups of cows classified as different temperaments in 'Low' (lower reactivity class, also referred to as calm animals), 'Inter' (intermediate reactivity class, also referred to as normal animals), and 'High' (higher reactivity class, also referred to as reactive or nervous or excitable or aggressive animals in the publications reviewed). The outcomes of interest were daily milk yield, total milk yield (whole lactation), and milk quality, but the present study will report only the results regarding yield, despite our database search having included all these measures (Figure 1). To be included in our SR, the publications had to assess at least one of the response variables of interest in association with dairy cows' temperament.

A search protocol was previously developed, and screening tools were adapted from forms used in previous studies (Canozzi et al., 2017, 2019) and tested prior to their application.

Search methods for the identification of publications

The systematic literature search was conducted from September to December 2020 in four electronic databases—CABI Abstracts (Thomson Reuters, 1910–2020), ISI Web of Science (Thomson Reuters, 1900–2020), PubMed (MEDLINE, 1940–2020), and Scopus (Elsevier, 1960–2020). Additional searches were carried out using the literature cited from the publications included in the MA to include peer-reviewed publications not identified by the literature search as well as abstracts published in conference proceedings that were relevant to the subject. All references were exported to EndNote Web software (Clarivate Analytics, Jersey, England) to organize and manually remove duplicate references.

Publications selection criteria and relevance screening

We applied the screening in all citations identified by the literature search using three stages. Before starting the screening, four reviewers were previously trained using 30 publications.

In the first stage, we aimed to identify possible citations of interest among those selected by the search. Each citation was evaluated by reading only the title and applying five simple questions (Supplementary material – S1). This stage was carried out by two researchers independently. In the next step, the remaining citations were evaluated by the same two reviewers, assessing the title, keywords, and abstract, based on eight questions (Supplementary material – S2). When both evaluators answered "no" to one or more questions, the citation was excluded, and, in case of conflicting answers, both evaluators would consensually make the decision. A citation was considered relevant when it was peer-reviewed or conference proceedings assessing dairy cows' temperament, and its relationships with milk yield. In this last stage, we did not apply any restrictions to language or year of publication. The Microsoft Excel software was used throughout all screening stages.

Methodological assessment and data collection process

The first and last authors were responsible for the extraction of data from the selected publications. The relevance of the previously selected publications was confirmed by reading them in full.

The evaluated publications were restricted to the languages in which the research team was fluent (English, Spanish, and Portuguese). Data extracted from each publication was divided into characteristics related to population, intervention, measures, and outcome data, in addition to journal name, author(s), year of publication, and original language. The data extraction forms were adapted from previous studies (Canozzi et al., 2017, 2019).

We need to highlight the diversity of methods found within the selected publications, with different ways to assess temperament and data analyses, hindering the summarization of results. Furthermore, some of these papers allowed for only a qualitative analysis of data (Breuer et al., 2000; Rousing et al., 2004; Bertenshaw et al., 2008; Szentléleki et al., 2008;

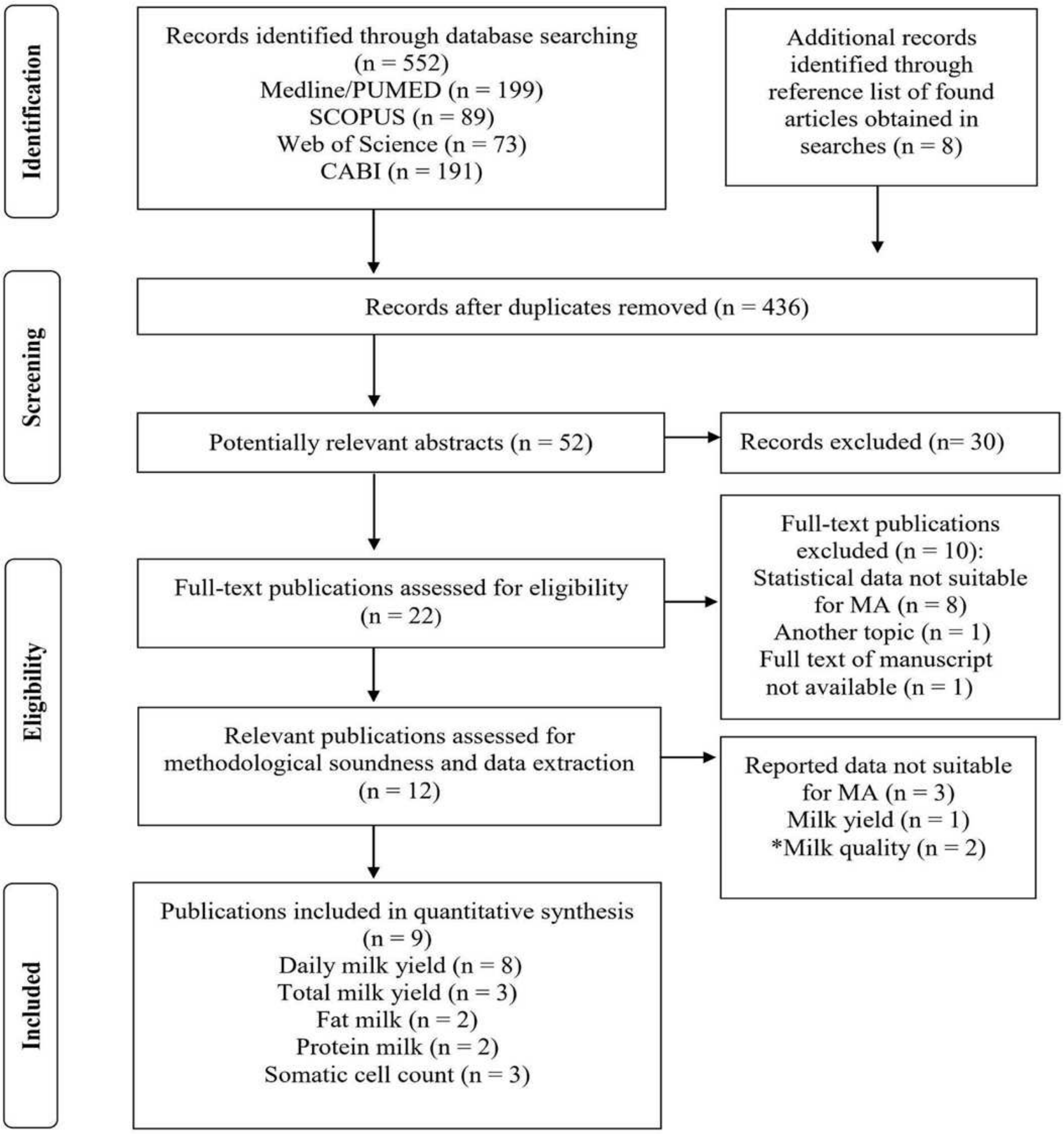

Figure 1. Flow diagram indicating the number of citations and publications included and excluded in each level of the systematic review on temperament of dairy cows and milk yield and milk quality, adapted from PRISMA guidelines (Page et al. 2021). All search results are included in the diagram to allow a better understanding of the total number of records found. *Data from both procedures (milk yield and milk quality) are presented in the flow diagram to allow the researchers to update the same systematic review.

Dodzi and Muchenje, 2011; Sutherland and Dowling, 2014; Hedlund and L¢vlie, 2015; Cerqueira et al., 2017), as they presented results as correlations and/or regressions, making their inclusion in the MA impossible. Therefore, the included publications were divided into two groups: one for meta-analytical evaluation, and the other for qualitative evaluation.

Considerations for data collection and manipulation

A table with the data were created for each of the results of interest, including mean, standard deviation of mean or another dispersion measure, *P*-value, and the number of evaluated cows in each comparison: (Low vs. Inter), (Low vs. High), and (Inter vs. High), with each comparison for a temperament indicator (measure) being regarded as a 'trial'.

For daily yield results, the obtained values refer to the average daily milk yield (in kg/day); and total milk yield (sum of milk yield throughout the whole lactating period, in kg). Some publications presented a greater number of scores and distinct classifications for temperament (Orbán et al., 2011; Gergosvka et al., 2014; Neja et al., 2017), so we standardized them to consider only three temperament types (Low, Inter, High). With these three temperaments, we formed three comparison groups for the analysis of subgroups: group 1 (Low × Inter), group 2 (Low × High), and group 3 (Inter × High).

For two publications that reported only the means values and *P*-values for means comparisons, without a measure of dispersion (Neja et al., 2015; Sawa et al., 2017), an estimate of common standard deviation was calculated using *t*-statistics and assuming the data was normally distributed, based on the following equation (Ceballos et al., 2009; Mederos et al., 2012):

$$SP = \frac{(x2 - x1)}{t(\alpha dfE)\sqrt{(1/n2) + (1/n1)}}$$

were $\chi_2 - \chi_1$ represents the means difference; $t(\alpha d f E)$ is the percentile of the reference distribution, and n is the sample size of each group.

Quality assessment

The risk of publication bias in the publications was assessed using Newcastle–Ottawa Scale (**NOS**) (Wells et al., 2014). This is an appropriate tool to assess the quality of observational and not experimental randomized trials, based on three main criteria: 'Selection', 'Comparability', and 'Outcome'. The publications receive one 'star' for each quality item included in the criteria of selection and outcome and a maximum of two 'stars' for comparability. In the end, the quality of the publications is expressed on a 9-point scale (Wells et al., 2014).

Meta-analysis

The publications which presented qualitative data that allowed us to estimate the mean difference (**MD**) between the evaluated temperament types and a confidence interval of 95% (**95% CI**) were included in this MA. The statistical analyses were carried out using the Stata V 16.0 software (StataCorp., Texas, EUA).

In subgroup analysis, we carried out an MA separately with datasets consisting of, at least, two individual publications which investigated the same comparative group and the same outcome of interest. The MA results were shown considering MD and 95% CI. Cochran's Q (chi-square test for heterogeneity) and I^2 (percentage of total variation between publications due to heterogeneity and not by chance) were obtained based on the evaluated temperament type (groups 1, 2, and 3) and the outcome variable. The magnitude of I^2 was interpreted in the orders of 25%, 50%, and 75%, and considered as low, moderate, or high heterogeneity, respectively (Higgins et al., 2003).

Publication bias

Publication bias was assessed through a funnel plot and the statistical tests of Begg's correlation and Egger's linear regression. Bias was considered as present based on the visual analysis of the plot and if at least one of the statistical methods was significant ($P < 0.10$). In case there was any indication of the presence of bias, we used the "trim-and-fill" method to estimate its extension (Duval and Tweedie, 2000), which allows us to estimate the number of publications that should be included in the analysis in order for the graph to become symmetrical.

Meta-regression analysis

Univariate meta-regression was performed to identify possible sources of heterogeneity that could influence the results. The variables explored were: year of publication; geographic regions (North America, South America, Europe, Africa, Asia, Oceania); experiment time (days); sample size; racial group (European or Zebu); parity (primiparous or multiparous); lactation stage (beginning = first weeks of lactation or throughout lactation = over the whole lactation); observer effect (unfamiliar person, familiar person or milker); blinding (no, yes, not reported, or not applicable); clustering (no, yes, or not applicable); and identified and controlled confounders (no, yes, or not applicable). The results were reported only for variables that were significant.

Cumulative meta-analysis and influential publications

The cumulative MA was carried out to estimate the effect of the different temperament types on daily and total milk yield each time a new publication was published, to demonstrate the pattern of evidence over time (Borenstein et al., 2009). A sensibility analysis was carried out to check if a certain publication had influenced the effect measurement (MD), by successively removing manually one publication at a time and assessing if MD varied ± 30% after re-inserting the publication and removing the next one.

Results

Publication selection

Our database search identified 552 citations. From that total, 52 were potentially relevant abstracts and 22 were selected for eligibility. Finally, 12 publications were fully read, and among those, nine had their data extracted (Figure 1) and included in this MA, with a total of 84 trials. For daily milk yield, a total of eight publications reporting 75 trials were included, and for total milk yield, it was considered three publications reporting nine trials.

The main characteristics of the included publications are shown in Tables 1 and 2. Three publications were excluded for presenting insufficient data for quantitative analysis (Table 3). We contacted the authors, but no numerical data were obtained, and, since we could not extract them manually, the publication was excluded.

Eight publications evaluated daily milk yield, and three, total yield. The relationship of temperament with daily milk yield was assessed in 26,614 cows, and total milk yield in 23,885 cows.

Risk of bias

The NOS tool was used to analyze the risk of bias, considering the type of publications used in this MA (observational) (Table 4). Of the nine publications included, four (Sutherland and Huddart, 2012; Sutherland et al., 2012; Neja et al., 2015; Sawa et al., 2017) were considered of moderate quality (score between 5 and 7), and the other seven were scored as high quality (scores 8 or 9). This result indicates a moderate to high quality and moderate to low risk of bias in the publications included.

Meta-analysis

In our MA, nine publications were included, six of which evaluated only daily yield and three, daily and total milk yield. The number of publications and types of outcome measures are shown in Table 1. For the analyses, in addition to temperament, the influence of breed, parity, and stage of lactation on milk yield were also evaluated.

Effect of temperament on daily milk yield

The daily yield was the most frequently studied outcome and was shown in eight of the nine publications included in the MA

Table 1. A descriptive summary of each relevant study included in the meta-analysis (n = 9) for daily milk yield and total milk yield.

Reference	Country	Study population (breed/ sample size)	Temperament indicator	*Comparison groups	Outcome parameter
Praxedes et al. (2009)	Brazil	Zebu (Gyr)/ 2.507	Other	Group 1 Group 2 Group 3	Total milk yield
Orbán et al. (2011)	Hungry	Holstein Friesian/ 69 Jersey/ 283	Crush score (reactivity in score in the squeeze chute)	Group 1 Group 2 Group 3	Daily milk yield
Sutherland and Huddart (2012)	New Zealand	Holstein Friesian/ 40	Flight speed (in m/s)	Group 1 Group 2 Group 3	Daily milk yield
Sutherland et al. (2012)	New Zealand	Holstein Friesian/ 30	Flight speed (in m/s)	Group 1 Group 2 Group 3	Daily milk yield
Gergovska et al. (2014)	Bulgaria	Black and White/ 143	Reactivity in scores in the milking parlor	Group 1 Group 2 Group 3	Daily milk yield
Neja et al. (2015)	Poland	Holstein Friesian/ 11.629	Reactivity in scores in the milking parlor	Group 1 Group 2 Group 3	Daily milk yield/ Total milk yield
Neja et al. (2017)	Poland	Holstein Friesian/ 158	Reactivity in scores in the milking parlor	Group 1 Group 2 Group 3	Daily milk yield
Marçal-Pedroza et al. (2020)	Brazil	Zebu-crosses (Girolando)/ 31	Reactivity in scores in the milking parlor/ Steps or kicks/ FSK[1] (or MOV)/ Entrance time/ Crush score/ Flight speed/ Flight distance/ Novel object test	Group 1 Group 2 Group 3	Daily milk yield
Sawa et al. (2017)	Poland	Holstein Friesian/ 12.028	Reactivity in scores in the milking parlor	Group 1 Group 2 Group 3	Daily milk yield/ Total milk yield

[*] Comparison groups between temperament types, with group 1: low vs. inter; group 2: low vs. high; group 3: inter vs. high. [1]FSK or MOV: Score based on the performance of flinching, stepping, and kicking or sum of the number of kicks and steps during milking.

(I^2 = 99.9%). Mean difference (MD) in daily yield (n = 8 publications, 75 trials) among Group 3 (i.e. Inter vs. High cows) was −0.82 kg of milk/day (95% CI: −1.01, −0.63; P < 0.001), suggesting that Inter cows produced less milk than the High ones, with high heterogeneity among publications (I^2 = 99.4%).

Effect of temperament on daily milk yield considering breed, parity, and lactation stage

For the effect of breed on temperament, only studies with European breed (n = 7 publications, 35 trials) were evaluated, since only one publication assessed Zebu cows. The comparison among Group 1 (n = 6 publications, 35 trials) resulted in an MD of 0.67 kg/milk (95% CI: 0.10, 1.24; P = 0.020), indicating that daily milk yield was lower for Inter than for Low cows, with high heterogeneity between publications (I^2 = 99.9%). In the comparison among Group 3 (n = 6 publications, 35 trials), MD was −1.18 kg/milk (95% CI: −1.41, −0.95; P < 0.001), with Inter cows producing less milk than High. In summary, for studies with European breeds, cows with intermediate temperament produced less milk than the calm and reactive ones.

Among primiparous animals (n = 4 publications, 50 trials) in Group 3, Inter cows produced less milk (MD = −0.74 kg/milk; 95% CI: −0.93, −0.56; P < 0.001) than High ones, with high heterogeneity among publications (I^2 = 96.4%). Among multiparous (n = 6 publications, 25 trials) in Group 1 (n = 4 publications, 25 trials), Inter cows produced less milk (MD = 0.70 kg/milk; 95% CI: 0.07, 1.35; P = 0.032) than Low ones, with high heterogeneity among publications (I^2 = 99.7%). In Group 3 (n = 5 publications, 25 trials), Inter individuals produced less than High ones (MD = −1.08; 95% CI: −1.54, −0.61, P < 0.001), with a 99.8% heterogeneity. So, intermediate cows produced less than the calm and reactive ones, without difference between the last ones.

When assessing the influence of the lactation stage (n = 3 publications, 50 trials) on daily milk yield, we only found significance for experiments carried out throughout lactation, but not at the beginning of lactation. In Group 1 (n = 3 publications, 13 trials), MD was 0.73 kg/milk (95% CI: −0.09, 1.55; P = 0.081), that is, Low cows tended to have a greater daily milk yield than Inter ones, with high heterogeneity among publications (I^2 = 99.7%). In Group 2 (n = 3 publications, 13 trials), MD was −1.01 kg/milk (95% CI: −1.34, −0.68; P < 0.001), Low cows produced less milk than High, with high heterogeneity among publications (I^2 = 97.5%). In Group 3 (n = 3 publications, 13 trials), Inter cows were less productive (MD = −1.24 kg/milk; 95% CI: −1.99, −0.49; P = 0.001) than the High ones, with high heterogeneity among publications (I^2 = 98.2%). In summary, the daily milk yield was higher for reactive, followed by calm and intermediate cows, which had the lowest milk yield.

Effect of temperament on total milk yield

Results for total milk yield were found in three publications (n = 9 trials), with high heterogeneity among publications (I^2 = 99.9%). In Group 2 (n = 3 publications, 9 trials), we obtained

Table 2. Descriptive characteristics of nine publications included in the meta-analyses (MA).

Variable	Categories	Number of publications
Study design	Observational study	7
	Controlled trial	2
Publication type	Peer-reviewed	8
	Conference proceedings	1
Indicator temperament	Reactivity in scores in the milking parlor	5
	Steps or kicks	1
	FSK (or MOV)[1]	1
	Entrance time (in s)	1
	Crush score	2
	Flight speed (in m/s)	3
	Flight distance (in m)	1
	Novel object test	1
	Other	2
Treatment (type of temperament)	Low	9
	Intermediate	9
	High	9
Year of publication	2009-2014	5
	2014-2020	4
Breed	Not reported	0
	European	2
	Zebu/ Zebu-crosses	7
Calving order	Primiparous	3
	Multiparous	4
	Primiparous and multiparous	1
Lactation stage	Not reported	3
	Beginning of lactation	2
	Throughout lactation	4
Housing system	Not reported	3
	Free-stall or tie stall	3
	Loose housing/ open yard	1
	Pastures/ paddock	2
Milking system	Not reported	6
	Herringbone-milking parlor	2
	Parallel-milking parlor	0
	Tandem_milking parlor	0
	Rotary (Carousel) parlor	1
	Robotic milking parlor	0
Who performed the procedure	Not reported	5
	Unfamiliar person, technician, or researcher (authors)	4
	Familiar person or milker	0
	Other	
Outcome assessed	Daily milk yield	8
	Total milk yield	3
Continent	South America	2
	Oceania	2
	Europe	5
Sample size	$N < 100$	3
	$n \geq 100$ and $n < 1000$	3
	$n \geq 1000$	3

[1]FSK or MOV: Score based on the performance of flinching, stepping, and kicking or sum of the number of kicks and steps during milking.

Table 3. List of relevant publications excluded from the final dataset in the meta-analyses (MA).

Reference	Country	Indicator temperament	Temperament type	Outcome parameter	Reason for exclusion
Szentléleki et al. (2015)	Hungry	Reactivity in scores in the milking parlor	Low/ High	Total milk yield	Insufficient numerical data
Kalińska and Slósarz (2016)	Poland	Reactivity in scores in the milking parlour	Low/ Inter/ High	Fat milk/ Protein milk	Insufficient numerical data
Abdel et al. (2017)	Egypt	Reactivity in scores in the milking parlor	Low/ Inter/ High	Daily milk yield/ Total milk yield/ Fat milk/ Protein milk	Insufficient numerical data

Table 4. Risk of bias assessment in the nine studies included in the final dataset of the meta-analyses (MA).

Reference	Selections				Comparability	Outcome			Total
	Adequate definition of temperament groups	Representativeness of the cows used	Selection of divergent temperament groups	Control for disease or incidents that affected the outcome	Adjustment for confounders	Assessment of outcome	Enough time of outcome recording	Adequacy of outcome recording	
Praxedes et al (2009)	☆	☆	☆		☆☆	☆	☆	☆	8
Orbán et al. (2011)	☆	☆	☆		☆☆	☆	☆	☆	8
Sutherland and Huddart (2012)	☆	☆	☆	☆		☆	☆	☆	7
Sutherland et al. (2012)	☆	☆	☆			☆	☆	☆	6
Gergovska et al. (2014)	☆	☆	☆		☆☆	☆	☆	☆	8
Neja et al. (2015)	☆	☆	☆	☆		☆		☆	6
Neja et al. (2017)	☆	☆	☆		☆☆	☆	☆	☆	8
Sawa et al. (2017)	☆	☆	☆			☆		☆	5
Marçal-Pedroza et al. (2020)	☆	☆	☆	☆	☆☆	☆	☆	☆	9

an MD of −1,217.57 kg/milk (95% CI: −2,589.08, 153.94), indicating that Low cows tended (P = 0.082) to produce less milk than the High ones, with high heterogeneity among publications (I^2 = 99.9%). In Group 3 (n = 3 publications, 9 trials), Inter animals had a yield −1,062.45 kg/milk (95% CI: −1,288.35, −836.54; P < 0.001) lower when compared to High ones, with high heterogeneity among publications (I^2 = 99.9%). It indicates that reactive cows produced more milk than the calm and intermediate ones.

Effect of temperament on total milk yield considering breed, parity, and lactation stage

For breed effect, subgroup analysis was carried out only with European breeds (n = 2 publications, 6 trials), since only one publication evaluated Zebu animals. In Group 3 (n = 2 publications, 6 trials), cows of Inter temperament yielded less milk (MD = −414.97 kg/milk; 95% CI: −656.05, −173.90; P = 0.001) than High ones, with high heterogeneity among publications (I^2 = 99.9%).

For primiparous cows (n = 2 publications, 6 trials), we observed difference only for Group 3 (n = 2 publications, 6 trials). High cows produced 414.97 kg (98% CI: −656.05, 173.90; P = 0.001) more milk than Inter ones, with high heterogeneity between publications (I^2 = 99.9%). Among the three publications included, none assessed total milk yield in multiparous cows.

Regarding the lactation stage, only one of the three publications described it, which made such a comparison impossible.

Publication bias

The data included in this MA is quite heterogenous, therefore, results must be interpreted carefully. Both for daily and total milk yield, the asymmetry found in the funnel plot was confirmed by Egger's statistical test (P < 0.001 for both tests), and Begg's test was not significant (P = 0.14; P = 0.75, respectively), with no insertion of new publications by the "trim-and-fill" test.

Meta-regression analysis

Meta-regression results on daily milk yield

Eight publications (n = 75 trials) were inserted in this analysis. Results showed that 99.9% of the variation among publications was due to chance. None of the eight variables were significantly associated with daily yield, and only three contributed to explaining the variation among publications: sample size (4.6%), lactation stage (4.2%), and identified and controlled confounders (5.5%).

Meta-regression results on total milk yield

Three publications (n = 9 trials) were considered in the meta-regression, and it was evidenced that 99.9% of the variation among publications was due to chance. Meta-regression indicated that with the increase of one year in the year of publication, there was an increase of 233.83 kg in the predicted value (P = 0.050). Publications carried out in Europe showed a 1,905.75 kg (P = 0.019) increase in the predicted value for total milk yield when compared to publications conducted in South America. The number of evaluated animals showed a significant effect, and the increase of one experimental unit rose the predicted value of 0.20 kg of milk (P = 0.022). Publications with animals of Zebu breeds showed a decrease of 1.90 kg in the predicted value (P = 0.019) when compared to those carried out with European cattle. When clustering factors were considered, the predicted value increased by 1.90 kg (P = 0.019) (Table 5).

Cumulative MA and sensitivity analysis

Daily milk yield

In the cumulative MA (2011–2020) for daily yield, there was clear evidence of a change in the estimated yield between temperament groups, going from a positive (MD = 0.16 kg/milk) to a negative value (MD = −0.54 kg/milk). Sensibility analysis showed that removing two publications (Orbán et al., 2011; Sutherland et al., 2012) reduced MD from −0.24 kg to −0.34 and −0.31 kg/milk, respectively. Removing the publication by Neja et al. (2017) increased MD from −0.23 to −0.09 kg/milk.

Total milk yield

In the cumulative MA (2009–2017) for total yield, there was any evidence of changes through the years. Removing the publication by Neja et al. (2015) decreased MD from −796.10 kg to −1,291.86 kg/milk, while removing the publication by Praxedes et al. (2009) increased MD from −796.10 to −171.43 kg/milk.

Qualitative analysis

Some publications assessed the influence of temperament on milk yield using correlation and regression analyses, thus, they were not included in the MA. Due to their relevance, they were considered and analyzed in a qualitative way (Table 6).

All eight publications were carried out with European breeds and evidenced different patterns of relationship between temperament and milk yield. In one of them, milk yield was greater for reactive animals (Rousing et al., 2004), where cows that took more steps in the milking parlor yielded more milk (in kg/day), with Odds Ratios of 1.5 (20-to-30-liter production) and 2.2 (production of over 30 liters). In its turn, Szentleleki et al. (2008) did not find an association between temperament and milk yield using milking reactivity scores as temperament indicators.

Most of the publications (n = 6) reported a negative relationship between temperament and yield, that is, calmer cows produced more milk, as reported by Breuer et al. (2000) (r = −0.38; P < 0.05 for milking reactivity scores); Bertenshaw et al. (2008) (r = −0.25; P = 0.01 for steps); Dodzi and Muchenje (2011) (r = −0.17; P < 0.05 for kicks); Sutherland and Dowling (2014) (r = −0.23; P < 0.05 for milking reactivity scores); Hedlund and L¢vlie (2015) (R^2 = −0.32; P < 0.02 for steps); and Cerqueira et al. (2017) (r = −0.10; P = 0.00 for steps). Bertenshaw et al. (2008) report in the regression analysis, a 7.1% of the variation in productivity occurred due to the number of steps and kicks in the presence of humans (R^2 = 0.07; P < 0.001), which did not occur in the absence of humans (R^2 = 0.002; NS).

Discussion

An SR followed by MA was carried out to quantitatively assess the effects of dairy cows' temperament on milk yield. According to our MA results, calmer cows were not the most

Table 5. Univariate meta-regression results showing significant (P < 0.05) and marginally significant (0.05 ≤ P < 0.10) covariates investigated as potential sources of study heterogeneity for total milk yield. The explained results for each of the covariates included in the meta-analysis are presented for daily production.

No. of studies [1] (trials) [2]	Covariate (trials)	Estimate [3]	*95% CI* [4]	P-value	I^2 (%)	Adj-R^2 (%)
Total milk yield 3 (9)	Null model	−796.10	−1,765.62, −173.41	0.095	99.9	NA
	Publicarion year (9)	233.83	−0.52, −468.18	0.050	99.9	0
	Continent	–	–	0.019	99.9	0
	South America (9)	Reference				
	Europe (9)	1,905.75	413.93, 3,397.57	0.019		
	Sample size (9)	−2,563.72	0.04, 0.36	0.022	99.9	0
	Cattle group (9)	-	-	0.019	99.9	0
	Zebu (9)	Reference				
	Europe (9)	1,905.75	−3,397.57, −413.93	0.019	–	–
	Clustering (9)	-	-	0.019	99.9	0
	No (9)	Reference				
	Yes (9)	1,905.75	413.93, 3,397.57	0.019		

I^2 between-study residual variation; Adj-R2 percentage of the residual variation.
[1] Number of studies included in the meta-regression.
[2] Number of trials included in the meta-regression.
[3] Standard mean difference of the effect size.
[4] These values represent 95% confidence intervals (CI) for the effect size.

Table 6. A descriptive summary of each relevant study (*n* = 8) that was included in the qualitative synthesis (could not be included in the MA) for daily and total milk yield.

Reference	Country	Study population (breed/ sample size)	Temperament indicator	Outcome parameter
Breuer et al. (2000)	Australia	Holstein Friesian/ 100-200	Reactivity in scores in the milking parlor, steps, and other	Total milk yield
Rousing et al. (2004)	Denmark	Holstein Friesian/ 1.196	Steps, kicks, and other	Daily milk yield/
Bertenshaw et al. (2008)	United Kingdom	Holstein Friesian/ 148	Steps and kicks	Daily milk yield
Szentléleki et al. (2008)	Hungary	Holstein Friesian/ 17	Reactivity in scores in the milking parlour	Daily milk yield
Dodzi and Muchenje (2011)	South Africa	Holstein Friesian/ 7, Jersey/ 7, and crossbred/ 7	Steps, kicks, FD, and FS[1]	Total milk yield
Sutherland and Dowling (2014)	New Zealand	Holstein Friesian/ 150	FSK, FD[1]	Total milk yield
Hedlund and L¢vlie (2015)	Sweden	Holstein Friesian/ 29, and Swedish Red and White cattle/ 27	Steps, kicks, and NOT	Daily milk yield
Cerqueira et al. (2017)	Portugal	Holstein Friesian/ 2.903	Steps, and kicks	Total milk yield

[1]FD: Flight distance; FS: Flight speed; FSK: Score based on the performance of flinching, stepping, or kicking during milking; NOT: Novel object test.

productive for both daily and total milk yield, against our initial hypothesis. Despite the significant number of publications, only nine had enough information to be included in the quantitative synthesis (MA).

Effect of temperament on daily milk yield

In general, our MA results for daily milk yield evinced that cows classified as reactive (High) produced more than intermediates, and even more than the calm ones (Low), which differed from what we expected. According to Abdel-Hamid et al. (2017), reactive cows, possibly, spend more energy on motor activities, such as walking and standing. Additionally, reactive cows in the milking parlor drop teat cups more often and direct less liquid energy to lactating, which leads to a lower yield (Marçal-Pedroza et al., 2021). However, there are authors who argue that reactive cows are more aggressive during feeding and ingest greater amounts of food, resulting in greater productivity (Sawa et al., 2017). Despite our sensibility analysis not identifying it, the study by Marçal-Pedroza et al. (2020) could be influencing these results, since rumination frequency during milking was used as a temperament measurement. In this particular study, a significant relationship between temperament and milk yield was reported for the behavioral indicator of rumination in the milking parlor. In this specific case, cows classified as High ruminated more during milking, therefore being calmer and more relaxed, and reaching greater milk yield than the Low ones who spent less time ruminating. This classification was different from the other publications included in this MA, in which the High category animals were the most reactive.

The high variability found for the eight analyzed publications may be due to the different methods used to measure reactivity as an indicator of the cows' temperament. This makes it difficult to compare the data in published literature, since some methods may be more sensitive to recording the intensity of the behavioral responses of the animals than others (Sutherland and Huddart, 2012).

The effect of temperament on daily milk yield was assessed considering the subgroups of breed, parity, and lactation stage. Among the evaluated publications, only Marçal-Pedroza et al. (2020) studied Zebu cows. In the European cows, Inter animals produced less than the Low and High ones. For two (Orbán et al., 2011; Sutherland and Huddart, 2012) of the seven publications evaluated in the MA for European breeds, there was no evidence of any effect of temperament on daily milk yield, with only five publications leading to these results. Thus, it is evident that we need to be careful when interpreting results, mainly due to the low number of publications available.

Regarding the effect of parity, primiparous cows of Inter temperament yielded less than those of High temperament. Again, we highlight the work of Marçal-Pedroza et al. (2020), which, by using the frequency of rumination as temperament measurement, primiparous in the High category were the ones with the most rumination and higher milk yield. According to Sawa et al. (2017), the selection of animals to increase productivity may also increase the risk of selecting animals with undesirable temperaments, which might remain in the herd due to their greater milk yield (Praxedes et al., 2009).

Regarding multiparous cows, productivity was lower for Inter than for Low and High cows. In general, multiparous individuals are more used to the milking process, and their reaction to handling may be smaller, which possibly results in better productive performance for the calmer and for reactive ones compared to the intermediates (Sutherland and Huddart, 2012).

When considering lactation stage, the temperament classes differed only throughout the lactation, with a higher daily milk yield for reactive, followed by calm and intermediate cows that had the lowest milk yield. Among the four publications analyzed, two failed to find an influence of temperament on productivity (Orbán et al., 2011; Sutherland and Huddart, 2012), while the other two (Gergovska et al., 2014; Sawa et al., 2017) found greater productivity in High cows, in a total of 12,068 evaluated cows, and argued that High animals could have yielded more due to greater consumption. Whereas Gergovska et al. (2014) reported that High cows, despite their greater production, had an irregular lactation curve, which does not occur for Low cows.

Effect of temperament on total milk yield

Only three of the publications included in the MA evaluated the effect of animal temperament on total milk yield (over the whole lactation), which may compromise the interpretation of these findings. In general, High cows were more productive than Low and Inter ones. Regarding the breed effect, only two publications with European breeds were considered. In that case, High cows had greater productivity than Inter ones. Moreover, among the primiparous animals, also the High yielded more than Inter ones, possibly due to the previously mentioned relationship between greater feed intake and high milk yield in reactive animals.

Frequently used reactivity indicators for dairy cows have been the number of steps and kicks in the milking parlor (Rousing et al., 2004; Cerqueira et al. 2017; Marçal-Pedroza et al., 2020), but there is no consensus among authors regarding the real interpretation of these movements. Steps may represent a stress indicator, mainly for animals classified as aggressive (Wenzel et al., 2003), or have another meaning, e.g., younger animals with a high parasitic rate (ticks) may take more steps than those with a lower rate, signaling discomfort rather than a more excitable (or reactive) temperament (Rousing et al., 2004). This divergence of interpretation of the animals' temperament may lead to an incorrect association between temperament type and productivity variables. As highlighted by Sawa et al. (2017), the relationship between temperament and milk yield depends on several factors, such as the temperament indicator used, studied breed, age of the animals, and parity.

Meta-regression analysis

Of the eight covariables analyzed (year of publication, geographic region - continent, experiment duration, sample size, breed, parity, lactation stage, and controlled confounders), only three contributed to explaining the variation between publications: sample size, lactation stage, and controlled confounders have shown a direct correlation with the daily milk yield of cows. As for the total milk production, some variables showed an association with milk production, but none of them contributed to explaining the variability found between the publications.

Meta-regression indicated that with every one-year increase in the year of publication, there was an increase in MD, which is possibly related to the period of publication of the selected papers since all nine publications were published starting from the 2000s, a period of growing interest in issues related to behavior, productive performance, and welfare of farm animals (Hemsworth et al., 2000; Rousing et al., 2004; Broom, 2010; van Dijk et al., 2019). Another element we need to highlight is that most studies carried out in Europe showed an increase in MD for total milk yield when compared to studies conducted in South America (Praxedes et al., 2009; Marçal-Pedroza et al., 2020). It could be attributed to the longer period of selection for high productivity in the European breeds, resulting in higher productivity for these animals compared to the Zebu breeds and local crossbreeds used in Latin America (Brito et al., 2021). In spite of the lower milk production, the use of Zebu breeds and their crosses (such as Girolando), more adaptable to warm climates, would result in higher sustainability of dairy production in tropical regions (Canaza-Caio et al., 2016; Brito et al., 2021). The number of evaluated animals had a significant effect, which is probably because the publications had a great variation in sample size (from 30 to 12,028 animals).

For daily milk yield, there was clear evidence of change in the estimated MD, going from a positive value to a negative one, indicating that milk yield increases for the higher temperament classes (Inter and High). The exclusion of the publications by Orbán et al. (2011) and Sutherland et al. (2012) lead to a reduction in MD, but the daily yield of the reactive animals continues to be higher than that of calm and intermediate cows. Both publications together evaluated only 382 dairy cows, all of European breeds. In turn, the exclusion of Neja et al. (2017) resulted in increased MD, also maintaining greater production for reactive cows, and in their study, only 158 animals of European breed were evaluated.

Differently from daily yield, no tendencies were evidenced for total milk yield. The removal of Neja et al. (2015) decreased MD, and it was conducted with 11.629 cows of European breeds, but the total yield of the reactive cows remained higher than the intermediate and calm ones. The opposite happened when we excluded Praxedes et al. (2009), leading to an increase in MD, but the milk yield of reactive cows remained higher. Praxedes et al. (2009) investigated the production of 2,507 animals of Zebu breed, with a lower sample size when compared with the publications by Neja et al. (2015). The last one, published by Sawa et al. (2017), evaluated 12,028 cows. Neja et al. (2015) and Sawa et al. (2017) used European animals, which has possibly led to this variation alongside the fact that Zebu cows, in general, have lower milk yield than European breeds.

Qualitative analysis

The publication of Rousing et al. (2004), which evaluated the cows' temperament based on the number of steps in the milking pen, was the only one to find that High cows yielded more milk, in agreement with our results from MA. For these authors, the occurrence of steps is an indication of discomfort during the milking process, mainly in younger animals, and does not necessarily indicate reactive temperament, which could explain why High cows were more productive. In turn, Bertenshaw et al. (2008) and Dodzi and Muchenje (2011) reported that primiparous individuals which took more steps and kicks while milking were less productive. Hedlund and L¢vlie (2015) found the same pattern of association with nervous cows producing less milk, which was seen only in the first lactations. Cerqueira et al. (2017), who evaluated multiparous and primiparous cows, observed that the relationship between reactivity and production is associated with parity: cows with a greater number of calvings, i.e., the oldest of the herd, which took more steps, had a lower yield.

The quality of the human-animal relationship during the milking routine is possibly mediating the relationships between temperament and milk yield, as reported by Breuer et al. (2000) and Hemsworth (2003). Therefore, with high-quality handling, based on application of good practices, even the cows with the reactive temperament (more susceptible to stress) might express their best productive potential under adequate environmental conditions (Praxedes et al., 2009; Marçal-Pedroza et al., 2020).

Our SR/MA has some limitations that must be considered. Firstly, the low number of publications found on the subject.

Secondly, some publications which could have been included did not present the data in a format that allowed it to be extracted for a MA. Even after trying to contact the authors to obtain details, as suggested by Lean et al. (2009), we were not successful to reach the numerical data. Additionally, some publications were analyzed separately from the MA in a qualitative manner, due to the relevance of their results. Also, the lack of standardization of the methods of temperament assessment in dairy cows associated with the large variation in productive performance of the animals made the analysis and interpretation of the results a challenging task. Putting it all together, the results obtained in this MA, reporting the greater production by High cows, may be due to how the behavior is interpreted in these studies (reactivity considering the leg movement levels). It is important to highlight the fact that the animals being less agitated, or even still, during the milking procedures does not necessarily mean a calmer temperament, but a fear state (Munksgaard et al., 2001). Understanding animal reactivity as an indicator of temperament type requires, aside from objective measurements, an interpretation of the intrinsic traits of animals, what could be achieved based on the inclusion of physiological measures.

Conclusion

This is the first SR-MA that assessed results published in the scientific literature on the effect of dairy cows' temperament on productivity. Our results of the MA did not support the original hypothesis, as we obtained that reactive cows generally produce greater milk yield than those of calm and intermediate temperament. On the other hand, correlation and regression data support our hypothesis of calm cows being more productive. This contrast leads us to further questions: which indicators should we use to classify animal temperament? And when should this classification be applied? In addition to the need for standardization of protocols for behavioral assessments, in order to allow for a better understanding of the results, and the need for more studies reporting this type of assessment for cows of Zebu breeds.

Acknowledgments

We would like to express our gratitude to Jéssica Tatiana Morales-Pineyrúa (INIA, Uruguay) and Rogério Ribeiro Vicentini (UFJF, Brazil) for contributing to the selection of the publications covered in this review. This work was supported by the Fundação de Amparo à Pesquisa do Estado de Minas Gerais – Brasil (Fapemig) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq # 311794/2020-3). The study was part of the doctoral thesis of the lead author of the Graduate Program in Biodiversity and Nature Conservation at the Federal University of Juiz de Fora (UFJF), Juiz de Fora, Campus, Brazil.

Conflict of Interest Statement

The authors have declared no conflicts of interests.

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Animal 15 (2021) 100224

Contents lists available at ScienceDirect

Animal

The international journal of animal biosciences

Are dairy cows with a more reactive temperament less efficient in energetic metabolism and do they produce more enteric methane?

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ARTICLE INFO

Article history:
Received 6 November 2020
Revised 28 February 2021
Accepted 5 March 2021
Available online 25 May 2021

Keywords:
Behavior
Holstein-Gyr
Personality
Restlessness
Sustainability

ABSTRACT

It remains unknown whether dairy cows with more reactive temperament produce more enteric methane (**CH_4**) and are less bioenergetically efficient than the calmer ones. The objectives of this study were (a) to evaluate the relationship between cattle temperament assessed by traditionally used tests with energetic metabolism and enteric CH_4 emissions by crossbred dairy cows; (b) to assess how cows' restlessness in respiration chambers affects energetic metabolism and enteric CH_4 emissions. Temperament indicators were evaluated for 28 primiparous F1 Holstein-Gyr cows tested singly in the handling corral (entrance time, crush score, flight speed, and flight distance) and during milking (steps, kicks, defecation, rumination, and kick the milking cluster off). Cows' behaviors within respiration chambers were also recorded for each individual kept singly. Digestibility and calorimetry trials were performed to obtain energy partitioning and CH_4 measures. Cows with more reactive temperament in milking (the ones that kicked the milking cluster off more frequently) spent 25.24% less net energy on lactation (P = 0.04) and emitted 36.77% more enteric CH_4/kg of milk (P = 0.03). Furthermore, cows that showed a higher frequency of rumination at milking parlor allocated 57.93% more net energy for milk production (P < 0.01), spent 50.00% more metabolizable energy for milk production (P < 0.01) and 37.10% less CH_4/kg of milk (P = 0.04). Regarding the handling temperament, most reactive cows according to flight speed, lost 29.16% less energy as urine (P = 0.05) and tended to have 14.30% more enteric CH_4 production (P = 0.08), as well as cows with a lower entrance time (most reactive) that also lost 13.29% more energy as enteric CH_4 (P = 0.04). Temperament and restless behavior of Holstein-Gyr cows were related to metabolic efficiency and enteric CH_4 emissions. Cows' reactivity and rumination in the milking parlor, in addition to flight speed and entrance time in the squeeze chute during handling in the corral, could be useful measures to predict animals more prone to metabolic inefficiency, which could negatively affect the sustainability of dairy systems.

Implications

Livestock production plays an important role in the greenhouse gas emissions, part of them comes from enteric methane emissions of cattle. In this study, we assessed the effects of cows' behavior on their energetic metabolism and enteric methane emissions. We have found that environmental consequences might arise from the inefficient feeding resource use, increasing methane emissions by temperamental and reactive cattle. We recommend improving temperament throughout animal breeding and good practices of cattle handling as viable strategies for attaining a more sustainable dairy production.

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https://doi.org/10.1016/j.animal.2021.100224
1751-7311/

Introduction

Sustainable livestock production has been a theme of debates in the international scene, raising new challenges for the stakeholders of farm animal production chains (van Dijk et al., 2019). Public opinion has shown an increasing interest in the acquisition of high-quality animal products. It includes the requirement of information about the products' origin and the productive processes, comprising issues related to their impacts on animal welfare and environment (Risius and Hamm, 2017). This is related to a growing global demand for an ethical and sustainable way to develop the economic activities, including the livestock production. The concept of "One Welfare" seems to be a useful guide to achieve this since it proposes that the activities that affect (positively or negatively) animal welfare, human wellbeing, biodiversity, and environmental conservation are closely connected and are mutually dependent on each other (García et al., 2016; Tarazona et al., 2019).

In this context, one of the challenges is the efficient use of resources and the mitigation of greenhouse gas emissions by livestock (Herrero et al., 2016). Enteric methane (**CH_4**) is one of the greenhouse gasses produced during the digestive process of ruminants by the action of anaerobic microorganisms that colonize the rumen, through fermentation of plant carbohydrate (Beauchemin et al., 2008). In Brazil, estimates pointed out that ruminants' enteric fermentation was responsible for 11 352 (t) of methane produced in 2017, and the dairy industry contributed with 0.33 L of methane/kg of milk in the country (SEEG, 2018).

There is a variation in the amount of CH_4 emission by ruminants; thus, it is important to understand which factors affect the enteric CH_4 production by these animals. For example, quality of the diet (Cottle et al., 2011), level of dry matter intake (Dini et al., 2019), environmental temperature (Yadav et al., 2016) were reported to be associated with CH_4 emissions. Thus, some possible alternatives for CH_4 mitigation have been investigated, most of them including nutritional strategies (Haque, 2018), besides other alternatives, such as intensification of the productive systems (de Vries et al., 2015). Despite considerable recent progress in the nutritional field, several other factors related to animal physiology may contribute to their bioenergetic efficiency and reduction of greenhouse gas emissions (Ornelas et al., 2019), which still deserve to be better understood.

There is some evidence showing that physiological and behavioral responses to stress might be associated with a higher enteric CH_4 production (Yadav et al., 2016; Llonch et al., 2018) and lower productivity in dairy cows (Hedlund and Løvlie, 2015). The emissions of enteric CH_4 represent an environmental concern and a source of energetic efficiency reduction due to the loss of gross energy as CH_4 (Johnson and Johnson, 1995). The energy released as CH_4 gas could be allocated for weight gain (in beef cattle) and milk yield (in dairy cattle), ranging from 2% to 12% of the animals' energy intake, depending on the type of diet (Johnson and Johnson, 1995). Thus, strategies for enteric CH_4 mitigation should result in environmental and economic gains, optimizing the use of nutrients.

Temperament had been defined as individual differences in animals' behavioral responses to stressors (Fordyce et al., 1982; Koolhaas et al., 2010). Previous studies have shown that 'nervous' and restless cows produce less milk (Sutherland and Dowling, 2014; Hedlund and Løvlie, 2015); however, the metabolic mechanisms underlying this relationship are poorly understood. One could expect that animals with divergent temperaments would differ in their efficiency to convert the feed energy into milk, i.e., reactive cows could be less efficient than the reactive ones. Thus, cattle temperament could affect the energetic partition, decreasing the energy to milk yield. If reactive cows, in fact, lose a higher percentage of energy through feces, urine, heat production, and CH_4, the temperamental animals may show a more significant impact on the sustainability of the dairy industry. However, these hypotheses still lack empirical support for dairy animals, remaining unknown whether animals with more reactive temperament and restless behavior produce more CH_4 (Llonch et al., 2016) and are less bioenergetically efficient than the calmer ones.

Therefore, the aims of this study were (a) to evaluate the relationships between cattle temperament assessed by traditionally used tests with energetic metabolism and enteric CH_4 emission by Holstein-Gyr dairy cows; (b) to assess how cows' restlessness in the respiration chambers affects energetic metabolism and enteric CH_4 emissions. We hypothesize that individuals with a more reactive temperament and restless in a situation of physical restraint would be metabolically and bioenergetically less efficient than the calmer ones, showing higher enteric CH_4 emission.

Material and methods

Animals and housing conditions

Data were collected from April to November 2017, at the Multi-use Livestock Complex of Bioefficiency and Sustainability of the Brazilian Agricultural Research Corporation, Embrapa (Coronel Pacheco, Minas Gerais, Brazil), with twenty eight primiparous F1 Holstein-Gyr lactating cows, aging 30 ± 1.04 years (mean ± SD) and weighing 568 ± 41.50 kg. Cows were kept in a free-stall barn equipped with an electronic feeding system (AF-1000 Master Gate, Intergado Ltd., Contagem, MG, Brasil) and water troughs (WD-1000, Intergado Ltd., Contagem, Minas Gerais, Brazil). Twice a day, cows were milked in a fishbone milking parlor (2 × 4) (DeLaval, Tumba, Sweden), always by the same two stockpersons. More details about the animals and facilities were previously published in Marçal-Pedroza et al. (2020) that it is part of the same study. Individual daily milk yield data were recorded automatically on the days of the behavioral observations.

Temperament assessment

The cows' temperament was measured based on the cows' behavioral responses to being handled by humans, assessed during milking (i.e., milking temperament) and during handling in the corral (handling temperament). The temperament data used come from data collected in a previous study (Marçal-Pedroza et al., 2020). The milking temperament of the lactating cows was evaluated 45 days after calving, and the subsequent sessions with an average interval of 45 days, performing three sessions along the early lactation period. In each session, data collection was made on three consecutive days, always in the morning milking (a total of nine days of assessment). The following behavioral indicators of cattle temperament were recorded by a previously trained observer, as described in Marçal-Pedroza et al. (2020): number of Steps (STEPS), number of Kicks (KICKS) and the occurrences of behaviors defecation, rumination, and kick the milking cluster off (KOFF), from the time that the milking cluster was attached until its extraction when milking was finished.

The handling temperament was assessed on the last day of each milking evaluation session, in a total of three evaluations in the corral. The following measures were used: Entrance Time (in s), Crush Score, Flight Speed (in m/s), Flight Distance (in m). For the full description of the temperament methods used, please see Marçal-Pedroza et al. (2020).

Whole tract digestibility and energy partitioning

The digestibility assays took place every 45 days throughout all lactation, for a total of six sampling periods. For the digestibility assays, groups of eight cows were transferred to a tie-stall system with individual feeders and water troughs. Individual samplings of feces were collected for five days per group. Total urine was collected on the first two days of the fecal collection. Aliquots of silage, concentrate, and orts were daily collected along the five consecutive days and stored at −20 °C (Supplementary Table S1). The full description of the methods and equations used was included as Supplementary Material S1.

For the calculation of the energy partition, the gross energy intake (**GEI**), daily fecal (Fecal-E, Mcal/d) and urinary (Urine-E, Mcal/d) energy outputs were obtained by multiplying DM intake (**DMI**) and fecal and urinary dry matter excretion with their respective energy contents. Digestible energy intake (**DEI**, Mcal/d) was calculated as the difference between GEI and fecal energy excretion. Metabolizable energy intake (**MEI**, Mcal/d) was derived as the difference between DEI and the sum of Urine-E and CH_4 energy (CH_4-E, Mcal/d), which was assumed to be 45 Kcal/L (Brouwer, 1965). Energy retention was calculated as the difference between MEI and heat production (Heat-E). Heat-E (Kcal/d) was determined based on measurements of O_2 consumption (L/d), CO_2, and CH_4 production (L/d), using the equation of Brouwer (1965). The net energy of lactation (**NEL**) was also obtained based on the feed energy available for milk production after digestive and metabolic losses (in Mcal/kg). The additional measures were also used in the analyses: metabolizable energy/digestible energy (MEI/DEI), metabolizable energy/gross energy (MEI/GEI), energy balance (**EB**), and milk-energy/metabolizable energy (Milk-energy/MEI). These methods were described in Ornelas et al. (2019), carried out under the same conditions and installations of our study.

Respiration measurements

The open-circuit respiration chambers (n = 4) were used to measure gas exchanges. The full description of the chambers system used and its validation was previously published in Machado et al. (2016). Briefly, the net volume of each chamber is 21.10 m^3, containing a 2.26 × 1.26 m pen. The chambers have large double-glazed windows (150 cm high, 150 cm wide) to guarantee visual contact between the animals. Each chamber is fitted with one large back door for animal access and a smaller front door for operator access and feeding. The common gas analysis and data acquisition system were shared by the four chambers (Sable Systems International, Las Vegas, USA). Infrared technology was used to analyze CO_2 and CH_4 concentrations, whereas fuel cell technology was used for O_2. The injection of known volumes of CO_2 and CH_4 in each chamber was used to perform the recovery test of the whole system, using a mass flowmeter (MC-50SLPM-D, Alicat Scientific Inc., Tucson, AZ). The average recovery of the four chambers for CO_2 (mean ± SD) was 87.87 ± 0.04% and for CH_4 was 84. 75 ± 0.07%.

The animals were halter-trained, adapted to handling and went to respiration chambers for two to three days before the trials began. Six sessions of two days of respiratory measurements in chambers were done, performing a total of 12 days of evaluation per cow. The respiration chamber evaluation began on the 45th day after calving with a 45-day interval between sessions, for four cows at a time, as there were only four respiration chambers available. Groups of four animals went to respiration chambers; then, they were subjected to the digestibility assay in groups of eight cows; in sequence, the remaining four cows of the digestibility group went to the chambers after the digestibility. The sessions started immediately after morning feeding at 9:00 a.m. The respiratory indirect calorimetry reading was initiated, and gas exchanges were measured during 21–23 h, with an extrapolation of 24 h. The animals were randomly allocated to each chamber where they remained singly and then confined for 48 hours, leaving only for milking (morning and afternoon).

Data acquisition and analysis software (Expedata Data Analysis Software 1.8.5, version PRO, Sable Systems International) was used to calculate the consumption of O_2, CO_2, and CH_4 production (L/day). Individual enteric CH_4 production (g/day), CH_4 yield (g/kg DMI), and CH_4 intensity (g/kg milk) were calculated. Inside the chambers, there was a feeding and watering trough, and a video camera that recorded the behaviors of the animals throughout the experimental period.

Behavior within the respiration chambers

For the record of behavior, the videos (seven hours per cow, on average, performing a total of 196 h of video footages) captured by video cameras (VM 310 IR, an infrared camera from Intelbras S/A – Brazilian Electronic Telecommunications Industry, Manaus/AM, Brazil) between the two daily milking procedures at the first day of respiration chamber confinement were used. The videos of each one of the twenty eight cows were observed using focal-animal sampling and instantaneous sampling, with one-minute intervals. The following behavioral categories were used to measure cows' restlessness in the respiration chambers: lying, feeding, ruminating in the chamber, shaking ears, shaking the head, moving and being inactive, considering the time spent in each behavior, expressed in relative frequencies (%). A continuous recording was used to register the occurrences of steps, vocalization, and turning the head, expressed as number of occurrences.

Statistical analysis

First, to analyze the temperament indicators and energetic metabolism variables, a single individual measurement was obtained for each indicator, through the average of the sessions carried out throughout the study.

To assess the effects of temperament and behaviors in the chambers on the energetic metabolism and CH_4 emission measures, linear mixed models for longitudinal data were fitted by using PROC MIXED of SAS (version 9.2, SAS Institute Inc., Cary, NC). Models included the dependent variables of energetic metabolism (Fecal-E, Urine-E, CH_4-E, Heat-E, MEI/DEI, MEI/GEI, Milk-energy/MEI, NEL, EB) and CH_4 emission measures (production, yield, and intensity). Fixed effects of temperament and behavioral measures (one measure at a time), evaluation session, and their interactions, in addition to milking group, were included. The random effect of animal (subject) was considered as a repeated measure within the evaluation session. In all analyses, means were compared using posthoc Tukey Test, and P-values were assumed as significant when <0.05 and as a trend when <0.10.

For inclusion in the mixed models as fixed effects, the handling temperament, milking temperament indicators, and behavioral measures were categorized into three scores (low, average, and high). Most of the variables were classified based on the terciles of distribution (low = fist tercile, intermediate = second tercile, and high = third tercile), except by Entrance Time and Flight Distance, which were classified based on threshold values, as follows: Entrance Time ('low' = 0–9.9 s; 'intermediate' = 10–20 s; 'high' = over 20 s); Flight Distance ('low' = 0 cm; 'intermediate' = 0.1–0.9 9 cm; and 'high' = over 1 m). Finally, the behaviors such as Defecation, Rumination, KOFF that were binomial variables (occurs or not) were classified based on the number of occurrences across the 3-day session: 'low' = 0 occurrence; 'intermediate' = 1 occurrence; and 'high' = 2 or 3 occurrences. Behavioral measures in the respiration chambers (steps in the chamber, turning the head, lying, feeding, ruminating in the chamber, ear shaking, head shaking, vocation, and being inactive) were also categorized in terciles.

Results

Effects of temperament indicators on energetic metabolism and methane emissions

Regarding the effects of the milking temperament indicators, the number of STEPS showed a significant effect on Urine-E ($P = 0.02$), MEI/DEI ($P = 0.03$) and a tendency on DMI ($P = 0.06$) and GEI ($P = 0.07$) (Table 1). Similarly, a tendency for number of KICKS was found on CH_4-E ($P = 0.07$), CH_4 production ($P = 0.09$) and Heat-E ($P = 0.09$) (Table 1). Cows classified as intermediate for $STEPS_{Inter}$ had 26.96% lower loss of energy as urine, 2.35% higher MEI/DEI rate, and 8.98% higher gross energy intake than those classified as $STEPS_{Low}$. Either the cows defined as intermediate for $KICKS_{Inter}$ tended to show reduced losses of energy as CH_4-E, as Heat-E, and lower CH_4 production (differences of 9.19%, 7.24%, and 9.93%, respectively) than those defined as $KICKS_{Low}$ (Table 1).

The milking behaviors of rumination and kicking the milking cluster off affected NEL ($P < 0.01$, $P = 0.04$, respectively) and CH_4 intensity ($P = 0.04$, $P = 0.03$), in addition to a significant effect of rumination on Milk-energy/MEI ($P < 0.01$) (Table 1). Cows that kicked the milking cluster off more frequently ($KOFF_{High}$) and ruminated less frequently ($RUMINATION_{Low}$) allocated less net energy on lactation (differences of 25.24%, 57.93%, respectively) and more CH_4 intensity (36.77%, 37.10%, respectively) per liter of milk than cow classified as $KOFF_{Low}$ and $RUMINATION_{High}$, respectively. The animals classified as $RUMINATION_{High}$ had 50.00% greater Milk-energy/MEI than cows classified as $RUMINATION_{Low}$.

Concerning cows' temperament in the handling corral, Flight Speed showed a significant effect on Urine-E ($P = 0.05$) and a tendency on CH_4 production ($P = 0.08$) (Table 1). Additionally, Entrance Time affected CH_4-E ($P = 0.04$) and also showed a tendency on Urine-E ($P = 0.08$). Cows classified as Flight $Speed_{High}$ tended to lose 29.16% less energy as Urine-E and 14.29% more CH_4 production than Flight $Speed_{Low}$. Cows with Entrance $Time_{High}$ showed 35.18% more energy loss as Urine-E and 13.29% less energy loss as CH_4-E than cows with Entrance $Time_{Low}$.

Effects of behaviors in the respiration chambers on the energetic metabolism and methane emissions

The cows' behavior within the respiration chambers during the respiration assay affected some measures of energetic metabolism (Table 2). Cows that spent less time being inactive showed 2.35% less MEI/DEI ($P = 0.04$), and a higher frequency of vocalizations was related to 6.61% more energy loss as CH_4 (lower CH_4-E) ($P = 0.03$). Finally, cows that took more steps in the chamber showed a tendency of reduction of 5.65% in NEL ($P = 0.10$) and an increase of 12.95% in CH_4 intensity ($P = 0.09$) (Table 2).

Discussion

The objectives of the present study were to evaluate the effects of temperament and behavior in respiration chambers of dairy cows on energy metabolism and enteric methane emissions. Cows' temperament and behaviors in the chambers influenced energy metabolism and methane emissions, with more reactive cows allocating less energy for lactation and emitting more methane per liter of milk produced compared to calmer animals. In addition, cows with an intermediate temperament measured by steps and kicks in the milking parlor lost less energy as urine, heat and CH_4 and also produced less methane per day, compared to reactive cows.

Table 1
Effects of handling temperament and milking temperament indicators on energetic metabolism and methane emissions. Adjusted means (±SE) of energetic metabolism and methane emission measures for each temperament indicator are shown ($n = 28$ Holstein-Gyr dairy cows).

Item	Low	Intermediate	High	$F_{2,23}$	*P-value*
Handling Temperament Indicators					
FS (m/s)					
Urine-E (Mcal/d)	5.04 ± 0.38[a]	4.27 ± 0.27	3.57 ± 0.40[b]	3.52	0.05
CH4 Production (g/d)	229.31 ± 11.40[b]	261.43 ± 8.28[a]	262.10 ± 12.04[a]	2.88	0.08
ET (s)					
Urine-E (Mcal/d)	3.95 ± 0.32[b]	4.27 ± 0.30[b]	5.34 ± 0.49[a]	2.86	0.08
CH4-E (Mcal/d)	5.34 ± 0.14[a]	5.08 ± 0.13[a]	4.63 ± 0.22[b]	3.73	0.04
Milking Temperament Indicators					
KOFF					
NEL (Mcal/d)	12.68 ± 0.77[a]	14.37 ± 1.27[a]	9.48 ± 1.33[b]	3.67	0.04
CH4 Intensity (g/Kg milk)	19.17 ± 1.63[b]	15.49 ± 2.69[b]	26.22 ± 2.83[a]	3.92	0.03
RUMI					
NEL (Mcal/d)	9.51 ± 1.07[c]	12.41 ± 0.78[b]	15.02 ± 0.99[a]	7.19	< 0.01
Milk-energy/MEI	0.14 ± 0.01[c]	0.17 ± 0.01[b]	0.21 ± 0.01[a]	8.17	< 0.01
CH4 Intensity (g/kg milk)	25.39 ± 2.54[a]	19.07 ± 1.83[b]	15.97 ± 2.35[b]	3.83	0.04
KICKS					
CH4-E (Mcal/d)	5.33 ± 0.15[a]	4.84 ± 0.15[b]	5.30 ± 0.21[ab]	2.98	0.07
Heat-E (Mcal/d)	34.11 ± 0.83[a]	31.64 ± 0.80[b]	32.00 ± 1.16[ab]	2.65	0.09
CH4 Production (g/d)	261.54 ± 9.93[a]	235.57 ± 9.49[b]	268.68 ± 13.58[a]	2.68	0.09
STEPS					
DMI (Kg/d)	14.93 ± 0.39[b]	16.29 ± 0.41[a]	15.97 ± 0.52[ab]	3.09	0.06
GEI (Mcal/d)	66.24 ± 1.71[b]	72.19 ± 1.83[a]	70.78 ± 2.28[ab]	3.04	0.07
Urine-E (Mcal/d)	4.97 ± 0.30[a]	3.63 ± 0.32[b]	4.29 ± 0.40[ab]	4.47	0.02
MEI/DEI	0.85 ± 0.01[b]	0.87 ± 0.01[a]	0.86 ± 0.01[ab]	3.94	0.03

Abbreviations: Urine-E = % urine energy, CH_4-E = % methane energy, NEL = net energy of lactation, Milk-energy/MEI = milk-energy/ metabolizable energy intake, CH_4 intensity = methane emission, Heat-E = % heat energy, DMI = DM intake, GEI = gross energy intake, MEI/DEI = metabolizable energy intake/digestible energy intake, FS = flight speed (m/s), ET = entrance time (s), KOFF = kick off the milking cluster, RUMI = rumination, KICKS = number of kicks, STEPS = number of steps.
[a–c] Adjusted means without a common letter differ statistically from each other (Tukey test. $P < 0.10$).

Table 2
Effects of behaviors in the respiration chambers on the energetic metabolism and methane emissions. Adjusted means (±SE) of energetic metabolism and methane emissions measures for each behavior are shown (*n* = 28 Holstein-Gyr dairy cows).

Item	Low	Intermediate	High	$F_{2,50}$	*P-value*
Steps					
NEL (Mcal/d)	12.74 ± 0.66[a]	12.39 ± 0.68[ab]	12.02 ± 0.67[b]	2.42	0.10
CH_4 Intensity (g/kg milk)	18.37 ± 1.53[b]	20.50 ± 1.58[a]	20.75 ± 1.53[a]	2.60	0.09
Vocalization					
CH4-E (Mcal/d)	4.84 ± 0.14[b]	5.27 ± 0.12[a]	5.16 ± 0.14[a]	3.83	0.03
Inactive					
MEI/DEI	0.85 ± 0.006[b]	0.86 ± 0.005[a]	0.87 ± 0.006[a]	3.38	0.04

Abbreviations: NEL = net energy of lactation, CH_4-E = % methane energy, MEI/DEI = metabolizable energy intake/digestible energy intake.
[a–b] Adjusted means without a common letter differ statistically from each other (Tukey test. *P* < 0.10).

Effects of temperament indicators on energetic metabolism and methane emissions

Animals with temperament categorized as 'intermediate' for STEPS and KICKS lost less energy in the form of urine and had higher rates of MEI/DEI, besides presenting a tendency to produce less CH_4 and lower loss of energy as heat and CH_4. The number of leg movements has been considered a valid indicator of cows' reactivity in the milking parlor, with less reactive cows taking lower numbers of steps (Hemsworth, 2003). Nevertheless, Munksgaard et al. (2001) have observed that when some cows are kept under situations of tension and stress, they might have an opposite reaction, remaining immobile during milking. Under such perspective, it would be plausible that cows that took a few steps (as for cows in the 'intermediate' score) could be more relaxed than those that remained immobile (cows in 'low' score). Cows classified as intermediate for numbers of STEPS and KICKS showed higher DMI and could be considered more efficient as well, given the reduced losses of energy as Urine-E and CH_4-E, and lower CH_4 production. In a previous study conducted with the same animals of the present during the raising period, Ornelas et al. (2019) found a negative correlation between DMI and CH_4 production. Cows with a higher feed intake are more efficient if the metabolizable energy that exceeds maintenance is retained, associated with reduced losses of energy as urine, heat, and CH_4 (Chaokaur et al., 2014). It could explain the higher DMI in addition to lower loss of energy as urine, heat, CH_4, and higher MEI/DEI rate in cows classified as 'intermediate' for STEPS and KICKS that could be considered more efficient.

Cows that were more reactive in the milking ($KOFF_{High}$) and ruminated less in the milking parlor ($RUMINATION_{Low}$) were less efficient, allocating less net energy to milk production. Kicking the milking cluster off indicates cows' reactivity related to discomfort and emotional state of agitation (Marçal-Pedroza et al., 2020). Similarly, rumination was related to emotional states of relaxation, while its reduction could reflect tension and stress (Manteca et al., 2013). A previous study of our research group has shown that cows ruminating more frequently in the milking parlor produced 17.26% more milk than those with a lower frequency of rumination (17.59 vs. 15.00 kg/day) (Marçal-Pedroza et al., 2020). Based on the results of the present study, it is possible to infer that the increased production for more ruminating cows derives, in parts, from their better performance in allocating energy for milk production associated with lower losses as methane. This result might reveal the implications of cows' milking behaviors for the sustainability of milk production.

Cows' reactive temperament in the handling had also influenced the energy metabolism and methane emissions, with cows exiting the squeeze faster (Flight $Speed_{High}$) showed less energy in the urine and more CH_4 production, while the animals that entered faster (Entrance $Time_{Low}$) lost less energy as urine and produced more CH_4-E. It is worth to remember that the most reactive cows showed Flight $Speed_{High}$ (in m/s) and Entrance $Time_{Low}$ (in s), since they spent less time to enter into the squeeze and exit faster (high speed); thus, these measures were inversely correlated. Cows that entered and exited the squeeze chute faster (characterizing states of fear and agitation) tended to show higher losses of energy as CH_4-E and enteric CH_4 production. The flight speed and entrance time reflect an innate tendency of general fearfulness and high behavioral reactivity, revealing a susceptibility to stress in temperamental cows (the faster ones) (Cafe et al., 2011). The emotional state of fear has implications on the physiological control of metabolism, being a potential psychological stressor that leads to higher activation of the hypothalamic–pituitaryadrenal (HPA) axis, resulting in the release of glucocorticoids (Hemsworth, 2003). A relationship between reactive temperament (measured by flight speed) and susceptibility to stress was previously shown in several studies (Cafe et al., 2011). Reactive temperaments in cattle (high flight speed and crush score) were related to a more prolonged and more intense activation of HPA axis and sympatho-adrenomedullary system in responses to stress (Cafe et al., 2011). Both axes are involved in the control of catabolism, energetic homeostasis, energy balance, and storage of energy in the body. To the best of our knowledge, the present study is the first to assess the relationships between temperament, energy partitioning, and CH_4 emissions in cattle. In the study by Llonch et al. (2016), the authors investigated the relationships between beef cattle temperament (measured by flight speed and crush score), cortisol levels following transportation, and CH_4 emissions. Despite those authors not finding a relationship between flight speed and crush score with methane emissions, they reported a positive association between cortisol following transport and CH_4 emissions corrected for feed intake (g/kg DMI). Thus, the present study contributes to the scarce evidence that characteristics intrinsic to the behavior of ruminants, such as temperament, emotional states, and intensity of behavioral and physiological responses to stressors, should be taken into account in the development of alternatives to mitigate enteric CH_4 by cattle (Llonch et al., 2016, present study).

Effects of behaviors in the respiration chambers on the energetic metabolism and methane emissions

The behavior of cows in respiration chambers affected energy metabolism and methane emissions. Cows expressing behaviors indicative of restlessness (less time inactive, vocalized more and took more steps) had lower rates of MEI/DEI and lost more energy as CH_4, and tended to allocate less NEL and more CH_4 intensity. For confined beef cattle, Llonch et al. (2018) showed that a higher level of activity in the home pens (measured as number of steps per day) was related to lower feed efficiency (poorer residual feed intake), which the authors attributed to the higher energy expenditure for muscle activity in more active individuals. Additionally, in beef cattle, efficient animals show lower maintenance requirements as well as better usage of metabolizable energy for growth

(Cantalapiedra-Hijar et al., 2018). These results might explain the lower MEI/DEI and lower NEL in cows that took more steps, which probably were less efficient.

Vocalizations and steps in situations involving physical restraint can be used as indicators of cows' restlessness since confinement and social isolation are stressors for social animals (Llonch et al., 2018). Restless cows might lose more energy as CH_4-E, allocating less energy for milk yield, in parts, due to more intense physiological responses to stress in these animals. Stress responses are detrimental for efficiency in energy use, leading to reduced productivity and the rise of enteric CH_4 emissions (Hedlund and Løvlie, 2015; Llonch et al., 2018). On the other hand, calmer and relaxed cows might have the potential to be more productive and efficient in energy partitioning and use, along with CH_4 intensity reduction per unity of product (Yan et al., 2010).

Our study has some limitations that have to be taken into account. First, the measures of metabolism and methane emissions were taken in potentially stressful situations. Both tie-stall and respiration chambers involve physical restraint and reduced social interactions, in spite of the visual contacts were maintained. All the cows were exposed to the same experimental conditions when they were heifers (Ornelas et al., 2019). The heifers went through ten days of adaptation to the tie-stall and four days of adaptation in the respiration chambers, followed by a 5-day digestibility assay and two days in the respiration chambers. The feed intake was monitored by collecting and weighing feed leftovers to ensure they did not exceed 10%, as a measure of behavioral changes in tie-stall and chambers. Thus, we expect that all the cows were adapted to this study's conditions, leading us to consider our results valid; even so, caution is required when extrapolating our findings to non-experimental or commercial conditions. A second limitation was the lack of ruminal microbiome community assessment in our study. It is known that the ruminal microbiome composition plays an important role in cows' feed efficiency, energy utilization, and methane emissions (Difford et al., 2018; Schären et al., 2018) and should have affected our results.

In summary, reactive temperament, stress, and welfare problems potentially cause additional energy expenditure for animals to cope with such situations. Beyond the economic losses caused by the inefficient use of feeding resources and reduced milk yield, the reactive temperaments of cattle might cause concerns related to the risks of accidents and deteriorate the labor conditions in dairy farms (Hemsworth, 2003; Sutherland and Huddart, 2012). Finally, this study has shown that environmental consequences might arise from the increasing CH_4 emissions for temperamental cattle. All these factors are integrated within the perspective of 'One Welfare' (García et al., 2016; Tarazona et al., 2019). Thus, we recommend the improvement of temperament throughout animal breeding and good practices of cattle handling as viable strategies for attaining a more sustainable dairy production.

Conclusion

Cattle temperament assessed during milking and in the handling corral, in addition to cows' behaviors within the respiration chambers, were related to energy partitioning and CH_4 emissions by crossbred dairy cows under the experimental conditions of the present study. Animals classified as more reactive allocated less energy for lactation and emitted more enteric CH_4 per unity of product. All those impacts of reactive temperaments are undesirable for an efficient and sustainable livestock activity. A selection of calmer cows and the adoption of good practices of cattle handling could favor the welfare of cows, stockpeople, and the environment.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2021.100224.

Ethics approval

This research was approved by the Embrapa Dairy Cattle Animal Care and Use Committee, Juiz de Fora, MG, Brazil (Protocol 5201240417), being conducted according to the ethical principles of animal experimentation.

Data and model availability statement

None of the data was deposited in an official repository. Data and models used may be available upon request by contacting the corresponding author.

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Declaration of Interest

The authors declare no conflict of interest.

Acknowledgments

This study is part of the first author's doctoral thesis prepared for the Graduate Program in Biodiversity and Nature Conservation of the Federal University of Juiz de Fora (UFJF), Brazil.

Financial support statement

This work was supported by in part by the Fundação de Amparo à Pesquisa do Estado de Minas Gerais – Brasil (Fapemig) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Proc. # 409059/2016-1).

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RESEARCH ARTICLE

Is the temperament of crossbred dairy cows related to milk cortisol and oxytocin concentrations, milk yield, and quality?

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Citation: Marçal-Pedroza MG, Campos MM, Martins MF, Silva MVB, Paranhos da Costa MJR, Negrão JA, et al. (2023) Is the temperament of crossbred dairy cows related to milk cortisol and oxytocin concentrations, milk yield, and quality? PLoS ONE 18(6): e0286466. https://doi.org/10.1371/journal.pone.0286466

Editor: Julio Cesar de Souza, Universidade Federal de Mato Grosso do Sul, BRAZIL

Received: February 1, 2023

Accepted: May 16, 2023

Published: June 1, 2023

Data Availability Statement: All relevant data are within the paper and its Supporting information files.

Funding: This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (ACS Proc. # 409059/2016-1; # 311794/2020-3) and in part by the Fundação de Amparo à Pesquisa do Estado de Minas Gerais – Brasil (MGMP Fapemig). The funders had no role in study design, data collection and analysis,

Abstract

Reactive dairy cows are more susceptible to stress, and this may result in negative effects on milk yield and quality. The aims of this study were to investigate the relationships between temperament traits and concentration of milk cortisol and oxytocin, milk yield, milkability, and milk quality in Holstein-Gyr cows. Temperament traits were assessed in 76 Holstein-Gyr cows in the milking parlor (by scoring milking reactivity and recording the numbers of steps and kicks during pre-milking udder preparation and when fitting the milking cluster) and during handling in the corral (by measuring the time to enter in the squeeze chute, ET and flight speed, FS). Milk samples were collected for milk quality (% fat, % protein, % lactose, and somatic cell count, SCC), and milk cortisol and oxytocin. Milk yield, milking time, and average flow were also measured. The calmer cows during milking management (class 'low') produced milk with higher protein ($p = 0.028$) content and tendencies for lower fat ($p = 0.056$) and higher lactose ($p = 0.055$) contents. Regarding the hormones, the most reactive cows (class 'high') in the milking and handling corral produced milk with higher concentrations of cortisol ($p<0.001$) and oxytocin ($p = 0.023$). In addition, the temperament of the animals affected some of the productive measures evaluated. Cows with reactive temperament had lower milk flow and longer milking time than the intermediate ones and had higher fat and a tendency for lower protein percentage in milk compared to cows with intermediate temperaments. Calm and intermediate cows in the handling corral produced more milk and presented better milkability parameters, such as a shorter milking time and greater average milk flow. Our results suggest that the cows'

decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

behavioral reactivity can be related to the intensity of their response to stress during handling.

1. Introduction

Bovines, like other animals, present individual differences in behavior when exposed to challenging situations, and these behavioral differences are often described as temperament [1]. Temperament is expressed through a set of behavioral and physiological responses as a strategy to adapt to stressful situations in the environment [2]. However, most studies recognize that the characterization of temperament is complex since it can consider various traits, such as coping style, emotionality, and sociability [1, 3].

Studies have shown the importance of cattle temperament in livestock husbandry. Some studies have reported that calmer and more docile dairy cows in the milking parlor (milking temperament) produced greater milk yield [4–5], while others have found opposite results [6, 7] or did not find any association between milking temperament and milk yield [8, 9], showing a lack of consistency among results. It is important to highlight that these articles used different methods to assess milking temperament. Hedlund and Løvlie [10]; Marçal-Pedroza et al. [11]; and Neave et al. [5] used the number of steps and kicks as measures of reactivity during milking procedure. Breuer et al. [4]; Sutherland et al. [8]; Sutherland and Huddart [9] measured reactivity based on the intensity of leg movements, whereas Gergovska et al. [6, 12] and Sawa et al. [7] assigned subjective temperament scores.

Additionally, there is a lack of studies assessing the relationship between cows' temperament, milk quality [12], and milkability parameters [13, 14]. Some of these studies have indicated that calmer animals produced milk with greater contents of fat and protein [15, 16], while others showed contrasting results, with the most reactive cows showing higher percentages of fat in the milk [17]. It has also been reported that calmer cows had better milkability parameters, such as greater milk flow and lower milking time [13, 14]. Considering the small number of studies addressing these issues and the divergent results, more research is needed to clarify the underlying behavioral and physiological factors affecting the relationship between temperament and productivity of dairy cows. All these cited studies used reactivity scores in the milking parlor to measure the milking temperament.

It is of particular interest to assess the temperament of dairy cattle breeds known for expressing a more reactive temperament, reacting more intensely and with greater agitation to the handling procedures [18]. Among them, we highlight the dairy Gyr cattle [19], which are widely used for crossbreeding in tropical countries, like Brazil, where around 80% of the dairy herd are Holstein-Gyr crossbred cows [20]. Under such conditions, it is expected that the crossed dairy cows with a greater Zebu breed composition will be more reactive to milking management, which may result in negative effects on milk yield and quality. Along with a higher cortisol concentration, a reduction in plasma oxytocin concentration is also expected [21], which is responsible for milk ejection and maintenance of lactation [22]. Few studies have investigated the relationship between oxytocin concentration and the temperament of dairy cows and they have found contradictory results. Sutherland and Tops [23] showed that Zebu crossbred cows displaying higher levels of agitation (measured by a reactivity score during the milking cluster attachment) in a new milking environment tended to present a greater concentration of blood oxytocin, but Sutherland et al. [10] did not find any association between reactivity in the milking parlor and the concentration of plasmatic oxytocin.

Thus, this study aimed to investigate the relationships between temperament traits and concentration of milk cortisol and oxytocin, milk yield, milkability, and milk quality in Holstein-

Gyr cows. We hypothesized that more reactive cows in the milking parlor (with higher reactivity scores, more steps, and kicks) and in the handling corral (entered and exited the squeeze chute faster) would have higher concentrations of milk cortisol, oxytocin, and produce less milk with lower quality.

2. Material and methods

This study is in accordance with the ethical principles of animal experimentation and was approved by the Embrapa Dairy Cattle Animal Care and Use Committee, Juiz de Fora, MG, Brazil (Protocol n. 5201240417).

2.1. Animals and handling

The study was carried out in the Campo Experimental da Embrapa Gado de Leite 'José Henrique Bruschi' (Coronel Pacheco, MG), by evaluating 76 Holstein (H)-Gyr (G) primiparous and multiparous cows with 2.75 ± 1.35 lactations (mean ± SD), average daily milk yield 19.90 ± 6.30 kg, and days in lactation 138.56 ± 91.91 at the beginning of the study. The animals were classified in four breed compositions: $^{3}/_{8}$HG (n = 8); ½HG = F1 HG (n = 25); ¾HG (n = 35) and $^{7}/_{8}$HG (n = 8). Cows were kept on pasture and were milked twice a day in a herringbone milking parlor (2 × 6), beginning at 07.30 a.m. and 03.00 p.m., always by the same milker, who was previously trained in good handling practices.

2.2. Temperament assessment

The behavioral responses of all 76 animals were assessed during the handling routines in the milking parlor (milking temperament) and the corral (handling temperament). The milking temperament was assessed during the morning milking for three consecutive days per month from June to August 2018, resulting in nine repeated measurements per cow. Only one milker and one observer were present during the behavioral recordings. The milker prepared each cow individually to be milked, so the observer could record the behavior of each cow in a direct and individualized manner. The reactivity measurements were taken by only one previously trained observer, considering the movement of the hind legs based on the following criteria: a) Reactivity score which is a behavioural-based score of the type and intensity of leg movement, assessed during pre-milking udder preparation (RSprep, from the first contact of the milker with the cow's teats, pre-dipping, evaluation of forestripping milk until the drying of teats) and when fitting the milking cluster (RStca, from the beginning of attachment of the first until the attachment of the last teat cup), by attributing one of the following scores: 1 = hind legs remained immobile throughout the procedure; 2 = one or two slow and gentle movements (hoof elevated at less than 15 cm from the ground) performed with one or both hind legs; 3 = three or more inconstant slow and gentle movements; 4 = constant (most of the observation time) slow and gentle movements; 5 = vigorous (elevating hooves above 15 cm from the ground), but inconstant movements; 6 = constant (most of the observation time) and vigorous movement of the hind limbs; 7 = the cow kick (elevating the hind hoof above hock line and directing it laterally towards the stockperson) and 8 = had to have one or both hind legs tied to be milked; b) Number of STEPS (elevations of the hooves below the hock line): corresponds to the sum of steps the animals took during pre-milking udder preparation and when fitting the milking cluster; c) Number of KICKS (defined as elevations of the hind hoof above hock line and directing it laterally towards the stockperson): corresponds to the sum of kicks during pre-milking udder preparation and during when fitting the milking cluster.

The handling temperament was assessed one day after assessing milking temperament, totalling three recordings throughout the study (one per month). The behavioral recordings were performed by individual observations for each animal by another observer who was unfamiliar with the animals and had experience with handling temperament assessment. Briefly, after the morning milking, the farm workers took the cows to a handling corral close to the milking parlor in a calm manner, according to the good management practices used on the farm. The following measurements were taken: a) Entrance time (ET), by measuring the time (in seconds) that each animal takes to go through the single-file race until entering the squeeze chute. The cow was allowed to move alone for ten seconds, without using any mechanism to encourage it to move. After this interval, those cows who stopped and refused to move forward were encouraged to move using voice command and, if necessary, were gently touched until they entered the squeeze chute [24]; and b) Flight speed (FS), by measuring the speed that each cow left the squeeze chute. It was done using equipment (Duboi®, Campo Grande, Brazil) comprised of two pairs of photoelectric cells and a chronometer, one of them fixed just after the exit gate of the squeeze chute and the other 2 m away. When the cow went through, the first pair of cells and the chronometer were activated, and were stopped when she went through the second pair. The time interval displayed on the equipment was used to calculate the speed of each cow, in m/s (faster animals were considered the most reactive ones).

2.3. Milk cortisol and oxytocin

The samples used to measure the concentrations of oxytocin and cortisol were collected during the morning milking, simultaneously with the milk collections for milk quality assessment, and on the last day of each milking temperament session (the third day of each monthly assessment). For the cortisol and oxytocin analyses, only ½HG and ¾HG cows were included to reduce the variation due to genetic composition. Among the 60 cows available (½HG, n = 25; ¾HG, n = 35), some had more than 6 lactations, or more than 180 days in lactation, or had clinical signs of mastitis on the days of milking sampling, and therefore were excluded. Thus, a subsample of 38 cows (½HG, n = 19 and ¾HG, n = 19) were assessed for these analyses. Hormones were measured in milk by immunoassay analysis (EIA) using commercial kits according to the manufacturer's instructions (cortisol: Monobind, Lake Forest, CA, EUA; oxytocin: Mybiosource, San Diego, CA, EUA). As hormone concentrations in milk were substantially lower than those measured in plasma, we extracted milk samples. Briefly, we centrifuged the milk sample to separate the fatty and aqueous fractions. Each fraction was lyophilized, and the milk samples were 10-fold less diluted than the plasma samples. Regarding the milk, the intra-assay CVs were 4.8 and 6.5, and the inter-assay CV was 6.0 and 9.0% for cortisol and oxytocin, respectively.

2.4. Productive performance and milkability parameters

The individual daily milk production (kg/day), daily milking time (average of morning and afternoon milkings, in seconds), average milk flow (average of morning and afternoon milkings, in kg/s), and lactation days were manually recorded by the same observer who performed the behavioral observations, one day after performing the milking temperament assessment.

2.5. Milk quality indicators

To assess milk quality (percentage of fat, protein, lactose, and somatic cell count), individual milk samples were collected from all 76 cows, always on the last day of each of the three-monthly data collections in the milking parlor. The milk samples were kept in plastic containers of 50 mL each. The Centesimal Composition Analysis and Somatic Cell Count in Raw Milk Samples tests were performed at Embrapa Gado de Leite (Juiz de Fora, MG, Brazil). The analyses of fat, protein, and lactose content (% = g/100 g of raw milk) were carried out via absorption spectrometry in a mid-range infrared sensor (ISO 9622 | IDF 141) (Bentley Instruments, Bentley FTS, Id.: 85015); whereas the somatic cell count was performed via Flow cytometry (ISO 13366–2 | IDF 148–2); (Bentley Instruments, SomaCount FCM, Id.: 82015).

2.6. Data analysis

First, a descriptive statistical analysis of the data (S1 Data) from each evaluation month was carried out using the UNIVARIATE process of the SAS statistical package (SAS Inst. Inc. Cary. NC, version 9.3). Then, we used the Kolmogorov–Smirnov test to assess whether the distribution of milking temperament measures (RSprep, RStca, STEPS, and KICKS) and handling temperament measures (ET and FS), production and physiology variables met normality. We also checked if the temperament measures differed across the months and between the breed compositions, using linear mixed models for repeated measures, via PROC MIXED of SAS, including each temperament measurement as a dependent variable, and the fixed effects of breed composition ($^3/_8$HG, ½HG, ¾HG, and $^7/_8$HG), month (1 to 3), parity (1, 2, 3, and 4 or more calvings) and the random effect of animal. The temperament measures did not differ between the months of evaluation ($P > 0.05$ for all). Regarding the breed composition, we found a significant effect for RSprep ($p = 0.031$) and FS ($p = 0.002$), with $^3/_8$HG and $^1/_2$HG cows being more reactive (higher averages for both traits) than the other breed compositions. Parity did not affect any of the temperament measures evaluated ($P > 0.05$ for all).

To assess the relations of milking temperament with cortisol and oxytocin concentrations, milk yield, milkability parameters, and milk quality parameters, first, we calculated the individual monthly averages of milking temperament measures (RSprep, RStca, numbers of STEPS and KICKS), milk yield, and milkability to eliminate the 'day' effect and obtain a single monthly measure for all of the measures studied (3 repetitions, from June to August). Then we categorize the temperament to include them as fixed effects in the models (classes low, intermediate, and high). The categorization was done based on the tertiles of distribution for the 76 cows within each month (the first tertile was categorized as 'low', the second as 'intermediate', and the third tertile as 'high' for each temperament measure). Considering the low occurrence of KICKS its distribution was considered binomial, so this variable was categorized as "low" = no occurrence of kicks and "high" = 1 or more occurrence of kicks. We did a chi-square test in contingency table to determine if there were differences in the temperament categories distribution between the three months. Non-significant results ($P > 0.05$) were obtained for all of the temperament measures, showing that the temperament categories distributions did not change across the months.

Finally, linear mixed models were fitted using PROC MIXED of SAS when the residuals attained normality and generalized linear models using PROC GLIMMIX for somatic cell count, adopting lognormal distribution of dependent variable. The models included as dependent variables the concentration of cortisol and oxytocin, average daily milk production (in kg/day), milkability parameters (milking time and milk flow), milk quality (percentages of fat, protein, and lactose, and somatic cell count), and the fixed effects of temperament measurements (one trait included at a time), assessment month (1 to 3), breed composition, parity and days in lactation as covariates with linear effect. In all models, the random animal effect (*SUBJECT*) was considered as a repeated measurement within the evaluation month (1 to 3). In all of the analyses *P*-values ≤ 0.05 were considered as significant and ≤ 0.10 were discussed as trends.

3. Results

3.1. Relationships between temperament and concentrations of milk cortisol and oxytocin

Milk cortisol was related to the milking temperament, assessed by RSprep ($p<0.001$), RStca ($p<0.001$), STEPS ($p<0.001$), and a tendency for KICKS ($p = 0.087$) (Table 1). Cows with a greater reactivity during pre-milking udder preparation (RSprep-$_{High}$) had 95.05% more cortisol in their milk than calmer cows (RSprep-$_{Low}$). Animals classified in the RStca-$_{High}$ had a cortisol concentration 100.09% greater than the cows classified as RStca-$_{Low}$. Cows that took more steps during the milking (STEPS-$_{High}$) had 81.43% more cortisol in their milk than cows with a calm temperament (STEPS-$_{Low}$). Finally, animals that kicked during milking tended to have 28.40% more cortisol in their milk when compared to cows that did not kick. Regarding handling temperament, cows in the FS-$_{Inter}$ category tended to have 36.96% more cortisol than FS-$_{low}$ individuals ($p = 0.088$). These results indicate that reactive cows had a higher concentration of cortisol in milk.

The milking temperament was also related to oxytocin concentration, with significant effects for RStca ($p = 0.023$) and tendencies for the RSprep ($p = 0.083$) and FS ($p = 0.095$) measurements. The RSprep-$_{High}$ cows had 49.5% more oxytocin in milk than RSprep-$_{Low}$ cows (Table 1). The RStca-$_{High}$ cows had 46.9% more oxytocin in milk than RStca-$_{Inter}$ ones. Finally, milk from the animals in the FS-$_{High}$ category had 36.83% more oxytocin than milk from cows in the FS-$_{Low}$ category (Table 1). The ET was not related to milk cortisol and oxytocin concentrations ($P > 0.05$).

Table 1. Least-square means (± SE) of concentration of cortisol and oxytocin as a function of classes of temperament indicators (n = 38).

Dependent variables[1]	Temperament classes			$F_{2,104}$	*P*-value
	Low	Intermediate	High		
		RSprep			
Cortisol, ng/ml	6.23 ± 0.56 [b]	7.35 ± 0.54 [b]	12.15 ± 1.12 [a]	10.87	<0.001
Oxytocin, pg/ml	5.29 ± 0.49 [b]	5.75 ± 0.47 [b]	7.82 ± 0.99 [a]	2.54	0.083
		RStca			
Cortisol, ng/ml	5.44 ± 0.60 [b]	6.89 ± 0.54 [b]	10.88 ± 0.71 [a]	17.56	<0.001
Oxytocin, pg/ml	5.82 ± 0.55 [ab]	4.91 ± 0.49 [b]	7.21 ± 0.65 [a]	3.91	0.023
		STEPS			
Cortisol, ng/ml	6.03 ± 0.53 [b]	7.23 ± 0.63 [b]	10.93 ± 0.88 [a]	11.36	<0.001
Oxytocin, pg/ml	5.50 ± 0.50	6.56 ± 0.56	5.01 ± 0.79	1.52	0.225
		KICKS		$F_{1,105}$	
Cortisol, ng/ml	7.06 ± 0.44 [b]	-	9.06 ± 1.05 [a]	2.99	0.087
Oxytocin, pg/ml	5.76 ± 0.36	-	5.87 ± 0.87	0.01	0.910
		FS (m/s)			
Cortisol, ng/ml	6.19 ± 0.69 [b]	8.48 ± 0.70 [a]	7.88 ± 0.85 [ab]	2.49	0.088
Oxytocin, pg/ml	4.74 ± 0.57 [b]	6.49 ± 0.60 [a]	6.50 ± 0.70 [ab]	2.41	0.095
		ET (s)			
Cortisol, ng/ml	7.22 ± 0.83	7.16 ± 0.55	8.05 ± 0.85	0.40	0.673
Oxytocin, pg/ml	5.39 ± 0.68	5.74 ± 0.45	6.23 ± 0.70	0.36	0.699

[1]RSprep = reactivity scores during pre-milking udder preparation, RStca = reactivity scores when fitting the milking cluster, STEPS = number of steps, KICKS = number of kicks, ET = entrance time, FS = flight speed.

[a–b] Means followed by the same letters in the row are not statistically different ($P < 0.05$).

https://doi.org/10.1371/journal.pone.0286466.t001

3.2. Relationships of temperament with milk yield and milkability

The milking temperament was not related to milk yield, or the milkability parameters (Table 2). Regarding handling temperament, ET had a significant relationship with milk yield (p = 0.004). Cows classified in the ET-$_{Inter}$ category produced 27.62% more milk than ET-$_{High}$ cows (Table 2). Among the milkability parameters, milking time was influenced by ET (p<0.0001) and FS (p = 0.0002). Cows with both extreme temperaments (high and low) for ET and FS were more difficult to milk and took more time to be milked than the intermediate ones. Cows classified as ET-$_{High}$ spent 20.22% longer time being milked than ET-$_{Low}$ cows. The same happened for animals who left the squeeze chute more slowly (FS-$_{Low}$), which spent 19.91% longer being milked than FS-$_{High}$ cows (Table 2). ET had also a significant relationship (p = 0.046) with milking flow. The ET-$_{Inter}$ cows had a flow rate 14.80% faster than the ET-$_{Low}$ cows, which did not significantly differ from ET-$_{High}$.

3.3. Relationship between milk temperament and milk quality

The milking temperament measured by RStca showed a tendency in the percentage of fat (p = 0.056). The milk from cows categorized as RStca-$_{Inter}$ had 11.83% higher fat content than the milk from cows with lower reactivity (RStca-$_{Low}$) (Table 3).

Table 2. Least-square means (± SE) of milk yield and milkability traits as a function of the temperament indicators (n = 76).

Dependent variables[1]	Temperament classes			$F_{2,211}$	*P*-value
	Low	Intermediate	High		
		RSprep			
Milk yield, kg/d	20.10 ± 1.23	18.67 ± 1.39	19.25 ± 1.50	0.57	0.565
Milking time, s	420.81 ± 12.83	435.80 ± 14.45	465.14 ± 18.15	2.22	0.111
Flow, g/s	20.45 ± 1.27	18.80 ± 1.46	21.67 ± 1.56	1.36	0.259
		RStca			
Milk yield, kg/d	19.62 ± 1.24	19.19 ± 1.36	19.56 ± 1.39	0.05	0.951
Milking time, s	421.16 ± 14.08	439.44 ± 14.04	450.36 ± 16.22	1.09	0.337
Flow, g/s	20.87 ± 1.29	19.67 ± 1.41	20.43 ± 1.45	0.33	0.718
		STEPS			
Milk yield, kg/d	20.55 ± 1.20	18.69 ± 1.44	18.43 ± 1.35	1.31	0.273
Milking time, s	435.72 ± 13.33	439.37 ± 15.53	431.19 ± 15.65	0.08	0.921
Flow, g/s	21.21 ± 1.25	18.88 ± 1.49	20.31 ± 1.41	1.20	0.303
		KICKS			
Milk yield, kg/d	19.08 ± 1.06	-	20.90 ± 1.61	$F_{1,211}$ = 1.25	0.264
Milking time, s	432.91 ± 10.38	-	446.85 ± 19.50	$F_{1,210}$ = 0.46	0.497
Flow, g/s	19.95 ± 1.10	-	22.15 ± 1.71	1.63	0.203
		FS (m/s)			
Milk yield, kg/d	21.05 ± 1.52	18.79 ± 1.12	19.69 ± 1.65	1.03	0.360
Milking time, s	516.44 ± 19.42 [a]	435.89 ± 14.22 [b]	430.68 ± 21.07 [b]	8.77	0.0002
Flow, g/s	20.78 ± 1.61	20.00 ± 1.16	21.75 ± 1.74	0.58	0.562
		ET (s)			
Milk yield, kg/d	18.49 ± 1.18 [b]	21.77 ± 1.25 [a]	17.06 ± 1.71 [b]	5.78	0.004
Milking time, s	416.38 ± 15.30 [b]	494.35 ± 16.20 [a]	500.60 ± 21.92 [a]	10.34	<0.001
Flow, g/s	19.31 ± 1.24 [b]	22.18 ± 1.31 [a]	18.86 ± 1.79 [ab]	3.13	0.046

[1] RSprep = reactivity score during pre-milking udder preparation, RStca = reactivity score when fitting the milking cluster, STEPS = number of steps, KICKS = number of kicks, ET = entrance time, FS = flight speed.

[a–b] Means followed by the same letters in the row are not statistically different (P < 0.05).

https://doi.org/10.1371/journal.pone.0286466.t002

Table 3. Least-square means (± SE) of milk quality traits as a function of the temperament indicators (n = 76).

Dependent variables[1]	Temperament classes			$F_{2,203}$	*P-value*
	Low	Intermediate	High		
	RSprep				
Fat, %	1.12 ± 0.05	1.15 ± 0.05	1.26 ± 0.06	2.07	0.129
Protein, %	3.33 ± 0.05 [a]	3.33 ± 0.05 [a]	3.17 ± 0.06 [b]	3.63	0.028
Lactose, %	4.49 ± 0.06	4.47 ± 0.06	4.44 ± 0.07	0.20	0.817
SCC, log cel/ml	5.53 ± 0.20	5.16 ± 0.23	5.30 ± 0.25	1.40	0.249
	RStca				
Fat, %	1.12 ± 0.05 [b]	1.25 ± 0.05 [a]	1.19 ± 0.05 [ab]	2.92	0.056
Protein, %	3.27 ± 0.05	3.30 ± 0.05	3.27 ± 0.05	0.19	0.825
Lactose, %	4.48 ± 0.06	4.43 ± 0.06	4.49 ± 0.06	0.53	0.588
SCC, log cel/ml	5.38 ± 0.20	5.52 ± 0.23	5.22 ± 0.23	0.74	0.478
	STEPS				
Fat, %	1.13 ± 0.05	1.24 ± 0.05	1.18 ± 0.05	1.99	0.140
Protein, %	3.31 ± 0.05 [a]	3.19 ± 0.05 [b]	3.30 ± 0.05 [a]	2.46	0.088
Lactose, %	4.47 ± 0.05	4.42 ± 0.06	4.50 ± 0.06	0.70	0.498
SCC, log cel/ml	5.46 ± 0.20	5.44 ± 0.24	5.18 ± 0.23	0.73	0.481
	KICKS				
Fat, %	1.18 ± 0.04	-	1.14 ± 0.06	$F_{1,211}$ = 0.33	0.568
Protein, %	3.26 ± 0.04	-	3.35 ± 0.06	$F_{1,211}$ = 1.80	0.181
Lactose, %	4.46 ± 0.05	-	4.50 ± 0.07	$F_{1,208}$ = 0.33	0.565
SCC, log cel/ml	5.42 ± 0.18	-	5.20 ± 0.27	$F_{2,213}$ = 0.68	0.409
	FS (m/s)				
Fat, %	1.25 ± 0.06	1.14 ± 0.04	1.19 ± 0.06	1.86	0.158
Protein, %	3.23 ± 0.06	3.27 ± 0.04	3.32 ± 0.06	0.35	0.701
Lactose, %	4.56 ± 0.07	4.44 ± 0.05	4.43 ± 0.07	1.69	0.187
SCC, log cel/ml	5.21 ± 0.25	5.42 ± 0.19	5.45 ± 0.28	0.37	0.691
	ET (s)				
Fat, %	1.19 ± 0.05	1.12 ± 0.05	1.23 ± 0.07	1.98	0.140
Protein, %	3.33 ± 0.05 [a]	3.25 ± 0.05 [ab]	3.16 ± 0.07 [b]	2.66	0.073
Lactose, %	4.41 ± 0.05 [b]	4.55 ± 0.06 [a]	4.44 ± 0.08 [ab]	2.93	0.055
SCC, log cel/ml	5.45 ± 0.20	5.27 ± 0.21	5.33 ± 0.29	0.30	0.741

[1]RSprep = reactivity score during preparation for milking, RStca = reactivity score during milking cluster attachment, STEPS = number of steps, KICKS = number of kicks, ET = entrance time, FS = flight speed, SCC, somatic cell count.

[a–b] Means followed by the same letters in the row are not statistically different ($P < 0.05$).

https://doi.org/10.1371/journal.pone.0286466.t003

Regarding protein, cows with lower reactivity scores (RSprep-$_{Low}$) produced milk with 5.21% higher protein content (p = 0.028) than the milk produced by cows of a more reactive temperament (RSprep-$_{High}$). The cows classified as STEPS-$_{Inter}$ tended (p = 0.088) to produce milk with 3.45% lower protein content when compared to cows classified as STEPS-$_{Low}$ (Table 3). Protein content was also influenced by handling temperament, as the milk from cows with ET-$_{Low}$ tended (p = 0.073) to have 5.24% greater protein content than the milk from cows with ET-$_{High}$.

Lactose content tended to be related with ET (p = 0.055), as the milk from cows classified in the ET-$_{Inter}$ category had 3.17% more lactose than cows with ET-$_{Low}$ (Table 3). Finally, the SCC was not related to any of the temperament traits, either during milking or in the handling in the corral (Table 3).

4. Discussion

4.1. Relationships between temperament and concentrations of milk cortisol and oxytocin

The concentration of milk cortisol was greater for cows with a more reactive temperament during milking, as measured by our high reactivity scores during preparation and teat cup attachment, and by the high number of steps and tended to kick more during milking. It should indicate that these cows presented behavioral and physiological signs of stress during milking, suggesting that reactive cows are more susceptible to stress during routine handlings. This is similar to the findings by Wenzel et al. [25] and Gygax et al. [26] in which cows that kicked more or took more steps in the milking parlor produced milk with higher concentrations of cortisol when compared to their calmer counterparts. However, this differed from the results by Sutherland et al. [10] and Sutherland and Huddart [11], who evaluated the reactivity of the animals using reactivity scores similar to ours and did not find an association between the agitation of the cows in the milking parlor and the concentration of plasmatic cortisol. The same was reported by Van Reenen et al. [27], who did not find an association between the number of steps and kicks in milking and the concentration of plasmatic cortisol. These different results could be due to the cortisol sampling methods. In our study, we assessed the concentration of cortisol in the milk, as it is a less invasive method that does not cause additional stress during sampling collection. Van Reenen et al. [27]; Sutherland et al. [10] and Sutherland and Huddart [11] used blood sampling, which could increase the levels of plasmatic cortisol even in less reactive cows.

Blood cortisol is widely used to assess the neuroendocrine stress response [10, 11, 27, 28], but it is an invasive technique that could activate the HPA axis and cause an increase in plasma cortisol levels in cows [29]. A non-invasive alternative has been to measure cortisol in the milk. Cortisol, like other steroid hormones, can permeate and cross the epithelial layer between blood vessels and the alveoli of the mammary gland [29], resulting in a positive correlation between the concentration of cortisol in the blood and milk in response to different milking techniques [26, 30, 31]. Milk cortisol may be used as a biomarker to assess stress response to short- medium-term (12 h) environmental challenges in dairy cow [32].

Studies using ACTH challenge to investigate the changes in milk cortisol concentration found that the cortisol in milk might remain elevated until 8–10 h after receiving the stimulus, depending on the ACTH dosage [30, 31, 33]. In the study of Sgorlon et al. [34], the animals were milked twice a day (12 h intervals), as in the present study. In these situations, the cortisol concentration in the milk possibly reflects the variation of the plasma concentration in the interval of 10 to 14 h before the milk sampling, *i.e.* the previous milking [34].

Our results confirm the hypothesis that cows that are more reactive during milking are also more susceptible to physiological stress during handling and show a higher concentration of cortisol in milk. The high concentrations of cortisol and noradrenaline in the blood are associated with stress in the milking environment [35], as cortisol is one of the main hormones associated with physiological stress response in mammals [36]. A greater increase of this glucocorticoid occurs due to a stronger activation of the hypothalamic-pituitary-adrenal (HPA) axis in response to a stressing agent, that might be physical or emotional [36]. Individual differences in response to environmental stimuli are expected, and the variation in the glucocorticoid concentration has been associated with differences in temperament in beef cattle measured by the flight speed test [37].

The concentration of oxytocin was also higher in cows that presented greater reactivity scores during milking, as measured by high reactivity scores during teat cup attachment. Our results corroborate those of Sutherland and Tops [23], where cows with greater levels of RStca agitation in a new milking environment (psychological stressor) tended to present a greater concentration of blood oxytocin, suggesting that oxytocin may be related to the behavioral stress response in dairy cows. According to the authors, cows that present a heightened response to a psychological stressor and have higher concentrations of oxytocin could have greater stress coping mechanisms. In turn, Sutherland et al. [10] did not report any association between reactivity in a familiar milking parlor and concentrations of plasmatic oxytocin.

Oxytocin is the hormone responsible for milk ejection and maintenance of lactation [22], but has also been pointed to as a physiological reaction to stressing agents [10, 23]. In our study, the milk from reactive cows had higher cortisol and oxytocin concentrations, suggesting that a higher concentration of oxytocin might be part of the stress response in these cows, likely as a stress coping mechanism. That may occur as an attempt to mitigate the effects of stress during the milking process, as oxytocin has anti-stress [38] and anxiolytic effects [39], both associated with the HPA axis [37, 38]. However, some studies report that a high oxytocin concentration in female rodents leads to a decrease in cortisol concentration [39]. The same happens in dairy cows habituating to a new milking environment, where there is an increase in oxytocin release as the cows get used to the new environment [23], accompanied by a decrease in cortisol concentration. Sutherland et al. [10] found that in a new milking environment (psychological stressor), the blood cortisol concentration was greater before milking, and the oxytocin concentration was greater after milking. These results suggest that the level of cortisol before milking attenuated the oxytocin response to the new situation.

However, other studies have indicated that high levels of cortisol do not suppress the secretion of oxytocin [21, 40], similar to what occurred with the concentration of both hormones in the milk of our cows. Therefore, our results show that Holstein-Gyr crossbred cows with high reactivity had behavioral and physiological signs of stress during milking, even if they were milked in a familiar environment and by milkers using good handling practices, but the stress experienced by the cows seems not to affect the milk production. Reactive cows during milking had lower milk flow and longer milking time. They also showed an increase in oxytocin concentration during milking. Thus, a higher concentration of oxytocin does not necessarily mean a good milk ejection. That is, cows could release oxytocin and retain milk. Therefore, to analyze milking quality as a function of cows' temperament, it is necessary to gather data from oxytocin release, milk flow, milking time, and milk yield.

Unlike milking temperament, the cows with intermediate handling temperament measured by FS tended to have higher concentrations of milk cortisol and oxytocin compared to those with extreme temperaments (low and high). These results differ from those of Sutherland et al. [10], who found that the more reactive cows (with high FS) had a higher basal cortisol concentration in a familiar milking environment (*i.e.* a rotary milking parlor where the cows were usually milked), but there was no variation in the cortisol concentration between cows of different FS categories exposed to an exogenous ACTH challenge. When exposed to a novel milking environment (a herringbone parlor within the same farm), these cows did not show variation in the concentration of plasmatic cortisol in relation to FS. In the same study, Sutherland et al. [10], working with multiparous cows, found that the concentration of blood oxytocin was higher for cows in the novel environment, regardless of FS category. However, in primiparous cows, the concentration of plasmatic cortisol was higher in cows with high FS during the first milking sessions [11]. In general, the authors found that the heifers previously trained to be milked reached lower plasmatic cortisol concentration. Flight speed is commonly used to assess differences in temperament for beef cattle [37, 41], but fewer studies have used this indicator for dairy cattle [6, 10, 42]. Since the concentration of cortisol and oxytocin had a positive and linear relationship with the reactivity measures during milking (but non-linear relation with the reactivity to handling in the corral), we might infer that the cows had different perceptions of the stimuli in the two distinct handling locations and reacted distinctively, resulting in different patterns of relationships between behavioral and physiological responses.

4.2. Relationships between temperament, milk yield, and milkability

We hypothesized that milking temperament would be related to milk yield based on previous studies reporting that cows who are more reactive to milking (measured by the number of steps and kicks) produced less milk [4, 5, 7]. Nevertheless, none of the milking temperament measures assessed in the present study were related to milk yield. The lack of association between milking temperament and milk yield was previously reported by Van Reenen et al. [27]; Orbán et al. [43]; and Sutherland and Huddart [11].

In contrast to the results reported by Sutherland and Dowling [44], Sutherland and Huddart [11], we did not find any association between FS and milk yield. Regarding milkability parameters, FS was associated with milking time and average milk flow. The cows which exited the squeeze chute slowly, considered to have a calmer temperament, spent more time being milked than more reactive cows, contrary to what we expected, but similar to what was reported by Sutherland and Huddart [11].

Among the handling temperament measures assessed in this study, only ET was related to milk yield, with cows classified as intermediate producing more milk than those classified as low and high for ET. It is possible that among the cows with the highest values for ET, some refused to walk and need to be stimulated with voice commands and / or touch to go into the squeeze chute. In its turn, those with the lowest ET values should include cows that entered running (i.e., more reactive ones). In this specific case, the Intermediate class should include animals with a better temperament that entered walking the single-file race and did not need to be stimulated to walk. Both extremes (low and high) for this measure, could be regarded as undesirable behaviors in the production environment. The ET was also related to milkability parameters since the intermediate cows showed greater average flow than the low and high classes. Furthermore, cows that took longer to enter the squeeze chute (possibly including cows that refused to walk as a response to fear), were the ones that took longer to be milked. Contrasting results were reported by Sutherland et al. [10], who found that dairy cows of intermediate temperament (average exit time–i.e., between 2 and 4s) reached a lower average flow when compared to those of calmer (exit time $>$ 4s) and more reactive (exit time $<$ 2s) temperaments, revealing a lack of consensus, that is probably related to the different types of temperament measures used.

It is interesting to highlight that few studies [11, 44, the present] evaluated the relationships between handling temperament with productive parameters for lactating dairy cows. Most of the studies with dairy cows limited the temperament assessment to the milking reactivity. In future studies, assessing the temperament of dairy cows should include indicators from different handling situations (beyond the milking parlor) to evaluate if the temperament in a broader sense could be related to productive parameters.

4.3. Relationship between temperament and milk quality

Calmer cows, measured by reactivity score during preparation, produced milk with a higher protein content and calmer cows during teat cup attachment tended to produce lower fat content. Similar results were found by Morales Pineyrúa et al. [45] for Holstein cows, in which calmer cows based on a milking reactivity score similar to ours, had lower protein and fat content. The handling temperament also influenced the milk quality. Cows that entered the squeeze chute faster (i.e., low class for ET) tended to have higher protein content while cows that entered the chute calmly (intermediate ET) tended to produced milk with higher contents of lactose than the faster cows. Kruszynski et al. [16] found that calmer cows produced milk with higher protein and fat contents. In turn, Cziszter et al. [17] reported that the milk produced by more agitated cows in the milking parlor had greater fat percentages than the milk from cows of intermediate temperament, which had a lower content of protein than the calmer and more agitated ones. In contrast, Gergovska et al. [12] found that both more agitated and calmer cows produced milk with a higher fat content than those of intermediate temperament. Finally, Orbán et al. [43] failed to find a significant effect of temperament on the protein and fat contents in the milk of Jersey and Holstein cows. All of these studies assessed temperament based on the cows' reactivity during milking. The lack of consensus on the effect of dairy cows' temperament on fat and protein milk contents is likely due to differences in temperament assessment methods, breed, or handling conditions. In the present study, animals with a calmer temperament in the milking parlor produced milk that could be regarded as more desirable by consumers of fluid milk, that is, with higher protein content and lower fat content [46]. The relationship between temperament and milk quality should be further investigated in future research since there are few studies published on this topic.

Finally, the present study had some limitations that must be discussed. The research was conducted on an experimental farm where the animals are handled more frequently, which would make them more habituated to handling (being regarded as 'calmer') than the average Zebu cows in Brazilian commercial herds. Additionally, our sample varied in days in lactation, parity, and genetic group. To standardize these sources of variation we would have to exclude animals from our sample, leading to an even lower sample size. Therefore, we decided to include all of the cows available in the herd and control for these factors in the statistical analyses. Finally, we expected to find a genetic group effect in the temperament measures, but we were not able to investigate this relationship because of the low sample of animals within each genetic group. Future studies on this topic should include larger samples of crossed Zebu cows to allow for the assessment of genetic group effects on temperament and hormone concentration. It would also be of interest to integrate physiological and temperament indicators assessed in different handling situations (corral and milking parlor) [3]. The inclusion of other tests traditionally used to assess temperament in cattle should also be investigated in future studies, such as novel object, novel human, avoidance distance, and restraint tests [7]. It would allow for a broader view of the cows' temperament, including traits that go beyond milking reactivity. The integration of various temperament tests should be assessed using statistical methods for data dimensionality reduction, such as principal component analyses or factor analysis, which would help identify key components or factors that provide a better overall understanding of Zebu cows' temperament.

5. Conclusions

We conclude that handling temperament is related to milk yield and milkability, since calm and intermediate cows in the handling corral produced more milk and presented better milkability parameters, such as a shorter milking time and greater average milk flow. Additionally, the cows with better temperament in the milking parlor (calm and intermediate cows) produced milk with lower fat content and higher protein content. More reactive cows during milking produced milk with higher concentrations of cortisol and oxytocin, showing that behavioral reactivity could be related to the intensity of the physiological stress response. Future studies should investigate measures that lead to the improvement of temperament of crossbred Zebu cows, such as genetic selection and the use of good practices of handling, with the aim of reducing the cows' reactivity to handling and improving the welfare of the cows, the workers, and the productive indices, making the dairy industry more sustainable and efficient.

Supporting information

S1 Data. Data set used in the statistical analysis.
(XLSX)

Acknowledgments

This study is part of the first author's doctoral thesis prepared for the Graduate Program in Biodiversity and Nature Conservation of the Federal University of Juiz de Fora (UFJF), Brazil. The authors thank EMBRAPA Gado de Leite for kindly providing the animals and infrastructure necessary for the study, and the NEBEA students and Embrapa staff who collaborated with the study. We are grateful to the International Society for Applied Ethology for the English editing assistance as part of the English Language Help Service.

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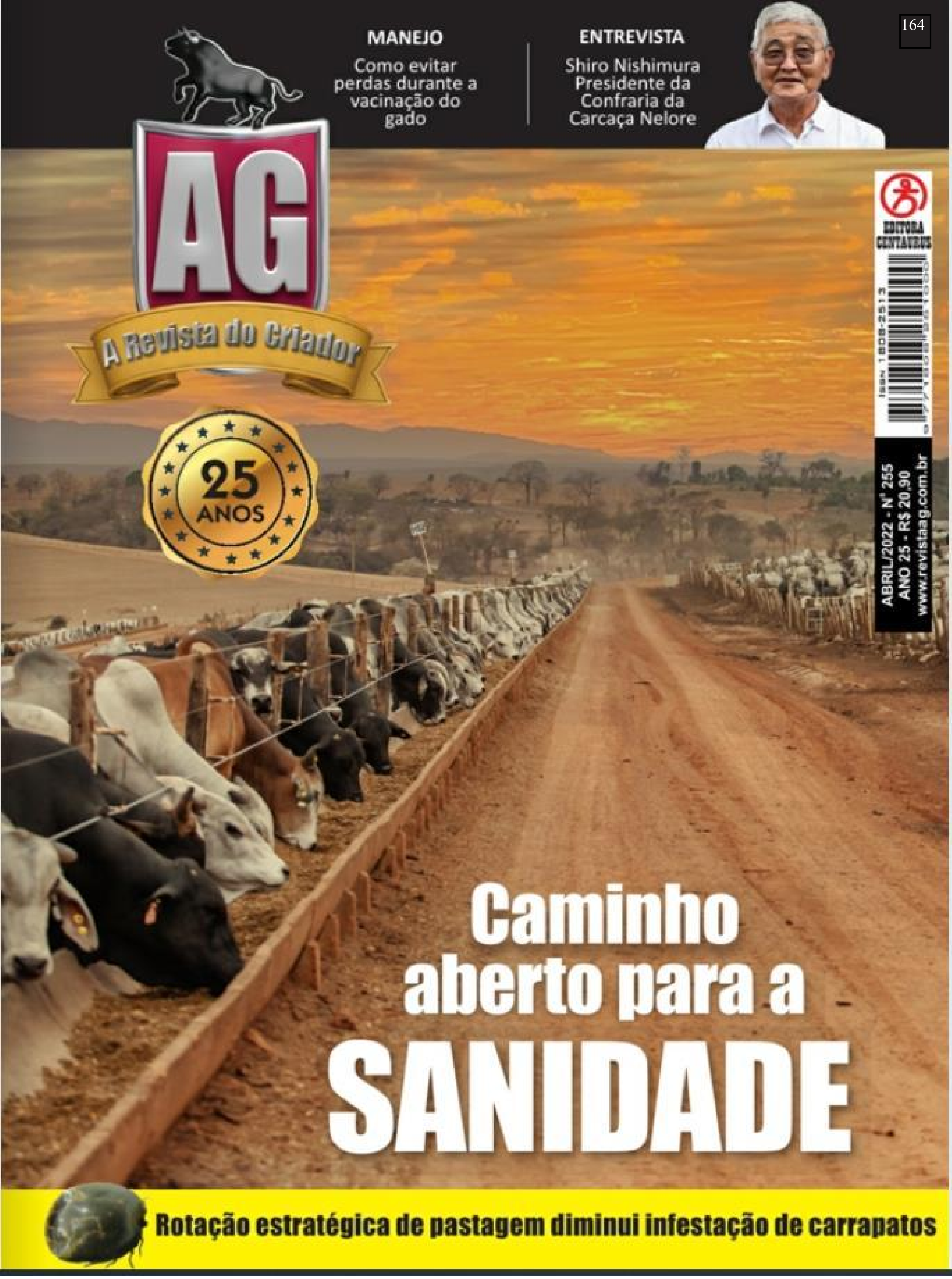
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O risco das ARISCAS

Vacas calmas destinam mais energia líquida para a produção e emitem menos metano entérico por litro de leite durante a ordenha

Maria Guilhermina Pedroza[1]

Ao longo das últimas décadas, a pecuária, no Brasil e no mundo todo, vem despertando um maior interesse dos consumidores e da população em geral, por questões relacionadas ao bem-estar dos animais e aos impactos ambientais gerados pela atividade. Nesse contexto, destacam-se as emissões de metano entérico (gás metano) proveniente da criação do gado. O metano entérico é um produto natural resultante do processo digestivo dos ruminantes, sendo produzido pela ação de micro-organismos que vivem no rúmen, os quais permitem que os bovinos possam digerir alimentos fibrosos. O metano é emitido, em sua grande maioria, por meio dos processos de respiração e da eructação (arroto) dos animais. A produção de metano entérico representa não apenas danos ambientais, mas também prejuízos para o metabolismo e a produtividade do animal, uma vez que as emissões diárias de gás metano podem chegar a representar de 2% a 12% de perda da energia bruta consumida por animal adulto, energia esta que poderia ser convertida em carne (gado de corte) ou leite (gado leiteiro). Dessa maneira, as emissões de metano entérico representam não só perdas ambientais, como também econômicas.

A bovinocultura vem sendo apontada como uma das grandes vilãs pelo aumento da temperatura no planeta, em decorrência das emissões de gás metano, que é um dos gases de efeito estufa (GEE). A redução das emissões desses gases foi um dos temas intensamente debatidos durante a realização da Conferência das Nações Unidas sobre mudanças climáticas (COP26), realizada em Glasgow, na Escócia, no final de 2021. Nesse encontro, no qual representantes de vários países estiveram presentes, mais de 100 deles, incluindo o Brasil, se comprometeram em reduzir em até 30% as emissões de gás metano até 2030. Um passo importante e necessário, visto que o Brasil possui cerca de 218 milhões de cabeças de gado. Desse total, 16,2 milhões foram vacas leiteiras ordenhadas em 2021, de acordo com dados do IBGE.

Assim, são necessárias e urgentes estratégias que visem à redução das emissões de metano entérico pela pecuária, principalmente medidas acessíveis que possam ser adotadas por grandes e pequenos produtores. Nesse sentido, alguns fatores já foram relatados como associados às emissões de metano, como a qualidade da dieta, o nível de ingestão de matéria seca, a temperatura ambiente, dentre outros, baseados em estudos realizados, em sua maioria, com gado de corte. As principais alternativas possíveis para mitigação de metano investigadas estão relacionadas às estratégias nutricionais, como a utilização de aditivos, além de alternativas como o uso de sistemas de confinamento para bovinos de corte.

Para gado leiteiro, ainda há menos pesquisas que investigam as emissões de metano, incluindo suas causas e estratégias de mitigação. Um estudo recentemente realizado pela Embrapa Gado de Leite e pela Universidade Federal de Juiz de Fora (UFJF), em Minas Gerais, mostrou que o temperamento de vacas leiteiras, ou seja, as diferenças comportamentais entre os animais, pode influenciar as emissões de metano e a produção de leite. A pesquisa faz parte da tese de doutorado de Maria Guilhermina Pedroza pelo Programa de Biodiversidade e Conservação da Natureza da UFJF. Segundo a doutoranda, vacas mais agitadas durante a ordenha (que derrubaram mais vezes o conjunto de teteiras) emitiram 36,77% mais metano entérico por litro de leite e destinaram 25,24% menos da energia líquida (energia consumida menos a energia gasta para manutenção do animal) para a lactação. O contrário aconteceu com as vacas mais calmas que ruminaram mais na sala de ordenha, demostrando estarem mais tranquilas e relaxadas durante o manejo. Elas emitiram 37,10% menos metano entérico por litro de leite e destinaram 57,93% mais energia líquida para a produção de leite. O temperamento das vacas no curral de manejo também se relacionou com as emissões de metano e a produtividade. Vacas que saíram mais rápido do tronco de contenção (consideradas mais reativas), em geral, tiveram 14,30% mais emissão de metano entérico por litro de leite, e as que entraram mais rapidamente no tronco (mais reativas) perderam 13,29% mais energia bruta na forma de metano entérico.

Foto: Maria Guilhermina Pedroza

Vacas mais calmas emitiram 37,1% menos metano entérico por litro de leite que as reativas

A pesquisa foi realizada no campo experimental da Embrapa Gado de Leite em Coronel Pacheco (MG), com 28 vacas primíparas cruzadas (F1) entre as raças Holandesa e Gir Leiteiro. Todas receberam treinamento para a ordenha no período pré-parto. Vacas cruzadas de origem zebuína representam 80% do rebanho leiteiro nacional, os animais 5/8 formam a raça Girolando, uma raça sintética desenvolvida para as condições tropicais, unindo duas raças de temperamentos diferentes. "O cruzamento dessas raças resultou em um animal rústico e com boa produção de leite; no entanto, são mais ariscos à ordenha", diz Mariana Campos, pesquisadora da Embrapa.

Foto: Maria Guilhermina Pedroza

Agitação foi medida pela movimentação do corpo, das orelhas e da cabeça

Avaliação individual

O comportamento das vacas foi avaliado individualmente, para que o temperamento de cada animal fosse

Foto: Maria Guilhermina Pedroza

Vacas que deram mais passos, coices ou derrubaram mais vezes as teteiras foram consideradas como as mais reativas

definido, variando desde vacas mais calmas, intermediárias, às mais reativas. A classificação ocorreu de acordo com o comportamento na sala de ordenha e no curral de manejo. No ambiente de ordenha, o comportamento avaliado foi a movimentação das patas traseiras, registrada por meio da contagem do número de passos e de coices durante a higienização dos tetos e o teste da caneca de fundo preto e durante a colocação do conjunto de teteiras, além do registro de ocorrência de defecação, micção, derrubada das teteiras e ruminação durante todo o processo de ordenha. As vacas que deram mais passos, coices ou derrubaram mais vezes as teteiras foram consideradas como as mais reativas, e as que foram menos agitadas e/ou ruminaram mais na sala de ordenha, as mais calmas.

No curral de manejo, as vacas foram conduzidas para o tronco de contenção, sendo registrado o tempo gasto por cada animal para entrar no tronco. As vacas que demoraram muito tempo para entrar demonstraram medo, e as que entraram correndo foram consideradas as mais reativas. Dentro do tronco, foi medido o nível de agitação das vacas por meio da movimentação do corpo, das orelhas e da cabeça, da respiração audível, sendo que cada animal recebeu uma nota para o seu grau de reatividade, em que as notas mais altas indicaram maior reação. Após essa avaliação, foi registrada a velocidade de saída das vacas do tronco (quanto mais rápidas, mais reativas) e, por último, foi medida a velocidade de fuga em relação a um observador desconhecido. Vacas que permitiram uma maior aproximação, ou se deixaram ser tocadas pelo observador, foram consideradas mais calmas e dóceis.

Medição

A medição das emissões de metano entérico foi realizada em câmaras respirométricas, nas quais as vacas ficaram por 48 horas, saindo apenas para serem ordenhadas. O procedimento foi repetido seis vezes durante todo o período de lactação. Dentro dessas câmaras, foi possível medir a quantidade de gases liberados e consumidos por cada animal e, assim, fazer as estimativas das emissões de gás metano por dia. Para as medidas metabólicas, foram realizados ensaios de digestibilidade, com coletas de amostras de alimento, sobras de alimento, urina e fezes, para estimar a quantidade de energia consumida e perdida (na forma de fezes, urina, metano e produção de calor) e a energia retida ou líquida destinada para a produção de leite. Além disso, também foi registrada a produção diária de cada vaca para fazer o cálculo da quantidade de metano emitido por litro de leite produzido. As emissões de gás metano têm sido calculadas por unidade de produto (carne ou leite), por ser uma medida mais adequada.

A reatividade e a ruminação das vacas na sala de ordenha, bem como os testes realizados no curral de manejo, podem ser medidas úteis para prever animais mais propensos às maiores emissões de metano entérico por litro de leite e à baixa produtividade, elementos que afetam negativamente a sustentabilidade e a eficiência da atividade leiteira. O temperamento indesejável dos animais no ambiente de criação pode ser amenizado com o uso de boas práticas de manejos desde o nascimento, passando pelo treinamento para a primeira ordenha antes do parto das novilhas e o trato gentil (tom de voz suave, chamar o animal pelo nome ou número, sem realizar movimentos bruscos nem utilizar-se de instrumentos de agressão) dentro e fora da sala de ordenha. Atitudes que também favorecem o manejo com os animais, pois animais mais calmos tornam o trabalho mais eficiente, e sobretudo mais seguro, aumentando, assim, o nível de bem-estar dos próprios trabalhadores e evitando acidentes e danos às instalações.

De acordo com Aline Cristina Sant'Anna, professora da UFJF e orientadora de Maria Guilhermina Pedroza, o temperamento dos animais é uma característica herdável, mas que sofre interferência das condições ambientais. Assim, a redução das emissões de gás metano na pecuária passa pela melhoria do manejo dos animais, o que traz também ganho para a eficiência produtiva e o bem-estar dos animais, dos trabalhadores e do planeta.

[1] Doutoranda pela UFJF, orientada pela professora Aline Cristina Sant'Anna (UFJF) e coorientada pela pesquisadora da Embrapa Gado de Leite, Mariana Campos

Comportamento, Manejo, Sanidade, Sustentabilidade

Vacas mais calmas emitem menos metano

Pesquisadores apontaram que o manejo racional transmite bem-estar e está associado à menor emissão de metano entérico e à maior produtividade das vacas leiteiras.

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Por Mariana Magalhães Campos, Maria Guilhermina Pedroza e Aline Cristina Sant'Anna individuais de comportamento, ou seja, à forma como cad[...]age às rotinas de manej[...] e ao ambiente de produção. É possível notar tendências de alguns indivíduos serem mais ou menos agitados, agressivos, ativos, curiosos, medrosos, mansos, dóceis e reativos. **De modo geral, os bovinos com maiores níveis de reatividade enfrentam problemas de bem-estar e são mais susceptíveis ao estresse, condição que pode acarretar prejuízos para a saúde e produtividade dos animais.**

Além disso, o trabalho com animais mais reativos implica em elevação dos custos associado[...] com: (1) necessidades de mais manejadores bem treinados; (2) riscos com a segurança dos colaboradores; (3) tempo despendido com o manejo dos animais; (4) necessidade de infraestrutura otimizada e que demanda manutenções mais frequentes; (5) lotes de animais mais heterogêneos; (6) perda em produção e qualidade de leite, devido ao estresse durante a ordenha; (7) redução da eficiência na detecção de cio em sistemas nos quais a inseminação artificial é utilizada. Ou seja, temperamentos indesejáveis acarretam danos ao bem-estar do animal e do homem, além de prejuízos econômicos.

É possível reduzir a reatividade dos animais, uma vez que o temperamento possui um componente hereditário, conforme explorado nos programas de melhoramento genético bovino. As condições ambientais também interferem no comportamento das vacas, por isso, o manejo racional deve ser adotado. E, embora a genética influencie o temperamento do animal, é possível moldar o fenótipo por meio da manutenção dos ambientes livres de estresse (da ordenha, em especial). Assim, a seleção de vacas mais calmas e a adoção de boas práticas na rotina da atividade favorecem o bem-estar tanto das vacas quanto dos trabalhadores.

As boas práticas requerem qualidade nas interações entre pessoas e animais, com certos níveis de contatos positivos, de forma a reduzir reações de medo dos bovinos e facilitar a ação do homem. **Partindo dessa premissa, torna-se cada vez mais comum o treinamento da novilhas para a primeira ordenha, técnica bastante adequada ao rebanho leiteiro brasileiro, composto por cerca de 80% de animais mestiços, principalmente da raça Girolando.** Os animais que possuem algum grau de sangue zebuíno são mais adaptados às condições tropicais – clima, alimentação e presença de agentes parasitários, além de atingirem melhores índices produtivos, comparados aos animais de raças europeias criados no Brasil. Porém, os zebuínos apresentam temperamento mais reativo, que pode ser afetado por genética, sexo, sistema de criação, tipo de manejo e experiências prévias.

<table>
<tr><td rowspan="3">
Foto 1. Massagem com bastão.</td><td>Dias 1 e 2</td><td>**junto aos gr**[...] e se desloca de modo calmo.</td></tr>
<tr><td>Dias 3 e 4</td><td>Uso de **bastão com material macio na extremidade,** para **estimulação tátil** inicial, à distância (10 minutos/animal, em média).</td></tr>
<tr><td>Dias 5 a 8</td><td>**Estimulação tátil com uso de escova:** inicialmente, no pescoço, no flanco e na inserção da cauda; posteriormente, no úbere, no interior das pernas e na virilha (10 minutos/animal, em média).</td></tr>
<tr><td rowspan="2">**Semana 2**

Foto 2. Passagem pela ordenha.</td><td>Dia 1</td><td>Primeira **passagem pela ordenha:** primeiramente, as novilhas permanecem soltas na sala de espera, para se habituarem ao local (10 minutos); em seguida, são levadas à contenção da ordenha, onde recebem estimulação tátil no úbere.</td></tr>
<tr><td>Dias 2 a 5</td><td>Condução até a sala de espera da ordenha, de onde são imediatamente direcionadas à contenção, para a realização de **escovação enquanto permanecem dentro da ordenha** (2 minutos/animal, em média): inicialmente, escovação de todo o corpo e, a partir do 5º dia, apenas do úbere, do interior das pernas e da virilha.</td></tr>
<tr><td>**Semana 3**

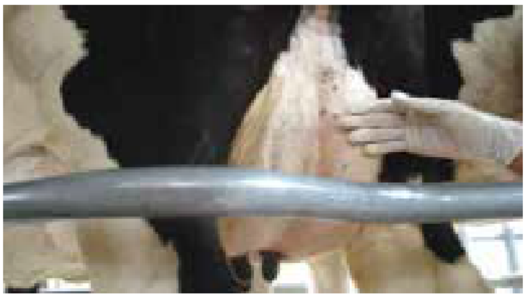



Foto 3. Massagem no úbere.
Semana 4

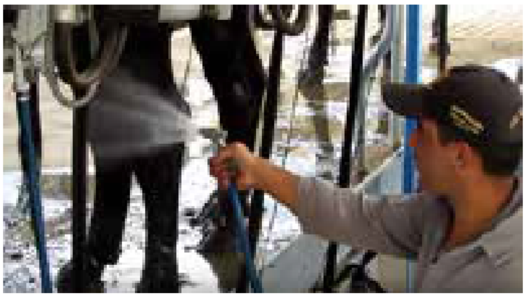



Foto 4. Uso de mangueira para lavagem e estímulos sonoros na ordenha.</td><td>Dias 1 a 5</td><td>Condução até a sala de espera da ordenha, de onde são imediatamente direcionadas à contenção, para a realização de **estimulação tátil do úbere (com as mãos)** e **lavagem (uso de mangueira),** de forma a simular a preparação para a ordenha: início da exposição das novilhas aos **estímulos sonoros presentes na ordenha** (ventiladores e ordenhadeira mecânica).</td></tr>
</table>

As boas práticas devem nortear todas as etapas da criação animal, do nascimento à fase adulta. No caso das vacas leiteiras, as quais mantêm contato frequente com humanos, o manejo racional ou gentil inicia-se na fase pré-parto e estende-se na rotina do período de lactação, com ações positivas, como: (1) conduzir para o local de ordenha com calma, sem calmas.

De acordo com os resultados do estudo, as vacas que deram menos coices e ruminaram ma durante a ordenha, produziram mais leite. **Logo, a ocorrência de coices durante a ordenha é indicadora de reatividade, ao passo que a ruminação indica relaxamento, o que sugere que as vacas que ruminam mais apresentam melhores índices de bem-estar no ambiente de produção.**

> **AS VACAS QUE DERAM MENOS COICES E RUMINARAM MAIS DURANTE A ORDENHA, PRODUZIRAM MAIS LEITE**

Mais Calma, Menos Metano

Os impactos ambientais associados à atividade pecuária, principalmente as emissões de metano (CH_4), um dos principais gases do efeito estufa (GEE), também desperta crescente interesse na população. As emissões de CH_4 pelos bovinos ganharam ainda mais destaque nas mídias sociais a partir do final de 2021, quando potências mundiais reuniram-se na Conferência das Nações Unidas sobre Mudanças Climáticas (COP26), em Glasgow, na Escócia.

No encontro, representantes de mais de 100 países, incluindo o Brasil, comprometeram-se a reduzir em até 30% as emissões do gás metano, até 2030. Boa parte da redução passa pelo desenvolvimento da pecuária mais sustentável, pautada em medidas que visam a mitigação do metano entérico emitido pelo rebanho nacional, um dos maiores do mundo, com cerca de 224,6 milhões de cabeças, em 2021, de acordo com dados do IBGE.

Para investigar esse assunto, outro estudo foi realizado pelos mesmos pesquisadores [2], os quais demonstraram que o temperamento das 31 vacas leiteiras F1 Holandês x Gir, dessa vez em lactação, também se relacionou com as emissões de CH_4. O temperamento das vacas fo avaliado na sala de ordenha e no curral de manejo, onde as vacas foram conduzidas ao tronc de contenção, enquanto era registrado o tempo para cada animal entrar no tronco. As vacas que demoraram mais para entrar demostraram medo; já as que entraram correndo foram consideradas mais reativas. Dentro do tronco, foi avaliado o nível de agitação das vacas, por meio da movimentação do corpo, das orelhas e da cabeça; da respiração tranquila ou audíve

Foto 5A. *Câmaras respirométricas da unidade experimental da Embrapa Gado de Leite de Coronel Pacheco/MG.*

digestibilidade, com coletas de amostras de alimento (for... ...bras), urina e fezes, para estimar a quantidade de energia consumida, perdida (nas formas de fezes, urina, metano e produção de calor) e retida (ou líquida), destinada à produção de leite (Figura 2). A produção diária de cada vaca foi registrada e a quantidade de metano emitido por litro de leite produzido foi calculada.

Segundo os resultados, as vacas mais agitadas durante a ordenha, as que derrubaram mais vezes o conjunto de teteiras, emitiram 36,77% mais metano entérico por litro de leite e destinaram 25,24% menos energia líquida para a lactação (energia consumida menos a energia gasta para manutenção do animal, nesse caso; Gráfico 1).

> **O TEMPERAMENTO DAS VACAS LEITEIRAS ESTÁ ASSOCIADO ÀS EMISSÕES DE CH4**

> ***Se liga: Em produção animal, a métrica mais adequada para representar a emissão do gás metano é o cálculo por unidade de produto (carne ou leite).***

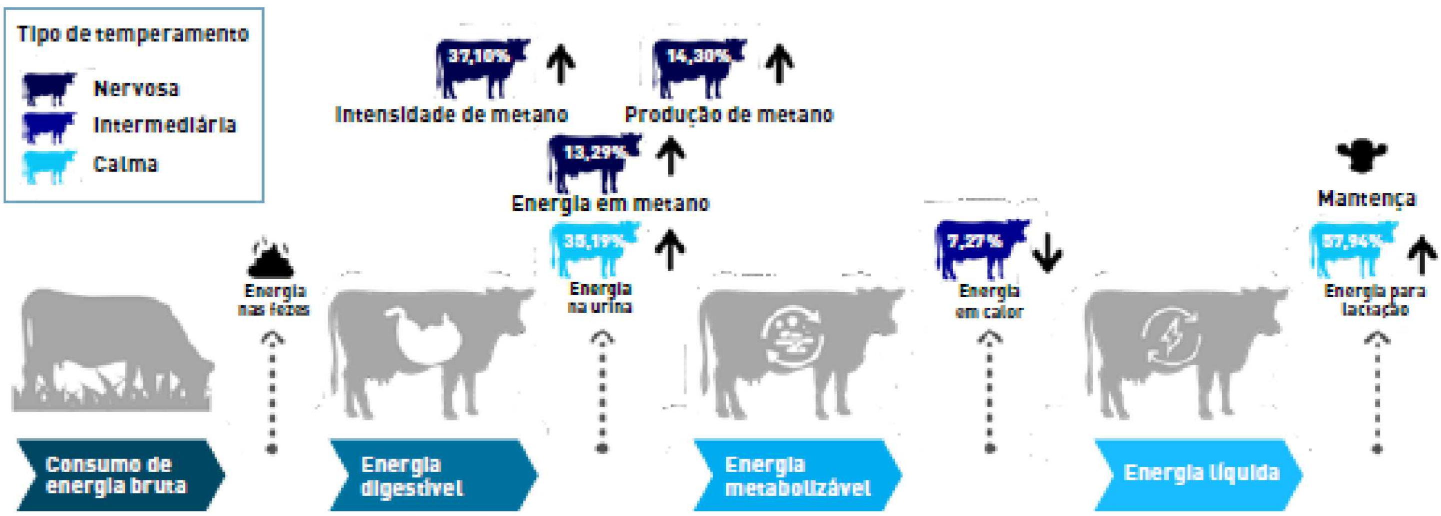


__Figura 2.__ Esquema do fluxo de energia e emissões de metano entérico, com base no tipo de temperamento das vacas leiteiras F1 Holandês X Gir.

Fonte: Rogério Vicentini e Maria Pedroza.

emitiram 37,10% menos metano entérico por litro de leite … **m 57,93% mais energia líquida para a produção de leite.** Logo, foram mais produtivas (Gráfico 2).

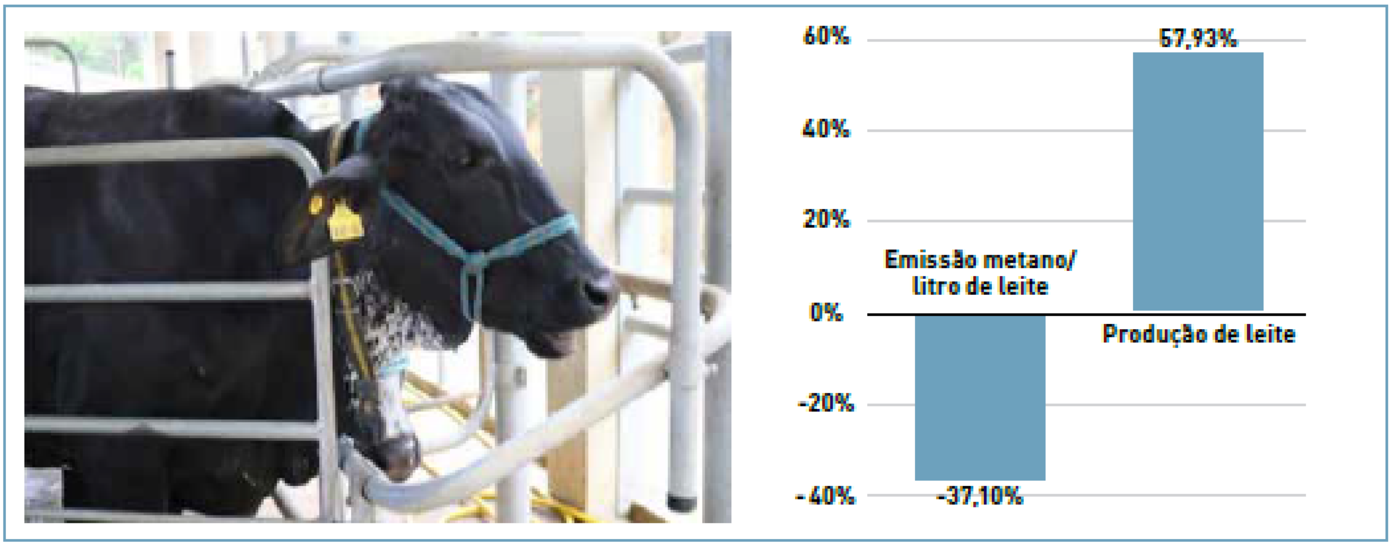
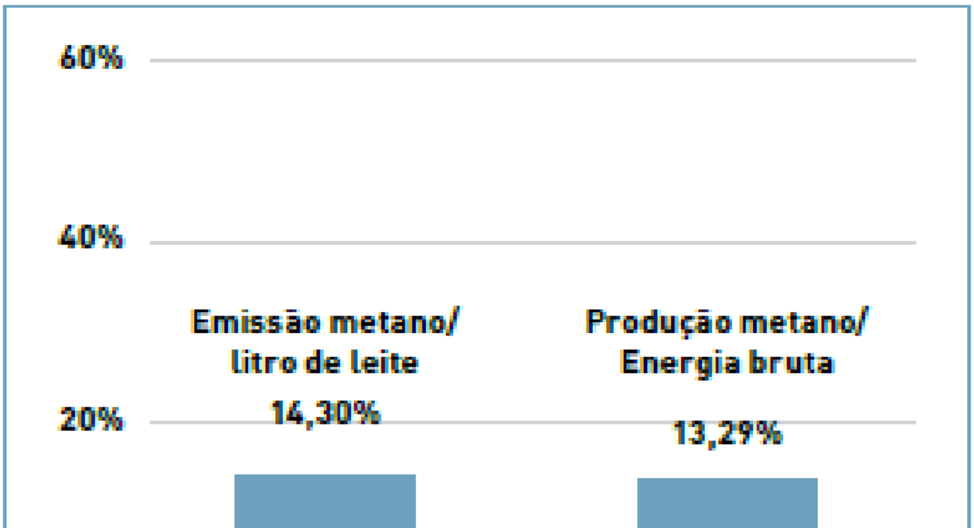


Gráfico 2. *Vacas que ruminam mais emitem menos metano e destinam mais energia para a produção de leite.*

O temperamento das vacas no curral de manejo também influenciou as emissões de metano: as vacas que saíram mais rapidamente do tronco de contenção, as mais reativas, tenderam a emitir 14,30% mais metano entérico por litro de leite, bem como as que entraram mais rapidamente no tronco (mais reativas) perderam 13,29% mais energia bruta na forma de metano entérico (Gráfico 3).



garanta a sustentabilidade do negócio.

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*Os estudos fazem parte da tese de doutorado de Maria Guilhermina Pedroza, aluna do Programa de Pós-graduação em Biodiversidade e Conservação da Natureza da UFJF, sob a orientação da pesquisadora Dra. Mariana Campos (Embrapa) e da professora Dra. Aline Cristina Sant'Anna (UFJF).

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04 de Outubro de 2022

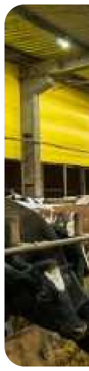
0 Comentários

Gestão, Sustentabilidade

A boa governança

04 de Outubro de 2022

0 Comentários



MENU

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VAIVÉM DAS COMMODITIES

Mauro Zafalon
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Vaca brava emite 40% mais gás metano e produz menos leite, afirma Embrapa

A pecuária entra de vez na lista dos setores mais checados quando se trata de redução de gás metano, após o país ter assinado o Compromisso Global de Metano, na COP26.

O Brasil e mais uma centena de países se comprometeram em reduzir em 30%, até 2030, as emissões de gás metano, provindas de aterros, decomposição de matéria orgânica e, principalmente, da pecuária.

Todo estudo é válido, e o mais recente da Embrapa indica que vacas bravas produzem menos leite e emitem 40% a mais de fermentação entérica (o arroto) por quilo de leite.

A pesquisa, conduzida pela Embrapa Gado de Leite, em Minas Gerais, e pela Universidade Federal de Juiz de Fora (MG), demonstra que os animais mais arredios à presença humana e à ordenha, além de gerar uma emissão maior de gás, produzem menos leite.

Um dos caminhos para a redução da emissão de gás metano na pecuária passa pela melhora do manejo dos animais e a busca por um sistema mais eficiente na produção de leite, segundo Mariana Campos, pesquisadora da Embrapa.

A manifestação das vacas mais bravas vem por meio de coices, agitação e menor velocidade na ordenha. E elas gastam energia com isso.

As mais calmas passam mais tempo ruminando e ficam mais tranquilas durante a ordenha, produzindo mais leite, segundo a pesquisadora.

Para a professora Aline Sant'Anna, da Universidade Federal de Juiz de Fora, o temperamento dos animais possui um componente herdável, mas as condições ambientais também interferem no caráter das vacas. É necessário um ambiente calmo durante a ordenha.

A redução da emissão de gás metano nas vacas leiteiras já ajuda no cumprimento do acordo. Segundo dados do IBGE, o rebanho brasileiro é de 218 milhões de cabeças de gado. Deste número, 16,2 milhões são vacas que foram ordenhadas no ano passado.

Maurício Antônio Lopes, pesquisador da Embrapa, afirma que a pecuária realmente é um problema na emissão de gás metano, mas que começam a surgir soluções para essa redução. E elas são necessárias porque o rebanho bovino mundial cresce ano a ano.

Dados do Usda (Departamento de Agricultura dos Estados Unidos) indicam que o rebanho mundial de bovinos é de 996,5 milhões de cabeças de gado e que, em um ano, estará em 1,01 bilhão.

O pesquisador da Embrapa afirma, no entanto, que o metano, o novo vilão da crise climática, não deve necessariamente ser encarado como um problema muitíssimo grave, como o CO_2.

"Além de ter vida curta na atmosfera terrestre, de 10 a 12 anos — o que nos permite manejar seu conteúdo em prazos reduzidos e a custos baixos—, o metano é um gás valioso, do ponto de vista do interesse humano."

Ele acredita que a crise climática possa aguçar o interesse da sociedade pelas qualidades do metano como fonte de energia e matéria-prima para múltiplas indústrias. Um desses usos, por exemplo, pode ser a substituição do gás natural, derivado do petróleo.

No próximo ano, a New Holland coloca no mercado um trator movido a biometano. Por meio de biodigestores, as propriedades agrícolas podem produzir seu próprio combustível, gerar energia elétrica e vender eventuais sobras para a rede de distribuidoras.

Segundo a New Holland, ao usar o biometano, o impacto de carbono da máquina é virtualmente zero.

Com relação aos desafios que a pecuária bovina terá, à medida que avançarem estratégias para redução do metano, o pesquisador já vê algumas soluções.

Serão necessários investimentos cada vez maiores no melhoramento genético para uma elevação da produtividade e garantia de carne e de leite. Essa evolução permite a existência de um número menor de bovinos e uma redução da emissão de metano.

Lopes afirma também que há evidências de variabilidade de genética para emissão de metano. No futuro, haverá linhagem de animais geneticamente propensos a emitir um menor volume do gás.

Linhagens de animais de certa composição genética tendem a se associar a microrganismos que produzem menos metano quando provocam a fermentação anaeróbia do rúmen.

Outro aspecto interessante, na avaliação de Lopes, é a possibilidade de manejar a composição do chamado microbioma bovino, que compõe a enorme população de microrganismos que habitam o rúmen e provocam a fermentação anaeróbica da matéria vegetal ingerida pelos bovinos, o que leva à emissão de metano.

O manejo da alimentação, por exemplo, com a introdução de aditivos alimentares em rações, capazes de reduzir a produção de metano pela inibição de organismos metanogênicos no rúmen.

Segundo o pesquisador, a DSM, empresa dedicada à nutrição animal, recebeu recentemente aprovação para comercializar no Brasil um aditivo para rações que reduz o metano para ruminantes.

Consultada, a DSM informou que desenvolve pesquisa há dez anos em 13 países e que, colocado na ração das vacas (e outros ruminantes), o aditivo reduz em aproximadamente em 30% a emissão de metano entérico.

A pesquisa da Embrapa e da Universidade Federal de Juiz de Fora foi a base da tese de doutorado de Maria Guilhermina Pedroza em biodiversidade e conservação da natureza.

14/12/2021 |

Vacas “reativas” emitem mais metano e produzem menos leite

Foto: Mariana Campos

(/image/journal/article?img_id=67767006&t=1642516583629)
Boas práticas de manejo favorecem o bem-estar das vacas e dos trabalhadores e contribuem para a descarbonização e sustentabilidade da pecuária

O temperamento interfere no metabolismo da vaca, influenciando a emissão do gás metano entérico, um dos principais causadores do efeito estufa. Essa é a conclusão de pesquisa da Embrapa Gado de Leite (http://www.embrapa.br/gado-de-leite) (MG) em parceria com o Departamento de Zoologia do Instituto de Ciências Biológicas da Universidade Federal de Juiz de Fora (UFJF (http://www.ufjf.br/)). Além disso, a pesquisa demonstra que vacas cujo temperamento é mais reativo à presença humana e à ordenha, produzem menos leite.

Vacas mais bravias chegam a emitir quase 40% a mais de metano entérico por quilo de leite comparadas às fêmeas mais calmas.

Resultado veio de pesquisa da Embrapa e da Universidade Federal de Juiz de Fora.

Verificou-se que as mais reativas destinaram 25,24% menos energia líquida para a lactação, enquanto as vacas mais calmas alocaram 57,93% mais essa energia para a produção de leite.

Pesquisa foi realizada com a raça Girolando, a principal produtora de leite no Brasil.

Adoção de manejo racional com ambiente calmo favorecem a produção e a sustentabilidade ambiental.

Segundo as pesquisadoras que conduziram o trabalho, mudanças climáticas e produtividade tornaram-se dois grandes argumentos para a adoção do manejo racional, prática que começa a ser utilizada com sucesso entre produtores que têm vacas das raças Gir Leiteiro e Girolando em seus rebanhos. “Outro argumento já conhecido na prática pelos produtores que adotam a técnica é a melhora no manejo dos animais, facilitando o trabalho de condução do gado, evitando acidentes e o descarte das vacas mais reativas”, relata a pesquisadora da Embrapa Mariana Campos (https://www.embrapa.br/equipe/-/empregado/329971/mariana-magalhaes-campos), que coordenou a pesquisa no Complexo Multiusuário de Bioeficiência e Sustentabilidade da Pecuária, na Embrapa em Coronel Pacheco (MG).

A preocupação com a emissão de gases de efeito estufa e o aquecimento global vem se destacando como uma preocupação ainda mais premente do setor. As pesquisas da Embrapa e da UFJF mostram que os bovinos leiteiros mais reativos chegam a emitir quase 40% a mais de metano entérico por quilo de leite, quando comparado às vacas mais calmas. Os experimentos que levaram a essa conclusão são parte da tese de doutorado em Biodiversidade e Conservação da Natureza de Maria Guilhermina Pedroza. Ela explica que os trabalhos foram feitos com 28 vacas Girolando (F1) de primeira cria.

(https://www.embrapa.br/documents/10180/58624202/211214_VacasReativas_câmara+respiratória/51765009-616f-2f51-9d3d-96be88dd4fc5?t=1639168414909)Todos os animais foram submetidos ao treinamento para a ordenha no período pré-parto e observados tanto no curral quanto na ordenha. “Analisamos o temperamento de cada indivíduo, identificando os mais calmos e os mais reativos por meio de comportamentos como passos, coices e a ocorrência de defecação e micção durante o processo de ordenha e no curral de manejo por meio indicadores como a agitação dos animais no tronco de contenção, a velocidade de saída dos animais do tronco e velocidade de fuga em relação a um observador desconhecido", explica a doutoranda.

A produção de leite também foi medida e, ao se realizar ensaios de digestibilidade e respirometria (em câmaras respirométricas), verificou-se que as mais reativas destinaram 25,24% menos energia líquida para a lactação, enquanto as mais calmas, que ruminam mais na sala de ordenha alocaram 57,93% mais energia líquida para a produção de leite.

(https://www.embrapa.br/documents/10180/58624202/211214_VacasReativas_Mariana+Campos/71d43c95-75f4-8864-ef35-4fef8882f02c?t=1639168478020)Mariana Campos (**foto à esquerda**) diz que o experimento é importante para a pecuária de leite brasileira devido à importância da raça Girolando para a produção nacional. O Girolando é uma raça sintética desenvolvida para as condições tropicais, unindo duas raças de temperamentos diferentes: Gir Leiteiro e Holandês. "O resultado do cruzamento dessas raças trouxe como consequência, um animal rústico e com boa produção de leite; no entanto são mais ariscos à ordenha. O treinamento de novilhas para a primeira ordenha é uma técnica bastante adequada aos rebanhos de leite no Brasil devido à utilização de animais mestiços ou zebuínos.

A professora Aline Sant'Anna, coordenadora do Núcleo de Pesquisa em Etologia e Bem-estar Animal (Nebea) da UFJF, que orientou Maria Guilhermina na tese, conta que o temperamento dos animais possui um componente herdável, mas as condições ambientais também interferem no caráter das vacas. Embora os programas de melhoramento genético bovino tenham obtido conquistas nesse aspecto, o manejo racional, aliado a um ambiente calmo no momento da ordenha, deve ser adotado. "Embora o genoma influencie o caráter do animal, é possível moldar o fenótipo por meio de um ambiente adequado", pondera a professora. A seleção de vacas mais calmas e a adoção de boas práticas de manejo favorecem o bem-estar tanto das vacas quanto dos trabalhadores.

No momento em que as empresas do setor lácteo estão trabalhando para neutralizar as emissões de carbono do setor, a pesquisa comprova que a adoção de protocolos de doma racional e o melhoramento animal focado na busca por animais mais dóceis podem ser importantes estratégias para que as metas de descarbonização sejam atingidas. "Animais com temperamento mais reativo são indesejáveis para uma pecuária eficiente e sustentável," conclui Campos.

(https://www.embrapa.br/documents/10180/58624202/211214_VacasReativas_ordenha/d b188-7ac5-8bd1-d72cc82df9dc?t=1639168445158)Vaca reativa X vaca calma

Após parir, algumas novilhas demonstram maior reatividade do que outras e a energia desperdiçada resulta em menos produção de leite e maior ı metano entérico, além de elevar o risco de acidentes. Entre as características de uma vaca ou novilha reativa na sala de ordenha estão:

- Urinam e defecam com maior frequência;
- dão coices;
- sapateiam;
- se mostram agitadas;
- derrubam o conjunto de teteiras;
- apresentam menor velocidade de ordenha.

Em oposição, as vacas mais calmas facilitam o manejo e diminuem o tempo de ordenha. As seguintes características são apreciáveis:

- Ficam mais tranquilas durante o procedimento de ordenha;
- passam mais tempo ruminando;
- raramente urinam e defecam na sala na ordenha;
- permanecem mais tempo no cocho.

Rubens Neiva (MTb 5.445/MG)
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Certificado

O Vice-coordenador dos cursos de Graduação em Ciências Biológicas da Universidade Federal de Juiz de Fora, Prof. Dr. Artur Andriolo, certifica que a MSc. Maria Guilhermina Marçal Pedroza coorientou o aluno Victor Nascimento Cerqueira Silva em seu trabalho de conclusão de curso, intitulado *Relações entre o comportamento e crescimento em bezerras leiteiras na fase de aleitamento mantidas em baias individuais* e apresentado no dia 19 de dezembro de 2023, às 14 horas, na sala online (https://meet.google.com/psg-eisv-qou) do Instituto de Ciências Biológicas.

Juiz de Fora, 27 de dezembro de 2023.

Prof. Dr. Artur Andriolo

Vice-coordenador dos cursos de Graduação em Ciências Biológicas

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Certificado

O Coordenador dos cursos de Graduação em Ciências Biológicas da Universidade Federal de Juiz de Fora, Prof. Dr. Aripuanã Sakurada Aranha Watanabe, certifica que a MSc. Maria Guilhermina Marçal Pedroza integrou a banca avaliadora do trabalho de conclusão de curso da aluna Ana Luíza de Almeida Cândido Vargas, intitulado *A Relação entre Temperamento e Comportamentos em Cativeiro de Papagaios do Gênero Amazona* realizado sob orientação da Prof.ª Dr.ª Aline Cristina Sant'Anna e coorientação da MSc. Gabriela de Araújo Porto Ramos e apresentado no dia 12 de julho de 2023, às 10 horas, na sala 01 do Departamento de Zoologia do Instituto de Ciências Biológicas.

Juiz de Fora, 01 de dezembro de 2023.

Prof. Dr. Aripuanã Sakurada Aranha Watanabe

Coordenador dos Cursos de Graduação em Ciências Biológicas

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Juiz de Fora, 01 de dezembro de 2023.

Prof. Dr. Aripuanã Sakurada Aranha Watanabe

Coordenador dos Cursos de Graduação em Ciências Biológicas