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The Look of Lameness

Behaviors and facial expressions associated with orthopedic pain in horses

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Abstract

There are increasing concerns about equine welfare in equestrian sports, where early detection of orthopedic pain remains a major challenge since reliable and valid pain assessment tools are lacking. Movement asymmetry may be present in horses perceived as free from lameness by their owners, as well as in horses with confirmed orthopedic pain. It is therefore important to differentiate movement asymmetry due to pain from that due to other reasons, which may be achievable by improving orthopedic pain assessment. The aim of this thesis was thus to identify body behaviors and changes in facial activity related to orthopedic pain and movement asymmetry in horses.

Progression and regression of movement asymmetry after induced orthopedic pain was monitored and measured with gait analysis in eight horses. A number of behaviors including altered posture, head position, location in the box stall, focus and human interaction were found to be associated with orthopedic pain, as were facial expressions. Only one of four equine pain scales tested detected orthopedic pain reliably and accurately. Dynamic and diverse facial displays were identified in resting and moving horses during pain, illustrating that the concept of one prototypical pain face may be a simplification of the full pain-related facial repertoire. Horses trotted by hand showed a great inter-individual variation in facial expressiveness, highlighting the need for further analysis of facial activity during motion before its use for pain detection. The new knowledge on the relationship between pain and movement asymmetry provided in this thesis, can lead to improved pain assessments, pain management and equine welfare.

Keywords: facial action unit, EquiFACS, movement asymmetry, equine, pain scale, pain assessment tool, LPS induction, objective gait analysis, reliability, prediction

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Preface

“The challenge is not to all applications of behavioral theory to the field of pain behavior, but to its weakness in explaining such behavior particularly in clinical setting, and to its shortcomings in relation to the interactive nature of pain expression, as in the systematic biases evident in certain observer judgements of pain.” (de C. Williams, 2002)

Dedication

This thesis is dedicated to my favorite people and animals.

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Ask K., Rhodin M., Tamminen L-M., Hernlund E. and Andersen P.H. (2020). Identification of body behaviors and facial expressions associated with induced orthopedic pain in four equine pain scales. *Animals* 10 (2155), 1-16.

II. Ask K., Andersen P.H., Tamminen L-M., Rhodin M. and Hernlund E. (2022). Performance of four equine pain scales and their association to movement asymmetry in horses with induced orthopedic pain. *Frontiers in Veterinary Science* 9 (938022), 1-14.

III. Ask K., Rhodin M., Rashid-Engström, M., Hernlund E. and Andersen P.H. (2022). Changes in the equine facial repertoire during different orthopedic pain intensities (manuscript).

IV. Ask K., Andersen P.H., Hernlund E. and Rhodin, M. (2022). Facial activities in trotted horses during progression and regression of induced lameness (manuscript).

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The contribution of Katrina Ask to the papers included in this thesis was as follows:

I. Participated in conceptualization and shared responsibility for designing the study and project administration. Main responsibility for data acquisition and curation. Shared responsibility for formal analysis and interpretation of results. Main responsibility for writing and critically revising the article, with input from the co-authors.

II. Participated in conceptualization and shared responsibility for designing the study and project administration. Main responsibility for data acquisition and curation. Shared responsibility for formal analysis and interpretation of results. Main responsibility for writing and critically revising the article, with input from the co-authors.

III. Shared responsibility for conceptualization. Main responsibility for project administration, data acquisition, and data curation. Shared responsibility for formal analysis and interpretation of results. Main responsibility for writing and critically revising the article, with input from the co-authors.

IV. Shared responsibility for conceptualization. Main responsibility for project administration, data acquisition, data curation, formal analysis and interpretation of results. Main responsibility for writing and critically revising the article, with input from the co-authors.

1. Introduction

The horse, a powerful and beautiful animal, is loved for its empathy, kindness, and fairness. After domestication during the Bronze Age, horses became an important part of human civilizations (Atsenova *et al.*, 2022). A broad diversity of breeds emerged to serve in warfare, transportation, and agriculture. During the 20th century, interest in equestrian sports increased and nowadays horses are mainly considered as companion or sports animals. The horse-human relationship is strong and the horse might even be one of our dearest friends (Dubois *et al*., 2018). With this relationship comes great responsibility for humans to take care of the horse and identify signs of sickness. Equestrian athletes may not consider a strong relationship necessary to achieve success in equestrian sports, and being emotionally distant to the horse might even be beneficial for success (Hogg & Hodgins, 2021). However, lack of knowledge and/or emotional distance carries the risk of focusing less on the welfare of the horse, and more on the goal of winning. Several welfare risks arising from inappropriate use of equipment and exposure to injury have been identified in equestrian sports, and concern about the welfare of sport horses have been raised (Holmes & Brown, 2022). If welfare is not improved, the whole concept of 'social license to operate' in equestrianism might be threatened (Douglas *et al*., 2022). It is therefore essential to develop validated tools and frameworks for monitoring and improving equine welfare (Campbell, 2021; Holmes & Brown, 2022), such as tools assessing the emotional state of horses during competition events, training, and daily life (Fletcher *et al*., 2021; Furtado *et al*., 2021). Welfare protocols aiming to quantify and categorize the welfare state of an individual animal, or a population of animals, by assessing quality-of-life aspects have already been developed (Fraser *et al*., 1997; Hockenhull & Whay, 2014). However, it is important to assess not only the animal's health and expressions of natural behaviors, but also its emotions, since sentient animals, including horses, can experience negative and positive affective states. A very important negative affective state is associated with the experience of pain. According to several systematic welfare measures, such as ‘five freedoms’, ‘five provisions’, and ‘five domains’ (FAWC, 1993; Mellor & Beausoleil, 2015), an animal should be free from pain, or should be treated as soon as possible to reduce pain. This may sound simple, but there are numerous challenges in identifying and assessing pain in animals. In addition, since horses are prey animals, it is natural for them to hide pain (Taylor *et al.*, 2002). As is the case for animal welfare, protocols specifically for assessing pain in animals have been developed. They aim to quantify the affective experience of pain, mainly by assessing face and body behaviors known to be related to pain, and measuring physiological parameters. Since the affective component of pain has no real gold standard in animals, the validation process is difficult, as is developing sensitive tools that can quantify mild pain. The lack of validated pain assessment tools has been highlighted, and it has been suggested that welfare assessment protocols would benefit from including such tools (Hockenhull & Whay, 2014).

The main clinical symptom of orthopedic pain, which refers to pain arising from pathologies in the locomotor apparatus, is lameness, whereby the horse alters its gait to reduce pain. Depending on the pathology, lameness can be difficult to treat and sometimes even difficult to detect. As a result, lameness is one of the most common causes of euthanasia of horses (Penell *et al.*, 2005; van Proosdij & Frietman, 2022). Even if lameness does not result in euthanasia, it may end the competitive career of a sports horse and the welfare of retired and geriatric horses has been debated. It has been suggested that environment and management changes may lead to negative emotions, such as frustration or boredom, and if the horse is chronically ill the experience of chronic pain will impair welfare further (Holmes & Brown, 2022). It is difficult for horse owners to assess different affective states objectively (Fletcher *et al.*, 2021), and they tend to underestimate or misinterpret clinical signs of health problems in geriatric horses (Ireland *et al.*, 2012). Introduction of welfare assessment tools prioritizing the emotions experienced by the horse has therefore been recommended, in order to improve decision making on euthanasia (Long *et al.*, 2022).

For moderate to severe orthopedic pain, for instance after orthopedic surgery, certain pain-related behaviors have been identified, such as altered posture (*i.e.*, weight shifting, non-weight bearing), pawing on the floor, and increased head movement (Bussières *et al.*, 2008). Changes in facial activity have also been demonstrated (Dalla Costa *et al.*, 2016; van Loon & Van Dierendonck, 2019). Based on these findings, pain assessment tools called pain scales have been constructed to assess orthopedic pain at rest and may be used by equine veterinarians. Whether these pain scales can detect mild orthopedic pain remains to be determined, and associated specific body behaviors and changes in facial activity have not yet been identified. Instead, mild orthopedic pain is primarily assessed by visually evaluating the lameness grade during motion. Equine veterinarians perform a thorough examination of the locomotor apparatus, identifying structures reacting to provocation (palpation and flexion tests), and apply local anesthetics to the region or structure suspected to be pathological. If pain is originating from the treated site, an analgesic effect is seen and the horse improves its gait. This may sound straight-forward, but there are several factors to consider when interpreting the effect of local anesthetics, as summarized in a review by Schumacher & Boone (2021). Furthermore, other clinical tools for assessing pain are lacking.

Years of systematic and in-depth equine biomechanical research have yielded extensive knowledge on gait adaptions during lameness and how to measure lameness with equine gait analysis systems. Lameness is usually measured objectively in trot, a symmetrical two-beat gait where the horse may decrease the load on the lame limb to reduce pain. This results in changes in ground reaction forces, where a reduction in peak vertical force of the lame limb has been identified during lameness, together with other compensatory mechanisms (Weishaupt *et al.*, 2004, 2006). During trot, the head, withers, and pelvis move up and down in sinusoidal patterns that change during lameness (Buchner *et al.*, 1996). This results in an asymmetrical pattern of vertical displacements of the head, withers, and pelvis, also referred to as ‘movement asymmetry’. Since visual lameness assessment is prone to bias and may not be sufficiently sensitive, systems for objective gait analysis, measuring movement asymmetries in a reliable and valid way in clinical settings, are recommended (Serra Bragança *et al.*, 2018).

According to the latest research, movement asymmetries are present in 53-88% of horses believed to be free from pain by their owners (Pfau *et al.*, 2016b, 2016c; Rhodin *et al.*, 2016, 2017; Pfau *et al.*, 2018; Kallerud *et al.*, 2020; Müller-Quirin *et al*., 2020; Hardeman *et al*., 2022; Scheidegger *et al*., 2022). There are two possible explanations: i) these horses are actually experiencing mild orthopedic pain, but hide it or it is overlooked by the owners, or ii) there are other reasons for movement asymmetries to occur, such as motor laterality. In relation to equine welfare, it is of great importance to understand how movement asymmetries due to pain can be distinguished from those arising due to other reasons. In horses with lameness or poor performance, locally applied diagnostic anesthesia can alter movement asymmetries caused by pain (Marunova *et al*., 2022) and is an important diagnostic tool for locating the site of the pain. However, applying local anesthetics to find the reason for movement asymmetry in horses perceived as sound by their owners has some disadvantages. For example, the process is time-consuming, demands equine veterinary expertise, and may be unsafe for both the horse and the veterinarian. It is also ethically questionable whether horses believed to be healthy should be subjected to several injections in order to locate a potential pain site. Finding a less invasive way to detect pain in horses with movement asymmetry should therefore be prioritized, while local diagnostic anesthesia remains a very important component in investigation of lameness.

Identifying which movement asymmetries are due to pain is laborious and difficult. Important gaps in the current understanding are that: i) the relationship between the level of pain and movement asymmetry has not been described, and ii) objective measures of movement asymmetry and pain have not been used in combination to investigate this (Serra Bragança *et al*., 2018; Egan *et al*., 2019). The reason for these research gaps may lie in the difficulty in detecting and assessing mild pain in horses. A lack of self-reporting of pain in animals, differences in individual responses to standardized painful stimuli, and fluctuations in pain intensity hamper animal pain research. This thesis takes its starting point in these challenges, with the overall aim of identifying body behaviors and changes in facial activity related to mild orthopedic pain and movement asymmetry in horses. The remaining sections of this chapter provide an overview of pain physiology and biomechanical adaptations during lameness, summarizing these extensive research areas in a way relevant for the work in this thesis. Current research on pain-related behaviors and facial activity is reviewed, and existing equine pain scales are described. Specific research objectives and hypotheses tested in the work are presented in Chapters 2 and 3. Chapter

4 provides an overview and comments on the materials and methods used in Papers I-IV. Chapter 5 summarizes the main results obtained in Papers I-IV and Chapter 6 presents a general discussion of these results. Chapter 7 presents some conclusions, reflections, and future perspectives.

1.1 Current understandings of pain and its physiology

Continuous learning and extensive pain research have resulted in frequent updates of the definition of pain, the latest acknowledging that humans and animals can feel pain despite not being able to verbalize it. The International Association for the Study of Pain (IASP) now identifies pain as "*an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage*" and has further expanded the definition by adding clarifying key notes. Importantly, pain is acknowledged as a personal experience where self-reporting of pain should be respected, and not reduced to the neurophysiological process of nociception. In addition, verbal description is acknowledged as only one way of expressing pain – "*inability to communicate does not negate the possibility that a human or a non-human animal experiences pain*" (Raja *et al.*, 2020). The updated definition highlights two key aspects of pain: the inter-individual variability in pain and the presence of other signs of pain apart from verbalization. This section focuses on inter-individual variation, by briefly describing research on pain physiology in humans. Sections 1.3 and 1.4 describe signs of pain in horses, such as body behaviors and facial expressions, and section 1.5 describes existing equine pain assessment tools.

Pain is a sensory and emotional experience because of the advanced perception of nerve signals in several parts of the brain, which explains why it is fundamental not to confuse nociception with pain, as clarified by IASP. Nociception involves the encoding of a noxious stimulus by nociceptors. Depending on the type of nociceptor, mechanical, thermic, and/or chemical stimuli activate the nociceptor through binding to receptors such as TRPV1 (capsaicin receptor) and TRPA1 (chemical and mechanical stimuli) (Basbaum *et al.*, 2009). Voltage-gated sodium channels then amplify the membrane potential to an action potential, and the electric signal is transported to the dorsal horn of the spinal cord by thicker Aδ fibers responsible for sharp, well-localized pain, and thinner, unmyelinated C fibers responsible for diffuse and dull pain. Nociceptor activation occurs at certain thresholds, which are then lowered when the nociceptor becomes sensitized. After activation, nociceptors can release inflammatory mediators such as serotonin, histamine, substance P, prostaglandins, and nerve growth factor, to further activate new nociceptors by binding to the receptors. Inflammatory mediators are also produced by the damaged tissue. During synapsing with second-order neurons in the spinal cord, voltage-gated sodium and calcium channels open, resulting in increased intracellular sodium and calcium. Synaptic vesicles start fusing with the presynaptic membrane and release neurotransmitters such as prostaglandins, glutamate, substance P and CGRP. Glutamate activates NMDA receptors on the postsynaptic neurons. After synapsing, the signal is projected via the spinothalamic and spinoreticular tracts to the thalamus and parabrachial nucleus in the brainstem. Here, synapsing with third-order neurons occurs and the signal is transported to several areas in the cortex for perception (Basbaum *et al.*, 2009; Meintjes, 2012).

Primary and secondary somatosensory cortices interpret the sensory dimension of pain, while the emotional and affective dimension of pain is interpreted by the anterior cingulate and rostral insular cortices (Xie *et al.*, 2009). The cognitive component of pain is mainly due to activation of the prefrontal cortex, including the ventrolateral orbital cortex (Apkarian *et al.*, 2005; Xie *et al.*, 2009; Khera & Rangasamy, 2021). Hence, the individual understands where the pain is located in the body and experiences a specific level of pain intensity. Importantly, motivation to take action against the pain occurs when the individual understands how ‘bad’ the pain is (Auvray *et al.*, 2010; Talbot *et al.*, 2019). This results in important learning processes, and pain aids in identification of behaviors an individual can perform to avoid injuries and increase survival (Seymour, 2019). The learning process, together with memory, attention, and decision making, are examples of cognitive parameters. Cognition and pain are closely linked to each other, where pain can affect the level of cognition and different cognitive parameters may affect the perception of pain, *e.g.*, the pain experience may be modulated by the emotional state (Villemure & Bushnell, 2002). Understanding the difference between pain and emotions has been the subject of research, where the sensory interpretation of nociception seems to separate pain from emotions. The individual then understands that the emotion felt is related to a body part (Gilam *et al.*, 2020). As for cognition, the presence of emotions may affect the experience of pain. For instance, watching unpleasant pictures during induction of spinal nociception has been found to result in increased subjective pain ratings and activation of several brain regions, such as the right anterior insula, thalamus, amygdala, and paracentral lobule (Roy *et al.*, 2009). Moreover, pain intensity ratings and activity in the anterior cingulate cortex were higher when patients experienced sadness compared to when they experienced happiness (Yoshino *et al.*, 2010). Stress is also known to affect the experience of pain and a short summary is provided in section 1.1.1.

The body has a well-developed endogenous modulation with descending pain pathways to modulate the pain experience (Millan, 2002; Ossipov *et al.*, 2010). The important gate-control theory illustrates how interneurons in the dorsal horn of the spinal cord synapse with Aβ fibers, bringing tactile information, instead of synapsing with primary afferent Aδ and C fibers, bringing nociceptive information. In this way, the nociceptive information does not reach the brain and pain is reduced. In a similar way, the descending pathways may inhibit an ongoing pain experience. This begins with the periaqueductal grey (PAG), located in the midbrain, receiving information from the thalamus, cortex, hypothalamus, and amygdala, and via descending enkephalin-releasing neurons transporting signals to the locus coeruleus. The signals are further transported through noradrenergic neurons to the nucleus raphe magnus (NRM) of the rostral ventral medulla (RVM), and via serotonin-releasing neurons down to the dorsal horn (Millan, 2002; Ossipov *et al.*, 2010). Neurons arising from NRM can also be GABAergic, as can the interneurons distributed in the dorsal horn of the spinal cord. When synapsing with primary afferent neurons, GABA binds to the GABA-B receptors, resulting in inhibited voltage-gated calcium channels and thereby inhibited release of neurotransmitters (Bowery, 2006; Ossipov *et al.*, 2010).

To summarize, the descendent pathways are important in pain modulation, but also in pain therapy (see section 1.5.1). The endogenous and exogenous opioid system plays a central role in both modulation and therapy. Four types of opioid receptors (mu, delta, kappa, and nociceptin), when activated, can reduce the release of neurotransmitters and inhibit voltage-gated calcium channels (Corder *et al.*, 2018). The receptors are expressed in the terminals of first-order neurons and in the majority of brain areas involved in pain perception (somatosensory cortex, anterior cingulate cortex, prefrontal cortex, insula, amygdala, and thalamus) and in pain modulation (PAG and NRM). The enkephalin-releasing neurons can activate especially the mu opioid receptors (endogenous opioid system), as can for instance morphine (exogenous opioid system) (Corder *et al*., 2018).

The physiological processes described so far mainly occur in acute pain and in nociceptive pain. However, a clinically challenging type of pain is chronic pain with its seven clinical categories of: chronic primary pain, chronic cancer-related pain, chronic postsurgical pain or posttraumatic pain, chronic neuropathic pain, chronic secondary headache or orofacial pain, chronic secondary visceral pain, and chronic secondary musculoskeletal pain (Treede *et al.*, 2019). Due to the complexity of chronic pain, the previous definition of "*pain that persists past normal healing time"* has been updated to *"pain that lasts or recurs for longer than 3 months*" (Treede *et al*., 2019). For chronic pain to arise, functional and structural plasticity may be present, illustrating complex alterations in the normal physiological pain process. Here, the concept of central sensitization, *i.e*., "*increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input*" defined by IASP (Loeser *et al*., 2020), plays a key role. It is similar to the phenomenon 'wind-up', where a repeated noxious stimulus of the same intensity results in hyperalgesia (Mendell, 2022). NMDA receptors on second-order neurons in the spinal cord are activated by glutamate and substance P, which also activate neurokinin receptors. This results in prolonged depolarization of the neurons and increased action potential, which are summarized during <1 minute to produce hyperalgesia during the wind-up process. During central sensitization, the temporal summation of depolarized neurons is not necessary, and instead activation of some second-order neurons results in activation of other second-order neurons, despite them not receiving a noxious signal (Mendell, 2022). High intracellular levels of calcium seem to maintain the activation of NMDA receptors and activate protein kinase C and calcium-calmodulin-dependent protein kinase II, so the threshold for excitation is decreased. This is a complicated process, only briefly described in this thesis (for details, see the review by Latremoliere & Woolf, 2009). Functional plasticity is present, where the overall aim is to increase membrane excitability and synapsing and decrease the effect of the descending inhibitory pathway in the spinal cord (Kuner, 2010). Structural plasticity also occurs, where neurons are degenerated or hypertrophic, and axons are degenerated or regenerated (Kuner, 2010). Extensive research has shown that the descending inhibitory pain pathway malfunctions during pain (Ossipov *et al*., 2010) and that a reorganization of the brain network occurs during chronic pain, where highly activated brain areas during pain, such as the insula, are down-regulated and where less activated areas are upregulated (Barroso *et al*., 2021).

Another challenging type of pain is neuropathic pain, *i.e.*, "*pain caused by a lesion or disease in the somatosensory nervous system*" according to the definition by IASP (Loeser *et al*., 2020). The lesion may be central or peripherally localized. When a nerve is damaged, expression of sodium channels is increased, perhaps due to released nerve growth factor, and action potentials begin firing. Nociceptors on surrounding intact nerves, innervating the same area as the damaged nerve, may also become sensitized. Alterations in pain modulation may occur and it seems that central sensitization also plays a role in neuropathic pain (Campbell & Meyer, 2006).

For all types of pain, the pain experience can vary greatly between individuals. Biological and psychosocial factors are important and the pain experience may depend on sex, race/ethnicity, age, and genetics, as summarized in the extensive review by Fillingim (2017). Recognizing these variations may lead to improved pain assessments in human healthcare. This raises the questions of how personalized pain treatments can be incorporated in animal pain assessments and how inter-individual variations in pain experience can be differentiated from those due to biological factors. These are complex questions that cannot be answered in this thesis. However, this brief summary on human pain research shows that there are multifaceted pain experiences, and it may be important to consider this in animal pain research.

1.1.1 The stress of pain

An important affective state is stress, known to be closely linked to pain, so it is also considered in this thesis. Stress has been defined in several ways, but the definition used here is that of König *et al*. (2017): "*the organism's non-specific response to challenges, such as situations that require or potentially require the individual to fight or flee, to cope with environmental conditions such as extreme temperatures, or to cope with psychological challenges*". The sympathetic-adrenal medulla (SAM) axis is first to be activated, initiating fight or flight and preparing the body for acute exercise by releasing adrenaline that increases cardiovascular and respiratory responses (Smith & Vale, 2006). Second, the hypothalamic-pituitary-adrenal cortex (HPA) axis is activated, where the paraventricular nucleus in the hypothalamus releases corticotropin-releasing factor (CRF) to bind to corticotropic receptors in the anterior pituitary. This stimulates synthesis of adrenocorticotropin hormone (ACTH) and production of cortisol by the adrenal cortex (Smith & Vale, 2006; Wagner, 2010). In addition, a cognitive component is present, making stress challenging to assess since changes in physiological parameters may not fully represent the stress experience. According to a review by Koolhaas *et al.* (2011), stress involves uncontrollable and unpredictable situations where environmental demands exceed natural regulatory capacity. In uncontrollable situations, regaining a normal physiological response is delayed, while in unpredictable situations there is a lack of anticipatory response (Koolhaas *et al*., 2011). In the cognitive component, the individual perceives the stress, which may result in proactive or reactive coping behavior.

Pain is considered to be a major stressor, resulting in an initial arousing and secondary recovering stress response as described in the review by Chapman *et al.*, (2008). When nociception occurs, the locus coeruleus activates the paraventricular nucleus after receiving nociceptive input, and CRF is released. The paraventricular nucleus, and hence the HPA axis, can also be activated by cytokines and other inflammatory mediators. Circulating cortisol downregulates the HPA axis, aiming to decrease further cortisol production. In chronic pain, the transition between the arousing stress response and the recovery is believed to fail, resulting in a dysfunctional recovery with different types of dysregulation (Chapman *et al*., 2008). Correspondingly, stress may affect the pain experience and induce hyperalgesia, *i.e*., "*increased pain from a stimulus that normally provokes pain*" (Loeser *et al*., 2020). Increased activity in the anterior cingulate cortex, changes in the descending inhibitory pain pathway on different levels, and altered synapsing in the spinal cord, for instance through decreased GABA release, may result in hyperalgesia (Jennings *et al*., 2014). Interestingly, stress may also activate the descending inhibitory pathway in several ways to achieve analgesia, for instance fear-conditioned analgesia (Butler & Finn, 2009). Levels of cortisol after acute stress manipulations have been correlated to increased heat pain thresholds, suggesting that the HPA axis is mainly responsible for stress-induced analgesia (Timmers *et al*., 2018).

To sum up, it is important to consider the interaction between stress and pain when evaluating pain, since pain can induce stress behaviors and stress may alter the level of pain experience. However, there has been very little research to date on stress and pain in horses.

1.2 Biomechanical adaptations during orthopedic pain

Gait adaptions during lameness can be assessed visually by an equine veterinarian or measured objectively by systems for gait analysis. There are several gait parameters that may change during lameness, and different gait analysis systems to choose between. Systems for kinetic gait analysis measure forces during motion, while systems for kinematic gait analysis measure how the position of body segments changes over time. One example of kinematic gait analysis is optical motion capture systems consisting of high-speed infrared cameras that record the absolute position in 3D of spherical reflex markers, attached to different body segments such as the forehead, withers, and tuber sacrale and tubera coxae of the pelvis (Serra Bragança *et al.*, 2018). The frequency of the cameras in Hz determines how often the positions are recorded per second. On plotting the measured points from head, withers, and tuber sacrale of a horse trotting, the characteristic sinusoidal pattern occurs. The position data are analyzed and vertical displacement asymmetry for each body segment is computed. For each stride, the lowest position of a marker during the right stance phase is compared with the lowest position of the same marker during the left stance phase, and the difference is MinDiff. Similarly, MaxDiff is the difference between the highest positions of a marker during the right and left stance phase, for each stride (Kramer *et al.*, 2004; Kelmer *et al.*, 2005). MinDiff and MaxDiff for the head and pelvis may also be referred to as HDmin, HDmax, PDmin, and PDmax (Keegan *et al.*, 2011) (Figure 1). For instance, a hindlimb impact lameness is characterized by increased MinDiff and a hindlimb push-off lameness is characterized by increased MaxDiff *(*Bell *et al.*, 2016). On evaluating these movement asymmetries, the equine veterinarian obtains detailed information about how the body moves. During the stance phase of the lame limb, the trunk and head are lowered less, to reduce the impact of the lame limb, and lifting of the trunk is made with less peak acceleration to reduce the push-off effect (Buchner *et al.*, 1996). As a result, right front limb lameness is characterized by positive MinDiff and/or MaxDiff of the head, where the head is less lowered and raised during the right stance phase compared with during the left stance phase. During a right hindlimb lameness, the pelvis is less lowered and raised, resulting in positive MinDiff and/or MaxDiff (Kramer *et al.*, 2004). However, gait adaptations during lameness are often more complicated and several studies have identified compensatory movement asymmetries. A primary forelimb lameness may be accompanied by compensatory mainly contralateral hindlimb asymmetry, and in primary hindlimb lameness an ipsilateral compensatory forelimb asymmetry often occurs. Thus, it can be difficult to determine the primary lameness, especially since the compensatory asymmetry tends to be of the same magnitude as the primary lameness (Kelmer *et al*., 2005; Rhodin *et al*., 2013). Recent research has shown that the movement asymmetry of the withers can add valuable information for the decision-making process, *e.g*., during forelimb lameness the movement asymmetry of the head and withers are synchronized, but during hindlimb lameness ipsilateral compensatory head asymmetry is seen together with contralateral withers asymmetry (Rhodin *et al*., 2018). A natural next step for future research would be to investigate whether certain movement asymmetries, such as PDmax, are more prevalent during specific causes of lameness (Pfau *et al*., 2016a). Identifying such relationships would improve understanding of the specific movement asymmetries caused by pain.

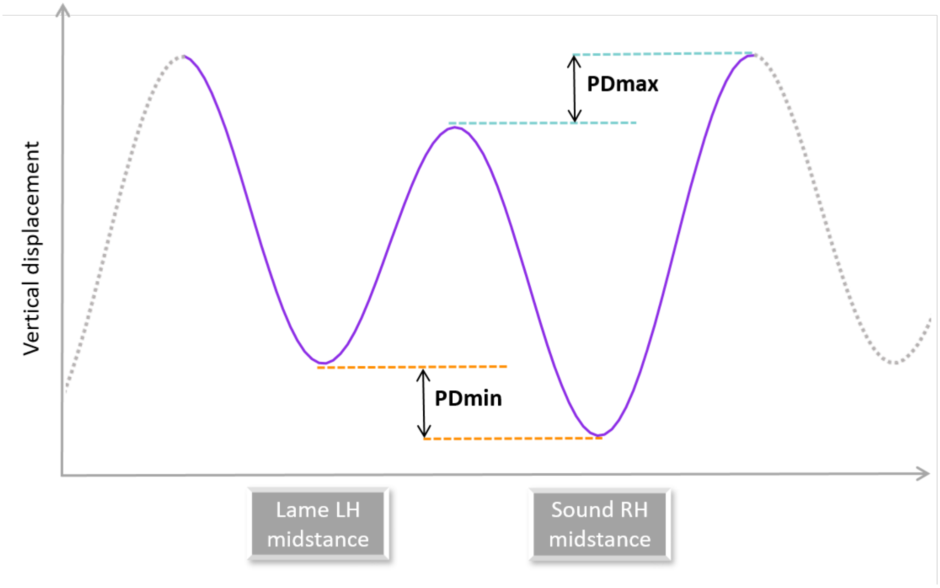

Figure 1. Vertical displacement of the pelvis during left hindlimb lameness in trot. The sinusoidal pattern for tuber sacrale during one stride is shown, where PDmin is the difference between the lowest position of tuber sacrale for the left and right hindlimb, and PDmax is the difference between the highest positions.

Research to date has identified movement asymmetries arising during experimentally induced pain and clinical pain in the locomotor apparatus. Systems for objective gait analysis have been used during straight-line trot (over ground or on a treadmill) and on the circle (lunging), to understand how movement asymmetries vary during lameness (Serra Bragança *et al.*, 2018). In addition, studies in horses moving symmetrical on the straight line have identified circle-induced asymmetry (Pfau *et al.*, 2016b; Rhodin *et al.*, 2016). Interestingly, those two studies also identified movement asymmetry in 61% and 53%, respectively, of horses perceived as sound by their owners. This was further evaluated in 222 riding horses considered sound by their owners, of which 72.5% were found to have movement asymmetries (Rhodin *et al.*, 2017). When comparing the mean HDmin, HDmax, PDmin, and PDmax to those previously reported in horses with clinical lameness (Maliye *et al.*, 2015; Maliye & Marshall, 2016), they were found to be of similar magnitude. This has also been observed in other studies of riding horses, racing thoroughbreds, and trotters in training (Pfau *et al.*, 2016c, 2018; Kallerud *et al.*, 2020; Müller-Quirin *et al.*, 2020; Hardeman *et al.*, 2022; Scheidegger *et al.*, 2022). It also appears that the degree of movement asymmetry may vary over time, especially HDmin and HDmax, in riding horses in training (Hardeman *et al.*, 2019).

Vertical displacement asymmetries may provide high sensitivity for ‘shifting of load’, but it is unknown how gait adaptations and pain are related. In human biomechanical research, there are varying results on how pain and gait adaptations are associated. Kinematic studies on walking patients with different types of pain have identified significant associations between gait asymmetry and fear and avoidance, with pain associated with fear and avoidance, but not gait asymmetry (Beebe *et al.*, 2021). Similar results have been reported in patients with lower back pain who participated in kinematic gait analyses over nine months, *i.e.*, no biomechanical parameters were associated with pain and range of motion was the only parameter associated with self-reported disability, while pain was associated with fear (Nordstoga *et al.*, 2019). In patients with patellofemoral pain, decreased knee flexion, less muscular activity, and increased difficulty in voluntarily contracting the quadriceps muscle have been found to be significantly associated with pain scores given with a numerical rating scale (NRS), but not other biomechanical parameters (Greuel *et al.*, 2019). However, in patients with knee osteoarthritis, several associations between pain scores and kinematic parameters have been identified (Bensalma *et al*., 2019). These studies show inconsistent results and indicate the difficulties in evaluating the relationship between pain and movement asymmetry. Consequently, a definitive answer to ‘Does it hurt when you move and how much?’ is lacking. In fact, a recent review on movement-evoked pain in humans highlighted the need to determine how such pain is related to other measures of pain, and the variation in pain over days, and suggested that pain should be evaluated specifically during motion, instead of using questionnaires where the patient has to recall a previous movement-evoked pain experience (Corbett *et al*., 2019).

In horses, the relationship between movement asymmetry and pain experienced during rest and motion in individuals with orthopedic pain is not yet understood, but two recent studies have touched upon the importance of understanding the relationship between movement asymmetry and pain. In one of these, a study by Scheidegger *et al*. (2022), it was theorized that if movement asymmetry increases after competition then the asymmetries are pain-related, since in competition horses are put through high-intensity exercise. However, on comparing measures from the veterinary examination and from the day after a cross-country event no increase in movement asymmetry was seen, and Scheidegger *et al*. (2022) concluded that it was not possible to state whether pain was present or not. The other study, by Persson-Sjodin *et al*. (2019), targeted the issue more directly and aimed to identify pain-related movement asymmetries in asymmetrical horses considered sound by their owners. Interestingly, treatment with meloxicam during four days did not reduce movement asymmetry in that study, raising the question of whether movement asymmetries are due to biological variation or to pain (Persson-Sjodin *et al*., 2019). Despite not knowing the true relationship between pain and movement asymmetries, it is clear that movement asymmetry increases when orthopedic pain, *i.e*., lameness, is present. Lameness is what the horse actually displays and is an outcome of orthopedic pain, and therefore this physical activity can be used as a proxy for orthopedic pain (De Vet *et al*., 2011). It is important to define what to measure when using a proxy. For instance, movement asymmetry cannot act as a proxy for pain in horses perceived as sound by their owners, since this relationship is unknown. However, in horses with induced lameness, movement asymmetries increase due to the lameness induction, so movement asymmetry can act as a proxy.

A final note is that pain research in humans can inspire and guide equine research, with the research on pain-related facial expressions as a great example (see section 1.4). However, research on human orthopedic pain seems to struggle to identify the relationship between pain and gait adaptations in the same way as equine research, despite their patients being able to verbalize their pain, so self-reporting is perhaps not required to understand the relationship. The complex nature of equine movement asymmetries indicates a need for combining different measures of pain in order to identify movement asymmetries caused specifically by pain. Assessment of body behaviors and facial expressions during rest, and assessments of facial expressions during motion, are examples of potential measures of orthopedic pain.

1.3 Body behaviors related to pain

When an animal is in pain, it performs some body behaviors with the purpose of relieving pain. As described in the review by Prunier *et al.*, (2013), behaviors resulting in altered posture and gait adaptations during lameness may lessen stimulation of the painful area, while behaviors of avoidance and defense, such as moving away and kicking, may reduce the noxious stimulus. Behaviors such as rubbing and/or licking the painful area may reduce pain, since Aβ fibers are activated through tactile stimulation (gate control theory). In addition, changes may occur in general behaviors such as eating, interaction, and attention, which have no purpose in reducing pain but are a consequence of the pain experience (Prunier *et al.*, 2013). Other factors that may influence performed behaviors are the environment and inter-individual variations (Price *et al.*, 2003). These variations may depend on how the animal is coping with the pain experience, *i.e.*, how it alters behavior and physiological components to manage a situation. Depending on their coping style, individuals may differ in vulnerability to a stressor such as pain (Koolhaas *et al.*, 1999). Proactive coping involves being prepared for certain situations, for instance when moving is painful, while reactive coping involves responses to a pain experience.

Many behaviors associated with discomfort and pain in horses have been described, and recently compiled in an equine discomfort ethogram (Torcivia & McDonnell, 2021). Sixty-four behaviors related to posture and weight-bearing, and movements of limbs, body, head, neck, mouth, lips, ears, and tail are included in the ethogram, as are attention to area, overall demeanor, altered eating/drinking, and vocalization/audible sounds. According to Torcivia & McDonnell (2021), these behaviors do not indicate discomfort on their own, but do so when co-occurring. In fact, most equine behaviors related to pain or discomfort are included in the normal behavioral repertoire, such as kicking, pawing, rolling, lowering the head, weight-shifting, and lying down. Whether these are associated with pain or discomfort is indicated by the frequency and duration of the behaviors, and co-occurrence with other behaviors (Taylor *et al.*, 2002). An important aspect is whether the behaviors are related to pain specifically, or to discomfort. Pain and discomfort are sometimes used interchangeably in animals, since pain results in discomfort. However, discomfort may be considered a broader term, including other physical and physiological states in addition to pain, such as fatigue, hunger, fear, stress, and anxiety (Ashkenazy & DeKeyser Ganz, 2019). This thesis focuses only on behaviors related to the experience of pain, but includes discomfort when literature sources cited use the term.

Some behaviors are indicative of certain types of pain, while others are seen during general pain. In horses with orthopedic pain, several changes in frequency of behaviors have been identified. Most research has focused on moderate to severe orthopedic pain, identifying less exploratory behavior, restlessness, and reduced locomotion in horses after arthroscopy (Price *et al.*, 2003), increased weight-shifting in laminitic horses (Rietmann *et al.*, 2004), and changed posture and increased pawing in horses with amphotericin-B induced synovitis (Bussières *et al.*, 2008). In addition, 33 discomfort behaviors have been described in orthopedic surgical equine patients, including changes in posture, limb and head movements and restlessness (Torcivia & McDonnell, 2020). In horses with chronic laminitis, stifle arthroscopy, and lipopolysaccharide (LPS) induced arthritis, increased lameness scores have been identified (Owens *et al.*, 1995; Goodrich *et al.*, 2002; Lindegaard *et al.*, 2010). Pain scales have been used to assess orthopedic pain and have revealed changes in pain scores (van Loon & Van Dierendonck, 2019), but have not be used to evaluate behaviors specifically present during orthopedic pain. Thus research to date has contributed to identifying important behaviors related to orthopedic pain, but the overall association between behaviors, lameness scores, and pain scores has yet to be established. This issue has already been highlighted in a previous study suggesting that association of these behaviors to the degree of movement asymmetry should be explored (Bussières *et al.*, 2008). Further research is needed to assess mild to moderate orthopedic pain and relate the pain experience during rest to that during motion.

In addition to distinct behavioral changes that can be objectively described, horses may show behaviors interpreted by the owners as diffuse, *e.g.*, spookiness, hyper-reactivity, or poor performance. Such diffuse behaviors are difficult to identify and assess, and may only be present during certain situations, so video surveillance over several hours may be required to identify behavioral changes (McDonnell, 2005). An example of a specific situation where discomfort behaviors may occur is when the horse is ridden. Behaviors indicating the occurrence of potentially negative affective states such as pain, fear, and anxiety have been reviewed in previous research. One such study emphasized the need for accurate interpretation of behavioral signs of comfort or discomfort and concluded that when these behaviors have been identified objectively, a validated ethogram can be applied to the ridden horse, thereby greatly improving equine athlete welfare (Hall *et al.*, 2013). An ethogram for ridden horse behavior covering 24 facial, body, and gait features was developed recently (Dyson *et al.*, 2018a). After applying the ethogram on blinded video recordings of sound and lame horses, those authors concluded that there was a strong correlation between most markers and lameness. Identified behaviors in lame horses were: ears back, repeated opening of the mouth and/or showing the tongue, and gait-related behaviors such as hindlimbs not following the tracks of forelimbs, incorrect canter, and unwillingness and resistances (Dyson *et al.*, 2018a). In a subsequent study, significant reductions in behaviors such as head tilted to one side, head tossing, mouth opening, and hindlimb not following the tracks of forelimbs were seen after local diagnostic anesthetics had removed the lameness (Dyson *et al.*, 2018b). These interesting findings are important for identifying pain-related behaviors in ridden horses and indicate that identification of orthopedic pain during motion based on behavioral changes may be possible. However, the ethogram needs further reliability and validity testing before it can be introduced as a welfare assessment tool (Ladewig *et al.*, 2022).

It seems that the animal can make active decisions on whether to perform pain-relieving behaviors or not. It is important to bear in mind that horses are considered genuine in their behavior and only behave in a certain way when discomfort is present, so 'stupidity' is seldom the cause of unwanted behaviors (McDonnell, 2005). However, they may appear stoic due to being prey animals, and can hide their pain in the presence of predators. A recent study illustrated that horses may even consider their caretakers to be predators, since they perform fewer discomfort behaviors in the presence of a caretaker (Torcivia & McDonnell, 2020). This raises the questions: i) When is a human considered a predator by the horse? ii) Are all behaviors hidden? and iii) Is lameness a behavior that can be hidden? Note that this thesis does not answer these questions, but they were borne in mind when interpreting the results and drawing conclusions.

1.4 Facial expressions related to pain

In addition to body behaviors, facial expressions have been linked to pain in horses, as in other animal species. While the main reasons for performing body behaviors seem logical and approachable, the reasons for performing facial expressions are the opposite. Facial expressions are neither pain-relieving nor protecting, and may reveal to the predator that the animal is in pain. Today, it is generally accepted that horses and other animals use facial expressions for communication of different affective states, and that the communications serve different purposes (Waller & Micheletta, 2013). Interesting associations have been reported between the level of facial mobility and the size of the herd, with *e.g.*, the larger the social group in primates, the higher the facial mobility (Dobson, 2009). This has not been investigated in horses but the equine facial nucleus seems to be well-developed, indicating rather high facial mobility, with several subnuclei responsible for ear and nasolabial movements, and mastication (Furutani & Sugita, 2008). In addition, muscles around the ears, nose, and lips have large muscular mass (Wathan *et al.*, 2015). In humans, the facial nucleus receives input from different cortical regions, such as the primary motor cortex, ventral and dorsal lateral premotor cortex, caudal cingulate motor cortex, supplementary motor cortex, and rostral midcingulate motor cortex (reviewed by Müri, 2016). Facial expressiveness during pain has been associated with activity in the primary motor cortex, supplementary motor area, and putamen, *i.e.*, areas in the brain responsible for movement control (Kunz *et al.*, 2011). It has also been shown in humans that stoicism, *i.e.*, less facial expressiveness, results in higher activity in the prefrontal cortex and nucleus caudatus during pain (Kunz *et al.*, 2011). These areas are responsible for learning and inhibitory control, so stoicism may be a learned behavior where facial expressions are inhibited to different extents. It has been debated why facial expressions of pain can be voluntarily controlled; from an evolutionary perspective, suppression of facial expressions can hide vulnerability, while amplification can improve the communication of pain to obtain help (de C. Williams, 2002). For instance, humans show fewer facial expressions of pain to strangers, and more to a partner (Karmann *et al*., 2014). When observed, individuals may report less pain, in addition to less facial expressiveness (Kleck *et al*., 1976). Nonetheless, facial expressions of pain seem to reflect the pain experience, since they increase when the pain stimulus increases and can be associated with the self-reported pain using the visual analogue scale (VAS) (Kunz *et al*., 2004). Moreover, facial expressiveness during pain can be reproduced (Prkachin & Solomon, 2009). How horses suppress and amplify their facial expressions during pain and whether facial expressions are affected by observer presence in the same way as body behaviors are not yet understood. However, horses and other animals are generally considered more honest in their facial expressions, and multiple benefits of including facial expressions in assessment of pain and welfare in animals have been reported (review by Descovich *et al*., 2017).

In the past decade, the volume of research on pain-related facial expressions in horses has increased substantially. The main focus of the work has been on constructing and validating pain assessment tools based on facial expressions, and on describing combinations of facial expressions occurring with certain types of pain during rest and motion. The Horse Grimace Scale (HGS) consists of six facial action units (FAUs), originally described in the Mouse Grimace Scale (Langford *et al*., 2010) and adapted to equine facial configuration. FAUs can be ‘not present’, ‘moderately present’, or ‘obviously present’, which are assigned 0, 1, and 2, respectively, on the score sheet (Dalla Costa *et al*., 2014). The Equine Utrecht University Scale for Facial Assessment of Pain (EQUUS-FAP) describes similar facial expressions, with nine categories that can be scored from 0 to 2 (van Loon & van Dierendonck, 2015). The Ridden Horse Pain Ethogram contains categories describing changes in the eye and mouth area and ear movements in ridden horses. When present, changes are assigned a score of 1 (Dyson *et al*., 2018a). In addition, the ‘equine pain face’ has been described, to illustrate facial expressions occurring together during pain (Gleerup *et al*., 2015). It is included in the Equine Pain Scale (EPS) (Gleerup & Lindegaard, 2016). The anatomical areas selected for scoring of facial features are very similar across pain assessment tools and are summarized in Table 1.

Using these tools, changes in facial expressions have been assessed during different types of pain in horses. Using HGS and EQUUS-FAP, facial expressions occurring during moderate to severe pain have been assessed in newly castrated horses (Dalla Costa *et al.*, 2014, 2021), in horses with colic (van Loon & van Dierendonck, 2015; van Dierendonck & van Loon, 2016), dental disorders (Coneglian *et al.*, 2020), and laminitis (Dalla Costa *et al.*, 2016), in horses after head-related (van Loon & van Dierendonck, 2017) and orthopedic surgery (van Loon & Van Dierendonck, 2019), and in foals with acute health problems (van Loon *et al.*, 2020). The equine pain face has been described for mild to moderate experimental pain (Gleerup *et al.*, 2015) and a modified EPS has been applied to horses with colic (Lawson *et al.*, 2019). Attempts have been made to evaluate the presence of pain-related facial expressions in ridden horses, where backwards ears, exposure of sclera, and intense stare seem to indicate lameness (Dyson *et al.*, 2018a), and mouth opening and shutting repeatedly seem to decrease significantly when lameness is reduced with diagnostic analgesia (Dyson *et al.*, 2018b). These findings demonstrate that horses, like humans and other animals, show recognizable and specific patterns of facial expressions during pain, resulting in increased pain scores with existing assessment tools. However, research to date has not revealed facial expressions that co-occur with other behaviors, how facial expressions vary with pain type and intensity, or whether these expressions can be suppressed by the horse during observer presence. Since only predefined facial expressions have been assessed, it is not known whether other changes in facial activity may occur during pain.

Table 1. *Overview of facial expressions of the horse included in different pain assessment tools (Horse Grimace Scale (HGS), Equine Utrecht University Scale for Facial Assessment of Pain (EQUUS-FAP), Ridden Horse Pain Ethogram (RHpE), the 'equine pain face' included in Equine Pain Scale (EPS)*

Facial area	**HGS**	**EQUUS-FAP**	**RHpE**	**Equine Pain Face (EPS)**
Ears	Stiffly backwards ears	Ears (orientation towards sound, delayed response, or backwards)	Ears rotated back or flat (both or only one) >5s, repeatedly lay flat	Asymmetrical/ low ears
Eyes	Orbital tightening Tension above the eye area	Eyelids (more opened eyes with visible sclera, or tightening of the eyelids)	Eyelids closed/ half closed 2-5s Sclera exposed Intense stare 5s	Withdrawn and tense stare Angled eye
Muscles of the face	Prominent strained chewing muscles	Muscle tone head (presence of fasciculation)	-	Tension of the mimic muscles
Muzzle/ mouth	Mouth strained and pronounced chin	Corners mouth/lips (relaxed or lifted) Flehmen and/or yawning Teeth grinding and/or moaning	Mouth opening and shutting repeatedly, for more than >10s Tongue exposed and/or moving in and out	Tension of the muzzle (increased tonus of the lips and tension of the chin)
Nostrils	Strained nostrils and flattening of the profile	Nostrils (relaxed or opened)	-	Square-like (dilated medio-laterally)

1.4.1 Decoding equine facial expressions during pain

With the purpose of describing emotions, a Facial Action Coding System (FACS) has been developed for humans to objectively assess all visible facial activities (Ekman & Friesen, 1971). That FACS contains 44 action units (AUs) and action descriptors (ADs) describing visible changes in the face, based on when facial muscles contract. Detailed instructions on how to recognize and to make the movement oneself are also provided (Ekman & Friesen, 1971). FACS have since become a highly suitable tool for describing pain-related facial expressions in humans. Important research over several decades has identified core AUs constituting the human pain face. As summarized in a review by Kunz *et al.*, (2019), these are: *lowering the brows*

(AU4), *cheek raise/lid tightening* (AUs 6_7), *nose wrinkling/raising the upper lip* (AUs 9_10), *opening of the mouth* (AUs 25_26_27), and *eye closure* (AU143) during clinical pain. Inspired by the possibilities provided by FACS, different animal FACS have been developed, *e.g.*, the Equine Facial Action Coding System (EquiFACS) (Wathan *et al.*, 2015). Recent research has identified *ear rotator* (EAD104), *nostril dilator* (AD38), *chin raiser* (AU17), *half blink* (AU47) and *chewing* (AD81) as important pain indicators in horses (Rashid *et al.*, 2020). That study was the first to map all facial activities in horses with experimental and clinical pain, allowing for objective and exhaustive description of the equine facial repertoire during pain. The approach has since been applied successfully on stressed horses during isolation and transportation (Lundblad *et al.*, 2021). Thus, EquiFACS is a powerful tool for describing all changes in facial activity during pain.

1.5 Pain assessment in horses

Body behaviors and facial expressions shown by the horse during a pain experience can be assessed in several ways, with the main goal of quantifying the pain experience. It is difficult to quantify such a complex experience, so it is important to cover all dimensions of pain, *i.e.*, the frequency, duration, and intensity (Ashley *et al.*, 2005). Subjective pain assessment is greatly influenced by several factors, such as the experience of pain in the observer and their relationship to the subject, with *e.g.*, observers with a family member experiencing chronic pain assigning higher pain scores than observers not related to the subject (Prkachin *et al.*, 2001). In contrast, observers who have watched a video containing clips of patients with high-intensity pain may rate test patients as experiencing less pain than observers who have not watched the video (Prkachin & Rocha, 2010). This underestimation effect has also been shown in nurses with much experience of patients in pain, who give lower pain scores than nurses with little experience (Wilson & McSherry, 2006), and in physicians and nurses at hospital emergency departments, who underestimate patients' pain experience (Marquie *et al.*, 2003; Puntillo *et al.*, 2003). In addition, observers may ascribe less pain to patients and show less sympathy for them when they do not believe that there is a medical reason for the pain (De Ruddere *et al.*, 2012). Similar underestimation effects have been reported among veterinarians, who may assign cows with different diagnoses lower pain scores than farmers (Thomsen *et al.*, 2012). Younger veterinarians seem to use analgesics more often than older veterinarians, and a gender effect is present whereby women ascribe more pain to the animal than men do (Raekallio *et al.*, 2003; Thomsen *et al.*, 2010; Lorena *et al.*, 2013; Canozzi *et al.*, 2022).

To overcome these biases, objective pain assessment with reliable and validated pain assessment tools can be performed and may aid in equine orthopedic pain detection. The gold standard is a validated assessment tool that correctly measures what it is intended to measure. If a tool meets the gold standard to a high degree, it has high criterion validity. When there is no gold standard, only construct validity can be estimated, *i.e.*, the tool reflects hypothesized measures of the true state (Mokkink *et al.*, 2010). Self-reporting of pain is considered the gold standard in human pain research, but is lacking in animals. Therefore, the validation process may be challenging in animals, for which both the construct and the hypothesized measures must be clearly defined. Reliability is more frequently evaluated for equine pain scales, by testing inter- and intra-observer agreement (reproducibility and repeatability, respectively), and internal consistency. These measures show "*the extent to which scores for patients who have not changed are the same for repeated measurement under several conditions*" (Mokkink *et al.*, 2010). Assessment is usually performed in an experimental setting, where observers are fully trained in using a pain scale prior to the experiment and may be fully blinded to the pain status of the horse. In contrast, a clinical setting may include expectation bias (knowledge of diagnosis and pain status), no expert training prior to using the pain scale, and stress/fatigue of the observer. One should therefore be careful about assuming the same degree of reliability during different settings (Mogil *et al.*, 2020). Furthermore, reliability and validity should not be confused. For instance, a scale item can be reliable without being a valid indicator of pain or can be reliable and/or valid for moderate to severe pain, but not for mild to moderate pain (De Vet *et al.*, 2003).

To conclude, reliable and validated pain scales for mild to moderate orthopedic pain in horses are lacking. In previous research, HGS, EQUUS-FAP, and Composite Orthopedic Pain Scale (CPS) (Bussières *et al.*, 2008) have been used for assessing moderate to severe orthopedic pain in horses, and have shown promising results. Reliability and validity of EPS for orthopedic pain have not been evaluated, but a modified version of the pain scale can successfully assess visceral pain in a clinical setting (Lawson *et al.*, 2019). These four pain scales can therefore potentially detect mild to moderate orthopedic pain. During lameness examination, the only pain assessment performed is subjective assessment of lameness score. However, this lameness score might not be correlated with the level of pain the horse experiences during rest and motion. From a welfare perspective, it may therefore be important to introduce another measure of pain.

1.5.1 Does it hurt? Seeking answers with analgesic testing

A common way of evaluating the validity of a pain assessment tool is to treat the horse with a systemic or local anesthetic and evaluate changes in lameness, and/or pain-related body behaviors and facial expressions. However, it must be clarified that the definition of the construct plays an important role here. A construct of evaluating a pain scale presupposes that the hypothesis that the anesthetic efficiently reduces pain is fulfilled. Conversely, a construct of evaluating an anesthetic presupposes that the pain score or lameness score correctly reflects the pain experienced by the horse. Since the effect of an anesthetic may differ depending on the type of pain, and the behavioral response from the horse also may differ, it can be more challenging than originally thought to use analgesic testing.

Local anesthetics are commonly used as a diagnostic tool during lameness examinations and may be administered intra-articularly or close to a sensory nerve, innervating the damaged tissue. Drugs such as bupivacaine, mepivacaine, and lidocaine block voltage-gate sodium channels along the axon membrane, resulting in attenuated action potential and conduction of impulses (Vadhanan *et al.*, 2015). The level of myelination of the blocked nerve determines how many impulses are blocked, but C fibers are in general less sensitive to local anesthetic than Aδ fibers (Vadhanan *et al.*, 2015). Importantly, the gait pattern seems to be unaffected on applying distal limb nerve blocks in sound horses, confirming that more symmetrical gait patterns after applying local anesthetics are due to pain relief (Keg *et al.*, 1996; Keegan *et al.*, 1997; Van de Water *et al.*, 2016). Thus, local anesthetics provides short-acting relief of acute and neuropathic pain, and is an important aid in equine pain detection.

While local diagnostic anesthesia is time-consuming, systemic anesthetics can quickly attenuate different parts of the physiological process of nociception or stimulate the descending inhibitory pain pathway

(Giovannitti *et al.*, 2015). Alpha-2 adrenergic receptor agonists can provide analgesia, but mainly result in sedation by obstructing bindings of noradrenaline to receptors in the locus coeruleus (Giovannitti *et al.*, 2015). Therefore, it may be difficult to evaluate changes in pain-related behavior. The same applies for opioids, which are a very important drug for acute and chronic pain relief in humans, but may result in excitation or behavioral changes, such as increased locomotor activity and incoordination (Clutton, 2010). Combining opioids and alpha-2 adrenergic receptor agonists seems to be suitable for mainly acute peri- and postoperative pain, since fewer side-effects are seen together with a potentiated sedative and analgesic effect (Clutton, 2010; Studer *et al.*, 2021). Opioids may also be administered locally into the joint or epidural space to obtain short-acting local analgesia (van Loon *et al.*, 2010).

Systemic analgesic therapy may be used to target certain inflammatory mediators important during nociceptive pain. Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit cyclooxygenase (COX), involved in the synthesis of prostaglandin by different tissue cells (COX-1) or by inflammatory cells (COX-2) (Matthews, 2009). The effect of NSAIDs in chronic pain is debated, since the presence of nociception and prostaglandin synthesis is not required for the perception of chronic pain, but for when inflammation is present during chronic pain, NSAIDs are successful in reducing pain (Ho *et al.*, 2018). Glucocorticoids are also used in horses, mainly to reduce inflammation locally in the joint, and may for instance up-regulate synthesis of anti-inflammatory proteins such as lipocortin, and inhibit pro-inflammatory cytokines (Barnes, 1998).

In conclusion, while there are options for treating nociceptive pain, chronic pain modulation in equines remains challenging. Answering the question ‘Does it hurt?’ through exploiting the positive effect of analgesic treatment is therefore challenging and may be limited to acute nociceptive and neuropathic pain.

2. Aims of the thesis

Identifying and assessing pain in horses is complex and beset by challenges. The overall aim of this thesis was to improve equine welfare by providing tools and know-how on detecting orthopedic pain in horses that can be applied by veterinarians, animal health personnel, and animal owners.

Specific objectives were to:

- Identify body behaviors and facial expressions in four equine pain scales that predict orthopedic pain and frequently occur together during pain (Paper I).

- Investigate the relationship between orthopedic pain at rest and degree of movement asymmetry during trot in horses (Paper II).

- Evaluate performance parameters of four equine pain scales for assessment of orthopedic pain at rest (Paper II).

- Explore changes in facial repertoire in resting horses with different intensities of induced orthopedic pain (Paper III).

- Identify changes in facial activities in horses trotted by hand with different degrees of induced orthopedic pain (Paper IV).

3. Hypotheses

The following hypotheses were tested:

- Specific combinations of body behaviors and facial expressions listed in four equine pain scales are associated with increased movement asymmetry in horses with induced orthopedic pain (Paper I).
- In resting horses with induced orthopedic pain, body behaviors can be assessed with higher reliability than facial expressions (Paper I).
- In resting horses with induced orthopedic pain, increased pain scores from four equine pain scales are associated with increased movement asymmetry (Paper II).
- Pain scales containing both body behaviors and facial expressions perform better than scales with only behavioral or facial items in assessing orthopedic pain at rest (Paper II).
- The reliability of four equine pain scales assessing orthopedic pain is consistent with reported values (Paper II).
- Previously described AUs/ADs (half blink, ear rotator, nostril dilator, chin raiser, chewing) can predict orthopedic pain, and co-occur more frequently in resting horses with orthopedic pain (Paper III).
- Previously described AUs/ADs (half blink, ear rotator, nostril dilator, chin raiser, chewing) co-occur less frequently in mild intensities of orthopedic pain than in moderate intensities (Paper III).
- Facial displays of pain in trotted horses with orthopedic pain are affected by the movement, but are associated with orthopedic pain intensities and co-occur more frequently during orthopedic pain (Paper IV).

4. Material and methods

The results presented in this thesis are based on data obtained in an experimental setting where fully reversible orthopedic pain was induced in eight clinically healthy horses. The aim was to create a standardized acute orthopedic pain experience where movement asymmetry was included as a proxy for the pain experience. The study was designed to allow for pain assessments in a clinical setting and for high-quality video recordings that could be annotated with EquiFACS. The study protocol was approved by the Swedish Ethics Committee (diary number 5.8.18-09822/2018). This chapter presents an overview of the experimental work and comments and reflections on the methods used in Papers I-IV.

4.1 Study design in Papers I-IV

Eight clinically healthy horses were included in the study after undergoing a full clinical examination, subjective lameness evaluation, and objective gait analysis. A subjective lameness score of ≤1 grade on an ordinal scale (ranging from 0 = sound, to 5 = non-weight bearing lameness) was accepted. All horses went through an acclimatization period of 10-12 days prior to the orthopedic pain induction. Baseline objective gait analyses and pain assessments were performed on the last day of acclimatization. One or two days later, orthopedic pain was induced by administration of lipopolysaccharides (LPS) from *E.coli* O55:B5 into the tarsocrural joint of the hindlimb with highest baseline movement asymmetry. A volume of 3 mL diluted LPS solution (1.167 ng/mL, *L5418 Sigma*) was injected, using routine aseptic techniques. After resting for 1.5 h in its box stall, each horse was subjected to a minimum of four objective gait analyses (OGA) before it returned to a movement asymmetry similar to that of the baseline. Before and after every OGA, a pain assessment was performed on the horse resting in its box stall. This was done by three observers standing outside the box stall, who assessed pain simultaneously and independently with the pain scales HGS, EQUUS-FAP, EPS, and CPS, in that fixed order. Thereafter, the horse was prepared for OGA. After OGA, the horse was returned to its box stall and a pain assessment was performed. If a horse was considered too lame to trot (corresponding to lameness grade >3/5), OGA was postponed until trotting was possible. Pain assessments were performed every 45 minutes during this period. Additionally, a rescue protocol was initiated to reduce intra-articular joint pressure by evacuating synovia.

4.2 Orthopedic pain induction

Intra-articular administration of LPS is a well-known model for induction of orthopedic pain in horses, resulting in a transient primary synovitis. Different grades of joint effusion, peri-articular swelling, and changes in synovial fluid parameters, such as increased total nucleated cell count and total protein, darkened color, and reduced viscosity, are present and appear to be dose-dependent (Palmer & Bertone, 1994). These local signs of inflammation may be accompanied by systemic signs such as fever, increased heart rate and respiratory rate, and hematological changes (Firth *et al.*, 1988; Andreassen *et al.*, 2017). Lameness is present around two hours after intra-articular administration of LPS and peaks within four hours, to then diminish within 48 hours (Andreassen *et al.*, 2017; Van de Water *et al.*, 2021). Other pain-related behaviors have also been identified, such as decreased time spent eating and reduced weight bearing (van Loon *et al.*, 2010), resulting in higher pain scores as lameness increases (Andreassen *et al.*, 2017). Thus, LPS induction is a highly suitable method for assessing acute orthopedic pain and related behaviors. The gradient of increase and decrease in lameness after LPS induction allows several assessments of lameness and pain behavior to be performed, where progression and regression can be closely monitored.

4.3 Objective gait analyses

In this thesis, progression and regression of lameness was measured objectively with an optical motion capture system (Qualisys AB, Gothenburg, Sweden), providing detailed information on biomechanical adaptations during lameness. The 3D positions of spherical markers attached to the poll of the head and the tubera sacrale were recorded with 13 infrared motion capture cameras at a sampling frequency of 200 Hz, and computed by the Qualisys Track Manager software (version 2.11-2019.3, Qualisys AB, Gothenburg, Sweden). After visual inspection, tracked position data were analyzed with custom-written scripts in MatLab (2018), where the signal was filtered through a high-pass digital fourth-order zero-phase Butterworth filter to remove low-frequency noise. The cut-off frequency was set to that of stride frequency. As a result, the first and second harmonics of the sinusoidal pattern were extracted and summed based on their original amplitudes and how they occur in relation to each other (Serra Bragança *et al*., 2020). The first harmonic illustrates the movement asymmetry, occurring once per stride cycle, and the second harmonic illustrates the normal biphasic movement in trot, occurring twice per stride cycle (Peham *et al*., 1996). Stride segmentation is performed by detecting left/right ground contact based on the vertical position of the pelvis and its rotations (Roepstorff *et al*., 2021). In Papers I-IV, vertical displacement data for the head and pelvis during hard straight-line trot were included. To estimate overall movement asymmetry, a total asymmetry score (TAS) was created by summing the mean trial HDmin/2 and PDmin values. In this way, the advanced compensatory mechanisms during motion were included. To prevent left-side negative values from neutralizing right-side positive values when added together, the left-side values were multiplied by -1. Baseline TAS was then set to 0 and increase in TAS from baseline was used as a proxy for orthopedic pain.

4.4 Pain assessments

Pain assessments in Papers I-IV were performed using four existing equine pain scales (HGS, EQUUS-FAP, EPS, CPS), selected based on previous literature. Since the use of pain scales in equine hospital settings may be affected by expectation bias, lack of prior experience in using the scale, stress/fatigue of the observer, and observer presence during live scoring, the assessments in Papers I-IV aimed to replicate these conditions. Five observers (three veterinarians, one agronomist, and one ethologist) were recruited for the study and were allowed to familiarize themselves with published score sheets, descriptions, and papers describing the pain scales. No expert training or reliability testing was performed, in order to replicate a clinical setting where the equine veterinarian applies a pain scale after studying published material. The observers were informed that they were assessing horses with induced orthopedic pain, since in hospital settings the veterinarian would have information on the type of pain being experienced by the patient. In addition, observer 1 had knowledge of the hindlimb in which pain was induced and how lameness progressed and regressed.

For each induction course, three observers were selected based on availability. Pain assessments were performed before and after each OGA, and horses were assessed from outside the box stall. The observers performed the assessments simultaneously and independently, using existing score sheets. Pain assessments with HGS, EQUUS-FAP, and EPS lasted for two minutes each, and pain assessments with CPS for five minutes. During the last two minutes, physiological parameters were measured. Different pain assessments and adjacent OGAs were included in Papers I-IV, as summarized in Table 2.

Table 2. *Overview of objective gait analyses (OGA) and related pain assessments (PA) included in the analysis in Papers I-IV*

Paper	Objective gait analyses	Pain assessments
I	Baseline	Before and after (all scales)
	Until reaching maximum TAS	Before and after (all scales)
II	Baseline	Before and after (all scales)
	All performed	Before and after (all scales)
III	Baseline	Before and after (CPS) Before induction (CPS)
	Selected based on highest pain score	Before and after (CPS) Between (CPS)
IV	Baseline All performed	Not included

4.5 Video recordings and annotations with EquiFACS

When acclimatization was initiated, each horse was moved to a box stall equipped with four infrared network surveillance cameras (WDR ExIR Turret Network Camera, Hikvision Digital Technology Co., Hangzhou, China). Each camera was installed on the wall at approximately 180 cm above ground in the corners of the box stall (Figure 2). To avoid shading of the face during video recording, the original lighting in each box stall was improved by installing nine strip lights (cold white, 18W, 4000 Kelvin) in the ceiling of the box stall (Figure 3). The surveillance cameras were set to record continuously from 12 hours before baseline measurements until the last OGA was performed. Every time a pain assessment started or ended with each pain scale, one of the observers signaled to the cameras. These signals were used to manually cut out video sequences for annotation, and automated horse face detection software (Rashid *et al.*, 2018) was applied to select video sequences with the face of the horse clearly visible. After blinding, the resulting 384 video sequences were randomly distributed among nine annotators with EquiFACS certification (Wathan *et al.*, 2015). In addition, each OGA was recorded with two handheld video cameras (Canon Legria HF R78). Recordings were made of the horse from the side, focusing on the head, and edited to show only the horse from the side when trotting. After blinding, the 103 video sequences were randomly distributed among two EquiFACS-certified annotators.

Annotators used modified annotation templates and viewed video sequences frame-by-frame and in normal speed in ELAN Linguistic Annotator (ELAN Linguistic Annotator, 2019).

Figure 2. (Left) Infrared network surveillance camera installed on the wall of the box stall, (center) the computer connected to the surveillance cameras, and (right) views of the computer screens during recordings in two box stalls.

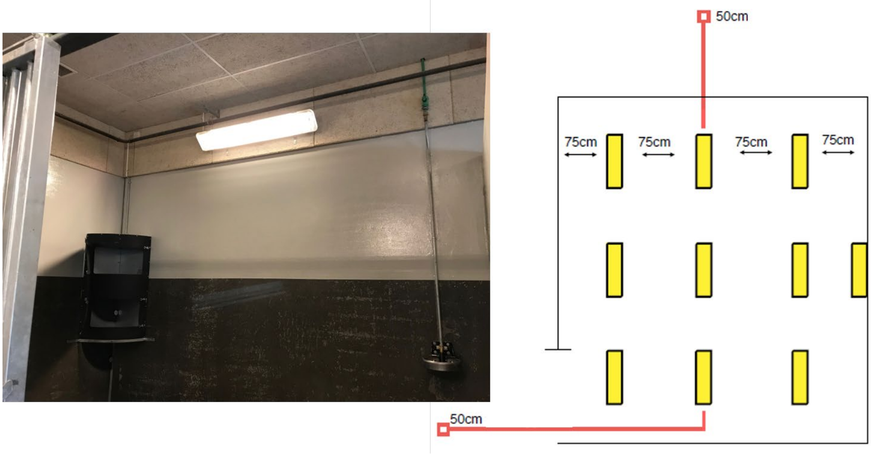

Figure 3. (Left) Original lighting in a box stall and (right) diagram showing the position of nine strip lights installed in the ceiling of each box stall.

4.6 Statistical methods

All statistical computations were performed in R (R Core Team, 2020) and plots were created with 'ggplot' (Wickham, 2016). Shapiro-Wilks tests ($p<0.05$) and histograms showed that the dataset was not normally distributed, so median and 1st and 3rd interquartile are presented for each paper. Papers I, III, and IV included predictive modeling to identify scale items or AUs/ADs that could predict different levels of pain intensity defined by movement asymmetry. Regularization and shrinking of estimated coefficients of variables are performed to identify associations between variables and the outcome. Those variables receiving a coefficient of 0 are not associated with the outcome and are ignored in the model. In Paper I, shrinkage of coefficient estimates was performed with Lasso regression, and variables (scale items) that predicted the outcome (increase in TAS) were selected by the models. The shrinkage parameter lambda was used and was determined by 10-fold cross-validation. In Papers III and IV, elastic net regression was performed, where two shrinkage parameters (alpha and lambda) were included based on 10-fold cross-validation. This model included all variables (EquiFACS codes), as is recommended when the variables are highly correlated. The outcome was binary in Paper III ('no pain' and 'pain') and multinomial in Paper IV ('sound', 'pain (inc)', 'pain (max)', and 'pain (dec)'). Horse was included as a variable in all three papers to estimate the effect of the individual horse on the predictions. In Paper I, multiple correspondence analysis (MCA) was used to identify dimensions of the data explaining the variation. These dimensions grouped different variables together depending on how much of the variation they explained. The dimensions were then used in linear regression to identify significant dimensions, with the help of Akaike information criterion (AIC).

In Paper II, generative additive mixed models (GAMM) were used to evaluate the relationship between the outcome (total pain scores) and the explanatory variable (increase in TAS). GAMM allow non-linear relationships to appear and the explained deviance (R^2 value) of the model illustrates how well an increase in TAS can explain the total pain scores. Prediction outcomes from GAMM comprised predictive values that were used to compute area under the curve (AUC) of receiver-operating characteristics (ROC) curves. AUC was then used as a measure of accuracy.

Reliability testing was performed in Papers I and II to evaluate inter-observer agreement. Kendall's coefficient of concordance (*W*) was used for ordinal ranked data (scale items ranging from 0-2), while Intraclass Correlation Coefficient (ICC) was used for total pain scores since they were considered continuous, ranging from 0-39.

In Papers III and IV, the temporal dynamics of facial activities were analyzed with a previously described method specifically developed for this purpose (Rashid *et al*., 2020). It performs data-driven selection of EquiFACS codes co-occurring within a specific observation window. An observation window size (OWS) of two seconds was chosen in Papers III and IV, since it was considered to represent a 'grimace' during live assessment.

5. Results

In this chapter, the main results from Papers I-IV are summarized. General results on lameness induction are presented first, followed by specific results for each paper (presented in sections 5.1-5.3). Increases in movement asymmetry and lameness were successfully induced in all eight horses. The experimental study resulted in 53 OGAs and 97 pain assessments, where mean (standard deviation, SD) number of OGAs per horse was 6.6 (1.2) and mean (SD) number of pain assessment per horse was 12.1 (2.4). Mean (SD) PDmin value was 3 (3) mm for baseline OGA and 46 (20) mm for OGAs where each horse reached its maximum increase in TAS. All horses returned to movement asymmetries similar to those at baseline OGA within 52 hours. Two horses were subjected to rescue analgesia, where synovia was evacuated to reduce intra-articular pressure and synovial volume.

5.1 Association between orthopedic pain and scale items

The four pain scales contained 37 scale items in total (six in HGS, nine in EQUUS-FAP, nine in EPS, and 13 in CPS). The distributions of scale item scores were plotted and this revealed that many scale items received a score of 0 despite an increase in TAS. However, some scale items received scores >0 when TAS increased. These were: ‘ears’ and ‘nostrils’ in HGS; ‘head’, ‘focus’, and ‘ears’ in EQUUS-FAP; ‘location’, ‘posture’, ‘pain face’, ‘gross pain behavior’, and ‘head’ in EPS; and ‘pawing’, ‘head’, ‘appearance’, ‘posture’, ‘response to palpation’, and physiological parameters in CPS. Scale items varied in reliability, where EPS and CPS had the most scale items with strong agreement ($W = 0.7\text{-}0.9$) between observers. Facial expression items had in general low to moderate agreement, except for ‘orbital tightening’ in HGS, which had strong agreement.

Predictive modeling with Lasso regression showed that horses were associated with TAS, together with several scale items. When all scale items were combined in one model, the scale items ‘posture’ in EPS and CPS, physiological parameters in CPS, and ‘focus’ in EQUUS-FAP were most associated with orthopedic pain. Follow-up MCA revealed that nine dimensions were significantly associated with orthopedic pain, particularly containing the scale items ‘posture’, ‘interaction’, ‘location’, and ‘head position’. Facial expressions co-occurred with most body behaviors, but one dimension showed that facial expressions were negatively associated with orthopedic pain when postural changes were positively associated with pain. Based on these results, five body behaviors (posture, head position, location in the box stall, focus, and interactive behavior) should be included in live orthopedic pain assessments in resting horses, together with facial expressions.

5.2 Performance of four pain scales

Distributions of total pain scores were plotted for each of the four pain scales, to examine how the total pain scores varied between horses, scales, and degree of TAS. The results showed that total pain scores were in general in the low end of each scale. Despite this, significant associations between total pain scores and increase in TAS were identified for all pain scales, although this varied between observers 1-5. CPS was the only scale where total pain scores from all observers were associated with an increase in TAS. Evaluation of partial effect plots showed non-linear relationships between total pain scores and increase in TAS, where a rather high TAS could be present before total pain score increased.

Comparison of performance parameters identified CPS as the only scale with good reliability and high accuracy based on AUC measures. With the other scales, at least one observer did not succeed in determining whether horses were in pain or not (AUC <0.5). Therefore it can be concluded that CPS is the most reliable and accurate pain scale in assessing orthopedic pain live in resting horses.

5.3 Facial displays of pain in resting and trotted horses

The total length of annotated video material in resting horses (Paper III) was 892.5 minutes, resulting in 20,208 annotations in ‘no pain’ video sequences and 16,864 annotations in ‘pain’ sequences. In trotted horses (Paper IV), 60 videos of 48 OGAs were annotated, resulting in a total of 1603 annotations. Descriptive statistics from Papers III and Paper IV are summarized in Table 3. Distribution plots of AUs/ADs per horse during ‘no pain’ and ‘pain’ for rest and trot were visually assessed. During trot in particular, some horses showed more facial activities during ‘pain’, while others showed less facial activities during ‘pain’.

Predictive modeling identified AUs/ADs associated with orthopedic pain during rest and trot, and assigned to each regression coefficients illustrating the predictive value. In Paper III, the model outcome was binomial (‘no pain’ and ‘pain’), while in Paper IV it was multinomial (sound (Sound), increasing pain intensity (Pain (inc)), maximum pain intensity (Pain (max)), and decreasing pain intensity (Pain (dec)). The co-occurrence method selected AUs/ADs co-occurring when ‘pain’ states were compared with ‘no pain’ states. In Paper III, orthopedic pain intensity was defined according to increase in absolute values of TAS. In Paper IV, the multinomial outcome was included, where intensity levels were defined based on individual progression and regression of lameness. AUs/ADs associated with different pain states in the predictive model and selected by the co-occurrence method are summarized in Figure 4.

These results allowed details of how facial activity varies during rest and motion, and during different pain intensities, to be identified. Notably, it appeared that the individual horse was important for the changes in facial activity that occurred. However, facial displays of pain were identified in both resting and trotting horses, where *lower lip depressor* (AU16) and *lips part* (AU25) co-occurred for both rest and trot.

Table 3**.** *Median, and 1st and 3rd interquartile, from Paper III (resting horses) and Paper IV (trotted horses) summarized for video sequences labeled 'no pain' and 'pain'.*

Descriptive statistics		**Median (1st and 3rd interquartile)**	
		No pain	Pain
Annotations per video	Resting horses	102.5 (65.8, 151.3)	84.0 (61.0-129.0)
	Trotted horses	25.0 (12.0, 41.0)	22.0 (12.0, 32.0)
Annotations per horse	Resting horses	2314.0 (1822.0, 3267.25)	1997.5 (1259.0, 2308.0)
	Trotted horses	70.0 (59.5, 87.8)	119 (71.8, 144.0)
Duration of annotations	Resting horses	0.466 (0.340, 0.700)	0.491 (0.350, 0.750)
	Trotted horses	0.30 (0.2, 0.43)	0.36 (0.23, 0.59)

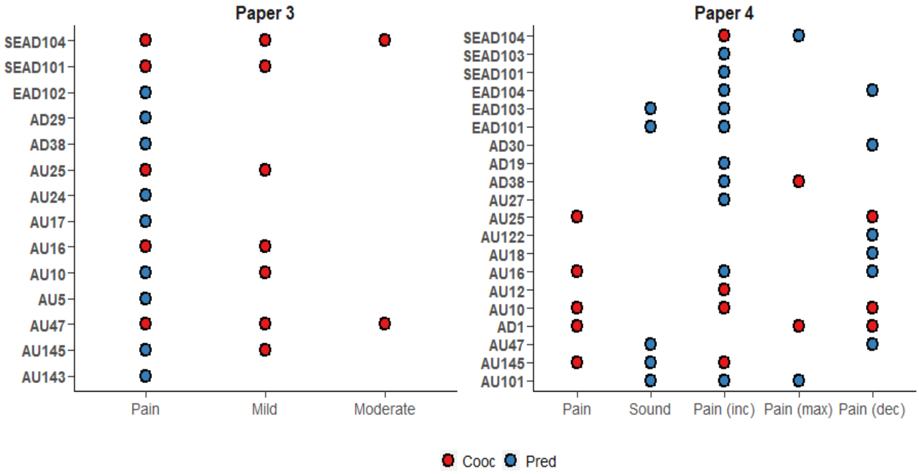

Figure 4. EquiFACS codes associated with, and co-occurring in, orthopedic pain intensities in Papers III and IV. Codes are shown on the y-axes and pain intensity levels on the x-axes. Cooc = co-occurrence method, Pred = predictive model.

6. General discussion

Through the work in this thesis, body behaviors and changes in facial activity associated with orthopedic pain and increase in movement asymmetry were identified. Previous findings on pain-related body behaviors in resting horses were confirmed, and novel findings were made regarding the dynamicity of facial activities in horses during pain. The importance of a nuanced view on the pain experience in horses was acknowledged by reviewing existing knowledge on humans and applying the findings to the study design, statistical computations, and interpretation of results. One result of this was that the definition of level of pain intensity was extended from a binary definition to a more individual gradual approach, which is rarely done in equine pain research. Pain scale performance was tested by comparing four existing pain scales under the same conditions. Thus, this thesis presents relevant information on how equine pain assessment can be refined, thereby contributing to improvement in equine welfare. In this chapter, the materials and methods used and the results obtained in Papers I-IV are discussed in an attempt to answer the critical question ‘Which movement asymmetries are due to pain?’.

6.1 Behavioral changes during orthopedic pain

When resting in their box stalls, horses with induced orthopedic pain performed body behaviors and facial expressions. Changing the posture, focus, and location in the box stall were behaviors strongly associated with orthopedic pain, as were several facial expressions defined in pain scales, such as ‘tension above the eye area’ and ‘stiffly backwards ears’ in HGS (Dalla Costa *et al.*, 2014), ‘eyelids’ and ‘nostrils’ in EQUUS-FAP (van Loon & van Dierendonck, 2015), and ‘pain face’ in EPS (Gleerup & Lindegaard, 2016). These findings in Paper I confirm previous knowledge about pain-related body behaviors and facial expressions in resting horses (as reviewed by Gleerup & Lindegaard, 2016). Further statistical computations in Paper I identified combinations of certain behaviors that were strongly associated with orthopedic pain. A combination of focus, location in the box stall, posture, and appearance was associated with orthopedic pain, as was a combination of facial expressions. Hence, horses perform both body behaviors and facial expressions when experiencing orthopedic pain. However, sometimes when posture-related behaviors were associated with pain, facial expressions were not. This indicates that facial expressions vary in their appearance and may not be present if the horse performs pain-relieving behaviors altering the posture. How behaviors influence each other has not been assessed in previous research in terms of behaviors that are not performed when a particular behavior is performed, but instead in terms of weighting during scale construction. Behaviors assigned higher weights impact the pain score more when present. However, the weights are often empirically based, with statistical weighting of pain-related behavior introduced only recently (Trindade *et al*., 2022). That study illustrated how statistical methods could be implemented in an ovine pain scale (USAPS), and it would be very interesting to apply similar statistics to the dataset in Paper I and Paper II, to further rank the identified behaviors and assign weights to them.

It is not known why facial expressions sometimes were less present during changed posture in Paper I. This might reflect a general fluctuation in pain-related facial expressions, as previously discussed in the study by Rashid *et al*. (2020) where only 6.1% of video frames contained three or more pain-specific AUs co-occurring. It might also reflect the pain-relieving effect of postural changes, resulting in less pain-related facial expressions, since facial expressions during pain are sensitive and decrease when pain is lessened. This has been seen in humans (Kunz *et al.*, 2021) and in horses, with HGS pain scores decreasing significantly after treatment (Dalla Costa *et al*., 2016; Coneglian *et al*., 2020). It can also be reasoned that orthopedic pain in general is a more fluctuant type of pain, since it is often located in the extremities where decreased loading of the affected limb reduces the pain. This further emphasizes the need for a better understanding of the relationship between increasing movement asymmetry and pain intensity.

The LPS induction model was selected specifically in this thesis to represent an acute inflammatory pain experience that progressed and regressed over time, so that intra-individual changes in behaviors could be assessed. Similar consideration has been given to this issue in other studies on equine LPS induced orthopedic pain (Egan *et al.*, 2021). Its importance was emphasized in Paper II, since great variations in baseline total pain scores were found, also illustrating that the baseline cannot show how the horse will react when in pain. Another reason for selecting the LPS induction model was its similarities with clinical synovitis and osteoarthritis (Ross *et al.*, 2012). Osteoarthritis is a very common cause of lameness in horses and is a disease characterized by low-grade joint inflammation that can flare up and progress into damaged articular cartilage. Therefore, it contains both an acute inflammatory pain component and a chronic pain component with central sensitization (van Weeren & de Grauw, 2010). Hence, the findings in this thesis may be applicable to horses with osteoarthritic pain of different degrees, but this needs to be verified in future research. In such research, osteoarthritic pain could be reduced with local diagnostic anesthesia and pain-related behaviors during rest could be assessed before and after applying the local anesthetics. Other drugs, such as NSAIDs and opioids, remain challenging to use if the goal is to reduce pain in a standardized way. Pain-related behaviors should be assessed objectively, preferably using CPS, as it was the pain scale that performed best for orthopedic pain in Paper II.

Another important consideration is whether the identified behaviors are to some extent a result of stress, a negative affective state that co-occurs with pain, since pain is an internal stressor (see section 1.1.1). This is difficult to determine during pain, but stress-related behaviors may be identified by high pain scores in baseline, as shown in Paper II, where baseline total pain scores ranged from 0-5 for EPS and CPS. In the dataset in Paper I (S2 File), horse 4, 6, and 8 distinguished themselves by having item scores >0 for ‘pain face’ and ‘activity’ in EPS, and ‘appearance’, ‘appetite’, and ‘sweating’ in CPS. The presence of pain face during baseline may be explained by the similarities between stress- and pain-related facial activities (Lundblad *et al.*, 2021). An activity score of 3 in EPS and an appearance score of 2 in CPS represent restlessness, while an appetite score of 2 in CPS represents little interest in eating, *i.e.*, signs of a medium stress level according to Young *et al.* (2012). In fact, in Paper I ‘activity’ in EPS was a negative predictor of pain, as were ‘appetite’ and ‘sweating’ in CPS.

Thus, the results presented here were based on successful orthopedic pain induction that may represent osteoarthritic pain. Pain-relieving body behaviors were identified and were associated with movement asymmetry more than stress.

6.2 Using movement asymmetry as a proxy for pain

In Papers I-IV, increase in movement asymmetry was used as a proxy for orthopedic pain. This is a physical outcome from LPS induction that can be objectively measured and hypothesized to represent the pain experience. The objectives in this thesis were to associate scale items (Paper I), total pain scores (Paper II), frequency and duration of AUs/ADs during rest (Paper III), and during trot (Paper IV) to orthopedic pain estimated by movement asymmetry. Thus, movement asymmetry was assumed to be the outcome best representing the pain experience, rather than pain scores and facial activity. Paper II was the first study to demonstrate both linear and non-linear relationships between total pain score and increase in movement asymmetry in horses. This indicates that pain experienced during rest and pain experienced during motion are associated, strengthening the assumption the movement asymmetry can act as a proxy. However, when pain scores and facial activity do not fully explain the variation in movement asymmetry in the statistical models, the question is whether this assumption is valid. In human research, the difficulties reported in correlating biomechanical parameters to self-reports of pain illustrate that movement asymmetry may not represent the full pain experience (as reviewed by Hutchison *et al.*, 2022). Instead, facial expressions of pain in humans are sensitive and specific (Kunz *et al.*, 2004; Prkachin & Solomon, 2009). Perhaps it is possible that facial activity in horses also reflects orthopedic pain better than movement asymmetry. However, this thesis identified challenges with assessing facial activity in horses because: i) there is great inter-individual variation, ii) facial activity may be influenced by other affective states, and iii) the relationship between pain intensity and facial activity is complex. Measuring movement asymmetry does not involve these challenges and can therefore be used as a proxy for orthopedic pain in future studies until evidence for rebuttal emerges.

In Papers III and IV, the levels of orthopedic pain intensity were defined in two ways: by absolute values of increase in TAS, and by calibrating the pain experience so that individual intensity estimates were obtained. To the author's knowledge, this is the first time that pain intensity in horses has been defined on multi-level, integrating mild and moderate intensities and increasing and decreasing pain intensities. A binary outcome of 'no pain' and 'pain' states is often used in equine pain research, since it is difficult to define a more nuanced pain experience without self-reporting or to find proxies for pain with a fully known relationship between the pain and the proxy. By including pain intensity levels in Papers III and IV, differences in facial activity between the intensity levels were identified during both rest and motion. This indicates that equine pain research may benefit from including pain intensity levels and that combining occurrences of facial expressions to estimate intensity may be misleading. However, considering the relationship between facial expressions of pain and self-reporting of pain in human research, the relationships are complex, especially during chronic pain. For instance, facial expressions in humans with chronic back pain may not fully reflect the self-reported intensity of the pain (Vachon-Presseau *et al*., 2016). This thesis evaluated intensity in acute experimental pain, and different facial displays may be present in horses with chronic pain.

It can be debated whether other physical outcomes of pain could have been used as proxies. Such pain biomarkers could assist in identifying predisposition to chronic pain and in diagnosing disorders in humans (Reckziegel *et al*., 2019). In horses, increased expression of nerve growth factor (NGF) receptors in synovial membranes varies in different stages of osteoarthritis (Kendall *et al*., 2022), and detection of these might aid in osteoarthritis detection. While research would benefit from being able to quantify the pain experience with other measures, the multifaceted pain experience probably cannot be represented by a single biomarker. A similar points has been made in the debate on using neuroimaging as a measure of pain, where neuroimaging biomarkers may identify groups of patients responding to treatment in a similar way, but do not directly reflect the pain experience (Mouraux & Iannetti, 2018). Since the aim in this thesis was to identify behaviors related to different orthopedic pain intensities in horses, biomarkers would most possibly not have been useful. A recent review on physiological and behavioral alterations in equine nociceptive pain suggests evaluation of behavioral changes together with cortisol, lactate, glucose, and catecholamines, and physiological parameters such as heart rate, respiratory rate, heart rate variability, parasympathetic tone activity index, and temperature (Hernández-Ávalos *et al.*, 2021). In a study where acute visceral pain was assessed with a modified EPS, significant correlations between total pain scores and cortisol levels were found (Lawson *et al.*, 2019). Weak correlations between heart and respiratory rate and cortisol were also found in that study, but the overall correlation between pain score and cortisol was only marginally affected by excluding the physiological parameters. However, both these papers point out that endocrine biomarkers such as cortisol and physiological parameters are indicators of stress, not pain specifically. Paper I revealed associations between temperature, heart rate, and increased movement asymmetry. As discussed, LPS is a pyrogen and body temperature is associated with heart rate (Firth *et al.*, 1988; Jensen *et al.*, 2019). Therefore, despite some calls for inclusion of physiological parameters in pain assessment, in this thesis changes in physiological parameters would have been interpreted as responses to the induction model, rather than to the pain experience itself. It can also be discussed whether the level of stress is directly correlated to the level of pain intensity, and thus whether physiological parameters or endocrine biomarkers indicating a certain level of stress automatically indicate a certain level of pain intensity.

In summary, this section assessed the advantages of using movement asymmetry as a proxy for pain and considered other options. The conclusions reached indicate that future research on equine pain assessment could benefit from including more proxies for pain, and especially movement asymmetry as a proxy for orthopedic pain.

6.3 Assessing orthopedic pain in resting horses

In Papers I-III in this thesis, the main focus was on assessing orthopedic pain in horses resting in their box stalls. In Paper II a method to test the performance of pain scales in a clinical setting was developed, by applying a unique study design comprising three observers who assessed pain at exactly the same time. Resting pain displays were associated with an increase in movement asymmetry and were detected with the four pain scales. The scales had been used previously to assess moderate to severe orthopedic pain during amphotericin-B induced synovitis (Bussières *et al.*, 2008), in laminitis (Dalla Costa *et al.*, 2016), and after orthopedic surgery (van Loon & Van Dierendonck, 2019), but had not been validated for mild to moderate orthopedic pain. Among the four pain scales, CPS proved to be the most reliable and accurate and can be recommended for assessment of orthopedic pain in resting horses. However, the varying linear and non-linear relationships between pain scores and movement asymmetry indicate that the size of the total pain score is not directly correlated to the level of pain intensity, in contrast to pain scores on visual analogue scales (VAS, 0-10) and numerical rating scales (NRS, 0-10) (Ferreira-Valente *et al*., 2011; Karcioglu *et al*., 2018). Instead, pain scores could be taken as probability coefficients for the presence of pain. The clinical interpretation of this is that the equine veterinarian can expect moderate lameness to be present in horses with total pain scores >0. This may improve pain assessment in equine patients during strict box rest, *e.g*., post-surgery or when kept in a support sling, and could also facilitate pain assessment in the home environment where the prey animal narrative may be eliminated. As a complement to this, the recommendation made in Paper I was to include scale items regarding posture, head position, location in the box stall, focus, and interactive behavior when assessing mild to moderate orthopedic pain. This may be achievable with future refinements of equine pain assessment, where *e.g*., video surveillance with alarm systems could be programmed to detect these specific behaviors.

Comparisons of the performance of the four pain scales in Paper II revealed that the face-based pain scales were the least accurate. This might be due to their poor to moderate overall reliability (Paper II). Additionally, scale items assessing lower facial activity in HGS and EQUUS-FAP, eyelid position and nostrils in EQUUS-FAP, and the overall pain face in EPS were not very reliable (Paper I). Some scale items assessing the same facial feature differed in reliability, indicating difficulties in interpreting the item. These findings of poor reliability in Papers I and II are in contradiction to previous results showing good reliability of the scales (Dalla Costa *et al*., 2014, 2016; van Loon & van Dierendonck, 2015). The level of observer training prior to the experiment did not seem to differ between Papers I-II and previous research, and the observers had similar experience of assessing pain. Therefore the discrepancy observed in scale performance is an important finding that confirms the relevance of re-evaluating reliability when scoring conditions or when a type of pain is new (de Grauw & van Loon, 2016).

In Paper III, changes in facial activity in resting horses were further explored since the face-based pain scales were not sufficiently reliable. EquiFACS was applied to the video-recorded time slots, which were pain-assessed live, and a dynamic facial repertoire consisting of asymmetrical ears (co-occurring *single ear forward* (SEAD101) and *single ear rotator* (SEAD104)), *half blink* (AU47), and lower face activity was identified. Facial expressions assessed in pain scales seemed more stoic and constant than the dynamicity observed in Paper III. For instance, 'mouth strained and pronounced chin' (Dalla Costa *et al.*, 2014), 'edged shape of the muzzle with the lips pressed together' (Gleerup *et al.*, 2015), and 'slightly/obviously lifted corners mouth/lips (van Loon & van Dierendonck, 2015) were in contrast to the 'mouth-playing' identified in Paper III.

The discrepancy in results may lie in the great differences between the two methods applied. When using pain scales to assess facial expressions, the assessment becomes subjective despite scoring criteria. For instance, the observer subjectively decides for how long a 'pain face' needs to be present during the observation period to register an item score of 2 in EPS. In EQUUS-FAP, the observer decides subjectively when nostrils are 'a bit more opened' and assigns an item score of 1, while in HGS, the observer subjectively decides when 'tension above the eye area' is moderate and assigns an item score of 1. This degree of subjectivity may be reflected in the poor-moderate reliability identified in Papers I and II. EquiFACS, on the other hand, leaves little room for subjectivity, as all facial activities are registered throughout the observation time, resulting in a substantial and objective dataset. The conclusion reached in Paper III was therefore that detection of facial expressions of pain in horses may benefit from video assessment, and that assessing still images will overlook dynamic facial activities. However, despite being a powerful and objective tool, EquiFACS is not suitable for pain assessment in clinical settings, due to the very time-consuming process of annotating, and is better used as a research tool (Wathan *et al.*, 2015).

To sum up, orthopedic pain in resting horses can be most accurately and reliably assessed with CPS, but estimating the level of pain intensity based on total pain scores is challenging. Face-based pain scales may struggle in assessing facial expressions of orthopedic pain in resting horses and tend to focus on more stoic expressions. However, a dynamic upper and lower facial repertoire is performed during pain, so the concept of one prototypical pain face may be a simplified version of a more advanced dynamic facial repertoire. Despite showing some drawbacks in the work in this thesis, pain scales remain central for clinical pain assessment by adding objectivity during assessment of pain progression and regression.

6.4 Assessing orthopedic pain in moving horses

In Paper IV, changes in facial activity in horses trotted by hand were described with EquiFACS for the first time. A grimace indicative of orthopedic pain in moving horses consisted of blinking (AU145) with visible sclera in between (AD1), together with moving the lips (AU10, AU16, AU25), all occurring together within two seconds. Co-occurrence of *nostril dilator* (AD38) may indicate moderate pain intensity. Similar changes in the eye and lower face areas are described in the Ridden Horse Pain Ethogram (Dyson *et al.*, 2018a), but over a longer time and without defining co-occurrence of facial activities (see Table 1 in section 1.4 for an overview). Only repeated mouth opening and shutting for ≥10 seconds decreases when lameness is reduced with local diagnostic anesthesia in a within-animal study design (Dyson *et al.*, 2018b). Additionally, mouth opening have been identified as a behavior performed to reduce the pressure of the bit on oral tissues (Eisersiö *et al.*, 2023) and can be influenced by the rider's skill (Dyson *et al.*, 2022), further indicating that evaluation of temporal dynamics of facial expressions can assist in defining facial displays of pain during movement. It can be argued that exhaustively coding all facial activities is the correct approach in identifying these facial displays of pain in trotted horses, since reliability is only slight-moderate when facial expressions are assessed on videos of ridden horses (Dyson & Van Dijk, 2018). This agrees with findings in Paper I of low-moderate reliability for facial scale items.

A core of two co-occurring AUs (*lower lip depressor* (AU16) and *lips part* (AU25)) was identified to be consistent between rest and motion, indicating that lower facial activity with lips separated is important for orthopedic pain detection. In humans, opening the mouth (AUs25_26_27) is considered important in pain, but it is theorized that it may be a preparation for vocalization (Kunz *et al.*, 2019). However, this was not the case for horses in the dataset used in this thesis, where *vocalization* (AD50) was not coded at all. Hence, how lower facial activity is related to pain in horses with lameness requires further research.

Paper IV identified individual differences in the distribution of AUs/ADs, with two horses in particular emerging as less expressive than the others. Due to the limited size of the dataset, this might be a coincidence, but stoicism is present in humans (Kunz *et al.*, 2011) and the social context (with different definitions) seems to influence facial expression of pain (Kappesser, 2019). As in the case of humans, several issues have not been tested in horses, such as the true purpose of facial expressions of pain and whether facial expressions of pain in experimental settings truly represent clinical and chronic pain. Therefore, future research should focus on describing facial expressions in moving healthy and lame horses in different settings, and thereby identify inter-individual variations in facial displays. If some horses are less expressive than others, this must be considered when including facial expressions in pain detection in moving horses.

Paper IV showed clearly that lame horses perform changes in their facial activities that differ from the prototypical pain face described in resting horses. Fully extrapolating what is known about facial displays of pain in resting horses to moving horses is therefore not recommended. Furthermore, there seem to be a large inter-individual variation, so further research is needed.

6.5 Methodological considerations

In this thesis, several methods that may be applicable in future equine pain research were developed. A within-animal study design was used, where the importance of including baseline measurements was illustrated by great variation in item scores (Paper I) and total pain scores (Paper II). Scores of 0 were present during increasing movement asymmetry and scores >0 were present during baseline. Great variation in facial activities was illustrated by distribution plots in Papers III and IV, underlining the importance of looking at data distributions. It was also shown that several statistical models involving prediction and data-driven selection can help to identify associations that are otherwise difficult to detect in small, non-normally distributed datasets with high inter-individual variation. Using these statistical methods, many previous findings on pain-related behaviors were confirmed, supporting use of these methods. In all papers, an attempt was made to interpret the statistics in a clinically relevant way, as exemplified by discussions on how to interpret an EquiFACS code as a predictor for pain in the model.

There were some limitations to the studies presented in Papers I-IV, such as small sample size, use of LPS induction to represent naturally occurring lameness, and the possibility that poor reliability of live pain assessments influenced the accuracy of the pain scales. Section 1.3 raised questions about the behaviors hidden by the horse when it is being observed. Observers were present during every pain assessment in this dataset and during video-recordings in motion, which was a major limitation of the work. It is likely that more prominent behavioral displays would have been observed if the horses had been assessed from video recordings, *i.e.*, with the observers not present. The fact that video sequences of horses during live pain assessments were annotated was possibly also a limitation. However, this thesis presents clinically relevant results representing the situation that the equine veterinarian encounters on a daily basis, namely: i) horses in environments new to them, ii) horses experiencing negative affective states such as stress and fear as a result of pain, iii) pain assessments during observer presence, and iv) lameness examinations during observer presence.

7. Concluding remarks

This thesis provides new research on body behaviors and facial activities that are performed by resting and moving horses with orthopedic pain. In the introduction of this thesis, concerns and shortages in equine welfare research are highlighted, and it stands clear that equine welfare will benefit from improved pain assessment, where pain is detected in a reliable and validated way. The main conclusions are:

- The assessment of orthopedic pain in resting horses may benefit from including behaviors where the horse alters the posture, head position, location in box stall, focus and interaction.
- The Composite Orthopedic Pain Scale (CPS) can reliably and accurately detect orthopedic pain in resting horses, where an increase in total pain score indicate moderate lameness.
- Facial expressions described in equine pain scales are associated with orthopedic pain. However, live assessments may result in low or moderate reliability.
- Resting horses with orthopedic pain show a dynamic facial display of pain, consisting of asymmetrical ears, *half blink* (AU47), *lower lip depressor* (AU16) and *lips part* (AU25) occurring together within 2 seconds.
- Horses with progression and regression of orthopedic pain show a dynamic facial display of pain when trotted by hand. *Lower lip depressor* (AU16) and *lips part* (AU25) co-occur with *blink* (AU145) and *eye white increase* (AD1) within 2 seconds.
- Level of pain intensity may be defined from increasing movement asymmetry, resulting in intensity-related differences in the facial displays of pain during rest and motion.

7.1 Which movement asymmetries are due to pain?

Finally, it is time to return to the critical question of which movement asymmetries are due to pain. Horses with movement asymmetry due to orthopedic pain may perform body behaviors and show a facial display of pain during rest and movement. This thesis identified some such behaviors that can be detected and associated pitfalls in detection, thereby advancing understanding of the relationship between pain and movement asymmetry. Based on the results, it can no longer be assumed that higher movement asymmetry equals higher pain intensity level for mild to moderate lameness, since the relationship is complex and nuanced and the experience of pain is most probably individual. The horse's ability to respond to the pain experience by creating a weight-shift that offloads the lame limb is most likely also individual. Vertical asymmetry might therefore not be a perfect depiction of this weight-shift.

By adding assessment of body behaviors and facial displays to lameness examination, veterinarians could obtain valuable information on the level of pain experienced by the horse. However, the results in this thesis do not make it possible to identify movement asymmetries due to pain in horses perceived as sound by their owners. Questions that remain to be explored include:

- Which body behaviors and facial expressions are associated with orthopedic pain in spontaneously lame horses?
- Do the behaviors differ between environments?
- Which behaviors occur in the healthy horse in these environments?
- Does the level of facial expressiveness differ between individual horses, breeds, and sexes?
- What is the optimal proxy for orthopedic pain in horses?
- How are facial displays of pain influenced by observer presence during rest and motion?

7.2 Future perspectives

While several research questions remain to be answered in order to fully understand the relationship between movement asymmetry and orthopedic pain, the conclusions drawn in this thesis can already be implemented in equine welfare assessments, not by specifically including identified behaviors, but by being aware of the complexity in assessing pain. One approach could be to avoid binary categories such as 'no pain' and 'pain', and instead introduce probabilities for the presence of pain, summarized by several proxies. It may even be slightly naïve to believe that welfare protocols comprising one measure, such as a subjective pain assessment, can successfully establish whether pain is present or not. A welfare tool including one pain assessment tool is obviously better than not assessing pain at all, but it would be inadvisable to believe that such a welfare tool is sensitive enough to identify most horses in pain. This is an important area that requires further research on how to adapt and develop welfare assessment tools with high sensitivity and specificity to pain, capturing the nuanced pain experience.

The results of this thesis may also be used in the emerging field of automated recognition of animal behavior, including computer vision and machine learning (Andersen *et al.*, 2021). Since live assessment of facial expressions is associated with low-moderate reliability, EquiFACS was found to be a powerful method for describing changes in facial activities in this thesis. However, it is not applicable in clinical settings, as most AUs/ADs identified in facial displays of pain are difficult to detect in real-time. With proper training and experience, the equine veterinarian might learn to identify grimaces that include asymmetrical ears, and rapid movements and separation of the upper and lower lips in resting horses, but they would not identify the *half blink* (AU47), which only occurs for <0.5 seconds. It would also be challenging to detect short shows of the sclera, interrupted by blinks, and co-occurring with lower face activity in trotted horses. In future, computer vision and machine learning can help to automatically detect the face in a video (Rashid *et al.*, 2018), as done in Paper III, and other facial key point detection software may be applied to detect AUs/ADs in the video material. Indeed, this is already happening, as summarized in a related paper to this thesis (Andersen *et al.*, 2021). In addition to details described in Chapter 4 of this thesis, as part of another study the horses in the dataset were video-recorded from the side when standing calmly outside the box stall directly before or after each live pain assessment (Broomé *et al.*, 2022). A machine learning model was trained using a previous pain dataset (Gleerup *et al.*, 2015) and then used for pain detection in the LPS dataset, while equine veterinarians were also allowed to subjectively assess the same videos as the machine learning model. It was found that the model outperformed the veterinarians in detecting pain based on facial activities (Broomé *et al.*, 2022). Video surveillance recordings of the horses in the present dataset have also been used to detect pain from pose estimation (Rashid *et al*., 2022). My hope is that the dataset in this thesis will assist in developing machine learning methods for equine pain detection, since research already shows promising results. Upcoming studies on induced pain in equines should also bear in mind how the dataset obtained may be used for machine learning, so that fewer animals are subjected to pain induction.

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Popular science summary

Orthopedic disorders are very common in horses, with lameness as the main clinical sign of pain. From an equine welfare perspective, early detection of orthopedic disorders is very important and serious concerns are continuously raised about the welfare of sports horses. Unfortunately, there are several challenges with assessing pain and wellbeing in horses, and reliable and valid assessment tools are lacking. It has previously been assumed that movement asymmetry in horses arises from pain and orthopedic disorders, but recent research shows that a large proportion of horses in training and perceived as sound by their owners, do not move symmetrically. It is therefore difficult to identify which horses with movement asymmetry are actually experiencing pain, and to determine whether degree of movement asymmetry is associated with pain intensity. One way to detect pain in these horses could be to assess changes in body behaviors and facial expressions, which are known to be associated with moderate to severe orthopedic pain and are used as a basis in pain scales for assessing this pain objectively. However, whether resting horses and moving horses with mild orthopedic pain, i.e. low-grade lameness, perform behaviors and facial expressions associated with orthopedic pain remains to be determined.

The overall aim of this thesis was to identify body behaviors and facial expressions associated with orthopedic pain in horses, evaluate the performance of pain scales for assessing orthopedic pain, and identify changes in facial activities that occur during different levels of orthopedic pain intensity and during rest and motion. Data were obtained in an experimental study where mild to moderate transient hindlimb lameness was induced in eight horses. Progression and regression of lameness in these horses was measured with objective gait analysis, and pain during rest was assessed with pain scales. The horses were monitored with surveillance cameras in their box stalls and video-recorded from the side when trotted during each objective gait analysis. Facial activities in the video material were annotated (coded) with EquiFACS (Equine Facial Action Coding System).

Body behaviors strongly associated with orthopedic pain in resting horses were: altered posture, head position, location in the box stall, focus, and level of interaction. Facial expressions were also associated with orthopedic pain, but less reliably than body behaviors. One of the pain scales had high reliability and accuracy in determining whether a horse was in pain or not. Pain scores were associated with increased movement asymmetry for all scales, but there was a delayed increase in pain scores relative to the increase in movement asymmetry.

Several changes in facial activities were associated with orthopedic pain and complex combinations were identified during rest and motion. Resting horses combined asymmetrical ears with half blink and moving the lips when they experienced pain. When trotted by hand, they combined moving the lips with blinks and showing the eye white when pain was experienced. These changes in facial activity varied with the level of pain intensity and between individuals, especially during motion.

The results obtained in this thesis improve understanding of behaviors to look for in resting and moving horses with orthopedic pain. Some challenges with assessing pain in horses were identified, for instance choosing the correct pain scale, the possible presence of low reliability and the fluctuating nature of orthopedic pain. Changes in facial activities can identify orthopedic pain in resting and moving horses, but may be difficult to observe and document in real-time. Many horses with orthopedic disorders experience chronic pain, so future research should focus on identifying body behaviors and facial expressions performed by these horses. Methods described in this thesis can be applied in future research with similar aims, while the extensive annotation dataset obtained in this thesis could assist in the development of computer vision and machine learning approaches for detection of pain in horses. Computer vision or machine learning approaches could then be applied to identify changes in facial activity in horses, ideally in combination measures of movement asymmetry. Furthermore, the findings obtained in this thesis can be applied by animal health personnel and/or horse owners to improve the accuracy of pain assessments, thereby contributing to improved equine welfare.

Populärvetenskaplig sammanfattning

Hästar drabbas ofta av ortopediska skador, där hälta är det vanligaste symptomet på smärta. Ur ett djurvälfärdsperspektiv är det viktigt att kunna upptäcka de hästar som är skadade och som har ont i ett tidigt skede av det ortopediska sjukdomsförloppet. Stort fokus läggs just nu på djurvälfärden inom hästsport och svårigheterna kring att bedöma hästarnas välmående. Det saknas verktyg för att på ett tillförlitligt och säkert sätt kunna avgöra när en häst har ont. Tidigare har man antagit att hästar som rör sig asymmetriskt har ont, men ny forskning visar att även ett stort antal hästar i träning, som antas vara friska av sina ägare, rör sig asymmetriskt. Det är oklart vilka hästar som har ont och om man kan förlita sig helt på graden av asymmetri för att avgöra om hästen har ont eller inte. Ett sätt att identifiera smärta är att bedöma förändringar i beteenden och ansiktsuttryck hos hästar, till exempel med smärtskalor. Förändringar i beteenden och ansiktsuttryck har tidigare kopplats till måttlig till kraftig ortopedisk smärta hos hästar, med det är okänt om och hur hästar med mild ortopedisk smärta, motsvarande en låggradig hälta, ändrar sina beteenden och ansiktsuttryck i vila och rörelse.

Syftet med denna avhandling var att undersöka vilka beteenden och ansiktsuttryck hästar med ortopedisk smärta visar, utvärdera vilka smärtskalor som kan användas för att bedöma denna smärta, samt förstå hur ansiktsuttryck varierar vid olika grader av smärta, samt i vila och rörelse. Avhandlingen baseras på data från ett experimentellt försök där mild till måttlig övergående bakbenshälta inducerades hos åtta hästar. Ökningen och minskningen av hältan mättes med objektiv rörelseanalys och smärtan hästarna upplevde i vila bedömdes med smärtskalor. Hästarna övervakades även av kameror i sina boxar samt filmades från sidan vid varje rörelseanalys. Ansiktsuttryck som förekom i videomaterialet kodades med kodsystemet EquiFACS (Equine Facial Action Coding System).

Vissa beteenden var starkt kopplade till ortopedisk smärta i vila, så som: om hästen avlastade det onda benet, hur huvudet hölls i förhållande till manken, var hästen stod i boxen, samt om den hade fokus på och interagerade med omgivningen. Ansiktsuttrycken förändrades också när hästarna hade ont, men bedömdes med lägre samstämmighet mellan bedömarna än beteendena. En utav smärtskalorna hade god samstämmighet mellan bedömarna och det var lättast att avgöra om en häst hade ont eller inte med den här skalan. Smärtpoängen var relaterad till graden av rörelseasymmetri för alla skalor, även om det sågs en försenad ökning i smärtpoäng jämfört med ökningen i rörelseasymmetri.

Flera ansiktsuttryck, kodade med EquiFACS, var relaterade till smärta där komplexa kombinationer sågs både i vila och rörelse. I vila kombinerade hästarna ett öra framåt och ett öra bakåt (asymmetriska öron) med en halv blinkning och rörelser av läpparna när de hade ont. I trav kombinerade hästarna rörelser av läpparna med blinkningar och synlig ögonvita när de hade ont. Kombinationen av ansiktsuttryck verkade även variera med graden av smärta och det sågs en stor individuell variation främst i rörelse i hur många ansiktsuttryck hästarna visade.

Resultaten från denna avhandling bidrar till en ökad förståelse kring vilka beteenden och ansiktsuttryck som ses hos hästar i vila och rörelse när de upplever ortopedisk smärta. Även svårigheterna kring smärtbedömning lyfts fram, såsom att välja rätt smärtskala, att låg samstämmighet kan uppstå mellan bedömare, samt att hästar upplever varierande grad av smärta när de står stilla kan påverka bedömningen. Ansiktsuttryck kan användas för att identifiera smärta i vila och rörelse, men är komplexa och kan vara svåra att se utan att titta på videomaterial. Många hästar med ortopediska skador upplever kronisk smärta, varför framtida forskning bör kartlägga vilka beteenden och ansiktsuttryck som ses i vila och rörelse hos dessa hästar. De metoder som beskrivits i den här avhandlingen kan då till fördel användas. Avhandlingens omfattande kodningsdata skulle även kunna bidra till att utveckla maskininlärningsmetoder för smärtdetektion hos häst, utifrån de ansiktsuttryck hästen visar. Därefter skulle ansiktsuttryck kunna undersökas vidare hos de hästar som rör sig asymmetriskt men som inte misstänks vara smärtpåverkade. Resultaten från denna avhandling kan även användas av djurhälsopersonal och djurägare så att bedömningen av ortopedisk smärta förbättras och därmed även djurvälfärden inom hästsport.

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animals

Article

Identification of Body Behaviors and Facial Expressions Associated with Induced Orthopedic Pain in Four Equine Pain Scales

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Simple Summary: Pain scales are tools developed to improve pain assessment in horses. They are based on behaviors and/or facial expressions, and the observer allocates a score based on the character of the behavior or facial expression. Little is known about behaviors and facial expressions at rest in horses with orthopedic pain since pain is mainly assessed by lameness evaluation during movement. The aim of this study was to describe how closely equine behaviors and facial expressions are associated with movement asymmetry and to identify combinations of behavior and expressions present in horses with induced orthopedic pain. Orthopedic pain was induced in eight horses and assessed in two ways; using four existing equine pain scales at rest, and by measuring movement asymmetry during movement. The association of behavior and facial expression items in the pain scales with actual lameness was analyzed. Posture-related behavior showed the strongest association, while facial expressions varied between horses. These results show that pain scales for orthopedic pain assessment would benefit from including posture, head position, location in the box stall, focus, interactive behavior, and facial expressions. This could improve orthopedic pain detection in horses during rest with mild lameness.

Abstract: Equine orthopedic pain scales are targeted towards horses with moderate to severe orthopedic pain. Improved assessment of pain behavior and pain-related facial expressions at rest may refine orthopedic pain detection for mild lameness grades. Therefore, this study explored pain-related behaviors and facial expressions and sought to identify frequently occurring combinations. Orthopedic pain was induced by intra-articular LPS in eight horses, and objective movement asymmetry analyses were performed before and after induction together with pain assessments at rest. Three observers independently assessed horses in their box stalls, using four equine pain scales simultaneously. Increase in movement asymmetry after induction was used as a proxy for pain. Behaviors and facial expressions commonly co-occurred and were strongly associated with movement asymmetry. Posture-related scale items were the strongest predictors of movement asymmetry. Display of facial expressions at rest varied between horses but, when present, were strongly associated with movement asymmetry. Reliability of facial expression items was lower than reliability of behavioral items. These findings suggest that five body behaviors (posture, head position, location in the box stall, focus, and interactive behavior) should be included in a scale for live assessment of mild orthopedic pain. We also recommend inclusion of facial expressions in pain assessment.

Keywords: horses; movement asymmetry; movement symmetry; lameness; reliability; pain predictor; pain indicator

1. Introduction

Pain scales are available as assessment tools for horses in pain and generally comprise composite-measure pain scales assessing pre-selected body behaviors and/or facial expressions. Body behaviors have been extensively studied and reviewed in horses for general pain or specific types of pain, such as orthopedic or visceral pain [1–3]. Facial expressions have been used for pain assessment in humans for many years, and have now been successfully introduced into pain assessment in horses, for acute pain types [4–7]. Behaviors and facial expressions are commonly seen together in horses experiencing pain [4], but can be difficult to assess. How well pain-related behaviors are expressed can depend on personality [8] and may be suppressed in response to an environment with possible threat [9]. Pain-related facial expressions can shift in presence as pain varies over time and can be influenced by the age of the animal, other affective states, or whether the pain is of an acute or chronic nature [10].

Scale reliability is important in clinical settings for scales to give reproducible results, independent of the observer. However, the level of reliability does not necessarily correlate to items that are good pain indicators. For lower degrees of pain, less distinct changes in behaviors or facial expressions may increase the variation in pain scores between observers, resulting in lower reliability. Good reliability obtained in studies with higher pain intensities should therefore not be generalized to studies with mild pain intensities, since the study groups are different [11]. So far, no study has evaluated reliability for scale items when low-degree orthopedic pain is assessed.

Current equine orthopedic pain scales are targeted towards horses with moderate to severe pain due to orthopedic surgery or laminitis [12–14]. Mild orthopedic pain of acute and chronic origin is mainly assessed subjectively, through evaluation of lameness grade during movement, and objectively, by kinetic or kinematic methods. It is commonly assumed that a higher lameness grade or movement asymmetry is equal to a higher degree of pain. However, this is not the case in human studies, where more complex relationships between pain and movement asymmetry are demonstrated. Both linear and non-linear positive relationships between biomechanical parameters (trunk asymmetry, vertebral motion, and range of motion in different joints) and pain have been shown [15–17], but also no relationship [18] or a negative relationship [19] between knee biomechanics and pain. Hence, a positive linear relationship between the magnitude of movement asymmetry or lameness and pain intensity should not be assumed. It may be assumed that horses at rest are in less pain, since they can decrease the load on the painful limb to a greater extent than is possible for horses in motion. The type of pain (acute or chronic) probably plays an important role in this regard. In acute pain, nociception occurs due to the inflammatory process and pain in an inflamed joint can be reduced by decreasing the load on the joint. This may result in reduced pain behaviors and facial expressions, suggesting that posture-related behaviors in acute orthopedic pain may be more stable than e.g., facial expressions. In chronic pain, central sensitization is often present, resulting in expansion of the painful area and an increase in pain intensity, which can be accompanied by stress, fatigue, and depression in humans [20,21]. Thus, decreased loading may not always be enough to alleviate pain, and horses at rest could then experience a high pain intensity, and display related pain behaviors and facial expressions. More research on pain behavior related to acute and chronic mild orthopedic pain in horses is needed to understand the relationship between different pain-related behaviors and facial expressions, and how they are affected by acute and chronic orthopedic pain. An improved assessment of pain behavior at rest could refine orthopedic pain detection.

Hence, for mild orthopedic pain, low-grade lameness during movement may be the only sign of pain observed and the lack of a gold standard for pain hampers determination of sensitivity and specificity for different scale items. In this study, we therefore used movement asymmetry as a proxy for orthopedic pain [11], assuming that increasing movement asymmetry post-induction was associated with presence of pain. The aim of this study was to investigate the relationship between scale items used in four equine pain scales and actual orthopedic pain. Specific objectives were to explore how well the scale item scores given at rest predicted lower degrees of movement asymmetry during movement, and to identify frequently occurring combinations of items in horses at rest with induced orthopedic pain. The reliability of each scale item was also evaluated. The first hypothesis tested was that a combination of behaviors and facial expressions is associated with movement asymmetry, since they commonly occur together during pain. We suspect that posture-related behaviors may reduce pain and other pain-related behaviors or facial expressions, why the second hypothesis tested was that assessment of body behaviors is more reliable than assessment of facial expressions.

2. Materials and Methods

The experimental protocol was approved by the Swedish Ethics Committee in accordance with the Swedish legislation on animal experiments (diary number 5.8.18-09822/2018). The study was designed to serve several purposes and the 3R's were thoroughly considered designing the study. As few horses as possible were included and a fully reversible lameness induction model was used. It was important to induce lameness and let each horse be its own control, to achieve a standardized design and limit the variation in pain behavior between the horses.

2.1. Subjects

Lameness was inducted in six mares and two geldings (seven Standardbred trotters and one warmblood; mean ± SD age = 14.5 ± 3.7 years, mean ± SD body mass = 552 ± 39 kg and mean ± SD height at withers = 160 ± 2.78 cm). All horses were owned by the university or bought for/donated to the experiment. Before the experiment, the horses underwent a full clinical examination and subjective and objective lameness evaluations. All showed no signs of disease or >1 grade of lameness on a 0–5 lameness ordinal scale, where 0 = sound and 5 = non-weight bearing lameness. The 10–12 days immediately preceding lameness induction consisted of an acclimatization period with daily turnouts in a paddock, walker exercise, and handling and training. The horses were housed individually in box stalls with sawdust bedding. They were fed with hay three times a day and concentrate twice a day. The handling and training focused on positive reinforcement, to acclimatize the horses to palpation of the limbs, handling in different environments and lunging. The horses were all dewormed and hoof-trimmed during the first days of acclimatization.

2.2. Experimental Design and Induction of Orthopedic Pain

The last day of the acclimatization period contained an objective movement analysis to determine baseline movement asymmetry and what hindlimb to induce. The hindlimb with highest movement asymmetry was chosen for induction. Pain assessments in the box stall were also performed to determine baseline pain scores. One or two days later, mild to moderate orthopedic pain was induced early in the morning. After induction, the horse was taken back to its box stall for rest for 1.5 h, before the first pain assessment and movement measurement were performed. A minimum of three occasions with movement measurements and pain assessments were performed post-induction. Measurements were considered complete when each horse had returned to movement asymmetry similar to that of the baseline measurement.

Lipopolysaccharides (LPS) from E. coli O55:B5 (stock solution 1 mg/mL) were used to induce an acute inflammatory arthritis. Ready-made LPS solution (*L5418 Sigma*) was diluted with 0.9% sodium chloride to a final volume of 3 mL and a stock concentration of 1.167 ng/mL. The diluted solution was stored at −20 °C until the day of induction, when it was thawed and vortexed vigorously before intra-articular administration into the dorsomedial pouch of the tarsocrural joint. Routine aseptic techniques were used, where the horses were clipped and scrubbed on both hindlimbs. If a bandage or wound plaster was used after injection, it was added to the other hindlimb as well. A minimum of 3 mL synovia was extracted from the joint before LPS administration. If the induction resulted in a lameness grade >3 at trot, a protocol for rescue analgesia was initiated, comprising arthrocentesis and evacuation of synovia to reduce the intra-articular joint pressure and inflammatory pain.

2.3. Objective Movement Analysis

A movement measurement consisted of straight line walk and trot on hard and soft surface and lunging on soft surface. If movement asymmetry increased during the measurement, a second hard straight-line trot was performed. Each horse was equipped with seven skin-mounted spherical markers (38 mm diameter, Qualisys AB, Gothenburg, Sweden). Thirteen infrared optical motion capture cameras (Qualisys AB, Gothenburg, Sweden) recorded marker positions in 3D at 200 Hz and QTM software (version 2.11-2019.3, Qualisys AB, Gothenburg, Sweden) was used to track the positions of the markers. After visual inspection of tracking results, the data were exported and analyzed with custom-written scripts in MatLab [22]. Filtering was performed with a fourth-order zero-phase Butterworth filter, where the cut-off frequency was adjusted to the stride frequency of the horse [23]. Stride segmentation was based on peak detection of the vertical movement of the tubera sacrale, and left and right stride detection were performed using algorithms based on expected pelvic roll and yaw rotations of the tubera coxae [24]. Hard straight-line trot data from one marker placed over the poll and one marker placed between the tubera sacrale were included for further analysis. Vertical displacement asymmetry of the tubera sacrale is a well-established measure for hindlimb lameness [25], where pelvis reaches a lower position during the sound hindlimb stance. Vertical displacement asymmetry of the poll can be seen in some horses that reduce the weight on the lame hindlimb by shifting the weight forward, and the head and neck is lowered during the lame diagonal stance. Data from the other markers were collected for studies of more biomechanical focus. The difference between the two vertical displacement minima for each stride was calculated for head (HD_{min}) and pelvis (PD_{min}) in a way that assigned negative values to left-sided asymmetries. Mean total asymmetry for each measurement was computed as the sum of the absolute values for $HD_{min}/2$ and PD_{min}. The change in total movement asymmetry between baseline and induced measurements was then calculated and defined as total asymmetry score for each measurement. In addition, one or two experienced equine veterinarians subjectively graded the lameness during each movement measurement. An ordinal 0–5 lameness scale was used.

2.4. Pain Assessments

Pain assessments were performed by direct observation at rest in the stable approximately 20 min before and 20 min after each movement asymmetry measurement. During this time, the horse was equipped/unequipped. For each pain assessment, three pain evaluators stood outside the box stall and performed simultaneous and independent live pain assessments on the same horse. Five pain evaluators took part in the study, two of whom (observers 1 and 2) were present for all assessments. Observers 3–5 changed between horses, based on availability. The observers consisted of three veterinarians, one agronomist, and one ethologist, and all had private or professional equestrian experience. Prior to pain assessments, the evaluators familiarised themselves thoroughly with the pain scales, using published available score sheets, descriptions, and scientific reports. Only observer 1 participated during the induction, while the other observers only saw the horses in their box stalls during pain assessment. They were therefore blinded to the limb of induction and the increase in movement asymmetry post-induction.

Four equine pain scales were used, in the following order: Horse Grimace Scale (HGS) [4], Equine Utrecht University Scale of Facial Assessment of Pain (EQUUS-FAP) [5,6,14], Equine Pain Scale (EPS) [26] and Composite Pain Scale (CPS) [12]. The HGS and EQUUS-FAP scales primarily assess facial expressions and have been used previously to assess orthopedic pain of moderate to a severe degree. EPS assesses body behavior and presence of pain face and has been recommended for general pain. CPS assesses body behavior and physiological parameters and has been used for assessment of post-surgical orthopedic pain. The scales contain six to 13 items (see Supplementary Materials, Table S1) and item scores range from 0 to 2 for HGS and EQUUS-FAP to 0–4 for EPS and 0–3 for CPS. The EQUUS-FAP, EPS, and CPS scales are designed for direct (live) scoring, while HGS is designed for indirect (video) scoring [4,13]. The pain assessments with HGS, EQUUS-FAP, and EPS lasted for two minutes each.

The assessments with CPS lasted for five minutes, where the last two minutes consisted of measuring physiological parameters and palpation of the limbs.

2.5. Statistics

Statistical computations and analyses were executed in R [27]. Data from movement measurements performed until each horse reached the maximum total asymmetry score, and associated pain assessments, were included for further analyses. The highest increase in movement asymmetry was determined manually for each individual. Pain assessments performed before and after selected movement measurements were included. Mean and standard deviation were computed for item scores and movement asymmetry data. Reliability of each scale item was analyzed with Kendall's coefficient of concordance (W) [28] for agreement of ordinal ranking data [29]. Physiological parameters of CPS were excluded for reliability testing since they were objectively measured. The role of scale items in predicting movement asymmetry was explored with Lasso regression models, that were fitted using the package 'glmnet' [30] (alpha = 1), to identify the combination of predictors associated with total asymmetry score for each scale and for all scales in the dataset. The models were fitted with 10-fold cross-validation. Scale items were analyzed as factors, while horse and observer were included as fixed effects to account for individual variation. Scores from pain assessments pre- and post-movement measurements were included. When there were two straight-line trot measurements, both were included and the first associated with the pre-pain assessment and the second with the post-pain assessment. The lambda generating models with the minimum mean cross-validated error was selected. To further assess the associations between scale items from different scales, multiple correspondence analysis (MCA) was performed using the package 'FactoMineR' [31]. The 33 largest dimensions (explaining a minimum of 1% of the variation within scale items) were then included in a linear regression with movement asymmetry as the outcome. The model was reduced using Akaike information criterion (AIC) and the components of the significant ($p < 0.01$) dimensions in the final model were interpreted. Horse and observer were included as random effects and the distribution of the residuals was controlled for signs of temporal autocorrelation in the final model. Results were plotted using 'ggplot2' [32].

3. Results

Mean (±SD) number of movement measurements per horse was 4.25 (±1.04) and mean number of pain assessments was 6.88 (±1.46). Three left and five right hindlimbs were induced and mean (±SD) maximum increase in total asymmetry score was 61 mm (±24 mm). Mean (± SD) PD_{min} value was 3 mm (±3 mm) for baseline measurements, and 46 mm (±20 mm) for measurements where the maximum movement asymmetry was reached. The baseline subjective lameness score was 0 for all horses, except two that had 0.25 and 0.5 grades respectively. The maximum subject lameness score varied between 2 and 4 grades (mean 2.94 and SD 0.78). Changes in total asymmetry score and PD_{min} can be seen in detail in Supplementary Materials File S2. Rescue analgesia (reducing synovial volume by arthrocentesis) was performed in two horses. A total of 37 scale items were included in the dataset, some of which were similar despite originating from different scales. Mean (±SD) item scores before and after induction are presented in Supplementary Materials Table S1. Figures 1–4 illustrate the distribution of item scores and that item scores of zero were present for all degrees of movement asymmetry. However, certain items stood out and only achieved scores above zero when movement asymmetry increased. This was seen especially for 'ears' and 'nostrils' in HGS (Figure 1); 'head', 'focus' and 'ears' in EQUUS-FAP (Figure 2); 'location', 'posture', 'pain face', 'gross pain behavior' and 'head' in EPS (Figure 3); and 'pawing', 'head', 'appearance', 'posture' and 'response to palpation' in CPS (Figure 4). Physiological parameters in CPS had also scores >0 when movement asymmetry increased (Figure 4).

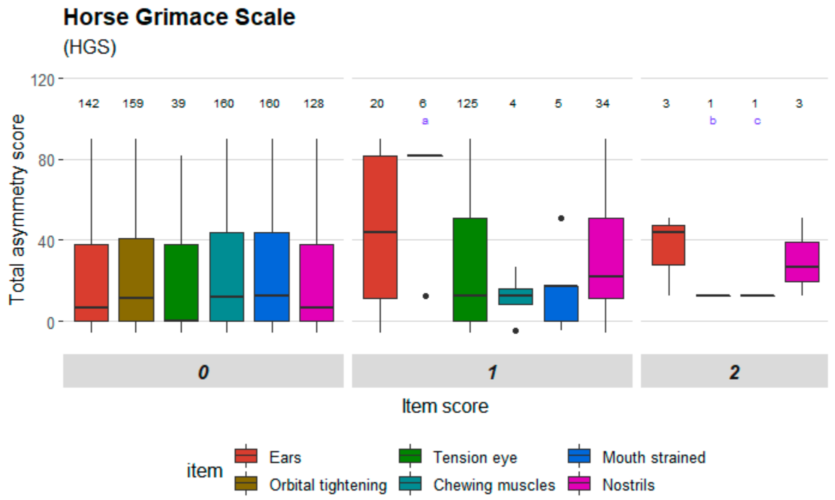

Figure 1. Distribution of item scores for Horse Grimace Scale (HGS). Total asymmetry score is presented on the y-axis. Scale items are presented on the x-axis and divided into the item scores given (ranging from 0 to 2). Item scores for all observers are included and number of scores (*n*) is stated above each box. Outliers are included and shown as black dots. The black line in the boxes shows the median and the upper and lower ends of the boxes show the upper and lower quartile. The upper and lower whiskers show the highest and lowest 25% of the data. Some of the boxes contain few observations and low spread, and appear as horizontal lines where the color is not visible. Instead, they are marked with letters in the diagram: a—orbital tightening, b—tension above the eye area, **c**—prominent strained chewing muscles.

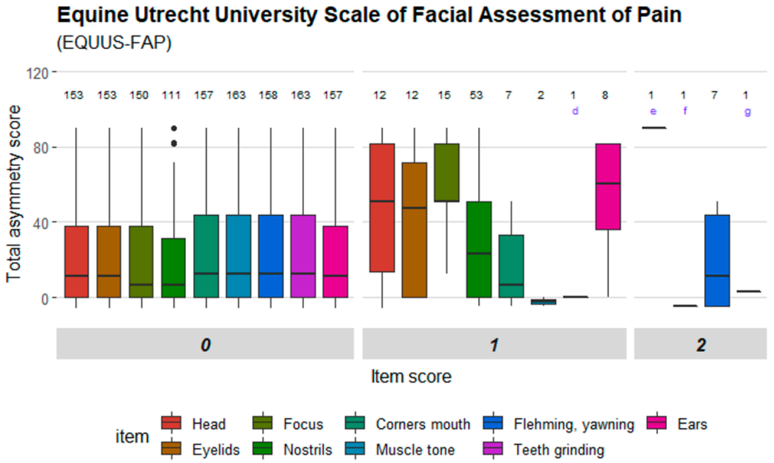

Figure 2. Distribution of item scores for Equine Utrecht University Scale of Facial Assessment of Pain (EQUUS-FAP). Total asymmetry score is presented on the y-axis. Scale items are presented on the x-axis and divided into the item scores given (ranging from 0 to 2). Letters in the diagram: d—teeth grinding/moaning, e—nostrils, f—corners of mouth/lips, g—teeth grinding/moaning.

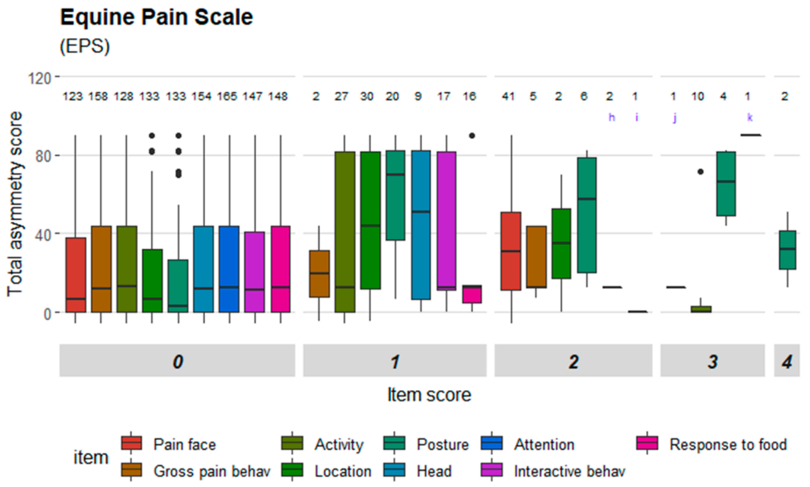

Figure 3. Distribution of item scores for Equine Pain Scale (EPS). Total asymmetry score is presented on the y-axis. Scale items are presented on the x-axis and divided into the item scores given (ranging from 0 to 4). Letters in the diagram: h—head position, i—interactive behavior, j—pain face, k—response to food.

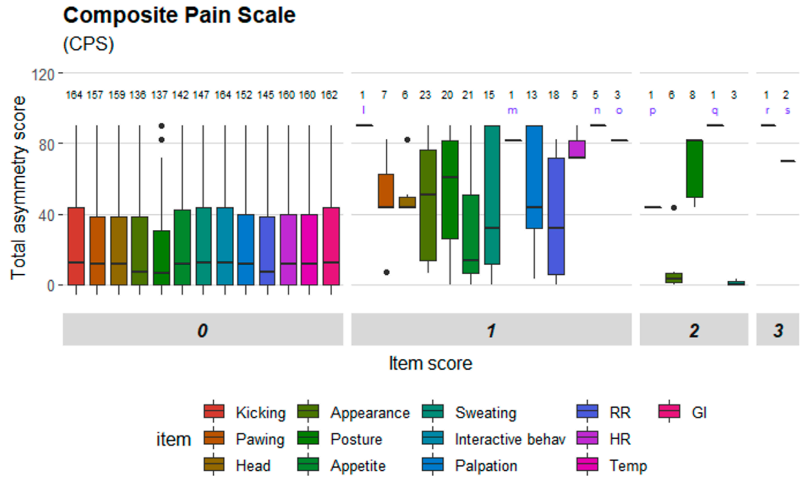

Figure 4. Distribution of item scores for Composite Pain Scale (CPS). Total asymmetry score is presented on the y-axis. Scale items are presented on the x-axis and divided into the item scores given (ranging from 0 to 3). Letters in the diagram: l— kicking abdomen, m—interactive behavior, n—rectal temperature, o—digestive sounds, p—pawing on the floor, q—appetite, r—appetite, s—respiratory rate.

Agreement between observers was considered very strong for W > 0.9, strong for W 0.7–0.9, moderate for W 0.5–0.7, and weak or very weak for W < 0.5 [33]. One scale item in HGS showed strong agreement ('orbital tightening'), while two scale items showed moderate agreement and three poor agreement. Two scale items in EQUUS-FAP showed strong agreement ('focus' and 'flehmen and/or yawning') and seven items had weak agreement. Five items in EPS had strong agreement ('gross pain behavior', 'activity', 'posture/weight bearing', 'interactive behavior' and 'response to food'), one item showed moderate agreement and three items weak agreement.

'Sweating' in CPS was the only item with very strong agreement, and four items in CPS had strong agreement ('pawing', 'posture', 'appetite', 'response to palpation'). Two items had moderate agreement and two items had weak agreement. The coefficients are presented in Table 1.

Table 1. Inter-observer agreement estimated with Kendall's coefficient of concordance (*W*) for the four pain scales tested. Pain scores for each scale item and three observers are included.

Scale	Scale Item	*W*	*p*-Value
Horse Grimace Scale (HGS)	Stiffly backward ears (ears)	0.567	<0.001 ***
	Orbital tightening (orb)	0.794	<0.001 ***
	Tension above the eye area (ten)	0.470	0.025 *
	Prominent strained chewing muscles (chew)	0.421	0.091
	Mouth strained and pronounced chin (mouth)	0.418	0.099
	Strained nostrils and flattening of the profile (nost)	0.575	<0.001 ***
Equine Utrecht University Scale of Facial Assessment of Pain (EQUUS-FAP)	Head (head)	0.383	<0.001 ***
	Eyelids (eye)	0.433	0.068
	Focus (focus)	0.819	<0.001 ***
	Nostrils (nost)	0.405	0.134
	Corners mouth/lips (mouth)	0.316	0.584
	Muscle tone head (tone)	0.329	0.500
	Flehmen and/or yawning (fleya)	0.751	<0.001 ***
	Teeth grinding and/or moaning (teeth)	0.333	0.474
	Ears (ears)	0.376	0.242
Equine Pain Scale (EPS)	Pain face (pf)	0.428	0.078
	Gross pain behavior (gross)	0.753	<0.001 ***
	Activity (act)	0.722	<0.001 ***
	Location in the stall (loc)	0.605	<0.001 ***
	Posture/weight bearing (pos)	0.743	<0.001 ***
	Head position (head)	0.409	0.122
	Attention towards painful area (att)	0.333	0.474
	Interactive behavior (int)	0.775	<0.001 ***
	Response to food (food)	0.881	<0.001 ***
Composite Pain Scale (CPS)	Kicking abdomen (kick)	0.333	0.474
	Pawing on the floor (paw)	0.763	<0.001 ***
	Head movement (head)	0.538	<0.003 **
	Appearance (app)	0.646	<0.001 ***
	Posture (pos)	0.741	<0.001 ***
	Appetite (app2)	0.837	<0.001 ***
	Sweating (sweat)	0.920	<0.001 ***
	Interactive behavior (int)	0.333	0.474
	Response to palpation of painful area (palp)	0.844	<0.001 ***

Significant coefficients are indicated as: ns = $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviations used for statistical analysis are stated in brackets for each item. Only behavioral items in CPS are included.

The results from the Lasso regression indicated that the combination of following scale items within each scale were most strongly associated with the total asymmetry score: 'orbital tightening' for HGS, 'focus' for EQUUS-FAP, 'posture' for EPS, and 'temperature' and 'posture' for CPS (Figure 5). For all scales, horses h1–h8 seemed to be relatively strongly associated with total asymmetry score and horse h4 in particular had a strong association on three scales. The effects of horse and observer were accounted for in scale items associated with total asymmetry score. Scale items negatively associated with total asymmetry score were 'teeth grinding' (EQUUS-FAP), 'gross pain behavior' (EPS) and 'sweating' (CPS). The model best describing changes in movement asymmetry with all scale items included was 'posture' in CPS and EPS, together with 'temperature' and 'heart rate' in CPS, and 'focus' in EQUUS-FAP (Figure 6).

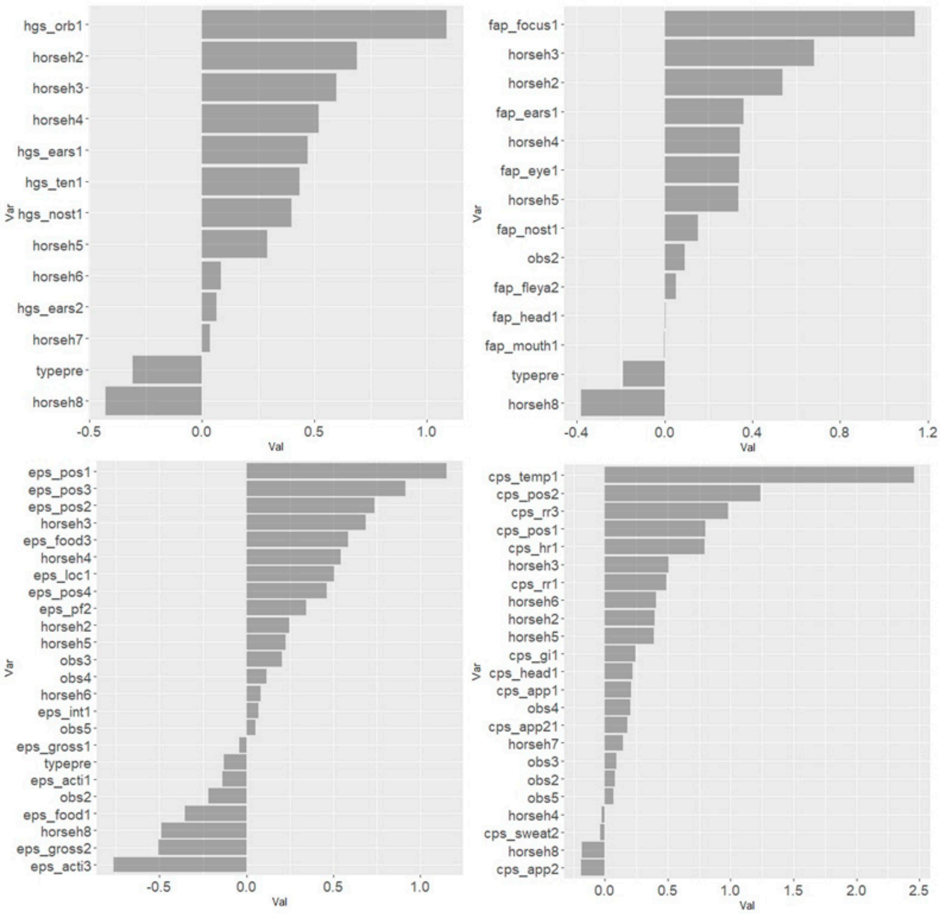

Figure 5. Coefficient of Lasso regression models with lambdas generating the minimum mean cross-validated error when using scale items to predict movement asymmetry. Scale items from the four equine pain scales (Horse Grimace Scale (HGS), Equine Utrecht University Scale of Facial Assessment of Pain (EQUUS-FAP), Equine Pain Scale (EPS) and Composite Pain Scale (CPS)) were analyzed in separate models. Scale items with a positive coefficient (to the right) were positively associated with total asymmetry score, used here as a proxy for orthopedic pain. The number after the scale item is the item score, for example hgs_orb1 means an item score of 1 for orbital tightening in HGS.

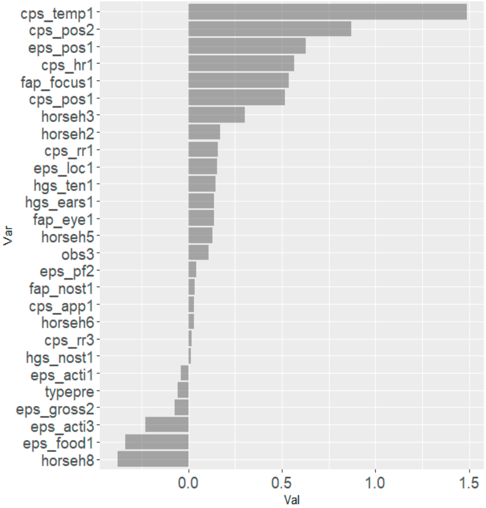

Figure 6. Coefficient of Lasso regression models with lambdas generating the minimum mean cross-validated error when using scale items of all scales to predict movement asymmetry. Scale items from the four equine pain scales (Horse Grimace Scale (HGS), Equine Utrecht University Scale of Facial Assessment of Pain (EQUUS-FAP), Equine Pain Scale (EPS) and Composite Pain Scale (CPS)) were analyzed in the same model.

Thirty-three MCA-dimensions explaining from 10% to 1% of the variation in the data were included in the linear regression. Nine dimensions were significantly associated with movement asymmetry (Table 2) and the composition of these dimensions is presented in Figure 7. Facial expressions and 'pain face' were seen together with 'interaction' and 'activity' parameters in horses with increased total asymmetry score in dimension 1, with 'gross pain behavior' and 'postural changes' in dimension 5, and 'gross pain behavior' in dimension 10. Dimension 29 was most strongly associated with movement asymmetry and involved an interesting combination of behaviors ('stiffly backwards ears', 'focus', 'posture', 'location' and 'appetite') and lack of pain-related facial expressions in horses with increased total asymmetry score. 'Interaction', 'gross pain behavior' and 'head position' were other behaviors not seen in horses in this dimension. A similar pattern was seen for dimension 8 and 9, where many facial expressions and 'gross pain behavior' had a negative association with movement asymmetry, while 'posture' and 'pawing' had a positive association. In dimension 2, facial expressions and 'pain face' were not seen in horses with increased total asymmetry score if they showed gross pain behaviors, were kicking, had lowered head and decreased appetite, and had increased temperature.

Table 2. Values of significant dimensions in multiple component analysis (MCA).

Dimension	Beta	SE	z-Value	*p*-Value
1	32.08	3.80	8.44	<0.001 ***
9	−28.66	6.30	−4.55	<0.001 ***
29	44.59	11.54	3.86	<0.001 ***
10	24.69	7.10	3.48	<0.001 ***
8	−21.84	6.39	−3.42	<0.001 ***
2	−16.97	5.32	−3.19	0.00141 **
5	17.28	5.87	2.94	0.00324 **
17	−24.34	8.62	−2.82	0.00477 **
11	19.42	7.54	2.58	0.01000 *

Significant coefficients are indicated as: ns = $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. SE = standard error of the mean. Variance of random effects: horse 0.25, observer 4.2×10^{-8}.

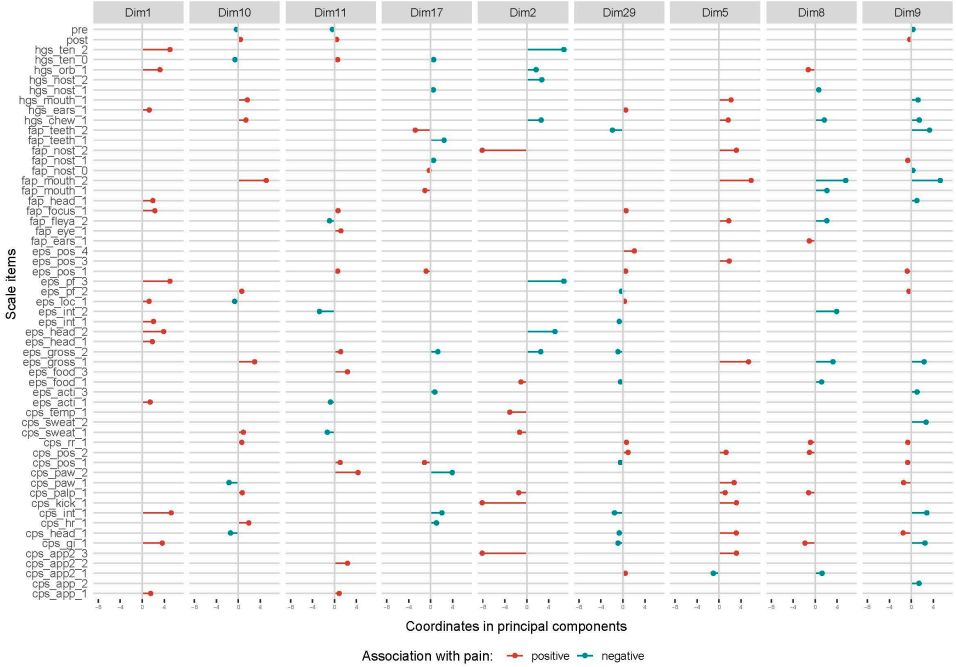

Figure 7. Results of multiple component analysis (MCA) illustrating the most significant dimensions. All scale items are included. The variance explained by each dimension is stated in the diagram. Items with red markers have a positive association with total asymmetry score, while items with blue markers have a negative association.

4. Discussion

The scale item most strongly associated with movement asymmetry when comparing all scale items in a Lasso regression model was 'body temperature' from CPS, closely followed by 'posture' (EPS and CPS) and 'heart rate' (CPS). Since movement asymmetry was induced by intra-articular administration of LPS, which is a pyrogen, it is not surprising that increased body temperature was a strong predictor of pain [34,35]. The association between heart rate and orthopedic pain is not consistent in studies [12,36], however heart rate is closely associated with body temperature [37,38]. This may indicate that increased heart rate in our subjects was associated with increased body temperature. The items 'focus' and 'flehmen and yawning' from EQUUS-FAP were also strong predictors. Facial expressions were in general not as strong pain predictors as behaviors. 'Orbital tightening' from HGS, and 'eyelids' and 'ears' from EQUUS-FAP had larger coefficients than other facial expressions in this study, but 'stiffly backwards ears', 'tension around the eye area', 'nostrils' and 'pain face' were also positively associated with pain. As the Lasso tends to select one variable in case of correlated variables, this indicates that separate expressions are important in themselves and that a combination of facial expressions indicates increased pain.

When association between scale items and pain was compared within each scale, 'posture' had large coefficients in both scales assessing posture (EPS and CPS). 'Tension around the eye area' and'stiffly backwards ears' from HGS, and 'eyelids' from EQUUS-FAP were the facial expressions with the largest coefficient, i.e., they were important variables for predicting change in movement asymmetry. Interestingly, 'gross pain behavior' from EPS was negatively associated with pain in this study, which contradicts published research results [26,36,39]. Behaviors included in 'gross pain behavior' in EPS are yawning, mouth playing, flehmen, stretching, kicking abdomen, tail swishing and sweating, where 'flehmen and/or yawning' from EQUUS-FAP was positively associated with pain. It is possible that some behaviors included in 'gross pain behavior' were present due to reasons other than pain, for instance emotional stress. When inspecting the scores given for 'gross pain behavior' in this study, three horses (h2, h3 and h8) had positive scores post-induction, but not during assumed maximum pain level, when total asymmetry score peaked. Since the frequency of positive scores was low, this can affect the statistical outcome and result in a negative prediction.

Interestingly, some of the individual horses' large coefficients in the Lasso regressions (Figure 5), are underlying the importance of individual characteristics when assessing pain. Theoretically, this is not surprising. Since pain is an experience and related to the *personality* of the horse [8], all horses cannot be expected to show the same frequency and intensity in pain behaviors, as is also the case for humans [40]. This adds to the limitations with small sample sizes in pain studies, where larger samples could have compensated for large individual variations.

Scale items occurring together varied greatly but, in general, body behaviors and facial expressions were seen together in horses experiencing pain. Facial expressions were positively associated with movement asymmetry in several of the dimensions derived from the MCA analysis, indicating that they are important indicators of pain. However, no dimension contained all facial expressions included in a pain face. Eye- and ear-related facial expressions were found in one dimension, and lower facial expressions in another. This is consistent with results from the Lasso regression indicating that facial expressions are not always correlated but add value individually. Four different combinations of facial expressions are reported to be present during pain in humans, some more stable than others [41], illustrating individual variations in how a pain face is expressed. The results in the present study may indicate similar variations in horses. In humans, upper facial expressions, such as brow lowering and nose wrinkling, are of more importance when assessing pain [42], and it is possible that the observer may subconsciously see these features more easily in horses but overlook other relevant facial expressions. Studies of facial expressions of pain in humans describe complex relationships between facial expressions and social context, with an unsafe environment or the presence of strangers sometimes seeming to decrease facial expressions, even during high pain intensity [43,44]. Whether such explanations are also valid for horses needs to be investigated further, for example by comparison of facial expressions with and without observers present. So far, discomfort behavior in general seem to decrease when caretaking staff are approaching equine patients [45]. We performed all pain assessments live, with three unknown observers present. Behaviors of an interactive character were seen together with upper facial expressions, while postural changes were seen with lower facial expressions (Figure 7). Lower facial expressions were also seen with gross pain behavior and may represent different pain intensities, since gross pain behavior is indicative of higher pain intensity. Facial expressions were not always present together with postural changes, even though total movement asymmetry was high. As discussed in the introduction, lame horses can be expected to modify their pain at rest, by simply avoiding situations that may increase pain intensity, for example loading of the painful limb. This may result in other behaviors occurring less frequently during pain [46,47]. The patterns seen in our MCA may confirm this theory since facial expressions rarely were seen when only posture-behaviors were present. Lowered head was also a behavior present in several dimensions and may indicate a depressed clinical state, a behavior often seen together with different pain intensities or in horses with pain for long duration and sleep deprivation [3]. Whether a depressive state is present in horses needs to be investigated for both acute and chronic pain. Scale items with few positive scores, such as 'temperature', 'sweating', 'response to palpation', 'kicking abdomen' and 'appetite', were clustered together. Behavioral changes in these items often indicate high pain intensity, but were seen in horses without pain face or gross pain behavior. However, little weight can be given to this clustering, due to the few positive scores.

Reliability was estimated for each scale item and surprisingly low agreement was found for three independent observers scoring all items from all scales. Low agreement may indicate difficulties in interpreting the scale items and/or difficulties in seeing what to score, leading to larger variance in the scores. If there were difficulties in seeing a facial expression, it can be argued that scale items assessing this expression still have the same level of reliability. Our results showed that for instance 'stiffly backward ears' from HGS and 'ears' from EQUUS-FAP have moderate vs. low agreement. This suggests that the item 'stiffly backwards ears' is easier to interpret than the item 'ears'. The same phenomenon was seen for some behavior items such as 'head position' in EPS, 'head' in EQUUS-FAP and 'head movement' in CPS, where low agreement was seen for items of EPS and EQUUS-FAP, while the item of CPS had moderate agreement. This may indicate and that more extensive introduction and training are needed to be able to interpret the items correctly [48] In addition, the long scoring sessions may have contributed to observer fatigue [49]. Nevertheless, there is a reason to believe that the pain scales may need improvement of the scale item definitions to be more user-friendly and to increase the reliability. The generally low reliability may affect the results in this study, and an important bias is that easily detected behaviors may have achieved higher scores compared to behaviors or facial expressions being harder to identify.

Inter-observer agreement for facial expressions was lower than that for body behaviors, which is an important finding for pain assessment quality in horses. It can be due to the scale limitations above, but it can also be due to other factors such as facial expressions being harder to identify for the human eye. It has been argued that humans have an innate tendency to focus on the face region, and that this could facilitate the use of facial expressions in monitoring welfare in rabbits [50]. This is apparently not the case for the species in this study. A possible bias may be the potential influence of observing different scale items, for example, lameness or gross pain behavior, when scoring other more difficult or subtle scale items. For instance, a facial expression may be scored differently depending on whether the observer has seen gross pain behavior or not. More analyses of the material are needed to investigate this potential bias effect and advice on whether facial expressions and body behaviors should be evaluated blinded to each other, or not. An option could be automated scoring of facial expressions from video or the application of more objective coding systems such as EquiFACS [51].

Of the 37 scale items evaluated in this study, several were included in more than one pain scale, but were weighted differently in the scale design. How items should be weighted may differ between pain intensities, since behaviors that are good indicators of pain at higher pain intensities are not necessarily good indicators at lower pain intensities. This could be overcome for instance by specifying the pain intensity for which a pain scale is designed or by introducing cut-off values for different pain intensities. The results of this study indicate that posture should be weighted higher than other behaviors for mild orthopedic pain at rest. It is however important to emphasize that only hindlimb lameness was induced in this study and that posture-related changes may show higher or lower associations with movement asymmetry if the lameness is located in the front limb. Whether the localization of the lameness should be included in an orthopedic pain scale cannot be determined from this study. Facial expressions seem to be of less value during rest, due to the variation observed together with postural changes.

5. Conclusions

This study showed that pain scale items related to posture at rest were the strongest behavioral predictors of movement asymmetry in horses with mild orthopedic pain. Behaviors and facial expressions commonly occurred together and were strongly associated with movement asymmetry. The study also showed that the presence of facial expressions at rest can vary in horses with low-grade lameness but, when present, facial expressions were strongly associated with movement asymmetry. Reliability was lower for facial expression items than for behavioral items, indicating that it can be difficult to assess facial expressions by direct observation.

The results obtained suggest that pain scales combining facial expressions with body behaviors should be used when performing direct pain assessment of horses with mild orthopedic pain at rest. We propose that five body behaviors (posture, head position, location in the box stall, focus, and interactive behavior) be included in an optimal scale for live assessment of mild orthopedic pain.

We also propose that the posture item be refined to include more levels. We recommend that facial expressions during pain assessment of mild orthopedic pain at rest should be considered together with other behaviors and that observers may need more extensive training to be able to assess them.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2615/10/11/2155/s1, Table S1: Mean (± SD) scores for each scale item, File S2: Dataset in an Excel book used for data analysis.

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Performance of four equine pain scales and their association to movement asymmetry in horses with induced orthopedic pain

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Objective: This study investigated the relationship between orthopedic pain experienced at rest, and degree of movement asymmetry during trot in horses with induced reversible acute arthritis. Orthopedic pain was assessed with the Horse Grimace Scale (HGS), the Equine Utrecht University Scale of Facial Assessment of Pain (EQUUS-FAP), the Equine Pain Scale (EPS), and the Composite Orthopedic Pain Scale (CPS). Reliability and diagnostic accuracy were evaluated with intraclass correlation coefficients (ICC) and area under the curve (AUC).

Study design and animals: Eight healthy horses were included in this experimental study, with each horse acting as its own control.

Methods: Orthopedic pain was induced by intra-articular lipopolysaccharide (LPS) administration. Serial pain assessments were performed before induction and during pain progression and regression, where three observers independently and simultaneously assessed pain at rest with the four scales. Movement asymmetry was measured once before induction and a minimum of four times after induction, using objective gait analysis.

Results: On average 6.6 (standard deviation 1.2) objective gait analyses and 12.1 (2.4) pain assessments were performed per horse. The ICC for each scale was 0.75 (CPS), 0.65 (EPS), 0.52 (HGS), and 0.43 (EQUUS-FAP). Total pain scores of all scales were significantly associated with an increase in movement asymmetry (R^2 values ranging from -0.0649 to 0.493); with CPS pain scores being most closely associated with movement asymmetry. AUC varied between scales and observers, and CPS was the only scale where all observers had a good diagnostic accuracy ($AUC > 0.72$).

Conclusions and clinical relevance: This study identified significant associations between pain experienced at rest and degree of movement asymmetry for all scales. Pain scores obtained using CPS were most closely associated with movement asymmetry. CPS was also the most accurate and reliable pain scale. All scales had varying linear and non-linear relations between total pain scores and movement asymmetry, illustrating challenges with orthopedic pain assessment during rest in subtly lame horses since movement asymmetry needs to be rather high before total pain score increase.

KEYWORDS

pain assessment, lameness, LPS induction, objective gait analysis, movement symmetry, reliability

Introduction

Painful pathology in the locomotor apparatus often leads to increased movement asymmetry, due to decreased loading of the painful limb, i.e., lameness. Nonetheless, horses perceived as sound by their owners commonly show movement asymmetry (1, 2), and it remains unclear how the degree of movement asymmetry is associated with the level of pain experienced. Changes in behavior and in facial expressions have been recognized and assessed with an ethogram in ridden horses with clinical orthopedic pain (3, 4), but have not yet been associated to different degrees of movement asymmetry detected by objective gait analysis.

Different types of orthopedic pain during rest, including moderate and severe post-surgical orthopedic pain (5), laminitis (6), and induced inflammatory arthritis (7), have been successfully assessed using different pain assessment tools. We recently showed that a number of body behaviors and facial expressions included in those tools predict mild orthopedic pain in resting horses (8). However, it is not known whether these pain assessment tools can recognize resting pain displays associated with movement asymmetry in a reliable and accurate way. A clinically relevant question in that regard is whether increased pain score and movement asymmetry occur simultaneously or not.

In addition, different pain pathologies may generate different pain displays (9) and a pain assessment tool may therefore only be valid for the pain types specified in the validation study. Pain *per se* is associated with a number of general features, but the anatomical location of the pain will induce different compensatory body behaviors, such as increased movement asymmetry due to decreased weight bearing during orthopedic pain. Facial displays of pain, on the other hand, are thought to be general for acute pain or acute exacerbations of chronic pain in most mammals, including horses (10). Indeed, grimace-based pain scales developed for horses experiencing post-surgical castration pain (11) and acute visceral pain (12) seem to identify laminitis (6), post-surgical orthopedic pain (5), and head-related pain (13) successfully. Whether a behavior- or grimace-based pain scale performs better on the same type of orthopedic pain has not been evaluated, but assessment of behaviors and facial expressions together has been recommended to optimize pain detection (14, 15).

Understanding the relationship between pain experienced at rest and degree of movement asymmetry during motion can aid the investigation of whether a movement asymmetry is caused by pain or not. Adding a pain assessment tool during rest to the lameness examination may thus be helpful in deciding the pain level in the equine orthopedic patient. For this use, proper validation of the pain assessment tool is essential, since validation and high observer reliability in experimental settings do not necessarily mean that a pain assessment tool performs well under clinical conditions (16). For instance, observers are commonly trained prior to pain assessment to improve reliability in experimental studies, while observer training may not be possible under clinical conditions, especially with the current lack of standardized training protocols and purpose-made teaching material. Blinding of observers to the animal's pain status in experiments is also common, but in a clinical setting the clinician very often has information or beliefs about the pain status of the patient, for example knowing the diagnosis or treatment, and thereby if the horse is lame or in post-surgical pain.

This study therefore had two aims: (1) to investigate the relationship between orthopedic pain experienced at rest and degree of movement asymmetry during trot in horses; and (2) to compare, under clinical conditions, the performance parameters of pain assessment tools containing varying categories of facial expressions and body behaviors.

Four existing pain assessment tools were applied simultaneously by three observers immediately before and after serial objective measurements of movement asymmetry ranging from baseline conditions to painful conditions, and back to baseline. The hypotheses tested were that increased pain scores are associated with increased movement asymmetry, and that scales containing both body behaviors and facial expressions perform better than scales with only behavioral or facial items. A final hypothesis was that the reliability of the pain assessment tools would be similar to previous published values.

Material and methods

Ethical approval

The study was approved by the Swedish Ethics Committee (diary number 5.8.18-09822/2018) in agreement with Swedish legislation on animal experiments. As outlined in EU Directive 2010/63/EU on animal experiments, replacement, reduction, and refinement were carefully considered in the study design. The ARRIVE guidelines were followed (17) and the data collected can be used for multiple purposes.

Animals and experimental design

The data were collected as part of a previous study (7). In brief, seven healthy Standardbred trotters and one Warmblood horse [mean (standard deviation, SD) age 14.5 (3.7) years, body mass 552 (39) kg, height at withers 160 (2.78) cm] were recruited for the experiment. Exclusion criteria were lameness grade >1, scored during straight line trot on a 0–5 ordinal scale (0 = sound and 5 = non-weight bearing lameness) or any significant clinical findings after a full clinical examination.

An experimental study was conducted with each horse as its own healthy control. Movement asymmetry was measured using objective gait analysis (section Objective gait analysis) on one occasion before induction of lameness (baseline) and a minimum of four times after induction, until each horse had returned to its baseline movement asymmetry. Pain was evaluated in the box stalls using four pain scales, directly before and after each objective gait analysis (section Pain assessment). Baseline measurements were performed after 10–12 days of acclimatization, and acute short-term inflammatory arthritis was induced 1 or 2 days later by administering lipopolysaccharides (LPS) into the tarsocrural joint of the pelvic limb with the highest pre-existing movement asymmetry. A 3 ml solution of LPS from *Escherichia coli* O55:B5 1 mg/ml (*L5418 Sigma*), with a stock concentration of 1.167 ng/ml, was administered to the dorsomedial pouch after evacuation of 3 ml synovia, using routine aseptic techniques.

If the horse was judged to be too lame to trot, corresponding to lameness grade >3/5 on a 0–5 ordinal scale, a protocol for rescue analgesia was initiated. This protocol consisted of evacuation of synovia to decrease joint distension and lessen inflammatory load and pain. Measurements were then continued when the lameness grade decreased.

Objective gait analysis

Movement asymmetry was measured at walk and trot, on a straight line on hard and soft surfaces and during lunging on a soft surface. For horses with subjectively increased movement asymmetry at the lunge, a second straight-line trot measurement was performed on the hard surface after lunging. During motion, the positions of seven spherical markers (38 mm diameter, Qualisys AB, Sweden) attached to the horse were recorded in 3D at 200 Hz, using 13 infrared optical motion capture cameras (Qualisys AB, Sweden) and tracked by the QTM software (version 2.11-2019.3, Qualisys AB, Sweden). Lameness was subjectively assessed during ongoing measurements by experienced equine veterinarians, one of whom also participated in the pain assessments. Data from the first and, when present, the second straight-line trot on hard surface were used for further analysis. Only the vertical traces from head and pelvic markers were extracted for calculation of lameness metrics, using custom-written scripts in MatLab (18). Details on filtering and stride segmentation can be found elsewhere (19, 20). To cover different strategies used by the horses to decrease loading of the pelvic limb in pain (impact lameness), differences in minimum height between the left and right stance phase of each stride were computed, resulting in HD_{min} for the head marker and PD_{min} for the pelvic marker. These are two variables that change in horses with weight-bearing pelvic limb lameness and with a compensatory head nod (21, 22). Trial means of HD_{min} and PD_{min} were computed and negative left-side means were converted to positive right-side means. To illustrate the change in overall movement asymmetry after induction, a total asymmetry score (TAS) in mm was calculated by adding together absolute differences in $HD_{min}/2$ and PD_{min} from baseline movement asymmetry. Subjective lameness scores were not included in calculation of TAS.

Pain assessment

Pain was evaluated directly from outside the box stall using the Horse Grimace Scale (HGS) (11), the Equine Utrecht University Scale of Facial Assessment of Pain (EQUUS-FAP) (12), the Equine Pain Scale (EPS) (23), and the Composite Orthopedic Pain Scale (CPS) (7). These scales consist of multiple items assessing facial expressions, behaviors, and/or physiological variables. Item scores are added to give a total pain score ranging from 0 to 12 (HGS), 0 to 18 (EQUUS-FAP), 0 to 30 (EPS), or 0 to 39 (CPS). HGS was originally designed for pain assessment from video or footage, while the other scales are applicable for live assessment. Observation time was 2 min for HGS, EQUUS-FAP, and EPS, and 5 min for CPS.

The same horse was observed by three pain assessors, simultaneously and independently assigning the horse a total pain score with each of the pain scales, always used in the same order (HGS, EQUUS-FAP, EPS, and CPS). This was defined as one pain assessment, and yielded HGS, EQUUS-FAP, EPS, and CPS pain scores from observer 1, from observer 2, and from a third observer. Observers 1 and 2 participated in all assessments, while the third observer was one of observer 3, 4, or 5. All observers, except observer 1 who participated during objective gait analyses, were blinded to limb of induction and lameness grade, and only observed the horses in their box stalls. Observers 1–3 were equine veterinarians, with experience of pain assessment, observer 4 was an agronomist, and observer 5 was an equine ethologist.

All had private and/or professional equestrian experience. Prior to the study, the observers familiarized themselves thoroughly with the pain scales, through reading published scientific papers and score sheets/descriptions, but did not train on videos or live horses.

Statistics

All statistical analyses were conducted in R (24). Descriptive statistics for pain assessment and movement symmetry data were calculated and plotted with "ggplot2" (25). Normality of the dataset was evaluated with Shapiro Wilks test ($p < 0.05$ indicating non-normality) and visually with histograms. Due to non-normality, median and 1st and 3rd interquartile were calculated for total pain scores. Reliability was analyzed with intraclass correlation (ICC) coefficient (26), by computing two-way random $\text{ICC}_{\text{agreement}}$ (ICC2, A1; "iccNA"). The level of reliability was categorized according to an existing system (27).

To estimate construct validity, the change in TAS was used as a proxy for pain intensity. To enable identification of non-linear associations, the association between total pain score and TAS was tested with generative additive mixed models ("gamm") (28, 29), with total pain score as dependent variable and TAS as explanatory variable. "Horse" was included as a random effect and an autocorrelation effect was added to handle similarity between observations over time. To enable comparison between horses, the effect of time was standardized by the use of a proportional time scale. The maximum change in TAS was set at 50%, the baseline at 0%, and the last measurement at 100%. Information on whether a pain assessment was performed before or after an objective gait analysis was also included. As the model could not handle crossed random effects, separate models were run for observers 1–5. The explained deviance (R^2 value) of the model for each scale was noted, and residuals were plotted and evaluated visually.

Performance of pain scales were further evaluated with area under the curve (AUC) generated from receiver-operating characteristic (ROC) curves. AUC is a measure of the probability that an observation classified as "pain" is ranked higher than an observation classified as "no pain" – the higher the probability the better accuracy (30). Prediction outcomes were computed from the generative additive mixed models ("predict") (31) and used as predictive values when computing ROC curves ("roc") and AUC with 95% confidence intervals (CI) ("auc," "ci") (32). The change in TAS defined the pain status of the horse in each observation, hence, TAS > 10 categorized the horse as in pain and TAS ≤ 10 categorized the horse as free from pain. This is a cut-off value, resembling a mild lameness grade. The AUC was classified according to previously described thresholds (33).

Results

Lameness was successfully induced in all horses (three right and five left pelvic limbs). Rescue protocol was initiated in two horses, where evacuation of synovia was sufficient to decrease joint distension and lameness grade. All objective gait analyses and pain assessments before and after evacuation of synovia were included in the analysis. In total, 53 measurements of objective gait analysis were performed, with a mean (SD) number of occasions of 6.6 (1.2). Mean (SD) increase in TAS after induction was 27 mm (26). The time points for measurement differed between horses, as did the time with increased TAS, due to individual responses to the induction (Figure 1). All horses returned to baseline movement asymmetry within 52 h after induction. Details of changes in asymmetry over time and absolute values of HD_{min} and PD_{min} are provided in Supplementary Materials (Supplementary File S1).

During the study, 97 pain assessments were performed, with a mean (SD) number of 12.1 (2.4) pain assessments per horse. There was considerable variation in total pain scores for both low and high total asymmetry scores (Figure 2), and total pain scores >0 were present for pain assessments before induction for all scales (Table 1). As illustrated in Figure 2 and Table 1, the majority of total pain scores were at the low end of each scale's range. The highest pain score reached 58.3% (HGS), 27.8% (EQUUS-FAP), 40% (EPS), or 23.1% (CPS) of each scale's maximum total pain score.

The only scale with good reliability was CPS, with an ICC coefficient (95% confidence interval, CI) of 0.753 (0.675–0.818). EPS and HGS were both moderately reliable with an ICC coefficient (95% CI) of 0.648 (0.548–0.736) and 0.522 (0.406–0.631), respectively. EQUUS-FAP showed poor reliability, with an ICC coefficient (95% CI) of 0.432 (0.310–0.552).

Generalized additive mixed models revealed significant associations between total pain scores and TAS on a normalized timeline for all scales, but not for all observers (Table 2). CPS had a significant association between total pain scores and TAS for all observers, while the other scales had significant associations for three (EPS and HGS) or two (EQUUS-FAP) observers. The R^2 values ranged from -0.0649 to 0.493, and showed that TAS explained higher variance in total CPS pain scores for most observers compared with pain scores of the other scales. On several occasions, total pain scores before performing objective gait analysis were significantly higher than pain scores given after objective gait analysis (Table 2). HGS had significantly higher pain scores before objective gait analysis for observers 1–4, EQUUS-FAP for observers 1 and 4, and EPS for observer 1. Partial effects plots were created to depict the changing linear and non-linear relationship between total pain scores and total asymmetry scores (Figures 3–6). Visual evaluation of the plots showed that many points did not follow the estimated line and confidence interval, indicating great variance in the data that

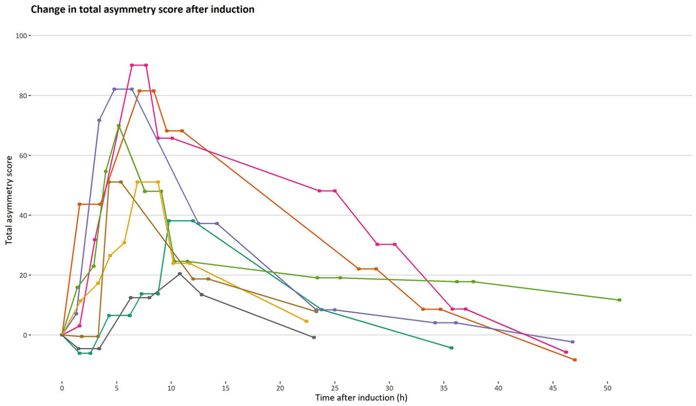

FIGURE 1
Progression and regression of total asymmetry score after induction of orthopedic pain. Total asymmetry score is presented on the *y*-axis. A timeline is presented on the *x*-axis to illustrate changes in total asymmetry unaffected by time. Each line represents one horse ($n = 8$) and each point on a line represents an occasion where an objective gait analysis was performed.

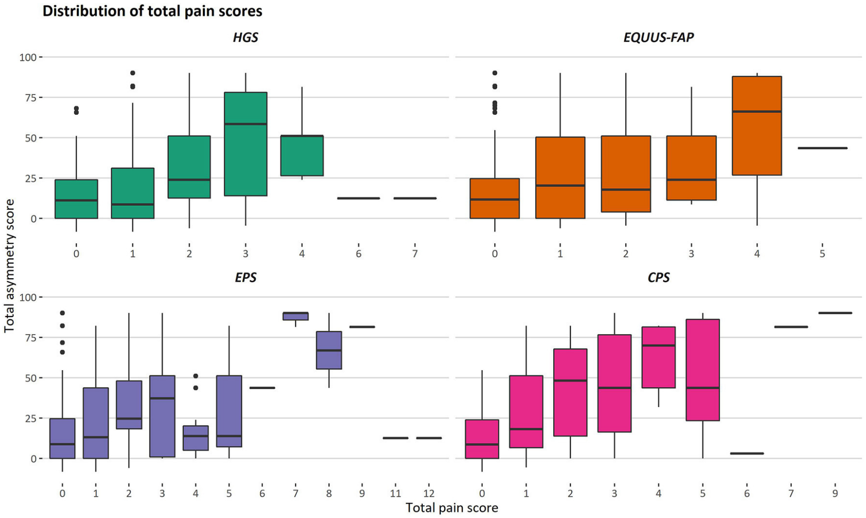

FIGURE 2
Distribution of total pain scores for different degrees of movement asymmetry. Total asymmetry scores are presented on the *y*-axis, where 0 is the objective gait analysis performed before induction. Total pain scores given with the Horse Grimace Scale (HGS), Equine Utrecht University Scale of Facial Assessment of Pain (EQUUS-FAP), Equine Pain Scale (EPS), and Composite Orthopedic Pain Scale (CPS) are presented on the *x*-axis. Each box illustrates the distribution of one pain score over different degrees of movement asymmetry, given by three observers. The median is presented as the black line in a box, and the lower and upper ends of the boxes show the lower and upper quartile. The lower and upper whiskers show the lowest and highest 25% of the data. Outliers are shown as black dots.

TABLE 1 Median, 1st and 3rd interquartile (IQ), minimum (min) and maximum (max) total pain score for pain assessments, made before and after pain induction, using the Horse Grimace Scale (HGS), Equine Utrecht University Scale of Facial Assessment of Pain (EQUUS-FAP), Equine Pain Scale (EPS), and Composite Orthopedic Pain Scale (CPS).

Scale	Pre-induction				Post-induction			
	Median	1st−3rd IQ	Min	Max	Median	1st−3rd IQ	Min	Max
HGS (0–12)	1.00	0.00–1.00	0.00	3.00	1.00	0.00–1.00	0.00	7.00
EQUUS-FAP (0–18)	0.00	0.00–1.00	0.00	2.00	0.00	0.00–1.00	0.00	5.00
EPS (0–30)	0.00	0.00–2.25	0.00	5.00	1.00	0.00–2.00	0.00	12.00
CPS (0–39)	0.00	0.00–0.00	0.00	5.00	0.00	0.00–1.00	0.00	9.00

The total pain score range is stated in brackets after each scale.

was not explained by the model. The plots also showed that pain scores above moderate level were rare and not necessarily present when TAS was high in our experimental model.

Area under the curve generated from ROC curves varied among observers and scales (Figure 7). In general, fitted models for observer 1 ($n = 97$ observations) and 2 ($n = 94$ observations) performed better than the models for observer 3, 4 and 5. Based on AUC, observer 1 and 2 could correctly identify horses in pain with HGS with 77%−89% chance, 84%−87% with EQUUS-FAP, 83%−99% with EPS and 92%−95% with CPS. Fitted models for observer 3 ($n = 55$), observer 4 ($n = 20$) and observer 5 ($n = 20$) varied greatly in AUC. For AUC <0.5, it is not possible to distinguish horses in pain from horses without pain, and the random chance is higher. Observer 3 did not succeed in discriminating between "pain" and "no pain" with EQUUS-FAP. Observer 4 did not succeed with EPS, and observer 5 did not succeed with HGS and EPS. Thus was CPS the only scale where all observers succeeded in correctly identifying horses in pain.

Discussion

Increased movement asymmetry was successfully induced with LPS in all horses – an induction method well-described in horses and known to result in lameness and pain behavior (34–37). This study showed varying performance of four pain scales when assessing low-degree orthopedic pain, but significant linear and non-linear relationships were identified between increases in movement asymmetry and total pain scores given at rest for all scales. Of the four pain scales studied, CPS performed best and pain scores obtained with CPS were most closely associated with movement asymmetry. Progression and regression of movement asymmetry was shown with serial movement asymmetry measurements, beginning 1.5 h post-induction. Lameness progressed and regressed in all horses, as expected from earlier studies (38, 39). Maximum increase in movement asymmetry varied greatly between horses (Figure 1), which is in agreement with previous findings of a highly individual inflammatory response in horses (40) and a wide range in maximum lameness grade (1–4 on an ordinal lameness scale of 0–5) (39). Pain is an experience influenced by external inputs from the surroundings, earlier experience of pain, and compensatory abilities, so individual variance in experienced pain is often present despite standardized pain induction protocols. Use of a within-animal study design where the animals are their own control, as in this study, is therefore recommended (41). To further evaluate the individual pain experience at rest, and since a gold standard for experienced pain is lacking (42), another measure of pain during rest could have been included in our study. Although nociception is different from pain, mechanical nociceptive thresholds in our horses could have been used to demonstrate presence of hyperalgesia around the induced joint as an indicator of inflammatory nociception (38, 43).

In parallel to this, varying degrees of pain behavior were observed at rest, with the majority of pain scores at the low end of each scale's score range. Total pain scores of 0 were sometimes seen post-induction, which may indicate that the horses in our study did not constantly experience pain at rest. Horses are able to decrease the load on the painful limb, resulting in reduced pain intensity and lower pain scores. For instance, facial expressions of pain have been found to be less often present when horses change their posture (8). However, horses with LPS-induced low-grade bilateral orthopedic pain are reported to show no specific behaviors during the presence of lameness (44), and horses with orthopedic disorders may hide their discomfort when observers are present (45). These results indicate that pain can be present despite lack of observed behavioral changes, and that a total pain score of 0 in our study may therefore not be equal to 'no pain'. In addition, it is often anticipated that the baseline should be zero, which can be misleading when interpreting the magnitude of the scores. In this study, total pain score was higher than 0 before induction on some occasions, especially for EPS. This is an issue rarely discussed in the literature, but positive baseline scores have been described for mice using the Mouse Grimace Scale (46). Further studies are needed to determine baseline intervals and cut-off values for pain in horses.

TABLE 2 Results of generalized additive mixed models for the Horse Grimace Scale (HGS), Equine Utrecht University Scale of Facial Assessment of Pain (EQUUS-FAP), Equine Pain Scale (EPS), and Composite Orthopedic Pain Scale (CPS), where each observer (1–5) is modeled separately.

	HGS			EQUUS-FAP		
	Association (*p*-value)	Type (*p*-value)	R^2 value	Association (*p*-value)	Type (*p*-value)	R^2 value
Observer 1 (*n* = 97)	0.00159**	0.00127**	0.125	<0.001***	0.0147*	0.13
Observer 2 (*n* = 94)	<0.001***	0.0273*	0.083	<0.001***	0.109	0.214
Observer 3 (*n* = 55)	<0.001***	0.019*	0.304	0.4	0.292	0.035
Observer 4 (*n* = 20)	0.189	0.0259*	0.0884	0.19	0.0267*	0.205
Observer 5 (*n* = 20)	0.692	0.326	−0.0649	0.115	0.603	0.0375

	EPS			CPS		
	Association (*p*-value)	Type (*p*-value)	R^2 value	Association (*p*-value)	Type (*p*-value)	R^2 value
Observer 1 (*n* = 97)	<0.001***	0.0208*	0.142	<0.001***	0.120	0.299
Observer 2 (*n* = 94)	<0.001***	0.513	0.133	<0.001***	0.571	0.433
Observer 3 (*n* = 55)	0.0479*	0.413	0.118	<0.001***	0.240	0.298
Observer 4 (*n* = 20)	0.508	0.0992	0.0608	<0.001***	0.140	0.493
Observer 5 (*n* = 20)	0.571	0.252	0.0173	0.0418*	0.780	0.142

p-values for association between total asymmetry score and total pain score (association), p-values for increase in pain scores before objective gait analysis compared with after (type), and R^2 values explaining the deviance. Number of pain assessments performed (n) is stated for each observer. Statistical significance is indicated as $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

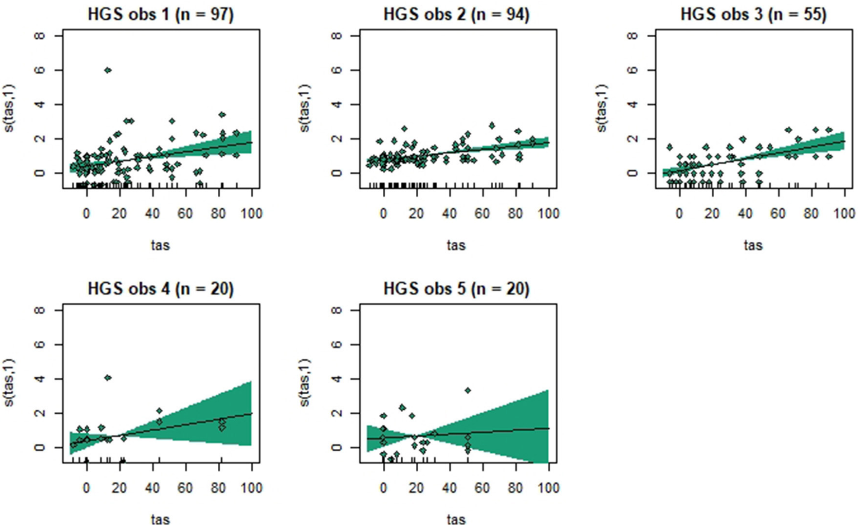

FIGURE 3
Association between total pain scores from the Horse Grimace Scale (HGS) and total asymmetry scores (TAS), analyzed using generalized additive mixed models. The *y*-axis on the partial effects plots shows total pain scores (maximum total pain score on the scale is 12), with the estimated degrees of freedom (EDF) in brackets. 1 indicates a linear relationship and 3 a cubic function. The *x*-axis shows the total asymmetry score (TAS) in mm, where 0 is the baseline objective gait analysis. Residuals are plotted in the graphs. The shaded areas indicate the 95% confidence intervals. The number of observations made by each observer is shown as *n*.

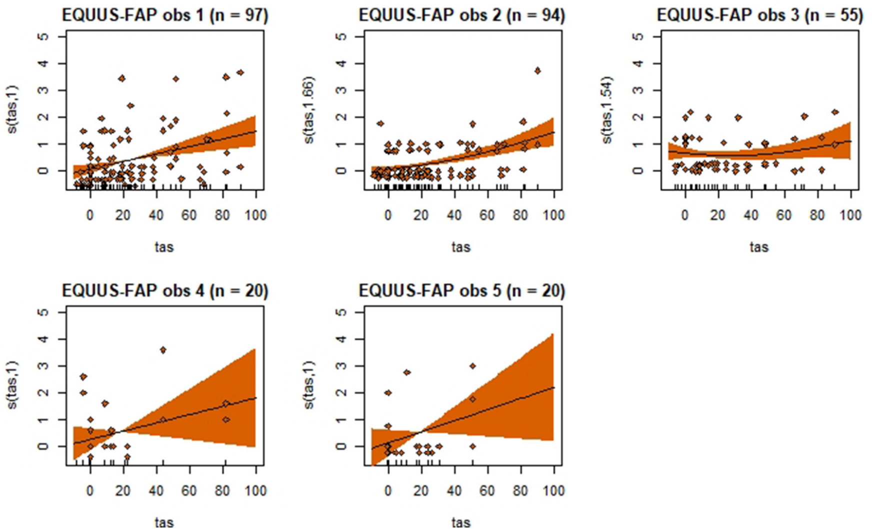

FIGURE 4
Association between total pain scores from the Equine Utrecht University Scale of Facial Assessment of Pain (EQUUS-FAP[[Inline Image]]) and total asymmetry scores (TAS), analyzed using generalized additive mixed models. See the caption to Figure 3 for more details. Maximum total pain score on the scale is 18.

Despite individual variations, total pain scores, especially those obtained with CPS, were significantly associated with the progression and regression in movement asymmetry, but the asymmetry explained <50% of the variance in pain scores (as illustrated by the R^2 values in Table 2). Based on visual evaluation of the partial effect plots in Figures 3–6, rather high movement asymmetry was present before pain scores increased. In effect, the curve approached a clinically relevant increase in pain score only when TAS reached around 60 mm (see CPS for observers 1–3 in Figure 6). A TAS of 60 mm is a moderate level of lameness, indicating that lower grades of lameness were assigned very low pain scores. Hence, when a resting lameness patient has a total pain score of CPS is >0, the clinician can anticipate that lameness during movement will be present.

When evaluating the AUC as performance parameter, all observers using CPS correctly identified horses in pain with a minimum chance of 72%, which is considered as good performance. This is comparable to the AUC presented for CPS when assessing different types of post-surgical pain in horses using the CPS and Unesp-Botucatu Horse Acute Pain Scale (UHAPS) (47). A difference in performance between observers was present for HGS, EQUUS-FAP and EPS resulting in failure of observer 3–5 to distinguishing between pain and no pain (AUC <0.5). Notably, these observers also had fewer observations than observer 1 and 2, who distinguished between pain and no pain using all scales. This may be interpreted as a need of training to develop skill in using HGS, EQUUS-FAP and EPS before these scales correctly identify pain (48).

Interestingly, both non-linear and linear relationships were seen in the plots in this study, varying between both scales and observers. Hence, an increase in pain score of 20% did not necessarily imply an increase in pain intensity of 20%. Therefore, more research is needed on the clinical meaning of a numerical pain score, especially during pain progression and regression. Furthermore, the relationship between movement asymmetry and LPS-induced pain identified in this study may be very different in horses with chronic lameness, such as osteoarthritis. LPS-induced pain is an acute pain experience not previously encountered by the horse, while most lameness types involve more long-lasting pain experiences where the horse has time to develop a coping behavior. Different degrees of pain may also be present depending on the pain process. For instance, osteoarthritic bone processes may only be painful during motion, whereas LPS-induced synovitis is painful during loading at rest and in motion. This will affect the outcome of pain assessment during rest.

The order of pain assessment and objective gait analysis seemed important for the results obtained using HGS, EQUUS-FAP and EPS. We tested the hypothesis that movement increases pain scores, but found that pain scores were significantly higher

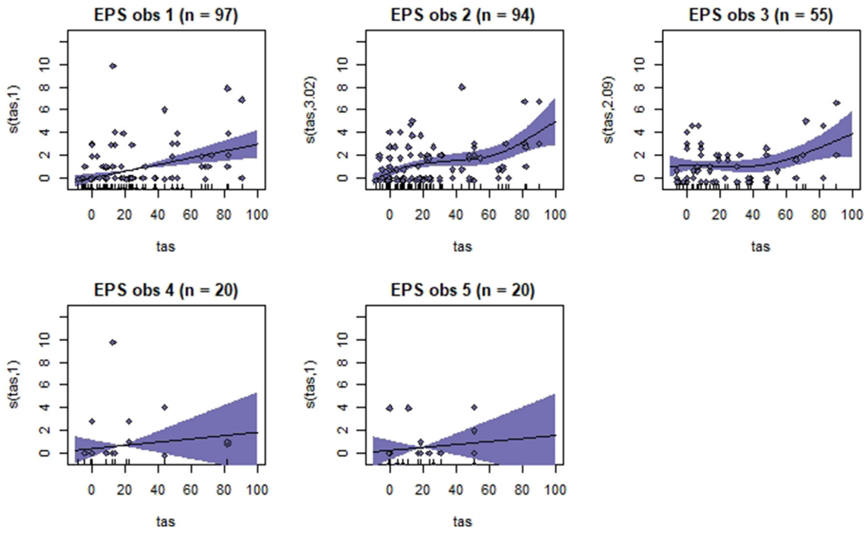

FIGURE 5
Association between total pain scores from the Equine Pain Scale (EPS) and total asymmetry scores (TAS), analyzed using generalized additive mixed models. See the caption to Figure 3 for more details. Maximum total pain score on the scale is 30.

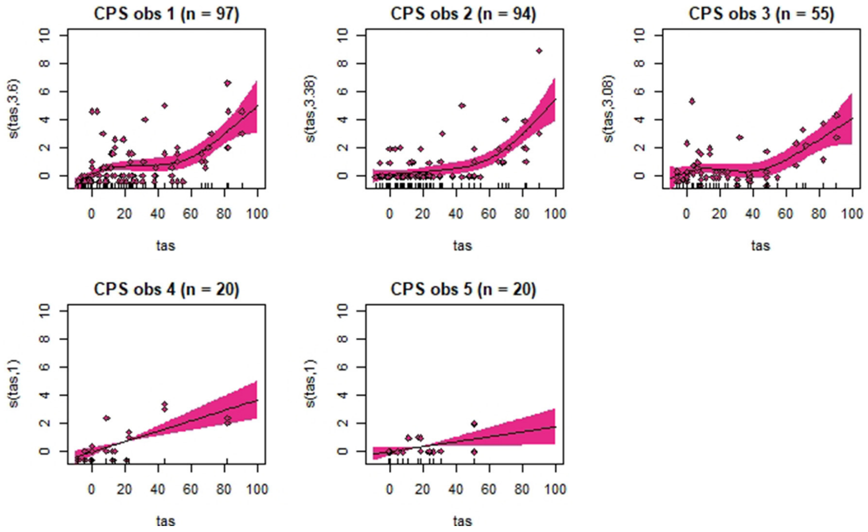

FIGURE 6
Association between total pain scores from the Composite Orthopedic Pain Scale (CPS) and total asymmetry scores (TAS), analyzed using generalized additive mixed models. See the caption to Figure 3 for more details. Maximum total pain score on the scale is 39.

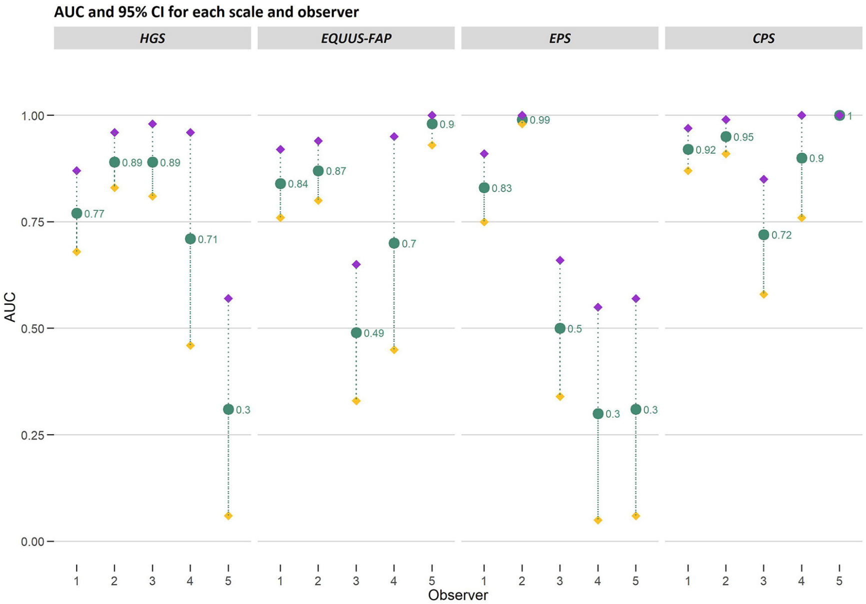

FIGURE 7
Area under the curve (AUC) and 95% confidence intervals (CI) generated from ROC curves for the Horse Grimace Scale (HGS), Equine Utrecht University Scale of Facial Assessment of Pain (EQUUS-FAP), Equine Pain Scale (EPS), and Composite Orthopedic Pain Scale (CPS). AUC and upper (purple filled diamond) and lower (yellow filled diamond) bounds of CI are presented on the *y*-axis and connected with gray dotted lines. Observer 1–5 are presented on the *x*-axis. The number of observations made by each observer was 97 (observer 1), 94 (observer 2), 55 (observer 3), 20 (observer 4) and 20 (observer 5). The AUC values are stated in the figure.

before objective gait analysis. This finding may be interpreted in different ways. Movement may decrease joint distension and result in transient pain relief. Alternatively, movement may contribute to concealment of facial or other cues, due to external input, tiredness, or stress during measurements. HGS had significantly higher scores before movement for all observers except observer 5, indicating that pain-related grimaces detected with HGS may decrease or be concealed after movement. If the horses in our study were stressed, there would have been high HGS scores after movement since facial expressions of pain are present in stressed horses experiencing pain (49), and significant increases in HGS scores have been recorded when applying HGS on stressed horses (50). However, the possible influence, especially of stress, on tool performance should be investigated further before pain assessment tools are incorporated into lameness evaluations.

We hypothesized that all scales are highly reliable. We found that the most reliable pain scale was CPS, where the strong agreement between observers is consistent with previous results (5, 7). EPS was moderately reliable, but has not been evaluated previously. The poor and moderate agreement seen for EQUUS-FAP and HGS is inconsistent with previous results showing good or excellent reliability (5, 6). These scales only assess facial expressions, which may affect the reliability since facial configuration seems to be more difficult to appraise than body movements (8). In addition, the more ambiguity there is in descriptions of a category and its scoring, the more training of observers is needed. It may be argued that scales should be designed in such a way that any observer can use them correctly. It has been suggested that before assuming that a pain scale is generalizable, it should be tested with untrained observers unfamiliar with the scale (51). Nonetheless, observers are often trained prior to reliability testing, but standardized training protocols are seldom published (51). The lack of supervised or reference-guided observer training in our study may have impaired the reliability, and evaluation of the reliability on a small set of horses prior to the experiment would perhaps have identified shortcomings in the training. As discussed earlier, especially training prior to using HGS, EQUUS-FAP and EPS might be needed since observers performing fewer pain assessments struggled more often to identify pain than did observers performing more pain assessments. Training on videos and live horses prior to using these scales might improve the reliability and accuracy of identifying pain. However, when comparing the level of observer training in our study with previous studies reporting high reliability for EQUUS-FAP, HGS, and CPS, they did not differ greatly. Observers using EQUUS-FAP familiarized themselves with the scale and trained on horses free from pain prior to reliability testing (5, 52) and observers using HGS had a detailed protocol containing pictures and descriptions during scoring (6, 11). In the study validating CPS (7), no information is given on observer training. The observers in the present study did not train on horses known to be free from pain prior to the experiment, but thoroughly familiarized themselves with the scales and used the same protocol as in the original studies, when available. The observers in previous studies had experience with scoring behaviors and/or horses, and some were veterinary students or veterinarians. This is comparable to the level of experience among observers in the present study (veterinarians, ethologist, and agronomist, all experienced with horses and some with pain scoring). Despite these similarities in observer training and experience, reliability for EQUUS-FAP and HGS was low or moderate in this study, corroborating the claim that re-evaluation of reliability (and validity) may be required when the disease category or the rating conditions are changed (53).

During the controlled circumstances of experimental studies, the presence of affective states of pain could have been documented further by adding certain physiological measures associated with negative valence affects such as pain, for instance heart rate and heart rate variability (54, 55). This is however not feasible during clinical conditions and in order not to disturb the horses more than necessary, such measures were not included in the present study. A limitation in the present study was the small sample size (eight horses), since horses displayed great variation in lameness and intensity of pain – as described in other studies (39). Including more horses might have led to better representation of different pain intensities, but individual variation should not be ignored for the data to be generalizable. A small sample size was selected, primarily due to ethical concerns regarding induction of pain. The association between pain scores at rest and degree of movement asymmetry has not been described previously; complicating sample size calculation prior to the study since the coefficient of determination (R^2) needs to be estimated. In previous studies in which orthopedic pain was induced in the same way as in this study, sample size ranged from 4 to 19, with most studies commonly involving 6–8 horses (7, 34, 36, 37, 39, 40, 56–66). Another limitation was the blinding level of the observers. Knowing that a horse was going to be subjected to induced pain might have resulted in expectation bias, with the observers anticipating that pain would be present and giving higher pain scores (67). The non-blinded observer 1 was the only observer that obtained significantly higher scores with HGS, EQUUS-FAP, and EPS before objective gait analysis. This indicates that seeing the horse move may lead to assigning it a higher pain score before assessment, compared to after. Interestingly, this situation corresponds to that clinical situation where repeated measurements are used, since every pain assessment gives the observer information on the pain status of the horse. The four pain scales were always used in the same order, which may also have induced expectation bias. Since observer 1 was not blinded, the observers were included separately in the statistical models, thereby preventing a potential blinding effect between observer 1 and the other observers from influencing the results. We found comparable results for the non-blinded observer and the blinded observers. However, this is not always the case and the effect of blinding and expectation bias is an important area that should be investigated further.

Conclusions

We identified significant associations between pain experienced at rest and degree of movement asymmetry for all scales. Pain scores obtained using CPS were most closely associated with movement asymmetry, but movement asymmetry only explained a minor part of the variation in pain scores at rest. Increases in pain score and movement asymmetry did not occur simultaneously and a horse may have rather high movement asymmetry before total pain scores increase. This is an important challenge when assessing orthopedic pain during rest in subtly lame horses, and underlines the relevance of identifying painful orthopedic lesions by other means, for example local or systemic analgesic testing.

All observers managed to distinguish correctly between horses in pain and without pain when using CPS, with excellent accuracy in four out of five observers. However, when using HGS, EQUUS-FAP and EPS some observers were not able to distinguish between horses in pain and without pain. CPS was also the most reliable scale, while low-moderate reliability for the other scales indicate that different pain assessors might assign the equine patient different pain scores despite being familiar with HGS, EQUUS-FAP and EPS.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Swedish Ethics Committee, diary number 5.8.18-09822/2018.

Author contributions

KA, PA, EH, and MR designed the study and contributed to the data collection. PA, EH, and MR were responsible for funding acquisition and supervised the project. L-MT and KA were responsible for the statistical analysis and data interpretation. KA prepared the initial draft. All authors made substantial contributions to data interpretation and manuscript revision, and have approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.938022/full#supplementary-material

SUPPLEMENTARY FILE S1
Dataset in an Excel book used for data analysis. Excel book containing two sheets. The sheet "Abbreviations" explains abbreviations used in column headers. The sheet "Dataset" contains symmetry data for all objective gait analyses, and total pain scores for pain assessments before and after the objective gait analyses.

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This thesis identifies body behaviors associated with orthopedic pain and movement asymmetry in resting horses and tests the performance of four pain scales in detecting orthopedic pain. Dynamic and diverse facial displays are further identified in both resting and moving horses during different orthopedic pain intensities. Thus, this thesis provides new knowledge on behaviors and facial activities to look for when assessing equine orthopedic pain.

Katrina Ask received here postgraduate education at the Department of Anatomy, Physiology and Biochemistry, Swedish University of Agricultural Sciences (SLU) in Uppsala. She obtained her degree in veterinary medicine at the University of Copenhagen.

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