UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO”
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

AVALIAÇÃO DA INFLUÊNCIA DA HORA DO DIA, ANESTESIA E ANALGESIA NOS COMPORTAMENTOS ESPONTÂNEOS DE DOR APÓS ORQUIECTOMIA EM CAVALOS

PEDRO HENRIQUE ESTEVES TRINDADE

Botucatu, São Paulo
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Tese de doutorado apresentada ao programa de pós-graduação em Biotecnologia Animal para obtenção do título de Doutor em Biotecnologia Animal, subárea de anestesiologia veterinária.

Orientador: Prof. Dr. Stelio Pacca Loureiro Luna

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Dedico esta tese

Aos familiares e ancestrais que possibilitaram minha existência

Aos animais, minha motivação

À ciência, minha vocação

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TRINDADE, Pedro Henrique Esteves. Avaliação da influência da hora do dia, anestesia e analgesia nos comportamentos espontâneos de dor após orquiectomia em cavalos, Orientador: Stelio Pacca Loureiro Luna, 2021. Tese (Doutorado em Biotecnologia Animal). Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista "Júlio de Mesquita Filho", Botucatu, 2021

Nas últimas duas décadas houve um crescente número de estudos empenhando esforços para diagnosticar a dor no *Equus caballus ferus* pela linguagem corporal. Entretanto, fatores de confusão ainda pouco esclarecidos ou não estudados podem estar dificultando a avaliação da dor em cavalos. Portanto, objetivou com a tese identificar comportamentos espontâneos de dor pós-orquiectomia em cavalos, independentemente dos efeitos da anestesia, analgesia e horário de registro do dia. No primeiro capítulo está apresentada uma introdução acerca da temática e uma breve revisão de literatura sobre a dinâmica da resposta comportamental diante do efeito residual de fármacos, interação com humano, horário do dia e intensidade da dor. Na segunda decomposição capitular está apresentado o estudo prospectivo e longitudinal. Vinte e quatro cavalos divididos em quatro grupos foram submetidos à: anestesia inalatória apenas (GA), ou combinada com analgesia prévia (GAA), orquiectomia sob analgesia pré (ACG) ou pós-operatória (GC). Os dados obtidos da subtração da frequência e / ou duração de 34 comportamentos registrados durante sete momentos de 60 minutos nas 24 horas após a anestesia daqueles registrados nos momentos espelhados nas 24 horas antes da anestesia (delta) foram comparados ao longo do tempo e entre os grupos pelos testes de Friedman e Kruskal-Wallis, respectivamente ($p < 0,05$). A hora do dia influenciou os comportamentos de caminhar, olhar pela janela, descansar o membro pélvico e ficar parado. Os únicos comportamentos relacionados à dor encontrados foram beber, comer, olhar para a ferida, retrair o membro pélvico, expor o pênis e olhar para o fundo da baia considerando o delta. Em conclusão, os fatores de confusão podem influenciar vários comportamentos relacionados à dor documentados na literatura. No terceiro capítulo está apresentado as considerações finais das informações dos capítulos anteriores, evidenciando os benefícios práticos dos achados desta tese.

Palavras-chave: avaliação de dor; bem-estar animal; cavalos.

Abstract

TRINDADE, Pedro Henrique Esteves. **Assessment of the influence of time of day, anesthesia, and analgesia on spontaneous pain behaviors after orchiectomy in horses**, Supervisor: Stelio Pacca Loureiro Luna, 2021. Tese (Doctor in Biotecnologia Animal). Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista "Júlio de Mesquita Filho", Botucatu, 2021

Over the past two decades there has been a growing number of studies making efforts to diagnose pain through body language in *Equus caballus ferus*. However, confounding factors that are still poorly understood or not studied may be making it difficult to assess pain in horses. Therefore, the current thesis aimed to identify spontaneous post-orchiectomy pain behaviors in horses, regardless of the effects of anesthesia, analgesia, and recording time of day. The first chapter presents an introduction to the subject and a brief literature review on the dynamics of the behavioral response to the residual effect of drugs, interactions with humans, time of day, and pain intensity. The second chapter presents the prospective and longitudinal study. Twenty-four horses divided into four groups were submitted to: inhalation anesthesia only (GA), or combined with previous analgesia (GAA), orchiectomy under pre (ACG) or postoperative (GC) analgesia. Data obtained by subtracting the frequency and/or duration of 34 behaviors recorded during seven 60-minute moments in the 24 hours after anesthesia from those recorded in the mirrored moments in the 24 hours before anesthesia (delta) were compared over time and between the groups by the Friedman and Kruskal-Wallis tests, respectively ($p < 0.05$). The time of day influenced the behaviors of walking, looking out the window, resting the pelvic limb, and standing still. Considering the delta, the only pain-related behaviors found were drinking, eating, looking at the wound, retracting the pelvic limb, exposing the penis, and looking at the back of the stall. In conclusion, confounding factors can influence several pain-related behaviors documented in the literature. The third chapter

presents the final considerations on the information from the previous chapters, highlighting the practical benefits of the findings of this thesis.
Keywords: animal welfare; horses; pain measurement.

CAPÍTULO 1

1. Introdução e Justificativa

A conscientização acerca da senciência nos animais tem despertado uma crescente preocupação das sociedades em relação ao bem-estar de animais não humanos (MELLOR et al., 2020). Definiu-se bem-estar como “o estado físico e mental de um animal em relação às condições em que vive e morre” (OIE, 2010), a qual engloba a dor como um dos fatores para seu empobrecimento. A dor por sua vez é entendida como uma “uma experiência sensorial e emocional desagradável associada a, ou semelhante àquela associada a, dano real ou potencial ao tecido” (RAJA et a., 2020). Portanto, além da dor ocasionar desconforto pela percepção somatossensorial, também afeta estados emocionais por provocar sofrimento e angústia quando não tratada apropriadamente, por isso, é imprescindível diagnosticá-la para tratá-la de forma adequada (MOLONY, 1997; HELLEBREKERS, 2000) e evitar o sofrimento desnecessário nos animais (GREEN; MELLOR, 2011).

Na última década, revisões apontam que houve um crescente número de estudos empenhando esforços para diagnosticar e reduzir os impactos negativos da dor no *status* de bem-estar do *Equus ferus caballus* (DE GRAUW; VAN LOON, 2016; GLEERUP; LINDEGAARD, 2016; VAN LOON; VAN DIERENDONCK, 2018). Nesse sentido, foram elaboradas escalas compostas contemplando o domínio fisiológico, comportamental e emocional dos cavalos. Tais instrumentos objetivaram quantificar a dor aguda em situações específicas, como a síndrome do abdômen agudo (DE GRAUW; VAN LOON, 2016; GRAUBNER et al., 2011; LAWSON et al., 2020; PRITCHETT et al., 2003; SELLON et al., 2004; SUTTON et al., 2013a, 2013b, 2016, 2019; VAN LOON et al., 2010), dor dentária (LAWSON et al., 2020; PEHKONEN; KARMA; RAEKALLIO, 2019), após orquiectomia (DALLA COSTA et al., 2014; LAWSON et al., 2020; TAFFAREL et al., 2015; VAN LOON et al., 2010) e após procedimentos ortopédicos (BUSSIÈRES et al., 2008; DUTTON; LASHNITS; WEGNER, 2009; LAWSON et al., 2020; LINDEGAARD et al., 2010; PRICE et al., 2003; RAEKALLIO; TAYLOR; BLOOMFIELD, 1997; VAN LOON; VAN DIERENDONCK, 2015, 2019). Apesar da motilidade gastrointestinal, taxa respiratória e cardíaca estarem contempladas em algumas dessas metodologias, sua capacidade diagnóstica foi considerada questionável devido à baixa especificidade (ANDERSEN et al., 2018; GLEERUP; LINDEGAARD, 2016; GRAUBNER et al., 2011; LINDEGAARD et al., 2010; PRICE et al., 2003; RAEKALLIO; TAYLOR; BLOOMFIELD, 1997; SELLON et al., 2004). Revisão aponta que a exclusão de parâmetros pouco sensíveis e/ou específicos como supracitados possa melhorar a validação e o tempo de aplicação das escalas compostas (DE GRAUW; VAN LOON, 2016). Além de ser considerada específica e sensível para diagnosticar a dor nos equinos, a avaliação comportamentos que exibidos espontaneamente sem a presença ou interferência humana representa o refinamento metodológico por não ser invasiva e intrusiva com base no princípio dos 4 R's (*reduce*, *refine*, *replacement and respect*) adotados na experimentação animal (HUBRECHT; CARTER, 2019).

Apesar da vasta literatura no que tange avaliações de dor em equinos, estudos sugerem que ainda não existe um método considerado como padrão-ouro para tal propósito (ANDERSEN et al., 2018; DODDS et al., 2017; GIGLIUTO et al., 2014; GUEDES, 2017). Isso pode ser parcialmente explicado pela característica multidimensional da dor, sendo ela constituída pela combinação dos componentes somatossensitivos, cognitivos e emocionais, tornando sua interpretação bastante complexa e individual (PRICE, 2000). Entretanto, em nosso entendimento, existem fatores de confusão pouco esclarecidos que poderiam interferir no reconhecimento da dor com base na linguagem corporal como, o efeito residual das drogas para anestesia e analgesia, e a intensidade da dor; ou mesmo fatores de confusão ainda não estudados como o efeito do horário de registro do comportamento. Tais fatores poderiam ocasionar sub ou superestimação do diagnóstico da dor, portanto requerem investigação detalhada para obter uma estimativa mais precisa e eficiente da dor nos equinos.

Portanto, diante do exposto, objetivou-se com esta tese identificar comportamentos espontâneos de dor pós-orquiectomia em cavalos, independentemente dos efeitos da anestesia, analgesia e horário de registro do dia.

2. Revisão de literatura

2.1 Definições, conceitos e contextualização

A primeira sessão desta revisão destina-se ao esclarecimento de definições e conceitos que serão largamente usados no decorrer do texto, bem como contextualizar a importância de tais entendimentos para o foco principal desta obra.

Nos últimos três anos o entendimento da dor para os animais humanos definida como “uma experiência sensorial e emocional desagradável associada a um dano real ou potencial ao tecido, ou descrita em termos de tal dano” pela *Associação Internacional para o Estudo da Dor* (IASP) em 1979 tem sido contestada. Tal questionamento está embasado na individualidade interpretativa do descritor “desagradável”, na omissão de dores desmembradas de qualquer dano tecidual e, por fim, nos pacientes dependentes de um observador para estimar a dor (COHEN; QUINTNER; VAN RYSEWYK, 2018).

Antecipando esta indagação, para os animais não humanos a dor tem sido melhor entendida desde o pretérito como “uma experiência sensorial e emocional aversiva que representa uma consciência por parte do animal de dano ou ameaça à integridade de seus tecidos” (MOLONY, 1997). A definição atualizada da de dor pela IASP em 2020 como sendo “uma experiência sensorial e emocional desagradável associada a, ou semelhante àquela associada a, dano real ou potencial ao tecido” está em harmonia com a definição estabelecida no final da década de 90 para os animais supracitada. Essas definições destacam a relevância da resposta comportamental relacionada a dor e elucida o conceito de multidimensionalidade da dor delineado pelo componente somatossensitivo, cognitivo e emocional, demonstrando a complexidade na estimativa da dor que pode ser potencializada pela singularidade de cada animal (PRICE, 2000).

A dor poder ser ordenada por diversas classificações, uma delas fraciona-a em três tipos de acordo com sua ação no organismo (nociceptiva, inflamatória e neuropática) (GUEDES, 2017). Em uma visão macroscópica, a dor classificada como nociceptiva representa na maioria dos casos um estímulo temporário com a função de alertar o organismo e protegê-lo de uma possível lesão; enquanto que a dor inflamatória representa um estímulo mais duradouro que objetiva diminuir a movimentação para restaurar o dano tecidual ocasionado, sendo evocada com a ativação dos nociceptores pelas células inflamatórias atraídas quimicamente para o local do dano tecidual; e por fim, a dor neuropática sem uma função discernível de proteção ou reparação ocasionada por uma disfunção ou dano ao sistema nervoso (GUEDES, 2017).

A dor pode ser considerada como uma de muitas das fontes de sofrimento animal, entendido por “um conjunto de emoções negativas, como medo, dor e tédio, e reconhecidas operacionalmente como estados causados por reforços negativos” (DAWKINS, 2008), que contribui para o empobrecimento do *status* de bem-estar animal. Uma das definições mais aceitas de bem-estar para a comunidade científica refere-se ao o “estado físico e mental de um animal em relação às condições em que vive e morre” (OIE, 2010). Já é claro que a nocicepção e a dor tiveram uma substancial importância adaptativa no processo evolutivo das espécies com a função básica de alerta para um potencial dano, estabelecendo motivações para própria proteção e aprendizado para evitar danos futuros (SNEDDON et al., 2014), sendo desta forma considerada como dor adaptativa ou fisiológica (GUEDES, 2017). No entanto, também existem dores consideradas mal adaptativas provenientes de sequelas de doenças, traumas cirúrgicos ou outras situações com maior intensidade e duração comparado com as dores adaptativas, que desencadeiam um *status* motivacional aversivo, como angústia e sofrimento (GUEDES, 2017; SNEDDON et al., 2014).

Nas últimas décadas a conscientização acerca da capacidade de sentir (*senciência*) dos animais tem despertado uma crescente preocupação das sociedades ao redor do mundo em relação ao bem-estar de animais não humanos (MELLOR et al., 2020). Um *status* de bem-estar “bom é quando o animal está saudável e tem o que deseja”, evidenciando a necessidade de preservar as funções biológicas e atividades de manutenção dos organismos (DAWKINS, 2003 e 2004). Tal cuidado também é essencial no cenário da dor pós-operatória cuja privação de sono, por exemplo, mostrou-se capaz de prejudicar o tempo de cura de ratos (LI et al., 2019). Tal ensejo engloba o eminente requerimento de dimensionar e tratar adequadamente a dor mal adaptativa (MOLONY, 1997; HELLEBREKERS, 2000) para evitar o sofrimento desnecessário nos animais (GREEN; MELLOR, 2011).

É importante saber que a maioria dos estudos com foco em diagnosticar a dor em equinos foram conduzidos após cirurgias ortopédicas ou celiotomia (HALL et al., 2018) o que pode representar apenas uma parcela (dor inflamatória de moderada a severa) da vasta abrangência dos tipos de dor levando em conta que a sensação dolorosa é uma experiência singular e multidimensional (PRICE, 2000) como apresentado no decorrer desta sessão. Além do amplo horizonte de estudos a serem conduzidos ao entorno dessa temática, o diagnóstico da dor em equinos ainda conta com diversos desafios para serem alcançados que serão apresentados nas sessões subsequentes.

2.2 Comportamentos relacionados à dor

O privilégio do autorrelato da dor para a maioria dos animais humanos, desde o princípio foi um grande desafio na avaliação da dor nos outros animais incorporando complexidade no diagnóstico da dor. Portanto, cientistas ao redor do mundo têm buscado por sinais que possam servir de base interpretativa para a estimativa da dor como, a resposta fisiológica, endócrina e comportamental passíveis de mensuração nos equinos (SNEDDON et al., 2014). A partir dos esclarecimentos supracitados será apresentado nessa sessão a aplicação do comportamento como instrumento diagnóstico da dor, assim como uma breve justificativa da sua escolha dele dentre outros indicadores.

Em cavalos que experimentem dor, observa-se comportamentos novos ou incomuns e diminuição de comportamentos normais, espontâneos ou evocados, acompanhados ou não de mudanças fisiológicas, como pressão arterial, motilidade gastrointestinal, frequências cardíaca e respiratória (GUEDES, 2017). Embora os indicadores fisiológicos sejam úteis para estimar a dor em animais, eles podem ser alterados por outros fenômenos diferentes da dor, como ocorre com a taxa cardíaca na presença de humanos não familiares, ambiente desconhecido, momentos de ansiedade, isolamento social e estresse térmico, demonstrando um grau baixo de especificidade para diagnóstico de dor (ALM, 2004; DAS et al., 2016; GRIPPO et al., 2007; LANSADE; BOUISSOU, 2008). Dessa forma, as informações provenientes da linguagem corporal podem fornecer subsídios mais específicos para melhor conhecer a dor, além de serem considerados métodos não invasivos, não intrusivos e práticos que podem ser avaliados em tempo real por vídeo aplicando escalas comportamentais já validadas sem a necessidade de equipamentos sofisticados como requerido para dosagens bioquímicas (DAWKINS, 2004; DESCOVICH, 2017).

Apesar de os cavalos evitarem a exibição de suas vulnerabilidades como os sinais de dor, eles exibem uma gama de posturas e movimentos corporais que podem ser utilizados para avaliar a dor (ASHLEY; WATERMAN-PEARSON; WHAY, 2005). Inicialmente os comportamentos relacionados com a dor foram divididos em duas classes: não específicos e específicos. Dentre a primeira classe destacam-se agitação ou ansiedade, relutância a locomover-se, posição corporal rígida, cabeça baixa, agressão contra humanos, objetos, outros animais ou contra si próprio (ASHLEY; WATERMAN-PEARSON; WHAY, 2005).

Os indicadores comportamentais específicos em cavalos relacionam-se com a localização da inflamação ou lesão. Dores nos membros podem ser detectadas por claudicação, troca de apoio do peso corporal entre os membros, evitar que se toque determinado membro, apoiar apenas a pinça dos cascos, suspender o membro torácico e rotacioná-lo e distribuição anormal do peso. De igual modo, a dor de origem visceral faz com que os cavalos exteriorizem comportamentos, como grunhir, rolar, chutar o abdome, olhar para o flanco e alongar-se. Já dores relacionadas à cabeça ou aos dentes estão associadas ao meneio da cabeça e comportamentos mastigatórios anormais (ASHLEY; WATERMAN-PEARSON; WHAY, 2005).

A partir do conhecimento sobre a linguagem corporal relacionada a dor foram desenvolvidos instrumentos para avaliar a dor como, a escala analógica visual representada por uma linha horizontal de 100 milímetros sem gradações, a escala de contagem numérica variando de 0 à 10 cuja o incremento na pontuação de ambas escalas sinaliza maior severidade da dor, e a escala descritiva simples classificando a dor em ausente, leve, moderada e severa; sendo que o julgamento da pontuação de todas elas baseia-se na interpretação da linguagem corporal de acordo com a expertise do avaliador (DE GRAUW; VAN LOON, 2016). Mais adiante, dois estudos avaliaram (PRICE et al., 2003; PRITCHETT et al., 2003) a resposta comportamental da dor por variáveis entendidas como mais objetivas do que as escalas unidimensionais mencionadas acima, como, por exemplo, um etograma composto por registro da frequência e duração dispensada em cada comportamento analisados por um longo período de tempo (dois momentos de 24-48 h), revelando a contribuição de cada comportamento relacionado a dores originárias por artroscopia e celiotomia. Estes dois etogramas mostraram que além da exibição dos comportamentos específicos de dor relacionados a área afetada, os equinos exibiram alterações em comportamentos de manutenção relativos a diminuição da locomoção, interesse pelo ambiente ao redor, ingestão hídrica e alimentar que poderiam ser utilizados para o diagnóstico da dor (PRICE et al., 2003; PRITCHETT et al., 2003).

Vários desses comportamentos foram usados para criar escalas compostas contemplando aspectos fisiológicos, comportamentais e emocionais para avaliar a dor nos cavalos. Para essas escalas compostas o comportamento foi avaliado por um período de tempo mais curto (de dois a seis momentos de 5-15 min) aplicando escores referentes a linguagem corporal atinente a atividade, interação, locomoção, postura, apetite, desconforto e atenção à área afetada e ao ambiente, com descrições preestabelecidas para quantificar a dor aguda em situações específicas. Um exemplo são dores originárias de síndrome do abdômen agudo (DE GRAUW; VAN LOON, 2016; GRAUBNER et al., 2011; LAWSON et al., 2020; PRITCHETT et al., 2003; SELLON et al., 2004; SUTTON et al., 2013a, 2013b, 2016, 2019; VAN LOON et al., 2010), dor dentária (LAWSON et al., 2020; PEHKONEN; KARMA; RAEKALLIO, 2019), após orquiectomia (DALLA COSTA et al., 2014; LAWSON et al., 2020; TAFFAREL et al., 2015; VAN LOON et al., 2010) e após procedimentos ortopédicos (BUSSIÈRES et al., 2008; DUTTON; LASHNITS; WEGNER, 2009; LAWSON et al., 2020; LINDEGAARD et al., 2010; PRICE et al., 2003; RAEKALLIO; TAYLOR; BLOOMFIELD, 1997; VAN LOON; VAN DIERENDONCK, 2015, 2019).

Segundo revisão recente, dentre os indicadores comportamentais, as contrações e tensões faciais têm sido consideradas como sinais mais fidedignos do estado interno dos animais, em comparação com toda a linguagem corporal (DESCOVICH, 2017). Recentemente, foram identificadas expressões faciais de dor em vários mamíferos, tais como ratos (LANGFORD et al., 2010), coelhos (KEATING et al., 2012), gatos (EVANGELISTA et al., 2020), bois (GLEERUP et al., 2015a), porcos (DI GIMINIANI et al., 2016; GÖRANSSON, 2016) e ovelhas

(GUESGEN et al., 2016; MCLENNAN et al., 2016). Em equinos a orientação das orelhas, abertura dos olhos, contração da musculatura dos lábios e ao redor dos olhos, tensão do focinho e dos músculos miméticos ou mastigatórios, apresentaram associação com processos dolorosos como pós-orquiectomia (DALLA COSTA et al., 2014), indução de dor por capsaicina tópica no antebraço (GLEERUP et al., 2015b), síndrome do abdômen agudo (VAN LOON; VAN DIERENDONCK, 2015) e durante atividade física (MULLARD et al., 2017; TRINDADE et al., 2020).

Em um estudo que investigou as características da face relacionadas apenas aos olhos dos cavalos, foram descritas seis medidas distintas com base nas contrações, formação de rugas e ângulos apresentados ao redor dos olhos (HINTZE et al., 2016), demonstrando a elevada expressividade da configuração facial nesta espécie. A compreensão das contrações e tensões da face parece ser de complexa interpretação, pois uma mesma característica da face pode ser observada em diferentes situações (TRINDADE; COSTA; PARANHOS DA COSTA, 2018). Por exemplo, os olhos parcialmente fechados foram relacionados com dor (DALLA COSTA et al., 2014; MULLARD et al., 2017; VAN LOON; VAN DIERENDONCK, 2015), mas também são características exibidas quando o cavalo está sonolento ou em descanso (MCDONNELL, 2003) ou cansado fisicamente (TRINDADE et al., 2020). Deste modo, é provável que uma análise conjunta das expressões da face seja mais eficiente que sua avaliação isolada de cada característica (DESCOVICH, 2017; TRINDADE; COSTA; PARANHOS DA COSTA, 2018). Apesar das expressões faciais serem recomendadas como boa ferramenta para diagnóstico da dor, as características faciais podem não ser indicadores práticos para aplicação na rotina clínica, visto que os animais precisam ser filmados para posterior extração de imagens ou videoclipes das gravações originais para só depois realizar as avaliações, além de necessitar de treinamento específico e avaliador experiente muito mais do que as posturas e movimentos corporais devido a sutileza de detalhes (ANDERSEN et al., 2018; ASK et al., 2020).

Apesar dos cientistas da área concordarem quanto a inexistência de um método padrão-ouro para diagnóstico da dor em equinos, o comportamento tem sido reconhecido como uma opção útil e mais utilizada para tal finalidade (DE

GRAUW; VAN LOON, 2016; GLEERUP; LINDEGAARD, 2016; VAN LOON; VAN DIERENDONCK, 2018). Muito embora, fatores de confusão pouco ou nunca examinados no registro comportamental podem ter forte influência na avaliação da dor e, portanto, serão apresentados nas sessões seguintes dessa revisão.

2.3 Efeitos de confusão no comportamento de dor

A dor aguda em equinos tem sido estimada com base na linguagem corporal da espécie (BUSSIÈRES et al., 2008; DALLA COSTA et al., 2014; GLEERUP et al., 2015b; PRICE et al., 2003; PRITCHETT et al., 2003; TAFFAREL et al., 2015; VAN LOON; VAN DIERENDONCK, 2015). Entretanto, o comportamento não está livre de efeitos de confusão (MARTIN; BATESON, 1993). c

2.3.1 Presença e interação com humanos

Estudo tem questionado o paradigma que animais considerados como presas são evitam demonstrar a dor porque cães e gatos também poderiam evitar tal demonstrativa (CARBONE et al., 2020). Desta maneira é possível interpretar que outros fatores atrelados a presença humana poderiam estar dificultando a estimativa da dor (CARBONE et al., 2020). Apesar disso, alguns dos instrumentos para diagnóstico da dor em cavalos foram desenvolvidos com base em observações comportamentais *in locu* (VAN LOON; VAN DIERENDONCK, 2015, 2019). A avaliação da linguagem corporal pode ser considerada não intrusiva (DESCOVICH, 2017), muito embora quando o observador não for familiar ao cavalo, a sua presença pode ocasionar mudanças comportamentais devido ao medo ou curiosidade (WAGNER, 2010), por isso é importante considerar um período de adaptação antes de iniciar avaliações comportamentais *in cito* (MARTIN; BATESON, 1993). Por exemplo, em ratos expostos a um objeto e recinto desconhecidos, denominados testes de novo objeto e de nova arena respectivamente, reduziram a linguagem corporal de dor nos cinco primeiros minutos após administração de formalina intraplantar (FORD et al., 2008). De maneira análoga ao objeto no estudo anterior, os humanos poderiam representar fonte de ‘novidade’ ao animal fazendo com que ele mude seu comportamento relativo à dor.

Uma possível explicação para a redução dos comportamentos relativos a dor durante os 5 primeiros minutos de exposição à um novo objeto é o estresse ou medo que estimulam a liberação de opioides endógenos e reduzem momentaneamente a percepção da sensação dolorosa para uma reação defensiva do animal (BUTLER; FINN, 2009; FANSELOW, 1986). Outros estudos demonstraram que estímulos visuais e auditivos que configuram distrações, reduzem a expressão de comportamentos relacionados à dor em animais de laboratório (MORIARTY; MCGUIRE; FINN, 2011), que para o caso dos equinos hospitalizados os humanos desconhecidos poderiam representar distrações análogas aos estímulos visuais e auditivos do estudo anterior. Um estudo com equinos reportou alterações na resposta comportamental relacionada a dor mediante apenas a presença do humano, sugerindo que a avaliação sem a presença do observador poderia ser mais fidedigna (TORCIVIA; MCDONNELL, 2020). Em coelhos, a presença do observador ocasionou subestimação dos comportamentos de dor após a cirurgia e inibiu comportamentos normais antes da cirurgia (PINHO et al., 2020). Em contrapartida, em estudo com equinos não se observou diferença comportamental em cavalos com dor induzida na presença ou ausência de avaliador durante quantificação da dor (GLEERUP et al., 2015b). Entretanto, o avaliador não estava ausente do recinto, ele permaneceu escondido atrás de uma parede a 1 m de distância do cavalo, podendo representar estímulo auditivo e ou olfativo. Além disso, trata-se de estudo experimental no qual os animais foram previamente habituados ao ambiente de coleta e aos avaliadores.

Ademais, a resposta comportamental de equinos mediante a humanos pode ser diferente quando comparamos equinos cujas relações humano-animal prévias tenham sido amigáveis com aqueles com histórico de relações prévias aversivas (SCOPA et al., 2019). Apesar disso, a interação humano animal é realizada nas rotinas hospitalares e proporciona importante subsídios para estimar a dor por meio da palpação, resposta ao experimentador e locomoção conduzida (comportamento induzido). Entretanto, tal manipulação para investigar claudicação ou sensibilidade na ferida poderia ocasionar aumento do estímulo doloroso (GLEERUP et al., 2015b). Para evitar esse potencial fator de confusão, a avaliação das posturas e movimentos corporais poderia valer-se apenas de comportamentos espontâneos, como já empregado em algumas das avaliações de expressões faciais em equinos (DALLA COSTA et al., 2014; GLEERUP et al., 2015b).

2.3.2 Drogas anestésicas e analgésicas

É sabido que a expressão comportamental da dor pós-operatória em equinos pode sofrer interferência das drogas para sedação e anestesia geral (ASHLEY; WATERMAN-PEARSON; WHAY, 2005; GLEERUP; LINDEGAARD, 2016). Em gatos submetidos a um protocolo de anestesia dissociativa, os comportamentos de dor relacionados à locomoção foram alterados indicando diagnóstico falso-positivo para dor (BUISMAN et al., 2016). Sabendo desse efeito de confusão, para cães (WAGNER; HECKER; PANG, 2017) e equinos (OLIVEIRA et al., 2021) foi desenvolvida escala comportamental para reconhecimento de sedação. A diminuição da resposta comportamental mediante à estímulos visuais e auditivos é um parâmetro estimado na escala para reconhecimento de sedação em equinos (OLIVEIRA et al., 2021), muito embora a diminuição da atenção aos estímulos ambientais também se relaciona com a presença da dor (GRAUBNER et al., 2011; LINDEGAARD et al., 2010; PRITCHETT et al., 2003; TAFFAREL et al., 2015). A convergência entre os comportamentos de interesse no ambiente originários pelo fenômeno da dor ou da sedação pode representar um fator de confusão na avaliação da dor em equinos que requer investigação futura.

Apesar disso, para cavalos, estudos prévios assumiram empiricamente que 4 (VAN LOON; VAN DIERENDONCK, 2015; VANDIERENDONCK; VAN LOON, 2016; ZUTT et al., 2014) ou 6h (VAN LOON; VAN DIERENDONCK, 2019) após a recuperação anestésica seria tempo suficiente para evitar o fator de confusão desses fármacos na estimativa da dor, sendo que a falta de um controle negativo foi uma limitação reconhecida pelos autores (VAN LOON; VAN DIERENDONCK, 2019). A investigação com foco em equinos apenas anestesiados não mostrou qualquer diferença na somatória total da escala comportamental relacionado a dor 4h pós-recuperação em comparação com animais não anestesiados (PRITCHETT et al., 2003) ou 8h pós-recuperação para aqueles anestesiados e operados (DALLA COSTA et al., 2014;

GRAUBNER et al., 2011). Apesar disso, em dois desses estudos (GRAUBNER et al., 2011; PRITCHETT et al., 2003) os equinos foram anestesiados para exames de imagem e o tempo de anestesia geral foi diferente entre os grupos que eles foram comparados (anestesiados para celiotomia versus anestesiados para exame de imagem), além disso, no outro estudo foi usado um número inferior de equinos apenas anestesiados (6 *versus* 19 *versus* 21) em relação aos grupos comparados (DALLA COSTA et al., 2014). Portanto existe a necessidade de mais estudos investigando os efeitos desses fármacos na exibição comportamental relacionada a dor.

2.3.3 Intensidade da dor

A intensidade do processo doloroso é um aspecto relevante para a avaliação da dor com base na linguagem corporal. Um estudo não conseguiu reconhecer a dor com base no comportamento de equinos submetidos a implante subcutâneo de microchip e marcação a fogo, apesar de confirmarem o processo inflamatório local por aspectos clínicos, fisiológicos e endócrinos (LINDEGAARD et al., 2009). Cavalos castrados com diferentes protocolos analgésicos também não mostraram diferença na escala comportamental facial e corporal 8h pós-castração (DALLA COSTA et al., 2014).

Por outro lado, experimentos demonstraram que indicadores comportamentais foram capazes de diferenciar ovelhas (PAULL et al., 2009, 2012; SMALL et al., 2020) e porcos (SUTHERLAND; DAVIS; MCGLONE, 2011; WHALIN et al., 2016) com dor devido a claudicação, após caudectomia ou orquiectomia que receberam protocolos analgésicos com diferentes qualidades. Isso pode ser explicado parcialmente por que as ovelhas e os porcos estavam alojados em grupo. Grupos menores de ovelhas apresentaram maiores concentrações de cortisol salivar e maior tempo de vigília em comparação a grupos mais numerosos, evidenciando que o tamanho do grupo pode influenciar o nível de estresse (MICHELENA et al., 2012). Já é sabido que animais gregários coordenam e sincronizam o estado de vigilância dentro do grupo para que indivíduos possam ter momentos de repouso, alimentação, cuidado parietal, comportamento sexual, enquanto outros vigiam, como sendo um valor adaptativo de estratégia anti-predatória sustentável a longo prazo (SIROT;

TOUZALIN, 2009), o que também ocorre com equinos (DURANTON; GAUNET, 2016). Talvez o isolamento social dos equinos em baia reduza a exibição de suas vulnerabilidades, como a dor. Ademais, vale destacar que estudos com suínos e ovinos são desenvolvidos sob uma homogeneidade amostral (mesma idade e raça), e já é sabido que a idade (TORREY et al., 2009) e a raça (SILVA et al., 2020) podem interferir na exibição comportamental relacionada a dor para suínos e ovinos.

Apenas dois estudos clínicos aplicaram escalas comportamentais em equinos hospitalizados por variadas causas e possivelmente diferentes intensidades da dor (LAWSON et al., 2020; VAN LOON et al., 2010). Como a causa da internação era dependente dos atendimentos, o número de animais para cada tipo de intervenção era desbalanceado. Por tanto, aparentemente o diagnóstico da dor em diferentes intensidades ainda não está totalmente esclarecido quanto nos resultados reportados previamente em suínos e ovinos, demonstrando requerimento de mais estudos com a espécie equina.

2.3.4 Horário do dia

Muito pouco se sabe sobre a influência do horário do dia no comportamento relacionado a dor para a espécie equina. Um único estudo avaliou equinos durante 24h antes da intervenção cirurgia (sem dor) sendo que todos os registros de um mesmo comportamento neste período foram somados, impossibilitando a interpretação considerando a hora do registro e o horário espelhado no período pós-operatório (PRICE et al., 2003). Apesar disso, em coelhos, já foi demonstrado que tremer, arquear as costas, pressionar e arrastar o abdômen no piso foram mais exibidos pela manhã do que à tarde no momento pré-operatório (sem dor), enquanto interagir com enriquecimento, comer, beber, pular, cavar, explorar e alerta foram mais evidentes pela tarde em relação a manhã (LEACH et al., 2009).

Para equinos não existe estudo com a abordagem supracitada, muito embora, pode-se extrapolar que essa interferência também ocorra nesta espécie, uma vez que já é esperado que a distribuição comportamental ao longo do dia seja heterogênea como, por exemplo, alerta e ativo predominantemente durante o dia e dormir deitado ou em pé de noite (MCDONNELL, 2003). É possível que exista um padrão comportamental cíclico nos cavalos, um estudo observando cavalos Przewalski de vida livre evidenciaram que atividade de locomoção e pastejo foram mais duradouras no verão em relação ao inverno, sendo que em ambas as estações tais comportamentos ocorreram predominante durante as horas de luz solar (BERGER et al., 1999). As diferenças no tempo de luz solar nas estações do ano também representam uma forte influência para sintetização de hormônios reprodutivos, aumento de libido, crescimento de pelos e por consequência influenciando no comportamento dos equinos (MURPHY, 2019). Tais achados demonstram que as condições ambientais exercem uma influência na resposta comportamental cíclica com duração de aproximadamente um ano (ritmo circanual) (MURPHY, 2019).

Além das pressões ambientais, substâncias endógenas sintetizadas com uma variação constante também influenciam na exibição comportamental de uma espécie. Por exemplo, o ritmo circadiano do cortisol nos equinos proporciona picos regulares de concentração de cortisol endógeno em momentos específicos do dia (BOHÁK et al., 2013). Nesse sentido, revisão apontou que o pico da taxa de cortisol no início da manhã em animais humanos, como decorrência do ciclo circadiano, pode ser uma das ondas hormonais causadoras do despertar após o sono e, consequentemente estar relacionada com o aumento de atividades (CLOW et al., 2010). Tais estudos evidenciam a importância de sintetizações endógenas cíclicas na exibição de um certo padrão comportamental ao longo do dia (ritmo circadiano).

Contudo, é possível assumir que condições externas e internas cíclicas diariamente ou anualmente tiveram substancial papel na modulação de um ritmo circadiano ou circanual da expressão comportamental nos *Equus caballos* ao longo de sua evolução (BERGER et al., 1999; SCHEIBE et al., 1999; MURPHY, 2019). Sendo assim, é esperado que o aumento ou diminuição na exibição de comportamentos regidos por tais fenômenos, pode estar associado a um padrão da expressão comportamental, como é o caso da maior atividade de pastejo e locomoção durante o dia em relação à noite (BERGER et al., 1999; SCHEIBE et al., 1999; MURPHY, 2019). Sabendo que atividade, locomoção, apetite, atenção ao ambiente e descanso são comportamentos usados no diagnóstico da dor, o horário de registro poderia representar um fator de confusão na avaliação comportamental para avaliação da dor que precisa ser investigado futuramente, pois nunca foi objeto de estudo.

2.4 Resumo dos fatores de confusão no comportamento de dor

Em resumo, a avaliação de dor aplicando a linguagem corporal está sujeita a fatores de confusão que precisam ser melhor investigados, sendo eles: o efeito residual de fármacos usados para anestesia e analgesia; a intensidade da dor; e o horário de registro do comportamento. Portanto, no capítulo seguinte será apresentado o artigo produto desta tese com foco em preencher as lacunas apresentadas na revisão acima.

3. Referências

ALM, P. A. Stuttering, emotions, and heart rate during anticipatory anxiety: A critical review. **Journal of fluency disorders**, v. 29, n. 2, p. 123-133, 2004.

ANDERSEN, P. H.; GLEERUP, K. B.; WATHAN, J.; COLES, B.; KJELLSTRÖM, H.; BROOMÉ, S.; LEE, Y. J.; RASHID, M.; SONDER, C.; ROSENBERG, E.; FORSTER, D. **Can a machine learn to see horse pain? An interdisciplinary approach towards automated decoding of facial expressions of pain in the horse**. Disponível em: <www.measuringbehavior.org>. Acesso em: 2 mar. 2021.

ASHLEY, F. H.; WATERMAN-PEARSON, A. E.; WHAY, H. R. Behavioural assessment of pain in horses and donkeys: application to clinical practice and future studies. **Equine veterinary journal**, v. 37, n. 6, p. 565-575, 2005.

ASK, K.; RHODIN, M.; TAMMINEN, L.-M.; HERNLUND, E.; HAUBRO ANDERSEN, P. Identification of body behaviors and facial expressions associated with induced orthopedic pain in four equine pain scales. **Animals**, v. 10, n. 11, p. 2155, 2020.

BROOM, D. M. Indicators of poor welfare. **British veterinary journal**, v. 142, n. 6, p. 524-526, 1986.

BERGER, A.; SCHEIBE, K. M.; EICHHORN, K.; SCHEIBE, A.; STREICH, J. Diurnal and ultradian rhythms of behaviour in a mare group of Przewalski horse (Equus ferus przewalskii), measured through one year under semi-reserve conditions. **Applied Animal Behaviour Science**, 64(1), 1-17, 1999.

BOHÁK, Z.; SZABÓ, F.; BECKERS, J. F.; DE SOUSA, N. M.; KUTASI, O.; NAGY, K.; SZENCI, O. Monitoring the circadian rhythm of serum and salivary cortisol concentrations in the horse. **Domestic animal endocrinology**, 45(1), 38-42, 2013.

BUISMAN, M.; WAGNER, M. C.; HASIUK, M. M. M.; PREBBLE, M.; LAW, L.; PANG, D. S. J. Effects of ketamine and alfaxalone on application of a feline pain assessment scale. **Journal of feline medicine and surgery**, v. 18, n. 8, p. 643-651, 2016.

BUSSIÈRES, G.; JACQUES, C.; LAINAY, O.; BEAUCHAMP, G.; LEBLOND, A.; CADORÉ, J.-L.; DESMAIZIÈRES, L.-M.; CUVELLIEZ, S. G.; TRONCY, E. Development of a composite orthopaedic pain scale in horses. **Research in veterinary science**, v. 85, n. 2, p. 294-306, 2008.

BUTLER, R. K.; FINN, D. P. Stress-induced analgesia. **Progress in neurobiology**, v. 88, n. 3, p. 184-202, 2009.

CARBONE, Larry. Do “Prey Species” Hide Their Pain? Implications for Ethical Care and Use of Laboratory Animals. **Journal of applied animal ethics research**, v. 2, n. 2, p. 216-236, 2020.

CLOW, A.; HUCKLEBRIDGE, F.; STALDER, T.; EVANS, P.; THORN, L. The cortisol awakening response: more than a measure of HPA axis function. **Neuroscience & Biobehavioral Reviews**, 35(1), 97-103, 2010.

COHEN, M.; QUINTNER, J.; VAN RYSEWYK, S. Reconsidering the International Association for the study of pain definition of pain. **Pain Reports**, v. 3, n. 2, p. 1–7, 2018.

DALLA COSTA, E.; MINERO, M.; LEBELT, D.; STUCKE, D.; CANALI, E.; LEACH, M. C. Development of the Horse Grimace Scale (HGS) as a Pain Assessment Tool in Horses Undergoing Routine Castration. **PLoS ONE**, v. 9, n. 3, p. e92281, 2014.

DAS, R.; SAILO, L.; VERMA, N.; BHARTI, P.; SAIKIA, J.; IMTIWATI; KUMAR, R. Impact of heat stress on health and performance of dairy animals: A review. **Veterinary world**, v. 9, n. 3, p. 260, 2016.

DAWKINS, M. S. Behaviour as a tool in the assessment of animal welfare. **Zoology**, v. 106, n. 4, p. 383–387, 2003.

DAWKINS, M. S. Using animal behaviour to assess animal welfare. **Animal Welfare**, v. 13, n. 1, p. 3–7, 2004.

DAWKINS, M. S. The science of animal suffering. **Ethology**, v. 114, n. 10, p. 937–945, 2008.

DE GRAUW, J. C.; VAN LOON, J. P. A. M. Systematic pain assessment in horses. **The Veterinary Journal**, v. 209, p. 14–22, 2016.

DESCOVICH, K.; Wathan, J.; Leach, M. C.; Buchanan-Smith, H. M.; Flecknell, P.; Framingham, D.; Vick, S. J. Facial expression: An under-utilised tool for the assessment of welfare in mammals. **ALTEX**, 2017. Disponível em: <http://www.altex.org/index.php/altex/article/view/37>. Acesso em: 31 jan. 2019.

DI GIMINIANI, P.; BRIERLEY, V. L. M. H.; SCOLLO, A.; GOTTARDO, F.; MALCOLM, E. M.; EDWARDS, S. A.; LEACH, M. C. The Assessment of Facial Expressions in Piglets Undergoing Tail Docking and Castration: Toward the Development of the Piglet Grimace Scale. **Frontiers in Veterinary Science**, v. 3, p. 100, 2016.

DODDS, L.; KNIGHT, L.; ALLEN, K.; MURRELL, J. The effect of postsurgical pain on attentional processing in horses. **Veterinary Anaesthesia and Analgesia**, v. 44, n. 4, p. 933–942, 2017.

DURANTON, C.; GAUNET, F. Behavioural synchronization from an ethological perspective: Overview of its adaptive value. **Adaptive Behavior**, v. 24, n. 3, p. 181–191, 2016.

DUTTON, D. W.; LASHNITS, K. J.; WEGNER, K. Managing severe hoof pain in a horse using multimodal analgesia and a modified composite pain score. **Equine Veterinary Education**, v. 21, n. 1, p. 37–43, 2009.

EVANGELISTA, M. C.; BENITO, J.; MONTEIRO, B. P.; WATANABE, R.; DOODNAUGHT, G. M.; PANG, D. S. J.; STEAGALL, P. V. Clinical applicability of the Feline Grimace Scale: Real-time versus image scoring and the influence of sedation and surgery. **PeerJ**, v. 2020, n. 4, p. e8967, 2020.

FANSELOW, M. S. Conditioned Fear-Induced Opiate Analgesia: A Competing Motivational State Theory of Stress Analgesia. **Annals of the New York Academy of Sciences**, v. 467, n. 1, p. 40-54, 1986.

FORD, G. K.; MORIARTY, O.; MCGUIRE, B. E.; FINN, D. P. Investigating the effects of distracting stimuli on nociceptive behaviour and associated alterations in brain monoamines in rats. **European Journal of Pain**, v. 12, n. 8, p. 970–979, 2008.

GIGLIUTO, C.; DE GREGORI, M.; MALAFOGLIA, V.; RAFFAELI, W.; COMPAGNONE, C.; VISAI, L.; PETRINI, P.; AVANZINI, M. A.; MUSCOLI, C.; VIGANÒ, J.; CALABRESE, F.; DOMINIONI, T.; ALLEGRI, M.; COBIANCHI, L. Pain assessment in animal models: do we need further studies? **Journal of pain research**, v. 7, p. 227, 2014.

GLEERUP, K. B.; ANDERSEN, P. H.; MUNKSGAARD, L.; FORKMAN, B. Pain evaluation in dairy cattle. **Applied Animal Behaviour Science**, v. 171, p. 25–32, 2015a.

GLEERUP, K. B.; FORKMAN, B.; LINDEGAARD, C.; ANDERSEN, P. H. An equine pain face. **Veterinary Anaesthesia and Analgesia**, v. 42, n. 1, p. 103–114, 2015b.

GLEERUP, K. B.; LINDEGAARD, C. Recognition and quantification of pain in horses: A tutorial review. **Equine Veterinary Education**, v. 28, n. 1, p. 47–57, 2016.

GÖRANSSON, L. Porcine pain face – identifying visible characteristics of pain in pigs, 2016. Disponível em: <https://stud.epsilon.slu.se/8871/>. Acesso em: 31 jan. 2019.

GRAUBNER, C.; GERBER, V.; DOHERR, M.; SPADAVECCHIA, C. Clinical application and reliability of a post abdominal surgery pain assessment scale (PASPAS) in horses. **Veterinary Journal**, v. 188, n. 2, p. 178–183, 2011.

GREEN, T. C.; MELLOR, D. J. Extending ideas about animal welfare assessment to include “quality of life” and related concepts. **New Zealand Veterinary Journal**, v. 59, n. 6, p. 263–271, 2011.

GRIPPO, A. J.; LAMB, D. G.; CARTER, C. S.; PORGES, S. W. Social Isolation Disrupts Autonomic Regulation of the Heart and Influences Negative Affective Behaviors. **Biological Psychiatry**, v. 62, n. 10, p. 1162–1170, 2007.

GUEDES, A. Pain management in horses. **Veterinary Clinics: Equine Practice**, v. 33, n. 1, p. 181-211, 2017.

GUESGEN, M. J.; BEAUSOLEIL, N. J.; LEACH, M.; MINOT, E. O.; STEWART, M.; STAFFORD, K. J. Coding and quantification of a facial expression for pain in lambs. **Behavioural Processes**, v. 132, p. 49–56, 2016.

HALL, C.; RANDLE, H.; PEARSON, G.; PRESHAW, L.; WARAN, N. Assessing equine emotional state. **Applied Animal Behaviour Science**, v. 205, p. 183–193, 2018.

HELLEBREKERS, L. J. Pathophysiology of pain in animals and its onsequence for analgesic therapy. **Animal Pain**, p. 71-83, 2000.

HINTZE, S.; SMITH, S.; PATT, A.; BACHMANN, I.; WÜRBEL, H. Are Eyes a Mirror of the Soul? What Eye Wrinkles Reveal about a Horse's Emotional State. **PLOS ONE**, v. 11, n. 10, p. e0164017, 2016.

HUBRECHT; CARTER. The 3Rs and Humane Experimental Technique: Implementing Change. **Animals**, v. 9, n. 10, p. 754, 2019.

KEATING, S. C. J.; THOMAS, A. A.; FLECKNELL, P. A.; LEACH, M. C. Evaluation of EMLA Cream for Preventing Pain during Tattooing of Rabbits: Changes in Physiological, Behavioural and Facial Expression Responses. **PLoS ONE**, v. 7, n. 9, p. e44437, 2012.

LANGFORD, D. J.; BAILEY, A. L.; CHANDA, M. L.; CLARKE, S. E.; DRUMMOND, T. E.; ECHOLS, S.; GLICK, S.; INGRAO, J.; KLASSEN-ROSS, T.; LACROIX-FRALISH, M. L.; MATSUMIYA, L.; SORGE, R. E.; SOTOCINAL, S. G.; TABAKA, J. M.; WONG, D.; VAN DEN MAAGDENBERG, A. M. J. M.; FERRARI, M. D.; CRAIG, K. D.; MOGIL, J. S. Coding of facial expressions of pain in the laboratory mouse. **Nature Methods**, v. 7, n. 6, p. 447–449, 2010.

LANSADE, L.; BOUISSOU, M. F. Reactivity to humans: A temperament trait of horses which is stable across time and situations. **Applied Animal Behaviour Science**, v. 114, n. 3–4, p. 492–508, 2008.

LAWSON, A. L.; OPIE, R. R.; STEVENS, K. B.; KNOWLES, E. J.; MAIR, T. S. Application of an equine composite pain scale and its association with plasma adrenocorticotropic hormone concentrations and serum cortisol concentrations in horses with colic. **Equine Veterinary Education**, v. 32, n. S11, p. 20–27, 2020.

LEACH, M. C.; ALLWEILER, S.; RICHARDSON, C.; ROUGHAN, J. V.; NARBE, R.; FLECKNELL, P. A. Behavioural effects of ovariohysterectomy and oral administration of meloxicam in laboratory housed rabbits. **Research in Veterinary Science**, v. 87, n. 2, p. 336–347, 2009.

LI, Q.; ZHU, Z. Y.; LU, J.; CHAO, Y. C.; ZHOU, X. X.; HUANG, Y.; CHEN, X. M.; SU, D. S.; YU, W. F.; GU, X. Y. Sleep deprivation of rats increases postsurgical expression and activity of L-type calcium channel in the dorsal root ganglion and slows recovery from postsurgical pain. **Acta Neuropathologica Communications**, v. 7, n. 1, p. 1–15, 2019.

LINDEGAARD, C.; THOMSEN, M. H.; LARSEN, S.; ANDERSEN, P. H. Analgesic efficacy of intra-articular morphine in experimentally induced radiocarpal synovitis in horses. **Veterinary Anaesthesia and Analgesia**, v. 37, n. 2, p. 171–185, 2010.

LINDEGAARD, C.; VAABENGAARD, D.; CHRISTOPHERSEN, M. T.; EKSTØM, C. T.; FJELDBORG, J. Evaluation of pain and inflammation associated with hot iron branding and microchip transponder injection in horses. **American Journal of Veterinary Research**, v. 70, n. 7, p. 840–847, 2009.

MARTIN, P.; BATESON, P. P. G. **Measuring behaviour: An introductory guide**. Cambridge University Press, Cambridge, United Kingdom. 1993.

MCDONNELL, S. **Practical field guide to horse behavior: The equid ethogram**. The Blood-Horse, Lexington, United States, 2003, v. 1, p. 39–58.

MCLENNAN, K. M.; REBELO, C. J. B.; CORKE, M. J.; HOLMES, M. A.; LEACH, M. C.; CONSTANTINO-CASAS, F. Development of a facial expression scale using footrot and mastitis as models of pain in sheep. **Applied Animal Behaviour Science**, v. 176, p. 19–26, 2016.

MELLOR, D.J.; BEAUSOLEIL, N.J.; LITTLEWOOD, K.E.; MCLEAN, A.N.; MCGREEVY, P.D.; JONES, B.; WILKINS, C. The 2020 five domains model: including human–animal interactions in assessments of animal welfare. **Animals**, 10(10), 1870, 2020.

MICHELENA, P.; PILLOT, M.-H.; HENRION, C.; TOULET, S.; BOISSY, A.; BON, R. Group size elicits specific physiological response in herbivores. **Biology Letters**, v. 8, n. 4, p. 537–539, 2012.

MOLONY, V. Comments on Anand and Craig (Letters to the editor). **Pain**, v. 70, p. 293, 1997.

MORIARTY, O.; MCGUIRE, B. E.; FINN, D. P. The effect of pain on cognitive function: a review of clinical and preclinical research. **Progress in neurobiology**, v. 93, n. 3, p. 385-404, 2011.

MULLARD, J.; BERGER, J. M.; ELLIS, A. D.; DYSON, S. Development of an ethogram to describe facial expressions in ridden horses (FEReq). **Journal of Veterinary Behavior**, v. 18, p. 7–12, 2017.

MURPHY, B. A. Circadian and circannual regulation in the horse: internal timing in an elite athlete. **Journal of equine veterinary science**, v. 76, p. 14-24, 2019.

DE OLIVEIRA, A. R.; GOZALO-MARCILLA, M.; RINGER, S. K.; SCHAUVLIEGE, S.; FONSECA, M. W.; TRINDADE, P. H. E.; PUOLI FILHO, J. N. P.; LUNA, S. P. L. Development, validation, and reliability of a sedation scale in horses (EquiSed). **Frontiers in Veterinary Science**, v. 8, 2021.

FAO OIE WHO. The FAO-OIE-WHO Collaboration: Tripartite Concept Note, 2010.

PAULL, D.; LEE, C.; COLDITZ, I.; FISHER, A. Effects of a topical anaesthetic formulation and systemic carprofen, given singly or in combination, on the cortisol and behavioural responses of Merino lambs to castration. **Australian Veterinary Journal**, v. 87, n. 6, p. 230–237, 2009.

PAULL, D. R.; SMALL, A. H.; LEE, C.; PALLADIN, P.; COLDITZ, I. G. Evaluating a novel analgesic strategy for ring castration of ram lambs. **Veterinary Anaesthesia and Analgesia**, v. 39, n. 5, p. 539–549, 2012.

PEHKONEN, J.; KARMA, L.; RAEKALLIO, M. Behavioral signs associated with equine periapical infection in cheek teeth. **Journal of Equine Veterinary Science**, v. 77, p. 144–150, 2019.

PINHO, R. H.; LEACH, M. C.; MINTO, B. W.; ROCHA, F. D. L.; LUNA, S. P. L. Postoperative pain behaviours in rabbits following orthopaedic surgery and effect of observer presence. **PloS one**, v. 15, n. 10, p. e0240605, 2020.

PRICE, D. D. Psychological and neural mechanisms of the affective dimension of pain. **Science**, v. 288, n. 5472, p. 1769-1772, 2000.

PRICE, J.; CATRIONA, S.; WELSH, E. M.; WARAN, N. K. Preliminary evaluation of a behaviour–based system for assessment of post–operative pain in horses following arthroscopic surgery. **Veterinary Anaesthesia and Analgesia**, v. 30, n. 3, p. 124–137, 2003.

PRITCHETT, L. C.; ULIBARRI, C.; ROBERTS, M. C.; SCHNEIDER, R. K.; SELLON, D. C. Identification of potential physiological and behavioral indicators of postoperative pain in horses after exploratory celiotomy for colic. **Applied Animal Behaviour Science**, v. 80, n. 1, p. 31–43, 2003.

RAEKALLIO, M.; TAYLOR, P. M.; BLOOMFIELD, M. A comparison of methods for evaluation of pain and distress after orthopaedic surgery in horses. **Journal of Veterinary Anaesthesia**, v. 24, n. 2, p. 17–20, 1997.

RAJA, S. N.; CARR, D. B.; COHEN, M.; FINNERUP, N. B.; FLOR, H., GIBSON, S.; KEEFE, F. K. J.; MOGIL, J. S.; RINGKAMP, M.; SLUKA, K. A.; SONG, X. J.; STEVENS, B.; SULLIVAN, MARK D.; TUTELMAN, P. R.; USHIDA, T.; VADER, K. The revised International Association for the Study of Pain definition of pain: concepts, challenges, and compromises. **Pain**, v. 161, n. 9, p. 1976-1982, 2020.

SCOPA, C.; CONTALBRIGO, L.; GRECO, A.; LANATÀ, A.; SCILINGO, E. P.; BARAGLI, P. Emotional transfer in human–horse interaction: New perspectives on equine assisted interventions. **Animals**, v. 9, n. 12, p. 1–21, 2019.

SCHEIBE, K. M.; BERGER, A.; LANGBEIN, J.; STREICH, W. J.; EICHHORN, K. Comparative analysis of ultradian and circadian behavioural rhythms for diagnosis of biorhythmic state of animals. **Biological rhythm research**, 30(2), 216-233, 1999.

SELLON, D. C.; ROBERTS, M. C.; BLIKSLAGER, A. T.; ULIBARRI, C.; PAPICH, M. G. Effects of continuous rate intravenous infusion of butorphanol on physiologic and outcome variables in horses after celiotomy. **Journal of Veterinary Internal Medicine**, v. 18, n. 4, p. 555–563, 2004.

SILVA, N. E. O. F.; TRINDADE, P. H. E.; OLIVEIRA, A. R.; TAFFAREL, M. O.; MOREIRA, M. A. P.; DENADAI, R.; ROCHA, P. B.; LUNA, S. P. L. Validation of the Unesp-Botucatu composite scale to assess acute postoperative abdominal pain in sheep (USAPS). **PLOS ONE**, v. 15, n. 10, p. e0239622, 2020.

SIROT, E.; TOUZALIN, F. Coordination and synchronization of vigilance in groups of prey: The role of collective detection and predators' preference for stragglers. **American Naturalist**, v. 173, n. 1, p. 47–59, 2009.

SMALL, A. H.; JONGMAN, E. C.; NIEMEYER, D.; LEE, C.; COLDITZ, I. G. Efficacy of precisely injected single local bolus of lignocaine for alleviation of behavioural responses to pain during tail docking and castration of lambs with rubber rings. **Research in Veterinary Science**, v. 133, p. 210–218, 2020.

SNEDDON, L. U.; ELWOOD, R. W.; ADAMO, S. A.; LEACH, M. C. Defining and assessing animal pain. **Animal Behaviour**, v. 97, p. 201–212, 2014.

SUTHERLAND, M. A.; DAVIS, B. L.; MCGLONE, J. J. The effect of local or general anesthesia on the physiology and behavior of tail docked pigs. **Animal**, v. 5, n. 8, p. 1237–1246, 2011.

SUTTON, G. A.; ATAMNA, R.; STEINMAN, A.; MAIR, T. S. Comparison of three acute colic pain scales: Reliability, validity and usability. **Veterinary Journal**, v. 246, p. 71–77, 2019.

SUTTON, G. A.; DAHAN, R.; TURNER, D.; PALTIEL, O. A behaviour-based pain scale for horses with acute colic: Scale construction. **Veterinary Journal**, v. 196, n. 3, p. 394–401, 2013a.

SUTTON, G. A.; PALTIEL, O.; SOFFER, M.; TURNER, D. Validation of two behaviour-based pain scales for horses with acute colic. **Veterinary Journal**, v. 197, n. 3, p. 646–650, 2013b.

SUTTON, G. A.; BAR, L. Refinement and revalidation of the Equine Acute Abdominal Pain Scale (EAAPS). **Israel Journal of Veterinary Medicine**, v. 71, n. 1, p. 15-23, 2016.

TAFFAREL, M. O.; LUNA, S. P. L.; DE OLIVEIRA, F. A.; CARDOSO, G. S.; DE MOURA ALONSO, J.; PANTOJA, J. C.; BRONDANI, J. T.; LOVE, E.; TAYLOR, P.; WHITE, K.; MURRELL, J. C. Refinement and partial validation of the UNESP-Botucatu multidimensional composite pain scale for assessing postoperative pain in horses. **BMC Veterinary Research**, v. 11, n. 1, p. 1–12, 2015.

TORCIVIA, C.; MCDONNELL, S. In-Person caretaker visits disrupt ongoing discomfort behavior in hospitalized equine orthopedic surgical patients. **Animals**, v. 10, n. 2, p. 210, 2020.

TORREY, S.; DEVILLERS, N.; LESSARD, M.; FARMER, C.; WIDOWSKI, T. Effect of age on the behavioral and physiological responses of piglets to tail docking and ear notching. **Journal of Animal Science**, v. 87, n. 5, p. 1778–1786, 2009.

TRINDADE, P. H. E.; COSTA, F. D. O.; PARANHOS DA COSTA, M. J. R. Expressões faciais em cavalos: uma abordagem sobre os métodos de avaliação e as limitações de seu uso. **Revista Acadêmica Ciência Animal**, v. 16, p. 1, 2018.

TRINDADE, P. H. E.; HARTMANN, E.; KEELING, L. J.; ANDERSEN, P. H.; FERRAZ, G. de C.; PARANHOS DA COSTA, M. J. R. Effect of work on body language of ranch horses in Brazil. **PLOS ONE**, v. 15, n. 1, p. e0228130, 2020.

VAN LOON, J. P. A. M.; BACK, W.; HELLEBREKERS, L. J.; VAN WEEREN, P. R. Application of a composite pain scale to objectively monitor horses with somatic and visceral pain under hospital conditions. **Journal of Equine Veterinary Science**, v. 30, n. 11, p. 641–649, 2010.

VAN LOON, J. P. A. M.; VAN DIERENDONCK, M. C. Monitoring acute equine visceral pain with the Equine Utrecht University Scale for Composite Pain Assessment (EQUUS-COMPASS) and the Equine Utrecht University Scale for Facial Assessment of Pain (EQUUS-FAP): A scale-construction study. **Veterinary Journal**, v. 206, n. 3, p. 356–364, 2015.

VAN LOON, J. P. A. M.; VAN DIERENDONCK, M. C. Objective pain assessment in horses (2014–2018). **The Veterinary Journal**, v. 242, p. 1-7, 2018.

VAN LOON, J. P. A. M.; VAN DIERENDONCK, M. C. Pain assessment in horses after orthopaedic surgery and with orthopaedic trauma. **Veterinary Journal**, v. 246, p. 85–91, 2019.

VANDIERENDONCK, M. C.; VAN LOON, J. P. A. M. Monitoring acute equine visceral pain with the Equine Utrecht University Scale for Composite Pain Assessment (EQUUS-COMPASS) and the Equine Utrecht University Scale for Facial Assessment of Pain (EQUUS-FAP): A validation study. **Veterinary Journal**, v. 216, p. 175–177, 2016.

WAGNER, A. E. Effects of stress on pain in horses and incorporating pain scales for equine practice. **The Veterinary clinics of North America. Equine practice**, v. 26, n. 3, p. 481-492, 2010.

WAGNER, M. C.; HECKER, K. G.; PANG, D. S. J. Sedation levels in dogs: A validation study. **BMC Veterinary Research**, v. 13, n. 1, p. 1–8, 2017.

WHALIN, L.; PAIRIS-GARCIA, M.; PROUDFOOT, K.; STALDER, K.; JOHNSON, A. Validating behavioral sampling techniques for lame sows administered flunixin meglumine and meloxicam. **Livestock Science**, v. 191, p. 103–107, 2016.

ZUTT, R.; VAN DER KOOI, A. J.; LINTHORST, G. E.; WANDERS, R. J. A.; DE VISSER, M. Rhabdomyolysis: Review of the literature. **Neuromuscular Disorders**, v. 24, n. 8, p. 651–659, 2014.

CAPÍTULO 2

Os achados apresentados neste capítulo à seguir foram submetidos para o periódico ‘Animals’ (ISSN: 2076-2615) em 07/04/21, aceito em 20/05/21.e publicado em 31/05/21

Spontaneous Behaviors of Post-Orchiectomy Pain in Horses Regardless of the Effects of Time of Day, Anesthesia, and Analgesia

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Simple Summary

Confounding factors may hinder the estimation of pain in horses. The current study aimed to identify spontaneous post-castration pain behaviors in horses regardless of the effects of anesthesia, analgesia, and recording time of day. Twenty-four horses divided into four groups were submitted to inhalation anesthesia only or combined with pre-operative analgesia, or castration under pre or postoperative analgesia. Thirty-four behaviors were evaluated in seven 60-min time-point recordings in the 24 h after anesthesia and at mirrored time-points in the 24 h before the anesthesia. Results showed changes in the behaviors of walk, look out the window, rest the pelvic limb, and rest standing still when assessed in the morning, afternoon, and night. The only pain-related behaviors observed regardless of the effects of time of the day, anesthesia, and analgesia, were a decrease in the mirrored proportional differences in time spent drinking and eating, and an increase in the mirrored proportional differences in the frequencies of look at the wound, retract the pelvic limb, expose the penis, and look at the back of the stall. In conclusion, confounding factors rather than pain may influence several suggestive pain behaviors documented in equine literature.

Abstract

This prospective and longitudinal study aimed to identify spontaneous post-orchiectomy pain behaviors in horses regardless of the effects of anesthesia, analgesia, and recording time of day. Twenty-four horses divided into four groups were submitted to: inhalation anesthesia only (GA), or combined with previous analgesia (GAA), or orchiectomy under pre (GCA), or postoperative (GC) analgesia. The data obtained from the subtraction of frequency and/or duration of 34 behaviors recorded during seven 60-min time-points in the 24 h after the anesthesia from those recorded in the mirrored time-points in the 24 h before the anesthesia (delta) were compared over time and among groups by Friedman and Kruskal–Wallis tests, respectively ($p < .05$). Time of day influenced the behaviors of walk, look out the window, rest the pelvic limb, and rest standing still. The only pain-related behaviors were decreased mirrored proportional differences in time spent drinking, and eating, and increased mirrored proportional differences in the frequency or duration of look at the wound, retract the pelvic limb, expose the penis, and look at the back of the stall. In conclusion, confounding factors rather than pain may influence several suggestive pain-related behaviors documented in the literature.

Keywords: animal welfare; pain measurement; horses

1. Introduction

Several instruments have been proposed to assess acute pain in *Equus caballus* [1–4]. Behaviors related to activity, locomotion, appetite, attention to the environment, rest, and discomfort have been included in instruments to diagnose the pain of acute abdomen syndrome [5–16], dental procedures [16,17], orchiectomy [13,16,18,19], and orthopedic procedures [13,16,20–25]. The physiological parameters included in some of these instruments present questionable results due to their low specificity and sensitivity [1,10,14,20,23,25,26], added to which, they usually require the presence of the evaluator and a certain degree of intrusion [27]. Facial expression is a promising alternative for recognizing pain in horses [12,19,28], however, it requires experienced appraisers, specific training, and high-quality footage [1] or the observer's presence [12]. The remote evaluation of body language using cameras, as in the case of pigs [29] and sheep [30], could also be reliable to assess pain in horses, as it is not invasive or intrusive [27,31,32].

Although efforts to develop a reliable and validated instrument to identify pain in horses are recognized, there is still no gold standard tool for this purpose [1–4,33,34]. Most studies evaluated acute pain after orthopedic surgery or celiotomy [5–25,35]. Only two studies assessed pain in hospitalized horses undergoing different painful conditions and intensities [13,16]. Some instruments were created in clinical situations where there was no possibility of controlling variables that can generate biases or confounders. One of these is the residual post-anesthetic effect of sedatives, anesthetics, and analgesics on behavior [7,12,15,21,26,36]. Moreover, on-site human evaluation is a common bias that may induce discomfort behaviors [27] or even inhibit both normal behavior expression before surgery and postoperative pain behaviors, as described in horses [27] and rabbits [37], suggesting that remote evaluation with a camera may be more appropriate to assess pain.

A confounding effect not yet studied in horses, and which could also interfere with pain assessment, is the period of the day in which the horse is evaluated. Throughout the day, the behavioral distribution is expected to be heterogeneous, for example, alert and active during the day and sleeping at night [38].

These biases could both maximize or minimize pain estimation and require investigation. To our knowledge, there are no studies in the literature on the effects of time of day on normal behavior and the implications of anesthesia, analgesia, and pain intensity on spontaneous behaviors of horses with acute postoperative pain, remotely assessed using a camera, without any human presence inside or in front of the stall. Therefore, the objective of this study was to identify the post-orchiectomy spontaneous pain behaviors in horses and the effects of anesthesia, analgesia, pain intensity, and time of day. The study hypothesis was that some spontaneous behaviors are related to acute postoperative pain, while others are confounded by the effects of anesthesia, analgesia, and recording time.

2. Material and Methods

The study is opportunistic of previous publications [18,39] approved by the institution's Ethics Committee on the Use of Animals (ID186/2009) on 8 December 2009, and carried out with the consent of all owners. The experimental procedures presented in this study were developed in the previous publications [18,39]. In all groups the same periodical clinical examination and pain assessment were performed, and the data were published in the previous study [18]. The present study includes analysis of unpublished data from remote video recordings without visual contact or human presence inside or in front of the stalls.

2.1. Animals and Groups

Twenty-four crossbred horses, considered healthy after clinical and laboratory evaluations, were submitted to the same inhalation anesthesia protocol. Twelve experimental horses from the host institution not submitted to orchiectomy were randomly assigned to inhalation anesthesia only (GA n = 6; three geldings and three mares aged 9 ± 3 years and weighing 332 ± 48 kg) or inhalation anesthesia and analgesia (GAA n = 6; four geldings and two mares; 10 ± 5 years and 369 ± 68 kg). These institution-owned horses were familiar with the stalls, facilities and, personnel and the gelding horses from these groups had been submitted to previous intravenous anesthesia when they were castrated. Twelve client-owned horses who underwent elective orchiectomy were randomly treated with pre (GCA n = 6; 4 ± 2 years and 319 ± 48 kg) or postoperative analgesia (GC; n = 6; 4 ± 2 years and 302 ± 27 kg), to simulate two pain intensities (GC possibly representing severe pain and GCA possibly representing mild pain). These horses were having their first contact with the hospital stalls, facilities, personnel, and anesthesia procedures.

2.2. Management and Procedures

Twenty-four hours before the end of the anesthetic recovery, the horses were placed in individual stalls (4 × 4 m). Water and the same type of hay were available ad libitum for all horses before and after anesthesia when the horses were in the stall. The front stall door was divided into two parts. The superior half of the door (window) remained open to allow the horse to put its head out. None of the horses were subjected to preoperative fasting. The horses left the stall in the morning (07:43 ± 00:19 h) to be sedated with 0.5 mg/kg of xylazine (Sedomin®, König, Buenos Aires, Argentina) intramuscularly (IM). The preoperative analgesia for the GAA and GCA horses was 0.2 mg/kg of morphine (Dimorf®, Cristalia, Lindóia, Brazil) IM, 10 mg/kg of dipyrone (metamizole; Finador®, Ourofino, Brazil), and 1.1 mg/kg of flunixin meglumine (Desflan®, Ourofino, Cravinhos, Brazil) intravenously (IV) administered immediately before sedation. Anesthesia was induced in all horses with 100 mg/kg of guaifenesin 10% (EGG PPU Eter gliceril guaiacol®, LPS Agrofarma, Mogi Mirim, Brazil) and 5 mg/kg of thiopental IV (Thiopentax®, Cristalia, Lindóia, Brazil). After orotracheal intubation, the horses were hoisted onto a surgical table, positioned in dorsal recumbency, and general anesthesia was maintained with isoflurane (Isoforine®, Cristalia, Lindóia, Brazil) vaporized by oxygen (O_2), under controlled ventilation (Mallard Medical Skypark Drive Redding®, United States) to maintain the anesthetic-surgical plane. Tidal volume was set at 15 mL/kg with a peak inspiratory pressure of 20 to 30 cm H_2O to maintain normocapnia [end-tidal carbon dioxide ($EtCO_2$) values between 4.66 and 6.00 kPa (35–45 mm Hg)]. Local anesthesia with 10 mL of 2% lidocaine with adrenaline (Lidocaina®, Cristalia, Lindóia, Brazil), injected into each spermatic cord before surgery, was performed only in GCA horses. The isoflurane vaporizer setting was adjusted by one anesthetist unaware of whether the animal had been treated (GCA) or not (GC) with pre-operative analgesia, to maintain an adequate anesthetic depth, confirmed by diminished, but not abolished, palpebral reflex, corneal reflex present, and stable heart rate and arterial blood pressure in response to surgical stimuli.

Since none of the horses required anesthetic supplementation, it was assumed that they received equivalent amounts of anesthetic. Monitoring included heart rate, peripheral arterial hemoglobin saturation by pulse oximetry, inspired and end-tidal partial pressures of CO_2, O_2, and inspired and end-tidal isoflurane concentrations (Monitor Cardiocap 5®, DatexOhmeda, Helsinki, Finland). The facial artery was catheterized (20G, Introcan Safety®, Melsungen, Germany) to measure systolic, diastolic, and mean arterial blood pressure (MAP).

The pressure transducer (TruWaveTM®, Edwards Lifesciences, San Cristobal, Dominican Republic), which was previously calibrated against a mercury column, was zeroed to atmospheric pressure and positioned at the right atrium level. When MAP was below 70 mm Hg, dobutamine (Dobutamina®, Biosintética Farmacêutica Ltd., São Paulo, Brazil) was administered intravenously as required, with a maximal dose of 5µg/kg/min. Fluid therapy was provided with 5 mL kg/h of lactate ringer. The half-closed surgical castration technique was used [40]. The duration of anesthesia was approximately the same for all groups (48 ± 4 min), 45 ± 2 min, 51 ± 4 min, 50 ± 2 min, and 47 ± 3 min for GA, GAA, GC, and GCA, respectively. All horses recovered in a padded box with the lights on, without O_2 supplementation, and were assisted with head and tail ropes when they attempted to stand up. After recovery from anesthesia and regaining the ability to stand, the horses were extubated. When the horses were able to move, they were guided to the same stall occupied before anesthesia where they remained for 24 h with free access to water and hay. Postoperative analgesia for GC horses was administered four hours and 20 min after anesthetic recovery, composed of morphine, dipyrone, and flunixin meglumine with the same dosage and route as described previously for GCA (Figure 1). Operated horses were treated with three administrations every 48 h of 30,000 IU/Kg of procaine penicillin G, benzathine penicillin G, and dihydrostreptomycin (Penfort®, Ourofino, Cravinhos, Brazil) IM.

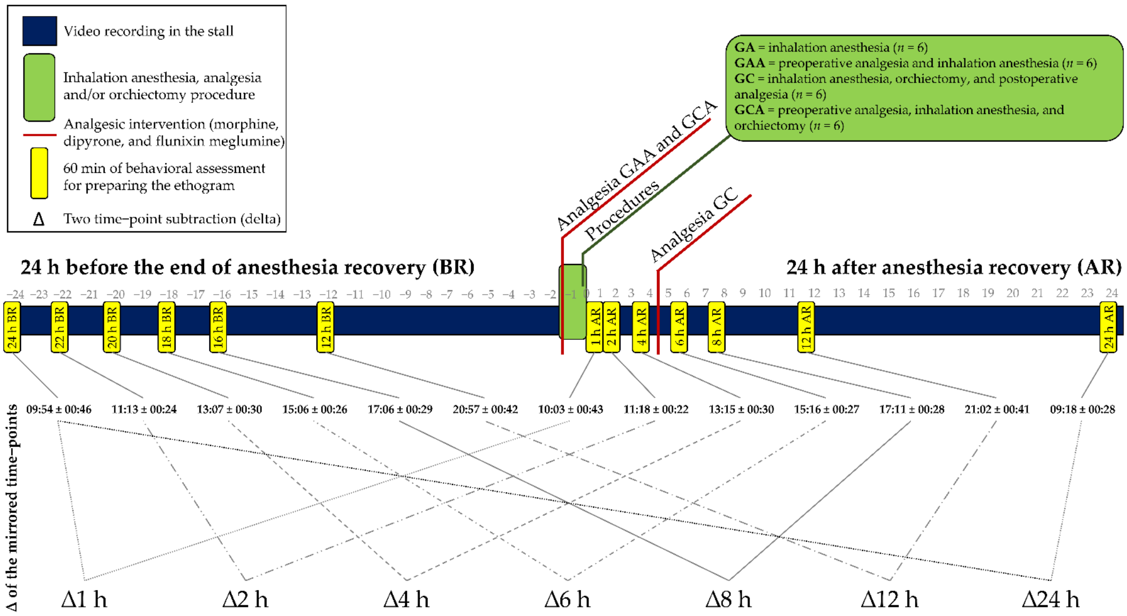

Figure 1. Timeline containing the 60-min behavior recording time-points, anesthetic, analgesic, surgical interventions, and identification of the mirrored time-points for extraction of the deltas. The delta (Δ) is the subtraction of the frequency and duration of the behaviors recorded after the anesthesia from those recorded in the mirrored hours before the anesthesia.

2.3. Behavioral Assessment

The horses were filmed uninterruptedly for the 48 h in the stall, except during anesthesia and orchiectomy, using two video cameras, one fixed in the upper right corner in front of the stall and the other in the upper left corner at the back of the stall. Seven continuous time-points of 60 min without any human presence inside or in front of the stall were selected (0–1 h, 1–2 h, 3–4 h, 5–6 h, 7–8 h, 11–12 h, and 23–24 h) after recovery from anesthesia. The same six 60-min time-points recorded on the previous day (before anesthesia) were selected, corresponding to the same times as the after-anesthesia time-points (Figure 1). The time-points from 1 h to 24 h after the anesthesia were mirrored in the 24 h before the anesthesia. An extra time-point was also recorded between 2 and 3 h before anesthesia (3 h BR; 06:43 ± 00:32) without a mirrored record, which was considered a baseline in a previous study [18].

An ethogram was developed by including the relevant behaviors to assess acute postoperative pain based on previous studies [1,7,11,12,20–22,36,41] and our recordings. One of the authors of the study (MOT) continuously recorded the frequency of 18 events (behaviors with shorter duration) and duration in minutes of 16 states (longer-lasting behaviors) of 34 behaviors divided into 9 categories according to pain diagnostic criteria reported in previous studies [7,11,12,18,20,22,23,36,41] (Table 1). The focal animal sampling method was used by observing and recording the frequency and duration of each horse's behaviors for a continuous period of time [42]. The evaluator was the same for all observations and was blinded to the GA vs. GAA and GC vs. GCA.

Table 1. Description and category of the recording of 34 spontaneous behaviors classified in 9 categories to compose the ethogram of 24 horses after anesthesia, analgesia, and orchiectomy ([d] duration; [f] frequency). The behavioral categories were based on previous studies [7,11,12,18,20,22,23,36,41].

Category	Spontaneous behaviors	Description
Feeding	Drink[d]	Immerse part of the mouth in the water in the drinking trough, suck, and swallow water.
	Eat[d]	Hold the hay with the lips, chew, and swallow.
Attention to the affected area	Swing tail[f]	Move the tail left or right, or at least 45 º upwards, and then back to the starting position.
	Kick[f]	Retract the pelvic limb quickly, flexing the tarsal joint to strike the abdomen, and the hoof may touch the abdomen.
	Retract pelvic limb[f]	Retract the pelvic limb, flex the tarsal joint without touching the hoof on the abdomen, and keep the retracted limb in suspension for a few seconds before returning to the initial position.
	Retract and extend pelvic limb[f]	Retract the pelvic limb, flexing the tarsal joint, stretch the limb back, and return the limb to the starting position.
	Look at the wound[f]	Direct the head and muzzle towards the inguinal region (groin).
Attention to the environment	Smell[f]	Touch the muzzle to a structure in the stall, inhaling, and exhaling air (smelling).
	Flehmen[f]	Raise the head forward, contracting the upper lip upwards towards the nostrils.
	Look at the back of the stall[d]	Stand in the middle of the stall with the head facing away from the stall door.
	Look out the window[d]	Stand in front of the stall door with the head positioned outside the stall through the window.

	Stare at the side of the stall[d]	Stand at the back or middle of the stall with the head facing one side of the stall.
	Stay at the back of the stall[d]	Stand on the opposite side from the stall door (back of the stall), however, with the head directed towards the stall door.
Self-care	Scratch[f]	Turn the neck to one side towards the body, lean the muzzle against the body, and scratch the body surface with movements of the upper lip (muzzle) or quick bites.
Rest	Lower head[f]	Move the upper edge of the head (occipital) down the withers.
	Head below withers[d]	Keep the upper edge of the head (occipital) below the withers.
	Rest standing still[d]	Stationary with the four members on the floor supporting the body weight (standing).
	Rest pelvic limb[d]	Standing supporting the bodyweight with three limbs resting on the floor, while one of the pelvic limbs remains relaxed, with no load and may have only the hoof tip resting on the floor.
Discomfort	Try to lie down[f]	Lower the head, smell the floor, and may or may not flex the thoracic limbs and touch the floor (kneeling), and return to the quadrupedal position.
	Yawn[f]	Open the mouth with the head extended forward and rotate the jaw before closing the mouth.
	Raise the tail[d]	Raise the tail's base upwards more than 45º and keep it raised for a few minutes and return to the starting position.
	Paw[f]	Raise one of the thoracic limbs forward, lean the hoof on the floor in front of the body, and drag the hoof across the floor until it returns to the initial position close to the body.
	Sternal decubitus[d]	Lie with the sternum resting on the floor.

	Lateral decubitus[d]	Lie with ribs resting on the floor.
	Movements with the tongue[f]	Put tongue out of the mouth and lick lips without eating or drinking water.
	Vertical movement of the head[f]	Move head up and down at least once.
	Masticatory movements[d]	Distance the mandible from the maxilla (chewing), open the mouth without eating or drinking water.
	Roll[f]	When lying on the floor in lateral decubitus move the limbs, neck, and head from side to side.
Excretion	Defecate[f]	Raise the tail and defecate.
	Urinate[f]	Eliminate a stream of urine.
Locomotion	Walk[d]	Move the four limbs at least once forward, backward, or sideways, changing position.
Relax	Expose the penis[d]	Completely expose the penis out of the prepuce.
	Stretch the body[f]	Stand supporting the bodyweight on the four limbs, stretch the neck down or forward, arching the spine upward, stretching.
	Shake[f]	Rotate the head, neck, and upper body quickly and rhythmically.

2.4. Statistical Analysis

All statistical analyses were performed by PHET in software R using the integrated development environment RStudio (Version 4.0.2 (2020−06−22), RStudio, Inc. Boston, MA, United States). The functions and packages were presented in the format "function {package}" and α was considered 5% in all analyses. The data distribution was investigated using histograms (hist {graphics}), quantile-quantile graphics (qqnorm {stats}), and boxes (ggboxplot {ggpubr}). According to the graphs, no variables were normally distributed. The behavior, expose the penis, was considered missing data for females.

Intergroup comparisons were performed to infer the effects of anesthesia only (GA), pre-operative analgesia and anesthesia (GAA), preoperative analgesia, anesthesia, and orchiectomy (GCA) possibly representing mild pain, and anesthesia, orchiectomy, and postoperative analgesia (GC), possibly representing severe pain. The analysis was based on the delta (Δ) extraction of the frequency and duration of each behavior by subtracting the values at each time-point after the anesthesia from its mirrored time-point at equivalent times before the anesthesia for each horse (Figure 1). Intergroup comparisons were investigated at each time-point using the Kruskal–Wallis test (kruskal {agricolae}) with multiple post-test comparisons with the false discovery rate. Delta results show the proportional increase or decrease in frequency or duration of each behavior recorded at each time-point in relation to the same time recorded the day before the anesthesia. The criterion for determining the effect of anesthesia, analgesia, and pain on behavior was based on differences between GC and the other groups for each time-point.

To investigate the effect of the recording time, alterations in behavior on the day before the anesthesia were analyzed using the Friedman test (friedman.test {stats}) with multiple post-test comparisons based on the false discovery rate (posthoc.friedman.nemenyi.test {PMCMR}). For this stage, behavior frequency, and duration, and not the delta, were used for all groups combined, because all horses were under the same conditions and had not yet undergone any procedure.

An extra analysis was conducted only with the raw data of frequency or duration of the six pain-related behaviors unaffected by the time of day and by the effects of anesthesia and analgesia. The Friedman test was used for within-group comparisons over time and the Kruskal–Wallis test with multiple post-test comparisons based on the false discovery rate was used for inter-group comparisons at each time point. The baseline time-point adopted was that used in the previous publication [18] (3 h BR) and following the time-points subsequences after anesthesia (1, 2, 4, 6, 8, 12, and 24 h AR).

3. Results

There were no differences in Δ between groups for any time-point for the following behaviors: flehmen, head below withers, kick, lateral decubitus, lower head, masticatory movements, movements with the tongue, paw, raise the tail, retract and extend pelvic limb, roll, scratch, shake, smell, stare at the side of the stall, sternal decubitus, stretch the body, swing tail, try to lie down, urinate, vertical movement of the head, and yawn. This means that the expression of these behaviors did not vary from the same time of the day before anesthesia in any groups, and these behaviors were not affected by analgesia, anesthesia, or surgery, and possibly are not associated with pain.

Horses from the GC exhibited proportionally increased frequency of behaviors of look at the wound (Δ2 h) and retract the pelvic limb (Δ24 h) compared to the other groups, while duration or frequency of drink (Δ4 h), eat (Δ4 h), walk (Δ6 h), look at the back of the stall (Δ1 h), and expose the penis (Δ1 h) were proportionally decreased in comparison with the other groups (Table 2 and Figures 2–5). The raw data of frequency or duration of the behaviors that showed Δ differences between groups (Table 2) are presented in Table S1. The intra and inter-group differences in frequency or duration of behaviors not affected by time of day, anesthesia, and analgesia are expressed in Table S2. Only the behaviors look at the wound and retract the pelvic limb showed similar differences compared to the analysis based on Δ (Table 2).

Table 2. Median and interquartile range (Q_1; Q_3) of the delta (Δ) of the frequency or duration of the behaviors that showed differences between groups of horses submitted to anesthesia (GA), previous analgesia and anesthesia (GAA), anesthesia, orchiectomy, and postoperative analgesia (GC) and preoperative analgesia, anesthesia, and orchiectomy (GCA). The delta (Δ) is the subtraction of the frequency and duration of the behaviors recorded after the anesthesia from those recorded in the mirrored hours before the anesthesia. Different uppercase letters and values in bold indicate a significant difference between groups at each time-point (A > B > C). The vertical dashed black line indicates when postoperative analgesia was performed for the GC; [d] indicates behaviors assessed as duration and [f] as frequency.

Behaviors	Groups	Time-Points						
		Δ1 h	**Δ2 h**	**Δ4 h**	**Δ6 h**	**Δ8 h**	**Δ12 h**	**Δ24 h**
Drink[d]	GA	**0 ^{AB} (−0.2; 0)**	0 (−0.2; 0.2)	**0.5 ^{A} (0; 1)**	**0 ^{AB} (−0.2; 0.2)**	0 (0; 0.2)	0 (−0.2; 0.2)	0 (−1; 1)
	GAA	**0 ^{A} (0; 0.2)**	−1 (−1; −0.7)	**0 ^{A} (−0.2; 0)**	**1 ^{A} (0; 1)**	0 (0; 1)	0 (−0.2; 0.2)	0 (0; 1.2)
	GC	**−0.5 ^{B} (−1.2; 0)**	−0.5 (−2.5; 0)	**−1 ^{B} (−1; 0.2)**	**−1 ^{B} (−1; 0)**	0 (−1.2; 0.5)	−0.5 (−1.2; 0.2)	1 (0; 1.2)
	GCA	**0 ^{AB} (0; 0)**	−0.5 (−1; 0)	**0 ^{A} (0; 0.2)**	**0 ^{B} (−0.2; 0)**	0 (0; 1)	0 (−0.2; 0.2)	0 (0; 0.2)
Eat[d]	GA	−3.5 (−11.7; 22)	10.5 (−11.7; 32)	**17.5 ^{A} (−6.2; 30.5)**	−7 (−11.2; 28.2)	−2 (−18.5; 11.2)	4 (−9; 9.5)	8.5 (−27.7; 26)
	GAA	−1.5 (−17; 14)	−9 (−18; 8.7)	**1 ^{A} (−5.5; 23.5)**	13.5 (−5.5; 20.2)	7.5 (−2.7; 26.7)	−5 (−19.2; 12.5)	−4.5 (−26; 6.5)
	GC	−11.5 (−28; −6)	−14.5 (−34.5; 0.5)	**−30.5 ^{B} (−38; −11.7)**	15 (−7.5; 22.7)	16.5 (5; 26.7)	1 (−12.5; 23.5)	5 (−10.5; 21.7)
	GCA	−6.5 (−19; 19.7)	25.5 (−23.7; 31.2)	**13 ^{A} (−15.2; 35)**	1 (−15.7; 10.2)	−22.5 (−36.2; 26.5)	−9 (−20.2; 18.7)	−8 (−13; 7.7)
Defecate[f]	GA	0 (−1; 1.2)	**0.5 ^{A} (0; 1)**	0.5 (0; 1)	0.5 (0; 1)	0 (−1; 0)	0 (−0.2; 1)	0 (−0.2; 1.2)
	GAA	−1 (−1.2; 0)	**0 ^{AB} (−1; 0.5)**	−1 (−1.2; 0)	0 (−0.2; 0.5)	0 (−3; 1.5)	0.5 (0; 1.2)	0 (−1; 1.2)
	GC	0.5 (−1; 1)	**−1 ^{AB} (−1; 0.2)**	−1 (−1.2; 0.2)	0 (−1; 0)	−1 (−1.5; 0)	0 (−1; 0.5)	0 (0; 1)
	GCA	−1 (−1; 0.2)	**−1 ^{B} (−1.2; −0.5)**	0 (−1; 0.5)	0 (−0.2; 0.2)	0 (−1; 0.2)	0 (−1; 0.2)	−0.5 (−1; 1)
Walk[d]	GA	0 (−2.7; 1)	0 (−0.5; 2.2)	1.5 (−1.5; 4)	**2.5 ^{A} (0; 6.2)**	−1.5 (−4.7; 1.5)	0 (−2.5; 0.5)	1 (−0.2; 4.5)
	GAA	0 (−1; 0)	0.5 (−0.2; 5)	3 (0.2; 6.7)	**1.5 ^{A} (0.7; 4.2)**	1 (−1; 2.2)	1 (0; 1.2)	2.5 (0.7; 9.2)
	GC	0 (−0.2; 1)	0.5 (−0.5; 2.5)	0 (−1.2; 0.5)	**−1 ^{B} (−2; −1)**	0 (−2.5; 1)	0.5 (−0.2; 1.5)	0 (0; 0.5)
	GCA	−1 (−2.5; 0.2)	0 (−2; 7)	0.5 (−1.7; 2.5)	**1 ^{A} (0; 3.2)**	0.5 (−1.5; 2)	0 (−0.2; 1.2)	0.5 (−2.5; 2.2)
Rest standing still[d]	GA	0 (−0.5; 0)	0 (−3.7; 0.2)	**2 ^{AB} (−7.2; 6.5)**	0 (−7; 5.2)	1.5 (−4.7; 5.5)	−9.5 (−20; 3.7)	0 (−0.5; 0.2)

	GAA	0 (−12.7; 0)	0 (−9.5; 2.7)	**−8.5 ^{B} (−19.7; 0.7)**	−7 (−14.5; 0.2)	0 (−0.7; 1.5)	−9.5 (−24.5; 14.7)	0.5 (−6.2; 3)
	GC	−14 (−25.5; 2.7)	7.5 (−2.5; 16.2)	**23.5 ^{A} (8.5; 32.5)**	−12.5 (−19; 8.7)	−8 (−13.2; 1.2)	2 (−33.5; 13.5)	−2 (−13.7; 7.5)
	GCA	−8 (−22.2; 0)	−4.5 (−16; 0.2)	**−5.5 ^{B} (−12.7; 0.2)**	2.5 (−13.7; 12.5)	0 (−3.7; 4)	4 (−3.2; 17.2)	1.5 (−7.7; 17)
Stay at the back of the stall[d]	GA	0 (0; 0)	0 (0; 0)	0 (−0.5; 0)	**0 ^{A} (0; 1.7)**	0 (−0.2; 1.5)	0 (0; 0.2)	0 (0; 0)
	GAA	0 (0; 0)	0 (−0.2; 0.5)	0 (0; 0)	**0 ^{AB} (0; 0)**	0 (−0.5; 0)	0 (0; 0)	0 (0; 0.2)
	GC	0 (−20.5; 0)	0 (−1; 0)	0 (−2.2; 1.7)	**−2 ^{C} (−7.5; 0)**	0 (0; 1)	0 (−0.7; 4)	0 (−14.2; 13)
	GCA	0 (−9.2; 0)	0 (−1.7; 0)	0 (−3.5; 0)	**0 ^{BC} (−9.2; 0)**	0 (0; 0.2)	1 (0; 5.5)	0 (−4; 3.7)
Look out the window[d]	GA	**−21 ^{BC} (−47.2; −3.5)**	**−17.5 ^{B} (−28.7; −0.7)**	−10 (−21; 11)	−1 (−23.7; 10.7)	4 (−2; 11)	1.5 (−5.2; 6.7)	−7.5 (−29.2; 27.7)
	GAA	**−20 ^{C} (−37.5; −12.2)**	**−8 ^{AB} (−17.2; 1.2)**	3 (−15.7; 18)	−4.5 (−13.5; 5.5)	−2 (−24.7; 4.5)	6 (0.7; 12)	0.5 (−16.2; 21)
	GC	**0.5 ^{A} (−3; 3.7)**	**2 ^{A} (−4.2; 9)**	1.5 (−3.7; 8.2)	−1.5 (−10; 1.2)	−0.5 (−11; 2.7)	0.5 (−2.5; 5)	2.5 (0.5; 6.2)
	GCA	**−4 ^{AB} (−11.7; 0)**	**−19 ^{B} (−26; −0.7)**	−5.5 (−22; 1.5)	6.5 (−7.2; 12.5)	1.5 (−9; 16)	2.5 (0; 11.7)	6 (1; 13)
Look at the back of the stall[d]	GA	**0 ^{A} (0; 0)**	**0 ^{B} (0; 0)**	0 (0; 0)	0 (0; 0.2)	0 (−1.7; 0)	0 (−10.7; 0.2)	**0 ^{A} (0; 0)**
	GAA	**0 ^{A} (0; 0)**	**0 ^{AB} (0; 2.7)**	0 (0; 0)	0 (−0.5; 2.2)	0 (−0.2; 0)	0 (0; 0)	**0.5 ^{A} (0; 2.2)**
	GC	**−8 ^{C} (−31.5; −1.5)**	**1.5 ^{A} (0; 11)**	0 (−2.5; 19)	−0.5 (−15.7; 0.2)	0 (−11.2; 0)	2.5 (0; 9.7)	**−4 ^{B} (−30; −0.7)**
	GCA	**−0.5 ^{B} (−5.5; 0)**	**0 ^{B} (0; 0)**	0 (−1.2; 0)	0 (−1.2; 0.2)	0 (0; 1)	0 (0; 0.7)	**0 ^{A} (−5.5; 0)**
Look at the wound[f]	GA	0 (0; 0)	**0 ^{B} (0; 0)**	0 (−0.5; 0)	0 (−0.5; 0)	0 (0; 0)	0 (0; 1.2)	0 (0; 0)
	GAA	0 (0; 0)	**0 ^{B} (0; 0)**	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	GC	0 (−0.2; 3.5)	**1 ^{A} (0; 7.7)**	0 (0; 3.5)	0 (0; 2.7)	0 (0; 1.2)	0 (0; 0.5)	0 (−0.5; 0)
	GCA	0 (0; 0)	**0 ^{B} (0; 0)**	0 (0; 2.7)	0 (0; 0.5)	0 (0; 0)	0 (0; 0)	0 (0; 0)
Rest pelvic limb[d]	GA	−11 (−19.5; 0.7)	−4.5 (−19; 3)	**8.5 ^{AB} (−13.7; 14.2)**	2 (−20.2; 9.2)	5.5 (0.5; 10.2)	4.5 (−11.5; 13.5)	−7.5 (−19.7; 4.2)
	GAA	−18 (−35; 0)	−5.5 (−19.5; 1.2)	**−15 ^{B} (−17.5; 3.7)**	−15 (−20.2; −10.7)	0 (−4.2; 5.2)	−3 (−20.5; 13.2)	−11 (−20.2; 0.2)
	GC	6 (−18; 9.2)	14 (−3; 25)	**29.5 ^{A} (1.5; 46.2)**	0.5 (−11.7; 8)	−3.5 (−11.2; 0)	−2 (−35.2; 3.2)	−13 (−22.7; 2)
	GCA	−0.5 (−15; 0)	−0.5 (−2.7; 0.2)	**−2 ^{B} (−22.7; 0.7)**	2.5 (−13; 6.5)	−2 (−6; 5.5)	1.5 (−5.2; 15.2)	−0.5 (−3.7; 8)
Retract pelvic limb[f]	GA	0 (0; 0)	0 (0; 0)	0 (−0.2; 0)	0 (0; 0)	0 (0; 0.2)	0 (−0.5; 0.2)	**0 ^{B} (0; 0)**
	GAA	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	**0 ^{B} (0; 0)**

	GC	0 (0; 0)	0 (0; 3.7)	3 (−0.5; 12)	1.5 (0; 10.5)	0 (0; 13.2)	0 (0; 0)	**1 [A] (0; 2)**
	GCA	0 (0; 0)	0 (0; 0)	0 (0; 1.2)	0 (0; 0.7)	0 (0; 0)	0 (0; 0.2)	**0 [B] (0; 0.2)**
Expose the penis[f]	GA	**0 [A] (0; 0)**	0 (0; 0)	0 (−0.2; 0)	0 (0; 0.2)	0 (−0.7; 0.5)	0 (0; 0.5)	0 (0; 0)
	GAA	**0 [A] (0; 0.2)**	0 (−2; 0)	0 (−1; 0)	0 (−0.5; 0)	0 (0; 0)	0 (−2.2; 0)	0 (0; 0.7)
	GC	**−3 [B] (−3; −1.5)**	−2.5 (−4; 0)	0 (0; 0.5)	−1 (−3.5; 0.5)	0 (−0.7; 4.5)	0 (−1.2; 0)	0 (−3; 2)
	GCA	**0 [A] (−0.5; 0.5)**	−1 (−2; 2.2)	−0.5 (−3; 3.7)	0 (0; 6)	−1 (−9.5; 1.2)	−2 (−2.2; 0.2)	0 (−1; 3)

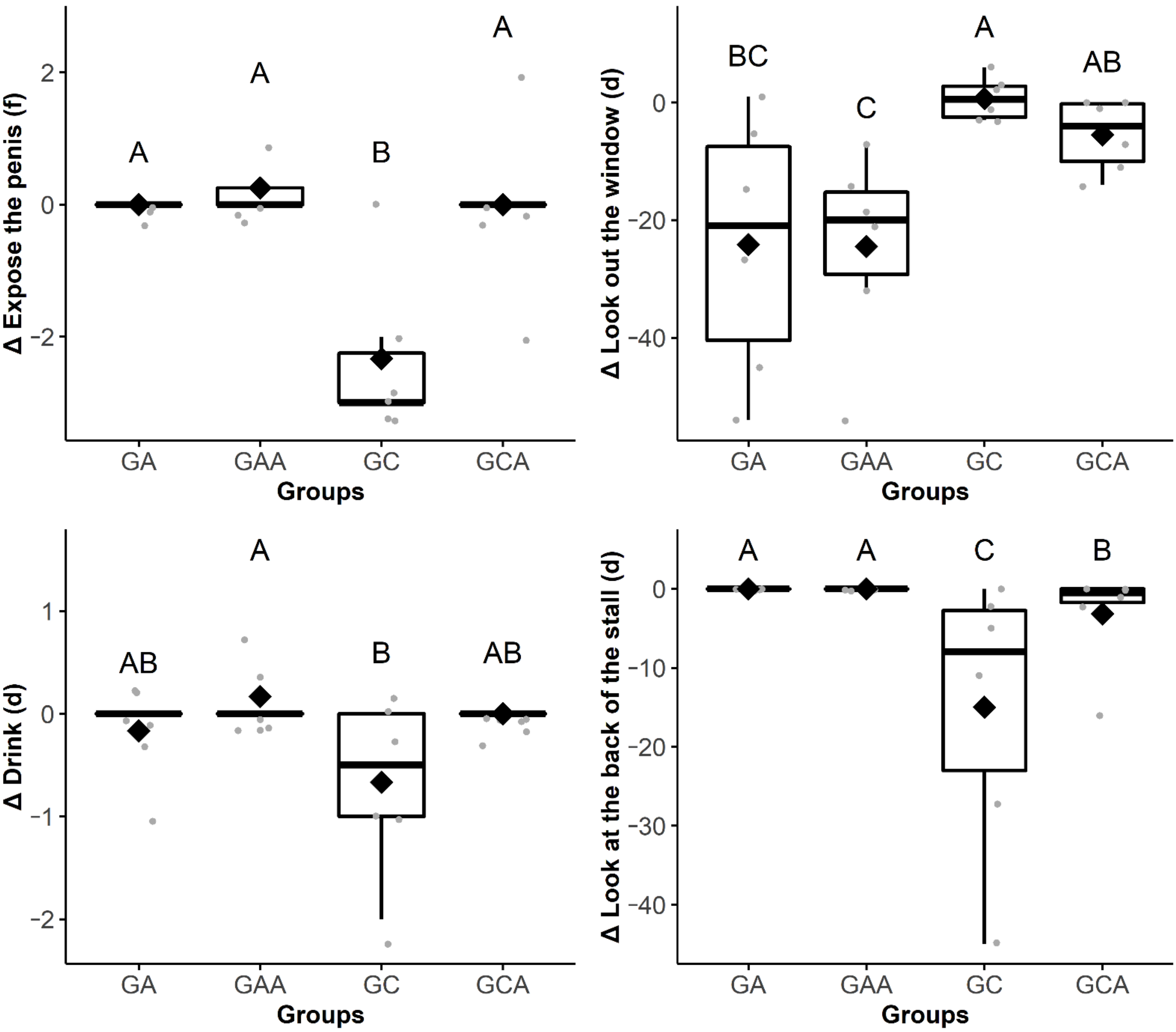

Figure 2. Boxplot of Δ1 h (10:03 ± 00:43) showing the differences in the frequency ([f]) or duration ([d]) of the behaviors expose the penis[f], drink[d], look out the window[d], and look at the back of the stall[d] among the groups of horses submitted to anesthesia (GA), previous analgesia and anesthesia (GAA), anesthesia, orchiectomy, and postoperative analgesia (GC) and preoperative analgesia, anesthesia, and orchiectomy (GCA). The delta (Δ) is the subtraction of the frequency or duration of the behaviors recorded after the anesthesia from those recorded in the mirrored hours before the anesthesia. Different uppercase letters indicate a significant difference over time (A > B > C). The gray dots represent each horse; “d” indicates behaviors assessed as duration and “f” as frequency.

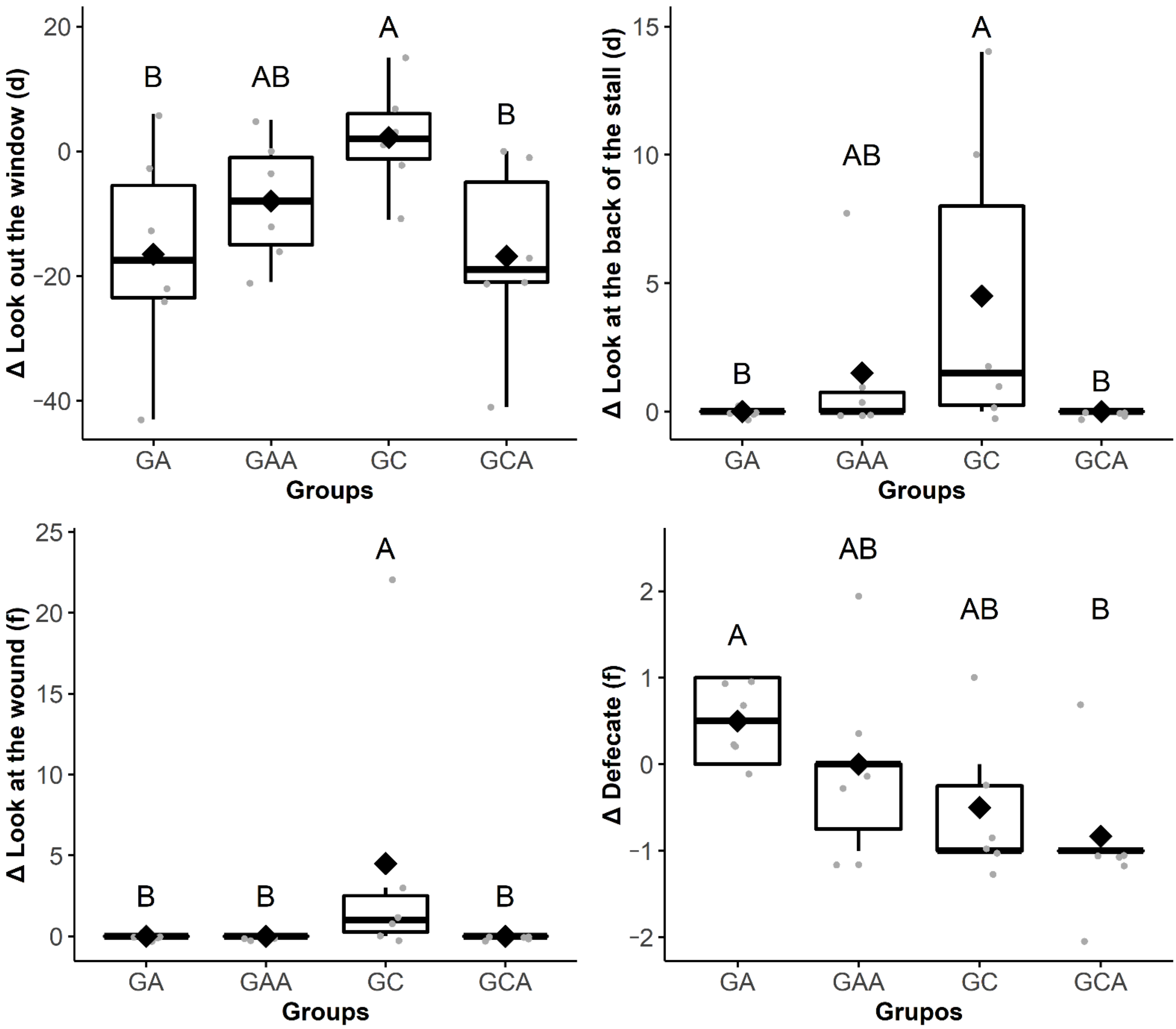

Figure 3. Boxplot Δ2 h (11:18 ± 00:22) showing the differences in the frequency (f) or duration (d) of the behaviors look out the window[d], at the back of the stall[d], and at the wound[f], and defecate[f] among the groups of horses submitted to anesthesia (GA), preoperative analgesia and anesthesia and analgesia (GAA), anesthesia, orchiectomy, postoperative analgesia (GC) and preoperative analgesia, anesthesia, orchiectomy (GCA). The delta (Δ) is the subtraction of the behaviors frequency or duration recorded after the anesthesia from those recorded in the mirrored hours before the anesthesia. Different uppercase letters indicate a significant difference over time (A > B). The gray dots represent each horse.

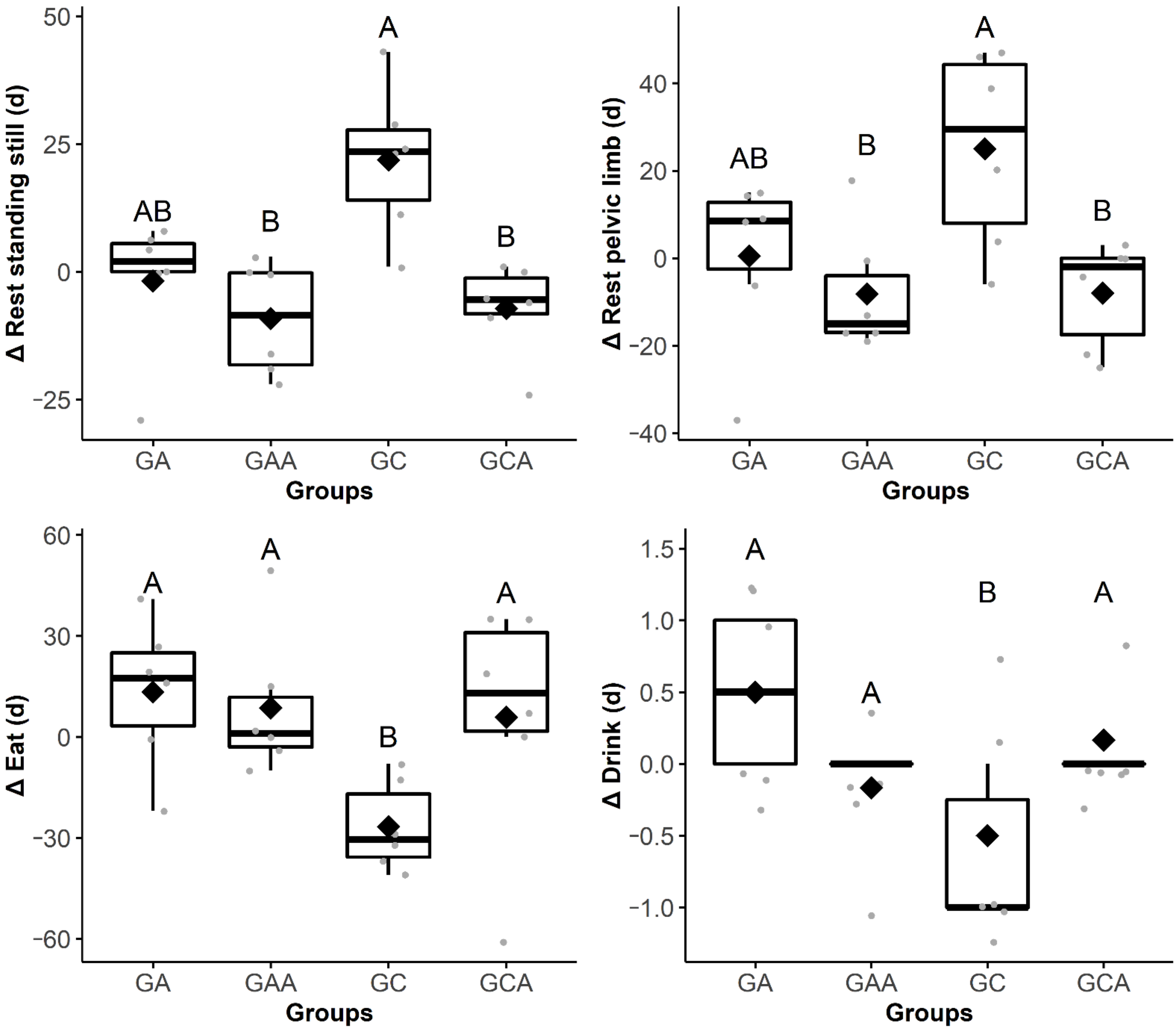

Figure 4. Boxplot Δ4 h (13:15 ± 00:30) showing the difference in the duration (d) of the behaviors rest standing still, eat, rest pelvic limb, and drink among the groups of horses submitted to anesthesia (GA), preoperative analgesia and anesthesia (GAA), anesthesia, orchiectomy, and postoperative analgesia (GC) and preoperative analgesia, anesthesia, and orchiectomy (GCA). The delta (Δ) is the subtraction of the behaviors frequency or duration recorded after the anesthesia from those recorded in the mirrored hours before the anesthesia. Different uppercase letters indicate a significant difference over time (A > B). The gray dots represent each horse.

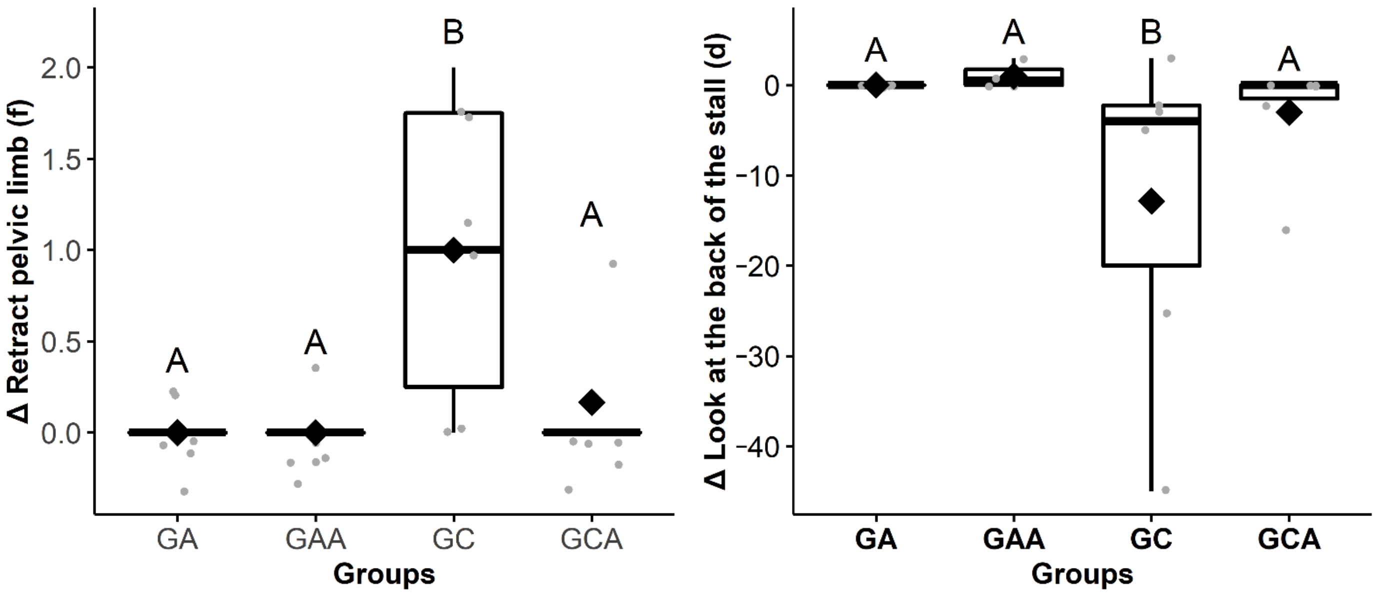

Figure 5. Boxplot Δ24 h (09:18 ± 00:28) showing the difference in the frequency (f) or duration (d) of the behaviors retract pelvic limb[f] and look at the back of the stall[d] among the groups of horses submitted to anesthesia (GA), anesthesia and analgesia (GAA), preoperative analgesia and anesthesia, orchiectomy, and postoperative analgesia (GC) and preoperative analgesia, anesthesia, and orchiectomy (GCA). The delta (Δ) is the subtraction of the behaviors frequency or duration recorded after the anesthesia from those recorded in the mirrored hours before the anesthesia. Different uppercase letters indicate a significant difference over time (A > B). The gray dots represent each horse.

When evaluating the effect of the recording time before the anesthesia, the horses walked and kept their heads out the window for less time and rested standing still and rested the pelvic limb for longer during the night, in relation to the daytime (Table 3). Table 4 combines the behaviors associated only with pain (and not affected by anesthesia and analgesia) and those affected by the time of the day (before anesthesia) in the current study and in studies available in the literature.

Table 3. Median and interquartile range (Q1; Q3) of the behavior duration of 24 horses recorded 24 h before anesthesia for all groups together. Different lowercase letters indicate significant difference over time (a > b > c). AR = after the end of the anesthetic recovery (to investigate the effect of the recording time, the frequency, and duration of behaviors were analyzed on the day before anesthesia combining all groups, because all horses were under the same conditions and had not undergone any procedure yet).

Behaviors	Behavioral Recording Time-Points before Anesthesia					
	-24 h (≈1 h AR)	**-22 h (≈2 h AR)**	**-20 h (≈4 h AR)**	**-18 h (≈6 h AR)**	**-16 h (≈8 h AR)**	**-12 h (≈12 h AR)**
	09:54 ± 00:46	**11:13 ± 00:24**	**13:07 ± 00:30**	**15:06 ± 00:26**	**17:06 ± 00:29**	**20:57 ± 00:42**
Walk	1 ^{ab} (0.25–2)	1 ^{ab} (0–1)	1 ^{ab} (0.25–3)	1 ^{ab} (1–2)	2 ^{a} (1–4)	0.5 ^{b} (0–1.75)
Rest standing still	0 ^{ab} (0–25.5)	0 ^{b} (0–13)	3 ^{b} (0–16.5)	11 ^{ab} (0–16.75)	2.5 ^{b} (0–11)	24 ^{a} (7–42.25)
Look out the window	9.5 ^{a} (3.25–22)	10.5 ^{a} (2–21)	13.5 ^{a} (5–38.5)	15.5 ^{a} (1.5–26)	8 ^{a} (1–23.25)	0 ^{b} (0–3.75)
Rest the pelvic limb	12 ^{ab} (0.25–21)	3 ^{b} (0–11.75)	7.5 ^{ab} (2–22.75)	12 ^{ab} (3–22.75)	6 ^{b} (1–11)	10 ^{a} (2–39)

Table 4. Summary of the changes in equine behaviors influenced by time of day*, not affected by analgesia or anesthesia and related only with pain in this study** and described in the literature in different equine pain conditions [1–25,36,41]. The delta (∆) of behaviors that proportionally increased (↑), decreased (↓), or both (↑↓) exclusively in the GC compared to the other groups in at least one time-point and the behaviors for which the duration or frequency were affected by the time of the day (before anesthesia). The delta (∆) is the subtraction of the frequency or duration of the behaviors recorded after the anesthesia from those recorded in the mirrored hours before the anesthesia. GC = horses submitted to anesthesia and orchiectomy with postoperative analgesia; ^{d} indicates behaviors assessed as duration and ^{f} as frequency.

Behaviors	Behavior Influenced by Time of Day (before Anesthesia-all Groups)*	Pain		
		Behavior Specifically Related to Pain in this Study (GC)**	**Pain Behavior according to Literature**	**Neither Described by Literature nor Present in this Study**
Drink^{d}		↓	X	
Eat^{d}		↓	X	
Swing tail			X	
Kick			X	
Retract pelvic limb^{f}		↑	X	
Retract and extend pelvic limb			X	
Look at the wound^{f}		↑	X	
Smell				X
Flehmen			X	
Look at the back of the stall^{d}		↓	X	
Look out the window^{d}	↓↑		X	
Stare at the side of the stall			X	

Stay at the back of the stall			X	
Scratch			X	
Lower head			X	
Head below withers			X	
Rest standing still[d]	↓↑		X	
Rest pelvic limb[d]	↓↑		X	
Try to lie down			X	
Yawn			X	
Raise the tail				X
Paw			X	
Sternal decubitus			X	
Lateral decubitus			X	
Movements with the tongue			X	
Vertical movement of the head			X	
Masticatory movements				X
Roll			X	
Defecate			X	
Urinate			X	
Walk[d]	↓↑	↓	X	
Expose the penis[f]		↓		
Stretch the body			X	
Shake			X	

4. Discussion

The novelty of the present study was the calculation of the Δ between mirrored post- and pre-anesthesia time-points to verify the most relevant behavior changes between horses submitted only to anesthesia or anesthesia and orchiectomy with or without analgesia in both cases. To our knowledge, this study is pioneering in identifying frequency and/or duration of spontaneous behaviors without any human presence inside or in front of the stall to assess acute post-orchiectomy pain in horses, considering the interference of anesthesia, analgesia, pain intensity, and recording time. Our findings suggest that the behaviors drink, eat, look at the back of the stall, look at the wound, retract the pelvic limb, and expose the penis are probably pain-related behaviors, independent of the time of day and the effects of anesthesia and analgesia. Walk, rest standing still, look out the window, and rest pelvic limb were relevant to the diagnosis of pain in previous studies [5–11,15,16,18–20,22,36], however, because they were influenced by the time of day in the present study, the observation time may be a confounding factor when these behaviors are used to assess postoperative pain in horses. On the other hand, other behaviors suggestive of pain in the literature (flehmen, head below withers, kick, lateral decubitus, lower head, paw, retract and extend pelvic limb, roll, scratch, shake, stare at the side of the stall, sternal decubitus, stretch the body, swing tail, try to lie down, urinate, vertical movement of the head, and yawn) [10–12,18,21–23,36] were not modified, suggesting that they are not associated with pain after orchiectomy. It was impossible to differentiate mild pain in castrated horses treated with preoperative analgesia (GCA) from severe pain in horses not treated with preventive analgesia (GC).

The time spent looking out the stall window in the immediate postoperative period in surgical horses not treated with preoperative analgesia was proportionally less affected compared to the other groups and may represent a confounder to assess pain in this period. Lack of interest in the environment is a possible indicator of pain [10,11,18,23], however, according to our results, caution should be taken when interpreting this behavior immediately after anesthetic recovery. Surprisingly, contrary to expectations for instruments assessing pain [11,12,17–18,20–22,28], at the first hour after recovery, the Δ duration of look out the window was not altered in the castrated horses, otherwise it was proportionally decreased in the other groups. This discrepancy could be related to the fact that in previous studies evaluations were not carried out immediately after anesthetic recovery. A possible explanation for the proportionally greater attention to the environment in surgical horses, compared to those only anesthetized, is anxiety as a non-specific response due to pain [36] or dislocated behavior, as reported in neonatal dogs after caudectomy without analgesia, who suckled more than those who received pain relief [43]. Another possible explanation is that the horses only submitted to anesthesia were from the host institution and were less curious, probably because they were more familiar with the environment. This result requires further investigation.

Although at the anticipated time-point of maximum pain (Δ4 h) [18], changes in various behaviors were expected, only the time spent eating and drinking proportionally decreased in horses submitted to surgery without previous analgesia compared to the other groups. As expected, horses receiving preventive pain relief from the analgesics and those only anesthetized ate proportionally more than horses without pain relief. Once pain relief was provided to the post-analgesia surgical group, eating behavior was like the other groups. In addition to pain relief, morphine increases food intake due to a direct effect of the opioid on neural mechanisms controlling feeding [44]. The importance of eating for pain assessment may be disputable because two of six horses ate even before analgesia, while two horses ate at 1 h and the other two ate only 4 h after analgesia. In this study, horses had continuous access to hay and were not fasted before anesthesia. Preoperative fasting is a possible confounding factor for interpreting the importance of appetite in pain assessment, since, after starvation, even horses experiencing pain tend to eat after surgery. Other confounders include variations in the routine of food offer, types, and quality, and individual preferences. Although pre-anesthetic starvation is a common practice in horses, the benefits of this procedure are disputable [45]. We decided not to deprive horses of food to avoid a possible effect of starvation increasing appetite after anesthesia.

Based on the Δ differences in the pain-related behaviors at different time-points (proportionally greater mirrored frequency of the behaviors look at the wound and retract the pelvic limb and proportionally smaller duration and/or frequency of drink, eat, look at the back of the stall, and expose the penis), only the last was not included in previously published pain scales [7,9,11,18,22]. These differences using the Δ approach for statistical analysis were not observed when the raw data of frequency and duration were used, suggesting that this method enhanced the possibility of detecting changes in behavior in response to pain, despite the trade-off of requiring more effort to understand the findings. In the present study, there were no expected significant alterations in possible pain behavior indicators reported in other studies [10–12,16,18,21–23,41] (flehmen, head below withers, kick, lateral decubitus, lower head, paw, retract and extend pelvic limb, scratch, shake, stare at the side of the stall, sternal decubitus, stretch the body, swing tail, urinate, vertical movement of the head, movements with the tongue, and yawn). The absence of changes in these behaviors, except roll and try to lie down, which are specific to abdominal pain [5–16], may be explained because (i) the pain model used in the present study is not expected to produce intense pain, and/or (ii) the behaviors are specific to a particular type of pain but do not occur in other conditions, and/or (iii) they are possibly irrelevant for diagnosing pain, and/or (iv) the sample number was small. Some other behaviors (masticatory movements, smell, and raise the tail) that we suspected would be associated with pain did not show changes in this or previous studies [1–25,36,41] and may not be important to diagnose pain.

This study mimicked a real scenario by comprising the usual transit of people during the clinical routine of a veterinary hospital. In all horses the same periodical clinical examination and pain assessment, including interaction with the observer, locomotion when led by the evaluator, and palpation to the affected area were performed and the data published in the previous study [18]. However, considering that human presence disturbs discomfort behavior in confined horses [27], in this study the recorded data were obtained remotely by video cameras, only when there was no presence of any human at the front of the door or inside the stall where the horse was housed. Our findings suggest that it is possible to diagnose post-orchiectomy pain based only on spontaneous behaviors, questioning the need for direct human pain assessment interventions [7,10,12,15,18,21,22].

Our results suggest that some adjustments could be performed in previously published pain assessment instruments to define the more specific pain behavioral indicators, as previously reported for pigs [29], sheep [30], cats [46], and oxen [47]. Except for the visceral pain scale for orchiectomy [18] and the orthopedic scale [22], other scales [6,8,9,12,21] did not consider the isolated analysis of the importance of each behavior according to internal consistency, item-total correlation, principal components analysis, sensitivity, and specificity, possibly leading to the inclusion of unnecessary behaviors in these instruments [48].

The quantification of pain is relevant to identify various degrees of suffering and aid decision-making for qualitative selection and dosage of analgesic intervention. Although it was expected that horses castrated with prior analgesia would express an intermediate degree of pain between those castrated without previous analgesia and those only anesthetized, this was not observed even 8 h after the anesthesia, when the analgesic effect of the morphine (2 h) [49], dipyrone (5 h) [50], and flunixin meglumine (8 h) would be fading [51]. It can be inferred that the analgesic protocol produced preventive analgesia. Horses treated with different quality analgesic protocols also did not show differences in the orthopedic composite behavioral scale after castration [19]. It is important to assess mild or moderate pain in practice to evaluate whether the horse requires analgesia or whether analgesia was adequate. The challenge of recognizing pain of mild or moderate intensity requires further investigation. In other species, this challenge has been unraveled by the definition of a cut-off point for rescue analgesia [29,30,46,47].

The second innovation of this study was the use of mirrored time-points, to control and assess the effect of the time of day on behavioral records 24 h before and after the interventions. This strategy identified the time-dependent behaviors before the anesthesia, as reported in rabbits [52]. The behaviors walk, rest standing still, look out the window, and rest pelvic limb have previously been applied to assess pain in horses [5,10–12,18,20,22,23], yet, according to our results, they are influenced by the time of day. Naturally, behaviors change throughout the day; horses are alert and active during the day and resting or sleeping at night [38]. These behavioral variations may also be related to greater movement by hospital staff in the daytime compared to at night. The practical implication of these findings is that it is advisable to relativize the importance of or disregard these behaviors to avoid false-positive results in interpreting pain intensity at night. Despite this, most behaviors pointed out in this and previous studies [9–12,18,21–23] did not suffer interference from the time of day.

Study limitations include the differences in numbers of each sex, between only male horses undergoing orchiectomy from animals that were only anesthetized, composed of castrated females and males. The normal daily behavior of stallions and free-living mares is different [53]. The estrous cycle influences the aggressive behavior (kicking, bucking, biting) in mares when handled [54], while stallions are more curious to an enriched environment in comparison to castrated and female horses [55]. In our study, except for the attention to the environment, the pain-related behaviors were related to feeding, relaxing, and attention to the affected area, which we believe suffer less sex influence. Although exposing the penis is unavailable in females, it is relevant for assessing pain, because time of the day, anesthesia, and analgesia did not influence exposing the penis and after surgery males castrated with postoperative analgesia exposed the penis proportionally less than those castrated with preoperative analgesia. Another limitation was that the non-operated horses, but not the operated ones, were familiar with the hospital environment because they belonged to the institution; therefore, the horses submitted to orchiectomy had no prior acclimatization to the hospital. Lack of adaptation to the environment reflects the real-life circumstances of an active equine hospital but may deflagrate curiosity and increase restlessness. New arena tests (open field) investigating fear or curiosity in a new environment are usually conducted during the first 20 min of horse exposure to a new environment [56], therefore it seems that the most important reactions occur for a short time. To partially overcome this limitation, the current study showed that behaviors related to environment familiarity, like look out the window, walk, and rest standing still, were different even between the groups of castrated horses with or without previous analgesia, that were equally acquainted with the surroundings, showing that they may be considered pain-related behaviors and are not only associated with environment adaptation.

The practical implications of this study are that the pain-related spontaneous behaviors identified may be used as a basis for future studies to build a shorter and simpler composite behavioral pain scale compared to those available to date [7,12,13,15–19,21,22], in order to reduce assessment time in a busy hospital routine. Our findings contribute to the avoidance of confounding factors and to guaranteeing a more accurate diagnosis of pain in this species.

The authors recognize that behavioral-based pain assessment is one of the best approaches to diagnose equine pain in the clinical and experimental setting [7,12,13,15–19,21,22]; nevertheless, future studies should consider that time of the day, anesthesia, and analgesia affect the frequency and/or duration of the pain-related spontaneous behaviors. Our results were based on 60 min periods of observation; prolonged observation periods may be necessary to identify the relevant, exclusive, and subtle equine pain behavioral changes [41]. Perhaps the current paradigm that it is possible to perform in-person equine pain assessment for up to 10−15 min demands further investigation. Artificial intelligence-based tools might help us to collect and analyze data automatically to build and implement a robust, reliable, and valid instrument in the clinical and experimental setting, to include detection of mild and severe pain.

5. Conclusion

Because the spontaneous behaviors drink, eat, look at the back of the stall, look at the wound, retract the pelvic limb, and expose the penis were not affected by anesthesia, analgesia, or time of the day, they may be considered pain-related behaviors and have the potential to be used as post-orchiectomy pain indicators in horses under remote video evaluation without human intervention. Because the time of day may be a confounding factor for the behaviors walk, rest standing still, look out the window, and rest pelvic limb, their importance should be relativized according to the time they are evaluated. Other behaviors considered suggestive of pain (flehmen, head below withers, kick, lateral decubitus, lower head, paw, retract and extend pelvic limb, scratch, shake, stare at the side of the stall, sternal decubitus, stretch the body, swing tail, urinate, vertical movement of the head, movements with the tongue, and yawn) were not significantly modified in horses submitted to orchiectomy and require further scrutiny in horses suffering pain under other conditions. No behaviors defined the effect of pain intensity.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1. Table S1: Raw data median and interquartile range (Q_1; Q_3) of the frequency (^{f}) or duration (^{d}) of the behaviors that showed differences in delta (Table 2) between groups of horses submitted to anesthesia (GA), preoperative analgesia and anesthesia (GAA), anesthesia, orchiectomy and postoperative analgesia (GC) and, anesthesia, preoperative analgesia and orchiectomy (GCA). BR = before the end of anesthesia recovery; AR = after the anesthesia recovery, and Table S2: Raw data median and interquartile range (Q1; Q3) of the frequency (^{f}) or duration (^{d}) of the six pain-related behaviors unaffected by the time of day, anesthesia, analgesia and surgery. Horses were submitted to anesthesia (GA), previous analgesia and anesthesia (GAA), preoperative analgesia, anesthesia and orchiectomy (GCA) and anesthesia, orchiectomy and postoperative analgesia (GC). Different lowercase letters and values in bold indicate a significant difference over time in each group (a > b > c), and different uppercase letters and values in bold indicate a significant difference between groups at each time-point (A > B > C).

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Data Availability Statement: The data presented in this study are available in the supplementary material according to "MDPI Research Data Policies" at https://www.mdpi.com/ethics.

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References

1. Andersen, P.H.; Gleerup, K.B.; Wathan, J.; Coles, B.; Kjellström, H.; Broomé, S.; Lee, Y.J., Rashid, M., Sonder, C., Resenberg, E. et. al. Can a machine learn to see horse pain? An interdisciplinary approach towards automated decoding of facial expressions of pain in the horse. *Measuring Behavior 2018-11th International Conference on Methods and Techniques in Behavioral Research*, Proceedings of Measuring Behavior, Manchester, UK, 6-8 June 2018 Available online: www.measuringbehavior.org (accessed on 2 March 2021).
2. Guedes, A. Pain management in horses. *Vet. Clin. North. Am. Equine Pract*. **2017**, *33*, 181–211, doi:10.1016/j.cveq.2016.11.006.

3. Dugdale, A.H.A. Progress in equine pain assessment? *Vet. J*. **2014**, *200*, 210–211, doi:10.1016/j.tvjl.2014.03.012.
4. Wagner, A.E. Effects of stress on pain in horses and incorporating pain scales for equine practice. *Vet. Clin. North. Am. Equine Pract*. **2010**, *26*, 481–492, doi:10.1016/j.cveq.2010.07.001.
5. Sutton, G.A.; Dahan, R.; Turner, D.; Paltiel, O. A behaviour-based pain scale for horses with acute colic: Scale construction. *Vet. J.* **2013**, *196*, 394–401, doi:10.1016/j.tvjl.2012.10.008.
6. Sutton, G.A.; Atamna, R.; Steinman, A.; Mair, T.S. Comparison of three acute colic pain scales: Reliability, validity and usability. *Vet. J*. **2019**, *246*, 71–77, doi:10.1016/j.tvjl.2019.01.004.
7. VanDierendonck, M.C.; van Loon, J.P.A.M. Monitoring acute equine visceral pain with the Equine Utrecht University Scale for Composite Pain Assessment (EQUUS-COMPASS) and the Equine Utrecht University Scale for Facial Assessment of Pain (EQUUS-FAP): A validation study. *Vet. J*. **2016**, *216*, 175–177, doi:10.1016/j.tvjl.2016.08.004.
8. Sutton, G.A.; Paltiel, O.; Soffer, M.; Turner, D. Validation of two behaviour-based pain scales for horses with acute colic. *Vet. J.* **2013**; *197*, 646–650, doi:10.1016/j.tvjl.2013.04.007.
9. Sutton, G.A.; Sutton, G.A.; Bar, L.; Sutton, G. Evaluation of pain in horses refinement and revalidation of the Equine Acute Abdominal Pain Scale (EAAPS). *Isr J. Vet. Med.* **2016**, *71*, 15–23.
10. Graubner, C.; Gerber, V.; Doherr, M.; Spadavecchia, C. Clinical application and reliability of a post abdominal surgery pain assessment scale (PASPAS) in horses. *Vet. J.* **2011**, *188*, 178–183, doi:10.1016/j.tvjl.2010.04.029.
11. Pritchett, L.C.; Ulibarri, C.; Roberts, M.C.; Schneider, R.K.; Sellon, D.C. Identification of potential physiological and behavioral indicators of postoperative pain in horses after exploratory celiotomy for colic. *Appl. Anim. Behav. Sci.* **2003**, *80*, 31–43, doi:10.1016/S0168−1591(02)00205−8.

12. van Loon, J.P.A.M.; Van Dierendonck, M.C. Monitoring acute equine visceral pain with the Equine Utrecht University Scale for Composite Pain Assessment (EQUUS-COMPASS) and the Equine Utrecht University Scale for Facial Assessment of Pain (EQUUS-FAP): A scale-construction study. *Vet. J.* **2015**, *206*, 356–364, doi:10.1016/j.tvjl.2015.08.023.
13. van Loon, J.P.A.M.; Back, W.; Hellebrekers, L.J.; van Weeren, P.R. Application of a composite pain scale to objectively monitor horses with somatic and visceral pain under hospital conditions. *J. Equine Vet. Sci*. **2010**, *30*, 641–649, doi:10.1016/j.jevs.2010.09.011.
14. Sellon, D.C.; Roberts, M.C.; Blikslager, A.T.; Ulibarri, C.; Papich, M.G. Effects of continuous rate intravenous infusion of butorphanol on physiologic and outcome variables in horses after celiotomy. *J. Vet. Intern. Med*. **2004**, *18*, 555–563, doi:10.1111/j.1939−1676.2004.tb02585.x.
15. Van Loon, J.P.A.M.; Jonckheer-Sheehy, V.S.M.; Back, W.; René van Weeren, P.; Hellebrekers, L.J. Monitoring equine visceral pain with a composite pain scale score and correlation with survival after emergency gastrointestinal surgery. *Vet. J.* **2014**, *200,* 109–115, doi:10.1016/j.tvjl.2014.01.003.
16. Lawson, A.L.; Opie, R.R.; Stevens, K.B.; Knowles, E.J.; Mair, T.S. Application of an equine composite pain scale and its association with plasma adrenocorticotropic hormone concentrations and serum cortisol concentrations in horses with colic. *Equine Vet. Educ.* **2020**, *32*, 20−27, doi.org/10.1111/eve.13143.
17. Pehkonen, J.; Karma, L.; Raekallio, M. Behavioral signs associated with equine periapical infection in cheek teeth. *J. Equine Vet. Sci.* **2019**, *77*, 144–150, doi:10.1016/j.jevs.2019.03.005.
18. Taffarel, M.O.; Luna, S.P.L.; de Oliveira, F.A.; Cardoso, G.S.; de Moura Alonso, J.; Pantoja, J.C.; Brondani, J.T.; Love, E.; Taylor, P.; White, K.; et al. Refinement and partial validation of the UNESP-Botucatu multidimensional composite pain scale for assessing postoperative pain in horses. BMC *Vet. Res*. **2015**, *11*, 1–12, doi:10.1186/s12917−015−0395−8.

19. Dalla Costa, E.; Minero, M.; Lebelt, D.; Stucke, D.; Canali, E.; Leach, M.C. Development of the Horse Grimace Scale (HGS) as a pain assessment tool in horses undergoing routine castration. **2014**, *9*, doi:10.1371/journal.pone.0092281.
20. Price, J.; Catriona, S.; Welsh, E.M.; Waran, N.K. Preliminary evaluation of a behaviour-based system for assessment of post-operative pain in horses following arthroscopic surgery. *Vet. Anaesth Analg*. **2003**, *30*, 124–137, doi:10.1046/j.1467−2995.2003.00139.x.
21. van Loon, J.P.A.M; Van Dierendonck, M.C. Pain assessment in horses after orthopaedic surgery and with orthopaedic trauma. Vet J. **2019**, *246*, 85–91, doi:10.1016/j.tvjl.2019.02.001.
22. Bussières, G.; Jacques, C.; Lainay, O.; Beauchamp, G.; Leblond, A.; Cadoré, J.-L.; Desmaizières, M.; Cuvelliez, S.G.; Troncy, E. Development of a composite orthopaedic pain scale in horses. *Res. Vet. Sci.* **2008**, *85*, 294–306, doi:10.1016/J.RVSC.2007.10.011.
23. Lindegaard, C.; Thomsen, M.H.; Larsen, S.; Andersen, P.H. Analgesic efficacy of intra-articular morphine in experimentally induced radiocarpal synovitis in horses. *Vet. Anaesth Analg*. **2010**, *37*, 171–185, doi:10.1111/J.1467−2995.2009.00521.X.
24. Dutton, D.W.; Lashnits, K.J.; Wegner, K. Managing severe hoof pain in a horse using multimodal analgesia and a modified composite pain score. *Equine Vet. Educ*. **2009**, *21*, 37–43, doi:10.2746/095777308X382669.
25. Raekallio, M.; Taylor, P.M.; Bloomfield, M. A comparison of methods for evaluation of pain and distress after orthopaedic surgery in horses. *J. Vet. Anaesth*. **1997**, *24*, 17–20, doi:10.1111/j.1467−2995.1997.tb00150.x.
26. Gleerup, K.B.; Lindegaard, C. Recognition and quantification of pain in horses: A tutorial review. *Equine Vet. Educ*. **2016**, *28*, 47–57, doi:10.1111/eve.12383.
27. Torcivia, C.; McDonnell, S. In-person caretaker visits disrupt ongoing discomfort behavior in hospitalized equine orthopedic surgical patients. *Animals* **2020**, *10*, 210, doi:10.3390/ani10020210.
28. Gleerup, K.B.; Forkman, B.; Lindegaard, C.; Andersen, P.H. An equine pain face. *Vet. Anaesth Analg*. **2015**, *42*, 103–114, doi:10.1111/vaa.12212.

29. Luna, S.P.L.; de Araújo, A.L.; da Nóbrega Neto, P.I.; Brondani, J.T.; de Oliveira, F.A.; Azerêdo LM dos, S.; Telles, F.G.; Trindade, P.H.E. Validation of the UNESP-Botucatu pig composite acute pain scale (UPAPS). *PLoS ONE*. **2020**, *15*, e0233552, doi:10.1371/journal.pone.0233552.
30. Silva, N.E.O.F.; Trindade, P.H.E.; Oliveira, A.R.; Taffarel, M.O.; Moreira, M.A.P.; Denadai, R.; Rocha, P.B.; Luna, S.P.L. Validation of the Unesp-Botucatu composite scale to assess acute postoperative abdominal pain in sheep (USAPS). *PLoS ONE*. **2020**, *15*, doi:10.1371/journal.pone.0239622.
31. Descovich, K.A.; Wathan, J.; Leach, M.C.; Buchanan-Smith, H.M.; Flecknell, P.; Farningham, D.; Vick, S.J. Facial expression: An under-utilized tool for the assessment of welfare in mammals. *Altex* **2017**, 409–429, doi:10.14573/altex.1607161.
32. Mcdonnell, S.M. Is it psychological, physical, or both? In Proceedings of the 51st Annual Convention of the American Association of Equine Practitioners, Seattle, WA, USA, 3−7 December 2005; pp. 231−238.
33. Dodds, L.; Knight, L.; Allen, K.; Murrell, J. The effect of postsurgical pain on attentional processing in horses. *Vet. Anaesth Analg.* **2017**, *44*, 933–942, doi:10.1016/j.vaa.2016.07.010.
34. Hall, C.; Randle, H.; Pearson, G.; Preshaw, L.; Waran, N. Assessing equine emotional state. *Appl. Anim. Behav. Sci*. **2018**, *205*, 183–193, doi:10.1016/j.applanim.2018.03.006.
35. Gigliuto, C.; De Gregori, M.; Malafoglia, V.; Raffaeli, W.; Compagnone, C.; Visai, L.; Petrini, P.; Avanzini, M.A.; Muscoli, C.; Viganò, J.; Calabrese, F.; Dominioni, T.; Allegri, M.; Cobianchi, L. Pain assessment in animal models: Do we need further studies? *J. Pain Res.* **2014**, *7*, 227–236, doi:10.2147/JPR.S59161.
36. Ashley, F.H.; Waterman-Pearson, A.E.; Whay, H.R. Behavioural assessment of pain in horses and donkeys: Application to clinical practice and future studies. *Equine Vet. J.* **2005**, *37*, 565–575, doi:10.2746/042516405775314826.

37. Pinho, R.H.; Leach, M.C.; Minto, B.W.; Rocha, F.D.L.; Luna, S.P.L. Postoperative pain behaviours in rabbits following orthopaedic surgery and effect of observer presence. *PLoS ONE*. **2020**, 15, e0240605, doi:10.1371/journal.pone.0240605.
38. McDonnell, S. *The Equid Ethogram: A practical field guide to horse behaviour*. Eclipse Pres*:* Lexington, KY, USA, 2003; Volume 1, pp. 39–58.
39. Taffarel, M.O.; Luna, S.P.L.; Cardoso, G.S.; de Oliveira, F.A.; Alonso, J. de, M.; Gozalo-Marcilla, M. Preemptive Analgesia, including morphine, does not affect recovery quality and times in either pain-free horses or horses undergoing orchiectomy. *J. Equine Vet.* Sci. **2017**, *48*, 82–85, doi:10.1016/j.jevs.2016.03.015.
40. Searle, D.; Dart, A.J.; Dart, C.M.; Hodgson, D.R. Equine castration: Review of anatomy, approaches, techniques and complications in normal, cryptorchid and monorchid horses. *Aust Vet. J*. **1999**, *77,* 428−434. doi.org/10.1111/j.1751−0813.1999.tb12083.x.
41. Torcivia, C.; Mcdonnell, S. Equine discomfort ethogram. *Animals* **2020**, *11*, 1–24.doi.org/10.3390/ani11020580.
42. Martin, P.; Bateson, P.P.G. *Measuring behaviour: An introductory guide*. Cambridge University Press: Cambridge, UK, 1993.
43. Steagall, P.; Luna, S.; Taylor, P.; Humm, K.F.T. Neurological, respiratory, bahavioural and endocrine effects of tail docking in newborn dogs submitted to epidural anesthesia. *Ars Vet.* **2009**, *25*, 58–062, doi:10.15361/2175−0106.2009v25n2p058−06251.
44. Valbrun, L.P.; Zvonarev, V. The opioid system and food intake: Use of opiate antagonists in treatment of binge eating disorder and abnormal eating behavior. *J. Clin. Med. Res*. **2020**, *12*, 41−63, doi:10.14740/jocmr4066.
45. Bailey, P.A.; Hague, B.A.; Davis, M.; Major, M.D.; Zubrod, C.J.; Brakenhoff, J.E. Incidence of post-anesthetic colic in non-fasted adult equine patients. *Can. Vet. J.* **2016**, *57*, 1263.

46. Brondani, J.T.; Mama, K.R.; Luna, S.P.L.; Wright, B.D.; Niyom, S.; Ambrosio, J.; Vogel, P.R.; Padovani, C.R. Validation of the English version of the UNESP-Botucatu multidimensional composite pain scale for assessing postoperative pain in cats. *BMC Vet. Res*. **2013**, *9*, 1–15, doi:10.1186/1746−6148−9−143.
47. de Oliveira, F.A.; Luna, S.P.L.; do Amaral, J.B.; Rodrigues, K.A.; Sant'Anna, A.C.; Daolio, M.; Brondani, J.T. Validation of the UNESP-Botucatu unidimensional composite pain scale for assessing postoperative pain in cattle. *BMC Vet. Res*. **2014**, *10*, 1–14, doi:10.1186/s12917−014−0200−0.
48. Streiner, D.L.; Norman, G.R.; Cairney, J. Health measurement scales—A practical guide to their development and use. Oxford University Press: Oxford, UK, **2015**, doi:10.1093/occmed/kqp179.
49. Devine, E.P.; Kukanich, B.; Beard, W.L. Pharmacokinetics of intramuscularly administered morphine in horses. *J. Am. Vet. Med. Assoc.* **2013**, *243*, 105–112, doi:10.2460/javma.243.1.105.
50. Klaus, A.; Schlingloff, Y.; Kleinitz, U.; Bottcher, M.; Hapke, H.J. Pharmacokinetic study of dipyrone metabolite 4-MAA in the horse and possible implications for doping control. *J. Vet. Pharmacol Ther***.** **1997**, *20*, 204–208, doi:10.1111/j.1365−2885.1997.tb00096.x.
51. Toutain, P.L.; Autefage, A.; Legrand, C.; Alvinerie, M. Plasma concentrations and therapeutic efficacy of phenylbutazone and flunixin meglumine in the horse: Pharmacokinetic/pharmacodynamic modelling. *J. Vet. Pharmacol Ther*. **1994**, *17*, 459–469, doi:10.1111/j.1365−2885.1994.tb00278.x.
52. Leach, M.C.; Allweiler, S.; Richardson, C.; Roughan, J.V.; Narbe, R.; Flecknell, P.A. Behavioural effects of ovariohysterectomy and oral administration of meloxicam in laboratory housed rabbits. *Res. Vet. Sci*. **2009**, *87*, 336–347, doi:10.1016/j.rvsc.2009.02.001.
53. Duncan, P. Determinants of the use of habitat by horses in a Mediterranean Wetland. *J Anim Ecol*. **1983**, *52*, 93, doi:10.2307/4590.
54. Melgaard, D.T.; Korsgaard, T.S.; Thoefner, M.S.; Petersen, M.R.; Pedersen, H.G. Moody mares—Is ovariectomy a solution? *Animals* **2020**, *10*, 1210, doi:10.3390/ani10071210.

55. Bulens, A.; Van Beirendonck, S.; Van Thielen, J.; Driessen, B. The enriching effect of non-commercial items in stabled horses. *Appl. Anim. Behav. Sci*. **2013**, *143*, 46–51, doi:10.1016/j.applanim.2012.11.012.
56. Forkman, B.; Boissy, A.; Meunier-Salaün, M.C.; Canali, E.; Jones, R.B. A critical review of fear tests used on cattle, pigs, sheep, poultry and horses. *Physiol. Behav*. **2007**, doi:10.1016/j.physbeh.2007.03.016.

CAPÍTULO 3

1. Considerações finais

A obra aqui apresentada teve enfoque em examinar lacunas na avaliação comportamental da dor em equinos. Tais lacunas são devido ao contexto de avaliação de dor perioperatório hospitalar que usualmente impõem condições que representam fatores de confusão na estimativa da dor. Nesse sentido, nós fomos pioneiros em estudar o efeito residual dos fármacos destinados a anestesia e analgesia, do horário de registro e da intensidade da dor em comportamentos espontâneos (sem a presença ou intervenção de humanos durante a avaliação) relacionados a dor pós-orquiectomia.

Os poucos estudos que investigaram o efeito residual farmacológico da anestesia e analgesia na resposta comportamental com a presença humana não observaram qualquer linguagem corporal relacionada ao efeito dessas drogas (PRITCHETT et al., 2003; GRAUBNER et al., 2011; DALLA COSTA et al., 2014). Mesmo assim, de maneira empírica os cavalos normalmente não são avaliados por outros estudos imediatamente após a recuperação anestésica para se esquivar do possível efeito residual farmacológico da anestesia e analgesia no comportamento (ZUTT et al., 2014; VAN LOON; VAN DIERENDONCK, 2015; VANDIERENDONCK; VAN LOON, 2016; VAN LOON; VAN DIERENDONCK, 2019). Nossos resultados demonstraram que a linguagem corporal atinente ao interesse no ambiente foi alterada apenas 1h após recuperação anestésica, indicando que esses fármacos podem representar um efeito de confusão quando a dor for avaliada imediatamente após a recuperação. Com esse achado é possível confirmar parcialmente o conhecimento empírico do efeito de tais drogas no comportamento relacionado a dor. Na prática, quando for estimar a dor imediatamente após a recuperação anestésica deve avaliar a atenção ao ambiente com cautela ou talvez a desconsiderar.

Apesar de parecer óbvio, o horário do dia em que o comportamento é registrado nunca havia sido considerado ou estudado anteriormente para os comportamentos relativos à dor. É esperado que o horário tenha efeito no comportamento uma vez que a distribuição comportamental equina é naturalmente heterogênea ao longo do dia (MCDONNELL, 2003; MURPHY, 2019). Portanto, não surpreendentemente, comportamentos relacionados a locomoção, interesse no ambiente e descanso foram diferentes quando comparados de manhã ou tarde com de noite em nosso estudo. Esse achado reforça nossa suspeita inicial de que o horário do dia pode ser um fator de confusão na avaliação comportamental da dor em equinos. Curiosamente, esses comportamentos estão contemplados na maioria dos estudos com objetivo de estimar a dor pós-operatória de cavalos pela linguagem corporal, desconsiderando qualquer efeito do horário em que o comportamento foi registrado (PRICE et al., 2003; PRITICHETT et al., 2003; BUSSIÈRES et al., 2008; LINDEGAARD et al., 2010; GRAUBNER et al., 2011; SUTTON et a., 2013 a e b; van LOON et al., 2015; TAFFAREL et al., 2015;). O óbvio pode ser invisível. A principal implicação prática desse resultado é a cautela necessária para quando avaliar a dor de um cavalo ao longo do dia e, talvez desconsiderar os resultados de comportamentos relacionados a locomoção, interesse no ambiente e descanso quando as avaliações forem feitas em períodos do dia diferentes.

A intensidade da dor não tem sido o foco principal dos estudos que avaliam a dor em equinos. Em linhas gerais, é esperado que procedimentos mais invasivos produzam uma dor de maior intensidade, sendo que nestes casos supõe-se que ocorra maior exibição de comportamentos relativos à dor. Apesar disso, os poucos estudos que fizeram tais investigações não conseguiram apontar diferenças entre as intensidades das dores (LINDEGAARD et al., 2009; VAN LOON et al., 2010; DALLA COSTA et al., 2014; LAWSON et al., 2020). Nesta tese duas intensidades de dor foram estabelecidas dadas as diferenças entre dois protocolos analgésicos. Em concordância com outros estudos, não encontramos evidências para distinguir as intensidades de dor estabelecidas usando o comportamento como ferramenta. Era esperado encontrar uma diferença entre a magnitude de frequência ou duração da expressão comportamental relativa à dor entre as duas intensidades de dor. Em um cenário real, é imprescindível diagnosticar a dor, tratá-la e reavaliá-la para saber se o tratamento admitido foi suficiente e, caso necessário tratar novamente. Todavia, aparentemente a capacidade discriminatória do comportamento sob a dor sofre interferência da intensidade, ou seja, quanto menor a dor menor a capacidade diagnóstica do comportamento. Tal achado evidencia o requerimento de mais estudos para diferenciar as intensidades de dor.

A inovação em registrar os comportamentos ao longo das 48h perioperatórias e analisá-las com o delta permitiu melhor entender o efeito do horário do dia na avaliação da dor nos equinos. O trade-off desta abordagem é a diminuição da variabilidade dos dados que incorre na menor chance de encontrar diferenças estatísticas, e a dificuldade de interpretação devido a perda da unidade de medida original, em nosso caso minutos ou contagem que são transformados em delta. Nós analisamos sete deltas para cada equino que foram obtidos mediante registro comportamental em 13 intervalos de 60 min, totalizando 780 min de observação por cavalo. Com estes dados nós conseguimos identificar comportamentos relacionados a dor independentes dos efeitos da anestesia, analgesia e horário do dia. Entretanto, tal quantidade de observações comportamentais é impraticável em uma rotina de atendimento e aparentemente seria impossível obter resultados análogos usando menos momentos ou menor tempo de avaliação. Uma solução para o futuro da avaliação de dor em equinos poderia ser o desenvolvimento de uma inteligência artificial para coletar a partir de filmagens na ausência de humanos e analisar automaticamente os dados comportamentais, permitindo registros mais prolongados.

Nessa perspectiva, em um futuro não tão distante é possível que seja indispensável baias enfermarias em hospitais equipadas com câmeras que estejam conectadas à algoritmos para análise em tempo real da linguagem corporal dos cavalos. Tal tecnologia já existe para diagnóstico de dor em animais humanos (YOUSSIF; ASKER, 2011; ABDULLAH; ABDULAZEEZ, 2021) e estudos têm empenhado esforços neste sentido com a espécie equina (ANDERSEN et al., 2021). Por fim, entendemos que no futuro a inteligência artificial irá executar a atividade repetitiva de coleta e análise de dados comportamentais, enquanto o médico veterinário irá direcionar sua atenção para interpretar os resultados produzidos pelo algoritmo e tomar a decisão final da conduta médica cabível à cada paciente.

2. Referências

ABDULLAH, S. M. S.; ABDULAZEEZ, A. M. Facial expression recognition based on deep learning convolution neural network: A review. **Journal of Soft Computing and Data Mining**, 2(1), 53-65, 2021.

ANDERSEN, P. H.; BROOMÉ, S.; RASHID, M.; LUNDBLAD, J.; ASK, K.; LI, Z.; KJELLSTRÖM, H. Towards Machine Recognition of Facial Expressions of Pain in Horses. **Animals**, 11(6), 1643, 2021.

DALLA COSTA, E.; MINERO, M.; LEBELT, D.; STUCKE, D.; CANALI, E.; LEACH, M. C. Development of the Horse Grimace Scale (HGS) as a Pain Assessment Tool in Horses Undergoing Routine Castration. **PLoS ONE**, v. 9, n. 3, p. e92281, 2014.

GRAUBNER, C.; GERBER, V.; DOHERR, M.; SPADAVECCHIA, C. Clinical application and reliability of a post abdominal surgery pain assessment scale (PASPAS) in horses. **Veterinary Journal**, v. 188, n. 2, p. 178–183, 2011.

LAWSON, A. L.; OPIE, R. R.; STEVENS, K. B.; KNOWLES, E. J.; MAIR, T. S. Application of an equine composite pain scale and its association with plasma adrenocorticotropic hormone concentrations and serum cortisol concentrations in horses with colic. **Equine Veterinary Education**, v. 32, n. S11, p. 20–27, 2020.

LINDEGAARD, C.; THOMSEN, M. H.; LARSEN, S.; ANDERSEN, P. H. Analgesic efficacy of intra-articular morphine in experimentally induced radiocarpal synovitis in horses. **Veterinary Anaesthesia and Analgesia**, v. 37, n. 2, p. 171–185, 2010.

LINDEGAARD, C.; VAABENGAARD, D.; CHRISTOPHERSEN, M. T.; EKSTØM, C. T.; FJELDBORG, J. Evaluation of pain and inflammation associated with hot iron branding and microchip transponder injection in horses. **American Journal of Veterinary Research**, v. 70, n. 7, p. 840–847, 2009.

MCDONNELL, S. **Practical field guide to horse behavior: The equid ethogram**. The Blood-Horse, Lexington, United States, 2003, v. 1, p. 39–58.

MURPHY, B. A. Circadian and circannual regulation in the horse: internal timing in an elite athlete. **Journal of equine veterinary science**, v. 76, p. 14-24, 2019.

PRICE, J.; CATRIONA, S.; WELSH, E. M.; WARAN, N. K. Preliminary evaluation of a behaviour–based system for assessment of post–operative pain in horses following arthroscopic surgery. **Veterinary Anaesthesia and Analgesia**, v. 30, n. 3, p. 124–137, 2003.

PRITCHETT, L. C.; ULIBARRI, C.; ROBERTS, M. C.; SCHNEIDER, R. K.; SELLON, D. C. Identification of potential physiological and behavioral indicators of postoperative pain in horses after exploratory celiotomy for colic. **Applied Animal Behaviour Science**, v. 80, n. 1, p. 31–43, 2003.

SUTTON, G. A.; DAHAN, R.; TURNER, D.; PALTIEL, O. A behaviour-based pain scale for horses with acute colic: Scale construction. **Veterinary Journal**, v. 196, n. 3, p. 394–401, 2013a.

SUTTON, G. A.; PALTIEL, O.; SOFFER, M.; TURNER, D. Validation of two behaviour-based pain scales for horses with acute colic. **Veterinary Journal**, v. 197, n. 3, p. 646–650, 2013b.

TAFFAREL, M. O.; LUNA, S. P. L.; DE OLIVEIRA, F. A.; CARDOSO, G. S.; DE MOURA ALONSO, J.; PANTOJA, J. C.; BRONDANI, J. T.; LOVE, E.; TAYLOR, P.; WHITE, K.; MURRELL, J. C. Refinement and partial validation of the UNESP-Botucatu multidimensional composite pain scale for assessing postoperative pain in horses. **BMC Veterinary Research**, v. 11, n. 1, p. 1–12, 2015.

VAN LOON, J. P. A. M.; VAN DIERENDONCK, M. C. Monitoring acute equine visceral pain with the Equine Utrecht University Scale for Composite Pain Assessment (EQUUS-COMPASS) and the Equine Utrecht University Scale for Facial Assessment of Pain (EQUUS-FAP): A scale-construction study. **Veterinary Journal**, v. 206, n. 3, p. 356–364, 2015.

VAN LOON, J. P. A. M.; VAN DIERENDONCK, M. C. Pain assessment in horses after orthopaedic surgery and with orthopaedic trauma. **Veterinary Journal**, v. 246, p. 85–91, 2019.

VANDIERENDONCK, M. C.; VAN LOON, J. P. A. M. Monitoring acute equine visceral pain with the Equine Utrecht University Scale for Composite Pain Assessment (EQUUS-COMPASS) and the Equine Utrecht University Scale for Facial Assessment of Pain (EQUUS-FAP): A validation study. **Veterinary Journal**, v. 216, p. 175–177, 2016.

YOUSSIF, A. A.; ASKER, W. A. Automatic facial expression recognition system based on geometric and appearance features. **Computer and Information Science**, 4(2), 115, 2011.

ZUTT, R.; VAN DER KOOI, A. J.; LINTHORST, G. E.; WANDERS, R. J. A.; DE VISSER, M. Rhabdomyolysis: Review of the literature. **Neuromuscular Disorders**, v. 24, n. 8, p. 651–659, 2014.

MATERIAL SUPLEMENTAR

Table S1. Median and interquartile range (Q_1; Q_3) of the frequency (^{f}) or duration (^{d}) of the behaviors in horses submitted to anesthesia (GA), preoperative analgesia and anesthesia (GAA), anesthesia, orchiectomy and postoperative analgesia (GC) and, anesthesia, preoperative analgesia and orchiectomy (GCA). BR = before the end of anesthesia recovery; AR = after the anesthesia recovery.

			Time-points						
Behaviors	**Groups**		**1 h AR and 24hBR**	**2hAR and 22hBR**	**4hAR and 20hBR**	**6hAR and 18hBR**	**8hAR and 16hBR**	**12hAR and 12hBR**	**24hAR and 24hBR**
Drink[d]	**GA**	**BR**	0 (0; 1)	0 (0; 1)	0 (0; 0.25)	0 (0; 1)	0 (0; 0.25)	0 (0; 1)	0 (0; 1)
		AR	0 (0; 0.25)	0 (0; 1)	0.5 (0; 1.25)	0 (0; 1)	0 (0; 1)	0 (0; 0.5)	0 (0; 1)
	GAA	**BR**	0 (0; 0)	1 (0.75; 1)	0.5 (0; 1)	0 (0; 0)	0 (0; 0.25)	0 (0; 0.25)	0 (0; 0)
		AR	0 (0; 0.25)	0 (0; 0)	0 (0; 1)	1 (0; 1)	0.5 (0; 1)	0 (0; 0.25)	0 (0; 1.25)
	GC	**BR**	0.5 (0; 1.25)	0.5 (0; 2.5)	1 (0; 1)	1 (0; 1)	0 (0; 1.25)	1 (0; 1.5)	0.5 (0; 1.25)
		AR	0 (0; 0)	0 (0; 0)	0 (0; 0.25)	0 (0; 0)	0 (0; 0.5)	0.5 (0; 1)	1 (0.75; 1.75)
	GCA	**BR**	0 (0; 0)	0.5 (0; 1)	0 (0; 0.25)	0.5 (0; 1)	0 (0; 0)	0 (0; 0.25)	0 (0; 0)
		AR	0 (0; 0)	0 (0; 0)	0 (0; 1)	0 (0; 1)	0 (0; 1)	0 (0; 0.25)	0 (0; 0.25)
Eat[d]	**GA**	**BR**	33.5 (8.25; 45.5)	30 (15.75; 39.75)	16 (8.25; 29.75)	30 (17.25; 41.75)	22 (16; 35.75)	22.5 (15.75; 43.25)	33.5 (8.25; 45.5)
		AR	30 (28.75; 36.75)	34.5 (25.75; 48.5)	35 (24.75; 38.75)	32 (18.5; 45.5)	23 (9; 33)	34.5 (11.75; 46.25)	28 (17.75; 53.75)
	GAA	**BR**	25.5 (13.5; 38.75)	39 (15; 45.75)	19.5 (9.5; 30)	22.5 (11.5; 45.25)	19.5 (9.75; 46.75)	18.5 (3; 39)	25.5 (13.5; 38.75)
		AR	25 (17.25; 30.5)	30.5 (17.25; 38)	24.5 (13.75; 47.25)	30.5 (19; 48.75)	33.5 (25.75; 47)	15.5 (9.75; 22.5)	15 (5.25; 34.5)
	GC	**BR**	25 (12.5; 38)	28.5 (10.5; 46)	45 (31.75; 51.25)	34 (24.25; 45.75)	33.5 (20.5; 41.75)	41.5 (9; 51.5)	25 (12.5; 38)
		AR	4.5 (0.75; 21.25)	2 (0; 21.5)	10 (2.25; 24.75)	51.5 (29.25; 57.25)	47 (44.5; 54)	42.5 (25.75; 48.25)	31.5 (18.75; 39.25)
	GCA	**BR**	32.5 (21.5; 43.75)	13.5 (7.5; 33.75)	28 (19.75; 62)	39.5 (24.75; 46)	38.5 (27.5; 46.75)	24 (7; 43)	32.5 (21.5; 43.75)
		AR	29 (22.75; 34.25)	34 (23.25; 45)	46.5 (29.5; 60.25)	34.5 (23.75; 52.5)	24 (3.75; 48.75)	26.5 (9.75; 38.5)	28.5 (18; 33.5)
Defecate[f]	**GA**	**BR**	1 (0; 1)	1 (0; 1)	1 (0.75; 1.25)	1 (0; 2)	1 (0; 2.75)	1 (0; 1.25)	1 (0; 1)
		AR	0.5 (0; 1.5)	1 (0.75; 2)	1 (1; 2.25)	1 (1; 2.25)	0.5 (0; 2.5)	1 (0; 2)	1 (0; 1.5)
	GAA	**BR**	1 (0; 1.25)	0.5 (0; 1)	1.5 (1; 2)	1 (0; 1)	2 (0; 4)	0 (0; 0)	1 (0; 1.25)
		AR	0 (0; 0)	0 (0; 0.75)	0.5 (0; 1.25)	0.5 (0; 1.5)	1 (0.75; 2.25)	0.5 (0; 1.25)	1 (0.75; 1.25)
	GC	**BR**	0.5 (0; 1)	1 (0; 1)	1 (0; 1.25)	0.5 (0; 1)	1 (0.75; 1.5)	1 (0; 1.25)	0.5 (0; 1)
		AR	0.5 (0; 1.25)	0 (0; 0.25)	0 (0; 0.25)	0 (0; 0.25)	0 (0; 0.25)	1 (0; 1.25)	1 (0.75; 1)
	GCA	**BR**	1 (0.75; 1.25)	1 (0.75; 1.25)	1 (0.75; 1)	1 (0; 1)	1 (1; 1.25)	1 (0.75; 1)	1 (0.75; 1.25)
		AR	0 (0; 0.75)	0 (0; 0.25)	1 (0; 1.25)	0.5 (0; 1.25)	1 (0; 2)	1 (0; 1)	1 (0; 1.25)
Walk[d]	**GA**	**BR**	2 (0.75; 3.5)	1 (0.75; 3)	2 (0.75; 6)	1 (1; 2.75)	4.5 (1.75; 6.25)	0.5 (0; 2.75)	2 (0.75; 3.5)
		AR	1 (0; 2.5)	1.5 (0.75; 5)	4 (1; 8)	5 (2.5; 7.25)	2 (0.75; 5)	0.5 (0; 1.25)	3.5 (1; 6.75)
	GAA	**BR**	0.5 (0; 3)	0.5 (0; 1.5)	0.5 (0; 3)	1.5 (0; 3)	2.5 (0; 4.25)	0 (0; 1.25)	0.5 (0; 3)
		AR	0 (0; 2.25)	0.5 (0; 6.5)	3.5 (1; 9)	3.5 (1; 6.25)	3 (0.75; 6)	1 (0.75; 2.25)	4 (0.75; 10.75)
	GC	**BR**	1 (1; 1.25)	1 (0.75; 1.25)	1 (0.75; 1.5)	1 (1; 2)	1 (1; 2.5)	0.5 (0; 1.25)	1 (1; 1.25)
		AR	1 (0.75; 2.25)	1.5 (0.75; 3.25)	1 (0.75; 1.25)	0 (0; 0)	1 (0; 2)	1 (1; 1.5)	1 (1; 1.75)

	GCA	**BR**	1 (0; 3.5)	1 (0; 2)	1 (0.75; 2.5)	1 (0; 1.25)	0.5 (0; 3.5)	1 (0; 2.5)	1 (0; 3.5)
		AR	0 (0; 1.75)	1 (0; 7)	1 (0; 3.75)	1.5 (0.75; 5)	1.5 (0; 4.25)	2 (0.75; 2.25)	1.5 (0.75; 2.5)
Rest standing still[d]	**GA**	**BR**	0 (0; 0.5)	0 (0; 3.75)	0 (0; 8.75)	0 (0; 13)	3 (0; 10.75)	24.5 (11.5; 40.75)	0 (0; 0.5)
		AR	0 (0; 0)	0 (0; 0.25)	3.5 (0; 6.75)	5 (0; 6.75)	2.5 (0; 12)	11.5 (0.75; 42.5)	0 (0; 0.25)
	GAA	**BR**	0 (0; 12.75)	0 (0; 9.5)	10 (0; 23.5)	11 (0; 19.75)	0 (0; 5)	35.5 (19.25; 57)	0 (0; 12.75)
		AR	0 (0; 0)	0 (0; 2.75)	1.5 (0; 4)	1.5 (0; 4.75)	0 (0; 6.25)	31 (21.5; 41.5)	3.5 (0.75; 8.25)
	GC	**BR**	28.5 (15.75; 37.5)	11.5 (0.75; 18.25)	8 (0; 13.25)	17 (10.5; 22)	12 (3; 15.5)	15 (3.75; 46.5)	28.5 (15.75; 37.5)
		AR	10 (3.75; 21.5)	17.5 (3.25; 28.75)	29.5 (20.5; 40.75)	1.5 (0; 15.75)	0 (0; 5)	11 (8.75; 26)	24.5 (4.5; 35.25)
	GCA	**BR**	8 (0; 22.25)	4.5 (0; 16)	5.5 (0; 12.75)	5.5 (0; 14)	0 (0; 7.5)	14 (4.5; 38.25)	8 (0; 22.25)
		AR	0 (0; 0)	0 (0; 0.25)	0 (0; 0.25)	3 (0; 20.25)	2 (0; 4.25)	31.5 (7; 41)	13.5 (0; 27.5)
Stay at the back of the stall[d]	**GA**	**BR**	0 (0; 0)	0 (0; 0)	0 (0; 0.5)	0 (0; 0)	0 (0; 2.75)	0 (0; 0)	0 (0; 0)
		AR	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 1.75)	0 (0; 3.5)	0 (0; 0.25)	0 (0; 0)
	GAA	**BR**	0 (0; 0)	0 (0; 0.25)	0 (0; 0)	0 (0; 0)	0 (0; 0.5)	0 (0; 0)	0 (0; 0)
		AR	0 (0; 0)	0 (0; 0.5)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0.25)
	GC	**BR**	0 (0; 20.5)	0 (0; 5.5)	0 (0; 10)	2 (0; 7.5)	0 (0; 3.5)	0 (0; 8)	0 (0; 20.5)
		AR	0 (0; 0)	0 (0; 4.5)	0 (0; 5)	0 (0; 0)	0 (0; 4.5)	0 (0; 9.75)	3.5 (0; 25)
	GCA	**BR**	0 (0; 9.25)	0 (0; 1.75)	0 (0; 3.5)	0 (0; 9.25)	0 (0; 0)	0 (0; 1.75)	0 (0; 9.25)
		AR	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0.25)	1 (0; 7.25)	0 (0; 5.5)
Look out the window[d]	**GA**	**BR**	23 (10.25; 49)	18.5 (9; 33.25)	32.5 (12; 40.5)	25 (13.25; 34)	16 (8.5; 25.5)	4.5 (0.75; 9)	23 (10.25; 49)
		AR	3.5 (0.75; 5)	4 (1; 12.75)	21 (15.5; 37.25)	16 (9; 31.75)	20.5 (8.5; 32.75)	5 (2.25; 10.5)	26.5 (3.75; 38)
	GAA	**BR**	21 (16; 37.5)	8 (3; 19.5)	24 (7; 51)	22 (12; 30)	15 (5.5; 34.25)	0 (0; 3.5)	21 (16; 37.5)
		AR	0 (0; 2.75)	0 (0; 4.5)	24 (5.75; 41)	20 (2.75; 27)	10.5 (6.25; 18.5)	10.5 (2.25; 12.75)	31 (9; 40)
	GC	**BR**	3.5 (2.25; 4.25)	0.5 (0; 5)	5 (0; 11.25)	2 (0; 12.25)	1 (0.75; 11)	0 (0; 2.5)	3.5 (2.25; 4.25)
		AR	4.5 (0; 7)	3 (1.5; 9)	6 (0.75; 13.25)	0.5 (0; 3)	0.5 (0; 3)	0.5 (0; 5)	5 (1.75; 10.25)
	GCA	**BR**	7.5 (0; 11.75)	19 (2.75; 26)	19 (1.5; 34.75)	4 (0.75; 19.75)	3.5 (0; 24)	0 (0; 3)	7.5 (0; 11.75)
		AR	0 (0; 1.75)	0 (0; 1.5)	7 (0; 21)	8.5 (1.5; 24.25)	11.5 (5.25; 22)	2.5 (1.5; 14.75)	12.5 (3.75; 18)
Look at the back of the stall[d]	**GA**	**BR**	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 1.75)	0 (0; 10.75)	0 (0; 0)
		AR	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0.25)	0 (0; 0)	0 (0; 0.25)	0 (0; 0)
	GAA	**BR**	0 (0; 0)	0 (0; 0.25)	0 (0; 0)	0 (0; 0.5)	0 (0; 0.25)	0 (0; 0)	0 (0; 0)
		AR	0 (0; 0)	0 (0; 3)	0 (0; 0)	0 (0; 2.25)	0 (0; 0)	0 (0; 0)	0.5 (0; 2.25)
	GC	**BR**	13 (1.5; 31.5)	0 (0; 5.5)	0 (0; 3)	0.5 (0; 16.5)	0 (0; 12.5)	0 (0; 12.5)	13 (1.5; 31.5)
		AR	0 (0; 2.5)	1.5 (0; 18.5)	1 (0; 19)	0 (0; 1)	0 (0; 0.75)	2.5 (0; 22.25)	2 (0; 3.5)
	GCA	**BR**	0.5 (0; 5.5)	0 (0; 0)	0 (0; 1.25)	0 (0; 1.25)	0 (0; 0)	0 (0; 0.75)	0.5 (0; 5.5)
		AR	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0.25)	0 (0; 1)	0 (0; 1.5)	0 (0; 0.25)
Look at the wound[f]	**GA**	**BR**	0 (0; 0)	0 (0; 0)	0 (0; 0.5)	0 (0; 0.5)	0 (0; 0)	0 (0; 0)	0 (0; 0)
		AR	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 1.25)	0 (0; 0)

	GAA	BR	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
		AR	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	GC	BR	0 (0; 0.5)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0.5)
		AR	0 (0; 4.25)	1 (0; 7.75)	0 (0; 3.5)	0 (0; 2.75)	0 (0; 1.25)	0 (0; 0.5)	0 (0; 0)
	GCA	BR	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
		AR	0 (0; 0)	0 (0; 0)	0 (0; 2.75)	0 (0; 0.5)	0 (0; 0)	0 (0; 0)	0 (0; 0)
Rest pelvic limb[d]	GA	BR	12 (4.75; 20.5)	7 (3; 19.25)	11 (3.5; 22)	4.5 (3.75; 24)	3.5 (1; 14)	22.5 (7.75; 36)	12 (4.75; 20.5)
		AR	1 (0; 4.5)	2.5 (0.75; 6)	16 (8.25; 19.5)	8.5 (2.75; 13.5)	12 (7.25; 15.5)	23 (4.75; 47.5)	3 (0; 9.5)
	GAA	BR	18.5 (0; 35)	8 (0; 19.5)	22.5 (14.5; 32)	21 (15.5; 28.75)	6.5 (0; 14.25)	36 (1.5; 53.25)	18.5 (0; 35)
		AR	0 (0; 0.25)	0 (0; 2.5)	10.5 (0; 30.25)	1 (0; 14.25)	9 (0; 14.5)	33.5 (20.25; 43)	7.5 (0.75; 14.25)
	GC	BR	15.5 (1.5; 22.75)	1 (0; 9)	3.5 (1.75; 10.25)	11 (2.5; 23.5)	8 (0.75; 11.25)	9.5 (3.75; 46.25)	15.5 (1.5; 22.75)
		AR	10.5 (1.5; 22.5)	15 (0; 27)	39.5 (6.5; 48)	2.5 (0; 24.75)	0 (0; 2.5)	9.5 (0.75; 15.75)	0 (0; 8.75)
	GCA	BR	0.5 (0; 15)	0.5 (0; 7.25)	3 (0; 26)	1.5 (0; 13)	7.5 (0; 12)	1.5 (0.75; 16)	0.5 (0; 15)
		AR	0 (0; 0)	0 (0; 5.25)	0.5 (0; 4.25)	2.5 (0; 8.25)	4.5 (0.75; 9.75)	10.5 (0; 23)	7 (0; 12.75)
Retract pelvic limb[f]	GA	BR	0 (0; 0)	0 (0; 0)	0 (0; 0.25)	0 (0; 0)	0 (0; 0)	0 (0; 0.5)	0 (0; 0)
		AR	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0.25)	0 (0; 0.25)	0 (0; 0)
	GAA	BR	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
		AR	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	GC	BR	0 (0; 0)	0 (0; 0.25)	0 (0; 0.5)	0 (0; 0.5)	0 (0; 0)	0 (0; 0)	0 (0; 0)
		AR	0 (0; 0)	0 (0; 4)	3 (0; 12)	1.5 (0; 12)	0 (0; 13.25)	0 (0; 0)	1 (0; 2)
	GCA	BR	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
		AR	0 (0; 0)	0 (0; 0)	0 (0; 1.25)	0 (0; 0.75)	0 (0; 0)	0 (0; 0.25)	0 (0; 0.25)
Expose the penis[f]	GA	BR	0 (0; 0)	0 (0; 0)	0 (0; 0.25)	0 (0; 0)	0 (0; 0.75)	0 (0; 0.25)	0 (0; 0)
		AR	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0.25)	0 (0; 0.5)	0 (0; 0.75)	0 (0; 0)
	GAA	BR	0 (0; 0)	0 (0; 2.75)	0 (0; 3.25)	0 (0; 0.5)	0 (0; 0)	0 (0; 2.25)	0 (0; 0)
		AR	0 (0; 0.25)	0 (0; 0.25)	0 (0; 2.25)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0.75)
	GC	BR	3 (1.5; 3)	2.5 (0; 4)	0 (0; 0)	2.5 (0; 3.5)	0 (0; 0.75)	0 (0; 2.75)	3 (1.5; 3)
		AR	0 (0; 0)	0 (0; 0)	0 (0; 0.5)	0 (0; 1.25)	0 (0; 4.5)	0 (0; 1.5)	1.5 (0; 4.25)
	GCA	BR	0 (0; 2.5)	1 (0; 2)	2.5 (0; 3.75)	0 (0; 3.75)	3 (0; 9.75)	2 (0; 2.25)	0 (0; 2.5)
		AR	0 (0; 1.5)	0 (0; 2.25)	0 (0; 8)	2.5 (0; 10.5)	1.5 (0; 2.75)	0 (0; 0.25)	0.5 (0; 3.75)

Table S2. Median and interquartile range (Q1; Q3) of the frequency (^{f}) or duration (^{d}) of the six pain-related behaviors unaffected by the time of day, anesthesia, analgesia and surgery. Horses were submitted to anesthesia (GA), previous analgesia and anesthesia (GAA), preoperative analgesia, anesthesia and orchiectomy (GCA) and anesthesia, orchiectomy and postoperative analgesia (GC). Different lowercase letters and values in bold indicate a significant difference over **time** in each group (a>b>c), and different uppercase letters and values in bold indicate a significant difference between groups at each time-point (A>B>C).

		Time-points							
Behaviors	**Groups**	**7 h BR**	**1 h AR**	**2 h AR**	**4 h AR**	**6 h AR**	**8 h AR**	**12 h AR**	**24 h AR**
Drink^{d}	**GA**	0 (0; 0)	0 (0; 0.25)	0 (0; 1)	0.5 (0; 1.25)	0 (0; 1)	0 (0; 1)	0 (0; 0.5)	0 (0; 1)
	GAA	0 (0; 0)	0 (0; 0.25)	0 (0; 0)	0 (0; 1)	1 (0; 1)	0.5 (0; 1)	0 (0; 0.25)	0 (0; 1.25)
	GC	0 (0; 1)	0 (0; 0)	0 (0; 0)	0 (0; 0.25)	0 (0; 0)	0 (0; 0.5)	0.5 (0; 1)	1 (0.75; 1.75)
	GCA	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 1)	0 (0; 1)	0 (0; 1)	0 (0; 0.25)	0 (0; 0.25)
Eat^{d}	**GA**	14 (3; 17.75)	**30 ^{A} (28.75; 36.75)**	34.5 (25.75; 48.5)	35 (24.75; 38.75)	32 (18.5; 45.5)	**23 ^{B} (9; 33)**	**34.5 ^{AB} (11.75; 46.25)**	28 (17.75; 53.75)
	GAA	5 (0; 22.75)	**25 ^{AB} (17.25; 30.5)**	30.5 (17.25; 38)	24.5 (13.75; 47.25)	30.5 (19; 48.75)	**33.5 ^{AB} (25.75; 47)**	**15.5 ^{B} (9.75; 22.5)**	15 (5.25; 34.5)
	GC	**32 ^{ab} (0.75; 49.5)**	**4.5 ^{bB} (0.75; 21.25)**	**2 ^{b} (0; 21.5)**	**10 ^{ab} (2.25; 24.75)**	**51.5 ^{a} (29.25; 57.25)**	**47 ^{aA} (44.5; 54)**	**42.5 ^{abA} (25.75; 48.25)**	**31.5 ^{ab} (18.75; 39.25)**
	GCA	18.5 (1.75; 29.5)	**29 ^{A} (22.75; 34.25)**	34 (23.25; 45)	46.5 (29.5; 60.25)	34.5 (23.75; 52.5)	**24 ^{B} (3.75; 48.75)**	**26.5 ^{AB} (9.75; 38.5)**	28.5 (18; 33.5)
Stay at the back of the stall^{d}	**GA**	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 1.75)	0 (0; 3.5)	0 (0; 0.25)	0 (0; 0)
	GAA	0 (0; 0.25)	0 (0; 0)	0 (0; 0.5)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0.25)
	GC	0 (0; 9.5)	0 (0; 0)	0 (0; 4.5)	0 (0; 5)	0 (0; 0)	0 (0; 4.5)	0 (0; 9.75)	3.5 (0; 25)
	GCA	0 (0; 0.25)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0.25)	1 (0; 7.25)	0 (0; 5.5)
Look at the back of the stall^{d}	**GA**	0 (0; 0)	0 (0; 0)	**0 ^{B} (0; 0)**	0 (0; 0)	0 (0; 0.25)	0 (0; 0)	0 (0; 0.25)	**0 ^{B} (0; 0)**
	GAA	0 (0; 0.5)	0 (0; 0)	**0 ^{AB} (0; 3)**	0 (0; 0)	0 (0; 2.25)	0 (0; 0)	0 (0; 0)	**0.5 ^{AB} (0; 2.25)**
	GC	2 (0; 29.75)	0 (0; 2.5)	**1.5 ^{A} (0; 18.5)**	1 (0; 19)	0 (0; 1)	0 (0; 0.75)	2.5 (0; 22.25)	**2 ^{A} (0; 3.5)**
	GCA	0 (0; 5)	0 (0; 0)	**0 ^{B} (0; 0)**	0 (0; 0)	0 (0; 0.25)	0 (0; 1)	0 (0; 1.5)	**0 ^{AB} (0; 0.25)**
Look at the wound^{f}	**GA**	0 (0; 0)	0 (0; 0)	**0 ^{B} (0; 0)**	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 1.25)	0 (0; 0)
	GAA	0 (0; 0)	0 (0; 0)	**0 ^{B} (0; 0)**	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0)
	GC	0 (0; 0)	0 (0; 4.25)	**1 ^{A} (0; 7.75)**	0 (0; 3.5)	0 (0; 2.75)	0 (0; 1.25)	0 (0; 0.5)	0 (0; 0)
	GCA	0 (0; 0)	0 (0; 0)	**0 ^{B} (0; 0)**	0 (0; 2.75)	0 (0; 0.5)	0 (0; 0)	0 (0; 0)	0 (0; 0)
Retract pelvic limb^{f}	**GA**	0 (0; 0)	0 (0; 0)	0 (0; 0)	**0 ^{B} (0; 0)**	**0 ^{B} (0; 0)**	0 (0; 0.25)	0 (0; 0.25)	**0 ^{B} (0; 0)**
	GAA	0 (0; 0)	0 (0; 0)	0 (0; 0)	**0 ^{B} (0; 0)**	**0 ^{B} (0; 0)**	0 (0; 0)	0 (0; 0)	**0 ^{B} (0; 0)**
	GC	0 (0; 0)	0 (0; 0)	0 (0; 4)	**3 ^{A} (0; 12)**	**1.5 ^{A} (0; 12)**	0 (0; 13.25)	0 (0; 0)	**1 ^{A} (0; 2)**
	GCA	0 (0; 0)	0 (0; 0)	0 (0; 0)	**0 ^{AB} (0; 1.25)**	**0 ^{AB} (0; 0.75)**	0 (0; 0)	0 (0; 0.25)	**0 ^{B} (0; 0.25)**
	GA	**0 ^{B} (0; 0)**	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0.25)	0 (0; 0.5)	0 (0; 0.75)	0 (0; 0)

Expose the penis[f]	**GAA**	**0 [B] (0; 0.25)**	0 (0; 0.25)	0 (0; 0.25)	0 (0; 2.25)	0 (0; 0)	0 (0; 0)	0 (0; 0)	0 (0; 0.75)
	GC	**1 [AB] (0; 3)**	0 (0; 0)	0 (0; 0)	0 (0; 0.5)	0 (0; 1.25)	0 (0; 4.5)	0 (0; 1.5)	1.5 (0; 4.25)
	GCA	**2 [A] (1.75; 3)**	0 (0; 1.5)	0 (0; 2.25)	0 (0; 8)	2.5 (0; 10.5)	1.5 (0; 2.75)	0 (0; 0.25)	0.5 (0; 3.75)