

UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ZOOTECNIA E ENGENHARIA DE ALIMENTOS

ASKA UJITA

**Protocolos de habituação de manejo em vacas leiteiras Gir e em novilhas taurinas de
corte sobre respostas fisiológicas e comportamentais**

Pirassununga
2022

ASKA UJITA

Protocolos de habituação de manejo em vacas leiteiras Gir e em novilhas taurinas de corte sobre respostas fisiológicas e comportamentais

VERSÃO CORRIGIDA

Tese de Doutorado apresentado à Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo, como parte dos requisitos para a obtenção do título de Doutor em Ciências.

Área de Concentração: Qualidade e Produtividade Animal.

Orientador: Prof. Dr. João Alberto Negrão

Co-orientadora: Prof^a. Dr^a. Lenira El Faro Zadra

**Pirassununga
2022**

Ficha catalográfica elaborada pelo
Serviço de Biblioteca e Informação, FZEA/USP,
com os dados fornecidos pelo(a) autor(a)

U33p Ujita, Aska
 Protocolos de habituação de manejo em vacas
 leiteiras Gir e em novilhas taurinas de corte sobre
 respostas fisiológicas e comportamentais / Aska
 Ujita ; orientador João Alberto Negrão ;
 coorientador Lenira El Faro. -- Pirassununga, 2022.
 107 f.

 Tese (Doutorado - Programa de Pós-Graduação em
 Zootecnia) -- Faculdade de Zootecnia e Engenharia
 de Alimentos, Universidade de São Paulo.

 1. Comportamento animal. 2. Estresse animal. 3.
 Cortisol. 4. Ocitocina. 5. Habituação. I. Negrão,
 João Alberto, orient. II. El Faro, Lenira,
 coorient. III. Título.

ASKA UJITA

Protocolos de habituação de manejo em vacas leiteiras Gir e em novilhas taurinas de corte sobre respostas fisiológicas e comportamentais

Tese de Doutorado apresentado à Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo, como parte dos requisitos para a obtenção do título de Doutor em Ciências.

Área de Concentração: Qualidade e Produtividade Animal.

Data de aprovação: ____/____/____

BANCA EXAMINADORA DA DEFESA DE TESE

Prof. Dr. João Alberto Negrão (President of the Committee)

Instituição Universidade de São Paulo

Prof.(a) Dr.(a) Aline Cristina Sant'Anna

Instituição Universidade Federal de Juiz de Fora

Prof. Dr. Henrique Barbosa Hooper

Instituição Centro Universitário Anhanguera

Prof.(a) Dr.(a) Luciane Silva Martello

Instituição Universidade de São Paulo

Prof.(a) Dr.(a) Márcia Saladini Vieira Salles

Instituição de Zootecnia

Prof. Dr. Vitor Rodrigues Gomes Mercadante

Instituição Virginia Tech

AGRADECIMENTOS

Aos meus queridos pais, Masaki e Midori Ujita, que me criaram com muitas broncas, limites e lições de vida, mas sem faltar amor. Fui capaz e sou o que sou hoje graças a vocês. Amo vocês!!

Ao meu orientador, Prof. Dr. João Negrão, pela orientação, ensinamentos e pelas oportunidades, além da confiança creditada e muitas vezes, permitir uma orientação à distância, os quais foram essenciais no meu trabalho.

À minha co-orientadora Lenira El Faro Zadra, pela confiança creditada neste projeto que tanto me deu, além da sua preocupação diária com minha pessoa e pela amizade.

Aos meus queridos amigos que fiz em Uberaba, Minas Gérias, especialmente ao Rogério e Dayanne, pessoas as quais mantenho sincera amizade e grande alegria sempre que os reencontro. Foram essenciais para que minha passagem nesta cidade fosse maravilhosa.

Aos meus queridos amigos de Pirassununga, São Paulo: Laura, Camilla, Salsicha, HB, Manu, Priscila, Gabi, Kuseco, Duda, Guilhermão, a família república Caipira... Tanta gente! Sinto muitas saudades dos nossos churrascos, nossas conversas, nossos dias de janta e claro, festas!

Ao meu namorado Juan, com quem compartilho e compartilhei muita coisa nesse meu doutorado. Tenho a certeza de que alguns dos caminhos ou melhor, algumas oportunidades, não teriam acontecido se não fosse você! Obrigada, por tanta ajuda, tanto apoio incondicional e carinho.

Aos funcionários da EPAMIG, principalmente o Bigode, Amauri, Cássio, Luiz, Jorge e Jarbinha que me ajudaram com muita disposição e alegria na execução do meu projeto.

Às pessoas, que de alguma forma fizeram parte de algum momento dessa jornada e me ajudaram nesse longo caminho: estagiários, pesquisadores e funcionários da USP/FZEA e da EPAMIG.

Aos pesquisadores da EPAMIG: Edilane, André, Leonardo, Renata, Fernando... Obrigada pela oportunidade, apoio e confiança para que eu pudesse executar o trabalho com tanta leveza e liberdade.

À Faculdade de Zootecnia e Engenharia de Alimentos, por ter me dado muitas oportunidades e me proporcionar o orgulho de fazer parte da história dessa instituição.

À FAPESP, EPAMIG, Finep e MCTI pelo apoio financeiro, oportunidade de trabalhar e executar o projeto que foi essencial à minha formação como doutora.

RESUMO

O objetivo deste trabalho foi avaliar o efeito de protocolos de habituação ao manejo em vacas de leite da raça Gir e em novilhas de corte de diversas raças europeias sobre respostas fisiológicas e comportamentais. No estudo com animais da raça Gir foram realizados dois experimentos para avaliar (1) o efeito do treinamento de rotina de ordenha em novilhas e (2) o efeito do estímulo tátil positivo em novilhas nulíparas e vacas múltíparas no corredor do curral sobre as características de comportamento, concentração de cortisol e ocitocina, expressão dos receptores chave de ejeção de leite e rendimento de leite. Ambos os protocolos foram realizados durante o período pré-parto por 14 dias. Em cada experimento foram utilizados 40 animais, metade pertencia ao grupo que recebia o protocolo de habituação e a outra metade ao grupo controle (sem protocolo de habituação). Em geral, o grupo com protocolo de habituação apresentou uma melhora no comportamento na entrada da sala de ordenha, na pré-ordenha e nos procedimentos de ordenha em comparação ao grupo controle ($p \leq 0,05$). Não foram observados efeitos dos protocolos de habituação na produção e composição do leite; entretanto, vacas habituadas apresentaram maiores níveis de ocitocina, menores níveis de cortisol e menor leite residual quando comparadas às vacas controle ($p \leq 0,05$). Além disso, vacas habituadas aumentaram a expressão dos genes dos receptores de prolactina, ocitocina e 5-hidroxitritamina e diminuiu a expressão do gene do receptor de cortisol nas células mamárias ($p \leq 0,05$). Neste primeiro estudo concluiu-se que a habituação diminuiu a expressão dos receptores de glicocorticóides, regulou positivamente a expressão de prolactina, ocitocina e 5-hidroxitritamina, melhorou o comportamento e temperamento na ordenha, e diminuiu o leite residual das vacas Gir. No estudo com novilhas taurinas, o objetivo foi determinar o impacto de diferentes protocolos de habituação no comportamento, fisiologia e temperamento de bovinos de corte em resposta ao manejo. Novilhas de corte foram expostas a três estratégias de habituação: (1) estimulação tátil no corredor do tronco por sete dias consecutivos (STI; $n = 18$); (2) passagem pelo corredor do tronco por sete dias consecutivos (CHU; $n = 19$) e; (3) sem habituação (CON; $n = 19$). A frequência respiratória individual das novilhas (RR; n/min), a temperatura vaginal interna (VAGT; $^{\circ}\text{C}$) e o cortisol sanguíneo foram medidos. Além disso, parâmetros de comportamento foram observados para gerar uma pontuação de comportamento, e a interação da novilha com os alunos e suas respostas comportamentais foram registradas. A habituação com STI e CHU resultou em melhores escores comportamentais em comparação com CON e latências de manuseio maiores ($p \leq 0,05$). A temperatura vaginal diminuiu no grupo STI em comparação com os grupos CHU e CONT ($p \leq 0,05$). A concentração de cortisol não diferiu entre os tratamentos, mas diminuiu ($p \leq 0,05$) desde o início do experimento até 14 dias após o início do tratamento. Neste estudo se concluiu que ambos os protocolos de habituação mostraram benefícios, entretanto, as novilhas que receberam a estimulação tátil no tronco tiveram melhorias de comportamento. Além disso, essas novilhas responderam com mais calma durante as interações humano-animal, o que é benéfico para a segurança. Em geral, os achados destes estudos mostram que é relevante aprimorar a interação humano-animal, com finalidade de melhorar a eficiência produtiva do animal, seu comportamento, facilitando o manejo e incluir o conceito de bem-estar na propriedade e instituições de ensino e pesquisa. Ainda, estudos com habituação ou treinamento de gado bovino são escassas, principalmente quando tratamos de animais leiteiros zebuínos ou de corte.

Palavras-chave: Comportamento animal; cortisol; estresse animal; ocitocina; produção de leite

ABSTRACT

The aim of this work was to evaluate the effect of habituation protocols in Gyr dairy cows and European beef heifers on physiological and behavioral responses. In the study with Gyr animals, two experiments were carried out to evaluate (1) the effect of routine milking training in heifers and (2) the effect of positive tactile stimulation in nuliparous heifers and multiparous cows in the corral corridor on the characteristics of behavior, cortisol level, oxytocin release, expression of key milk ejection receptors and milk yield. Both protocols were performed during the prepartum period for 14 days. In each experiment, 40 animals were used, half belonged to the group that received the habituation protocol and the other half to the control group (no habituation protocol). In general, the group with the habituation protocol showed an improvement in behavior when entering the parlor, pre-milking and milking procedures compared to the control group ($p \leq 0.05$). No effects of habituation protocols on milk production and composition were observed; however, habituated cows had higher levels of oxytocin, lower levels of cortisol and lower residual milk when compared to control cows ($p \leq 0.05$). In addition, habituated cows increased the expression of the prolactin, oxytocin and 5-hydroxytryptamine receptor genes and decreased the expression of the cortisol receptor gene in mammary cells ($p \leq 0.05$). In this first study, it was concluded that habituation decreased the expression of glucocorticoid receptors, positively regulated the expression of prolactin, oxytocin and 5-hydroxytryptamine, improved behavior and temperament in milking, and decreased residual milk in Gir cows. Our objective was to determine the impact of different habituation protocols on beef cattle behavior, physiology, and temperament in response to human handling. Beef heifers were exposed to three habituation strategies: (1) tactile stimulation (brushing) in the working chute for seven consecutive days (STI; $n = 18$); (2) passage through the working chute for seven consecutive days (CHU; $n = 19$) and (3) control group (CON; $n = 19$). Individual heifer respiratory rate (RR; n/min), internal vaginal temperature (VAGT; °C), and blood cortisol were measured. Further, behavior parameters were observed to generate a behavior score, and heifer interaction with students and their behavioral responses were recorded. Habituation with STI and CHU resulted in improved numerical behavior scores compared to CON, and greater ($p \leq 0.05$) handling latencies. Vaginal temperature was decreased in STI compared to CHU and CONT ($p \leq 0.05$). Cortisol concentration did not differ among treatments but decreased ($p \leq 0.05$) from the start of the experiment to 14 days after treatment initiation. Both habituation protocols showed benefits, but heifers that received the tactile stimulation in the chute had the greatest behavior improvements. Furthermore, these heifers responded more calmly during student-animal interactions in class, which is beneficial for the students' and animals' safety. In general, the findings of these studies show that it is relevant to improve human-animal interaction, in order to improve the productive efficiency of the animal, its behavior, facilitating handling and including the concept of welfare in the commercial farms and educational and research institutions. Still, studies with habituation or training of cattle are few, especially when dealing with dairy zebu or beef animals.

Keywords: animal behavior; animal stress; cortisol; milk production; oxytocin.

SUMÁRIO

1. INTRODUÇÃO	10
1.1. OBJETIVO GERAL	11
1.1.1. <i>Objetivos Específicos</i>	11
1.2. REVISÃO DE LITERATURA.....	11
1.2.1. <i>Relação Humano-Animal</i>	11
1.2.2. <i>Bem-estar animal</i>	12
1.2.3. <i>Comportamento e temperamento animal</i>	13
1.2.4. <i>Particularidades da raça zebuína</i>	16
1.2.5. <i>Principais indicadores fisiológicos de estresse</i>	17
1.2.5.1. <i>Temperatura e frequência respiratória</i>	18
1.2.5.2. <i>Hormônios – cortisol, ocitocina e prolactina</i>	18
1.2.6. <i>Expressão gênica</i>	20
1.2.7. <i>Protocolos de habituação como solução prática</i>	21
REFERÊNCIAS	23
2. EFFECT OF TRAINING MILKING ROUTINE ON BEHAVIOR, CORTISOL AND OXYTOCIN RELEASE, EXPRESSION OF KEY RECEPTORS OF MILK EJECTION AND MILK YIELD IN PRIMIPAROUS GYR COWS	33
ABSTRACT	33
2.1. INTRODUCTION.....	34
2.2. MATERIALS AND METHODS	35
2.2.1. <i>Animals and daily management</i>	35
2.2.2. <i>Treatments</i>	36
2.2.3. <i>Behavior scores attributed during milking routine.</i>	37
2.2.3.1. <i>Overall milking behavior score</i>	37
2.2.4. <i>Milk yield</i>	39
2.2.5. <i>Milk analysis</i>	39
2.2.6. <i>Gene expression</i>	39
2.2.7. <i>Statistical analysis</i>	40
2.3. RESULTS.....	41

2.3.1. <i>Behavior</i>	41
2.3.2. <i>Milk yield, hormones in milk, and gene expression</i>	43
2.4. DISCUSSION.....	45
ACKNOWLEDGMENTS	48
REFERENCES	49
3. EFFECT OF POSITIVE TACTILE STIMULATION AND PREPARTUM MILKING ROUTINE TRAINING ON BEHAVIOR, CORTISOL AND OXYTOCIN IN MILKING, MILK COMPOSITION, AND MILK YIELD IN GYR COWS IN EARLY LACTATION	52
ABSTRACT.....	52
3.1. INTRODUCTION.....	53
3.2. MATERIALS AND METHODS	54
3.2.1. <i>Study place and animals</i>	54
3.2.2. <i>Arrangement of the experiments</i>	55
3.2.2.1. Experiment 1: Positive tactile stimulation in the corral	55
3.2.2.2. Experiment 2: Milking routine training in the milking parlor	56
3.2.3. <i>Experimental milking</i>	56
3.2.4. <i>Data collection</i>	57
3.2.4.1. Animal behavior in the milking room.....	57
3.2.4.1.1. Experiment 1	59
3.2.4.1.2. Experiment 2	59
3.2.4.2. Productive performance.....	59
3.2.4.3. Laboratory analysis of milk	59
3.2.5. <i>Statistical analysis</i>	59
3.3. RESULTS	60
3.3.1. <i>Experiment 1</i>	60
3.3.2. <i>Experiment 2</i>	61
3.4. DISCUSSION.....	64
3.5. CONCLUSION	67
ACKNOWLEDGEMENT	67
REFERENCES	68

4. HABITUATION PROTOCOLS IMPROVE BEHAVIORAL AND PHYSIOLOGICAL RESPONSES OF BEEF CATTLE EXPOSED TO STUDENTS IN AN ANIMAL HANDLING CLASS.....	78
SIMPLE SUMMARY	78
ABSTRACT	79
4.1. INTRODUCTION.....	79
4.2. MATERIALS AND METHODS	80
4.2.1. <i>Experimental treatments</i>	81
4.2.1.1. Tactile stimulation (sti group)	81
4.2.1.2. Passage through the chute (chu group)	82
4.2.1.3. Control (con group)	82
4.2.2. <i>Halter breaking practice class (HBPC)</i>	83
4.2.3. <i>Experimental measurements</i>	83
4.2.3.1. Respiratory Rate and Vaginal Internal Temperature.....	84
4.2.3.2. Cortisol concentration in blood plasma	84
4.2.3.3. Behavioral parameters in the working chute	84
4.2.3.4. Behavioral measurements in the holding pen	85
4.2.4. <i>Statistical analysis</i>	85
4.3. RESULTS.....	89
4.3.1. <i>Behavioral indicators</i>	89
4.3.2. <i>Physiological parameters</i>	90
4.3.3. <i>Student-animal interaction during HBPC</i>	91
4.4. DISCUSSION.....	92
4.4.1. <i>Student-animal interaction during HBPC</i>	95
4.5. CONCLUSIONS	95
REFERENCES	97

1. INTRODUÇÃO

A interação humano-animal é um fator de grande importância quando tratamos de animais de produção. Uma interação negativa afeta o comportamento desejável, tornando o animal agitado e/ou arredios, intervindo principalmente no manejo, segurança e bem-estar animal, além de influenciar as características de produção e qualidade do produto final. Estes comportamentos indesejáveis podem ser demonstrados pelos animais quando estão diante de uma situação nova ou desafiadora (Haskell et al., 2014). Vacas conduzidas negativamente podem apresentar comportamento mais aversivo ao manejador (Breuer et al., 2003; Munksgaard et al., 2001) acarretando um menor desempenho e pior qualidade leiteira (Hemsworth et al., 2000). Novilhos de corte que possuem velocidade de fuga mais rápida são propensos a produzirem carne mais dura e com reduzida deposição de gordura intramuscular (Hall et al., 2011). Além disso, existem os problemas de manejo que causam custos mais altos de mão-de-obra, além de ferimentos ou acidentes mortais (Brouček et al., 2008).

Por outro lado, a interação humano-animal não ocorre apenas em sistemas de produção. Existem muitas instituições educativas que possuem um rebanho bovino que, além de visar um desempenho eficiente dos animais, se objetiva usá-los para práticas de manejo envolvendo discentes. Por tal motivo, é de grande importância ter animais com comportamentos positivos para facilitar o manejo e aprendizado dos estudantes.

Entretanto, entendemos que o comportamento do bovino é fruto da interação entre fatores genéticos e ambientais (Réale et al., 2007), existindo a necessidade de elaborar métodos de criação, desenvolver técnicas e treinamentos de manejo que sejam de curto prazo e eficientes para a redução da reatividade animal (Petherick, 2005). Assim, a adoção de práticas positivas de manejo pode reduzir a reatividade dos animais aos humanos, e consequentemente às instalações e instrumentos. Sendo de grande importância aplicar estas estratégias em animais jovens para ter um relacionamento positivo ao longo da sua vida (Haskell et al., 2014; Probst et al., 2013, 2012a). Situações novas podem causar insegurança e medo nos animais, levando-os a terem maior reatividade durante o manejo. Por outro lado, animais jovens podem ser mais suscetíveis a interações positivas que adultos, talvez por curiosidade.

À vista disso, o treinamento com estímulos não negativos e simulação de manejo, pode se tornar uma importante ferramenta para melhorar a relação humano-animal por meio da habituação dos animais à rotina e/ou manejo. Principalmente para animais jovens que não estão habituadas à rotina e à aproximação e com a interação humana (Van Reenen et al., 2002). A

estimulação tátil pode ser uma importante estratégia de manejo para melhorar essa interação, sendo esta relação formada através de estímulos positivos decorrentes da escovação, afago com as mãos, uma conversa e/ou gestos de carinho realizados pelo homem ao animal (Boivin and Braastad, 1996; Lansade et al., 2004; Ligout et al., 2008; Schmied et al., 2008)

Dessa forma, há a necessidade do entendimento de como o comportamento, a fisiologia e temperamento podem responder ao manejo e treinamento dos animais, com a finalidade de criar soluções para melhorar a eficiência do manejo, bem-estar animal e a interação homem-animal.

1.1. OBJETIVO GERAL

O objetivo deste estudo foi avaliar o efeito de protocolos de habituação ao manejo em novilhas de leite da raça zebuína Gir e em novilhas de corte de raça europeia e revelar seus benefícios no comportamento durante a interação humano-animal, além de avaliar parâmetros hormonais, ligados ao estresse e à facilidade de manejo.

1.1.1. Objetivos Específicos

- a) Investigar o efeito do treinamento na liberação de OT e CORT, expressão de receptores de oxitocina glicocorticoide e mineralocorticoides (OTR, GR e MR, respectivamente), além de alfa-adrenérgicos ($ADR\alpha1A$, $ADR\alpha2AD$, $ADB\beta2$) e receptores de serotonina (HTR1B, HTR2A, HTR2B, HTR4, HTR7) na glândula mamária e sua relação com a produção e ejeção de leite.
- b) Investigar o impacto da estimulação tátil positiva no curral e na habituação da rotina de ordenha durante o período pré-parto no comportamento e na produtividade de vacas Gir no início da lactação.
- c) Determinar o impacto de diferentes protocolos de habituação no comportamento, fisiologia e temperamento de bovinos de corte em resposta ao manuseio de chutes e interação humana.

1.2. REVISÃO DE LITERATURA

1.2.1. Relação Humano-Animal

A humanidade sempre domesticou animais para seus próprios propósitos (Hunter, 2018), sendo assim, o interesse em entender todos os aspectos que influenciam a relação

humano e animal vem aumentando. Nesta relação, a pecuária se destaca como uma das representações da domesticação, sendo a bovinocultura, um dos principais pilares da produção animal (Zliobaite, 2019).

A relação homem-animal ocorre em diferentes aspectos. E em uma propriedade pecuária, essa relação se desenvolve ao longo das interações diárias na fazenda, sendo elas agradáveis ou não ao animal (Kling-Eveillard et al., 2020). Na bovinocultura, os animais estão em constante interação com humanos durante atividades rotineiras como manejos de ordenha, alimentação, sanidade, dentre outros (Paul and Coleman, 2010). Sendo assim, a qualidade dessa relação pode ser definida como o grau de proximidade ou distância entre um animal e uma pessoa (Kling-Eveillard et al., 2020; Waiblinger et al., 2006), sendo possível ser detectada pela resposta que o animal tem ao estímulo que o humano oferece.

A preocupação com a qualidade da relação entre humanos e animais na pecuária ainda é escassa. Todavia, pesquisas vêm mostrando que esta relação tem alto impacto sobre o comportamento e bem-estar animal, consequentemente nas suas respostas fisiológicas, com implicações na sua saúde e também na produtividade e qualidade do produto final (Krohn et al., 2001).

Ainda, a interação humano-animal não ocorre apenas nos sistemas de produção. É importante ter em mente que o gado não é apenas uma atividade econômica, mas também uma atividade recreativa e educacional. Existem muitas instituições de ensino que possuem um rebanho bovino que, além de visar o desempenho animal eficiente, os utiliza para práticas que envolvem os alunos, que interagem com os animais em diferentes atividades de ensino. Sendo importante garantir a segurança nessas interações, principalmente nas práticas acadêmicas no campo, tão importantes para o desenvolvimento pessoal e profissional dos alunos.

Dessa forma, é importante ter animais com uma boa relação humana para apresentarem comportamentos positivos, facilitando o manuseio com interações humanas com animais, seguras.

1.2.2. Bem-estar animal

Os animais podem modificar o seu comportamento para atender algumas necessidades fisiológicas, essa capacidade é chamada de plasticidade comportamental, limitada apenas na situação de comportamento inato (sucção na mamada, por exemplo) (Phillips, 2002). E, apesar dos bovinos serem animais bem adaptáveis, a qualidade e sistema de manejo podem prejudicar essa capacidade, resultando em problemas comportamentais, que consequentemente afetam negativamente a produção e bem-estar dos mesmos (Bouissou et al., 2009). Sendo assim, muitas

regiões do mundo têm feito mudanças em relação ao bem-estar dos animais de criação, abordando principalmente o tratamento e manejo dos animais (von Keyserlingk and Hötzel, 2014).

Segundo a “Farm Animal Welfare Council”, o bem-estar animal é caracterizado pela qualidade de vida que o animal tem na propriedade. Entretanto, apesar dos vários conceitos, a qualidade da interação entre humano e animal é uma das mais consideradas na ciência do bem-estar animal e usada na prática (Adamczyk, 2018), principalmente em propriedades pecuárias.

O tipo de sistema de produção associado às condições de criação e manejo podem afetar negativamente o bem-estar animal (Cozzi et al., 2009; Tarantola et al., 2020). A atitude do manejador quando está na lida dos animais (Ceballos et al., 2018a; Cozzi et al., 2009), sistemas de alimentação e ambiente de criação são alguns dos fatores que afetam os animais no comportamento e também fisiologicamente (Tarantola et al., 2020).

Logo, como cada propriedade possui um tipo de sistema de produção e uma forma particular de lidar com seu gado, é necessário avaliar individualmente onde e como elaborar estratégias para melhorar o bem-estar animal. E, para melhorar o relacionamento entre seres humanos e gado no dia-a-dia da fazenda, podem não ser necessárias grandes mudanças ou investimentos, sendo preciso apenas um melhor entendimento do comportamento dos animais criados, adaptando o sistema e as instalações às características dos mesmos (Ujita et al., 2020).

1.2.3. Comportamento e temperamento animal

É de grande importância o conhecimento das características comportamentais dos animais, principalmente para a obtenção e manutenção do seu bem-estar, sendo possível, através destes, determinar os limites éticos de manejo e exploração animal pelos humanos (D'Eath et al., 2010).

A atenção ao bem-estar animal nos sistemas de produção atende a uma demanda dos consumidores. O conhecimento do comportamento animal permite que os produtores identifiquem desvios comportamentais e construam sistemas para minimizar o desconforto, aumentando consequentemente a produtividade do sistema de produção, além de atender às necessidades dos animais (Coelho e Franzoni, 2012). Nas fazendas de gado com baixos níveis de produção, se são feitos investimentos em melhorias de produtividade, ocorrem incrementos no bem-estar animal. Podendo-se esperar uma relação positiva entre aumento da produção e bem-estar, que gera melhorias na nutrição, controle de doenças e instalações da fazenda (McInerney, 2004).

No rebanho, comportamentos indesejáveis podem ser demonstrados diante de uma

situação desconfortável ao animal (Haskell et al., 2014) acarretando a um manejo negativo como consequência, e ainda, resultando em um animal mais aversivo (Breuer et al., 2003; Munksgaard et al., 2001). Isso porque, como explicado anteriormente, o tipo de relação humano-animal e a maneira que o manejo ocorre na propriedade influencia diretamente no seu comportamento, influenciando sua produtividade e qualidade de seus produtos (Breuer et al., 2000), e se essas interações foram negativas, pode resultar em maior reatividade (Breuer et al., 2000; Peters et al., 2010) podendo se potencializar quando associadas ao mau temperamento do animal.

Existem diferenças individuais na resposta comportamental dos animais perante situações alarmantes ou desafiadoras, e em bovinos, a magnitude da resposta e a diferença entre os animais têm grande importância principalmente onde a interação humana é constante na rotina da propriedade (Haskell et al., 2014). Esse conjunto de comportamentos dos animais em relação ao homem, normalmente atribuído ao medo (Fordyce et al., 1982), determina sua agressividade, agilidade, docilidade, reatividade e teimosia (da Costa et al., 2002), sendo definido como temperamento animal, na qual recentemente se tornou um tema de interesse na produção animal.

O temperamento é um conjunto de respostas que o indivíduo apresenta em relação a uma determinada situação (Friedrich et al., 2015), uma individualidade do animal que é expressado através do comportamento. Em bovinos, podemos evidenciar essa resposta comportamental quando manejados, o qual pode impactar sua rotina diária assim como a lucratividade da propriedade. Dessa forma, manejos negativos podem influenciar o animal de forma comportamental e, assim, fisiologicamente, afetando a produtividade, a performance do animal e a qualidade dos produtos finais, além do aumento de custo assim como a incidência de lesões e/ou acidentes fatais (Brouček et al., 2008). Logo, o temperamento envolve complexa combinação de características como o medo, a teimosia, a agressividade, a timidez, a curiosidade e o nervosismo, que resultam em tendências individuais dos animais, ou seja, um bovino leiteiro com temperamento difícil pode significar que ele tem alto grau de agitação ou de agressividade, sendo geralmente muito reativo ao manejo (da Costa et al., 2015). Além do mais, esta característica está relacionada ao desempenho produtivo dos animais, à qualidade de seus produtos e, ainda, à acidentes de trabalho envolvendo animais e pessoas, impactando consequentemente nos custos de produção (Peixoto et al., 2011).

A maneira mais clara e rápida de avaliar a interação humano-animal ou ambiente-animal é através da observação das respostas comportamentais manifestados pelo animal a cada estímulo que aquele manejo fornece, ou seja, sua reatividade. Ainda, existem alguns fatores a

serem considerados que podem contribuir com a reatividade do animal, por exemplo, a idade. Em primíparas, estes efeitos podem ser ainda maiores, devido à sua inexperiência, levando-as a demonstrar mais medo e maior reatividade, por exemplo, durante a ordenha, reações que têm influência negativa na secreção do leite (Van Reenen et al., 2002). Em contrapartida, as primíparas podem ser mais suscetíveis a interações positivas que as multíparas, talvez por curiosidade, propiciando uma melhor oportunidade para melhorar o relacionamento entre humanos e animais (Lürzel et al., 2016a), destacando a importância do manejo positivo antes do parto nesta fase (Bertenshaw et al., 2008).

Outro fator que pode influenciar a reatividade do animal é a raça do animal. Por questão de produtividade e facilidade no manejo, raças especializadas têm sido fortemente selecionadas para um comportamento maternal menos rígido, enquanto raças especializadas para produção de carne têm sido selecionadas para o reconhecimento precoce do bezerro e a manutenção do vínculo materno-filial (Kiley-Worthington et al., 1983). Dessa forma, a utilização de raças especializadas como a Holandesa para leite é muito comum pelo mundo todo, entretanto, a adaptação dessa raça aos trópicos para a produção leiteira tem se mostrado difícil devido aos problemas climáticos, doenças, tipo de manejo e alimentação (Alvarez et al., 1980), o que resultou na utilização da raça zebuína para cruzar e melhoramento genético para adaptabilidade e resistência à essas condições.

Na pecuária, principalmente na área leiteira, existe uma estreita interação entre humanos e animais, devido ao contato diário durante o desenvolvimento de atividades de rotina, como ordenha, alimentação e cuidados sanitários (Coleman et al., 1998).

Existem vários parâmetros comportamentais possíveis de serem analisados durante um manejo: deslocamento (Ujita et al., 2020); movimentação (Breuer et al., 2000; De Felicio Porcionato et al., 2009; Lefcourt et al., 1986; Néri et al., 2016; Peters et al., 2010), número de coices (Hemsworth and Barnett, 1989; Néri et al., 2016; Porcionato et al., 2009), reatividade ao toque (Néri et al., 2016; Peters et al., 2010; Porcionato et al., 2009), necessidade do uso de instrumentos para conduzir a vaca (Peters et al., 2010; Ujita et al., 2020), distância de fuga (Hötzel et al., 2005), micção e defecação (Peters et al., 2010; Porcionato et al., 2009), vocalização (Porcionato et al., 2009; Ujita et al., 2020), reatividade na aproximação (Burdick Sanchez et al., 2014), entre outros. Estes, podem ser avaliados de forma descritiva ou por escores durante o manejo através da observação dos animais.

O medo pode ser o fator principal que altera o comportamento no manejo (Coleman et al., 1998), tendo em vista que os comportamentos considerados negativos aumentam conforme o medo e a reatividade do animal (Breuer et al., 2000; Peters et al., 2010), podendo

consequentemente causar estresse animal e humano. E, ainda, em vacas leiteiras, ocorre a retenção de leite ocasionado pelo estresse, portanto, a maneira de como o homem conduz essa interação humano-animal é de extrema importância na resposta comportamental, principalmente para as recém-paridas (Bremner, 1997; Soardi et al., 2017; Tancin et al., 2001), no período de adaptação (Bertenshaw et al., 2008), devido à nova rotina e estímulos ambientais.

1.2.4. Particularidades da raça zebuína

A raça zebuína (*Bos taurus indicus*) é comumente utilizada nos países tropicais. Sua genética confere vantagens quanto à resistência ao calor e a ectoparasitas, além da maior capacidade de digerir forragens com alto teor de fibra, sendo considerado um animal rústico e de boa adaptabilidade às condições do país (Leão et al., 2013). Em países tropicais com ampla área agricultável, o uso de sistemas extensivos ou semi-intensivos é uma prática muito comum, mantendo, dessa forma, o rebanho a pasto durante o maior tempo de sua vida produtiva (da Costa et al., 2015), existindo assim uma interação humano-animal limitada (Ujita et al., 2020), criando animais mais arredios e difíceis de serem manejadas.

As vacas zebuínas têm menor produção leiteira e um pior temperamento comparado às vacas taurinas (*Bos taurus taurus*), associando-se à uma dificuldade no momento da ordenha (da Costa et al., 2015). E isto, com o sistema de produção tropical, ocorre um impacto considerável na pecuária leiteira onde, países como o Brasil, a participação das raças zebuínas explorados para leite é significativa (Facó et al., 2002), sendo comumente baseadas em vacas mestiças de dupla aptidão (Madalena et al., 2012), combinando a alta produção de leite do taurino com a adaptabilidade do zebuino às condições à pasto em clima tropical (Facó et al., 2002; Madalena et al., 2012).

Outra particularidade desta raça é sua habilidade materna. A vaca zebuína tem um vínculo mais forte com sua cria, o que dificulta a aproximação do homem ao bezerro e o manejo na ordenha devido sua necessidade de proteger o bezerro. Assim como elas têm uma melhor ejeção do leite quando ordenhadas com bezerro ao pé (Alvarez et al., 1980; Assan, 2015; Negrão and Marnet, 2003; Negrão and Marnet, 2002; Perez et al., 1983). Dessa forma, se a vaca zebuína em sistema de produção a pasto é primípara, a aproximação e interação repentina com o homem, a nova rotina e as novas instalações e manejo de ordenha, ela se torna um animal agitado e nervoso, dificultando a ordenha, tornando-a estressante e perigosa para o manejador e para o próprio animal e, consequentemente, aumentando a retenção de leite, aumentando o risco à mastite e lucratividade da propriedade. Portanto, não apenas devido ao temperamento e maior reatividade, a vaca zebuína deve receber uma atenção especial em relação ao manejo e

interação humano-animal, principalmente em uma propriedade leiteira.

Reconhecendo que o gado zebuino é mais reativo que o gado europeu, precisamos racionalizar os métodos de criação e desenvolver técnicas de manejo que influenciam positivamente o comportamento e temperamento do animal (Menezes et al., 2017), pois a forma como o gado se comporta durante o manejo é resultado da interação entre fatores genéticos e ambientais (da Costa et al., 2015). Uma estratégia que tem mostrado eficiente para a redução da reatividade é a adoção de Boas Práticas de Manejo que visa reduzir o medo dos animais aos humanos e às instalações (da Costa et al., 2015). Além disso, as boas práticas acrescentam positivamente conceitos de bem-estar animal no sistema de produção, melhorando a visão da atividade pela sociedade.

Dentre as várias opções de raças zebuínas, a Gir se destaca, como raça pura ou em cruzamentos com raças europeias, nos rebanhos leiteiros brasileiros (Madalena et al., 2012), devido a sua adaptação ao ambiente tropical e bom potencial para a produção de leite. A raça Gir, assim como a maioria das outras raças zebuínas (*Bos taurus indicus*) criadas no Brasil, é originária da Índia e entrou oficialmente no Brasil através de importações diretas da Índia, na primeira década do século 20, concentrando-se inicialmente no triângulo mineiro, região que já era tradicional na criação de gado zebu. Dentre os cruzamentos com Gir, temos em destaque o Girolando (Holandesa x Gir), correspondendo, aproximadamente, 80% da produção leiteira nacional (Brasil et al., 2013).

Alguns estudos, com Gir leiteiro, afirmam que o treinamento de escovação foi eficaz para acalmar os animais e melhorar a interação com os humanos, melhorando assim a relação humano-animal (Ujita et al., 2020), além de reduzir o leite residual no início da lactação (Ujita et al., 2021).

1.2.5. Principais indicadores fisiológicos de estresse

As interações agonísticas nas instalações de manejo e seus procedimentos (Sutherland and Huddart, 2012; Wicks et al., 2004) podem resultar em respostas comportamentais e também aumento da frequência cardíaca e respiratória e, liberação do cortisol relacionadas ao estresse (Eicher et al., 2007; Rushen et al., 1999; Van Reenen et al., 2002). Diante de tudo isso, temos mais que claro a importância de estudar os fatores que influenciam o estresse do animal na pecuária e como isso intervêm no seu comportamento, facilidade de manejo e produção.

1.2.5.1. *Temperatura e frequência respiratória*

Uma das defesas biológicas do animal perante uma situação de estresse é a ativação do sistema nervoso autônomo, realizado através de uma resposta rápida, preparando o organismo do animal para lutar ou fugir (Baldwin, 2013; Cannon, 1929; Chen et al., 2015), causando respostas fisiológicas como aumento de frequência cardíaca, temperatura corpórea, pressão arterial, frequência respiratória e redução da atividade gastrointestinal (Chen et al., 2015). A frequência respiratória e temperatura corporal (superfície corporal, retal ou vaginal) são comumente utilizadas para avaliar estresse animal em um determinado manejo, devido sua praticidade e facilidade de avaliação, sendo geralmente positivamente correlacionadas com o cortisol, assim como o temperamento.

Em seu estudo, Sánchez-Rodríguez et al. (2013) observaram que a temperatura e cortisol foram capazes de refletir diferenças fisiológicas associadas ao temperamento em bovinos de corte, tendo valores mais altos em animais mais excitáveis (temperamentais). Outros estudos relatam resultados similares com cortisol (Curley et al., 2006a) e temperatura retal (Gruber et al., 2010). Sugerindo, dessa forma, que podem ser parâmetros para avaliar estresse agudo e temperamento de animais durante o manejo. Em um estudo avaliando a evolução comportamental de gado zebuino durante um protocolo de habituação com estimulação tátil, foi observado a redução de frequência respiratória e de temperatura retal, tendo maiores valores nos primeiros dias da habituação e, reduzindo a partir de uma semana do protocolo, quando os animais demonstraram comportamento mais calmo e relaxados (Ujita et al., 2020).

1.2.5.2. *Hormônios – cortisol, ocitocina e prolactina*

Várias técnicas têm sido utilizadas para avaliar o temperamento animal, sendo muitas delas de natureza subjetiva (Burrow, 1997), o que permite variabilidade de resultados ou baixa confiabilidade sobre a metodologia adotada, afetando, dessa forma, a avaliação feita no estudo (Curley et al., 2006a). Com isso, medidas fisiológicas hormonais indicadores de estresse são comumente adotados para dar suporte a esses resultados subjetivos. Há uma relação entre o tipo de manejo (Grandin, 1997) e temperamento animal com o cortisol plasmático, o qual apresenta um aumento nas concentrações em resposta ao manejo. Em resumo, como resposta ao estresse, a glândula hipófise é estimulada, sintetizando e liberando hormônio adrenocorticotrófico na circulação, estimulando consequentemente a produção de glicocorticoides pelo córtex adrenal, principalmente cortisol (glicocorticoide primário), com a finalidade de disponibilizar energia para que o animal possa recuperar a homeostase (Burdick et al., 2011). Sendo assim, o cortisol é considerado um indicador de estresse a curto prazo, permitindo avaliar o estresse agudo

causado durante os procedimentos de manejo (Grandin, 1997).

O cortisol, é o principal indicador biológico do estresse, sendo conhecido como hormônio do estresse, e suas grandes concentrações circulantes podem prejudicar o desempenho do animal de várias maneiras. Animais em estresse podem apresentar menor desempenho, consumo de ração, menor crescimento e deposição de gordura quando comparados aos animais calmos e, conseqüentemente, ter seu desempenho reduzido (Llonch et al., 2016). Ainda, existem estudos que o temperamento negativo associado a altas concentrações de cortisol além de atrasar a puberdade, impacta negativamente o desempenho reprodutivo em vacas, reduzindo a eficiência reprodutiva e taxa de desmame (Cooke et al., 2016).

Outros hormônios importantes para avaliar o comportamento animal, por exemplo a prolactina e ocitocina, são relacionados ao comportamento materno e facilidade de ordenha (Abdel-Hamid et al., 2017; Bruckmaier et al., 1993; Ujita et al., 2021). Por outro lado, a ocitocina é conhecida como o hormônio responsável pela ejeção do leite. No úbere dos ruminantes, 20% do leite é armazenado na cisterna (Bruckmaier and Wellnitz, 2008; Bruckmaier and Blum, 1998) e sua ejeção alveolar depende da concentração do hormônio ocitocina liberada e de receptores localizados nas células mioepiteliais, permitindo a contração das células e dos alvéolos e, conseqüentemente, a ejeção do leite (Bruckmaier, 2003; Bruckmaier and Wellnitz, 2008; Bruckmaier and Blum, 1998). Do mesmo modo, a síntese de leite depende da interação entre a prolactina e seu receptor na glândula mamária (Lacasse et al., 2011; Svennersten-Sjaunja and Olsson, 2005). Contudo, a interação entre ocitocina, prolactina e seus receptores podem se alterar em função da fase da lactação, conforme a reflexo neuroendócrino de ejeção de leite evolui durante a lactação (Svennersten-Sjaunja and Olsson, 2005). Se no momento da ordenha, os animais estão estressados ocorre um aumento anormal de cortisol, a adrenalina (epinefrina) e a noradrenalina (noraepinefrina) (Blum and Bruckmaier, 1989; Tancin et al., 1995), impedindo a ligação da ocitocina aos seus receptores localizados nas células mioepiteliais dos alvéolos do úbere (Borghese et al., 2007) inibindo dessa forma, a ejeção de leite (Bruckmaier et al., 1997), condição mais frequente em primíparas devido ao estresse gerado pelas primeiras ordenhas (Negrão and Marnet, 2003; Van Reenen et al., 2002).

Outros estudos com ruminantes demonstraram associação da elevação dos níveis de cortisol com o estresse de ordenha (Rushen et al., 2001) e retenção do leite (Bruckmaier et al., 1993; Hemsworth et al., 2000; Negrão and Marnet, 2003; Tancin et al., 1995) devido à menor liberação de ocitocina em ambientes desconhecidos, troca de ordenhadores ou mudança de rotina de ordenha (Bruckmaier et al., 1993; Tancin et al., 1995), resultando em redução da produção leiteira (Bruckmaier et al., 1997).

1.2.6. Expressão gênica

O estudo da expressão gênica pode fornecer informações importantes sobre as diferentes vias moleculares que controlam as respostas celulares em diferentes tecidos relacionados à produção animal. De fato, alguns autores vêm relacionando a expressão gênica na glândula mamária às taxas de proliferação e apoptose celular, síntese de diferentes constituintes do leite e produção de leite (Bomfim et al., 2018; Hooper et al., 2020; Ouellet et al., 2021). Tendo em vista que as vacas Gir são reputadas temperamentais, pouco adaptadas à ordenha mecânica e difíceis de ordenhar, no presente estudo um conjunto genes relacionados ao estresse (receptores do cortisol: MR e GR; da adrenalina e noradrenalina: $ADR\alpha1A$, $ADR\alpha2AD$, $ADB\beta2$), ejeção de leite (receptor de ocitocina: OTR, receptor de prolactina: PRLR) e inibidores da lactação (receptores de serotonina: HTR1B, HTR2A, HTR2B, HTR4, HTR7) foram analisados nas células epiteliais mamárias.

Classicamente, a síntese de leite depende da interação de estímulos sistêmicos e locais (Bruckmaier and Wellnitz, 2008; Collier et al., 2012). Por exemplo, a liberação de prolactina e ocitocina causada pela amamentação ou ordenha controlam os processos de síntese e ejeção de leite, porém seu efeito biológico depende da presença de seus receptores na glândula mamária (Negrão, 2008). Deste modo, uma interação adequada entre hormônio-receptor no tecido alvo facilita a síntese de leite, sua ejeção tendo reflexos sobre inclusive a persistência da lactação. Alguns autores argumentam que a liberação de adrenalina, noradrenalina e suas interações com os receptores adrenérgicos presentes na glândula mamária podem influenciar negativamente a ligação da ocitocina aos receptores presentes nas células mioepiteliais dificultando assim a ejeção do leite (Bruckmaier and Wellnitz, 2008; Negrão, 2008). Porém, não foram encontradas na literatura informações relacionando as ações do cortisol, ocitocina, prolactina aos receptores de serotonina na glândula mamária.

Neste contexto, fatores locais como os receptores da adrenalina, noradrenalina ($ADR\alpha1A$, $ADR\alpha2AD$, $ADB\beta2$) e serotonina podem respectivamente prejudicar a ejeção do leite e inibir a síntese do leite contribuindo para diminuir a persistência de lactação. De modo geral, a retenção de leite é associada a uma ordenha ineficiente e relacionada negativamente ao aumento das concentrações de cortisol, adrenalina e noradrenalina e baixa produção de leite. Deste modo, a execução de ordenhas eficientes são fundamentais para aumentar a produção de leite das vacas Gir e os estudos da expressão dos genes OTR, PRLR, GR, MR, $ADR\alpha1A$, $ADR\alpha2AD$, $ADB\beta$, HTR1B, HTR2A, HTR2B, HTR4, HTR7 podem fornecer informações relacionadas à adaptação a ordenha.

1.2.7. Protocolos de habituação como solução prática

Para melhorar o relacionamento entre o ser humano e o gado no dia a dia da fazenda, não são necessárias mudanças nas condições do mercado ou investimentos substanciais, bastando que os produtores entendam melhor o comportamento dos animais criados, adaptando o sistema de manejo e as instalações às características do seu rebanho (da Costa et al., 2002; Ujita et al., 2020). Conhecendo os animais que criamos e melhorando seu bem-estar, podemos obter melhores resultados econômicos com um sistema de criação mais eficiente e/ou produtos de melhor qualidade (da Costa, 2000). Logo, aprimorar as condições de manejo dos animais ocasionará um incremento de produtividade e de bem-estar animal.

Bovinos que tiveram pouco contato com o manejo, apresentam respostas de estresse tanto comportamentais quanto fisiológicas ao tratador e ambiente (Arnold et al., 2007; Ujita et al., 2020), principalmente as novilhas primíparas (Bertenshaw et al., 2008), sendo uma fase de grande importância na qual se recomenda um manejo positivo para influenciar o comportamento durante o manejo, como redução de coices, medo da presença humana e chances de lesões (Bertenshaw and Rowlinson, 2001). Contudo, bovinos são animais que se condicionam a uma rotina, o que demanda interações positivas diárias animais com os humanos.

A percepção de diferentes sentidos pode estar envolvida na relação entre humanos e animais (Waiblinger et al., 2006), portanto, poderíamos usar diferentes estratégias para melhorar essa resposta negativa. Técnicas como acariciar (Lensink et al., 2000; Lürzel et al., 2016a; Ujita et al., 2020), escovar (Silva et al., 2017; Ujita et al., 2020), tocar (Probst et al., 2012a), conversar (Lürzel et al., 2016a) e presença humana positiva (Boivin et al., 2000) pode reduzir o medo dos humanos e, consequentemente, a distância de fuga. Assim, poderia reduzir o estresse na vida futura (Boivin and Braastad, 1996; Grandin, 1997).

Embora a eficiência deste treinamento pode ser determinada por diversos fatores como genética dos indivíduos, duração das interações, tipo de manejo, ambiente físico, comportamento social e experiências vividas anteriormente (Grignard et al., 2000; X Boivin et al., 2003), estímulos positivos melhoram a relação homem-animal. Ainda, a repetição do manejo pode reduzir a reação dos animais às exposições específicas (Grandin, 1997). Com isto, o treinamento ou habituação de rebanho que visa reduzir o medo de novilhas dos seres humanos e instalações, pode reduzir o comportamento negativo desses animais (Andrade et al., 2001; Fonsêca et al., 2019; Kutzer et al., 2015; Wicks et al., 2004), e a estimulação tátil pode ser uma importante ferramenta de manejo para melhorar essa interação (Néri et al., 2016; Polikarpus et

al., 2014; Ujita et al., 2020). Trazendo, dessa forma, benefícios positivos ao comportamento dos animais, tornando-os mais relaxados e confiantes em relação ao tratador e às instalações (Ujita et al., 2020).

Deste modo, há o interesse crescente de aprimorar a interação humano-animal, com finalidade de melhorar a eficiência do animal, facilitando o manejo e incluir o conceito de bem-estar na propriedade. Ainda, estudos com habituação ou treinamento de gado bovino são escassos, principalmente quando tratamos de animais leiteiros zebuínos ou de corte. Dessa forma, a hipótese desta tese de doutorado foi que a habituação do manejo em bovinos tem influência sobre o comportamento, reduzindo a reatividade do animal e consequentemente o estresse do mesmo. Logo, o objetivo deste estudo foi descrever o efeito do treinamento em novilhas Gir sobre o comportamento e produção leiteira e em novilhos europeus sobre o comportamento e interação humano-animal.

REFERÊNCIAS

- Abdel-Hamid, S.E., Fattah, D.M.A., Ghanem, H.M., Eman Abd-Allah Manaa, 2017. Temperament during Milking Process and its Effect on Behavioral, Productive Traits and Biochemical Parameters in Friesian Dairy Cows. *Adv. Anim. Vet. Sci.* 5, 508–513. <https://doi.org/http://dx.doi.org/10.17582/journal.aavs/2017/5.12.508.513>
- Adamczyk, K., 2018. Dairy cattle welfare as a result of human-animal relationship – a review. *Ann. Anim. Sci.* <https://doi.org/10.2478/aoas-2018-0013>
- Alvarez, F.J., Saucedo, G., Arriaga, A., Preston, T.R., 1980. Effect on Milk Production and Calf Performance of Milking Cross Bred European / Zebu Cattle in the Absence or Presence of. *Trop. Anim. Prod.* 5, 25–37.
- Andrade, O., Orihuela, A., Solano, J., Galina, C.S., 2001. Some effects of repeated handling and the use of a mask on stress responses in zebu cattle during restraint. *Appl. Anim. Behav. Sci.* 71, 175–181. [https://doi.org/10.1016/S0168-1591\(00\)00177-5](https://doi.org/10.1016/S0168-1591(00)00177-5)
- Assan, N., 2015. Influence of suckling and/or milking method on yield and milk composition in dairy animals. *Sci. J. Zool.* 4, 1–7. <https://doi.org/10.14196/sjz.v4i1.1792>
- Baldwin, D. V., 2013. Primitive mechanisms of trauma response: An evolutionary perspective on trauma-related disorders. *Neurosci. Biobehav. Rev.* <https://doi.org/10.1016/j.neubiorev.2013.06.004>
- Bar-Pelled, U., Maltz, E., Bruckental, I., Folman, Y., Kali, Y., Gacitua, H., Lehrer, A.R., Knight, C.H., Robinson, B., Voet, H., Tagari, H., 1995. Relationship Between Frequent Milking or Suckling in Early Lactation and Milk Production of High Producing Dairy Cows. *J. Dairy Sci.* 78, 2726–2736. [https://doi.org/10.3168/jds.S0022-0302\(95\)76903-X](https://doi.org/10.3168/jds.S0022-0302(95)76903-X)
- Becker, B.G., Lobato, J.F.P., 1997. Effect of gentle handling on the reactivity of zebu crossed calves to humans. *Appl. Anim. Behav. Sci.* 53, 219–224. [https://doi.org/10.1016/S0168-1591\(96\)01091-X](https://doi.org/10.1016/S0168-1591(96)01091-X)
- Benus, R.F., Bohus, B., Koolhaas, J.M., van Oortmerssen, G.A., 1991. Heritable variation for aggression as a reflection of individual coping strategies. *Experientia.* <https://doi.org/10.1007/BF01923336>
- Bertenshaw, C., Rowlinson, P., 2001. The influence of positive human-animal interaction during rearing on the approach behaviour of young dairy heifers. *Proc. Br. Soc. Anim. Sci.* <https://doi.org/10.1017/s1752756200006530>
- Bertenshaw, C., Rowlinson, P., Edge, H., Douglas, S., Shiel, R., 2008. The effect of different degrees of “positive” human-animal interaction during rearing on the welfare and subsequent production of commercial dairy heifers. *Appl. Anim. Behav. Sci.* 114, 65–75. <https://doi.org/10.1016/j.applanim.2007.12.002>
- Bertucci, P.Y., Quaglino, A., Pozzi, A.G., Kordon, E.C., Pecci, A., 2010. Glucocorticoid-induced impairment of mammary gland involution is associated with STAT5 and STAT3 signaling modulation. *Endocrinology* 151, 5730–5740. <https://doi.org/10.1210/en.2010-0517>
- Blum, J.W., Bruckmaier, R., 1989. Catecholamines, oxytocin and milk removal in dairy cows. *J. Dairy Res.* <https://doi.org/10.1017/S0022029900026388>
- Boivin, X., Braastad, B.O., 1996. Effects of handling during temporary isolation after early weaning on goat kids’ later response to humans. *Appl. Anim. Behav. Sci.* 48, 61–71. [https://doi.org/10.1016/0168-1591\(95\)01019-X](https://doi.org/10.1016/0168-1591(95)01019-X)
- Boivin, X., Tournadret, H., Le Neindre, P., 2000. Hand-feeding and gentling influence early-weaned lambs’ attachment responses to their stockperson. *J. Anim. Sci.* 78, 879–884. <https://doi.org/10.2527/2000.784879x>
- Bomfim, G.F., Merighe, G.K.F., de Oliveira, S.A., Negrao, J.A., 2018. Effect of acute stressors, adrenocorticotrophic hormone administration, and cortisol release on milk yield, the expression of

- key genes, proliferation, and apoptosis in goat mammary epithelial cells. *J. Dairy Sci.* 101, 6486–6496. <https://doi.org/10.3168/jds.2017-14123>
- Bondarenko, E., Beig, M.I., Hodgson, D.M., Braga, V.A., Nalivaiko, E., 2015. Blockade of the dorsomedial hypothalamus and the perifornical area inhibits respiratory responses to arousing and stressful stimuli. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* <https://doi.org/10.1152/ajpregu.00415.2014>
- Borghese, A., Rasmussen, M., Thomas, C.S., 2007. Milking management of dairy buffalo. *Ital. J. Anim. Sci.* <https://doi.org/10.4081/ijas.2007.s2.39>
- Bouissou, M.F., Boissy, A., Neindre, P. le, Veissier, I., 2009. The social behaviour of cattle., in: *Social Behaviour in Farm Animals*. <https://doi.org/10.1079/9780851993973.0113>
- Brasil, B.S.A.F., Coelho, E.G.A., Drummond, M.G., Oliveira, D.A.A., 2013. Genetic diversity and differentiation of exotic and American commercial cattle breeds raised in Brazil. *Genet. Mol. Res.* <https://doi.org/10.4238/2013.November.18.2>
- Bremner, K.J., 1997. Behaviour of dairy heifers during adaptation to milking. *Proc. New Zeal. Soc. Anim. Prod.*
- Breuer, K., Hemsworth, P.H., Barnett, J.L., Matthews, L.R., Coleman, G.J., 2000. Behavioural response to humans and the productivity of commercial dairy cows. *Appl. Anim. Behav. Sci.* 66, 273–288. [https://doi.org/10.1016/S0168-1591\(99\)00097-0](https://doi.org/10.1016/S0168-1591(99)00097-0)
- Breuer, K., Hemsworth, P.H., Coleman, G.J., 2003. The effect of positive or negative handling on the behavioural and physiological responses of nonlactating heifers. *Appl. Anim. Behav. Sci.* 84, 3–22. [https://doi.org/10.1016/S0168-1591\(03\)00146-1](https://doi.org/10.1016/S0168-1591(03)00146-1)
- Bristow, D.J., Holmes, D.S., 2007. Cortisol levels and anxiety-related behaviors in cattle. *Physiol. Behav.* 90, 626–628. <https://doi.org/10.1016/j.physbeh.2006.11.015>
- Brouček, J., 2014. Effect of Noise on Performance, Stress, and Behaviour of Animals. *Slovak J. Anim. Sci.* 47, 111–123.
- Brouček, J., Uhrinčat, M., ŠoCh, M., Kišac, P., UhRinČat, M., ŠoCh, M., Kišac, P., 2008. Genetics of behaviour in cattle. *Slovak J. Anim. Sci.* 41, 166–172. <https://doi.org/10.1079/9781780642215.0234>
- Bruckmaier, R.M., 2003. Chronic oxytocin treatment causes reduced milk ejection in dairy cows. *J. Dairy Res.* <https://doi.org/10.1017/S0022029902005940>
- Bruckmaier, R.M., Pfeilsticker, H.-U., Blum, J.W., 2009. Milk yield, oxytocin and β -endorphin gradually normalize during repeated milking in unfamiliar surroundings. *J. Dairy Res.* 63, 191. <https://doi.org/10.1017/s0022029900031691>
- Bruckmaier, R.M., Schams, D., Blum, J.W., 1993. Milk removal in familiar and unfamiliar surroundings: concentrations of oxytocin, prolactin, cortisol and β -endorphin. *J. Dairy Res.* 60, 449–456.
- Bruckmaier, R.M., Wellnitz, O., 2008. Induction of milk ejection and milk removal in different production systems. *J. Anim. Sci.* 86, 15–20. <https://doi.org/10.2527/jas.2007-0335>
- Bruckmaier, R.M., Wellnitz, O., Blum, J.W., 1997. Inhibition of milk ejection in cows by oxytocin receptor blockade, α -adrenergic receptor stimulation and in unfamiliar surroundings. *J. Dairy Res.* 64, 315–325. <https://doi.org/10.1017/S002202999700232X>
- Bruckmaier, R.M.M., Blum, J.W.W., 1998. Oxytocin Release and Milk Removal in Ruminants. *J. Dairy Sci.* 81, 939–949. [https://doi.org/10.3168/jds.S0022-0302\(98\)75654-1](https://doi.org/10.3168/jds.S0022-0302(98)75654-1)
- Burdick, N.C., Randel, R.D., Carroll, J.A., Welsh, T.H., 2011. Interactions between temperament, stress, and immune function in cattle. *Int. J. Zool.* <https://doi.org/10.1155/2011/373197>
- Burdick Sanchez, N.C., Carroll, J.A., Randel, R.D., Vann, R.C., Welsh, T.H., 2014. Associations

- between endotoxin-induced metabolic changes and temperament in Brahman bulls,. *J. Anim. Physiol. Anim. Nutr. (Berl)*. 98, 178–190. <https://doi.org/10.1111/jpn.12074>
- Burrow, H.M., 1997. Measurement of temperament and their relationship with performance traits of beef cattle. *Anim Breed Abstr*. <https://doi.org/10.1002/tea.20309>
- Cannon, W.B., 1929. Bodily Changes in Pain, Hunger, Fear and Rage. *South. Med. J.* <https://doi.org/10.1097/00007611-192909000-00037>
- Cavallina, R., Roncoroni, C., Campagna, M.C., Minero, M., Canali, E., 2008. Buffalo behavioural response to machine milking in early lactation. *Ital. J. Anim. Sci.* 7, 287–295. <https://doi.org/10.4081/ijas.2008.287>
- Ceballos, M.C., Sant’Anna, A.C., Boivin, X., Costa, F. de O., Carvalhal, M.V. d. L., Paranhos da Costa, M.J.R., 2018a. Impact of good practices of handling training on beef cattle welfare and stockpeople attitudes and behaviors. *Livest. Sci.* <https://doi.org/10.1016/j.livsci.2018.06.019>
- Ceballos, M.C., Sant’Anna, A.C., Góis, K.C.R., Ferraudo, A.S., Negrao, J.A., da Costa, M.J.R.P., 2018b. Investigating the relationship between human-animal interactions, reactivity, stress response and reproductive performance in Nellore heifers. *Livest. Sci.* 217, 65–75. <https://doi.org/10.1016/j.livsci.2018.08.001>
- Chen, Y., Arsenault, R., Napper, S., Griebel, P., 2015. Models and methods to investigate acute stress responses in cattle. *Animals* 5, 1268–1295. <https://doi.org/10.3390/ani5040411>
- Chida, D., Miyoshi, K., Sato, T., Yoda, T., Kikusui, T., Iwakura, Y., 2011. The role of glucocorticoids in pregnancy, parturition, lactation, and nurturing in melanocortin receptor 2-deficient mice. *Endocrinology* 152, 1652–1660. <https://doi.org/10.1210/en.2010-0935>
- Coelho, S.G., Franzoni, A.P.S., 2012. Comportamento e Bem-estar de bezerras na bovinocultura leiteira, in: Junior, A. de P.M., Heinemann, M.B., Garcia, S.K., Ângela Mara Leite Drumond (Eds.), *Cadernos Técnicos de Veterinária e Zootecnia*. Belo Horizonte, pp. 135–151.
- Coleman, G.J., Hemsworth, P.H., Hay, M., 1998. Predicting stockperson behaviour towards pigs from attitudinal and job-related variables and empathy. *Appl. Anim. Behav. Sci.* 58, 63–75. [https://doi.org/10.1016/S0168-1591\(96\)01168-9](https://doi.org/10.1016/S0168-1591(96)01168-9)
- Collier, R.J., Hernandez, L.L., Horseman, N.D., 2012. Serotonin as a homeostatic regulator of lactation. *Domest. Anim. Endocrinol.* 43, 161–170. <https://doi.org/10.1016/j.domaniend.2012.03.006>
- Connelly, M.K., Weaver, S.R., Kuehnl, J.M., Fricke, H.P., Klister, M., Hernandez, L., 2021. Elevated serotonin coordinates mammary metabolism in dairy cows. *Physiol. Rep.* 9, 1–12. <https://doi.org/10.14814/phy2.14798>
- Cooke, R.F., Schubach, K.M., Peres, R.F.G., Cipriano, R.S., Marques, R., Carvalho, R., Bohnert, D.W., Biehl, M. V., Pires, A. V., Vasconcelos, J.L.M., 2016. 1275 Effects of temperament on physiological and reproductive responses of Bos Indicus beef cows. *J. Anim. Sci.* <https://doi.org/10.2527/jam2016-1275>
- Cozzi, G., Brscic, M., Gottardo, F., 2009. Main critical factors affecting the welfare of beef cattle and veal calves raised under intensive rearing systems in Italy: A review. *Ital. J. Anim. Sci.* <https://doi.org/10.4081/ijas.2009.s1.67>
- Crowder, M.J., Hand, D.J., 1990. *Analysis of Repeated Measures*. Chapman and Hall, London.
- Curley, K.O., Paschal, J.C., Welsh, T.H., Randel, R.D., 2006a. Technical note: Exit velocity as a measure of cattle temperament is repeatable and associated with serum concentration of cortisol in Brahman bulls. *J. Anim. Sci.* 84, 3100–3103. <https://doi.org/10.2527/jas.2006-055>
- Curley, K.O., Paschal, J.C., Welsh, T.H., Randel, R.D., 2006b. Technical note: Exit velocity as a measure of cattle temperament is repeatable and associated with serum concentration of cortisol in Brahman bulls. *J. Anim. Sci.* <https://doi.org/10.2527/jas.2006-055>
- D’Eath, R.B., Conington, J., Lawrence, A.B., Olsson, I.A.S., Sandøe, P., 2010. Breeding for behavioural

- change in farm animals: Practical, economic and ethical considerations. *Anim. Welf.*
- da Costa, M.J.R.P., 2000. Ambiência na produção de bovinos de corte a pasto. *An. Etol.* 18, 26–42.
- da Costa, M.J.R.P., Costa e Silva, E.V., Chiquitelli Neto, M., Rosa, M.S., 2002. Contribuição dos estudos de comportamento de bovinos para implementação de programas de qualidade de carne. *Encontro Anu. Etol.* 71–89.
- da Costa, M.J.R.P., Sant’Anna, A.C., Silva, L.C.M., 2015. Temperamento de bovinos Gir e Girolando : efeitos genéticos e de manejo. *Inf. Agropecuário, Belo Horiz.* 36, 100–107.
- de Passillé, A.M., Rushen, J., Martin, F., 1995. Interpreting the behaviour of calves in an open-field test: a factor analysis. *Appl. Anim. Behav. Sci.* [https://doi.org/10.1016/0168-1591\(95\)00622-Y](https://doi.org/10.1016/0168-1591(95)00622-Y)
- Doerfler, R.L., Lehermeier, C., Kliem, H., Möstl, E., Bernhardt, H., 2016. Physiological and behavioral responses of dairy cattle to the introduction of robot scrapers. *Front. Vet. Sci.* 3, 1–11. <https://doi.org/10.3389/fvets.2016.00106>
- Eicher, S.D., Schutz, M., Kearney, F., Willard, S., Bowers, S., Gandy, S., Graves, K., 2007. Prepartum milking effects on parlour behaviour, endocrine and immune responses in Holstein heifers. *J. Dairy Res.* 74, 417–424. <https://doi.org/10.1017/S0022029907002695>
- El. Abdel-Hamid, S., Fattah, D.M.A., Ghanem, H.M., Manaa, E.A.A., 2017. Temperament during milking process and its effect on behavioral, productive traits and biochemical parameters in Friesian dairy cows. *Adv. Anim. Vet. Sci.* 5, 508–513. <https://doi.org/10.17582/journal.aavs/2017/5.12.508.513>
- Facó, O., Lôbo, R.N.B., Martins Filho, R., Moura, A.D.A.A., Nonato, R., Lôbo, B., Filho, R.M., 2002. Análise do Desempenho Produtivo de Diversos Grupos Genéticos Holandês x Gir no Brasil 1 Analysis of Productive Performance of Different Holstein x Gir Genetic Groups in Brazil. *Rev. Bras. Zootec.* 31, 1944–1952. <https://doi.org/10.1590/S1516-35982002000800010>
- Fonsêca, V.F.C., Saraiva, E.P., Maia, A.S.C., de Melo Costa, C.C., dos Santos, S.G.C.G., Morais, L.K.C., Fuller, A., Chiquitelli Neto, M., 2019. Training sheep for indirect calorimetry trials. *Small Rumin. Res.* 177, 36–43. <https://doi.org/10.1016/j.smallrumres.2019.06.010>
- Fordyce, G., Goddard, M.E., Seifert, G.W., 1982. The measurement of temperament in cattle and the effect of experience and genotype. *Proc. Aust. Soc. Anim. Prod.*
- Friedrich, J., Brand, B., Schwerin, M., 2015. Genetics of cattle temperament and its impact on livestock production and breeding – a review. *Arch. Anim. Breed.* <https://doi.org/10.5194/aab-58-13-2015>
- Geverink, N.A., Kappers, A., Van De Burgwal, J.A., Lambooij, E., Blokhuis, H.J., Wiegant, V.M., 1998. Effects of Regular Moving and Handling on the Behavioral and Physiological Responses of Pigs to Preslaughter Treatment and Consequences for Subsequent Meat Quality. *J. Anim. Sci.* <https://doi.org/10.2527/1998.7682080x>
- Grandin, T., 1997. Assessment of Stress during Handling and Transport. *J. Anim. Sci.* 75, 249–257. <https://doi.org/10.2527/1997.751249x>
- Grandin, T., 1990. Design of loading facilities and holding pens. *Appl. Anim. Behav. Sci.* 28, 187–201. [https://doi.org/10.1016/0168-1591\(90\)90053-G](https://doi.org/10.1016/0168-1591(90)90053-G)
- Grandin, T., Deesing, M.J., 2014. Genetics and Behavior During Handling, Restraint, and Herding, Second Edi. ed, *Genetics and the Behavior of Domestic Animals*. Elsevier Inc. <https://doi.org/10.1016/B978-0-12-394586-0.00004-4>
- Grandin, T., Shivley, C., 2015. How farm animals react and perceive stressful situations such as handling, restraint, and transport. *Animals* 5, 1233–1251. <https://doi.org/10.3390/ani5040409>
- Grignard, L., Boissy, A., Boivin, X., Garel, J.P., Le Neindre, P., 2000. The social environment influences the behavioural responses of beef cattle to handling. *Appl. Anim. Behav. Sci.* [https://doi.org/10.1016/S0168-1591\(00\)00085-X](https://doi.org/10.1016/S0168-1591(00)00085-X)

- Gruber, S.L., Tatum, J.D., Engle, T.E., Chapman, P.L., Belk, K.E., Smith, G.C., 2010. Relationships of behavioral and physiological symptoms of preslaughter stress to beef longissimus muscle tenderness. *J. Anim. Sci.* <https://doi.org/10.2527/jas.2009-2183>
- Hakan, K., Demirci, S., 2012. Livestock-Handling Related Injuries and Deaths, in: *Livestock Production*. <https://doi.org/10.5772/50834>
- Hall, N.L., Buchanan, D.S., Anderson, V.L., Ilse, B.R., Carlin, K.R., Berg, E.P., 2011. Working chute behavior of feedlot cattle can be an indication of cattle temperament and beef carcass composition and quality. *Meat Sci.* <https://doi.org/10.1016/j.meatsci.2011.03.020>
- Haskell, M.J., Simm, G., Turner, S.P., 2014. Genetic selection for temperament traits in dairy and beef cattle. *Front. Genet.* 5, 1–18. <https://doi.org/10.3389/fgene.2014.00368>
- Hearnshaw, H., Morris, C.A., 1984. Genetic and environmental effects on a temperament score in beef cattle. *Aust. J. Agric. Res.* 35, 723–733. <https://doi.org/10.1071/AR9840723>
- Hemsworth, P.H., Barnett, J.L., 1989. Relationships between fear of humans, productivity and cage position of laying hens. *Br. Poult. Sci.* 30, 505–518. <https://doi.org/10.1080/00071668908417175>
- Hemsworth, P.H., Coleman, G.J., Barnett, J.L., Borg, S., 2000. Relationships between human-animal interactions and productivity of commercial dairy cows. *J. Anim. Sci.* 78, 2821–2831. <https://doi.org/10.2527/2000.78112821x>
- Hooper, H.B., Silva, P. dos S., de Oliveira, S.A., Meringhe, G.K.F., Lacasse, P., Negrão, J.A., 2020. Effect of heat stress in late gestation on subsequent lactation performance and mammary cell gene expression of Saanen goats. *J. Dairy Sci.* 103, 1982–1992. <https://doi.org/10.3168/jds.2019-16734>
- Hötzel, M.J., Machado Filho, L.C.P., Yunes, M.C., Da Silveira, M.C.A.C., 2005. Influência de um ordenhador aversivo sobre a produção leiteira de vacas da raça holandesa. *Rev. Bras. Zootec.* 34, 1278–1284.
- Hunter, P., 2018. The genetics of domestication. *EMBO Rep.* <https://doi.org/10.15252/embr.201745664>
- Inderwies, T., Pfaffl, M.W., Bruckmaier, R.M., 2003. Milking characteristics and their relation to adrenergic receptor mRNA expression and ligand binding in the mammary gland of dairy cows. *Domest. Anim. Endocrinol.* 25, 275–286. [https://doi.org/10.1016/S0739-7240\(03\)00059-6](https://doi.org/10.1016/S0739-7240(03)00059-6)
- Johnsen, J.F., Zipp, K.A., Kälber, T., Passillé, A.M. de, Knierim, U., Barth, K., Mejdell, C.M., 2016. Is rearing calves with the dam a feasible option for dairy farms?—Current and future research. *Appl. Anim. Behav. Sci.* 181, 1–11. <https://doi.org/10.1016/j.applanim.2015.11.011>
- Jones, R.B., 1996. Fear and adaptability in poultry: Insights, implications and imperatives. *Worlds. Poult. Sci. J.* <https://doi.org/10.1079/wps19960013>
- Kasimanickam, R.K., Hall, J.B., Estill, C.T., Kastelic, J.P., Joseph, C., Abdel Aziz, R.L., Nak, D., 2018. Flunixin meglumine improves pregnancy rate in embryo recipient beef cows with an excitable temperament. *Theriogenology* 107, 70–77. <https://doi.org/10.1016/j.theriogenology.2017.10.043>
- Kiley-Worthington, M., de la Plain, S., Kiley-Worthington, M., de la Plain, S., 1983. Fostering and Adoption in Beef Cattle, in: *The Behaviour of Beef Suckler Cattle (Bos Taurus)*. https://doi.org/10.1007/978-3-0348-6782-5_8
- Kling-Eveillard, F., Allain, C., Boivin, X., Courboulay, V., Créach, P., Philibert, A., Ramonet, Y., Hostiou, N., 2020. Farmers' representations of the effects of precision livestock farming on human-animal relationships. *Livest. Sci.* <https://doi.org/10.1016/j.livsci.2020.104057>
- Kolb, D.A., 2015. *Experiential Learning- Experience as the Source of Learning and Development (2nd Edition)*, Person Education, Inc. <https://doi.org/10.1017/CBO9781107415324.004>
- Krohn, C.C., Jago, J.G., Boivin, X., 2001. The effect of early handling on the socialisation of young calves to humans. *Appl. Anim. Behav. Sci.* [https://doi.org/10.1016/S0168-1591\(01\)00161-7](https://doi.org/10.1016/S0168-1591(01)00161-7)
- Kutzer, T., Steilen, M., Gyga, L., Wechsler, B., 2015. Habituation of dairy heifers to milking routine-

- Effects on human avoidance distance, behavior, and cardiac activity during milking. *J. Dairy Sci.* 98, 5241–5251. <https://doi.org/10.3168/jds.2014-8773>
- Lacasse, P., Lollivier, V., Bruckmaier, R.M., Boisclair, Y.R., Wagner, G.F., Boutinaud, M., 2011. Effect of the prolactin-release inhibitor quinagolide on lactating dairy cows. *J. Dairy Sci.* 94, 1302–1309. <https://doi.org/10.3168/jds.2010-3649>
- Lacasse, P., Ollier, S., Lollivier, V., Boutinaud, M., 2016. New insights into the importance of prolactin in dairy ruminants. *J. Dairy Sci.* 99, 864–874. <https://doi.org/10.3168/jds.2015-10035>
- Lammoglia, M., Alarcón, M., Mancera, B., Cabrera, A., Daniel, A., 2015. Effects of Routinely Oxytocin Injection to Induce Milk Ejection on Some Reproductive Parameters of Crossbred Cows in the Tropics of Veracruz. *Annu. Res. Rev. Biol.* 6, 297–303. <https://doi.org/10.9734/arrb/2015/15902>
- Lansade, L., Bertrand, M., Boivin, X., Bouissou, M.F., 2004. Effects of handling at weaning on manageability and reactivity of foals. *Appl. Anim. Behav. Sci.* 87, 131–149. <https://doi.org/10.1016/j.applanim.2003.12.011>
- Laurence, M., Barnes, A., Collins, T., Hyndman, T., Musk, G.C., 2016. Assessing and mitigating post-operative castration pain in *Bos indicus* cattle. *Anim. Prod. Sci.* 58, 909–919. <https://doi.org/10.1071/AN16210>
- Leão, G.F.M., Pivatto, D.R.D., Carniel, H., Rodrigues, M.G.K., Braga, R.A., Silva, M.R.H. da, Teixeira, P.P.M., 2013. Melhoramento Genético Em Zebuínos Leiteiros – Uma Revisão. *Agropecuária Científica No Semiárido*.
- Lee, C., Cafe, L.M., Robinson, S.L., Doyle, R.E., Lea, J.M., Small, A.H., Colditz, I.G., 2018. Anxiety influences attention bias but not flight speed and crush score in beef cattle. *Appl. Anim. Behav. Sci.* 205, 210–215. <https://doi.org/10.1016/j.applanim.2017.11.003>
- Lefcourt, A.M., Kahl, S., Akers, R.M., 1986. Correlation of Indices of Stress with Intensity of Electrical Shock for Cows. *J. Dairy Sci.* 69, 833–842. [https://doi.org/10.3168/jds.S0022-0302\(86\)80473-8](https://doi.org/10.3168/jds.S0022-0302(86)80473-8)
- Lensink, B.J., Boivin, X., Pradel, P., Le Neindre, P., Veissier, I., 2000. Reducing veal calves' reactivity to people by providing additional human contact. *J. Anim. Sci.* 78, 1213–1218. <https://doi.org/10.2527/2000.7851213x>
- Ligout, S., Bouissou, M.F., Boivin, X., 2008. Comparison of the effects of two different handling methods on the subsequent behaviour of Anglo-Arabian foals toward humans and handling. *Appl. Anim. Behav. Sci.* 113, 175–188. <https://doi.org/10.1016/j.applanim.2007.12.004>
- Lima, M.L.P., Negrão, J.A., de Paz, C.C.P., Grandin, T., 2018. Minor corral changes and adoption of good handling practices can improve the behavior and reduce cortisol release in Nellore cows. *Trop. Anim. Health Prod.* 50, 525–530. <https://doi.org/10.1007/s11250-017-1463-9>
- Llouch, P., Somarriba, M., Duthie, C.A., Haskell, M.J., Rooke, J.A., Troy, S., Roehe, R., Turner, S.P., 2016. Association of temperament and acute stress responsiveness with productivity, feed efficiency, and Methane emissions in beef cattle: An observational study. *Front. Vet. Sci.* <https://doi.org/10.3389/fvets.2016.00043>
- Losada-Espinosa, N., Villarroel, M., María, G.A., Miranda-de la Lama, G.C., 2018. Pre-slaughter cattle welfare indicators for use in commercial abattoirs with voluntary monitoring systems: A systematic review. *Meat Sci.* 138, 34–48. <https://doi.org/10.1016/j.meatsci.2017.12.004>
- Lürzel, S., Windschnurer, I., Futschik, A., Waiblinger, S., 2016a. Gentle interactions decrease the fear of humans in dairy heifers independently of early experience of stroking. *Appl. Anim. Behav. Sci.* 178, 16–22. <https://doi.org/10.1016/j.applanim.2016.02.012>
- Lürzel, S., Windschnurer, I., Futschik, A., Waiblinger, S., 2016b. Gentle interactions decrease the fear of humans in dairy heifers independently of early experience of stroking. *Appl. Anim. Behav. Sci.* <https://doi.org/10.1016/j.applanim.2016.02.012>
- Maçal-Pedroza, M.G., Campos, M.M., Pereira, L.G.R., Machado, F.S., Tomich, T.R., da Costa,

- M.J.R.P., Sant'Anna, A.C., 2019. Consistency of temperament traits and their relationships with milk yield in lactating primiparous F1 Holstein - Gyr cows. *Appl. Anim. Behav. Sci.* 112061. <https://doi.org/https://doi.org/10.1016/j.applanim.2019.104881> This
- Macedo, G.G., Zúccari, C.E.S.N., de Abreu, U.G.P., Negrão, J.A., da Costa e Silva, E.V., 2011. Human-animal interaction, stress, and embryo production in *Bos indicus* embryo donors under tropical conditions. *Trop. Anim. Health Prod.* 43, 1175–1182. <https://doi.org/10.1007/s11250-011-9820-6>
- MacKay, J.R.D., Haskell, M.J., Deag, J.M., van Reenen, K., 2014. Fear responses to novelty in testing environments are related to day-to-day activity in the home environment in dairy cattle. *Appl. Anim. Behav. Sci.* <https://doi.org/10.1016/j.applanim.2013.12.008>
- Macuhová, J., Tancin, V., Kraetzl, W.D., Meyer, H.H.D., Bruckmaier, R.M., 2002. Inhibition of oxytocin release during repeated milking in unfamiliar surroundings: The importance of opioids and adrenal cortex sensitivity. *J. Dairy Res.* 69, 63–73. <https://doi.org/10.1017/S0022029901005222>
- Madalena, F.E., Peixoto, M.G.C.D.C.D., Gibson, J., 2012. Dairy cattle genetics and its applications in Brazil. *Livest. Res. Rural Dev.* 24, 1–49. <https://doi.org/http://www.lrrd.org/lrrd24/6/made24097.htm>
- May, T.M., Page, M.J., Fleming, P.A., 2016. Predicting survivors: Animal temperament and translocation. *Behav. Ecol.* 27, 969–977. <https://doi.org/10.1093/beheco/arv242>
- McInerney, J.P., 2004. Animal welfare, economics and policy. *J. R. Agric. Soc. Engl.* 165.
- Menezes, L.D.M., Cardoso, F.F., Barbosa Silveira, I.D., 2017. Temperamento em bovinos de corte: características genéticas, metodologias de mensuração e desempenho. *Rev. Electron. Vet.*
- Mohamed, R.A., Abou-Ismael, U.A., Shukry, M., Elmoslemany, A., Abdel-Maged, M., 2018. Effects of blindfolding and tail bending of Egyptian water buffaloes on their behavioural reactivity and physiological responses to pain induction. *Vet. Anim. Sci.* 5, 38–43. <https://doi.org/10.1016/j.vas.2018.06.001>
- Mota-Rojas, D., Broom, D.M., Orihuela, A., Velarde, A., Napolitano, F., Alonso-Spilsbury, M., 2020. Effects of human-animal relationship on animal productivity and welfare. *J. Anim. Behav. Biometeorol.* <https://doi.org/10.31893/JABB.20026>
- Müller, R., von Keyserlingk, M.A.G., 2006. Consistency of flight speed and its correlation to productivity and to personality in *Bos taurus* beef cattle. *Appl. Anim. Behav. Sci.* <https://doi.org/10.1016/j.applanim.2005.05.012>
- Munksgaard, L., DePassillé, A.M., Rushen, J., Herskin, M.S., Kristensen, A.M., 2001. Dairy cows' fear of people: Social learning, milk yield and behaviour at milking. *Appl. Anim. Behav. Sci.* 73, 15–26. [https://doi.org/10.1016/S0168-1591\(01\)00119-8](https://doi.org/10.1016/S0168-1591(01)00119-8)
- Negrão, J.A., 2008. Hormone release and behavior during suckling and milking in Gir, Gir x Holstein, and Holstein cows. *J. Anim. Sci.* 21–26. <https://doi.org/10.2527/jas.2007-0304>
- Negrão, J.A., Marnet, P.G., 2003. Cortisol, adrenalin, noradrenalin and oxytocin release and milk yield during first milkings in primiparous ewes. *Small Rumin. Res.* 47, 69–75. [https://doi.org/10.1016/S0921-4488\(02\)00247-X](https://doi.org/10.1016/S0921-4488(02)00247-X)
- Negrão, J.A., Marnet, P.G., 2002. Effect of calf suckling on oxytocin, prolactin, growth hormone and milk yield in crossbred Gir ´ Holstein cows during milking João. *Reprod. Nutr. Dev.* 43, 373–380. <https://doi.org/10.1051/rnd>
- Negrao, J.A., Porcionato, M.A. de F., de Passille, A.M., Rushen, J., 2010. Behavioural responses of heifers to ACTH injections. *Appl. Anim. Behav. Sci.* 128, 18–22. <https://doi.org/10.1016/j.applanim.2010.09.008>
- Néri, N.R.M., Toledo, L.M., Acaro Júnior, I., Ambrósio, L.A., 2016. Estimulação tátil em novilhas leiteiras: efeitos no comportamento e na produção de leite após o parto. *Bol. Indústria Anim.* 73,

- 171–179. <https://doi.org/10.17523/bia.v73n3p171>
- Odorčić, M., Rasmussen, M.D., Paulrud, C.O., Bruckmaier, R.M., 2019. Review: Milking machine settings, teat condition and milking efficiency in dairy cows. *Animal* 13, S94–S99. <https://doi.org/10.1017/S1751731119000417>
- Ouellet, V., Negrao, J., Skibieli, A.L., Lantigua, V.A., Fabris, T.F., Marrero, M.G., Dado-Senn, B., Laporta, J., Dahl, G.E., 2021. Endocrine signals altered by heat stress impact dairy cow mammary cellular processes at different stages of the dry period. *Animals* 11, 1–16. <https://doi.org/10.3390/ani11020563>
- Pajor, E.A., Rushen, J., De Passillé, A.M.B., Passille, A.M.B. De, 2000. Aversion learning techniques to evaluate dairy cattle handling practices. *Appl. Anim. Behav. Sci.* 69, 89–102. [https://doi.org/10.1016/S0168-1591\(00\)00119-2](https://doi.org/10.1016/S0168-1591(00)00119-2)
- Paul, H., Coleman, G.J., 2010. Human-livestock interactions: The stockperson and the productivity and welfare of intensively farmed animals, *Human-Livestock Interactions: The Stockperson and the Productivity and Welfare of Intensively Farmed Animals*. <https://doi.org/10.1079/9781845936730.0000>
- Peixoto, M.G.C.D., Pires, M. de F.Á., Pereira, M.C., Ribeiro, M.R.S.C.G.C., Brito, L.F., Verneque, R. da S., Bergmann, J.A.G., 2011. Integrando o temperamento às características de importância para o melhoramento de bovinos de leite : resultados de um estudo com fêmeas Guzerá. *Rev. Bras. Zootec.* 40, 26–37.
- Peters, M.D.P., Barbosa Silveira, I.D., Machado Filho, L.C.P., Machado, A.A., Pereira, L.M.R., 2010. Bem-Estar , Comportamento E Aspectos Produtivos Aversive Management in Dairy Cattle and Effects on Well-Being ,. *Arch. Zootec.* 59, 435–442.
- Petherick, J.C., 2005. Animal welfare issues associated with extensive livestock production: The northern Australian beef cattle industry. *Appl. Anim. Behav. Sci.* 92, 211–234. <https://doi.org/10.1016/j.applanim.2005.05.009>
- Phillips, C., 2002. Cattle Behaviour and Welfare, in: *Cattle Behaviour and Welfare: Second Edition*. <https://doi.org/10.1002/9780470752418>
- Polikarpus, A., Napolitano, F., Grasso, F., Di Palo, R., Zicarelli, F., Arney, D., De Rosa, G., 2014. Effect of pre-partum habituation to milking routine on behaviour and lactation performance of buffalo heifers. *Appl. Anim. Behav. Sci.* 161, 1–6. <https://doi.org/10.1016/j.applanim.2014.10.003>
- Ponchon, B., Zhao, X., Ollier, S., Lacasse, P., 2017. Relationship between glucocorticoids and prolactin during mammary gland stimulation in dairy cows. *J. Dairy Sci.* 100, 1521–1534. <https://doi.org/10.3168/jds.2016-11490>
- Porcionato, M.A.D.F., Negrão, J.A., De Paiva, F.A., Delgado, T.F.G., 2009. Respostas produtivas e comportamentais durante a ordenha de vacas Holandesas em início de lactação. *Acta Sci. - Anim. Sci.* 31, 447–451. <https://doi.org/10.4025/actascianimsci.v31i4.6326>
- Probst, J.K., Hillmann, E., Leiber, F., Kreuzer, M., Spengler Neff, A., 2013. Influence of gentle touching applied few weeks before slaughter on avoidance distance and slaughter stress in finishing cattle. *Appl. Anim. Behav. Sci.* 144, 14–21. <https://doi.org/10.1016/j.applanim.2012.12.007>
- Probst, J.K., Spengler Neff, A., Leiber, F., Kreuzer, M., Hillmann, E., 2012a. Gentle touching in early life reduces avoidance distance and slaughter stress in beef cattle. *Appl. Anim. Behav. Sci.* 139, 42–49. <https://doi.org/10.1016/j.applanim.2012.03.002>
- Probst, J.K., Spengler Neff, A., Leiber, F., Kreuzer, M., Hillmann, E., 2012b. Gentle touching in early life reduces avoidance distance and slaughter stress in beef cattle. *Appl. Anim. Behav. Sci.* <https://doi.org/10.1016/j.applanim.2012.03.002>
- Réale, D., Reader, S.M., Sol, D., McDougall, P.T., Dingemanse, N.J., Sol, D., RMcDougall, P.T., Eader, S.M., 2007. Integrating animal temperament within ecology and evolution. *Biol. Rev.* 82, 291–318. <https://doi.org/10.1111/j.1469-185X.2007.00010.x>

- Rushen, J., De Passillé, A.M.B., Munksgaard, L., 1999. Fear of people by cows and effects on milk yield, behavior, and heart rate at milking. *J. Dairy Sci.* 82, 720–727. [https://doi.org/10.3168/jds.S0022-0302\(99\)75289-6](https://doi.org/10.3168/jds.S0022-0302(99)75289-6)
- Rushen, J., Munksgaard, L., Marnet, P.G., DePassillé, A.M., 2001. Human contact and the effects of acute stress on cows at milking. *Appl. Anim. Behav. Sci.* 73, 1–14. [https://doi.org/10.1016/S0168-1591\(01\)00105-8](https://doi.org/10.1016/S0168-1591(01)00105-8)
- Sánchez-Rodríguez, H.L., Vann, R.C., Youngblood, R.C., Baravik-Munsell, E., Christiansen, D.L., Willard, S., Ryan, P.L., 2013. Evaluation of pulsatility index and diameter of the jugular vein and superficial body temperature as physiological indices of temperament in weaned beef calves: Relationship with serum cortisol concentrations, rectal temperature, and sex. *Livest. Sci.* <https://doi.org/10.1016/j.livsci.2012.10.019>
- Sanger, D.J., Joly, D., 1985. Anxiolytic drugs and the acquisition of conditioned fear in mice. *Psychopharmacology (Berl)*. <https://doi.org/10.1007/BF00428188>
- Sant’Anna, A.C., da Costa, M.J.R.P., 2013. Validity and feasibility of qualitative behavior assessment for the evaluation of Nellore cattle temperament. *Livest. Sci.* 157, 254–262. <https://doi.org/10.1016/j.livsci.2013.08.004>
- Sant’Anna, A.C., da Costa, M.J.R.P., Baldi, F., Rueda, P.M., Albuquerque, L.G., 2012. Genetic associations between flight speed and growth traits in nellore cattle. *J. Anim. Sci.* 90, 3427–3432. <https://doi.org/10.2527/jas.2011-5044>
- Sawa, A., Bogucki, M., Neja, W., Krężel-Czopek, S., 2017. Effect of temperament on performance of primiparous dairy cows. *Ann. Anim. Sci.* 17, 863–872. <https://doi.org/10.1515/aoas-2016-0085>
- Schmied, C., Boivin, X., Waiblinger, S., 2008. Stroking Different Body Regions of Dairy Cows: Effects on Avoidance and Approach Behavior Toward Humans. *J. Dairy Sci.* 91, 596–605. <https://doi.org/10.3168/jds.2007-0360>
- Shahin, M., 2018. The effects of positive human contact by tactile stimulation on dairy cows with different personalities. *Appl. Anim. Behav. Sci.* 204, 23–28. <https://doi.org/10.1016/j.applanim.2018.04.004>
- Silva, L.P., Sant’Anna, A.C., Silva, L.C.M., da Costa, M.J.R.P., 2017. Long-term effects of good handling practices during the pre-weaning period of crossbred dairy heifer calves. *Trop. Anim. Health Prod.* 49, 153–162. <https://doi.org/10.1007/s11250-016-1174-7>
- Soardi, K., Vilanova, M.S., Anami, J.M., Fernandes, R.C., 2017. EFEITO DA CATEGORIA ANIMAL NO COMPORTAMENTO EM SALA DE ORDENHA E NA PRODUÇÃO DE LEITE. *Rev. Científica Rural* 19, 184–194.
- Sutherland, M.A.A., Huddart, F.J.J., 2012. The effect of training first-lactation heifers to the milking parlor on the behavioral reactivity to humans and the physiological and behavioral responses to milking and productivity. *J. Dairy Sci.* 95, 6983–6993. <https://doi.org/10.3168/jds.2011-5211>
- Svennersten-Sjaunja, K., Olsson, K., 2005. Endocrinology of milk production. *Domest. Anim. Endocrinol.* 29, 241–258. <https://doi.org/10.1016/j.domaniend.2005.03.006>
- Tancin, V., Harcek, L., Broucek, J., Uhrincat, M., Mihina, S., 1995. Effect of Suckling During Early Lactation and Changeover to Machine Milking on Plasma Oxytocin and Cortisol Levels and Milking Characteristics in Holstein Cows. *J. Dairy Res.* 62, 249–256. <https://doi.org/10.1017/S0022029900030958>
- Tancin, V., Kraetzl, W.D., Schams, D., Bruckmaier, R.M., 2001. The effects of conditioning to suckling, milking and of calf presence on the release of oxytocin in dairy cows. *Appl. Anim. Behav. Sci.* 72, 235–246. [https://doi.org/10.1016/S0168-1591\(01\)00113-7](https://doi.org/10.1016/S0168-1591(01)00113-7)
- Tarantola, M., Biasato, I., Biasibetti, E., Biagini, D., Capra, P., Guarda, F., Leporati, M., Malfatto, V., Cavallarín, L., Miniscalco, B., Mioletti, S., Vincenti, M., Gastaldo, A., Capucchio, M.T., 2020. Beef cattle welfare assessment: use of resource and animal-based indicators, blood parameters and

- hair 20 β -dihydrocortisol. *Ital. J. Anim. Sci.* <https://doi.org/10.1080/1828051X.2020.1743783>
- Ujita, A., El Faro, L., Vicentini, R.R., Pereira Lima, M.L., de Oliveira Fernandes, L., Oliveira, A.P., Veroneze, R., Negrão, J.A., 2021. Effect of positive tactile stimulation and prepartum milking routine training on behavior, cortisol and oxytocin in milking, milk composition, and milk yield in Gyr cows in early lactation. *Appl. Anim. Behav. Sci.* <https://doi.org/10.1016/j.applanim.2020.105205>
- Ujita, A., Ribeiro Vicentini, R., Pereira Lima, M.L., Negrão, J.A., de Oliveira Fernandes, L., Penido Oliveira, A., Veroneze, R., El Faro Zadra, L., 2020. Improvements in the behaviour of Gir dairy cows after training with brushing. *J. Appl. Anim. Res.* <https://doi.org/10.1080/09712119.2020.1754217>
- Van Reenen, C.G., Engel, B., Ruis-Heutinck, L.F.M., Van Der Werf, J.T.N., Buist, W.G., Jones, R.B., Blokhuis, H.J., 2004. Behavioural reactivity of heifer calves in potentially alarming test situations: A multivariate and correlational analysis. *Appl. Anim. Behav. Sci.* <https://doi.org/10.1016/j.applanim.2003.09.007>
- Van Reenen, C.G., Van der Werf, J.T.N., Bruckmaier, R.M., Hopster, H., Engel, B., Noordhuizen, J.P.T.M., Blokhuis, H.J., 2002. Individual differences in behavioral and physiological responsiveness of primiparous dairy cows to machine milking. *J. Dairy Sci.* 85, 2551–61. [https://doi.org/10.3168/jds.S0022-0302\(02\)74338-5](https://doi.org/10.3168/jds.S0022-0302(02)74338-5)
- Vicentini, R.R., Montanholi, Y.R., Veroneze, R., Oliveira, A.P., Lima, M.L.P., Ujita, A., El Faro, L., 2020. Infrared thermography reveals surface body temperature changes during proestrus and estrus reproductive phases in Gyr heifers (*Bos taurus indicus*). *J. Therm. Biol.* 92, 102662. <https://doi.org/10.1016/j.jtherbio.2020.102662>
- Waiblinger, S., Boivin, X., Pedersen, V., Tosi, M.V., Janczak, A.M., Visser, E.K., Jones, R.B., 2006. Assessing the human-animal relationship in farmed species: A critical review. *Appl. Anim. Behav. Sci.* 101, 185–242. <https://doi.org/10.1016/j.applanim.2006.02.001>
- Wang, S., Zhang, H., Tian, H., Chen, X., Li, S., Lu, Y., Li, L., Wang, D., 2020. Alterations in vaginal temperature during the estrous cycle in dairy cows detected by a new intravaginal device—a pilot study. *Trop. Anim. Health Prod.* <https://doi.org/10.1007/s11250-020-02199-5>
- Weaver, S.R., Bohrer, J.C., Prichard, A.S., Perez, P.K., Streckenbach, L.J., Olson, J.M., Cook, M.E., Hernandez, L.L., 2016. Serotonin deficiency rescues lactation on day 1 in mice fed a high fat diet. *PLoS One* 11, 1–18. <https://doi.org/10.1371/journal.pone.0162432>
- Wicks, H.C.F., Carson, A.F., McCoy, M.A., Mayne, C.S., 2004. Effects of habituation to the milking parlour on the milk production and reproductive performance of first calving Holstein-Friesian and Norwegian dairy herd replacements. *Anim. Sci.* 78, 345–354.
- Windschnurer, I., Barth, K., Waiblinger, S., 2009. Can stroking during milking decrease avoidance distances of cows towards humans?, in: *Animal Welfare*.
- Wulff-Risner, L., Stewart, B., 1997. Using Experiential Learning To Teach Evaluation Skills. *J. Agric. Educ.* <https://doi.org/10.5032/jae.1997.03043>
- X Boivin, Lensink, J., Tallet, C., Veissier, I., 2003. Report on stockmanship and farm animal welfare [WWW Document]. *Anim. Welf.* URL <https://www.gov.uk/government/publications/fawc-report-on-stockmanship-and-farm-animal-welfare>
- Zliobaite, I., 2019. Revisiting biogeography of livestock animal domestication. *bioRxiv*. <https://doi.org/10.1101/786442>

2. EFFECT OF TRAINING MILKING ROUTINE ON BEHAVIOR, CORTISOL AND OXYTOCIN RELEASE, EXPRESSION OF KEY RECEPTORS OF MILK EJECTION AND MILK YIELD IN PRIMIPAROUS GYR COWS

ABSTRACT

Training dairy cows for routine milking can improve their behavior during milking sessions. The aim of this study was to evaluate the effect of prepartum training during the first 60 days of lactation of primiparous Gyr on milking behavior in addition to investigating the relationship between cortisol and oxytocin (CO and OT, respectively) in milk and expression of genes related to milk ejection and synthesis in mammary cells. Forty Gyr heifers were subjected to routine milking (TR) or control (CT) training sessions for 14 days, 40 days before the expected calving date. The training was performed in three progressive steps: (1) milking parlor entrance, (2) pre-milking, and (3) milking handling. Behavioral data were observed during the from parturition to day 60 of lactation: when the animal entered the milking parlor (stalled, needed help, and attempted to escape), during milking preparation (reaction to udder massage and to the strip cup test), and during milking (reaction to teat cups and whether the animal pulled off the teat cups). Milk yield, milk composition, CO and OT concentrations in milk were evaluated individually on days 5, 10, 15, 30, 45, and 60 of lactation, and gene expression analyses in mammary cells were performed on days 7, 15, 30, and 60 of lactation. At the first milking session, the percentage of restlessness was higher for CT than TR cows (24% versus 0%). In fact, training yielded a significant and positive effect on behavior measured in milking parlor entrance, pre-milking, and milking procedures in TR cows compared to CT cows. In the same way, TR showed that a higher percentage of trained cows were very calm, which was higher compared to control cows. However, the percentage of cows classified as very calm were similar for both treatments on days 45 and 60 of lactation. No treatment effects on milk yield and milk composition were observed; however, TR cows presented greater OT and lower CO levels and lower residual milk when compared to CT cows. Furthermore, the results also show that training caused an increase in expression of prolactin, oxytocin, and 5-hydroxytryptamine receptor genes (PRLR, OTR, and HTR1B, respectively) and caused a decrease in cortisol receptor (COR) gene expression in mammary cells. Finally, training routine milking significantly downregulated the expression of glucocorticoid receptors, upregulated the expression of PRLR, OTR and HTR1B, and led to an improvement in Gyr behavior and milking behavior score and a reduction in residual milk.

Keywords: behavior, cortisol, mammary cells, milking, oxytocin, receptors.

2.1. INTRODUCTION

In several countries, heifers are kept in pasture-based production facilities, have few positive interactions with humans, and do not have access to housing facilities (Macedo et al., 2011; Pajor et al., 2000; Petherick, 2005). Furthermore, primiparous cows are exposed to several stressors during the transition period, when they are introduced a new social group and subject to milking facilities and routines (Kutzer et al., 2015; Polikarpus et al., 2014; Ujita et al., 2020). Thus, in this new environment, primiparous cows need to interact several times a day with their handlers and milkers (Porcionato et al., 2009; Ujita et al., 2020). Consequently, Gyr cows are afraid of this new environment and show flight or threatening behavior when entering the milking parlor and aggressive behavior with the milkers during the first milking session (Ujita et al., 2020). These stressful situations also affect the hypothalamic–hypophysis–adrenal axis and cause the release of adrenaline, noradrenaline, and cortisol (AD, NOR, and CO, respectively), hormones that concurrently play a role in inhibition of milk ejection by these cows (Bruckmaier and Blum, 1998; Negrão, 2008; Odorčić et al., 2019).

Indeed, negative behavior during milking sessions are associated with inadequate oxytocin (OT) release and milk ejection (El. Abdel-Hamid et al., 2017). On one hand, inefficient milk ejection produces changes in milk synthesis due accumulation of the feedback inhibitor of lactation (FIL) in the alveoli, and serotonin is one of FIL that influence mammary synthesis and milk production in dairy cows (Collier et al., 2012; Connelly et al., 2021; Weaver et al., 2016). Although local actions of OT, CO, AD, NOR, and serotonin (SER) are regulated by presence of their receptors in different target tissues, the interactions between these hormones and their receptors in mammary gland (Collier et al., 2012; Inderwies et al., 2003; Weaver et al., 2016) are not clear. On the other hand, the first milking session did not induce effective OT release and milk ejection; however, significant OT release was measured just 2–3 weeks after the start of milking (Inderwies et al., 2003; Macuhová et al., 2002; Negrão, 2008; Odorčić et al., 2019).

Furthermore, zebu cows reputed as hard to milk have been exposed to a suckling–milking system to improve milk ejection (Negrão, 2008; Sant’Anna et al., 2012; Ujita et al., 2020). Although, suckling–milking stimulation causes an increase in dairy cow milk yield (Alvarez et al., 1980; Bar-Pelled et al., 1995; Negrão, 2008), suckling–milking management is a cumbersome and labor-intense system, and the time spent to milk large herds is a trouble. For this reason, many farmers use exogenous OT to improve milk ejection of Gyr cows exclusively during milking (Negrão, 2008). In this context, different strategies have been proposed with the

aim of training dairy cows and facilitating milk ejection during the first milking. Previously, we have demonstrated that positive handling and milking routine training will cause an improvement in behavior and OT release and a reduction in residual milk (Ujita et al., 2021).

In this context, our hypothesis stated that training will change the responsiveness of the mammary gland and improve the milk ejection and milking behavior in primiparous Gyr cows. Although OT, prolactin and serotonin are secreted by neurons and its receptor are described in brain and related to social, sexual, and maternal behavior, we studied the training routine milking on expression of oxytocin prolactin, serotonin and catecholamines receptors in mammary cells. For this reason, the objective of this study was to investigate training effect on milking behavior, OT and CORT release, expression of oxytocin glucocorticoid, and mineralocorticoid receptors (OTR, GR, and MR, respectively) in addition to alpha-adrenergic ($ADR\alpha1A$, $ADR\alpha2AD$, $ADB\beta2$) and serotonin (HTR1B, HTR2A, HTR2B, HTR4, HTR7) receptors in the mammary gland and their relationship with milk yield and milk ejection.

2.2. MATERIALS AND METHODS

2.2.1. Animals and daily management

All animal procedures complied with the Brazilian laws and ethical codes (Approval No. 230-16) of the Getúlio Vargas Experimental Station of Empresa de Pesquisa Agropecuária de Minas Gerais (EPAMIG), located in Uberaba, MG (19° 44' 54" S latitude, 47° 55' 55" W longitude, altitude of 801 m). According to the Köppen classification, the climate in this region is subtropical (*Cwa) with warm, rainy summers and relatively dry winters.

The 40 experimental Gyr heifers were kept on *Urochloa brizanta* grass in a rotational stocking with free access to water and supplemented with a vitamin and mineral mix. The pasture management maintained the pasture's ideal height, and during the dry season, the cows receive corn silage (32 kg of green forage/cow/day) and concentrate (1 kg of concentrate for each 4 kg of milk produced). Furthermore, lactating cows that produce over 10 kg/day receive 1 kg of concentrate for each 4 kg of milk produced.

During the last 60 days before expected date of parturition, all heifers remained in the paddock near the milking facilities. Colostrum suckling by calves was allowed, and all cows were subjected to milking–suckling management twice daily (at 5 and 17 h) in a tandem parlor with 12 sets for cows and calves. During all milking sessions, the calves suckled the anterior

teats of their respective mothers. After each milking, the calves were separated and brought to the collective calf housing facility in which calves had free access to water, silage, and mixed grains; the leftovers were observed but not weighed. All calves underwent the same management and standard procedures as indicated for their age, and calves were monitored from birth to weaning at the end of lactation.

The milking routine was performed in accordance with a specific protocol: (1) the experimental cows were conducted to the milking facilities, (2) the cows entered the parlor in tandem, (3) the calves entered the parlor in tandem with their mothers, (4) the strip cup test was performed, and (5) afterwards, milkers cleaned and pre-dipped the teats before starting the milking machine. During mechanical milking of all experimental cows, their respective calves had access to the mother cow to suckle one of the anterior teats. After milking, the calves were separated and brought to the collective calf house. Post-dipping of the teats was then performed. The lactating cows were released from the milking parlor and brought to their respective pasture paddocks. All experimental procedures were performed by a trained team (handling and milkers).

2.2.2. Treatments

Forty healthy heifers were randomly assigned to one of two treatments: (1) milking routine training (TR, $n = 20$) and (2) control (CT, $n = 20$) during the prepartum period, according to body weight (BW), body condition score (BCS) BW, and previous milk yield of their mothers.

The treatment was initiated 40 days before the expected calving date and was maintained for 14 consecutive days. At 10 h, the heifers from the TR group were trained to enter the milking parlor and progressively submit to tactile stimulation (by brushes and hands) in the milking parlor after which the heifers were trained with respect to the milking procedures. The heifers from the CT group did not receive any type of training, tactile stimuli or training and remained in the paddock near the milking facilities without human interaction until calving except during feeding.

For 14 consecutive days, the TR group was handled in three stages in the milking parlor by trained milkers:

- 1) Phase 1 (days 1–5), passage of the heifers through the milking parlor; the TR group was conducted into the operating milking parlor in which heifers entered the milking aisle and were released to the exit. A multiparous cow was included in group to facilitate animal

handling,

- 2) Phase 2 (days 6–10), tactile stimulation of heifers in the milking parlor; in addition to the procedures described for phase 1, one side of the body of each animal was brushed for 2 min, emphasizing mainly the region of the udder and hind legs, and
- 3) Phase 3 (days 11–14): training of heifer to milking procedures: in addition to the procedures described for phases 1 and 2, the hind legs of the heifers were restrained, and a pre-dipping was performed (i.e., asepsis of the teats by immersion in antiseptic solution and drying).

Throughout these 14 days, the side on which the heifers entered the milking parlor was altered; on one day the heifers entered on the left side, and on the next day, they entered on the right side.

2.2.3. Behavior scores attributed during milking routine.

After parturition, cow behavior after TR and CT treatments were measured during morning milking on days 1, 7, 15, 30, 45, and 60 of lactation. Observations were performed by one trained observer who was responsible for up to two animals. All data were recorded in real-time and scored according to the definitions described in Table 1. Briefly, several behavioral parameters were observed and scored: (1) during entry into the milking parlor (balked, needed help, and attempted to escape), (2) during preparation for milking (response to tying the hind legs and the strip cup test), and (3) during milking (reactivity to the teat cups, milking behavior, and whether the animal pulled off the teat cups).

2.2.3.1. Overall milking behavior score

Overall milking behavior score analyses of Gyr cows were performed according to the score of the behavior evaluated during the milking as described in Table 1. In fact, cows' milking behavior scores were calculated by taking in account the sum of behavior scores measured during milking routine handlings. The milking behavior score of each cow was divided into five categories: (1) very calm when < 16 , (2) calm when ≥ 16 and < 20 , (3) adequate when ≥ 20 and < 24 , (4) restless ≥ 24 and < 28 , and (5) very restless when ≥ 28 . Overall milking behavior score were calculated for each experimental cow on days 1, 7, 15, 30, 45, and 60 of lactation.

Table 1. Description of the behavioral parameters evaluated during the milking procedure and their corresponding scores.

PARAMETERS ¹	DESCRIPTION	SCORE ²				
		1	2	3	4	5
Balk	Balked to enter the parlor	No	Yes, because of being tame (calm, walking slowly)	Yes, because of fear (desperate and suspicious)	-	-
Help	Needed help to enter the parlor	No	Voice command	Patting	Poking the animal one time with a tool	Poking the animal two or more times with a tool
Escape	If there was an attempt to escape	No	Yes	-	-	-
Tying	Reaction to the placement and use of a restraint	Did not react	Low, no kicking	Low, kicking	High, no kicking	High, kicking
Strip	Reaction to the strip cup test	Did not react	Low, no kicking	Low, kicking	High, no kicking	High, kicking
Teat cups	Reaction to teat cup attachment or detachment	Did not react	Low, no kicking	Low, kicking	High, no kicking	High, kicking
Milking	Behavior during milking	Calm	Median	Restless	-	-
Removal of teat cups	If the cow pulled the teat cups off the teats	No	Yes	-	-	-

¹Adapted from Lima et al., 2018; Maçal-Pedroza et al., 2019; Ujita et al., 2020.

²A cow with a lower score was considered easy to milk, and cow with a high score was considered the most difficult to milk.

2.2.4. Milk yield

Individual milk yield was recorded daily, and individual samples for milk quality were taken on days 5, 10, 15, 30, 45, and 60 of lactation. As their respective calves suckled the right anterior teats during all milking, milk yield was corrected for the four teats. On days 15, 30, 45, and 60 of lactation, the effectiveness of milk ejection was evaluated by measuring the residual milk of all experimental cows. In this case, after the usual morning milking, the residual milk measurement was performed by intramuscular administration of 2 mL of exogenous OT (10 IU/mL) and another milking of four teats 10 min after OT administration. The resulting residual milk volume was transformed into a percentage in relation to the measured milk at the usual morning milking.

2.2.5. Milk analysis

Milk samples were taken from the milk collector after the morning milking for fat, protein, lactose, somatic cell count, CO, and OT analyses. The somatic cell count (SCC) in the milk was determined via a direct method (using Carnoy solution and methyl green pyronin) and counted under an optical microscope (Raynal-Ljutovac et al., 2007). The percentages of fat, protein, and lactose in milk were measured using an Ultrason (MilkoScope Expert Analyzer, Razgrad, Bulgaria). The SCC was taken as the average of two fields containing mammary cells and leukocytes and then multiplied by the microscope factor. The SCC was determined using 10 μ L of milk by counting the cells on the microscope slide, multiplying their number by the dilution factor, and converting the result into a log scale. CO and OT in milk were analyzed using an enzyme immunoassay assay (EIA) method according to the specifications of the manufacturers (cortisol: Monobind, Lake Forest, CA, USA; oxytocin: Mybiosource, San Diego, CA, USA).

2.2.6. Gene expression

A part of the total milk sample obtained during the morning milking was taken to obtain mammary cell and RNA expression. The selected method was based on previous studies that showed that isolation of mammary epithelial cells from milk is a non-invasive technique for studying the gene expression in mammary glands (Boutinaud et al., 2015; Toral et al., 2016). Briefly, the separation of epithelial cells from other milk cells was performed by centrifugation at low speeds to pellet the milk cells after which washing, suspending, and immunomagnetic separation using specific antibodies to separate of mammary cells from other milk cells were

performed. Sequential washings, suspensions, and centrifugations to pellet the purified mammary cells were performed.

The RNA of these samples was extracted and purified using a PureLink RNA Mini Kit (Invitrogen, Burlington, ON, Canada). The quality of each RNA sample was evaluated based on the optical density at 260 and 280 nm absorbance wavelengths, and the 260/280 absorption ratio was approximately 2. RNA concentrations were determined using Qubit 2.0 Fluorometric Quantification (Thermo Fisher Scientific, Waltham, MA). Total RNA samples were treated with RNase-Free DNase (Promega, Madison, WI) to exclude genomic DNA contamination from the analysis. The RNA was reverse transcribed into cDNA using the GoScript Reverse Transcriptase kit (Promega). The expression of target genes was determined using reverse transcriptase polymerase chain reaction (RT-PCR) equipment (Invitrogen) with primers for GR, MR, OXTR, PRLR, HTR1B, HTR2A, HTR2B, HTR4, HTR7, ADR α 1A, ADR α 2AD, ADB β 2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the housekeeping gene described in Table 1. These genes were amplified in a separate reaction, and each reaction was performed in duplicate. Each reaction was performed with a 20 μ L reaction mixture composed of 1 μ L of cDNA sample (mean concentration, 10 ng/ μ L), 10 μ L of SYBR Green (Invitrogen), 0.8 μ L of the primer pairs (final concentration, 0.4 μ M), and 8.2 μ L of ultrapure water. The thermocycling conditions for all genes were set for specific steps: (1) incubation stage at 95 °C for 10 min, (2) 40 cycles of 95 °C for 15 s, and (3) 60 °C for 1 min. The treatment was finished with establishment of a dissociation (melting) curve. For each set of primers, the PCR efficiency was close to 100%. RT-PCR efficiency was confirmed for all selected primer pairs, and specific products were verified using melt curve analysis and 1.5% agarose gel electrophoresis. Gene expression of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase (YWHAZ), ubiquitin C (UBC), and GAPDH were determined in mammary cells samples to identify the most stable reference gene. The YWHAZ gene was not expressed, and the UBC gene showed a high cycle threshold variability among samples. The GAPDH gene was identified as the most stably expressed and was then selected as the reference gene. The expression of target genes was calculated by Livak's method (Livak and Schmittgen, 2001) by comparing the expression levels of target genes with the reference (GAPDH) and the cycle threshold of the control at each specific point (Schmittgen and Livak, 2008).

2.2.7. Statistical analysis

Initially, simple descriptive statistics were applied using the FREQ and MEANS

procedures (SAS Institute, Inc., Cary, NC, USA), and the analysis of variance were performed using the MIXED procedure (SAS Institute, Inc., Cary, NC, USA) in a completely randomized design. The effects of treatment (milking training or control) were included as a fixed effect, and data were evaluated as repeated measures over time (represented by days of lactation). The covariance matrices were tested for each trait and the structure showing the best fit was used (Crowder and Hand, 1990). Gene expression of training cows was calculated as fold change relative to the placebo cows ($\Delta\Delta Ct = \Delta Ct \text{ training} - \Delta Ct \text{ control}$). Each animal was considered an experimental unit. Means were estimated using the LSMeans procedure. When significant differences were found ($P \leq 0.05$), means were compared using the Student's t-test at a level of significance of 5%.

2.3. RESULTS

2.3.1. Behavior

Training had a significant and positive effect on behavior score as measured in the milking parlor entrance for primiparous cows (Table 2), and the lower score showed that TR cows balked less, did not try to escape, and needed less help to access the milking parlor when compared with CT cows. A significant interaction between treatment and lactation days on behavior score was measured during pre-milking and milking procedures (Table 3), and TR cows were significantly less reactive to their milkers than CT cows during the first lactation week (Table 3).

Table 2. Behavior score (means \pm standard error of the mean [sem]) observed at the milking parlor entrance and during pre-milking and milking procedures of Gyr cows subject to training (TR) or control (CT) treatments from 3 to 60 days of lactation. Data are presented as means \pm sem.

TRAIT	TRAINING (N = 120)	CONTROL (N = 120)	SEM	P VALUE		
				TREAT	DAY	TREAT- DAY
Milking parlor Entrance	3.3 ^b	4.0 ^a	0.11	0.01	0.01	0.01
Pré-milking Procedures	2.9	2.8	0.13	0.58	0.01	0.03
Milking procedures	3.4	3.2	0.10	0.21	0.89	0.02

^{a,b} Means within a row with different superscripts differ ($P \leq 0.05$).

Table 3. Behavior score (means \pm sem) observed at the milking parlor entrance and pre-milking and milking procedures on days 3, 7, 15, 30, 45, and 60 of lactations.

BEHAVIOR SCORE	DAY	TRAINING (N=20)	CONTROL (N=20)	SEM	VALOR P TREAT-DAY
Milking parlor entrance	3	3.6 ^a	6.8 ^b	0.28	0.01
	7	3.3 ^a	3.9 ^b		
	15	3.4	3.5		
	30	3.2	3.4		
	45	3.1	3.5		
	60	2.9	3.1		
Pre-milking procedures	3	3.5 ^a	5.2 ^a	0.35	0.03
	7	2.9	2.6		
	15	2.7	3.0		
	30	2.6	2.2		
	45	2.4	2.3		
	60	2.8	2.4		
Milking procedures	3	2.8 ^a	4.2 ^b	0.27	0.02
	7	3.4	3.3		
	15	3.5	3.3		
	30	3.6	3.1		
	45	3.5	3.2		
	60	3.0	3.1		

^{a,b} Means within a row with different superscripts differ ($P \leq 0.05$). TREAT: treatment

Furthermore, a significant interaction between treatment and lactation day on overall milking behavior score of Gyr cows was found (Table 4). In fact, on day 3 of lactation, CT cows presented a significantly greater percentage of restlessness than TR cows (24% versus 0%). In the same way, during the first lactation week a higher percentage of TR cows could be classified as very calm or calm when compared with CT cows (Table 4). However, the percentage of cows classified as very calm was similar for both treatments on days 45 and 60 of lactation (Table 4).

Table 4. Effect of training of Gyr cows on milking behavior score during milking on days 3, 7, 15, 30, 45, and 60 of lactation.

DAY	OVERALL MILKING BEHAVIOR	CONTROL (N = 20)	TRAINING (N = 20)	P-VALUE TREAT-DAY
3	Very calm (%)	14 ^a	94 ^b	0.01
	Calm (%)	43 ^a	6 ^b	
	Adequate (%)	19 ^a	0 ^b	
	Restless (%)	24 ^a	0 ^b	
	Very restless (%)	-	-	
7	Very calm (%)	81	88.9	0.05
	Calm (%)	14	11.1	
	Adequate (%)	5 ^a	0 ^b	
	Restless (%)	-	-	
	Very restless (%)	-	-	
15	Very calm (%)	90	89	0.01
	Calm (%)	5 ^a	11 ^b	
	Adequate (%)	5 ^a	0 ^b	
	Restless (%)	-	-	
	Very restless (%)	-	-	
30	Very calm (%)	95	94	0.01
	Calm (%)	5 ^a	0 ^b	
	Adequate (%)	0 ^a	6 ^b	
	Restless (%)	-	-	
	Very restless (%)	-	-	
45	Very calm (%)	95	90	0.10
	Calm (%)	5 ^a	10 ^b	
	Adequate (%)	-	-	
	Restless (%)	-	-	
	Very restless (%)	-	-	
60	Very calm (%)	90	94	0.29
	Calm (%)	10 ^a	5 ^b	
	Adequate (%)	-	-	
	Restless (%)	-	-	
	Very restless (%)	-	-	

^{a,b} Means within a row with different superscripts differ ($P \leq 0.05$).

2.3.2. Milk yield, hormones in milk, and gene expression

No treatment effects were found on milk yield and milk composition (Table 5). However, TR cows presented lower residual milk values than CT cows (Table 5). Indeed, TR cows presented significantly greater OT and lower CO concentrations in milk when compared with CT cows (Table 5). In mammary cells, training had a significant effect on expression of PRLR gene expression with higher gene expression for TR cows than CT cows (Table 6). At the same time, there was interaction between treatment and lactation days for GR, OXTR and HTR1B genes (Figure 1). In fact, OXTR and HTR1B genes were significantly higher for TR cows in first week of lactation when compared to CT cows (Figure 1). Finally, during all

experimental days the expression of GR gene was significant lower to TR than those measured to MT cows (Figure 1).

Table 5. Milk yield, residual milk, protein, fat, and lactose percentage, somatic cell count (SCC), of Gyr cows subject to TR or CT treatments from 3 to 60 days of lactation. Data are presented as means \pm sem.

TRAIT	CONTROL (N = 20)	TRAINING (N = 20)	SEM	P VALUE		
				TREAT	DAY	T*D
Milk yield (kg)	10.33	12.76	0.50	0.15	0.01	0.40
Residual Milk (%) ¹	16.04	8.50	2.20	0.02	0.60	0.80
Protein (%)	3.46	3.47	0.06	0.53	0.01	0.79
Fat (%)	4.55	4.62	0.11	0.81	0.01	0.86
Lactose (%)	4.74	4.84	0.05	0.43	0.01	0.83
Somatic cell count (cells/mL) ²	222,920	232,133	18,070	0.23	0.01	0.26
Cortisol (ng/mL)	24.10 ^a	16.23 ^b	1.02	0.01	0.36	0.11
Oxytocin (pg/mL)	19.39 ^a	27.54 ^b	1.15	0.01	0.17	0.57

¹The residual milk was measured after OT administration and calculated as a percentage of milk measured after morning milking; ²Values $\times 10^3$ /mL. SCC were presented as the average of teats measured at morning milking. ^{a,b} Means within a row with different superscripts differ ($P \leq 0.05$).

Table 6. Effects of treatment on oxytocin and cortisol concentration in milk and gene expression in mammary cells.

TRAIT	CONTROL (N = 20)	TRAINING (N = 20)	SEM	P VALUE		
				TREAT	DAY	T*D
GR (mRNA)	8.17	5.92	1.08	0.09	0.18	0.04
OXTR (mRNA)	2.04	2.18	0.11	0.26	0.01	0.01
PRLR (mRNA)	2.14 ^a	4.04 ^b	0.22	0.01	0.56	0.31
MR (mRNA)	1.88	0.55	0.54	0.34	0.80	0.87
HTR1B (mRNA)	1.02 ^a	3.94 ^b	0.44	0.01	0.01	0.01
HTR2A (mRNA) ¹	-	-	-	-	-	-
HTR2B (mRNA)	1.00	1.28	0.56	0.79	0.86	0.81
HTR4 (mRNA) ¹	-	-	-	-	-	-
HTR7 (mRNA)	1.00	1.05	0.32	0.93	0.28	0.98
ADRA1A (mRNA) ¹	-	-	-	-	-	-
ADRA2AD (mRNA)	1.01	0.34	0.20	0.10	0.52	0.74
ADBB2 (mRNA)	1.02	2.59	0.73	0.24	0.59	0.32

¹For both treatments, the expression of HTR2A, HTR4 and ADRA1A genes in mammary cells was insufficient for statistical analysis; ^{a,b} Means within a row with different superscripts differ ($P \leq 0.05$).

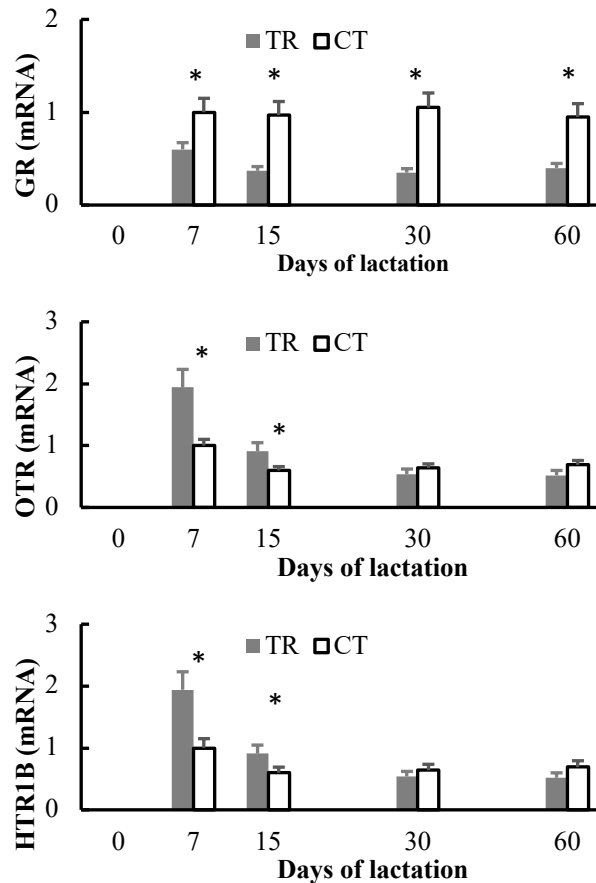


Figure 1. Gene expression (mRNA. $2^{-\Delta\Delta CT}$ method) of Gyr cows subjected to training (TR) or control treatments (CT). Data are presented as means \pm standard error of mean. Means within days with * differ ($P \leq 0.05$).

2.4. DISCUSSION

The first milking sessions are stressful for both cows and milkers as primiparous dairy cows are more difficult to milk than multiparous cows (Negrão, 2008; Odorčić et al., 2019; Ujita et al., 2020); therefore, it can be argued that training for the milking routine during late gestation may facilitate milking management during lactation. Furthermore, Gyr cows are reputed not well adapted to milk and subjected to OT administration to avoid incomplete milk removal during all lactation (Negrão, 2008; Negrão and Marnet, 2002). For these reasons, introducing farm management practices, such as training cows for routine milking can facilitate cow adaptation and welfare during subsequent lactation periods (Cavallina et al., 2008; Kutzer et al., 2015; Ujita et al., 2020). Ours results confirm that training could cause an improvement in cow behavior at the milk parlor and during pre-milking and milking handlings during first week of lactation as shown in previous studies (Polikarpus et al., 2014; Ujita et al., 2020). Indeed, the higher percentage of TR cows classified as very calm and calm when compared to

CT cows confirmed that training during late gestation could facilitate the milking process at the start of lactation. As demonstrated in the present study, training for the routine milking led to an improvement in the milking behavior of Gyr primiparous cows, and training during late gestation can be a boost to facilitate the milking without the use of daily OT administration.

Furthermore, the first milking sessions are associated to stress, higher CO release, higher percentage of residual milk, and lower milk yield (Negrao, 2008; Odorčić et al., 2019; Rushen et al., 1999). Although CO is considered the main indicator of stress, some authors have argued that CO action in different tissues is regulated by glucocorticoid receptor (GR) in target cells (Bertucci et al., 2010; Chida et al., 2011). In our study, training cows for the routine milking caused a significant decrease in CO levels in milk and the expression of GR in mammary cells. Together, our results also suggest that training has a positive effect on milk ejection thus reducing the percentage of residual milk TR cows when compared to CT cows. For these reasons, it is possible argue that training caused positive improvements in the relationship between CO–GR and milking effectiveness in primiparous Gyr cows.

Classical suckling and milking induce OT release and promote contractions of myoepithelial cells surrounding mammary alveolus and small ducts causing milk ejection and its accumulation in large ducts, cisterns of udder, and teat facilitating the milking (Bruckmaier and Blum, 1998; Negrao, 2008; Odorčić et al., 2019). This dynamic process is fundamental to maintaining milk synthesis because alveolar emptying via OT action avoids the increase caused by chemical inhibitors (considered the negative feedback of milk synthesis, FIL) due a long interruption of suckling or milking (Collier et al., 2012; Negrao, 2008). On the other hand, the biological effectiveness of OT release by milking depends on the presence of its receptor on mammary cells (Bruckmaier and Blum, 1998; Odorčić et al., 2019). In fact, our results concerning residual milk and expression of the OTR gene demonstrated that training has a positive effect on milk ejection because we can argue that a lower percentage of residual milk in cows subject to training was due a better relationship between OT release in milk and the OTRs on mammary cells during early lactation. However, further studies are necessary to understand how the relationship between OT and its receptor modulates the molecular pathways of milk ejection.

By other side, PRL is fundamental to start milk synthesis in transition period and daily milkings induces PRL release and maintain milk synthesis (Negrao, 2008; Ponchon et al., 2017). However, PRL release decrease during the lactation consequently its action on milk synthesis depends on the presence of its receptor in mammary cells (Hooper et al., 2020). Other

authors have argued that different environmental factors may change the presence of PRLR on mammary gland during the lactation (Lacasse et al., 2016; Ponchon et al., 2017). As our results showed, training cows for routine milking led to a significant increase in PRLR gene expression in mammary cells when compared to CT cows; it is possible to argue that training improves the responsiveness of this receptor on mammary cells. In contrast, this enhanced expression of the PRLR gene did not increase milk yield during our study; in fact, our results underscore the importance of studying PRL responsiveness in mammary glands.

Other authors have reported that an effective milk ejection and milk removal caused by suckling or milking are also related to lower HT concentrations in alveolar milk, and a lower concentration of this hormone involved in FIL in the alveolus was associated with copious milk synthesis (Collier et al., 2012; Connelly et al., 2021; Weaver et al., 2016). Although in our study a training effect on expression of HTB1 during the first lactation week was observed, the milk yield was not affected by training. Consequently, in our study we cannot speculate on the interaction of training with HTB1 and milk synthesis. Milking characteristics in ruminants are also associated with catecholamine release and milk flow rate and can be associated with AD, NOR, and ADR in mammary gland (Inderwies et al., 2003; Macuhová et al., 2002; Odorčić et al., 2019). Furthermore, the connection between these catecholamines and α - and β -adrenergic receptors were related to contraction or relaxation of myoepithelial cells and the smooth muscles of mammary gland (Inderwies et al., 2003; Odorčić et al., 2019). Although the present study demonstrated a decrease in the percentage of residual milk after training, no treatment effects on expression of $ADR\alpha2AD$ and $ADB\beta2$ genes in mammary cells were found. Consequently, we cannot speculate on the interaction of residual milk with the expression of adrenergic receptors genes in mammary cells.

Finally, our study showed that training yielded a significant decrease in CO concentration in milk, led to a downregulation of tGR expression, increase in OT concentration in milk, and upregulation of OTR and PRLR expression in mammary tissue compared to the CT. Indeed, training cows for routine milking produced significant improved behavior scores, milking behavior scores, and milk ejections of TR cows when compared to CT cows. Further studies are necessary to elucidate the action of training on molecular pathways of milk ejection in cows.

ACKNOWLEDGMENTS

This work was financed by the São Paulo Research Foundation (FAPESP Sao Paulo, SP, Brazil, grants 2015/24.174-3, 2016/00123-3) and Minas Gerais Agriculture Research Institute (EPAMIG); postgraduate students were funded by Coordination for the Improvement of Greater Education Personnel (CAPES, Brasilia, DF, Brazil) or National Counsel of Technological and Scientific Development (CNPq, Brasilia, DF, Brazil). Was supported by the CNPq. The authors declare no conflict of interest.

REFERENCES

- Alvarez, F.J., Saucedo, G., Arriaga, A., Preston, T.R., 1980. Effect on Milk Production and Calf Performance of Milking Cross Bred European / Zebu Cattle in the Absence or Presence of. *Trop. Anim. Prod.* 5, 25–37.
- Bar-Pelled, U., Maltz, E., Bruckental, I., Folman, Y., Kali, Y., Gacitua, H., Lehrer, A.R., Knight, C.H., Robinson, B., Voet, H., Tagari, H., 1995. Relationship Between Frequent Milking or Suckling in Early Lactation and Milk Production of High Producing Dairy Cows. *J. Dairy Sci.* 78, 2726–2736. [https://doi.org/10.3168/jds.S0022-0302\(95\)76903-X](https://doi.org/10.3168/jds.S0022-0302(95)76903-X)
- Bertucci, P.Y., Quaglino, A., Pozzi, A.G., Kordon, E.C., Pecci, A., 2010. Glucocorticoid-induced impairment of mammary gland involution is associated with STAT5 and STAT3 signaling modulation. *Endocrinology* 151, 5730–5740. <https://doi.org/10.1210/en.2010-0517>
- Bruckmaier, R.M.M., Blum, J.W.W., 1998. Oxytocin Release and Milk Removal in Ruminants. *J. Dairy Sci.* 81, 939–949. [https://doi.org/10.3168/jds.S0022-0302\(98\)75654-1](https://doi.org/10.3168/jds.S0022-0302(98)75654-1)
- Cavallina, R., Roncoroni, C., Campagna, M.C., Minero, M., Canali, E., 2008. Buffalo behavioural response to machine milking in early lactation. *Ital. J. Anim. Sci.* 7, 287–295. <https://doi.org/10.4081/ijas.2008.287>
- Chida, D., Miyoshi, K., Sato, T., Yoda, T., Kikusui, T., Iwakura, Y., 2011. The role of glucocorticoids in pregnancy, parturition, lactation, and nurturing in melanocortin receptor 2-deficient mice. *Endocrinology* 152, 1652–1660. <https://doi.org/10.1210/en.2010-0935>
- Collier, R.J., Hernandez, L.L., Horseman, N.D., 2012. Serotonin as a homeostatic regulator of lactation. *Domest. Anim. Endocrinol.* 43, 161–170. <https://doi.org/10.1016/j.domaniend.2012.03.006>
- Connelly, M.K., Weaver, S.R., Kuehnl, J.M., Fricke, H.P., Klister, M., Hernandez, L., 2021. Elevated serotonin coordinates mammary metabolism in dairy cows. *Physiol. Rep.* 9, 1–12. <https://doi.org/10.14814/phy2.14798>
- Crowder, M.J., Hand, D.J., 1990. Analysis of Repeated Measures. Chapman and Hall, London.
- El. Abdel-Hamid, S., Fattah, D.M.A., Ghanem, H.M., Manaa, E.A.A., 2017. Temperament during milking process and its effect on behavioral, productive traits and biochemical parameters in Friesian dairy cows. *Adv. Anim. Vet. Sci.* 5, 508–513. <https://doi.org/10.17582/journal.aavs/2017/5.12.508.513>
- Hooper, H.B., Silva, P. dos S., de Oliveira, S.A., Meringhe, G.K.F., Lacasse, P., Negrão, J.A., 2020. Effect of heat stress in late gestation on subsequent lactation performance and mammary cell gene expression of Saanen goats. *J. Dairy Sci.* 103, 1982–1992. <https://doi.org/10.3168/jds.2019-16734>
- Inderwies, T., Pfaffl, M.W., Bruckmaier, R.M., 2003. Milking characteristics and their relation to adrenergic receptor mRNA expression and ligand binding in the mammary gland of dairy cows. *Domest. Anim. Endocrinol.* 25, 275–286. [https://doi.org/10.1016/S0739-7240\(03\)00059-6](https://doi.org/10.1016/S0739-7240(03)00059-6)
- Kutzer, T., Steilen, M., Gygax, L., Wechsler, B., 2015. Habituation of dairy heifers to milking routine- Effects on human avoidance distance, behavior, and cardiac activity during milking. *J. Dairy Sci.* 98, 5241–5251. <https://doi.org/10.3168/jds.2014-8773>
- Lacasse, P., Ollier, S., Lollivier, V., Boutinaud, M., 2016. New insights into the importance of prolactin in dairy ruminants. *J. Dairy Sci.* 99, 864–874. <https://doi.org/10.3168/jds.2015-10035>
- Lima, M.L.P., Negrão, J.A., de Paz, C.C.P., Grandin, T., 2018. Minor corral changes and adoption of good handling practices can improve the behavior and reduce cortisol release in Nellore cows. *Trop. Anim. Health Prod.* 50, 525–530. <https://doi.org/10.1007/s11250-017-1463-9>
- Maçal-Pedroza, M.G., Campos, M.M., Pereira, L.G.R., Machado, F.S., Tomich, T.R., da Costa, M.J.R.P., Sant’Anna, A.C., 2019. Consistency of temperament traits and their relationships with

- milk yield in lactating primiparous F1 Holstein - Gyr cows. *Appl. Anim. Behav. Sci.* 112061. <https://doi.org/https://doi.org/10.1016/j.applanim.2019.104881> This
- Macedo, G.G., Zúccari, C.E.S.N., de Abreu, U.G.P., Negrão, J.A., da Costa e Silva, E.V., 2011. Human-animal interaction, stress, and embryo production in *Bos indicus* embryo donors under tropical conditions. *Trop. Anim. Health Prod.* 43, 1175–1182. <https://doi.org/10.1007/s11250-011-9820-6>
- Macuhová, J., Tancin, V., Kraetzl, W.D., Meyer, H.H.D., Bruckmaier, R.M., 2002. Inhibition of oxytocin release during repeated milking in unfamiliar surroundings: The importance of opioids and adrenal cortex sensitivity. *J. Dairy Res.* 69, 63–73. <https://doi.org/10.1017/S0022029901005222>
- Negrão, J.A., 2008. Hormone release and behavior during suckling and milking in Gir, Gir x Holstein, and Holstein cows. *J. Anim. Sci.* 21–26. <https://doi.org/10.2527/jas.2007-0304>
- Negrão, J.A., Marnet, P.G., 2002. Effect of calf suckling on oxytocin, prolactin, growth hormone and milk yield in crossbred Gir ´ Holstein cows during milking João. *Reprod. Nutr. Dev.* 43, 373–380. <https://doi.org/10.1051/rnd>
- Odorčić, M., Rasmussen, M.D., Paulrud, C.O., Bruckmaier, R.M., 2019. Review: Milking machine settings, teat condition and milking efficiency in dairy cows. *Animal* 13, S94–S99. <https://doi.org/10.1017/S1751731119000417>
- Pajor, E.A., Rushen, J., De Passillé, A.M.B., Passille, A.M.B. De, 2000. Aversion learning techniques to evaluate dairy cattle handling practices. *Appl. Anim. Behav. Sci.* 69, 89–102. [https://doi.org/10.1016/S0168-1591\(00\)00119-2](https://doi.org/10.1016/S0168-1591(00)00119-2)
- Petherick, J.C., 2005. Animal welfare issues associated with extensive livestock production: The northern Australian beef cattle industry. *Appl. Anim. Behav. Sci.* 92, 211–234. <https://doi.org/10.1016/j.applanim.2005.05.009>
- Polikarpus, A., Napolitano, F., Grasso, F., Di Palo, R., Zicarelli, F., Arney, D., De Rosa, G., 2014. Effect of pre-partum habituation to milking routine on behaviour and lactation performance of buffalo heifers. *Appl. Anim. Behav. Sci.* 161, 1–6. <https://doi.org/10.1016/j.applanim.2014.10.003>
- Ponchon, B., Zhao, X., Ollier, S., Lacasse, P., 2017. Relationship between glucocorticoids and prolactin during mammary gland stimulation in dairy cows. *J. Dairy Sci.* 100, 1521–1534. <https://doi.org/10.3168/jds.2016-11490>
- Porcionato, M.A.D.F., Negrão, J.A., De Paiva, F.A., Delgado, T.F.G., 2009. Respostas produtivas e comportamentais durante a ordenha de vacas Holandesas em início de lactação. *Acta Sci. - Anim. Sci.* 31, 447–451. <https://doi.org/10.4025/actascianimsci.v31i4.6326>
- Rushen, J., De Passillé, A.M.B., Munksgaard, L., 1999. Fear of people by cows and effects on milk yield, behavior, and heart rate at milking. *J. Dairy Sci.* 82, 720–727. [https://doi.org/10.3168/jds.S0022-0302\(99\)75289-6](https://doi.org/10.3168/jds.S0022-0302(99)75289-6)
- Sant’Anna, A.C., da Costa, M.J.R.P., Baldi, F., Rueda, P.M., Albuquerque, L.G., 2012. Genetic associations between flight speed and growth traits in nellore cattle. *J. Anim. Sci.* 90, 3427–3432. <https://doi.org/10.2527/jas.2011-5044>
- Ujita, A., El Faro, L., Vicentini, R.R., Pereira Lima, M.L., de Oliveira Fernandes, L., Oliveira, A.P., Veroneze, R., Negrão, J.A., 2021. Effect of positive tactile stimulation and prepartum milking routine training on behavior, cortisol and oxytocin in milking, milk composition, and milk yield in Gyr cows in early lactation. *Appl. Anim. Behav. Sci.* <https://doi.org/10.1016/j.applanim.2020.105205>
- Ujita, A., Ribeiro Vicentini, R., Pereira Lima, M.L., Negrão, J.A., de Oliveira Fernandes, L., Penido Oliveira, A., Veroneze, R., El Faro Zadra, L., 2020. Improvements in the behaviour of Gir dairy cows after training with brushing. *J. Appl. Anim. Res.* <https://doi.org/10.1080/09712119.2020.1754217>

Weaver, S.R., Bohrer, J.C., Prichard, A.S., Perez, P.K., Streckenbach, L.J., Olson, J.M., Cook, M.E., Hernandez, L.L., 2016. Serotonin deficiency rescues lactation on day 1 in mice fed a high fat diet. PLoS One 11, 1–18. <https://doi.org/10.1371/journal.pone.0162432>

3. EFFECT OF POSITIVE TACTILE STIMULATION AND PREPARTUM MILKING ROUTINE TRAINING ON BEHAVIOR, CORTISOL AND OXYTOCIN IN MILKING, MILK COMPOSITION, AND MILK YIELD IN GYR COWS IN EARLY LACTATION

Applied Animal Behaviour Science 234 (2021) 105205



Contents lists available at ScienceDirect

Applied Animal Behaviour Science

journal homepage: www.elsevier.com/locate/applanim



Effect of positive tactile stimulation and prepartum milking routine training on behavior, cortisol and oxytocin in milking, milk composition, and milk yield in Gyr cows in early lactation

Aska Ujita^{a,*}, Lenira El Faro^b, Rogério Ribeiro Vicentini^b, Maria Lucia Pereira Lima^b, Leonardo de Oliveira Fernandes^c, André Penido Oliveira^c, Renata Veroneze^c, João Alberto Negrão^a

^a Basic Science Department, Faculty of Animal Science and Food Engineering (FZEA), University of São Paulo (USP), Av. Duque de Caxias Norte 225, 13635- 900, Pirassununga, SP, Brazil

^b Institute of Animal Science (IZ), Beef Cattle Center of Agribusiness Technology Agency of São Paulo (APTA), 14174-000, Sertãozinho, São Paulo, Brazil

^c Agricultural Research Company of Minas Gerais (EPAMIG), Rua Afonso Rato 1301, 38001-970, Uberaba, Minas Gerais, Brazil

ABSTRACT

Positive interactions with humans can improve cow behavior and facilitate farm management, especially in dairy production systems in which milking occurs daily. Therefore, this study aimed to investigate the impact of positive tactile stimulation on behavior, and its effect on the milking routine, milk ejection, and milk yield of Gyr cows. Experiment 1 was performed with 40 cows: 20 cows (5 primiparous and 15 multiparous) received positive tactile stimulation (by hands and brushes) in the aisle of the corral during the prepartum period for 14 days; the other 20 cows (5 primiparous and 15 multiparous) did not receive any kind of tactile stimulation (control). Experiment 2 was performed with 40 heifers: 20 heifers were trained to enter the milking parlor and were progressively submitted to positive tactile stimulation (by hands and brushes) and to the milking routine during the prepartum period in the milking parlor for 14 days; the other 20 heifers did not receive any kind of training or tactile stimulation (control). Behavioral data were observed during the beginning of lactation: when the animal entered the milking parlor (stalled, needed help, and attempted to escape), during milking preparation (reaction to udder massage and to the strip cup test), and during milking (reaction to teat cups and whether the animal pulled off the teat cups). Milk yield and residual milk volume were measured, and milk samples were collected for analysis of milk composition, somatic cell count, and cortisol and oxytocin in milk. Entry into the milking parlor and preparation of the animal for milking are the most critical moments for detecting reactivity to milking procedures. At the same time, the two positive stimulations studied (i.e., handling in the corral or training in the milking parlor) were efficient in improving the behavior of Gyr cows at the beginning of lactation, thus demonstrating that prepartum positive stimulation was effective at reducing the negative behavior of cows during the firsts milkings. Indeed, the percentual of residual milk in

primiparous cows subjected to positive milking training was significantly lower than those measured in untrained primiparous and multiparous cows. This result is very promising because it indicates that Gyr heifers, when positively stimulated and trained in the milking routine during the prepartum period, will maintain adequate behavior and excellent milk ejection during their first milkings.

Keywords: behavior; cortisol; tactile stimulus; Gyr; heifers; residual milk.

3.1. INTRODUCTION

In most tropical countries, a dairy herd is composed mainly of crossbred cattle that combine the high milk production of taurine breeds (*Bos taurus taurus*) and the excellent adaptation of zebu breeds (*Bos taurus indicus*) for the pasture-based production conditions in tropical regions (Madalena et al., 2012; Pajor et al., 2000; Petherick, 2005). Among the various options in terms of zebu breeds that are part of the dairy herds in Brazil, the Gyr breed plays an important role in the crosses system; in fact, the Gyr/Holstein cross-breed accounts for 70% of all dairy cows in the country (Madalena et al., 2012; Negrão, 2008). In these pasture-based systems, heifers are kept in large pastures and have regular interactions with humans (Macedo et al., 2011; Petherick, 2005), mainly during farm management (vaccination, weighing, weaning, vermifugation, etc.). Consequently, the zebu breed is more distant from humans and, as a class, is difficult to handle. For these reasons, zebu frequently exhibit flight-or-threat behavior and aggressiveness due to their fear of humans (Pajor et al., 2000; Sant'Anna et al., 2012).

At the beginning of lactation, dairy cows are subjected to several new environmental stimuli related to milking (new housing conditions, new social groups, new management, new farm equipment, new handlers) that are considered stressful (Kutzer et al., 2015; Polikarpus et al., 2014). These stressors can negatively affect the animals' adaptation to different environmental challenges (May et al., 2016). In dairy cattle, the magnitude of their behavior responses is very important, especially in terms of the quality of the animal-human interaction (Breuer et al., 2000; Haskell et al., 2014), which is part of the routine of farm management. Furthermore, these different interactions with the handler can be used to determine the temperament of the cattle and can become a very important tool when a farmer works with a zebu-dominated herd (Ceballos et al., 2018b; Sant'Anna and da Costa, 2013).

Some authors have argued that zebu and crossbred cows are more difficult to milk from a behavioral point of view as compared to cows of specialized dairy breeds (Lammoglia

et al., 2015; Porcionato et al., 2009). In general, zebu cows are more agile, have a more pronounced behavior of protecting their calves, and are considered more temperamental and reactive than taurine cows (Lima et al., 2018; Sant'Anna et al., 2012). Frequently, these negative characteristics are related to an inadequate milking routine and inadequate milk ejection (Abdel-Hamid et al., 2017). In general, zebu cows have the reputation of not adapting to exclusively machine milking; for these reasons, zebu cows are milked with their calves (Alvarez et al., 1980; Assan, 2015; Negrão and Marnet, 2003, 2002). For Gyr heifers, their relationship with humans becomes intense during milking procedures, and their fear of humans can increase their reactivity to the milking routine, thereby increasing the duration of the milking process (Rushen et al., 2001, 1999; Sawa et al., 2017) and the risk of accidents involving humans and animals. However, a positive relationship between humans and animals can be achieved through stimuli such as brushing, stroking with the hands, and calm sounds spoken by a human voice (Boivin and Braastad, 1996; Lansade et al., 2004; Ligout et al., 2008; Shahin, 2018). In fact, positive tactile stimulation during the prepartum period might be an important tool to improve the cow-human interaction (Schmied et al., 2008). In the same way, prepartum training may reduce the impact of new environmental stressors such as the milking routine, milking installation, equipment, and human contact (Cavallina et al., 2008; Kutzer et al., 2015; Polikarpus et al., 2014) for Gyr heifers maintained in a pasture-based system. Hence, we hypothesized that these practices can positively change cows' behavior, facilitate milking, and increase the milk yield and milk ejection of Gyr cows during milking. For this reason, this study aimed to investigate the impact of positive tactile stimulation in the corral (Experiment 1) and in the milking routine habituation (Experiment 2) during the prepartum period in terms of the behavior and productivity of Gyr cows at the beginning of lactation.

3.2. MATERIALS AND METHODS

3.2.1. Study place and animals

The study was approved by the institutional Animal Care and Use Committee (Approval No. 230-16) and was conducted at the Getúlio Vargas Experimental Station of Empresa de Pesquisa Agropecuária de Minas Gerais (EPAMIG), located in the municipality of Uberaba – MG (19° 44' 54" S latitude, 47° 55' 55" W longitude, altitude of 801 m). According to the Köppen classification, the climate in the region is subtropical (*CWa), with warm and rainy summers and relatively dry winters.

The property maintains cows on *Urochloa brizanta* grass under rotational stocking, always observing the pasture's ideal height. During the rainy season, the animals are kept only in the pasture and receive a mineral mixture ad libitum. Lactating cows that produce over 10 kg/day receive 1 kg of concentrate for each 4 kg of milk produced. During the dry season, in addition to the mineral mixture ad libitum, the cows receive corn silage (32 kg of green forage/cow/day) and concentrate (1 kg of concentrate for each 3.8 kg of milk produced).

3.2.2. Arrangement of the experiments

In Experiment 1 (EXP1), tactile stimulation (by hands and brushes) of Gyr cows was performed in the aisle of the corral, located in front of the squeeze chute, during the prepartum period. The aim of EXP1 was to determine whether positive manual stimulation in the corral would facilitate the milking of Gyr cows at the beginning of lactation.

In Experiment 2 (EXP2), Gyr cows were trained to enter the milking parlor and were progressively submitted to tactile stimulation (by hands and brushes) and to the milking routine in the milking parlor, during the prepartum period. The aim of EXP2 was to determine whether habituation to the milking routine would facilitate the milking of Gyr cows at the beginning of lactation.

3.2.2.1. Experiment 1: Positive tactile stimulation in the corral

Forty Gyr cows were divided into two groups. The handling group consisted of 20 cows (5 primiparous and 15 multiparous), which received positive tactile stimuli (by hands and brushes) during the prepartum period. The control group consisted of 20 cows (5 primiparous and 15 multiparous) that were not submitted to any positive tactile stimulation during the prepartum period.

Experimental handling started 40 days before the expected calving date. The cows in the handling group were brushed and massaged for 20 consecutive days, twice a day (7:00 and 11:00 h), in the aisle in front of the squeeze chute. Each experimental animal was stimulated for 5 minutes, especially in the region of the udder and hind legs. The cows in the control group remained in the paddock near the corral and did not receive any type of tactile stimulus.

Guiding the animals from the pasture to the squeeze chute facility and providing tactile stimulation were acts performed only by the experimenters. There was no interaction between the milkers and experimental cows during this phase of the experiment.

3.2.2.2. *Experiment 2: Milking routine training in the milking parlor*

Forty Gyr heifers were divided into two groups. The training group consisted of 20 heifers that were progressively habituated to the milking routine and parlor. These animals received positive tactile stimuli (by hands and brushes) and were submitted to a milking simulation. The control group consisted of 20 heifers that did not receive any type of training or tactile stimulus and that remained in the paddock near the corral.

The experimental handling in the milking parlor started 40 days before the expected calving date and occurred for 14 consecutive days in the morning. Handling was performed by the milkers of the property and the experimenters in three phases:

- 1) Phase 1 (days 1 to 5): Passage of the animals through the milking parlor, simulating the entire milking routine. The experimental animals were directed in groups into the operating milking parlor. They entered the milking aisle and were released to the exit. A multiparous cow was part of the experimental group to facilitate animal handling.
- 2) Phase 2 (days 6 to 10): In the milking parlor, in addition to the procedures described for phase 1, each animal was brushed for 2 minutes, with the whole body being brushed but, in particular, the region of the udder and hind legs.
- 3) Phase 3 (days 11 to 15): In the milking parlor, in addition to the procedures described for phase 1, the hind legs of the cows were restrained. Each animal was brushed with a short-handled brush for 2 minutes (particularly the region of the udder and hind legs). In addition, pre-dipping was performed (i.e., asepsis of the teats by immersion in antiseptic solution and drying).

Throughout these 14 days, the side on which the cows entered the milking parlor was altered, i.e., on one day the cows entered on the left side and on another day they entered on the right side.

3.2.3. Experimental milking

During the colostrum phase, the cows of EXP1 were milked in a bucket milking machine sets. After this stage, these animals were milked in a conventional tandem parlor with 12 sets of milking machines. The cows of EXP2 were milked in a conventional tandem parlor with 12 sets of milking machines throughout lactation.

The animals of EXP1 and 2 were directed to the milking parlor in batches according

to their milk yield. Milking was performed twice a day by three milkers who took turns in the milking parlor. During all milkings, the handlers cleaned and pre-dipped the teats, let the calf suckle, and attached the teat cups. During mechanical milking of all experimental cows, their respective calves were allowed to suckle the right anterior teat. After milking, the calves were separated and brought to the collective calf housing facility, where they received complementary feed. Post-dipping of the teats was then performed and the milk yield was recorded. The lactating cows were released from the milking parlor and brought to their respective pasture paddocks.

The strip cup test was performed daily. If the test was positive, somatic cell count (SCC) and microbiological analysis were performed. Mastitis was treated with specific antibiotics when necessary.

3.2.4. Data collection

The behavioral and production data were collected during lactation only in the morning. Throughout the experiment, all milking parlors were monitored 24 hours a day with camcorders. All participants in the experiment were trained and prepared to collect the respective experimental data.

3.2.4.1. *Animal behavior in the milking room*

The following parameters were observed in both experiments: behaviors during entry into the milking parlor (stalled, needed help, and attempted to escape), during preparation for milking (response to tying the hind legs and to the strip cup test), and during milking (reactivity to the teat cups, milking behavior, and whether the animal pulled off the teat cups). The strip cup test was not performed in the milking parlor for the removal of colostrum. All data were recorded in real time according to the definitions described in Table 1, with one trained observer responsible for up to two animals.

Table 1. Description of the behavioral parameters evaluated during the milking procedure and their corresponding scores.

Time	Parameter	Description	Score*				
			1	2	3	4	5
Entry into the milking parlor	Stalled	Stalled to enter the parlor	No	Yes, because of being tame (calm, walking slowly)	Yes, because of fear (desperate and suspicious)	-	-
	Help	Needed help to enter the parlor	No	Voice command	Patting	Poking the animal one time with a tool	Poking the animal two or more times with a tool
	Escape	If there was an attempt to escape	No	Yes	-	-	-
Preparation for milking	Tying	Reaction to the placement and use of a restraint	Did not react	Low, no kicking	Low, no kicking	High, no kicking	High, no kicking
	Strip	Reaction to the strip cup test	Did not react	Low, no kicking	Low, no kicking	High, no kicking	High, no kicking
During milking	Teat cups	Reaction to teat cup attachment or detachment	Did not react	Low, no kicking	Low, no kicking	High, no kicking	High, no kicking
	Milking	Behavior during milking	Calm	Median	Very restless	-	-
	Removal of teat cups	If the cow pulled the teat cups off the teats	No	Yes	-	-	-

*The scores measured during entry, preparation and milking were used to obtain the total score, which corresponds to the overall behavior observed during milking.

3.2.4.1.1. Experiment 1

All animals (handling and control groups) were evaluated throughout the milking procedure, in the morning, on days 1, 3, 5, 7, 9, 11, 13, 15, 17, and 19 of lactation.

3.2.4.1.2. Experiment 2

All animals (training and control groups) were evaluated throughout the milking procedure, in the morning, on days 1, 3, 7, 15, 30, 45, and 60 of lactation.

3.2.4.2. Productive performance

The milk yield was measured on days 15, 30, 45, and 60 of lactation in EXP1, and on days 1, 3, 7, 15, 30, 45, and 60 in EXP2, using containers coupled to the milking machine. The residual milk in all quarters was measured in the morning on days 15, 30, 45, and 60 of lactation by intramuscular administration of 2 mL of exogenous oxytocin (10 IU/mL) immediately after the usual morning milking. Ten minutes after oxytocin administration, another milking was performed. The residual milk volume obtained was transformed into a percentage in relation to the total yield of the morning milking.

3.2.4.3. Laboratory analysis of milk

All laboratory analyses were carried out at the Laboratory of Animal Physiology, FZEA/USP. Milk composition, SCC, and hormonal parameters were analyzed in total milk samples collected on days 15, 30, 45, and 60 of lactation in EXP1, and on days 3, 7, 15, 30, 45, and 60 of lactation in EXP2.

The milk components were measured by ultrasound using the MilkoScope Expert Automatic milk analyzer (Scope Electric Ltd., Razgrad, BG). The SCC was obtained by direct microscopic somatic cell counting of 100 fields/smear per animal. Cortisol and oxytocin were analyzed in milk using an enzyme immunoassay (EIA) according to the specifications of the kits' manufacturers (cortisol: Monobind, Lake Forest, CA, USA; oxytocin: Mybiosource, San Diego, CA, USA).

3.2.5. Statistical analysis

For evaluation of the behavioral variables, the scores observed were converted into values on a reactivity scale ranging from 0 to 10, with 10 corresponding to most reactive and 0 to least reactive. Values for each time point evaluated (entry into the milking parlor, preparation

for milking, and during milking) and a total value considering all behavioral parameters and time points were calculated and submitted for analysis of variance.

Initially, simple descriptive statistics were applied using the FREQ and MEANS procedures (SAS Institute, Inc., Cary, NC, USA). The EXP1 data were analyzed by analysis of variance using the MIXED procedure (SAS Institute, Inc., Cary, NC, USA) in a completely randomized design and 2 x 2 factorial scheme (treatment and calving order). The effect of treatment (with and without tactile stimulation in the squeeze chute) and calving order (primiparous and multiparous) and their interaction were evaluated as fixed effects. The EXP2 data were submitted for analysis of variance using the MIXED procedure (SAS Institute, Inc., Cary, NC, USA) in a completely randomized design. The effect of treatment (with and without training in the milking parlor) was included as a fixed effect.

The data of the two experiments were evaluated as repeated measures over time (days of lactation). The covariance matrices were tested for each trait and the structure showing the best fit was used (Crowder and Hand, 1990). Each animal was considered the experimental unit. Means were estimated using the LSMeans procedure. When significant differences were found ($P \leq 0.05$), means were compared using the Student t-test at a level of significance of 5%.

3.3. RESULTS

3.3.1. Experiment 1

Positive tactile stimulation (by hands and brushes) performed in the aisle of the corral during the prepartum period exerted a significant effect ($P \leq 0.05$) on the behaviors evaluated during the milking procedure (Table 2). In fact, the lower scores (the values closer to 0, defined as less reactive) obtained during entry into the milking parlor, during preparation for milking, and during milking, as well as overall milking behavior (overall score), indicate that positive stimulation facilitated the milking routine of Gyr cows as compared to control cows (without tactile stimulation). In addition, significant interactions were observed between positive stimulation, calving order, and days of lactation for entry, preparation, milking, and overall score at the beginning of lactation. However, prepartum tactile stimulation had no significant effect on the release of cortisol and oxytocin or on productive performance. The residual milk volume was lower in the group receiving positive tactile stimulation than in the control group (Table 2).

3.3.2. Experiment 2

Prepartum training (habituation, positive tactile stimulation, and milking simulation) of primiparous Gyr cows in the milking parlor had a significant and positive effect ($P \leq 0.05$) on the behaviors measured during entry into the milking parlor, overall score, cortisol and oxytocin concentration, and residual milk volume as compared to control animals (Table 3).

Table 2. Effects of treatment, calving order, day of lactation and its interactions on behavior scores, milk yield, residual milk, cortisol and oxytocin during morning milking of the Gyr cows of Experiment 1.

Trait	Handling (n = 20)	Control (n = 20)	T	P-values		
				CO	DL	T*CO*DL
Entry	4.0 ± 0.19	4.6 ± 0.22	0.05	0.04	0.00	0.02
Preparation	1.6 ± 0.04	1.8 ± 0.05	0.00	0.01	0.00	0.01
Milking	3.0 ± 0.06	3.3 ± 0.07	0.02	0.95	0.01	0.00
Overall	4.2 ± 0.13	4.7 ± 0.15	0.00	0.03	0.00	0.01
Cort (ng/mL)	15.09 ± 1.46	18.05 ± 1.76	0.20	0.29	0.85	0.29
Oxyt (pg/mL)	20.06 ± 1.69	15.66 ± 2.03	0.10	0.20	0.00	0.82
MY (kg/milking)	7.02 ± 0.73	7.03 ± 0.65	0.18	0.85	0.64	0.21
RM (%/ milking)	26.3 ± 5.36	35.73 ± 6.56	0.27	0.34	0.95	0.11

LSMeans ± standard error and P value. Entry: behavior during entry into the milking parlor; Preparation: behavior during preparation for milking; Milking: behavior during milking; Overall: overall milking behavior; Cort: cortisol; Oxyt: oxytocin; MY: milk yield; RM: residual milk; T: treatment; CO: calving order; DL: day of lactation.

There was also an interaction between the treatment and the day of lactation for the behaviors measured during entry, preparation, and milking and for the overall score (Figure 2). Indeed, cows that were trained and stimulated in the milking parlor exhibited less reactivity on the first day of lactation for all behaviors evaluated. However, the behavioral differences between trained and untrained cows decreased across lactation, with all the experimental cows exhibiting low reactivity during preparation and milking from days 15 to 60 of lactation (Figure 2).

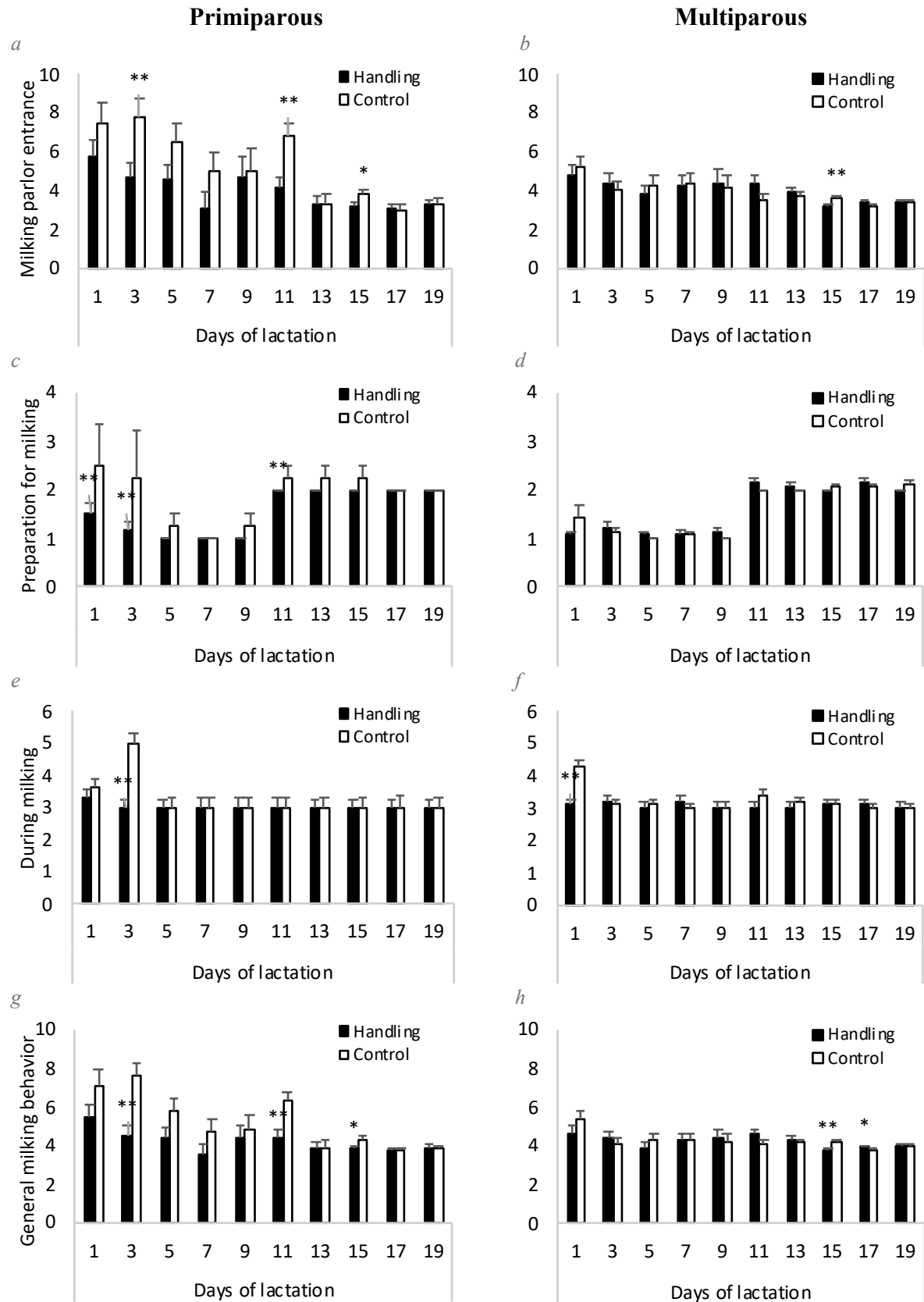


Figure 1. Scores of the behaviors during entry into the milking parlor (a and b), during preparation for milking (c and d), during milking (e and f) and overall milking behavior (g and h) according to the day of lactation in primiparous and multiparous Gyr cows submitted (or not) to tactile stimulation in the squeeze chute. Mean values with * and ** differ at $P \leq 0.05$ and $P \leq 0.01$, respectively.

Table 2. LSMeans \pm standard error of somatic cell count and milk fat, protein and lactose percentage in Gyr cows from EXP1 and EXP2.

	SCC/mL	% F	% P	% L
EXP1 (n = 20)	359,034 \pm 66823	4.89 \pm 0.10	3.33 \pm 0.04	4.49 \pm 0.03
EXP2 (n = 20)	329,164 \pm 23316	4.48 \pm 0.08	3.47 \pm 0.02	4.76 \pm 0.03

SCC: somatic cell count ($\times 1000$ cells/mL milk); %F: fat percentage in milk; %P: protein percentage in milk; %L: lactose percentage in milk.

Table 3. Effects of treatment, day of lactation and its interactions on behavior scores, milk yield, residual milk, cortisol and oxytocin during morning milking of the Gyr heifers of Experiment 2.

Trait	Training (n=20)	Control (n=20)	P-values		
			T	DL	T*DL
Entry	3.3 \pm 0.11	4.0 \pm 0.11	0.00	0.01	0.01
Preparation	2.8 \pm 0.10	2.9 \pm 0.13	0.58	0.01	0.03
Milking	3.2 \pm 0.11	3.4 \pm 0.10	0.21	0.89	0.02
Overall	3.1 \pm 0.08	3.4 \pm 0.08	0.01	0.01	0.01
Cort (ng/mL)	16.23 \pm 1.11	24.10 \pm 0.94	0.01	0.36	0.11
Oxyt (pg/mL)	25.46 \pm 1.44	20.32 \pm 1.21	0.01	0.17	0.57
MY (kg/milking)	6.67 \pm 0.29	6.43 \pm 0.26	0.55	0.30	0.96
RM (%/milking)	7.16 \pm 2.26	14.11 \pm 2.04	0.03	0.58	0.84

LSMeans \pm standard error and P value. Entry: behavior during entry into the milking parlor; Preparation: behavior during preparation for milking; Milking: behavior during milking; Overall: overall milking behavior; Cort: cortisol; Oxyt: oxytocin; MY: milk yield; RM: residual milk; T: treatment; DL: day of lactation.

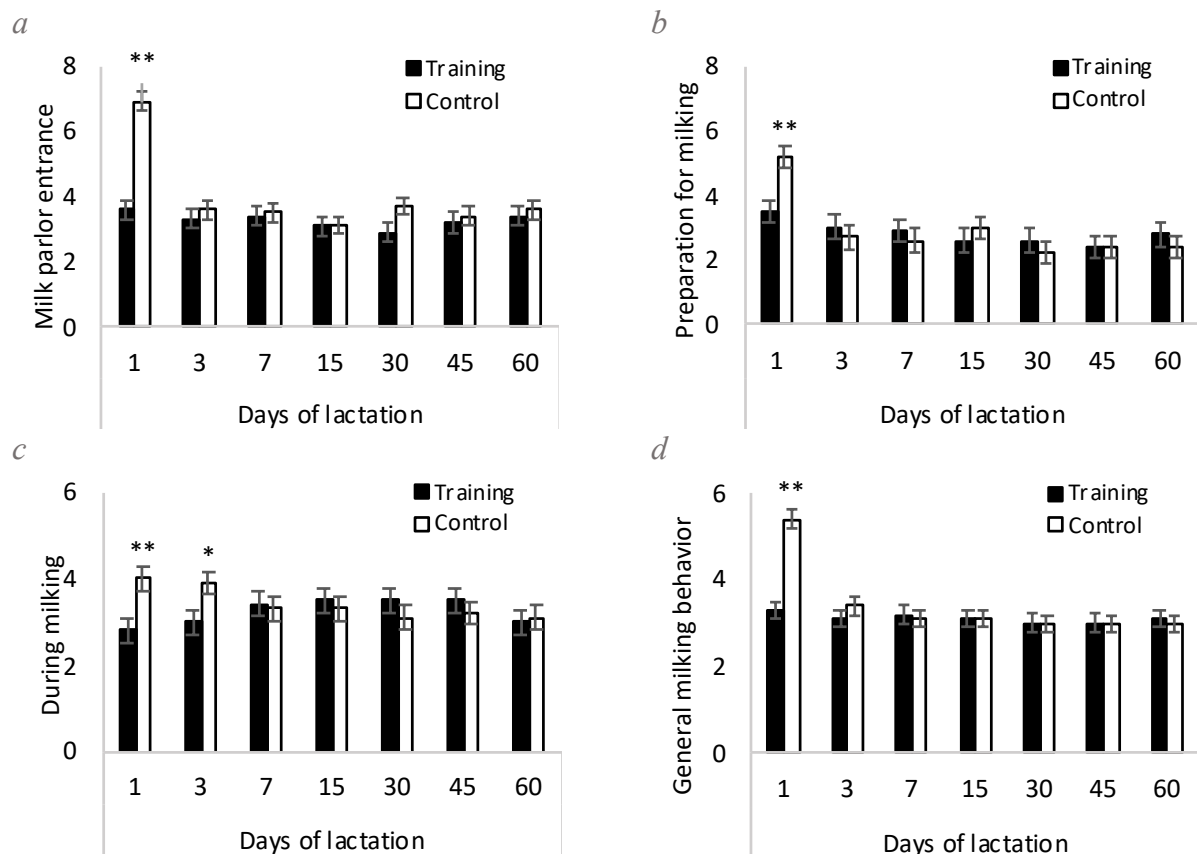


Figure 2. Scores of the behaviors during entry into the milking parlor (a), during preparation for milking, (b) during milking (c) and overall milking behavior (d) according to day of lactation in Gyr heifers submitted (or not) to habituation with tactile stimulation in the milking parlor. Mean values with * and ** differ at $P \leq 0.05$ and $P \leq 0.01$, respectively.

3.4. DISCUSSION

The results of this study reinforce the importance of prepartum positive handling and training to facilitate the milking routine of Gyr cows. As Gyr cows had previous minimal contact with the facilities, equipment, and humans, their first contact with this new environment could affect negatively the entry of the animal into the milking parlor (Breuer et al., 2000; Peters et al., 2010). Indeed, dairy cows are subjected to various challenging stimuli that must be overcome for them to enter the milking parlor for the first time; these stimuli include the gate, aisle, containment, type of floor, lighting, shadows (Grandin, 1990), and noises and sounds of the equipment and milkers. These stimuli together could negatively affect the cow's willingness to spontaneously enter the parlor, and some animals could show inadequate behavior (Brouček, 2014; Pajor et al., 2000). In addition, zebu breeds present more reactive behaviors than do taurine animals (da Costa et al., 2002; Hearnshaw and Morris, 1984). Zebu cows are also more agitated and difficult to bring into the milking parlor at the beginning of lactation (Negrão,

2008; Porcionato et al., 2009), and the first milkings are considered stressful by milkers. However, in the present study, prepartum training significantly improved the behaviors observed during entry, facilitating the flow of trained cows into the milking parlor as compared to control cows.

Milking preparation depends on the milker-cow interaction, as the cows are exposed to tactile stimulation by objects and the hands of the milkers. Consequently, the quality of the human-animal interaction can significantly influence, positively or negatively, the behavior of dairy cows (Breuer et al., 2000; Peters et al., 2010). As compared to trained cows, untrained cows reacted negatively to the milking preparation, particularly during the first days of lactation in both experiments. The higher reactivity to milking preparation was most frequently observed in primiparous cows as compared to multiparous cows, especially during the firsts milkings, when all experimental cows moved away when handled by the milker, demonstrating their reactivity to this routine (Grandin and Deesing, 2014; Polikarpus et al., 2014). However, on subsequent days, the reactivity to the milking preparation of trained cows decreased significantly as compared to control cows, showing the positive effect of prepartum training. In fact, in the present study, trained cows exhibited lower scores in terms of all behaviors studied and lower reactivity during the milking routine, demonstrating that prepartum training accompanied by tactile stimulation was effective in reducing the negative behavior of cows during milking.

In addition, our results demonstrated that entry into the milking parlor and milking preparation were the most critical moments of the milking routine in Gyr cows. In EXP1, primiparous cows were significantly more reactive than were multiparous cows; these results confirm the impact of positive previous experience on the behavior of animals (Cavallina et al., 2008; Sutherland and Huddart, 2012). However, the negative behavior of primiparous cows decreased significantly throughout lactation, while the behavior and reactivity of multiparous cows remained stable. These results highlight the importance of prepartum training of primiparous Gyr cows to facilitate the milking routine at the onset of first lactation. As observed in other studies, prepartum habituation-training positively modified the behavior of buffalo heifers (Polikarpus et al., 2014) and cows (Kutzer et al., 2015; Sutherland and Huddart, 2012), as well as facilitated the milking routine of Gyr cows in both experiments of the present study. At the same time, the stable behavior of multiparous cows observed in the present study suggests that cows with worse behavior scores maintain their negative behavior, which could increase the milking duration (Sawa et al., 2017) and the risk of accidents involving humans

and animals.

Although the two types of trainings that were studied had a positive effect on the behavior and temperament of Gyr cows during milking, no significant difference was observed in milk cortisol and oxytocin concentration in EXP1. Within this context, it is possible to argue that habituation, training, and tactile stimulation in the milking parlor in EXP2 had the most effectiveness. In fact, trained primiparous Gyr cows (EXP2) had significantly lower cortisol and higher oxytocin concentrations and lower residual milk volume compared to control cows. The better hormone release and milk ejection demonstrated the positive effect of prepartum training on the physiological responses at the starting of lactation. On the other hand, other authors have reported that exposure to different stressors before and during milking (Bruckmaier et al., 1977, 2009; Wicks et al., 2004; Sutherland and Huddart, 2012) increases the frequency of inappropriate behaviors, heart and respiratory rates, cortisol release, and milk retention (Eicher et al., 2007; Rushen et al., 1999; Van Reenen et al., 2002), with a negative impact on the productivity of dairy cows (Negrao et al., 2010). Likely, this lower milk yield is a consequence of the reduction of animal welfare in the milking parlor. In this context, even our experimental cows, maintained on a pasture and having had little human contact, showed a significant response to positive interactions with humans before calving. In addition, primiparous cows responded better to training than did multiparous cows, highlighting the importance of positive training at the onset of first lactation.

Although there were no significant differences in milk yield or quality between trained and control cows, these results show that the quality of management from pasture to milking installation and the quality of milker-cow interaction were adequate for all experimental cows. On the other hand, the percentage of residual milk was low in cows that received prepartum training in both experiments. Indeed, in EXP2, the residual milk volume measured in trained primiparous cows was significant and lower than 10% – a percentage similar to that described for dairy cows with excellent milk ejection (Bruckmaier and Blum, 1998). These findings confirmed that the habituation, handling, and training of Gyr heifers in the milking parlor using tactile stimulation improved milk ejection during milking. According to other authors, ideal milking promotes the release of oxytocin and reduces cortisol release and residual milk volume at the beginning of lactation (Bruckmaier and Blum, 1998; Negrão, 2008; Rushen et al., 2001, 1999). The behavior observed, and the hormone release and milk ejection measured, in the present study showed that the same milking routine was more effective for trained animals.

In fact, during both experiments, Gyr cows that were exposed to positive stimulation

had better behavior scores during entry, preparation, and milking than did control cows, as also observed by other authors (Cavallina et al., 2008; Kutzer et al., 2015; Polikarpus et al., 2014). However, it is important to note that all experimental cows were simultaneously milked and suckled by their own calf, so trained cows exhibited better milk ejection than did untrained cows. This result is interesting in terms of confirming the impact of positive training related to milking during the end-gestation period, as different studies have reported that the milking-suckling system per se significantly improves milk ejection and increases milk yield (Alvarez et al., 1980; Assan, 2015; Negrão and Marnet, 2002) just for Holstein cows (Bar-Pelled et al., 1995). As several farmers continue to administer daily exogenous oxytocin to zebu cows reputed to be poorly adapted to machine milking (Johnsen et al., 2016; Lammoglia et al., 2015), the significant and positive effect of training on residual milk is very promising, as it indicates that a primiparous cow, when habituated to the milking routine in the prepartum period, will have excellent milk ejection starting at the onset of first lactation.

3.5. CONCLUSION

The entry into the milking parlor and the preparation of the animal for milking are the most critical moments for detecting reactivity to the milking routine. The two types of trainings studied (in the corral and in the milking parlor) were effective in improving the behavior of Gyr cows in terms of milking at the beginning of lactation. However, our results demonstrated that training in the milking parlor was more effective because it also exerted a positive and significant effect on cortisol and oxytocin release and on residual milk volume, thereby improving milk ejection in trained animals from the onset of first lactation.

ACKNOWLEDGEMENT

The authors acknowledge FAPESP (process 2015/24.174-3) for financial support; EPAMIG, Finep and MCTI to support the structure for carrying out the research Project; and CAPES (Finance Code 001), that financed the scholarships.

REFERENCES

- Abdel-Hamid, S.E., Fattah, D.M.A., Ghanem, H.M., Eman Abd-Allah Manaa, 2017. Temperament during Milking Process and its Effect on Behavioral, Productive Traits and Biochemical Parameters in Friesian Dairy Cows. *Adv. Anim. Vet. Sci.* 5, 508–513. <https://doi.org/http://dx.doi.org/10.17582/journal.aavs/2017/5.12.508.513>
- Adamczyk, K., 2018. Dairy cattle welfare as a result of human-animal relationship – a review. *Ann. Anim. Sci.* <https://doi.org/10.2478/aoas-2018-0013>
- Alvarez, F.J., Saucedo, G., Arriaga, A., Preston, T.R., 1980. Effect on Milk Production and Calf Performance of Milking Cross Bred European / Zebu Cattle in the Absence or Presence of. *Trop. Anim. Prod.* 5, 25–37.
- Andrade, O., Orihuela, A., Solano, J., Galina, C.S., 2001. Some effects of repeated handling and the use of a mask on stress responses in zebu cattle during restraint. *Appl. Anim. Behav. Sci.* 71, 175–181. [https://doi.org/10.1016/S0168-1591\(00\)00177-5](https://doi.org/10.1016/S0168-1591(00)00177-5)
- Assan, N., 2015. Influence of suckling and/or milking method on yield and milk composition in dairy animals. *Sci. J. Zool.* 4, 1–7. <https://doi.org/10.14196/sjz.v4i1.1792>
- Baldwin, D. V., 2013. Primitive mechanisms of trauma response: An evolutionary perspective on trauma-related disorders. *Neurosci. Biobehav. Rev.* <https://doi.org/10.1016/j.neubiorev.2013.06.004>
- Bar-Pelled, U., Maltz, E., Bruckental, I., Folman, Y., Kali, Y., Gacitua, H., Lehrer, A.R., Knight, C.H., Robinson, B., Voet, H., Tagari, H., 1995. Relationship Between Frequent Milking or Suckling in Early Lactation and Milk Production of High Producing Dairy Cows. *J. Dairy Sci.* 78, 2726–2736. [https://doi.org/10.3168/jds.S0022-0302\(95\)76903-X](https://doi.org/10.3168/jds.S0022-0302(95)76903-X)
- Becker, B.G., Lobato, J.F.P., 1997. Effect of gentle handling on the reactivity of zebu crossed calves to humans. *Appl. Anim. Behav. Sci.* 53, 219–224. [https://doi.org/10.1016/S0168-1591\(96\)01091-X](https://doi.org/10.1016/S0168-1591(96)01091-X)
- Benus, R.F., Bohus, B., Koolhaas, J.M., van Oortmerssen, G.A., 1991. Heritable variation for aggression as a reflection of individual coping strategies. *Experientia*. <https://doi.org/10.1007/BF01923336>
- Bertenshaw, C., Rowlinson, P., 2001. The influence of positive human-animal interaction during rearing on the approach behaviour of young dairy heifers. *Proc. Br. Soc. Anim. Sci.* <https://doi.org/10.1017/s1752756200006530>
- Bertenshaw, C., Rowlinson, P., Edge, H., Douglas, S., Shiel, R., 2008. The effect of different degrees of “positive” human-animal interaction during rearing on the welfare and subsequent production of commercial dairy heifers. *Appl. Anim. Behav. Sci.* 114, 65–75. <https://doi.org/10.1016/j.applanim.2007.12.002>
- Bertucci, P.Y., Quaglino, A., Pozzi, A.G., Kordon, E.C., Pecci, A., 2010. Glucocorticoid-induced impairment of mammary gland involution is associated with STAT5 and STAT3 signaling modulation. *Endocrinology* 151, 5730–5740. <https://doi.org/10.1210/en.2010-0517>
- Blum, J.W., Bruckmaier, R., 1989. Catecholamines, oxytocin and milk removal in dairy cows. *J. Dairy Res.* <https://doi.org/10.1017/S0022029900026388>
- Boivin, X., Braastad, B.O., 1996. Effects of handling during temporary isolation after early weaning on goat kids’ later response to humans. *Appl. Anim. Behav. Sci.* 48, 61–71. [https://doi.org/10.1016/0168-1591\(95\)01019-X](https://doi.org/10.1016/0168-1591(95)01019-X)
- Boivin, X., Tournadret, H., Le Neindre, P., 2000. Hand-feeding and gentling influence early-weaned lambs’ attachment responses to their stockperson. *J. Anim. Sci.* 78, 879–884. <https://doi.org/10.2527/2000.784879x>
- Bomfim, G.F., Merighe, G.K.F., de Oliveira, S.A., Negrao, J.A., 2018. Effect of acute stressors, adrenocorticotrophic hormone administration, and cortisol release on milk yield, the expression of

- key genes, proliferation, and apoptosis in goat mammary epithelial cells. *J. Dairy Sci.* 101, 6486–6496. <https://doi.org/10.3168/jds.2017-14123>
- Bondarenko, E., Beig, M.I., Hodgson, D.M., Braga, V.A., Nalivaiko, E., 2015. Blockade of the dorsomedial hypothalamus and the perifornical area inhibits respiratory responses to arousing and stressful stimuli. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* <https://doi.org/10.1152/ajpregu.00415.2014>
- Borghese, A., Rasmussen, M., Thomas, C.S., 2007. Milking management of dairy buffalo. *Ital. J. Anim. Sci.* <https://doi.org/10.4081/ijas.2007.s2.39>
- Bouissou, M.F., Boissy, A., Neindre, P. le, Veissier, I., 2009. The social behaviour of cattle., in: *Social Behaviour in Farm Animals*. <https://doi.org/10.1079/9780851993973.0113>
- Brasil, B.S.A.F., Coelho, E.G.A., Drummond, M.G., Oliveira, D.A.A., 2013. Genetic diversity and differentiation of exotic and American commercial cattle breeds raised in Brazil. *Genet. Mol. Res.* <https://doi.org/10.4238/2013.November.18.2>
- Bremner, K.J., 1997. Behaviour of dairy heifers during adaptation to milking. *Proc. New Zeal. Soc. Anim. Prod.*
- Breuer, K., Hemsworth, P.H., Barnett, J.L., Matthews, L.R., Coleman, G.J., 2000. Behavioural response to humans and the productivity of commercial dairy cows. *Appl. Anim. Behav. Sci.* 66, 273–288. [https://doi.org/10.1016/S0168-1591\(99\)00097-0](https://doi.org/10.1016/S0168-1591(99)00097-0)
- Breuer, K., Hemsworth, P.H., Coleman, G.J., 2003. The effect of positive or negative handling on the behavioural and physiological responses of nonlactating heifers. *Appl. Anim. Behav. Sci.* 84, 3–22. [https://doi.org/10.1016/S0168-1591\(03\)00146-1](https://doi.org/10.1016/S0168-1591(03)00146-1)
- Bristow, D.J., Holmes, D.S., 2007. Cortisol levels and anxiety-related behaviors in cattle. *Physiol. Behav.* 90, 626–628. <https://doi.org/10.1016/j.physbeh.2006.11.015>
- Brouček, J., 2014. Effect of Noise on Performance, Stress, and Behaviour of Animals. *Slovak J. Anim. Sci.* 47, 111–123.
- Brouček, J., Uhrinčat, M., ŠoCh, M., Kišac, P., UhRinČat, M., ŠoCh, M., Kišac, P., 2008. Genetics of behaviour in cattle. *Slovak J. Anim. Sci.* 41, 166–172. <https://doi.org/10.1079/9781780642215.0234>
- Bruckmaier, R.M., 2003. Chronic oxytocin treatment causes reduced milk ejection in dairy cows. *J. Dairy Res.* <https://doi.org/10.1017/S0022029902005940>
- Bruckmaier, R.M., Pfeilsticker, H.-U., Blum, J.W., 2009. Milk yield, oxytocin and β -endorphin gradually normalize during repeated milking in unfamiliar surroundings. *J. Dairy Res.* 63, 191. <https://doi.org/10.1017/s0022029900031691>
- Bruckmaier, R.M., Schams, D., Blum, J.W., 1993. Milk removal in familiar and unfamiliar surroundings: concentrations of oxytocin, prolactin, cortisol and β -endorphin. *J. Dairy Res.* 60, 449–456.
- Bruckmaier, R.M., Wellnitz, O., 2008. Induction of milk ejection and milk removal in different production systems. *J. Anim. Sci.* 86, 15–20. <https://doi.org/10.2527/jas.2007-0335>
- Bruckmaier, R.M., Wellnitz, O., Blum, J.W., 1997. Inhibition of milk ejection in cows by oxytocin receptor blockade, α -adrenergic receptor stimulation and in unfamiliar surroundings. *J. Dairy Res.* 64, 315–325. <https://doi.org/10.1017/S002202999700232X>
- Bruckmaier, R.M.M., Blum, J.W.W., 1998. Oxytocin Release and Milk Removal in Ruminants. *J. Dairy Sci.* 81, 939–949. [https://doi.org/10.3168/jds.S0022-0302\(98\)75654-1](https://doi.org/10.3168/jds.S0022-0302(98)75654-1)
- Burdick, N.C., Randel, R.D., Carroll, J.A., Welsh, T.H., 2011. Interactions between temperament, stress, and immune function in cattle. *Int. J. Zool.* <https://doi.org/10.1155/2011/373197>
- Burdick Sanchez, N.C., Carroll, J.A., Randel, R.D., Vann, R.C., Welsh, T.H., 2014. Associations

- between endotoxin-induced metabolic changes and temperament in Brahman bulls. *J. Anim. Physiol. Anim. Nutr. (Berl)*. 98, 178–190. <https://doi.org/10.1111/jpn.12074>
- Burrow, H.M., 1997. Measurement of temperament and their relationship with performance traits of beef cattle. *Anim Breed Abstr*. <https://doi.org/10.1002/tea.20309>
- Cannon, W.B., 1929. Bodily Changes in Pain, Hunger, Fear and Rage. *South. Med. J.* <https://doi.org/10.1097/00007611-192909000-00037>
- Cavallina, R., Roncoroni, C., Campagna, M.C., Minero, M., Canali, E., 2008. Buffalo behavioural response to machine milking in early lactation. *Ital. J. Anim. Sci.* 7, 287–295. <https://doi.org/10.4081/ijas.2008.287>
- Ceballos, M.C., Sant’Anna, A.C., Boivin, X., Costa, F. de O., Carvalhal, M.V. d. L., Paranhos da Costa, M.J.R., 2018a. Impact of good practices of handling training on beef cattle welfare and stockpeople attitudes and behaviors. *Livest. Sci.* <https://doi.org/10.1016/j.livsci.2018.06.019>
- Ceballos, M.C., Sant’Anna, A.C., Góis, K.C.R., Ferraudo, A.S., Negrao, J.A., da Costa, M.J.R.P., 2018b. Investigating the relationship between human-animal interactions, reactivity, stress response and reproductive performance in Nellore heifers. *Livest. Sci.* 217, 65–75. <https://doi.org/10.1016/j.livsci.2018.08.001>
- Chen, Y., Arsenault, R., Napper, S., Griebel, P., 2015. Models and methods to investigate acute stress responses in cattle. *Animals* 5, 1268–1295. <https://doi.org/10.3390/ani5040411>
- Chida, D., Miyoshi, K., Sato, T., Yoda, T., Kikusui, T., Iwakura, Y., 2011. The role of glucocorticoids in pregnancy, parturition, lactation, and nurturing in melanocortin receptor 2-deficient mice. *Endocrinology* 152, 1652–1660. <https://doi.org/10.1210/en.2010-0935>
- Coelho, S.G., Franzoni, A.P.S., 2012. Comportamento e Bem-estar de bezerras na bovinocultura leiteira, in: Junior, A. de P.M., Heinemann, M.B., Garcia, S.K., Ângela Mara Leite Drumond (Eds.), *Cadernos Técnicos de Veterinária e Zootecnia*. Belo Horizonte, pp. 135–151.
- Coleman, G.J., Hemsworth, P.H., Hay, M., 1998. Predicting stockperson behaviour towards pigs from attitudinal and job-related variables and empathy. *Appl. Anim. Behav. Sci.* 58, 63–75. [https://doi.org/10.1016/S0168-1591\(96\)01168-9](https://doi.org/10.1016/S0168-1591(96)01168-9)
- Collier, R.J., Hernandez, L.L., Horseman, N.D., 2012. Serotonin as a homeostatic regulator of lactation. *Domest. Anim. Endocrinol.* 43, 161–170. <https://doi.org/10.1016/j.domaniend.2012.03.006>
- Connelly, M.K., Weaver, S.R., Kuehnl, J.M., Fricke, H.P., Klister, M., Hernandez, L., 2021. Elevated serotonin coordinates mammary metabolism in dairy cows. *Physiol. Rep.* 9, 1–12. <https://doi.org/10.14814/phy2.14798>
- Cooke, R.F., Schubach, K.M., Peres, R.F.G., Cipriano, R.S., Marques, R., Carvalho, R., Bohnert, D.W., Biehl, M. V., Pires, A. V., Vasconcelos, J.L.M., 2016. 1275 Effects of temperament on physiological and reproductive responses of Bos Indicus beef cows. *J. Anim. Sci.* <https://doi.org/10.2527/jam2016-1275>
- Cozzi, G., Brscic, M., Gottardo, F., 2009. Main critical factors affecting the welfare of beef cattle and veal calves raised under intensive rearing systems in Italy: A review. *Ital. J. Anim. Sci.* <https://doi.org/10.4081/ijas.2009.s1.67>
- Crowder, M.J., Hand, D.J., 1990. *Analysis of Repeated Measures*. Chapman and Hall, London.
- Curley, K.O., Paschal, J.C., Welsh, T.H., Randel, R.D., 2006a. Technical note: Exit velocity as a measure of cattle temperament is repeatable and associated with serum concentration of cortisol in Brahman bulls. *J. Anim. Sci.* 84, 3100–3103. <https://doi.org/10.2527/jas.2006-055>
- Curley, K.O., Paschal, J.C., Welsh, T.H., Randel, R.D., 2006b. Technical note: Exit velocity as a measure of cattle temperament is repeatable and associated with serum concentration of cortisol in Brahman bulls. *J. Anim. Sci.* <https://doi.org/10.2527/jas.2006-055>
- D’Eath, R.B., Conington, J., Lawrence, A.B., Olsson, I.A.S., Sandøe, P., 2010. Breeding for behavioural

- change in farm animals: Practical, economic and ethical considerations. *Anim. Welf.*
- da Costa, M.J.R.P., 2000. Ambiência na produção de bovinos de corte a pasto. *An. Etol.* 18, 26–42.
- da Costa, M.J.R.P., Costa e Silva, E.V., Chiquitelli Neto, M., Rosa, M.S., 2002. Contribuição dos estudos de comportamento de bovinos para implementação de programas de qualidade de carne. *Encontro Anu. Etol.* 71–89.
- da Costa, M.J.R.P., Sant’Anna, A.C., Silva, L.C.M., 2015. Temperamento de bovinos Gir e Girolando : efeitos genéticos e de manejo. *Inf. Agropecuário, Belo Horiz.* 36, 100–107.
- de Passillé, A.M., Rushen, J., Martin, F., 1995. Interpreting the behaviour of calves in an open-field test: a factor analysis. *Appl. Anim. Behav. Sci.* [https://doi.org/10.1016/0168-1591\(95\)00622-Y](https://doi.org/10.1016/0168-1591(95)00622-Y)
- Doerfler, R.L., Lehermeier, C., Kliem, H., Möstl, E., Bernhardt, H., 2016. Physiological and behavioral responses of dairy cattle to the introduction of robot scrapers. *Front. Vet. Sci.* 3, 1–11. <https://doi.org/10.3389/fvets.2016.00106>
- Eicher, S.D., Schutz, M., Kearney, F., Willard, S., Bowers, S., Gandy, S., Graves, K., 2007. Prepartum milking effects on parlour behaviour, endocrine and immune responses in Holstein heifers. *J. Dairy Res.* 74, 417–424. <https://doi.org/10.1017/S0022029907002695>
- El. Abdel-Hamid, S., Fattah, D.M.A., Ghanem, H.M., Manaa, E.A.A., 2017. Temperament during milking process and its effect on behavioral, productive traits and biochemical parameters in Friesian dairy cows. *Adv. Anim. Vet. Sci.* 5, 508–513. <https://doi.org/10.17582/journal.aavs/2017/5.12.508.513>
- Facó, O., Lôbo, R.N.B., Martins Filho, R., Moura, A.D.A.A., Nonato, R., Lôbo, B., Filho, R.M., 2002. Análise do Desempenho Produtivo de Diversos Grupos Genéticos Holandês x Gir no Brasil 1 Analysis of Productive Performance of Different Holstein x Gir Genetic Groups in Brazil. *Rev. Bras. Zootec.* 31, 1944–1952. <https://doi.org/10.1590/S1516-35982002000800010>
- Fonsêca, V.F.C., Saraiva, E.P., Maia, A.S.C., de Melo Costa, C.C., dos Santos, S.G.C.G., Morais, L.K.C., Fuller, A., Chiquitelli Neto, M., 2019. Training sheep for indirect calorimetry trials. *Small Rumin. Res.* 177, 36–43. <https://doi.org/10.1016/j.smallrumres.2019.06.010>
- Fordyce, G., Goddard, M.E., Seifert, G.W., 1982. The measurement of temperament in cattle and the effect of experience and genotype. *Proc. Aust. Soc. Anim. Prod.*
- Friedrich, J., Brand, B., Schwerin, M., 2015. Genetics of cattle temperament and its impact on livestock production and breeding – a review. *Arch. Anim. Breed.* <https://doi.org/10.5194/aab-58-13-2015>
- Geverink, N.A., Kappers, A., Van De Burgwal, J.A., Lambooij, E., Blokhuis, H.J., Wiegant, V.M., 1998. Effects of Regular Moving and Handling on the Behavioral and Physiological Responses of Pigs to Preslaughter Treatment and Consequences for Subsequent Meat Quality. *J. Anim. Sci.* <https://doi.org/10.2527/1998.7682080x>
- Grandin, T., 1997. Assessment of Stress during Handling and Transport. *J. Anim. Sci.* 75, 249–257. <https://doi.org/10.2527/1997.751249x>
- Grandin, T., 1990. Design of loading facilities and holding pens. *Appl. Anim. Behav. Sci.* 28, 187–201. [https://doi.org/10.1016/0168-1591\(90\)90053-G](https://doi.org/10.1016/0168-1591(90)90053-G)
- Grandin, T., Deesing, M.J., 2014. Genetics and Behavior During Handling, Restraint, and Herding, Second Edi. ed, *Genetics and the Behavior of Domestic Animals*. Elsevier Inc. <https://doi.org/10.1016/B978-0-12-394586-0.00004-4>
- Grandin, T., Shivley, C., 2015. How farm animals react and perceive stressful situations such as handling, restraint, and transport. *Animals* 5, 1233–1251. <https://doi.org/10.3390/ani5040409>
- Grignard, L., Boissy, A., Boivin, X., Garel, J.P., Le Neindre, P., 2000. The social environment influences the behavioural responses of beef cattle to handling. *Appl. Anim. Behav. Sci.* [https://doi.org/10.1016/S0168-1591\(00\)00085-X](https://doi.org/10.1016/S0168-1591(00)00085-X)

- Gruber, S.L., Tatum, J.D., Engle, T.E., Chapman, P.L., Belk, K.E., Smith, G.C., 2010. Relationships of behavioral and physiological symptoms of preslaughter stress to beef longissimus muscle tenderness. *J. Anim. Sci.* <https://doi.org/10.2527/jas.2009-2183>
- Hakan, K., Demirci, S., 2012. Livestock-Handling Related Injuries and Deaths, in: *Livestock Production*. <https://doi.org/10.5772/50834>
- Hall, N.L., Buchanan, D.S., Anderson, V.L., Ilse, B.R., Carlin, K.R., Berg, E.P., 2011. Working chute behavior of feedlot cattle can be an indication of cattle temperament and beef carcass composition and quality. *Meat Sci.* <https://doi.org/10.1016/j.meatsci.2011.03.020>
- Haskell, M.J., Simm, G., Turner, S.P., 2014. Genetic selection for temperament traits in dairy and beef cattle. *Front. Genet.* 5, 1–18. <https://doi.org/10.3389/fgene.2014.00368>
- Hearnshaw, H., Morris, C.A., 1984. Genetic and environmental effects on a temperament score in beef cattle. *Aust. J. Agric. Res.* 35, 723–733. <https://doi.org/10.1071/AR9840723>
- Hemsworth, P.H., Barnett, J.L., 1989. Relationships between fear of humans, productivity and cage position of laying hens. *Br. Poult. Sci.* 30, 505–518. <https://doi.org/10.1080/00071668908417175>
- Hemsworth, P.H., Coleman, G.J., Barnett, J.L., Borg, S., 2000. Relationships between human-animal interactions and productivity of commercial dairy cows. *J. Anim. Sci.* 78, 2821–2831. <https://doi.org/10.2527/2000.78112821x>
- Hooper, H.B., Silva, P. dos S., de Oliveira, S.A., Meringhe, G.K.F., Lacasse, P., Negrão, J.A., 2020. Effect of heat stress in late gestation on subsequent lactation performance and mammary cell gene expression of Saanen goats. *J. Dairy Sci.* 103, 1982–1992. <https://doi.org/10.3168/jds.2019-16734>
- Hötzel, M.J., Machado Filho, L.C.P., Yunes, M.C., Da Silveira, M.C.A.C., 2005. Influência de um ordenhador aversivo sobre a produção leiteira de vacas da raça holandesa. *Rev. Bras. Zootec.* 34, 1278–1284.
- Hunter, P., 2018. The genetics of domestication. *EMBO Rep.* <https://doi.org/10.15252/embr.201745664>
- Inderwies, T., Pfaffl, M.W., Bruckmaier, R.M., 2003. Milking characteristics and their relation to adrenergic receptor mRNA expression and ligand binding in the mammary gland of dairy cows. *Domest. Anim. Endocrinol.* 25, 275–286. [https://doi.org/10.1016/S0739-7240\(03\)00059-6](https://doi.org/10.1016/S0739-7240(03)00059-6)
- Johnsen, J.F., Zipp, K.A., Kälber, T., Passillé, A.M. de, Knierim, U., Barth, K., Mejdell, C.M., 2016. Is rearing calves with the dam a feasible option for dairy farms?—Current and future research. *Appl. Anim. Behav. Sci.* 181, 1–11. <https://doi.org/10.1016/j.applanim.2015.11.011>
- Jones, R.B., 1996. Fear and adaptability in poultry: Insights, implications and imperatives. *Worlds. Poult. Sci. J.* <https://doi.org/10.1079/wps19960013>
- Kasimanickam, R.K., Hall, J.B., Estill, C.T., Kastelic, J.P., Joseph, C., Abdel Aziz, R.L., Nak, D., 2018. Flunixin meglumine improves pregnancy rate in embryo recipient beef cows with an excitable temperament. *Theriogenology* 107, 70–77. <https://doi.org/10.1016/j.theriogenology.2017.10.043>
- Kiley-Worthington, M., de la Plain, S., Kiley-Worthington, M., de la Plain, S., 1983. Fostering and Adoption in Beef Cattle, in: *The Behaviour of Beef Suckler Cattle (Bos Taurus)*. https://doi.org/10.1007/978-3-0348-6782-5_8
- Kling-Eveillard, F., Allain, C., Boivin, X., Courboulay, V., Créach, P., Philibert, A., Ramonet, Y., Hostiou, N., 2020. Farmers' representations of the effects of precision livestock farming on human-animal relationships. *Livest. Sci.* <https://doi.org/10.1016/j.livsci.2020.104057>
- Kolb, D.A., 2015. *Experiential Learning- Experience as the Source of Learning and Development (2nd Edition)*, Person Education, Inc. <https://doi.org/10.1017/CBO9781107415324.004>
- Krohn, C.C., Jago, J.G., Boivin, X., 2001. The effect of early handling on the socialisation of young calves to humans. *Appl. Anim. Behav. Sci.* [https://doi.org/10.1016/S0168-1591\(01\)00161-7](https://doi.org/10.1016/S0168-1591(01)00161-7)
- Kutzer, T., Steilen, M., Gyax, L., Wechsler, B., 2015. Habituation of dairy heifers to milking routine-

- Effects on human avoidance distance, behavior, and cardiac activity during milking. *J. Dairy Sci.* 98, 5241–5251. <https://doi.org/10.3168/jds.2014-8773>
- Lacasse, P., Lollivier, V., Bruckmaier, R.M., Boisclair, Y.R., Wagner, G.F., Boutinaud, M., 2011. Effect of the prolactin-release inhibitor quinagolide on lactating dairy cows. *J. Dairy Sci.* 94, 1302–1309. <https://doi.org/10.3168/jds.2010-3649>
- Lacasse, P., Ollier, S., Lollivier, V., Boutinaud, M., 2016. New insights into the importance of prolactin in dairy ruminants. *J. Dairy Sci.* 99, 864–874. <https://doi.org/10.3168/jds.2015-10035>
- Lammoglia, M., Alarcón, M., Mancera, B., Cabrera, A., Daniel, A., 2015. Effects of Routinely Oxytocin Injection to Induce Milk Ejection on Some Reproductive Parameters of Crossbred Cows in the Tropics of Veracruz. *Annu. Res. Rev. Biol.* 6, 297–303. <https://doi.org/10.9734/arrb/2015/15902>
- Lansade, L., Bertrand, M., Boivin, X., Bouissou, M.F., 2004. Effects of handling at weaning on manageability and reactivity of foals. *Appl. Anim. Behav. Sci.* 87, 131–149. <https://doi.org/10.1016/j.applanim.2003.12.011>
- Laurence, M., Barnes, A., Collins, T., Hyndman, T., Musk, G.C., 2016. Assessing and mitigating post-operative castration pain in *Bos indicus* cattle. *Anim. Prod. Sci.* 58, 909–919. <https://doi.org/10.1071/AN16210>
- Leão, G.F.M., Pivatto, D.R.D., Carniel, H., Rodrigues, M.G.K., Braga, R.A., Silva, M.R.H. da, Teixeira, P.P.M., 2013. Melhoramento Genético Em Zebuínos Leiteiros – Uma Revisão. *Agropecuária Científica No Semiárido*.
- Lee, C., Cafe, L.M., Robinson, S.L., Doyle, R.E., Lea, J.M., Small, A.H., Colditz, I.G., 2018. Anxiety influences attention bias but not flight speed and crush score in beef cattle. *Appl. Anim. Behav. Sci.* 205, 210–215. <https://doi.org/10.1016/j.applanim.2017.11.003>
- Lefcourt, A.M., Kahl, S., Akers, R.M., 1986. Correlation of Indices of Stress with Intensity of Electrical Shock for Cows. *J. Dairy Sci.* 69, 833–842. [https://doi.org/10.3168/jds.S0022-0302\(86\)80473-8](https://doi.org/10.3168/jds.S0022-0302(86)80473-8)
- Lensink, B.J., Boivin, X., Pradel, P., Le Neindre, P., Veissier, I., 2000. Reducing veal calves' reactivity to people by providing additional human contact. *J. Anim. Sci.* 78, 1213–1218. <https://doi.org/10.2527/2000.7851213x>
- Ligout, S., Bouissou, M.F., Boivin, X., 2008. Comparison of the effects of two different handling methods on the subsequent behaviour of Anglo-Arabian foals toward humans and handling. *Appl. Anim. Behav. Sci.* 113, 175–188. <https://doi.org/10.1016/j.applanim.2007.12.004>
- Lima, M.L.P., Negrão, J.A., de Paz, C.C.P., Grandin, T., 2018. Minor corral changes and adoption of good handling practices can improve the behavior and reduce cortisol release in Nellore cows. *Trop. Anim. Health Prod.* 50, 525–530. <https://doi.org/10.1007/s11250-017-1463-9>
- Llouch, P., Somarriba, M., Duthie, C.A., Haskell, M.J., Rooke, J.A., Troy, S., Roehe, R., Turner, S.P., 2016. Association of temperament and acute stress responsiveness with productivity, feed efficiency, and Methane emissions in beef cattle: An observational study. *Front. Vet. Sci.* <https://doi.org/10.3389/fvets.2016.00043>
- Losada-Espinosa, N., Villarroel, M., María, G.A., Miranda-de la Loma, G.C., 2018. Pre-slaughter cattle welfare indicators for use in commercial abattoirs with voluntary monitoring systems: A systematic review. *Meat Sci.* 138, 34–48. <https://doi.org/10.1016/j.meatsci.2017.12.004>
- Lürzel, S., Windschnurer, I., Futschik, A., Waiblinger, S., 2016a. Gentle interactions decrease the fear of humans in dairy heifers independently of early experience of stroking. *Appl. Anim. Behav. Sci.* 178, 16–22. <https://doi.org/10.1016/j.applanim.2016.02.012>
- Lürzel, S., Windschnurer, I., Futschik, A., Waiblinger, S., 2016b. Gentle interactions decrease the fear of humans in dairy heifers independently of early experience of stroking. *Appl. Anim. Behav. Sci.* <https://doi.org/10.1016/j.applanim.2016.02.012>
- Maçal-Pedroza, M.G., Campos, M.M., Pereira, L.G.R., Machado, F.S., Tomich, T.R., da Costa,

- M.J.R.P., Sant'Anna, A.C., 2019. Consistency of temperament traits and their relationships with milk yield in lactating primiparous F1 Holstein - Gyr cows. *Appl. Anim. Behav. Sci.* 112061. <https://doi.org/https://doi.org/10.1016/j.applanim.2019.104881> This
- Macedo, G.G., Zúccari, C.E.S.N., de Abreu, U.G.P., Negrão, J.A., da Costa e Silva, E.V., 2011. Human-animal interaction, stress, and embryo production in *Bos indicus* embryo donors under tropical conditions. *Trop. Anim. Health Prod.* 43, 1175–1182. <https://doi.org/10.1007/s11250-011-9820-6>
- MacKay, J.R.D., Haskell, M.J., Deag, J.M., van Reenen, K., 2014. Fear responses to novelty in testing environments are related to day-to-day activity in the home environment in dairy cattle. *Appl. Anim. Behav. Sci.* <https://doi.org/10.1016/j.applanim.2013.12.008>
- Macuhová, J., Tancin, V., Kraetzl, W.D., Meyer, H.H.D., Bruckmaier, R.M., 2002. Inhibition of oxytocin release during repeated milking in unfamiliar surroundings: The importance of opioids and adrenal cortex sensitivity. *J. Dairy Res.* 69, 63–73. <https://doi.org/10.1017/S0022029901005222>
- Madalena, F.E., Peixoto, M.G.C.D.C.D., Gibson, J., 2012. Dairy cattle genetics and its applications in Brazil. *Livest. Res. Rural Dev.* 24, 1–49. <https://doi.org/http://www.lrrd.org/lrrd24/6/made24097.htm>
- May, T.M., Page, M.J., Fleming, P.A., 2016. Predicting survivors: Animal temperament and translocation. *Behav. Ecol.* 27, 969–977. <https://doi.org/10.1093/beheco/arv242>
- McInerney, J.P., 2004. Animal welfare, economics and policy. *J. R. Agric. Soc. Engl.* 165.
- Menezes, L.D.M., Cardoso, F.F., Barbosa Silveira, I.D., 2017. Temperamento em bovinos de corte: características genéticas, metodologias de mensuração e desempenho. *Rev. Electron. Vet.*
- Mohamed, R.A., Abou-Ismael, U.A., Shukry, M., Elmoslemany, A., Abdel-Maged, M., 2018. Effects of blindfolding and tail bending of Egyptian water buffaloes on their behavioural reactivity and physiological responses to pain induction. *Vet. Anim. Sci.* 5, 38–43. <https://doi.org/10.1016/j.vas.2018.06.001>
- Mota-Rojas, D., Broom, D.M., Orihuela, A., Velarde, A., Napolitano, F., Alonso-Spilsbury, M., 2020. Effects of human-animal relationship on animal productivity and welfare. *J. Anim. Behav. Biometeorol.* <https://doi.org/10.31893/JABB.20026>
- Müller, R., von Keyserlingk, M.A.G., 2006. Consistency of flight speed and its correlation to productivity and to personality in *Bos taurus* beef cattle. *Appl. Anim. Behav. Sci.* <https://doi.org/10.1016/j.applanim.2005.05.012>
- Munksgaard, L., DePassillé, A.M., Rushen, J., Herskin, M.S., Kristensen, A.M., 2001. Dairy cows' fear of people: Social learning, milk yield and behaviour at milking. *Appl. Anim. Behav. Sci.* 73, 15–26. [https://doi.org/10.1016/S0168-1591\(01\)00119-8](https://doi.org/10.1016/S0168-1591(01)00119-8)
- Negrão, J.A., 2008. Hormone release and behavior during suckling and milking in Gir, Gir x Holstein, and Holstein cows. *J. Anim. Sci.* 21–26. <https://doi.org/10.2527/jas.2007-0304>
- Negrão, J.A., Marnet, P.G., 2003. Cortisol, adrenalin, noradrenalin and oxytocin release and milk yield during first milkings in primiparous ewes. *Small Rumin. Res.* 47, 69–75. [https://doi.org/10.1016/S0921-4488\(02\)00247-X](https://doi.org/10.1016/S0921-4488(02)00247-X)
- Negrão, J.A., Marnet, P.G., 2002. Effect of calf suckling on oxytocin, prolactin, growth hormone and milk yield in crossbred Gir ´ Holstein cows during milking João. *Reprod. Nutr. Dev.* 43, 373–380. <https://doi.org/10.1051/rnd>
- Negrao, J.A., Porcionato, M.A. de F., de Passille, A.M., Rushen, J., 2010. Behavioural responses of heifers to ACTH injections. *Appl. Anim. Behav. Sci.* 128, 18–22. <https://doi.org/10.1016/j.applanim.2010.09.008>
- Néri, N.R.M., Toledo, L.M., Acaro Júnior, I., Ambrósio, L.A., 2016. Estimulação tátil em novilhas leiteiras: efeitos no comportamento e na produção de leite após o parto. *Bol. Indústria Anim.* 73,

- 171–179. <https://doi.org/10.17523/bia.v73n3p171>
- Odorčić, M., Rasmussen, M.D., Paulrud, C.O., Bruckmaier, R.M., 2019. Review: Milking machine settings, teat condition and milking efficiency in dairy cows. *Animal* 13, S94–S99. <https://doi.org/10.1017/S1751731119000417>
- Ouellet, V., Negrao, J., Skibieli, A.L., Lantigua, V.A., Fabris, T.F., Marrero, M.G., Dado-Senn, B., Laporta, J., Dahl, G.E., 2021. Endocrine signals altered by heat stress impact dairy cow mammary cellular processes at different stages of the dry period. *Animals* 11, 1–16. <https://doi.org/10.3390/ani11020563>
- Pajor, E.A., Rushen, J., De Passillé, A.M.B., Passille, A.M.B. De, 2000. Aversion learning techniques to evaluate dairy cattle handling practices. *Appl. Anim. Behav. Sci.* 69, 89–102. [https://doi.org/10.1016/S0168-1591\(00\)00119-2](https://doi.org/10.1016/S0168-1591(00)00119-2)
- Paul, H., Coleman, G.J., 2010. Human-livestock interactions: The stockperson and the productivity and welfare of intensively farmed animals, *Human-Livestock Interactions: The Stockperson and the Productivity and Welfare of Intensively Farmed Animals*. <https://doi.org/10.1079/9781845936730.0000>
- Peixoto, M.G.C.D., Pires, M. de F.Á., Pereira, M.C., Ribeiro, M.R.S.C.G.C., Brito, L.F., Verneque, R. da S., Bergmann, J.A.G., 2011. Integrando o temperamento às características de importância para o melhoramento de bovinos de leite : resultados de um estudo com fêmeas Guzerá. *Rev. Bras. Zootec.* 40, 26–37.
- Peters, M.D.P., Barbosa Silveira, I.D., Machado Filho, L.C.P., Machado, A.A., Pereira, L.M.R., 2010. Bem-Estar , Comportamento E Aspectos Produtivos Aversive Management in Dairy Cattle and Effects on Well-Being ,. *Arch. Zootec.* 59, 435–442.
- Petherick, J.C., 2005. Animal welfare issues associated with extensive livestock production: The northern Australian beef cattle industry. *Appl. Anim. Behav. Sci.* 92, 211–234. <https://doi.org/10.1016/j.applanim.2005.05.009>
- Phillips, C., 2002. Cattle Behaviour and Welfare, in: *Cattle Behaviour and Welfare: Second Edition*. <https://doi.org/10.1002/9780470752418>
- Polikarpus, A., Napolitano, F., Grasso, F., Di Palo, R., Zicarelli, F., Arney, D., De Rosa, G., 2014. Effect of pre-partum habituation to milking routine on behaviour and lactation performance of buffalo heifers. *Appl. Anim. Behav. Sci.* 161, 1–6. <https://doi.org/10.1016/j.applanim.2014.10.003>
- Ponchon, B., Zhao, X., Ollier, S., Lacasse, P., 2017. Relationship between glucocorticoids and prolactin during mammary gland stimulation in dairy cows. *J. Dairy Sci.* 100, 1521–1534. <https://doi.org/10.3168/jds.2016-11490>
- Porcionato, M.A.D.F., Negrão, J.A., De Paiva, F.A., Delgado, T.F.G., 2009. Respostas produtivas e comportamentais durante a ordenha de vacas Holandesas em início de lactação. *Acta Sci. - Anim. Sci.* 31, 447–451. <https://doi.org/10.4025/actascianimsci.v31i4.6326>
- Probst, J.K., Hillmann, E., Leiber, F., Kreuzer, M., Spengler Neff, A., 2013. Influence of gentle touching applied few weeks before slaughter on avoidance distance and slaughter stress in finishing cattle. *Appl. Anim. Behav. Sci.* 144, 14–21. <https://doi.org/10.1016/j.applanim.2012.12.007>
- Probst, J.K., Spengler Neff, A., Leiber, F., Kreuzer, M., Hillmann, E., 2012a. Gentle touching in early life reduces avoidance distance and slaughter stress in beef cattle. *Appl. Anim. Behav. Sci.* 139, 42–49. <https://doi.org/10.1016/j.applanim.2012.03.002>
- Probst, J.K., Spengler Neff, A., Leiber, F., Kreuzer, M., Hillmann, E., 2012b. Gentle touching in early life reduces avoidance distance and slaughter stress in beef cattle. *Appl. Anim. Behav. Sci.* <https://doi.org/10.1016/j.applanim.2012.03.002>
- Réale, D., Reader, S.M., Sol, D., McDougall, P.T., Dingemanse, N.J., Sol, D., RMcDougall, P.T., Eader, S.M., 2007. Integrating animal temperament within ecology and evolution. *Biol. Rev.* 82, 291–318. <https://doi.org/10.1111/j.1469-185X.2007.00010.x>

- Rushen, J., De Passillé, A.M.B., Munksgaard, L., 1999. Fear of people by cows and effects on milk yield, behavior, and heart rate at milking. *J. Dairy Sci.* 82, 720–727. [https://doi.org/10.3168/jds.S0022-0302\(99\)75289-6](https://doi.org/10.3168/jds.S0022-0302(99)75289-6)
- Rushen, J., Munksgaard, L., Marnet, P.G., DePassillé, A.M., 2001. Human contact and the effects of acute stress on cows at milking. *Appl. Anim. Behav. Sci.* 73, 1–14. [https://doi.org/10.1016/S0168-1591\(01\)00105-8](https://doi.org/10.1016/S0168-1591(01)00105-8)
- Sánchez-Rodríguez, H.L., Vann, R.C., Youngblood, R.C., Baravik-Munsell, E., Christiansen, D.L., Willard, S., Ryan, P.L., 2013. Evaluation of pulsatility index and diameter of the jugular vein and superficial body temperature as physiological indices of temperament in weaned beef calves: Relationship with serum cortisol concentrations, rectal temperature, and sex. *Livest. Sci.* <https://doi.org/10.1016/j.livsci.2012.10.019>
- Sanger, D.J., Joly, D., 1985. Anxiolytic drugs and the acquisition of conditioned fear in mice. *Psychopharmacology (Berl)*. <https://doi.org/10.1007/BF00428188>
- Sant’Anna, A.C., da Costa, M.J.R.P., 2013. Validity and feasibility of qualitative behavior assessment for the evaluation of Nellore cattle temperament. *Livest. Sci.* 157, 254–262. <https://doi.org/10.1016/j.livsci.2013.08.004>
- Sant’Anna, A.C., da Costa, M.J.R.P., Baldi, F., Rueda, P.M., Albuquerque, L.G., 2012. Genetic associations between flight speed and growth traits in nellore cattle. *J. Anim. Sci.* 90, 3427–3432. <https://doi.org/10.2527/jas.2011-5044>
- Sawa, A., Bogucki, M., Neja, W., Krężel-Czopek, S., 2017. Effect of temperament on performance of primiparous dairy cows. *Ann. Anim. Sci.* 17, 863–872. <https://doi.org/10.1515/aoas-2016-0085>
- Schmied, C., Boivin, X., Waiblinger, S., 2008. Stroking Different Body Regions of Dairy Cows: Effects on Avoidance and Approach Behavior Toward Humans. *J. Dairy Sci.* 91, 596–605. <https://doi.org/10.3168/jds.2007-0360>
- Shahin, M., 2018. The effects of positive human contact by tactile stimulation on dairy cows with different personalities. *Appl. Anim. Behav. Sci.* 204, 23–28. <https://doi.org/10.1016/j.applanim.2018.04.004>
- Silva, L.P., Sant’Anna, A.C., Silva, L.C.M., da Costa, M.J.R.P., 2017. Long-term effects of good handling practices during the pre-weaning period of crossbred dairy heifer calves. *Trop. Anim. Health Prod.* 49, 153–162. <https://doi.org/10.1007/s11250-016-1174-7>
- Soardi, K., Vilanova, M.S., Anami, J.M., Fernandes, R.C., 2017. EFEITO DA CATEGORIA ANIMAL NO COMPORTAMENTO EM SALA DE ORDENHA E NA PRODUÇÃO DE LEITE. *Rev. Científica Rural* 19, 184–194.
- Sutherland, M.A.A., Huddart, F.J.J., 2012. The effect of training first-lactation heifers to the milking parlor on the behavioral reactivity to humans and the physiological and behavioral responses to milking and productivity. *J. Dairy Sci.* 95, 6983–6993. <https://doi.org/10.3168/jds.2011-5211>
- Svennersten-Sjaunja, K., Olsson, K., 2005. Endocrinology of milk production. *Domest. Anim. Endocrinol.* 29, 241–258. <https://doi.org/10.1016/j.domaniend.2005.03.006>
- Tancin, V., Harcek, L., Broucek, J., Uhrincat, M., Mihina, S., 1995. Effect of Suckling During Early Lactation and Changeover to Machine Milking on Plasma Oxytocin and Cortisol Levels and Milking Characteristics in Holstein Cows. *J. Dairy Res.* 62, 249–256. <https://doi.org/10.1017/S0022029900030958>
- Tancin, V., Kraetzl, W.D., Schams, D., Bruckmaier, R.M., 2001. The effects of conditioning to suckling, milking and of calf presence on the release of oxytocin in dairy cows. *Appl. Anim. Behav. Sci.* 72, 235–246. [https://doi.org/10.1016/S0168-1591\(01\)00113-7](https://doi.org/10.1016/S0168-1591(01)00113-7)
- Tarantola, M., Biasato, I., Biasibetti, E., Biagini, D., Capra, P., Guarda, F., Leporati, M., Malfatto, V., Cavallarin, L., Miniscalco, B., Mioletti, S., Vincenti, M., Gastaldo, A., Capucchio, M.T., 2020. Beef cattle welfare assessment: use of resource and animal-based indicators, blood parameters and

- hair 20 β -dihydrocortisol. *Ital. J. Anim. Sci.* <https://doi.org/10.1080/1828051X.2020.1743783>
- Ujita, A., El Faro, L., Vicentini, R.R., Pereira Lima, M.L., de Oliveira Fernandes, L., Oliveira, A.P., Veroneze, R., Negrão, J.A., 2021. Effect of positive tactile stimulation and prepartum milking routine training on behavior, cortisol and oxytocin in milking, milk composition, and milk yield in Gyr cows in early lactation. *Appl. Anim. Behav. Sci.* <https://doi.org/10.1016/j.applanim.2020.105205>
- Ujita, A., Ribeiro Vicentini, R., Pereira Lima, M.L., Negrão, J.A., de Oliveira Fernandes, L., Penido Oliveira, A., Veroneze, R., El Faro Zadra, L., 2020. Improvements in the behaviour of Gir dairy cows after training with brushing. *J. Appl. Anim. Res.* <https://doi.org/10.1080/09712119.2020.1754217>
- Van Reenen, C.G., Engel, B., Ruis-Heutinck, L.F.M., Van Der Werf, J.T.N., Buist, W.G., Jones, R.B., Blokhuis, H.J., 2004. Behavioural reactivity of heifer calves in potentially alarming test situations: A multivariate and correlational analysis. *Appl. Anim. Behav. Sci.* <https://doi.org/10.1016/j.applanim.2003.09.007>
- Van Reenen, C.G., Van der Werf, J.T.N., Bruckmaier, R.M., Hopster, H., Engel, B., Noordhuizen, J.P.T.M., Blokhuis, H.J., 2002. Individual differences in behavioral and physiological responsiveness of primiparous dairy cows to machine milking. *J. Dairy Sci.* 85, 2551–61. [https://doi.org/10.3168/jds.S0022-0302\(02\)74338-5](https://doi.org/10.3168/jds.S0022-0302(02)74338-5)
- Vicentini, R.R., Montanholi, Y.R., Veroneze, R., Oliveira, A.P., Lima, M.L.P., Ujita, A., El Faro, L., 2020. Infrared thermography reveals surface body temperature changes during proestrus and estrus reproductive phases in Gyr heifers (*Bos taurus indicus*). *J. Therm. Biol.* 92, 102662. <https://doi.org/10.1016/j.jtherbio.2020.102662>
- Waiblinger, S., Boivin, X., Pedersen, V., Tosi, M.V., Janczak, A.M., Visser, E.K., Jones, R.B., 2006. Assessing the human-animal relationship in farmed species: A critical review. *Appl. Anim. Behav. Sci.* 101, 185–242. <https://doi.org/10.1016/j.applanim.2006.02.001>
- Wang, S., Zhang, H., Tian, H., Chen, X., Li, S., Lu, Y., Li, L., Wang, D., 2020. Alterations in vaginal temperature during the estrous cycle in dairy cows detected by a new intravaginal device—a pilot study. *Trop. Anim. Health Prod.* <https://doi.org/10.1007/s11250-020-02199-5>
- Weaver, S.R., Bohrer, J.C., Prichard, A.S., Perez, P.K., Streckenbach, L.J., Olson, J.M., Cook, M.E., Hernandez, L.L., 2016. Serotonin deficiency rescues lactation on day 1 in mice fed a high fat diet. *PLoS One* 11, 1–18. <https://doi.org/10.1371/journal.pone.0162432>
- Wicks, H.C.F., Carson, A.F., McCoy, M.A., Mayne, C.S., 2004. Effects of habituation to the milking parlour on the milk production and reproductive performance of first calving Holstein-Friesian and Norwegian dairy herd replacements. *Anim. Sci.* 78, 345–354.
- Windschnurer, I., Barth, K., Waiblinger, S., 2009. Can stroking during milking decrease avoidance distances of cows towards humans?, in: *Animal Welfare*.
- Wulff-Risner, L., Stewart, B., 1997. Using Experiential Learning To Teach Evaluation Skills. *J. Agric. Educ.* <https://doi.org/10.5032/jae.1997.03043>
- X Boivin, Lensink, J., Tallet, C., Veissier, I., 2003. Report on stockmanship and farm animal welfare [WWW Document]. *Anim. Welf.* URL <https://www.gov.uk/government/publications/fawc-report-on-stockmanship-and-farm-animal-welfare>
- Zliobaite, I., 2019. Revisiting biogeography of livestock animal domestication. *bioRxiv*. <https://doi.org/10.1101/786442>

4. HABITUATION PROTOCOLS IMPROVE BEHAVIORAL AND PHYSIOLOGICAL RESPONSES OF BEEF CATTLE EXPOSED TO STUDENTS IN AN ANIMAL HANDLING CLASS



animals



Article

Habituation Protocols Improve Behavioral and Physiological Responses of Beef Cattle Exposed to Students in an Animal Handling Class

Aska Ujita ¹, Zachary Seekford ², Michelle Kott ², Guillermo Goncherenko ³, Nicholas W. Dias ², Erica Feuerbacher ², Luciana Bergamasco ², Leonie Jacobs ², Dan E. Eversole ², João A. Negrão ¹ and Vitor R. G. Mercadante ^{2,4,*}

¹ Basic Science Department, Faculty of Animal Science and Food Engineering, University of São Paulo, Pirassununga, SP 13635-900, Brazil; aska_ujita@hotmail.com (A.U.); jnegrao@usp.br (J.A.N.)

² Department of Animal and Poultry Sciences, College of Agriculture and Life Sciences, Virginia Tech, Blacksburg, VA 24060, USA; zseekford@ufl.edu (Z.S.); mkott@vt.edu (M.K.); nichw16@vt.edu (N.W.D.); enf007@vt.edu (E.F.); lucianab@vt.edu (L.B.); jacobs1@vt.edu (L.J.); deversol@vt.edu (D.E.E.)

³ Polo Agroflorestal CENUR-Noroeste, Facultad de Veterinaria, Universidad de la Republica, Melo, CL 91500, Uruguay; guillermogoncherenko@gmail.com

⁴ Department of Large Animal Clinical Sciences, VA-MD College of Veterinary Medicine, Virginia Tech, Blacksburg, VA 24060, USA

* Correspondence: vrgm@vt.edu

SIMPLE SUMMARY

Students in agricultural programs have the opportunity to interact with animals during different teaching activities. However, students' interactions with livestock may be distressing to the animals and can affect the students' and animals' safety. We investigate whether two human-animal habituation strategies, one with positive tactile stimulation and one without, would improve behavioral and physiological responses of beef heifers during a livestock handling class. Overall, heifers that received a habituation treatment had more positive behavior responses, and decreased physiological stress responses in comparison to heifers that were not exposed to habituation. Furthermore, the heifers exposed to the habituation with a positive tactile stimulation had the greatest improvements in behavior in comparison to control and non-stimulated heifers, exhibiting more positive behaviors when interacting with humans. Strategies to habituate cattle to human interaction with positive stimulation aligned with training humans that handle and interact with cattle on best practices and cattle behavior can improve behavior, reduce stress-related physiological responses and enhance safety for both humans and animals.

ABSTRACT

Our objective was to determine the impact of different habituation protocols on beef cattle behavior, physiology, and temperament in response to human handling. Beef heifers were exposed to three habituation strategies: (1) tactile stimulation (brushing) in the working chute for seven consecutive days (STI; $n = 18$); (2) passage through the working chute for seven consecutive days (CHU; $n = 19$) and; (3) no habituation (CON; $n = 19$). Individual heifer respiratory rate (RR; n/min), internal vaginal temperature (VAGT; °C), and blood cortisol were measured. Further, behavior parameters were observed to generate a behavior score, and heifer interaction with students and their behavioral responses were recorded. Habituation with STI and CHU resulted in improved numerical behavior scores compared to CON, and greater ($p \leq 0.05$) handling latencies. Vaginal temperature was decreased in STI compared to CHU and CON ($p \leq 0.05$). Cortisol concentration did not differ among treatments but decreased ($p \leq 0.05$) from the start of the experiment to 14 days after treatment initiation. Both habituation protocols showed benefits, but heifers that received the positive tactile stimulation in the chute had the greatest behavior improvements. Furthermore, these heifers responded more calmly during student-animal interactions in class, which is beneficial for the students' and animals' safety.

Keywords: beef cattle; behavior; habituation; human handling; cortisol; positive stimulation

4.1. INTRODUCTION

Cattle behavior is the result of an interaction between genetic and environmental factors (Réale et al., 2007), thus it is important to develop efficient handling techniques that reinforce positive cattle behavior and low reactivity to humans. Accordingly, the adoption of positive handling practices may prevent the animal from associating humans with a negative experience, especially when introduced at young age. Animals that experience a positive human-animal interaction earlier in life are prone to be less reactive and more accustomed to human handling (Ujita et al., 2020). Thus, habituating young animals could result in a long-term positive human-animal relationship (Haskell et al., 2014; Probst et al., 2013, 2012a).

Human-animal interaction is a major factor when dealing with animals, frequently observed during any kind of handling. In addition to an agricultural setting, cattle are used in educational institutions, such as universities with agricultural programs. These educational institutions may use cattle for experiential learning and as a source of income. Experiential learning activities are important to strengthen students' cognitive learning as well as development and practice of animal handling techniques (Kolb, 2015; Wulff-Risner and Stewart, 1997).

Students in agricultural programs have the opportunity to interact with animals during different teaching activities. However, students' interactions with livestock, including cattle may be distressing and difficult due to students' lack of experience and knowledge of animal behavior, and infrequent exposure and interaction of humans and animals to each other. Therefore, student-herd interactions may result in negative and dangerous experiences for both humans and animals. Cattle may respond with undesirable or aversive behaviors when faced with a new or challenging situation (Breuer et al., 2003; Haskell et al., 2014; Munksgaard et al., 2001), such as student interactions. These negative interactions may lead to increased labor costs, injuries or fatal accidents (Brouček et al., 2008), as well as decreased profits due to reduced performance and poor product quality.

Novelty it is a potentially stressful stimulus that can impact animals negatively (Doerfler et al., 2016). Cattle that are infrequently handled, such as in teaching herds, show both behavioral and physiological stress responses to a handler. Previous research showed that a combination of brushing or stroking, a conversation, and affectionate gestures from humans directed to the animals results in positive animal behavior and also easier handling (Becker and Lobato, 1997; Lürzel et al., 2016a; Probst et al., 2013, 2012a; Silva et al., 2017; Ujita et al., 2021, 2020). Such strategies could also be applied at educational institutions, safeguarding both animal productivity and animal welfare. However, this has not yet been researched in an educational context, with students interacting with young cattle.

The study objective was to determine the impact of different habituation protocols on beef cattle behavior, physiology, and temperament in response to chute handling and human interaction. We hypothesized that European (*Bos taurus taurus*) beef heifers in a teaching herd that received the positive tactile stimulation as a habituation to chute handling and human interactions during a student halter break class, would show more docile behavior when interacting with students (handling, touching and approach), compared to those heifers without habituation or those only habituated to the chute without tactile stimulation.

4.2. MATERIALS AND METHODS

The study (Institutional Animal Care and Use Committee (IACUC) number 19-215), was conducted at the Virginia Tech Beef Cattle Center in Blacksburg (VA, USA), cattle of Angus, Simmental, Hereford and Charolais breeds were enrolled in the study. Prior to the initiation of the study, all animals were maintained on a single pasture and fed ad libitum with

silage and hay and had limited human interaction, except in standard farm managements: such as parturition, and pre-weaning weighing and vaccination.

4.2.1. Experimental treatments

Cattle enrolled in this study were part of a halter breaking and cattle handling class. Three weeks prior to the start of the class (day 21), 56 heifers from the university teaching herd (12 ± 2 months of age and 398.70 ± 5.08 kg body weight) were first scored in the chute according to the methods described in Table 1 and the individual measures were summed into a final score. These final scores were classified as high, medium and low reactivity, and cattle from each reactivity class were then randomly assigned to one of three human-animal interaction treatments: (1) habituation group with tactile stimulation (brushing) in the working chute (Stimulated group—STI, $n = 18$) during seven consecutive days; (2) habituation group with passage through the working chute (Chute group—CHU, $n = 19$) during seven consecutive days and; (3) a control group with no habituation, which represents the standard procedure for the teaching herd (Control group—CON, $n = 19$).

Figure 1 shows a timeline of the trial. At day 21, all animals were scored for reactivity classification (Table 1). Habituation treatments took place starting 14 days prior to the scheduled student-animal interactions (halter breaking practice class: HBPC, Day 0).

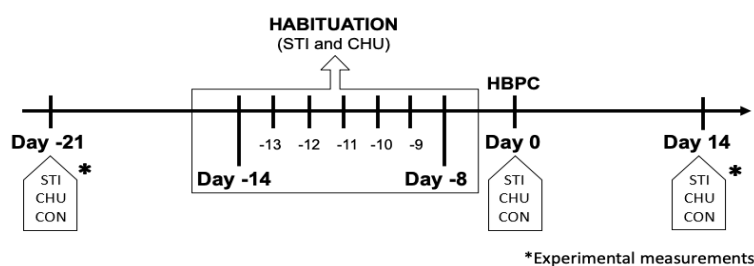


Figure 1. Timeline of the experiment activities at the Beef Cattle Center, relative to the start of the halter breaking practice class (HBPC). The habituation occurred on seven consecutive days Day -14 to -8) for the chute (CHU) and stimulated (STI) group. The Control group (CON) did not receive any habituation or handling prior to HBPC.

4.2.1.1. Tactile stimulation (sti group)

The tactile stimulation treatment was repeated for seven consecutive days (day 14 to day 8), applied once a day starting at 8:00 AM, for 10 min per animal. Heifers were guided to the working chute and were held in between the squeeze chute and the crowd box stall (Figure 2), with the sliding gates closed. Four animals were habituated simultaneously by four trained

people. The tactile stimulation consisted of brushing and touching the animal with a long handle broom, short handle broom, and hands one method at a time and equally distributed during the 10 min period. After all the STI animals received their stimulation, the herd was returned to their respective pasture.

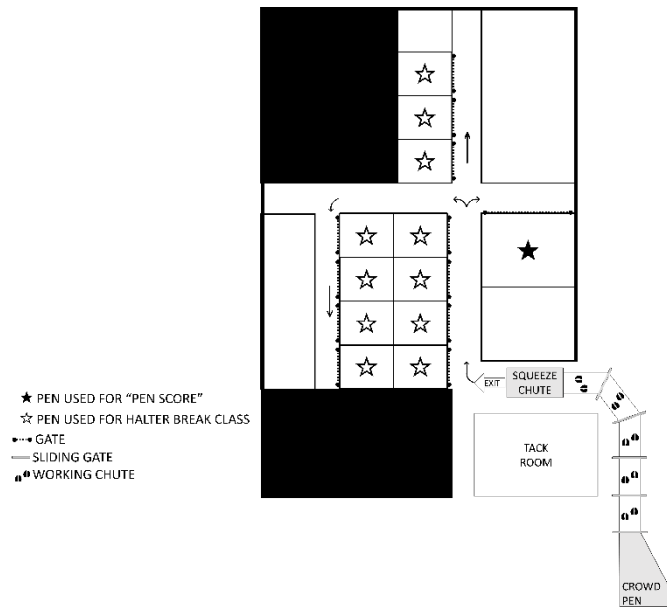


Figure 2. Corral layout at the university's Beef Cattle Center. The arrows represent the direction that heifers were moved in, from the squeeze chute to the box stall used for the pen score (each F or ☆ represents one box stall). Cattle received tactile stimulation in the first 4 working chutes.

4.2.1.2. *Passage through the chute (chu group)*

The chute treatment consisted of heifers being habituated to the squeeze chute (Figure 2). The cattle were guided to the chute and were allowed to walk through the working and squeeze chutes without restraint or any human interaction, taking less than one minute per animal. The herd were moved as a group in the working facility, but individually moved through the working chute and squeeze chute. After all CHU animals completed their habituation, the herd was returned to their respective pasture. This activity occurred on the same days as the STI group habituation and was repeated for seven consecutive days (day 14 to day 8). Chronologically, CHU animals received their treatments after the STI group on all 7 days.

4.2.1.3. *Control (con group)*

The animals from the CON treatment were not handled until the halter breaking practice class (HBPC) on day 0. In other words, they were kept on pasture throughout the habituation period of STI and CHU groups (day 14 to day 8) and did not undergo any human-

animal interactions that could be considered habituation to handling or to the chute.

4.2.2. Halter breaking practice class (HBPC)

Forty-nine students were enrolled in the “Livestock Management and Handling Laboratory” class (APSC 2114) with the objective of learning through hands-on activities safety in livestock handling, animal behavior, care, housing, and managerial practices related to beef cattle, sheep and swine. Students provided prior consent to participate in the study and be recorded (Institutional Review Board (IRB) protocol number 19-933).

For the HBPC, each student needs to work with one animal. Students were randomly paired with one of the initial 56 heifers enrolled in the study resulting in 17 heifer-student pairs from CHU, and 16 pairs from both STI and CON treatments.

Animal behavioral responses to student handling were collated from video footage recorded with monitoring cameras (IP Bullet camera FLPB133F, FLIR Systems Inc., OR, USA). Human behavior was not scored but rather the animals’ reactivity to the handling. Measurements (Table 2) were taken during four stages of student-animal interactions:

- 1) Stage 1: the student brought the animal through the working chute and into the squeeze chute for rope halter placement, which was performed by the student.
- 2) Stage 2: the student trailed the animal from the squeeze chute to the respective box stall (Figure 1), allowing the animal to move freely (dragging the halter’s rope without any tension from the student).
- 3) Stage 3: the student tied the animal to the fence inside a box stall as part of halter habituation. The student did not interact with the heifer for 5 min. Thereafter, the student scratched the animal with a long wooden stick with a small hook at the tip (often called a show stick). The scratching continued during the remainder of the class, but only the first two minutes were evaluated.
- 4) Stage 4: After being haltered for approximately 30 min, the student led the animal to exit the box stall and enter the pasture.

4.2.3. Experimental measurements

All data were collected in the squeeze chute in the mornings on days 21 and 14 (Figure 2).

4.2.3.1. *Respiratory Rate and Vaginal Internal Temperature*

Respiratory rate (RR; n/min) and internal vaginal temperature (VAGT; °C) were collected from animals while they were restrained in the squeeze chute. The RR was measured by counting the flank breathing movements for 15 s, then multiplying it by four to obtain the respiratory rate per minute. The VAGT was measured by placing a conventional thermometer (Digital Thermometer KD-1760, 180 INNOVATIONS, Denver, CO, USA) in the heifer's vagina until the temperature stabilized. For the STI treatment group, these measurements were collected in the squeeze chute right after tactile stimulation in the working chute.

4.2.3.2. *Cortisol concentration in blood plasma*

Immediately as the heifer entered the squeeze chute and was restrained blood samples were taken on days 21 and 14 from the coccygeal vein using vacuum collecting tubes (Vacutainer®, 10 mL; Becton Dickinson, Franklin Lakes, NJ, USA) containing sodium heparin (148 USP units), placed immediately on ice for < 4 h, and centrifuged at 2400 g for 15 min at room temperature. Plasma was collected and stored at 20 °C until further analysis. Plasma cortisol concentrations were determined in duplicates by using a chemiluminescence assay (Immulite 2000 Xpi, Siemens Medical Solutions, Princeton, NJ, USA) with an intra and inter-assay CV of 5.3% and 4.8%, respectively.

4.2.3.3. *Behavioral parameters in the working chute*

Behavioral parameters are defined in Table [1](#) and were assessed through direct observation from the moment heifers entered the working chute until they exited the squeeze chute (Figure [1](#)) on days 21 and 14. Balking during squeeze chute entry (BALK_ENT), velocity score to enter the squeeze chute (VEL_ENT) were determined as well as locomotion from working chute to squeeze chute and exit (LOC) and squeeze chute exit behavior as balking (BALK_EXT), velocity score to enter the squeeze chute (VEL_EXT) and latency to enter squeeze chute (LAT_ENT). On days of blood collection in the squeeze chute, behavior score was evaluated during restraint (BC)

Flight time (FT, sec/m) was measured as time taken to pass two pairs of infrared sensors (Wireless Electric Eyes, FarmTek Inc., North Wylie, TX, USA, Figure 3). The flight time was automatically recorded for each heifer as it exited the squeeze chute (Lee et al., 2018). The latency to enter the working chute (sec) was recorded from videos taken, using a predetermined point of reference as starting point located at the first sliding gate after the crowd

pen.

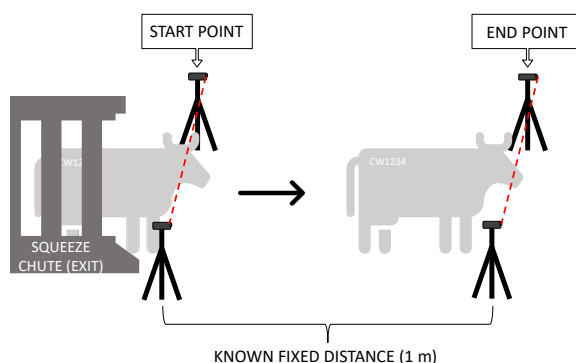


Figure 3. Flight time measurement. The sensors were placed on tripods, one sensor in each side (left and right) of the exit the way that they are parallel and facing each other. One pair of sensors (start point) were placed right after the headgate of squeeze chute (exit). The second pair (end point) were placed 1m in front of the first pair of sensors.

4.2.3.4. Behavioral measurements in the holding pen

Pen score (PS) methodology was adapted (Burdick Sanchez et al., 2014) and executed as shown in Table 2. After exiting the squeeze chute, the heifer was brought to a box stall individually, and scored for reactivity to a human presence and approach. In the first minute, the human would stand still and assign score 1 if the heifer approached voluntarily (Table 2). Thereafter, the human approached the heifer calmly, moving at approximately 0.7m/sec, to assign the other behavior scores (2 to 7). The procedure was executed in pairs: one person inside the pen, and another outside the box stall assigning the scores. All animals were scored by the same two observers.

4.2.4. Statistical analysis

Data analysis was performed using SAS 9.4 (SAS Institute, INC., Cary, NC, USA). We used a randomized block design. The treatments (STI, CHU, CON), sampling day (days -21 and 14) and their interaction were used as fixed effects. The data were evaluated as repeated measures over time (sampling day). The covariance matrices were tested for each trait and the structure showing the best fit was used (Crowder and Hand, 1990). Each animal was considered the experimental unit. When differences between treatments were found, a comparison of means by the t-test was performed, with a significance of 5%. No breed effect was found, but this factor was maintained in the model as a random effect for the best representation of the experimental design. The delta values (Δ) of behavioral parameters, RR, VAGT and cortisol over time were calculated as an additional response variable and were analyzed the same as the other response variables. The results were organized in two sections: a comparison between

treatments (STI vs CHU vs CON groups) before and after habituation protocols (days –21 and 14) to determine the treatment effect on behavioral and physiological parameters over time; and a comparison between treatments (STI vs CHU vs CON groups) to determine the habituation effect on behavioral parameters during HBPC.

Table 1. Animal's behavioral measurements from the chute entrance to chute exit measured on days –21 and 14 in the chute facility.

Moment	Parameter	Score	Animal's Behavior Description	Reference
Working chute entry	Balk (BALK_ENT)	1	Entered voluntarily when the gate opens	Adapted from (Laurence et al., 2016; Ujita et al., 2020)
		2	Entered with a voice command or light tap on the rump	
		3	A hard slap on the rump or tail twisting was required to induce the animal to enter	
		4	The animal did not enter with the techniques described above	
	Velocity (VEL_ENT)	1	Walking—Defined as a four-beat gait characterized by progression of the alternate lateral legs, i.e., each hoof takes off from and strikes the ground independently	(Lima et al., 2018)
		2	Trotting—Defined as a two-beat diagonal gait in which the legs move in diagonal pairs, but not quite simultaneously	
		3	Running—Defined as a three-beat gait in which the front hooves strike the ground, one after the other in a fast manner, followed by the rear hooves	
	Latency (LAT_ENT)	*	Time to enter the chute, having the body completely inside the chute	Adapted from (Maçãl-Pedroza et al., 2019)
From working chute to exit	Locomotion (LOC)	1	Calm (quiet tail, eyes and ears), walked slowly without problem or balking	Adapted from (Kasimanickam et al., 2018; Laurence et al., 2016)
		2	Calm (quiet tail, eyes and ears), walked slowly and balks sometimes	
		3	Calm (quiet tail, eyes and ears), walked fast without problem or balking	
		4	Calm (quiet tail, eyes and ears), walked fast and balks sometimes	
		5	Excited (restless tail, eyes and ears), walked slowly without problem or balking	
		6	Excited (restless tail, eyes and ears), walked slowly and balks sometimes	
		7	Excited (restless tail, eyes and ears), walked fast without problem or balking	
		8	Excited (restless tail, eyes and ears), walked fast and balks sometimes	
During blood sampling in squeeze chute **	Restraint (BC)	1	Animal remained calm, with no movement of body or hooves, during blood sampling	(Lima et al., 2018)
		2	Animal moved its head and body gently during blood sampling	
		3	Animal moved a lot, including movement of the hooves, and gently displaced the body	
		4	Animal moved briskly, shaking the body a lot, and moving the hooves simultaneously	
		5	Was struggling to escape—Animal was panicky or infuriated and struggles all the time to escape from the restraint during blood sampling	
Squeeze chute exit	Balk (BALK_EXT)	1	Exited voluntarily when the gate opens	Adapted from (Laurence et al., 2016; Ujita et al., 2020)
		2	Exited with a voice command or light tap on the rump	
		3	A hard slap on the rump or tail twisting was required to induce the animal to exit	
		4	The animal did not exit with the techniques described above	
	Velocity (VEL_EXT)	1	Walking—Defined as a four-beat gait characterized by progression of the alternate lateral legs, i.e., each hoof takes off from and strikes the ground independently	(Lima et al., 2018)
		2	Trotting—Defined as a two-beat diagonal gait in which the legs move in diagonal pairs, but not quite simultaneously	
		3	Running—Defined as a three-beat gait in which the front hooves strike the ground, one after the other in a fast manner, followed by the rear hooves	
	Flight time (FT)	***	Time taken to cut two pairs of electric eyes recorded electronically in the chute exit	(Lee et al., 2018)

* Was from the videos recorded during the experiment; ** Evaluated at the time of blood collection; *** Was measured in real-time by infrared sensors.

Table 2. Behavioral measurements of the animals during the human-animal interaction in handling on the first day of halter breaking habituation.

Human-Animal Interaction Description	Parameter	Score	Animal's Behavior Description
Student halted the heifer in the squeeze chute	Reaction to haltering	1	Calm, easy to halter the animal
		2	Excited (alert), but still, being easy to halter the animal
		3	Excited (alert), moving the head gently away, but easy to halter
		4	Excited (alert), moving the head gently away, but difficult to halter
		5	Excited (alert), moving the head abruptly away, but easy to halter
		6	Excited (alert), moving the head abruptly away, but difficult to halter
Student conducted the animal from the squeeze chute to the pen	Velocity when leaving the squeeze chute	1	Walking calmly
		2	Walking excited or fast
		3	Trotting or running
	Velocity when entered the pen	1	Walking calmly
		2	Walking excited or fast
		3	Trotting or running
Student tied the halter's rope of the animal to the pen's fence	Reaction during first 5 min after tied	1	Standing still
		2	Pulling few times for the first seconds
		3	Pulling few times for a long time, but pulled less than 5 times
		4	Pulling and was struggling to escape all the time
		5	Pulling and was struggling to escape all the time; laying or turning the neck but not necessary to untie
		6	Pulling and was struggling to escape all the time; laying or turning the neck being necessary to untie
	Pulling behavior	-	How many times the animal pulled the rope during five minutes observation
Student started the tactile stimulation with the show stick	Reaction during two minutes when scratching started	1	Calm
		2	Excited (wagging the tail and/or moving the body) during approach, but soon calmed down
		3	Excited, wagging the tail all the time, but standing still
		4	Excited, wagging the tail, displacing the body once or twice
		5	Excited, wagging the tail, displacing the body all the time
Student conducted the animal from the box stall to the pasture field	Velocity when left the pen	1	Walking calmly
		2	Walking excited or fast
		3	Trotting or running

Table 2. Pen score methodology adapted from (Burdick Sanchez et al., 2014).

Score	Animal Behavior Description
1	Very docile, approached the observer by itself (measured in the first minute after entering the pen).
2	Docile, did not react to the observer movements and allowed him/she to approach, even touch.
3	Docile, did not react to the observer movements and allowed him/she to approach but not to touch.
4	Slightly flighty, was aware of the observer and likely stand in a corner away from the observer.
5	Moved away from the observer and run with a raised head alongside the fence, fully aware of the observer's position.
6	Flighty and was aware of the observer, may run along the fence or even run into gates or fences.
7	Very flighty, often called 'crazy' and often run at gates, fences and humans in an attempt to exit the pen.

4.3. RESULTS

4.3.1. Behavioral indicators

The effect of treatment (STI, CHU, CON), day of sampling and their interaction on behavioral and physiological parameters are presented in Table 3.

Habituation with tactile stimulation (STI) resulted in improved numerical behavior scores (BALK_ENT, LAT_ENT, VEL_ENT, LOC, BALK_EXT, FT and PS) compared to the habituation without tactile stimulation (CHU) but only LAT_ENT and BC were significant ($p < 0.05$). Control heifers had worse (greater) values compared to habituated heifers on VEL_ENT, LOC, BC, VEL_EXT, and PS. Latency to enter the working chute (LAT_ENT) and reactivity during BC differed among habituation treatment groups ($p < 0.05$), with shorter latencies in the CON treatment compared to STI and CHU, and greater scores for BC compared to STI and CHU (Table 3).

Reactivity improved on day 14 compared to day 21 (BALK_ENT, VEL_ENT, BC, BALK_EXT, VEL_EXT and PS; $p < 0.05$). In contrast, LAT_ENT was longer on day 14 compared to day 21 ($p < 0.05$).

The interaction between treatment and sampling day showed a significant effect on BALK_ENT and VEL_EXT ($p < 0.05$) (Table 3; Figure 4A,C), starting with similar values on day 21 (Figure 4) and showing a significant decrease on day 14, except for the VEL_EXT duration of the CON group (Figure 4C). The delta values (Figure 4D,F) showed that the STI

group had the greatest decrease compared to CHU and CON groups, with lesser values (estimates and delta) and significant difference ($p \leq 0.05$), except on exit velocity, which differed only from CON group ($p \leq 0.05$).

4.3.2. Physiological parameters

Treatments affected VAGT ($p 0.05$), with reduced temperatures in the STI group compared to CHU and CON (Table 3). There was an effect of day, but not habituation treatment, on VAGT, RR and CORT with lesser values on day 14 compared to day 21 (Table 3; $p 0.05$). VAGT was affected by treatment depending on sampling day ($p 0.05$; Table 3), with treatments groups showing similar temperatures on day 21 (Figure 4B) and a greater temperature decrease in STI compared to CHU and CON on day 14 (Figure 4F).

Table 3. Effects of treatment, day of sampling and their interaction on behavioral and physiological parameters of *Bos taurus* beef cattle (n = 56).

Trait	Unit	STI (n = 18)	CHU (n = 19)	CON (n = 19)	DAY -21 (n = 56)	DAY 14 (n = 56)	p-Value		
							TRT	DAY	TRT * DAY
BALK_ENT	Score	1.50 ± 0.09	1.55 ± 0.08	1.55 ± 0.08	1.82 ± 0.08	1.25 ± 0.05	0.88	<0.0001	0.01
LAT_ENT	Seconds	2.03 ± 0.26 a	2.99 ± 0.24 b	1.92 ± 0.26 a	2.00 ± 0.18	2.63 ± 0.24	0.01	0.04	0.24
VEL_ENT	Score	1.06 ± 0.07	1.13 ± 0.07	1.16 ± 0.07	1.23 ± 0.05	1.00 ± 0.05	0.54	<0.01	0.54
LOC	Score	4.69 ± 0.33	4.71 ± 0.32	5.45 ± 0.32	4.69 ± 0.27	5.21 ± 0.27	0.18	0.17	0.13
RR	Respiratory cycles/min	44.00 ± 1.53	43.80 ± 1.51	46.63 ± 1.49	47.02 ± 1.24	42.60 ± 1.23	0.34	0.01	0.35
VAGT	°C	38.83 ± 0.06 a	39.10 ± 0.06 b	38.89 ± 0.06 a	39.04 ± 0.05	38.84 ± 0.05	<0.01	<0.01	0.01
BC	Score	1.72 ± 0.16 a	1.24 ± 0.16 b	1.87 ± 0.16 a	1.93 ± 0.17	1.29 ± 0.07	0.02	<0.01	0.62
CORT	µg/dL	1.94 ± 0.20	1.94 ± 0.19	2.34 ± 0.19	2.63 ± 0.16	1.52 ± 0.10	0.25	<0.0001	0.37
BALK_EXT	Score	1.19 ± 0.10	1.21 ± 0.10	1.18 ± 0.10	1.32 ± 0.10	1.07 ± 0.03	0.98	0.02	0.64
VEL_EXT	Score	1.42 ± 0.15	1.34 ± 0.15	1.45 ± 0.15	1.52 ± 0.10	1.28 ± 0.10	0.88	0.02	0.01
FT	Sec/m	1.42 ± 0.13	1.84 ± 0.14	1.58 ± 0.14	1.52 ± 0.11	1.71 ± 0.11	0.08	0.25	0.27
PS	Score	4.11 ± 0.17	4.24 ± 0.16	4.34 ± 0.16	4.57 ± 0.14	3.89 ± 0.14	0.62	<0.01	0.21

LSMeans ± standard error and p value. BALK_ENT (score): animal's balking behavior when entering the working chute; LAT_ENT (seconds): latency to enter completely inside the working chute (starting the timer when the animal's nose passed one point and ending the timer when the animal's body passed that point completely); VEL_ENT (score): animal's walking velocity score to enter the working chute; LOC (score): animal's locomotion score from the working chute to squeeze chute; RR: number of respiratory cycle during one minute; VAGT (°C): vaginal temperature; BC (score): animal's reactivity score during blood sampling; CORT (µg/dL): cortisol concentration in blood plasma; VEL_EXT (score): animal's walking velocity score to exit the squeeze chute; FT (s/m): animal's flight speed to exit the squeeze chute; PS (score): animal's reactivity score in

human presence and his approach inside the pen; STI: stimulated group; CHU: chute group; CON: control group; Day -21: 21 days before the halter breaking practice class and 7 days before habituation; Day 14: 14 days after the halter breaking practice class and 21 days after the habituation. TRT: treatment; DAY: day of sampling. a, b letters in the rows indicate statistical difference ($p \leq 0.05$) and equal letters do not differ significantly ($p > 0.05$).

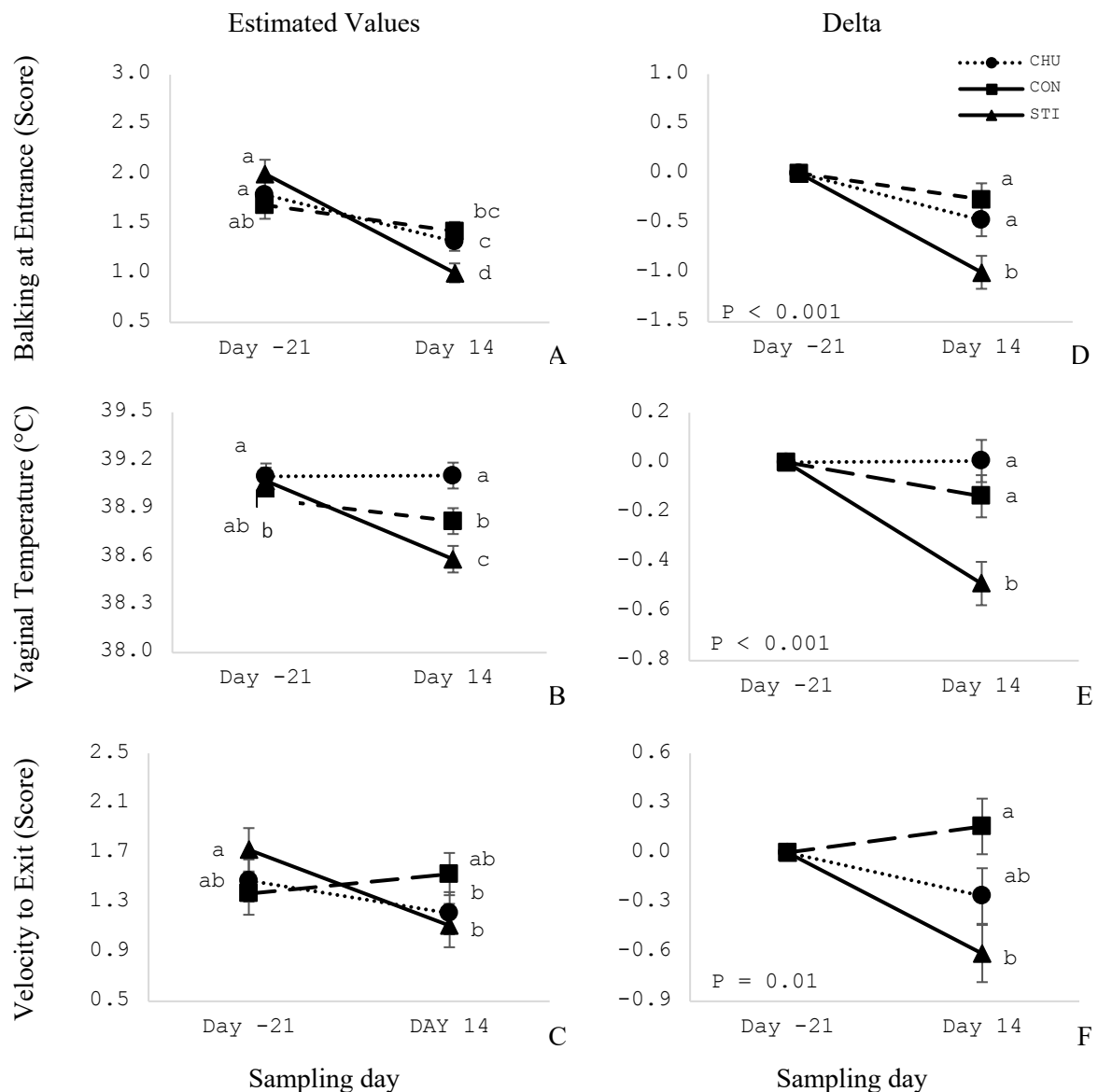


Figure 4. Estimated values (left column; A–C) with interaction effect of treatment and sampling day and, delta values (right column; D–F) with treatment effect of Stimulated (STI), Chute (CHU) and Control (CON) groups on sampling days -21 and 14. a, b: different lowercase letters indicate statistical difference ($p \leq 0.05$) and equal letters do not differ significantly ($p > 0.05$).

4.3.3. Student-animal interaction during HBPC

The STI treatment resulted in lesser values compared to the CON treatment for pen exit velocity and halter scores ($p 0.05$; Table 4). STI and CHU values for those variables did not differ. CHU heifers' exit velocity was slower compared to CON heifers (Table 4). Other behavioral responses during class did not differ among habituation treatments (Table 4).

Table 4. Effect of habituation treatment on behavioral parameters during the interaction between the students and their respective heifer (*Bos taurus*) in the halter break practice class.

Trait	STI (n = 16)	CHU (n = 17)	CON (n = 16)	p-Value
HALTER	2.63 ± 0.34 b	3.50 ± 0.36 ab	4.38 ± 0.34 a	p < 0.01
CH_EXT_VEL	1.81 ± 0.18	1.88 ± 0.18	2.00 ± 0.18	0.76
PEN_ENT_VEL	1.81 ± 0.21	1.88 ± 0.21	1.88 ± 0.21	0.97
PULL_N	8.13 ± 3.64	17.24 ± 3.53	16.31 ± 3.64	0.16
FIVEMIN	3.13 ± 0.25	3.24 ± 0.24	3.00 ± 0.25	0.80
SCRATCH	2.75 ± 0.32	2.82 ± 0.31	3.63 ± 0.32	0.11
PEN_EXT_VEL	1.69 ± 0.18 a	1.80 ± 0.18 a	1.13 ± 0.18 b	0.02

LSMeans ± standard error and p value. HALTER (score): animal's reactivity during the haltering; CH_EXT_VEL (score): animal's walking velocity score to exit the squeeze chute; PEN_ENT_VEL (score): animal's walking velocity score to enter the pen; PULL_N (n): number of times that the animal pulled the rope during the first 5 min after being tied; FIVEMIN: animal's reactivity during the first 5 min after being tied; SCRATCH (score): animal's reactivity during scratching with show stick; PEN_EXT_VEL (score): animal's walking velocity score to exit the pen. a, b: different lowercase letters in the line indicate statistical difference ($p \leq 0.05$) and equal letters do not differ significantly ($p > 0.05$).

4.4. DISCUSSION

Although habituation effect was significant only in three (LAT_ENT, VAGT and BC) of all traits assessed, these results indicate that seven days of habituation (STI and CHU) is a useful tool to decrease reactivity of heifers and increase ease of handling in working and squeeze chutes. This is in agreement with results from others (Ujita et al., 2020) that also indicated seven days of habituation would improve the ease of cattle movement through a handling facility and squeeze chute. Thus, our results reinforce previous findings showing the beneficial impact of positive reinforcement (STI) or just habituation to handling and facilities (CHU) on animal reactivity (Néri et al., 2016; Ujita et al., 2021, 2020).

Latency to enter the working chute can directly influence and be influenced by balking and velocity score to enter the working chute. An animal that balks (undesirable behavior) will have greater latency, but an exacerbated velocity to enter (also undesirable behavior) will decrease latency. The greater decrease in velocity in STI treatment indicates that brushing was more effective in improving this trait and heifers were calmer on day 14 in comparison to day 1. Our findings are similar to others where 36% of beef cattle showed slower velocities (walking rather than running) after 14 days of chute habituation with brushing (Ujita et al., 2020), indicating a reduced fear of handling and improved entry behavior. Furthermore, in other species latency to approach or move away are associated with temperament during handling and exposure to handling facilities (Geverink et al., 1998; Sanger and Joly, 1985). Therefore, it

is important to associate latency with velocity and balking measures, since together these measurements better indicate desirable and undesirable behaviors, such as trust or fear that may prolong or not latency.

Although LOC did not differ, results show that CON heifers were more excited than habituated heifers. Few studies working with novelty tests affirm that fear motivates action in behavior such as locomotion (de Passillé et al., 1995; MacKay et al., 2014; Van Reenen et al., 2004). In addition, previous research supports that a passive animal would show silence and inactivity to confront an unknown or uncomfortable situation, while active animals would be rely on fighting, struggling, vocalization and locomotion (Benus et al., 1991; Jones, 1996; Van Reenen et al., 2004). In our study, no passive heifers were identified and observed, suggesting that the herd is characterized by active or proactive animals. Thus, the exited locomotion of CON heifers indicates that this group was likely uncomfortable and stressed with handling, which may be the reason for the numerically increased RR. Stress is associated with physiological changes and respiratory rate is used to study aspects of stress and cognition (Bondarenko et al., 2015; Losada-Espinosa et al., 2018; Mohamed et al., 2018; Ujita et al., 2020). Following this reasoning, we expected VAGT to be greater in CON heifers, but surprisingly CHU heifers had greater VAGT compared to other treatments. Nonetheless, vaginal temperature can vary during the different stages of the estrous cycle (Vicentini et al., 2020; Wang et al., 2020) and we did not evaluate puberty status or followed the estrous cycle of the heifers enrolled in this study which could have affected our results.

Concentration of cortisol was increased in heifers without habituation. Cortisol production and release is often increased in response to stress, being a predictive of anxiety-related behaviors and its concentration is relevant for animal welfare (Bristow and Holmes, 2007). Other studies found that positive handling and habituation can reduce concentration of cortisol (Mota-Rojas et al., 2020; Probst et al., 2012a). However, exact thresholds for concentration of cortisol to determine different levels of stress do not exist in cattle. Reports in the literature vary significantly, one study observed cortisol concentrations of 4.97 µg/dL in a control group and 2.94 µg/dL in the group exposed to habituation (Probst et al., 2012a) while others reported cortisol concentrations of 0.70 µg/dL in non-stressed and 2.95 µg/dL in stressed groups (Bristow and Holmes, 2007). Differences in environment, breed, age, animal category and even method of sampling and analysis need to be considered when comparing and contrasting cortisol results.

Flight time, flight speed and pen score are commonly used to assess behavior and temperament in cattle. The stimulated heifers in our study tended to have a decreased flight time, which is a desirable behavior and indicative of reduced stress and fear. Pen score was similar among treatments, however it decreased between day -21 and day 14 independent of treatment, indicating that all heifers improved their behavior when interacting with a human inside of small space. Previous research with lactating dairy cows indicates that spending even a short-time stroking the cows during milking can positively affect their response to humans, reducing avoidance distances of the animals at the feeding place, the barn and in the milking parlor (Windschnurer et al., 2009). Another study found that gently touching young pre-weaning beef calves resulted in lesser avoidance distance, greater voluntary approaches and fewer backward movements inside the stunning box at slaughter at 10 months of age (Probst et al., 2012a). Clearly positive interactions such as stroking, brushing and talking with a gentle voice are necessary to maintain a positive human-animal relationship with improved trust and reduced fear from both sides (Lürzel et al., 2016a).

In the case of our study, seven days of habituation (with or without tactile stimulation) might not be enough to present a statistically significant differences in all traits observed, but could still improve the general behavior of the herd. Even a short interactions and handling events separated by long periods of time in between can improve cattle habituation to handling. The overall reduction in PS and cortisol concentration between day -21 and 14, independent of treatment highlights the importance of exposing young cattle to handling events and facilities. These findings are corroborated by other studies. Temperament scores decrease as the number of times an animal goes through a working facility increased (Curley et al., 2006b; Grandin and Shivley, 2015; Ujita et al., 2020).

There was treatment by day interaction for three traits assessed (BALK_ENT, VAGT and VEL_EXT) and the delta values, which represents the difference in the variables between day -21 and day 14, also differed. No difference in behavior and temperament assessment was detected at the beginning of the experiment among treatments. These results suggest that tactile stimulation is an alternative to facilitate handling and improve animal behavior, which might be linked to a decreased fear of human or facilities as other studies suggest (Lürzel et al., 2016b; Probst et al., 2013, 2012b; Silva et al., 2017; Ujita et al., 2020)

4.4.1. Student-animal interaction during HBPC

The HALTER trait represents the reactivity score when the student interacts with the heifer trying to place the halter in the heifer at the squeeze chute. Both habituation treatments resulted in decreased reactivity and increased ease to halter, suggesting that habituation is also a tool to reduce fear of human approach and handling. The PULL_N, that consists in how many times the animal pulled the rope during the first five minutes after being tied, did not differ statistically. However, STI heifers had a reduction in pull of approximately 50% in comparison to the other treatments.

Livestock involves a large variety of activities such as feeding, reallocation of animals (by moving from field to field or by loading animals on trucks/trailers), artificial insemination, hoof care, dehorning, roping animals, medicating, surgery procedures, ear tagging, milking, birth assistance and others (Hakan and Demirci, 2012) which are often taught in education institutions such as animal science or veterinary courses. These HALTER and PULL_N results highlight the importance of habituating animals in educational institutions herd used in skill practice activities. Knowing that tactile stimulation is frequently used as strategy to desensitize the animal to human touching specially in dairy cattle (Müller and von Keyserlingk, 2006; Shahin, 2018; Ujita et al., 2020; Windschnurer et al., 2009) where the interaction between human and animal is very close and with constant tactile stimuli, this could also be used as a method to habituate animals in teaching herds before classes start. Providing a safer environment for both students and animals.

4.5. CONCLUSIONS

The current study shows that habituation of beef cattle in a teaching herd improves some animal behavior and physiology responses to human-animal interactions. In general, both habituation protocols showed benefits, but heifers that received the positive tactile stimulation in the chute showed the greatest improvement for chute enter and exit behavior. Furthermore, these heifers responded more calmly during student-animal interactions in class, which is beneficial for the students' safety. To our knowledge, this is the first study that established beneficial effects habituation for cattle in an educational setting, in relation to student-animal interactions. More research is needed to optimize student learning whilst ensuring animal

welfare.

Author Contributions: Conceptualization, A.U., L.J., D.E.E., L.B., E.F., J.A.N. and V.R.G.M.; method- ology, A.U., L.J., D.E.E., L.B., E.F., J.A.N. and V.R.G.M.; statistical analysis, A.U. and L.J.; investigation, A.U., Z.S., M.K., G.G. and N.W.D.; resources, A.U., L.J., D.E.E. and V.R.G.M.; writing—original draft preparation, A.U.; writing—review and editing, A.U., L.J., D.E.E., J.A.N. and V.R.G.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received financed scholarship from CAPES (Finance Code 001).

Institutional Review Board Statement: The study was conducted at the Virginia Tech Beef Cattle Center in Blacksburg (VA, USA) under approval of the Institutional Animal Care and Use Committee (IACUC); protocol number 19-215.

Data Availability Statement: The data presented in this study are available on request from the cor- responding author. The data are not publicly available due to the presence of identifiable information from human subjects.

Acknowledgments: The authors acknowledge the Virginia Polytechnic Institute and State University and the Department of Animal and Poultry Science for carrying out the research project. Financial support from CAPES (Finance Code 001). This work is supported by the USDA National Institute of Food and Agriculture Hatch project VA-160080.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

REFERENCES

- Abdel-Hamid, S.E., Fattah, D.M.A., Ghanem, H.M., Eman Abd-Allah Manaa, 2017. Temperament during Milking Process and its Effect on Behavioral, Productive Traits and Biochemical Parameters in Friesian Dairy Cows. *Adv. Anim. Vet. Sci.* 5, 508–513. <https://doi.org/http://dx.doi.org/10.17582/journal.aavs/2017/5.12.508.513>
- Adamczyk, K., 2018. Dairy cattle welfare as a result of human-animal relationship – a review. *Ann. Anim. Sci.* <https://doi.org/10.2478/aoas-2018-0013>
- Alvarez, F.J., Saucedo, G., Arriaga, A., Preston, T.R., 1980. Effect on Milk Production and Calf Performance of Milking Cross Bred European / Zebu Cattle in the Absence or Presence of. *Trop. Anim. Prod.* 5, 25–37.
- Andrade, O., Orihuela, A., Solano, J., Galina, C.S., 2001. Some effects of repeated handling and the use of a mask on stress responses in zebu cattle during restraint. *Appl. Anim. Behav. Sci.* 71, 175–181. [https://doi.org/10.1016/S0168-1591\(00\)00177-5](https://doi.org/10.1016/S0168-1591(00)00177-5)
- Assan, N., 2015. Influence of suckling and/or milking method on yield and milk composition in dairy animals. *Sci. J. Zool.* 4, 1–7. <https://doi.org/10.14196/sjz.v4i1.1792>
- Baldwin, D. V., 2013. Primitive mechanisms of trauma response: An evolutionary perspective on trauma-related disorders. *Neurosci. Biobehav. Rev.* <https://doi.org/10.1016/j.neubiorev.2013.06.004>
- Bar-Pelled, U., Maltz, E., Bruckental, I., Folman, Y., Kali, Y., Gacitua, H., Lehrer, A.R., Knight, C.H., Robinson, B., Voet, H., Tagari, H., 1995. Relationship Between Frequent Milking or Suckling in Early Lactation and Milk Production of High Producing Dairy Cows. *J. Dairy Sci.* 78, 2726–2736. [https://doi.org/10.3168/jds.S0022-0302\(95\)76903-X](https://doi.org/10.3168/jds.S0022-0302(95)76903-X)
- Becker, B.G., Lobato, J.F.P., 1997. Effect of gentle handling on the reactivity of zebu crossed calves to humans. *Appl. Anim. Behav. Sci.* 53, 219–224. [https://doi.org/10.1016/S0168-1591\(96\)01091-X](https://doi.org/10.1016/S0168-1591(96)01091-X)
- Benus, R.F., Bohus, B., Koolhaas, J.M., van Oortmerssen, G.A., 1991. Heritable variation for aggression as a reflection of individual coping strategies. *Experientia*. <https://doi.org/10.1007/BF01923336>
- Bertenshaw, C., Rowlinson, P., 2001. The influence of positive human-animal interaction during rearing on the approach behaviour of young dairy heifers. *Proc. Br. Soc. Anim. Sci.* <https://doi.org/10.1017/s1752756200006530>
- Bertenshaw, C., Rowlinson, P., Edge, H., Douglas, S., Shiel, R., 2008. The effect of different degrees of “positive” human-animal interaction during rearing on the welfare and subsequent production of commercial dairy heifers. *Appl. Anim. Behav. Sci.* 114, 65–75. <https://doi.org/10.1016/j.applanim.2007.12.002>
- Bertucci, P.Y., Quaglino, A., Pozzi, A.G., Kordon, E.C., Pecci, A., 2010. Glucocorticoid-induced impairment of mammary gland involution is associated with STAT5 and STAT3 signaling modulation. *Endocrinology* 151, 5730–5740. <https://doi.org/10.1210/en.2010-0517>
- Blum, J.W., Bruckmaier, R., 1989. Catecholamines, oxytocin and milk removal in dairy cows. *J. Dairy Res.* <https://doi.org/10.1017/S0022029900026388>
- Boivin, X., Braastad, B.O., 1996. Effects of handling during temporary isolation after early weaning on goat kids’ later response to humans. *Appl. Anim. Behav. Sci.* 48, 61–71. [https://doi.org/10.1016/0168-1591\(95\)01019-X](https://doi.org/10.1016/0168-1591(95)01019-X)
- Boivin, X., Tournadret, H., Le Neindre, P., 2000. Hand-feeding and gentling influence early-weaned lambs’ attachment responses to their stockperson. *J. Anim. Sci.* 78, 879–884. <https://doi.org/10.2527/2000.784879x>

- Bomfim, G.F., Merighe, G.K.F., de Oliveira, S.A., Negrao, J.A., 2018. Effect of acute stressors, adrenocorticotrophic hormone administration, and cortisol release on milk yield, the expression of key genes, proliferation, and apoptosis in goat mammary epithelial cells. *J. Dairy Sci.* 101, 6486–6496. <https://doi.org/10.3168/jds.2017-14123>
- Bondarenko, E., Beig, M.I., Hodgson, D.M., Braga, V.A., Nalivaiko, E., 2015. Blockade of the dorsomedial hypothalamus and the perifornical area inhibits respiratory responses to arousing and stressful stimuli. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* <https://doi.org/10.1152/ajpregu.00415.2014>
- Borghese, A., Rasmussen, M., Thomas, C.S., 2007. Milking management of dairy buffalo. *Ital. J. Anim. Sci.* <https://doi.org/10.4081/ijas.2007.s2.39>
- Bouissou, M.F., Boissy, A., Neindre, P. le, Veissier, I., 2009. The social behaviour of cattle., in: *Social Behaviour in Farm Animals*. <https://doi.org/10.1079/9780851993973.0113>
- Brasil, B.S.A.F., Coelho, E.G.A., Drummond, M.G., Oliveira, D.A.A., 2013. Genetic diversity and differentiation of exotic and American commercial cattle breeds raised in Brazil. *Genet. Mol. Res.* <https://doi.org/10.4238/2013.November.18.2>
- Bremner, K.J., 1997. Behaviour of dairy heifers during adaptation to milking. *Proc. New Zeal. Soc. Anim. Prod.*
- Breuer, K., Hemsworth, P.H., Barnett, J.L., Matthews, L.R., Coleman, G.J., 2000. Behavioural response to humans and the productivity of commercial dairy cows. *Appl. Anim. Behav. Sci.* 66, 273–288. [https://doi.org/10.1016/S0168-1591\(99\)00097-0](https://doi.org/10.1016/S0168-1591(99)00097-0)
- Breuer, K., Hemsworth, P.H., Coleman, G.J., 2003. The effect of positive or negative handling on the behavioural and physiological responses of nonlactating heifers. *Appl. Anim. Behav. Sci.* 84, 3–22. [https://doi.org/10.1016/S0168-1591\(03\)00146-1](https://doi.org/10.1016/S0168-1591(03)00146-1)
- Bristow, D.J., Holmes, D.S., 2007. Cortisol levels and anxiety-related behaviors in cattle. *Physiol. Behav.* 90, 626–628. <https://doi.org/10.1016/j.physbeh.2006.11.015>
- Brouček, J., 2014. Effect of Noise on Performance, Stress, and Behaviour of Animals. *Slovak J. Anim. Sci.* 47, 111–123.
- Brouček, J., Uhrinčat, M., ŠoCh, M., Kišac, P., UhRinČať, M., ŠoCh, M., Kišac, P., 2008. Genetics of behaviour in cattle. *Slovak J. Anim. Sci.* 41, 166–172. <https://doi.org/10.1079/9781780642215.0234>
- Bruckmaier, R.M., 2003. Chronic oxytocin treatment causes reduced milk ejection in dairy cows. *J. Dairy Res.* <https://doi.org/10.1017/S0022029902005940>
- Bruckmaier, R.M., Pfeilsticker, H.-U., Blum, J.W., 2009. Milk yield, oxytocin and β -endorphin gradually normalize during repeated milking in unfamiliar surroundings. *J. Dairy Res.* 63, 191. <https://doi.org/10.1017/s0022029900031691>
- Bruckmaier, R.M., Schams, D., Blum, J.W., 1993. Milk removal in familiar and unfamiliar surroundings: concentrations of oxytocin, prolactin, cortisol and β -endorphin. *J. Dairy Res.* 60, 449–456.
- Bruckmaier, R.M., Wellnitz, O., 2008. Induction of milk ejection and milk removal in different production systems. *J. Anim. Sci.* 86, 15–20. <https://doi.org/10.2527/jas.2007-0335>
- Bruckmaier, R.M., Wellnitz, O., Blum, J.W., 1997. Inhibition of milk ejection in cows by oxytocin receptor blockade, α -adrenergic receptor stimulation and in unfamiliar surroundings. *J. Dairy Res.* 64, 315–325. <https://doi.org/10.1017/S002202999700232X>
- Bruckmaier, R.M.M., Blum, J.W.W., 1998. Oxytocin Release and Milk Removal in Ruminants. *J. Dairy Sci.* 81, 939–949. [https://doi.org/10.3168/jds.S0022-0302\(98\)75654-1](https://doi.org/10.3168/jds.S0022-0302(98)75654-1)

- Burdick, N.C., Randel, R.D., Carroll, J.A., Welsh, T.H., 2011. Interactions between temperament, stress, and immune function in cattle. *Int. J. Zool.* <https://doi.org/10.1155/2011/373197>
- Burdick Sanchez, N.C., Carroll, J.A., Randel, R.D., Vann, R.C., Welsh, T.H., 2014. Associations between endotoxin-induced metabolic changes and temperament in Brahman bulls. *J. Anim. Physiol. Anim. Nutr. (Berl)*. 98, 178–190. <https://doi.org/10.1111/jpn.12074>
- Burrow, H.M., 1997. Measurement of temperament and their relationship with performance traits of beef cattle. *Anim Breed Abstr.* <https://doi.org/10.1002/tea.20309>
- Cannon, W.B., 1929. Bodily Changes in Pain, Hunger, Fear and Rage. *South. Med. J.* <https://doi.org/10.1097/00007611-192909000-00037>
- Cavallina, R., Roncoroni, C., Campagna, M.C., Minero, M., Canali, E., 2008. Buffalo behavioural response to machine milking in early lactation. *Ital. J. Anim. Sci.* 7, 287–295. <https://doi.org/10.4081/ijas.2008.287>
- Ceballos, M.C., Sant’Anna, A.C., Boivin, X., Costa, F. de O., Carvalhal, M.V. d. L., Paranhos da Costa, M.J.R., 2018a. Impact of good practices of handling training on beef cattle welfare and stockpeople attitudes and behaviors. *Livest. Sci.* <https://doi.org/10.1016/j.livsci.2018.06.019>
- Ceballos, M.C., Sant’Anna, A.C., Góis, K.C.R., Ferraudo, A.S., Negrao, J.A., da Costa, M.J.R.P., 2018b. Investigating the relationship between human-animal interactions, reactivity, stress response and reproductive performance in Nellore heifers. *Livest. Sci.* 217, 65–75. <https://doi.org/10.1016/j.livsci.2018.08.001>
- Chen, Y., Arsenault, R., Napper, S., Griebel, P., 2015. Models and methods to investigate acute stress responses in cattle. *Animals* 5, 1268–1295. <https://doi.org/10.3390/ani5040411>
- Chida, D., Miyoshi, K., Sato, T., Yoda, T., Kikusui, T., Iwakura, Y., 2011. The role of glucocorticoids in pregnancy, parturition, lactation, and nurturing in melanocortin receptor 2-deficient mice. *Endocrinology* 152, 1652–1660. <https://doi.org/10.1210/en.2010-0935>
- Coelho, S.G., Franzoni, A.P.S., 2012. Comportamento e Bem-estar de bezerros na bovinocultura leiteira, in: Junior, A. de P.M., Heinemann, M.B., Garcia, S.K., Ângela Mara Leite Drumond (Eds.), *Cadernos Técnicos de Veterinária e Zootecnia*. Belo Horizonte, pp. 135–151.
- Coleman, G.J., Hemsworth, P.H., Hay, M., 1998. Predicting stockperson behaviour towards pigs from attitudinal and job-related variables and empathy. *Appl. Anim. Behav. Sci.* 58, 63–75. [https://doi.org/10.1016/S0168-1591\(96\)01168-9](https://doi.org/10.1016/S0168-1591(96)01168-9)
- Collier, R.J., Hernandez, L.L., Horseman, N.D., 2012. Serotonin as a homeostatic regulator of lactation. *Domest. Anim. Endocrinol.* 43, 161–170. <https://doi.org/10.1016/j.domaniend.2012.03.006>
- Connelly, M.K., Weaver, S.R., Kuehnl, J.M., Fricke, H.P., Klister, M., Hernandez, L., 2021. Elevated serotonin coordinates mammary metabolism in dairy cows. *Physiol. Rep.* 9, 1–12. <https://doi.org/10.14814/phy2.14798>
- Cooke, R.F., Schubach, K.M., Peres, R.F.G., Cipriano, R.S., Marques, R., Carvalho, R., Bohnert, D.W., Biehl, M. V., Pires, A. V., Vasconcelos, J.L.M., 2016. 1275 Effects of temperament on physiological and reproductive responses of Bos Indicus beef cows. *J. Anim. Sci.* <https://doi.org/10.2527/jam2016-1275>
- Cozzi, G., Brscic, M., Gottardo, F., 2009. Main critical factors affecting the welfare of beef cattle and veal calves raised under intensive rearing systems in Italy: A review. *Ital. J. Anim. Sci.* <https://doi.org/10.4081/ijas.2009.s1.67>
- Crowder, M.J., Hand, D.J., 1990. *Analysis of Repeated Measures*. Chapman and Hall, London.
- Curley, K.O., Paschal, J.C., Welsh, T.H., Randel, R.D., 2006a. Technical note: Exit velocity as a measure of cattle temperament is repeatable and associated with serum concentration of cortisol in

- Brahman bulls. *J. Anim. Sci.* 84, 3100–3103. <https://doi.org/10.2527/jas.2006-055>
- Curley, K.O., Paschal, J.C., Welsh, T.H., Randel, R.D., 2006b. Technical note: Exit velocity as a measure of cattle temperament is repeatable and associated with serum concentration of cortisol in Brahman bulls. *J. Anim. Sci.* <https://doi.org/10.2527/jas.2006-055>
- D'Eath, R.B., Conington, J., Lawrence, A.B., Olsson, I.A.S., Sandøe, P., 2010. Breeding for behavioural change in farm animals: Practical, economic and ethical considerations. *Anim. Welf.*
- da Costa, M.J.R.P., 2000. Ambiência na produção de bovinos de corte a pasto. *An. Etol.* 18, 26–42.
- da Costa, M.J.R.P., Costa e Silva, E.V., Chiquitelli Neto, M., Rosa, M.S., 2002. Contribuição dos estudos de comportamento de bovinos para implementação de programas de qualidade de carne. *Encontro Anu. Etol.* 71–89.
- da Costa, M.J.R.P., Sant'Anna, A.C., Silva, L.C.M., 2015. Temperamento de bovinos Gir e Girolando : efeitos genéticos e de manejo. *Inf. Agropecuário, Belo Horiz.* 36, 100–107.
- de Passillé, A.M., Rushen, J., Martin, F., 1995. Interpreting the behaviour of calves in an open-field test: a factor analysis. *Appl. Anim. Behav. Sci.* [https://doi.org/10.1016/0168-1591\(95\)00622-Y](https://doi.org/10.1016/0168-1591(95)00622-Y)
- Doerfler, R.L., Lehermeier, C., Kliem, H., Möstl, E., Bernhardt, H., 2016. Physiological and behavioral responses of dairy cattle to the introduction of robot scrapers. *Front. Vet. Sci.* 3, 1–11. <https://doi.org/10.3389/fvets.2016.00106>
- Eicher, S.D., Schutz, M., Kearney, F., Willard, S., Bowers, S., Gandy, S., Graves, K., 2007. Prepartum milking effects on parlour behaviour, endocrine and immune responses in Holstein heifers. *J. Dairy Res.* 74, 417–424. <https://doi.org/10.1017/S0022029907002695>
- El. Abdel-Hamid, S., Fattah, D.M.A., Ghanem, H.M., Manaa, E.A.A., 2017. Temperament during milking process and its effect on behavioral, productive traits and biochemical parameters in Friesian dairy cows. *Adv. Anim. Vet. Sci.* 5, 508–513. <https://doi.org/10.17582/journal.aavs/2017/5.12.508.513>
- Facó, O., Lôbo, R.N.B., Martins Filho, R., Moura, A.D.A.A., Nonato, R., Lôbo, B., Filho, R.M., 2002. Análise do Desempenho Produtivo de Diversos Grupos Genéticos Holandês x Gir no Brasil 1 Analysis of Productive Performance of Different Holstein x Gir Genetic Groups in Brazil. *Rev. Bras. Zootec.* 31, 1944–1952. <https://doi.org/10.1590/S1516-35982002000800010>
- Fonsêca, V.F.C., Saraiva, E.P., Maia, A.S.C., de Melo Costa, C.C., dos Santos, S.G.C.G., Morais, L.K.C., Fuller, A., Chiquitelli Neto, M., 2019. Training sheep for indirect calorimetry trials. *Small Rumin. Res.* 177, 36–43. <https://doi.org/10.1016/j.smallrumres.2019.06.010>
- Fordyce, G., Goddard, M.E., Seifert, G.W., 1982. The measurement of temperament in cattle and the effect of experience and genotype. *Proc. Aust. Soc. Anim. Prod.*
- Friedrich, J., Brand, B., Schwerin, M., 2015. Genetics of cattle temperament and its impact on livestock production and breeding – a review. *Arch. Anim. Breed.* <https://doi.org/10.5194/aab-58-13-2015>
- Geverink, N.A., Kappers, A., Van De Burgwal, J.A., Lambooij, E., Blokhuis, H.J., Wiegant, V.M., 1998. Effects of Regular Moving and Handling on the Behavioral and Physiological Responses of Pigs to Preslaughter Treatment and Consequences for Subsequent Meat Quality. *J. Anim. Sci.* <https://doi.org/10.2527/1998.7682080x>
- Grandin, T., 1997. Assessment of Stress during Handling and Transport. *J. Anim. Sci.* 75, 249–257. <https://doi.org/10.2527/1997.751249x>
- Grandin, T., 1990. Design of loading facilities and holding pens. *Appl. Anim. Behav. Sci.* 28, 187–201. [https://doi.org/10.1016/0168-1591\(90\)90053-G](https://doi.org/10.1016/0168-1591(90)90053-G)
- Grandin, T., Deesing, M.J., 2014. Genetics and Behavior During Handling, Restraint, and Herding, Second Edi. ed, Genetics and the Behavior of Domestic Animals. Elsevier Inc.

<https://doi.org/10.1016/B978-0-12-394586-0.00004-4>

- Grandin, T., Shivley, C., 2015. How farm animals react and perceive stressful situations such as handling, restraint, and transport. *Animals* 5, 1233–1251. <https://doi.org/10.3390/ani5040409>
- Grignard, L., Boissy, A., Boivin, X., Garel, J.P., Le Neindre, P., 2000. The social environment influences the behavioural responses of beef cattle to handling. *Appl. Anim. Behav. Sci.* [https://doi.org/10.1016/S0168-1591\(00\)00085-X](https://doi.org/10.1016/S0168-1591(00)00085-X)
- Gruber, S.L., Tatum, J.D., Engle, T.E., Chapman, P.L., Belk, K.E., Smith, G.C., 2010. Relationships of behavioral and physiological symptoms of preslaughter stress to beef longissimus muscle tenderness. *J. Anim. Sci.* <https://doi.org/10.2527/jas.2009-2183>
- Hakan, K., Demirci, S., 2012. Livestock-Handling Related Injuries and Deaths, in: *Livestock Production*. <https://doi.org/10.5772/50834>
- Hall, N.L., Buchanan, D.S., Anderson, V.L., Ilse, B.R., Carlin, K.R., Berg, E.P., 2011. Working chute behavior of feedlot cattle can be an indication of cattle temperament and beef carcass composition and quality. *Meat Sci.* <https://doi.org/10.1016/j.meatsci.2011.03.020>
- Haskell, M.J., Simm, G., Turner, S.P., 2014. Genetic selection for temperament traits in dairy and beef cattle. *Front. Genet.* 5, 1–18. <https://doi.org/10.3389/fgene.2014.00368>
- Hearnshaw, H., Morris, C.A., 1984. Genetic and environmental effects on a temperament score in beef cattle. *Aust. J. Agric. Res.* 35, 723–733. <https://doi.org/10.1071/AR9840723>
- Hemsworth, P.H., Barnett, J.L., 1989. Relationships between fear of humans, productivity and cage position of laying hens. *Br. Poult. Sci.* 30, 505–518. <https://doi.org/10.1080/00071668908417175>
- Hemsworth, P.H., Coleman, G.J., Barnett, J.L., Borg, S., 2000. Relationships between human-animal interactions and productivity of commercial dairy cows. *J. Anim. Sci.* 78, 2821–2831. <https://doi.org/10.2527/2000.78112821x>
- Hooper, H.B., Silva, P. dos S., de Oliveira, S.A., Meringhe, G.K.F., Lacasse, P., Negrão, J.A., 2020. Effect of heat stress in late gestation on subsequent lactation performance and mammary cell gene expression of Saanen goats. *J. Dairy Sci.* 103, 1982–1992. <https://doi.org/10.3168/jds.2019-16734>
- Hötzel, M.J., Machado Filho, L.C.P., Yunes, M.C., Da Silveira, M.C.A.C., 2005. Influência de um ordenhador aversivo sobre a produção leiteira de vacas da raça holandesa. *Rev. Bras. Zootec.* 34, 1278–1284.
- Hunter, P., 2018. The genetics of domestication. *EMBO Rep.* <https://doi.org/10.15252/embr.201745664>
- Inderwies, T., Pfaffl, M.W., Bruckmaier, R.M., 2003. Milking characteristics and their relation to adrenergic receptor mRNA expression and ligand binding in the mammary gland of dairy cows. *Domest. Anim. Endocrinol.* 25, 275–286. [https://doi.org/10.1016/S0739-7240\(03\)00059-6](https://doi.org/10.1016/S0739-7240(03)00059-6)
- Johnsen, J.F., Zipp, K.A., Kälber, T., Passillé, A.M. de, Knierim, U., Barth, K., Mejdell, C.M., 2016. Is rearing calves with the dam a feasible option for dairy farms?—Current and future research. *Appl. Anim. Behav. Sci.* 181, 1–11. <https://doi.org/10.1016/j.applanim.2015.11.011>
- Jones, R.B., 1996. Fear and adaptability in poultry: Insights, implications and imperatives. *Worlds. Poult. Sci. J.* <https://doi.org/10.1079/wps19960013>
- Kasimanickam, R.K., Hall, J.B., Estill, C.T., Kastelic, J.P., Joseph, C., Abdel Aziz, R.L., Nak, D., 2018. Flunixin meglumine improves pregnancy rate in embryo recipient beef cows with an excitable temperament. *Theriogenology* 107, 70–77. <https://doi.org/10.1016/j.theriogenology.2017.10.043>
- Kiley-Worthington, M., de la Plain, S., Kiley-Worthington, M., de la Plain, S., 1983. Fostering and Adoption in Beef Cattle, in: *The Behaviour of Beef Suckler Cattle (Bos Taurus)*. https://doi.org/10.1007/978-3-0348-6782-5_8

- Kling-Eveillard, F., Allain, C., Boivin, X., Courboulay, V., Créach, P., Philibert, A., Ramonet, Y., Hostiou, N., 2020. Farmers' representations of the effects of precision livestock farming on human-animal relationships. *Livest. Sci.* <https://doi.org/10.1016/j.livsci.2020.104057>
- Kolb, D.A., 2015. *Experiential Learning- Experience as the Source of Learning and Development* (2nd Edition), Person Education, Inc. <https://doi.org/10.1017/CBO9781107415324.004>
- Krohn, C.C., Jago, J.G., Boivin, X., 2001. The effect of early handling on the socialisation of young calves to humans. *Appl. Anim. Behav. Sci.* [https://doi.org/10.1016/S0168-1591\(01\)00161-7](https://doi.org/10.1016/S0168-1591(01)00161-7)
- Kutzer, T., Steilen, M., Gygas, L., Wechsler, B., 2015. Habituation of dairy heifers to milking routine- Effects on human avoidance distance, behavior, and cardiac activity during milking. *J. Dairy Sci.* 98, 5241–5251. <https://doi.org/10.3168/jds.2014-8773>
- Lacasse, P., Lollivier, V., Bruckmaier, R.M., Boisclair, Y.R., Wagner, G.F., Boutinaud, M., 2011. Effect of the prolactin-release inhibitor quinagolide on lactating dairy cows. *J. Dairy Sci.* 94, 1302–1309. <https://doi.org/10.3168/jds.2010-3649>
- Lacasse, P., Ollier, S., Lollivier, V., Boutinaud, M., 2016. New insights into the importance of prolactin in dairy ruminants. *J. Dairy Sci.* 99, 864–874. <https://doi.org/10.3168/jds.2015-10035>
- Lammoglia, M., Alarcón, M., Mancera, B., Cabrera, A., Daniel, A., 2015. Effects of Routinely Oxytocin Injection to Induce Milk Ejection on Some Reproductive Parameters of Crossbred Cows in the Tropics of Veracruz. *Annu. Res. Rev. Biol.* 6, 297–303. <https://doi.org/10.9734/arrb/2015/15902>
- Lansade, L., Bertrand, M., Boivin, X., Bouissou, M.F., 2004. Effects of handling at weaning on manageability and reactivity of foals. *Appl. Anim. Behav. Sci.* 87, 131–149. <https://doi.org/10.1016/j.applanim.2003.12.011>
- Laurence, M., Barnes, A., Collins, T., Hyndman, T., Musk, G.C., 2016. Assessing and mitigating post-operative castration pain in Bos indicus cattle. *Anim. Prod. Sci.* 58, 909–919. <https://doi.org/10.1071/AN16210>
- Leão, G.F.M., Pivatto, D.R.D., Carniel, H., Rodrigues, M.G.K., Braga, R.A., Silva, M.R.H. da, Teixeira, P.P.M., 2013. Melhoramento Genético Em Zebuínos Leiteiros – Uma Revisão. *Agropecuária Científica No Semiárido*.
- Lee, C., Café, L.M., Robinson, S.L., Doyle, R.E., Lea, J.M., Small, A.H., Colditz, I.G., 2018. Anxiety influences attention bias but not flight speed and crush score in beef cattle. *Appl. Anim. Behav. Sci.* 205, 210–215. <https://doi.org/10.1016/j.applanim.2017.11.003>
- Lefcourt, A.M., Kahl, S., Akers, R.M., 1986. Correlation of Indices of Stress with Intensity of Electrical Shock for Cows. *J. Dairy Sci.* 69, 833–842. [https://doi.org/10.3168/jds.S0022-0302\(86\)80473-8](https://doi.org/10.3168/jds.S0022-0302(86)80473-8)
- Lensink, B.J., Boivin, X., Pradel, P., Le Neindre, P., Veissier, I., 2000. Reducing veal calves' reactivity to people by providing additional human contact. *J. Anim. Sci.* 78, 1213–1218. <https://doi.org/10.2527/2000.7851213x>
- Ligout, S., Bouissou, M.F., Boivin, X., 2008. Comparison of the effects of two different handling methods on the subsequent behaviour of Anglo-Arabian foals toward humans and handling. *Appl. Anim. Behav. Sci.* 113, 175–188. <https://doi.org/10.1016/j.applanim.2007.12.004>
- Lima, M.L.P., Negrão, J.A., de Paz, C.C.P., Grandin, T., 2018. Minor corral changes and adoption of good handling practices can improve the behavior and reduce cortisol release in Nellore cows. *Trop. Anim. Health Prod.* 50, 525–530. <https://doi.org/10.1007/s11250-017-1463-9>
- Llouch, P., Somarriba, M., Duthie, C.A., Haskell, M.J., Rooke, J.A., Troy, S., Roehe, R., Turner, S.P., 2016. Association of temperament and acute stress responsiveness with productivity, feed efficiency, and Methane emissions in beef cattle: An observational study. *Front. Vet. Sci.* <https://doi.org/10.3389/fvets.2016.00043>

- Losada-Espinosa, N., Villarroel, M., María, G.A., Miranda-de la Lama, G.C., 2018. Pre-slaughter cattle welfare indicators for use in commercial abattoirs with voluntary monitoring systems: A systematic review. *Meat Sci.* 138, 34–48. <https://doi.org/10.1016/j.meatsci.2017.12.004>
- Lürzel, S., Windschnurer, I., Futschik, A., Waiblinger, S., 2016a. Gentle interactions decrease the fear of humans in dairy heifers independently of early experience of stroking. *Appl. Anim. Behav. Sci.* 178, 16–22. <https://doi.org/10.1016/j.applanim.2016.02.012>
- Lürzel, S., Windschnurer, I., Futschik, A., Waiblinger, S., 2016b. Gentle interactions decrease the fear of humans in dairy heifers independently of early experience of stroking. *Appl. Anim. Behav. Sci.* <https://doi.org/10.1016/j.applanim.2016.02.012>
- Maçal-Pedroza, M.G., Campos, M.M., Pereira, L.G.R., Machado, F.S., Tomich, T.R., da Costa, M.J.R.P., Sant’Anna, A.C., 2019. Consistency of temperament traits and their relationships with milk yield in lactating primiparous F1 Holstein - Gyr cows. *Appl. Anim. Behav. Sci.* 112061. <https://doi.org/https://doi.org/10.1016/j.applanim.2019.104881> This
- Macedo, G.G., Zúccari, C.E.S.N., de Abreu, U.G.P., Negrão, J.A., da Costa e Silva, E.V., 2011. Human-animal interaction, stress, and embryo production in *Bos indicus* embryo donors under tropical conditions. *Trop. Anim. Health Prod.* 43, 1175–1182. <https://doi.org/10.1007/s11250-011-9820-6>
- MacKay, J.R.D., Haskell, M.J., Deag, J.M., van Reenen, K., 2014. Fear responses to novelty in testing environments are related to day-to-day activity in the home environment in dairy cattle. *Appl. Anim. Behav. Sci.* <https://doi.org/10.1016/j.applanim.2013.12.008>
- Macuhová, J., Tancin, V., Kraetzl, W.D., Meyer, H.H.D., Bruckmaier, R.M., 2002. Inhibition of oxytocin release during repeated milking in unfamiliar surroundings: The importance of opioids and adrenal cortex sensitivity. *J. Dairy Res.* 69, 63–73. <https://doi.org/10.1017/S0022029901005222>
- Madalena, F.E., Peixoto, M.G.C.D.C.D., Gibson, J., 2012. Dairy cattle genetics and its applications in Brazil. *Livest. Res. Rural Dev.* 24, 1–49. <https://doi.org/http://www.lrrd.org/lrrd24/6/made24097.htm>
- May, T.M., Page, M.J., Fleming, P.A., 2016. Predicting survivors: Animal temperament and translocation. *Behav. Ecol.* 27, 969–977. <https://doi.org/10.1093/beheco/arv242>
- McInerney, J.P., 2004. Animal welfare, economics and policy. *J. R. Agric. Soc. Engl.* 165.
- Menezes, L.D.M., Cardoso, F.F., Barbosa Silveira, I.D., 2017. Temperamento em bovinos de corte: características genéticas, metodologias de mensuração e desempenho. *Rev. Electron. Vet.*
- Mohamed, R.A., Abou-Ismael, U.A., Shukry, M., Elmoslemany, A., Abdel-Maged, M., 2018. Effects of blindfolding and tail bending of Egyptian water buffaloes on their behavioural reactivity and physiological responses to pain induction. *Vet. Anim. Sci.* 5, 38–43. <https://doi.org/10.1016/j.vas.2018.06.001>
- Mota-Rojas, D., Broom, D.M., Orihuela, A., Velarde, A., Napolitano, F., Alonso-Spilsbury, M., 2020. Effects of human-animal relationship on animal productivity and welfare. *J. Anim. Behav. Biometeorol.* <https://doi.org/10.31893/JABB.20026>
- Müller, R., von Keyserlingk, M.A.G., 2006. Consistency of flight speed and its correlation to productivity and to personality in *Bos taurus* beef cattle. *Appl. Anim. Behav. Sci.* <https://doi.org/10.1016/j.applanim.2005.05.012>
- Munksgaard, L., DePassillé, A.M., Rushen, J., Herskin, M.S., Kristensen, A.M., 2001. Dairy cows’ fear of people: Social learning, milk yield and behaviour at milking. *Appl. Anim. Behav. Sci.* 73, 15–26. [https://doi.org/10.1016/S0168-1591\(01\)00119-8](https://doi.org/10.1016/S0168-1591(01)00119-8)
- Negrão, J.A., 2008. Hormone release and behavior during suckling and milking in Gir, Gir x Holstein, and Holstein cows. *J. Anim. Sci.* 21–26. <https://doi.org/10.2527/jas.2007-0304>

- Negrão, J.A., Marnet, P.G., 2003. Cortisol, adrenalin, noradrenalin and oxytocin release and milk yield during first milkings in primiparous ewes. *Small Rumin. Res.* 47, 69–75. [https://doi.org/10.1016/S0921-4488\(02\)00247-X](https://doi.org/10.1016/S0921-4488(02)00247-X)
- Negrão, J.A., Marnet, P.G., 2002. Effect of calf suckling on oxytocin, prolactin, growth hormone and milk yield in crossbred Gir ´ Holstein cows during milking João. *Reprod. Nutr. Dev.* 43, 373–380. <https://doi.org/10.1051/rnd>
- Negrao, J.A., Porcionato, M.A. de F., de Passille, A.M., Rushen, J., 2010. Behavioural responses of heifers to ACTH injections. *Appl. Anim. Behav. Sci.* 128, 18–22. <https://doi.org/10.1016/j.applanim.2010.09.008>
- Néri, N.R.M., Toledo, L.M., Acaro Júnior, I., Ambrósio, L.A., 2016. Estimulação tátil em novilhas leiteiras: efeitos no comportamento e na produção de leite após o parto. *Bol. Indústria Anim.* 73, 171–179. <https://doi.org/10.17523/bia.v73n3p171>
- Odorčić, M., Rasmussen, M.D., Paulrud, C.O., Bruckmaier, R.M., 2019. Review: Milking machine settings, teat condition and milking efficiency in dairy cows. *Animal* 13, S94–S99. <https://doi.org/10.1017/S1751731119000417>
- Ouellet, V., Negrao, J., Skibiél, A.L., Lantigua, V.A., Fabris, T.F., Marrero, M.G., Dado-Senn, B., Laporta, J., Dahl, G.E., 2021. Endocrine signals altered by heat stress impact dairy cow mammary cellular processes at different stages of the dry period. *Animals* 11, 1–16. <https://doi.org/10.3390/ani11020563>
- Pajor, E.A., Rushen, J., De Passillé, A.M.B., Passille, A.M.B. De, 2000. Aversion learning techniques to evaluate dairy cattle handling practices. *Appl. Anim. Behav. Sci.* 69, 89–102. [https://doi.org/10.1016/S0168-1591\(00\)00119-2](https://doi.org/10.1016/S0168-1591(00)00119-2)
- Paul, H., Coleman, G.J., 2010. Human-livestock interactions: The stockperson and the productivity and welfare of intensively farmed animals, *Human-Livestock Interactions: The Stockperson and the Productivity and Welfare of Intensively Farmed Animals*. <https://doi.org/10.1079/9781845936730.0000>
- Peixoto, M.G.C.D., Pires, M. de F.Á., Pereira, M.C., Ribeiro, M.R.S.C.G.C., Brito, L.F., Verneque, R. da S., Bergmann, J.A.G., 2011. Integrando o temperamento às características de importância para o melhoramento de bovinos de leite : resultados de um estudo com fêmeas Guzerá. *Rev. Bras. Zootec.* 40, 26–37.
- Peters, M.D.P., Barbosa Silveira, I.D., Machado Filho, L.C.P., Machado, A.A., Pereira, L.M.R., 2010. Bem-Estar , Comportamento E Aspectos Produtivos Aversive Management in Dairy Cattle and Effects on Well-Being ,. *Arch. Zootec.* 59, 435–442.
- Petherick, J.C., 2005. Animal welfare issues associated with extensive livestock production: The northern Australian beef cattle industry. *Appl. Anim. Behav. Sci.* 92, 211–234. <https://doi.org/10.1016/j.applanim.2005.05.009>
- Phillips, C., 2002. Cattle Behaviour and Welfare, in: *Cattle Behaviour and Welfare: Second Edition*. <https://doi.org/10.1002/9780470752418>
- Polikarpus, A., Napolitano, F., Grasso, F., Di Palo, R., Zicarelli, F., Arney, D., De Rosa, G., 2014. Effect of pre-partum habituation to milking routine on behaviour and lactation performance of buffalo heifers. *Appl. Anim. Behav. Sci.* 161, 1–6. <https://doi.org/10.1016/j.applanim.2014.10.003>
- Ponchon, B., Zhao, X., Ollier, S., Lacasse, P., 2017. Relationship between glucocorticoids and prolactin during mammary gland stimulation in dairy cows. *J. Dairy Sci.* 100, 1521–1534. <https://doi.org/10.3168/jds.2016-11490>
- Porcionato, M.A.D.F., Negrão, J.A., De Paiva, F.A., Delgado, T.F.G., 2009. Respostas produtivas e comportamentais durante a ordenha de vacas Holandesas em início de lactação. *Acta Sci. - Anim.*

- Sci. 31, 447–451. <https://doi.org/10.4025/actascianims.v31i4.6326>
- Probst, J.K., Hillmann, E., Leiber, F., Kreuzer, M., Spengler Neff, A., 2013. Influence of gentle touching applied few weeks before slaughter on avoidance distance and slaughter stress in finishing cattle. *Appl. Anim. Behav. Sci.* 144, 14–21. <https://doi.org/10.1016/j.applanim.2012.12.007>
- Probst, J.K., Spengler Neff, A., Leiber, F., Kreuzer, M., Hillmann, E., 2012a. Gentle touching in early life reduces avoidance distance and slaughter stress in beef cattle. *Appl. Anim. Behav. Sci.* 139, 42–49. <https://doi.org/10.1016/j.applanim.2012.03.002>
- Probst, J.K., Spengler Neff, A., Leiber, F., Kreuzer, M., Hillmann, E., 2012b. Gentle touching in early life reduces avoidance distance and slaughter stress in beef cattle. *Appl. Anim. Behav. Sci.* <https://doi.org/10.1016/j.applanim.2012.03.002>
- Réale, D., Reader, S.M., Sol, D., McDougall, P.T., Dingemanse, N.J., Sol, D., RMcDougall, P.T., Eader, S.M., 2007. Integrating animal temperament within ecology and evolution. *Biol. Rev.* 82, 291–318. <https://doi.org/10.1111/j.1469-185X.2007.00010.x>
- Rushen, J., De Passillé, A.M.B., Munksgaard, L., 1999. Fear of people by cows and effects on milk yield, behavior, and heart rate at milking. *J. Dairy Sci.* 82, 720–727. [https://doi.org/10.3168/jds.S0022-0302\(99\)75289-6](https://doi.org/10.3168/jds.S0022-0302(99)75289-6)
- Rushen, J., Munksgaard, L., Marnet, P.G., DePassillé, A.M., 2001. Human contact and the effects of acute stress on cows at milking. *Appl. Anim. Behav. Sci.* 73, 1–14. [https://doi.org/10.1016/S0168-1591\(01\)00105-8](https://doi.org/10.1016/S0168-1591(01)00105-8)
- Sánchez-Rodríguez, H.L., Vann, R.C., Youngblood, R.C., Baravik-Munsell, E., Christiansen, D.L., Willard, S., Ryan, P.L., 2013. Evaluation of pulsatility index and diameter of the jugular vein and superficial body temperature as physiological indices of temperament in weaned beef calves: Relationship with serum cortisol concentrations, rectal temperature, and sex. *Livest. Sci.* <https://doi.org/10.1016/j.livsci.2012.10.019>
- Sanger, D.J., Joly, D., 1985. Anxiolytic drugs and the acquisition of conditioned fear in mice. *Psychopharmacology (Berl)*. <https://doi.org/10.1007/BF00428188>
- Sant’Anna, A.C., da Costa, M.J.R.P., 2013. Validity and feasibility of qualitative behavior assessment for the evaluation of Nellore cattle temperament. *Livest. Sci.* 157, 254–262. <https://doi.org/10.1016/j.livsci.2013.08.004>
- Sant’Anna, A.C., da Costa, M.J.R.P., Baldi, F., Rueda, P.M., Albuquerque, L.G., 2012. Genetic associations between flight speed and growth traits in nellore cattle. *J. Anim. Sci.* 90, 3427–3432. <https://doi.org/10.2527/jas.2011-5044>
- Sawa, A., Bogucki, M., Neja, W., Krężel-Czopek, S., 2017. Effect of temperament on performance of primiparous dairy cows. *Ann. Anim. Sci.* 17, 863–872. <https://doi.org/10.1515/aoas-2016-0085>
- Schmied, C., Boivin, X., Waiblinger, S., 2008. Stroking Different Body Regions of Dairy Cows: Effects on Avoidance and Approach Behavior Toward Humans. *J. Dairy Sci.* 91, 596–605. <https://doi.org/10.3168/jds.2007-0360>
- Shahin, M., 2018. The effects of positive human contact by tactile stimulation on dairy cows with different personalities. *Appl. Anim. Behav. Sci.* 204, 23–28. <https://doi.org/10.1016/j.applanim.2018.04.004>
- Silva, L.P., Sant’Anna, A.C., Silva, L.C.M., da Costa, M.J.R.P., 2017. Long-term effects of good handling practices during the pre-weaning period of crossbred dairy heifer calves. *Trop. Anim. Health Prod.* 49, 153–162. <https://doi.org/10.1007/s11250-016-1174-7>
- Soardi, K., Vilanova, M.S., Anami, J.M., Fernandes, R.C., 2017. EFEITO DA CATEGORIA ANIMAL NO COMPORTAMENTO EM SALA DE ORDENHA E NA PRODUÇÃO DE LEITE. *Rev. Científica Rural* 19, 184–194.

- Sutherland, M.A.A., Huddart, F.J.J., 2012. The effect of training first-lactation heifers to the milking parlor on the behavioral reactivity to humans and the physiological and behavioral responses to milking and productivity. *J. Dairy Sci.* 95, 6983–6993. <https://doi.org/10.3168/jds.2011-5211>
- Svennersten-Sjaunja, K., Olsson, K., 2005. Endocrinology of milk production. *Domest. Anim. Endocrinol.* 29, 241–258. <https://doi.org/10.1016/j.domaniend.2005.03.006>
- Tancin, V., Harcek, L., Broucek, J., Uhrincat, M., Mihina, S., 1995. Effect of Suckling During Early Lactation and Changeover to Machine Milking on Plasma Oxytocin and Cortisol Levels and Milking Characteristics in Holstein Cows. *J. Dairy Res.* 62, 249–256. <https://doi.org/10.1017/S0022029900030958>
- Tancin, V., Kraetzl, W.D., Schams, D., Bruckmaier, R.M., 2001. The effects of conditioning to suckling, milking and of calf presence on the release of oxytocin in dairy cows. *Appl. Anim. Behav. Sci.* 72, 235–246. [https://doi.org/10.1016/S0168-1591\(01\)00113-7](https://doi.org/10.1016/S0168-1591(01)00113-7)
- Tarantola, M., Biasato, I., Biasibetti, E., Biagini, D., Capra, P., Guarda, F., Leporati, M., Malfatto, V., Cavallarini, L., Miniscalco, B., Mioletti, S., Vincenti, M., Gastaldo, A., Capucchio, M.T., 2020. Beef cattle welfare assessment: use of resource and animal-based indicators, blood parameters and hair 20 β -dihydrocortisol. *Ital. J. Anim. Sci.* <https://doi.org/10.1080/1828051X.2020.1743783>
- Ujita, A., El Faro, L., Vicentini, R.R., Pereira Lima, M.L., de Oliveira Fernandes, L., Oliveira, A.P., Veroneze, R., Negrão, J.A., 2021. Effect of positive tactile stimulation and prepartum milking routine training on behavior, cortisol and oxytocin in milking, milk composition, and milk yield in Gyr cows in early lactation. *Appl. Anim. Behav. Sci.* <https://doi.org/10.1016/j.applanim.2020.105205>
- Ujita, A., Ribeiro Vicentini, R., Pereira Lima, M.L., Negrão, J.A., de Oliveira Fernandes, L., Penido Oliveira, A., Veroneze, R., El Faro Zadra, L., 2020. Improvements in the behaviour of Gir dairy cows after training with brushing. *J. Appl. Anim. Res.* <https://doi.org/10.1080/09712119.2020.1754217>
- Van Reenen, C.G., Engel, B., Ruis-Heutinck, L.F.M., Van Der Werf, J.T.N., Buist, W.G., Jones, R.B., Blokhuis, H.J., 2004. Behavioural reactivity of heifer calves in potentially alarming test situations: A multivariate and correlational analysis. *Appl. Anim. Behav. Sci.* <https://doi.org/10.1016/j.applanim.2003.09.007>
- Van Reenen, C.G., Van der Werf, J.T.N., Bruckmaier, R.M., Hopster, H., Engel, B., Noordhuizen, J.P.T.M., Blokhuis, H.J., 2002. Individual differences in behavioral and physiological responsiveness of primiparous dairy cows to machine milking. *J. Dairy Sci.* 85, 2551–61. [https://doi.org/10.3168/jds.S0022-0302\(02\)74338-5](https://doi.org/10.3168/jds.S0022-0302(02)74338-5)
- Vicentini, R.R., Montanholi, Y.R., Veroneze, R., Oliveira, A.P., Lima, M.L.P., Ujita, A., El Faro, L., 2020. Infrared thermography reveals surface body temperature changes during proestrus and estrus reproductive phases in Gyr heifers (*Bos taurus indicus*). *J. Therm. Biol.* 92, 102662. <https://doi.org/10.1016/j.jtherbio.2020.102662>
- Waiblinger, S., Boivin, X., Pedersen, V., Tosi, M.V., Janczak, A.M., Visser, E.K., Jones, R.B., 2006. Assessing the human-animal relationship in farmed species: A critical review. *Appl. Anim. Behav. Sci.* 101, 185–242. <https://doi.org/10.1016/j.applanim.2006.02.001>
- Wang, S., Zhang, H., Tian, H., Chen, X., Li, S., Lu, Y., Li, L., Wang, D., 2020. Alterations in vaginal temperature during the estrous cycle in dairy cows detected by a new intravaginal device—a pilot study. *Trop. Anim. Health Prod.* <https://doi.org/10.1007/s11250-020-02199-5>
- Weaver, S.R., Bohrer, J.C., Prichard, A.S., Perez, P.K., Streckenbach, L.J., Olson, J.M., Cook, M.E., Hernandez, L.L., 2016. Serotonin deficiency rescues lactation on day 1 in mice fed a high fat diet. *PLoS One* 11, 1–18. <https://doi.org/10.1371/journal.pone.0162432>
- Wicks, H.C.F., Carson, A.F., McCoy, M.A., Mayne, C.S., 2004. Effects of habituation to the milking

- parlour on the milk production and reproductive performance of first calving Holstein-Friesian and Norwegian dairy herd replacements. *Anim. Sci.* 78, 345–354.
- Windschnurer, I., Barth, K., Waiblinger, S., 2009. Can stroking during milking decrease avoidance distances of cows towards humans?, in: *Animal Welfare*.
- Wulff-Risner, L., Stewart, B., 1997. Using Experiential Learning To Teach Evaluation Skills. *J. Agric. Educ.* <https://doi.org/10.5032/jae.1997.03043>
- X Boivin, Lensink, J., Tallet, C., Veissier, I., 2003. Report on stockmanship and farm animal welfare [WWW Document]. *Anim. Welf.* URL <https://www.gov.uk/government/publications/fawc-report-on-stockmanship-and-farm-animal-welfare>
- Zliobaite, I., 2019. Revisiting biogeography of livestock animal domestication. *bioRxiv.* <https://doi.org/10.1101/786442>