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I feel your pain: Individual differences in welfare indicators after castration in horses

Sabrina Briefer-Freymond [a,1], Emanuela Dalla Costa [b,2], Aurelie Jolivald [c,3], Rupert M. Bruckmaier [d,4], Elie Atallah [b,5], Maria Giorgia Riva [b,6], Carrie Ijichi [c,e,*,7,8]

[a] Swiss National Stud SNS, Les Longs-Pres, Avenches 1580, Switzerland
[b] Università degli Studi di Milano, Department of Veterinary Medicine and Animal Sciences, Via dell'Università 6, Lodi 26900, Italy
[c] School of Animal, Rural & Environmental Science, Nottingham Trent University, Southwell, Nottingham NG25 0QF, United Kingdom
[d] Vetsuisse Faculty, University of Bern, Bern 3001, Switzerland
[e] School of Veterinary Medicine, University of Lancashire, Fylde Rd, Preston PR1 2HE, United Kingdom

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ABSTRACT

Personality affects both experience and expression of pain and the welfare impact of castration on horses is poorly understood. Therefore, the current study observed 19 horses to determine: the welfare impact of standard castration on horses; whether individuals consistently vary in their behavioural and emotional responses to pain; the influence of personality on behavioural and physiological responses to pain; whether Horse Grimace Scale (HGS) indicates how individuals feel about painful experiences. Eye temperature (IRT), salivary cortisol, HGS and a pain ethogram were measured at intervals before, throughout and during recovery from castration. IRT ($p < 0.005$), Cortisol ($p < 0.024$), HGS ($p < 0.03$) and Maintenance behaviour ($p < 0.004$) significant changed from baseline. Physiological and behavioural responses to castration were varied but not consistent within individuals. Veterinarian influenced responses, presumably reflecting the importance of clinician's skill. Personality explained differences in cortisol responses with Neuroticism negatively (estimate=-0.275; $p = 0.035$), and Extroversion positively (estimate=0.406; $p = 0.001$) associated with the magnitude of response to castration. HGS was not confounded by personality suggesting that this pain indicator may be resilient to individual differences in pain expression and appears to reflect underlying affective pain states as it was associated with cortisol ($r = 0.568$, $p = 0.027$). Therefore, it is potentially an important tool in recognition of pain at an individual level. Further research should be done utilising a larger sample with greater standardisation of castration method to determine both the effect of baseline welfare on pain resilience and the sensitivity of Grimace Scales as an indicator of suffering during painful experiences.

1. Introduction

Animals are unable to verbally convey their emotions to human caregivers, which makes the measurement of pain difficult (Reid et al., 2013). Therefore, behaviour-based scales are utilised to quantify pain levels in animals, assisting the administration of the correct dosage of analgesic drugs and informing decisions on humane end-points (Ashley et al., 2010). Consequently, it is vital that these scales are both sensitive and valid, to reduce the welfare implications that could occur through the incorrect assessment of pain (Rutherford, 2002). However,

* Corresponding author at: School of Veterinary Medicine, University of Central Lancashire, Fylde Rd, Preston PR1 2HE, United Kingdom.
E-mail address: cijichi@lancashire.ac.uk (C. Ijichi).
[1] 0000–0002-7988–6929
[2] 0000–0002-5037–3759
[3] 0000–0002-9440–4107
[4] 0000–0002-9374–5890
[5] 0000–0002-1892–7803
[6] 0000–0002-3266–0236
[7] Permanent Address
[8] 0000–0003-1271–8813

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personality - defined as individual differences in behaviour which are stable over time and across contexts (Koolhaas et al., 1999) - may confound this (Ijichi et al., 2014; Lush and Ijichi, 2018). Animals differ in their ability to tolerate pain and in how they express that pain in much the same way that humans do (Ijichi et al., 2014; Lush and Ijichi, 2018). Correct assessment of individual pain experiences is critical for the ethical and effective consideration of humane end- points, treatment options and pain relief. Differences in pain expression make it easier to detect and manage pain in some individuals whilst others may not receive adequate pain relief or veterinary attention (Ijichi et al., 2014). Yet animals are often treated as interchangeable and the full implications of their individual differences may not be acknowledged (Richter and Hintze, 2019).

Accurate pain recognition is particularly important when caring for horses. Horses may continue to be worked if their owner does not detect that they have a painful condition. It is reported that on a sample of "healthy riding horses" 14.5 % showed irregularity of motion, 4.8 % were lame and 31 % showed negative responses to back palpation (Visser et al., 2014). Continued work is likely to worsen any injury or result in delayed veterinary attention. In fact, horses that are more tolerant to pain present at veterinary clinics with more serious injuries or degenerative conditions (Ijichi et al., 2014). Further, a horse's behavioural responses to pain can be highly dangerous, resulting in risks to people if they are not correctly recognised (Fureix et al., 2010; Story et al., 2021). Wastage due to behavioural problems or injuries is problematic in the industry which may result in the equine industry having its Social Licence to Operate revoked with serious consequences (Douglas et al., 2022; Duncan et al., 2018).

Castration provides an ideal model to study pain responses in animals without causing unnecessary suffering (Dalla Costa et al., 2014; Dalla Costa et al., 2018; Lush and Ijichi, 2018). It is routinely conducted electively for the benefit of the individual using relatively standardised procedures (Kilcoyne, 2013; Sjöberg., 2025) and is known to cause pain (Marquette et al., 2023). While the welfare impact of castration has been studied in livestock species extensively recently reviewed in Marquette et al. (2023), there appears to be scant evidence of the welfare impact of this standard procedure in horses. Yet castration is one of the most commonly performed elective equine surgical procedures (Kilcoyne, 2013). Therefore, the majority of male horses experience castration during a formative time in their learning about human interactions and so investigation into their subjective experience of this process is warranted.

Although we now know that animals differ in their behavioural and physiological responses to pain (Lush and Ijichi, 2018), behavioural indicators that accurately reflect differences in emotional responses have not been identified. It is crucial that we understand whether pain behaviour indicates underlying affective states since we rely on behaviour to assess the need for pain relief and veterinary attention (Ashley et al., 2010). Whilst gross motor patterns are not good indicators of tissue damage in horses (Ijichi et al., 2014) or dogs (Lush and Ijichi, 2018), species-specific facial expressions of pain, such as the Horse Grimace Scale (HGS) (Dalla Costa et al., 2014; Dalla Costa et al., 2016), may be and in addition may provide more sensitive indicators of the affective state of the subject. Whilst it is understood that HGS is sensitive to tissue damage (Dalla Costa et al., 2016; Marcantonio Coneglian et al., 2020), it is not yet known whether HGS or more general pain behaviour reflects affective responses to that tissue damage.

The current study aimed to determine 1) the welfare impact of standard castration on horses 2) whether individuals consistently vary in their behavioural and emotional responses to pain 3) the influence of personality on behavioural and physiological responses to pain and 4) if HGS accurately indicates how the individual feels about their painful experience. To achieve this, 19 colts undergoing routine castration were filmed during castration and recovery. Prior to castration, the owners completed a translated version of a validated personality questionnaire (Ijichi et al., 2013; Jolivald et al., 2022) which measures five factors including Extraversion and Neuroticism. These two traits have previously been shown to relate to pain expression in both horses (Ijichi et al., 2014) and humans (Harkins et al., 1989; Vassend et al., 2013; Wade et al., 1992). Infrared thermography of ocular temperature (Whittaker et al., 2023) and salivary cortisol (Contreras-Aguilar et al., 2018; Kang et al., 2022) were measured at intervals before and throughout castration and recovery as indicators of affective states. The Horse Grimace Scale (Dalla Costa et al., 2014) and a pain specific ethogram (Gleerup and Lindegaard, 2016) were used to measure behavioural expressions of pain. Since the painful procedure is routine and carried out according to one of two standardised castration guidelines, if individual differences do not affect pain experience, it would be expected that both pain behaviour and arousal would be relatively consistent across individuals. In addition, if pain behaviour accurately and sensitively reflects internal states, it would be expected to correlate with physiology. If variation in behaviour and physiological responses are observed, it is hypothesised these will be associated with Neuroticism and Extroversion.

2. Method

A sample of 19 colts were sourced from three locations (referred to as Home Farm) around Avenches, Switzerland. Subjects were aged between 9 and 10 months old. Subjects were housed as per normal protocol for their individual facilities and the study had no effect on their feeding or management. Routine care of the study animals was provided by the farm owners. The horses were fed with hay ad libitum and with concentrate as per individual requirements. They were housed in group of foals of the same age in a housing system with an inside and outside area and spent some hours in pastures, depending on the weather conditions.

The study took place in the spring castration season between March and April in 2021–2023. Subjects were kept in their social groups during the study to prevent stress caused by social isolation to both ensure the highest possible welfare standard and prevent confounding the study results. This study was an opportunistic sample of colts undergoing routine castration as part of normal management.

2.1. *Ethics*

Before data collection, this study was approved by Nottingham Trent University Ethics Committee (ARE918). In addition, a licence was granted by the Swiss Veterinary Office to conduct this research (approval number National 33307, cantonal VD3655a; Switzerland). During the study, no changes were made to the animal's routine management or veterinary care due to data collection. Colts were housed in groups, as it is mandatory in Switzerland, following the Animal Welfare Act. Measures of welfare used are non-invasive. Colts were habituated to saliva sampling and infrared thermography measurement. Should a subject take anything more than minor evasive action, such as rearing, or backing away rapidly, the sample was be abandoned, though this did not occur. The infrared thermography camera takes an image from 1 m away from the eye and is not known to cause stress in horses. Informed consent of the owner of the subjects and the veterinarians undertaking the surgery was recorded.

2.2. *Habituation*

As subjects were relatively naïve young horses, a systematic habituation period was used to accustom them to the data collection protocol one week beforehand (Supplementary Material 1). Subjects were fitted with a headcollar and leadrope and loosely retrained by a handler. The handler then gently restrained the colt and sham swabbed the inside of their mouth using Resano forceps which held a carrot, rather than a cortisol swab. Three images of each eye were then taken using the Infrared Thermography camera at approximately 1 m and at a 90-degree angle (Ijichi et al., 2020), taking breaks if subjects appeared agitated.

Colts were regularly rewarded throughout this process using small quantities of hard feed delivered by hand and handled calmly by competent handlers (Ijichi et al., 2018). This procedure was repeated on two different days.

2.3. Castration process

The day of castration the colts were castrated one after the other, either using the clamping technique (open castration) for 15 foals or with ligatures around the vascular spermatic cord (variation of the open castration) for 4 foals, all under general anaesthetic. It was conducted on a bed of thick straw indoors during warm weather conditions. Active warming strategies were not used. The castration procedure is detailed in Supplementary Material 4. The surgical procedure was performed by four different veterinarians, two of them using the open castration method and two of them using a variation of the open castration method with insertion of a drain and ligatures around the spermatic cord. Castration was conducted according to the clinician's judgement and total standardisation could not be mandated by the research for ethical reasons. Therefore, individual veterinarian and method (open or variation in the open method) were included in statistical models to account for differences in the clinician's approach. Pain relief (NSAID: Meloxicam or Flunixin meglumine) was administered on the day of castration to all colts in addition to the anaesthesia protocol (outlined in Supplementary Material 4) and were given up to 48 h and 72 h after castration depending on the method and the clinician. The administration of pain relief varied between veterinarians but was consistent for each veterinarian and was therefore accounted for within the model as Veterinarian was included in statistical model.

Time from first incision until the colt stood after surgery was 27.5 + /- 14 min for the open castration method and 41.5 + /- 12.6 min variation of the open castration method with ligatures around the spermatic cord. Surgery time therefore varied individually but also depending on the method chosen. Increased surgery time may impact inflammation and tissue damage and thereby pain experienced. Individual horse (and thereby individual experience of castration) and method (open versus variation in the open method) were included in statistical models to account for differences in castrations.

2.4. Data collection

Data collection was conducted as per the schedule outlined in Supplementary Material 1. One week before castration, baseline recordings were taken. To this end, an arena of 6 by 9 m was set up directly next to the home stalls. At two locations, there was an enclosure of these dimensions beside the home stalls for the colts. At the third location a temporary arena was made using electrical tape without electrical charge to the same dimensions which was also beside the home stables. Subjects were brought to the testing arena and allowed 15 min to explore the area and habituate to it. Following this, a second experimenter recorded the behaviour of the subject for 30 min with two GoPro Hero8 black fixed on each side of the arena to record behaviour for ethogram analysis. Subjects were then restrained using a headcollar and leadrope. Subsequently, a salivary swab was taken for cortisol analysis and three images were taken of each eye using the IRT camera. Video recording of the colt's lateral head either of the left or the right side (randomly assigned) (4 K HDR FDR-AX700 camcorder) was used to provide 1 min of clearly visible footage of the head of the subject for subsequent HGS analysis.

2.4.1. Cortisol

Saliva was collected with Salivette cotton rolls placed loosely onto the tongue of the horse for one minute each using forceps. At the end of each day, the Salivettes were centrifuged for six minutes at 1400 X g with a Hettich EBA 20 centrifuge. Samples were stored at —20°C until analysis. Concentrations of cortisol were determined with the Salimetrics® High Sensitivity Salivary Cortisol Enzyme Immunoassay kit, a direct enzyme immunoassay without extraction that has been validated for equine saliva (Schmidt et al., 2010). Baseline cortisol was collected on two days during the baseline data collection period (Gleerup and Lindegaard, 2016) and the average of the two used for analysis to account for any fluctuations that might occur. This was taken to indicate individual baselines in cortisol. A sample was taken immediately prior to castration and at 20 min post-incision (referred to as "Castration"), 1, 2, 3, 4, 8, 24, 48 and 72-hours post-castration (Supplementary Material 1). This is referred to as Cortisol throughout.

2.4.2. Infrared thermography

Eye temperatures were taken (Flir™ E5) from approximately one metre and a 90-degree angle to the sagittal plane of the subject's head (Ijichi et al., 2020). Although this should ideally be standardised using ground poles and markers (Ijichi et al., 2020; Squibb, 2018), this was not possible. The necessary materials were not available and it would have required additional handling of foals post-castration that was deemed to be detrimental to their welfare. FLIR tools software (version 5.9.16284.1001, FLIR Systems Inc.) was used to analyse IRT images. The maximum temperature found between the lateral commissure and the lacrimal caruncle of the palpebral fissure was recorded using the elliptical target function which captured no less than 1 cm around the eye area. The highest absolute values noted from the 3 available images for each eye were recorded. The right and left eye were averaged for each time point: baseline, immediately prior to castration, on eyes opening post-castration and 1, 2, 3, 4, 8, 24, 48 and 72-hours thereafter (Supplementary Material 1). This is referred to as IRT throughout. The discrepancy between the left and right eye was also recorded (highest right eye temperature – highest left eye temperature) as these were previously noted to be related to personality post-castration in dogs (Lush and Ijichi, 2018). This is referred to as IRT Discrepancy throughout.

2.4.3. Ethogram

Behavioural assessment of pain was conducted using an ethogram including both pain-related and maintenance behaviours. 30-min videos of the behaviour of each horse was recorded using two cameras (Go Pro Hero 8) in the same time-points as other recordings, a blind observer analysed videos with a focal animal continuous recording method, using the software Boris Inc® (Behavioural Observation Research Interactive Software). The ethogram used for behavioural analysis was adapted from Torcivia and McDonnell, (2020) and Ashley et al. (2005). To better define some specific behaviours additional definitions were used (Zeitler-Feicht, 2003; McDonnell, 2003). The resulting ethogram is reported in Supplementary Material 2. Depending on the behaviour, they were distinguished in "event" or "state"; then respectively evaluated for frequency or duration. Behaviours were also grouped in three categories: maintenance (explorative behaviour, interaction with other horses, grooming), stress (defecation, urination, tail swish) and pain-related behaviours (low head carriage, foot stamping, vocalization/hyperresponsiveness, rigid stance and reluctance to move, aggression toward handlers, horses, objects and self-looking). A specific key was added for those moments in which the horse was not clearly visible ("not visible"). Behavioural assessment of pain was conducted using an ethogram including both pain-related and maintenance behaviours. 30-min videos of the behaviour of each horse was recorded using two cameras (Go Pro Hero 8) in the same time-points as other recordings, a blind observer analysed videos with a focal animal continuous recording method, using the software Boris Inc® (Behavioural Observation Research Interactive Software). The ethogram used for behavioural analysis was adapted from Torcivia and McDonnell (2020), and Ashley et al. (2005). To better define some specific behaviours additional definitions were used (Zeitler-Feicht, 2003; McDonnell, 2003). The resulting ethogram is reported in Supplementary Material 2. Depending on the behaviour, they were distinguished in "event" or "state"; then respectively evaluated for frequency or duration. Behaviours were also grouped in three categories: maintenance (explorative behaviour, interaction with other horses, grooming), stress (defecation, urination, tail swish) and pain-related behaviours (low head carriage, foot stamping, vocalization/hyperresponsiveness, rigid stance and reluctance to move, aggression toward handlers, horses, objects and self-looking). A specific key was added for those moments in which the horse was not clearly visible ("not visible").

Maintenance and Pain behaviour was observed during baseline (one week prior) and immediately post-castration, 4, 8, 24, 48 and 72-hours thereafter (Supplementary Material 1). Change from baseline was calculated to measure response to castration.

2.4.4. Horse grimace scale analysis

The Horse Grimace Scale (HGS), validated to be used in recognising pain in horses through facial expression (Dalla Costa et al., 2014), was scored at different time point during the study (Supplementary Material 1) through a 1-minute video of the left or the right (randomly assigned) lateral face of the colts filmed using a 4 K HDR FDR-AX700 camcorder, as described in Ijichi et al. (2023). The videos were taken to enable the face of the horse to remain in view of the camera for the duration of the recording. HGS scoring was done by a trained observer blinded to the timing of the recordings (pre- or post-castration). HGS incorporates six Facial Action Units (FAUs) that are independently scored on a three-point scale, with zero indicating that the assessor is confident that the action unit is not present, one indicating that the assessor is confident that the action unit is only moderately present, and two indicating that the assessor is confident that the action unit is obviously present (Supplementary Material 3). A FAU is classified as obviously present if it was clearly observable for the majority of the video duration as described by Ijichi et al. (2023). FAUs included are: stiffly backwards ears, orbital tightening, tension above the eye area, prominent strained chewing muscles, mouth strained and pronounced chin, and strained nostrils as reported in Supplementary Material 3. The HGS total score for each video consisted of the sum of the scores across the six FAUs.

Baseline HGS was observed twice on two different days (one week prior to castration) and averaged to calculate baseline HGS. It was then observed upon opening eyes, 1, 2, 3, 4, 8, 24, 48 and 72-hours post castration (Supplementary Material 1). Change in HGS total score from baseline was used to measure the response to castration.

2.4.5. Subjective personality assessment

Personality questionnaires were completed by the horse caretaker of each farm using a fully validated subjective questionnaire (Ijichi et al., 2013; Jolivald et al., 2022). Full reporting of this method is available in Ijichi et al. (2013) but in summary this questionnaire measures horses on traits relating to personality factors Neuroticism, Extroversion, Agreeableness, Gregarious towards people and Gregarious towards other horses. Due to the sampling location, the original questionnaire was translated from English into French, the predominant language spoken in this region of Switzerland. For the purpose of this study, only Neuroticism and Extroversion were included in analysis. Raters were blind to subject personality scores when assessing behavioural and physiological variables.

2.5. Statistical analysis

Data were statistically analysed in R version 4.2.1 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria); all Linear Mixed Models (LMMs) were fitted using the ***lme4*** package (Bates et al., 2015). Throughout the analysis, the fit of all models was checked using residual plots generated using the performance package (Lüdecke et al., 2021).

To determine the welfare impact of standard castration on horses (aim 1), Gaussian LMMs were used to determine whether IRT, IRT Discrepancy, Cortisol, HGS, Maintenance behaviour and Pain behaviour differed from baseline at each time point post-castration. Time points, Breed and Castration Method were included as categorical fixed effects in all models, while Individual horse, Veterinarian and Home Farm were included as random effects. Only variables which showed significant changes from baseline for at least one time point post-castration were included in further analysis for Aims 2–4.

Aims 2 and 3 focused on welfare indicators' responses to castration; analyses were therefore restricted to post-castration time points when a response was observed, i.e. when absolute values differed significantly from baseline. For each variable X and individual horse H, the response to castration at time point T was calculated as $X_{H,T} - X_{H,Baseline}$.

To determine whether personality accounts for individual variation in response to castration (aim 3), Gaussian LMMs were used to model the response to castration of IRT, Cortisol, HGS, Maintenance behaviour and Pain behaviour as a function of Neuroticism and Extroversion scores. Both personality factors were entered as fixed effects in the LMMs, after mean-centring and standardising to improve the interpretability of regression coefficients (Schielzeth, 2010). Time point, Breed and Castration Method were also entered into the models as categorical fixed effects, and Individual horse, Veterinarian and Home Farm as random effects.

To quantify the variability of all variables during the castration process and inter-individual variation in response to castration (aim 2), median and interquartile ranges were calculated for each variable at each timepoint. In addition, the adjusted repeatability (Schielzeth, 2010) of each variable's response to castration was derived from Aim 3 Gaussian LMMs as the proportion of total variance in response to castration explained by individual horse identity, once fixed and random effects were accounted for (Dingemanse and Dochtermann, 2013; Hertel et al., 2020). The proportion of variance explained by Veterinarian and Home Farm were also calculated to quantify the importance of these variables in explaining response to castration.

To determine if HGS score accurately indicates how the individual feels about their painful experience (aim 4), the relationship between HGS and IRT and Cortisol, respectively, at 4hrs post-castration was investigated using Spearman Rank Correlations (SRC). Simple correlations were chosen over more complex models for this analysis due to the reduced size of the dataset when considering a single time point ($n = 17$ data points for IRT, $n = 15$ for Cortisol), which did not allow the accurate modelling of multiple fixed and random effects. SRC was chosen as data was non-parametric. Each variable had a differing latency to reach maximum change from baseline (see Supplementary Material 5), however four hours post castration was chosen as the time point when the variables of interest were at or approaching their peak (Lush and Ijichi, 2018).

3. Results

3.1. Aim 1 - welfare impact of standard castration on horses

IRT values were highly significantly decreased from baseline until five hours post-castration, when individual, veterinarian and farm were included as random effects (Table 1).

IRT discrepancies were not significantly changed from baseline to post-castration, when Individual Horse, Veterinarian and Home Farm were included as random effects (Supplementary Material 6). IRT Discrepancy was excluded from further analysis as it did not show changes in response to castration.

Salivary cortisol values significantly increased from baseline from 2-hours until 24-hours post-castration when Individual Horse, Veterinarian and Home Farm were included as random effects (Table 2).

HGS values significantly increased from baseline from 1-hour until 48-hours post-castration when Individual Horse, Veterinarian and Home Farm were included as random effects (Table 3).

Maintenance behaviour significantly decreased from baseline from 4 h until 72-hours post-castration when Individual Horse, Veterinarian and Home Farm were included as random effects (Table 4).

Table 1
Model of IRT over time from baseline when Individual Horse, Veterinarian and Home Farm are included as random effects.

Fixed Effect	Estimate (°C)	Std Error	Deg Freedom	T Value	p-Value
(Intercept)	36.16	0.194	45.847	186.632	< 0.001
Castration	-0.654	0.191	206.455	-3.431	**0.001**
1 h Post Cast.	-0.751	0.191	206.509	-3.939	**< 0.001**
2 h Post Cast	-0.834	0.194	206.719	-4.296	**< 0.001**
3 h Post Cast	-0.796	0.185	206.074	-4.303	**< 0.001**
4 h Post Cast	-0.58	0.185	206.074	-3.137	**0.002**
5 h Post Cast	-0.54	0.191	206.455	-2.834	**0.005**
6 h Post Cast	-0.228	0.185	206.074	-1.231	0.22
7 h Post Cast	0.102	0.188	206.241	0.543	0.588
8 h Post Cast	0.222	0.185	206.074	1.202	0.231
24 h Post Cast	0.262	0.185	206.074	1.415	0.158
48 h Post Cast	-0.214	0.185	206.074	-1.159	0.248
72 h Post Cast	-0.141	0.185	206.074	-0.761	0.447
Swiss Warmblood	0.1	0.181	16.135	0.552	0.589
Franches-Montagnes	0.346	0.389	15.835	0.891	0.386

Table 2
Model of salivary cortisol over time from baseline when Individual Horse, Veterinarian and Home Farm are included as random effects.

Fixed Effect	Estimate (°C)	Std Error	Deg Freedom	T Value	p-Value
(Intercept)	0.586	0.237	6.848	2.473	0.043
Pre Castration	-0.004	0.185	134.656	-0.022	0.983
Castration	0.224	0.207	137.229	1.082	0.281
2 h Post Cast	0.791	0.246	136.415	3.22	**0.002**
3 h Post Cast	0.606	0.246	136.415	2.465	**0.015**
4 h Post Cast	0.431	0.188	135.083	2.291	**0.023**
8 h Post Cast	0.447	0.185	134.656	2.412	**0.017**
24 h Post Cast	0.469	0.185	134.656	2.534	**0.012**
48 h Post Cast	0.269	0.185	134.656	1.453	0.148
72 h Post Cast	0.088	0.185	134.656	0.474	0.636
Swiss Warmblood	0.087	0.149	16.744	0.582	0.568
Franches-Montagnes	0.462	0.301	13.385	1.537	0.148

Table 3
Model of HGS over time from baseline when individual, veterinarian and farm are included as random effects.

Fixed Effect	Estimate (°C)	Std Error	Deg Freedom	T Value	p-Value
(Intercept)	1.412	0.57	6.455	2.476	0.045
Castration	0.206	0.519	143.077	0.397	0.692
1 h Post Cast	1.158	0.527	143.467	2.196	**0.03**
2 h Post Cast	1.559	0.519	143.077	3.005	**0.003**
3 h Post Cast	1.147	0.519	143.077	2.211	**0.029**
4 h Post Cast	2.029	0.519	143.077	3.913	**< 0.001**
8 h Post Cast	3.265	0.519	143.077	6.294	**< 0.001**
24 h Post Cast	1.676	0.519	143.077	3.232	**0.002**
48 h Post Cast	1.206	0.519	143.077	2.325	**0.021**
72 h Post Cast	0.559	0.519	143.077	1.077	0.283
Swiss Warmblood	-0.606	0.376	12.117	-1.611	0.133
Franches-Montagnes	0.002	0.723	10.675	0.003	0.998

Pain behaviour increased from 4 to 48 h post-castration compared to baseline. However, these changes were not statistically significant when Individual Horse, Veterinarian and Home Farm were included as random effects (Table 5).

Table 4
Model of Maintenance Behaviour over time from baseline when Individual Horse, Veterinarian and Home Farm are included as random effects.

Fixed Effect	Estimate (°C)	Std Error	Deg Freedom	T Value	p-Value
(Intercept)	8.715	1.966	4.526	4.434	0.009
4 h Post Cast	-8.655	1.506	80	-5.746	**< 0.001**
8 h Post Cast	-7.927	1.506	80	-5.263	**< 0.001**
24 h Post Cast	-7.75	1.506	80	-5.145	**< 0.001**
48 h Post Cast	-4.445	1.506	80	-2.951	**0.004**
72 h Post Cast	-4.884	1.506	80	-3.242	**0.002**
Swiss Warmblood	0.92	1.568	12.479	0.587	0.568
Franches-Montagnes	-0.59	3.04	10.417	-0.194	0.85

Table 5
Model of Pain Behaviour over time from baseline when Individual Horse, Veterinarian and Home Farm are included as random effects.

Fixed Effect	Estimate (°C)	Std Error	Deg Freedom	T Value	p-Value
(Intercept)	1.032	2.054	43.221	0.502	0.618
4 h Post Cast	4.174	2.437	79.963	1.713	0.091
8 h Post Cast	4.494	2.437	79.963	1.844	0.069
24 h Post Cast	4.022	2.437	79.963	1.651	0.103
48 h Post Cast	4.735	2.437	79.963	1.943	0.056
72 h Post Cast	0.514	2.437	79.963	0.211	0.834
Swiss Warmblood	-1.392	1.584	13.849	-0.879	0.395
Franches-Montagnes	-1.521	3.211	13.815	-0.474	0.643

3.2. Aim 2 - consistent individual variation in behavioural and emotional responses to pain

Median behaviour and physiology at baseline and at the peak change timepoint are reported in Table 6. Interquartile ranges are presented to illustrate variation. Complete results for the effect of Individual Horse, Home Farm and Veterinarian on behaviour and physiology response to pain are presented in Table 6. Adjusted repeatability reflects the percentage of variation accounted for by each factor.

3.3. Aim 3 - the influence of personality on individual behavioural and physiological responses to pain

Personality did not have an effect on IRT, HGS, Maintenance or Pain Behaviour, in response to castration (see Supplementary Material 7). Neuroticism significantly negatively predicted Cortisol response to castration (estimate = −0.309, SE = 0.134, p = 0.04). Extroversion significantly positively predicted Cortisol response to castration (estimate = 0.312, SE = 0.122, p = 0.028).

Table 6
Median and Interquartile ranges (IQR) are presented for each variable at baseline and at the peak change timepoint. Adjusted repeatability for each variable when considering Individual Horse, Home Farm and Veterinarian are included and indicate the percentage of variability accounted for by each factor.

			Adjusted Repeatability		
	Baseline Median (IQR)	Peak Median (IQR)	Individual Horse	Home Farm	Vet
IRT	35.55(0.5)	35.4(0.88)	0.002	0.436	0.16
Cortisol	0.672(0.27)	1.52(0.89)	0.027	0.209	0
HGS	1(1)	4(3)	0.026	0.15	0.095
Maintenance Behaviour	8.49(8.45)	0.2(0.83)	0.206	0	0.468
Pain Behaviour	0(0)	0(4.16)	0.007	0	0

3.4. Aim 4 - associations between HGS and physiological responses to pain

HGS was not significantly associated with IRT (SRC: n = 15, r = -0.0258, p = 0.317), but was significantly positively associated with Cortisol (SRC: n = 13, r = 0.568, p = 0.027) at 4 h post-castration (Fig. 1).

4. Discussion

The current study aimed to provide the first evidence for the welfare impact of standard castration on horses and, in particular, whether individuals vary in their behavioural and emotional responses to pain using castration as a consistent source of pain. The potential impact of personality on individual behavioural and physiological responses to pain was investigated to develop on from previous work in horses (Ijichi et al., 2014) and dogs (Lush and Ijichi, 2018). Finally, Horse Grimace Scale was compared to physiology to determine if this particular pain behaviour accurately indicates how the individual feels about their painful experience, since previous studies indicated that other behaviour may not (Ijichi et al., 2014; Lush and Ijichi, 2018). Results indicate castration is a significant welfare challenge and several factors affect individual response to this, but this should be interpreted with some caution as the sample size was relatively small and complete standardisation of castration was not possible.

4.1. Aim 1 - the welfare impact of standard castration on horses

Physiological measures showed pronounced responses to castration. IRT showed a highly significant decrease post castration which was maintained until five hours post-castration. This decrease may be influenced by anaesthesia, however this is typically associated with longer (45-minute or longer) procedures (Mayerhofer et al., 2005). The surgery here was completed in less than 20 min with typical subjects recovering from anaesthesia and standing between 27 and 47-minutes post-incision. Horses undergoing sedation with no painful treatment had an average decrease of only 0.5°C after an hour of sedation when not provided active warming methods and this was not different at this time point from those under a warming blanket (Florczyk et al., 2020). The decrease seen here was greater in a much shorter time and may be attributed to the addition of painful stimuli. Further, the observed reduction is consistent with existing literature on species such as sheep not under sedation (Stubsjøen et al., 2009) and dogs (Lush and Ijichi, 2018) and supports the use of core temperature decrease as a non-invasive indicator of pain. IRT Discrepancy did not show any significant difference in response to castration. If individual eye temperature reflects activation of the lateral hemisphere as suggested by Evans et al. (2024), Submitteda, Submittedb; Lush and Ijichi, 2018), it might be expected that the right eye would be hotter than the left as a result of painful castration. Though there are several primary hypotheses on the function of each hemisphere, there is general agreement that the right hemisphere would be activated in response to a negative, arousing experience, where withdrawal is beneficial (Goursot et al., 2021) but no corresponding increase in right eye temperature was observed here. While the use of discrepancy is consistent with prior castration research in dogs (Lush and Ijichi, 2018), recent studies noting potential lateralised temperature differences in horses use the absolute temperature of each eye (Evans et al., 2024; Evans et al., Submitted). In contrast, salivary cortisol significantly increased post-castration, peaking at 2 h but remaining significantly elevated for 24 h, remaining non-significantly elevated for 48 h. IRT and both absolute cortisol values and the latency to return to baseline cortisol values indicate that castration presented a significant welfare challenge and the pain relief administered may not be sufficient.

In support of physiological measures, behaviour measures of pain also revealed highly significant and long-lasting responses to pain. HGS was very highly significantly elevated, peaking at 8 h, and remaining significantly elevated for 48 h post-castration. While the effect of sedation on HGS during early observations cannot totally be discounted with regards to orbital tightening, the remaining FAUs are linked to an increase in muscular tension which is at odds with the relaxation of facial muscles in response to sedation (Oliveira et al., 2021). Therefore, the observed increase in HGS immediately post-castration is particularly notable. Maintenance behaviour was highly significantly reduced post-castration, though some of this response is likely explained by recovery from general anaesthesia. However, this reduction was still

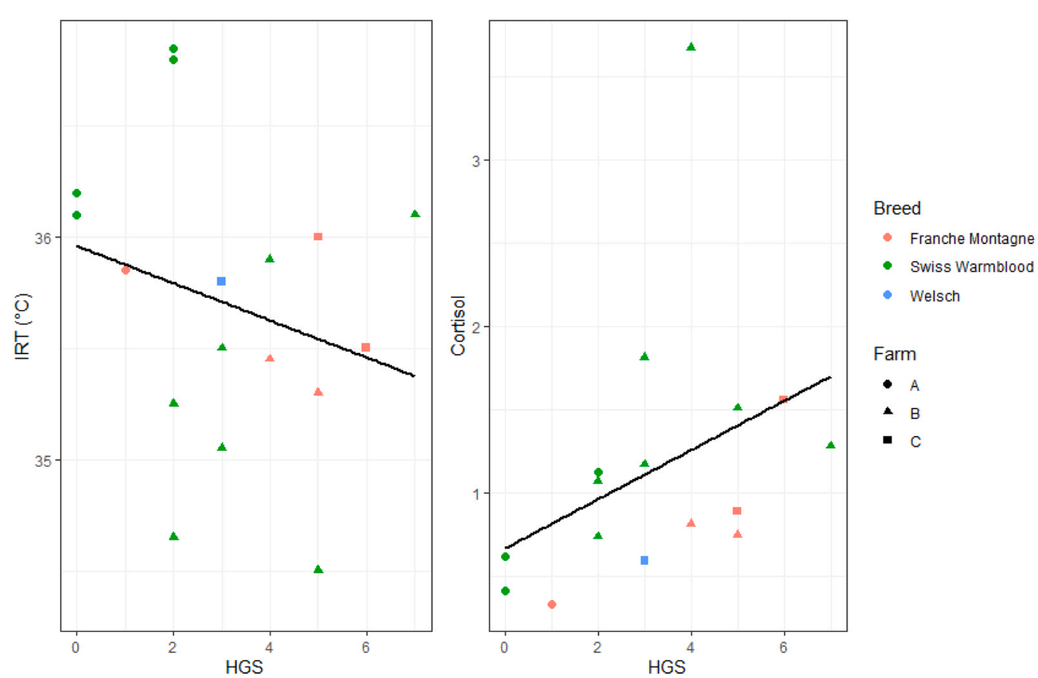

Fig. 1. Scatterplots showing no association between HGS and IRT but a significant, positive association between HGS and Cortisol (P = 0.027) at 4 h post-castration. The Breed and Home Farm for each subject are indicated.

pronounced even at 72 h post-castration which indicates long-term disruption to behavioural function indicative of pain (Ashley et al., 2005; Torcivia and McDonnell, 2020). Pain behaviour was observed over the three-day period but at relatively low durations, resulting in many zero values. Therefore, though increases in Pain behaviour were observed, particularly at four, eight and forty-eight hours post-castration, these were not statistically significant. these were not statistically significant. Therefore, this behavioural indicator may be useful in an applied setting in terms of presence/absence but may have limited application in research.

4.2. Aim 2 – consistent individual variation in behavioural and emotional responses to pain

Notable variation around the median was observed for most variables post-castration, with the exception of Maintenance Behaviour which reduced to such a significant effect that very little variation was reported. Therefore, there appears to be wide variation in the welfare impacts of castration on horses, despite being a standard procedure. However, IRT, Cortisol, HGS and Pain behaviour show little to no consistent individual variation in response to castration. This indicates that the observed variation in responses to castration were not well explained by consistent individual differences.

Maintenance Behaviour showed around 23 % consistency across individuals suggesting modestly consistent individual differences. Farm explained small amounts of residual variation in IRT and HGS (11 % and 15 % respectively), while individual clinician explained a notable percentage of residual variation in IRT (37 %), Maintenance Behaviour (41 %) and a smaller proportion of HGS (14 %). Therefore, taken together, observed behavioural and physiological responses to pain show relatively large variation around the median but this was not well explained by consistent individual differences. Instead, Veterinarian explained more of the variation. This suggested that particular approach of the clinicians may result in differences in thermal and behavioural disruption. Indeed, these two parameters are the most obviously influenced by anaesthesia, the administration of which varies dependent on clinical judgement. Further research to refine techniques for conducting castration based on minimising behavioural and physiological disruption are warranted.

4.3. Aim 3 the influence of personality on behavioural and physiological responses to pain

The inclusion of personality in models did not improve the proportion of variance explained for IRT. That is, individual differences in these values were not predicted by personality scores. This is not consistent with a potential association with peak temperature increase post castration in dogs (Lush and Ijichi, 2018). However, personality did explain individual variation in Cortisol response to castration with both Neuroticism and Extroversion predicting changes.

Increasing Neuroticism scores were associated with reduced cortisol response to castration. That is, the magnitude of the response was lesser for more Neurotic horses. While an association between Neuroticism and cortisol would be expected, the reduction in cortisol response is somewhat at odds with human literature. Highly neurotic people have a higher emotional stress response to pain when compared to those who have a low score for neuroticism (Goubert et al., 2004; Koenig et al., 2015). Neuroticism is conceptually linked with stress sensitivity and physiological reactivity to stressors though it is acknowledged that this relationship is surprisingly inconsistent and reflective of the complexity of wider cortisol psychophysiology (Garcia-Banda et al., 2014). Further, since more Neurotic horses are rated less tolerant to pain by their owners and score lower for Stoicism calculated from clinical data, an increased cortisol response to a painful stimuli might be anticipated. It is possible that more Neurotic horses have higher baseline cortisol which may limit the response that can be observed due to a ceiling effect and/or via negative feedback on the HPA axis (Henckens et al., 2016). Indeed, post-hoc analysis revealed that Neuroticism was significantly associated with higher baseline cortisol, supporting this possibility (Supplementary Material 8).

Extroversion score was highly significantly positively associated with the magnitude of cortisol response to castration. This is consistent with the previous dog study, in which it was Extroversion that predicted physiological responses to castration pain (Lush and Ijichi, 2018). With regards the previous research in horses and dogs, the association between Extroversion and cortisol is consistent with greater behavioural indicators of pain, if that expression reflects greater suffering. However, in humans, Extroversion is associated with increased expression, but not underlying suffering, in response to pain. For example, human subjects scoring more highly for Extraversion express their experiences of pain more clearly (Harkins et al., 1989), though they may actually experience pain less intensively (Ramírez-Maestre and Esteve, 2013). Interestingly, Extroversion in the current study did not explain variation in behavioural responses to pain as it does in the human literature, horse and dog studies. Further, Extroversion was not associated with increased IRT responses as was observed in the dog study (Lush and Ijichi, 2018).

Personality scores did not explain individual variation in HGS, Maintenance or Pain Behaviour in response to castration. With regards Pain Behaviour this should be interpreted with caution since there were many zero values resulting in only tendencies to differ in response to castration. While, in castrated dogs, Neuroticism was not an important factor in predicting either behavioural or physiological responses to pain in dogs, Extroversion was (Lush and Ijichi, 2018). In contrast, Neuroticism was associated with both owner ratings of pain tolerance and clinically calculated Stoicism (Lush and Ijichi, 2018). The current study suggests that Maintenance and Grimace indicators may not be vulnerable to the confounding effects of personality and are therefore potentially more suitable measures of pain and tissue damage than lameness (Ijichi et al., 2014). Alternatively, it is possible that personality was not associated with these specific pain behaviours because pain itself did not vary. However, our results for aims 1, 2 and 3 demonstrate that there is individual variation in behavioural and physiological responses to the castration and that Neuroticism and Extroversion are associated with differences in at least one marker of affective state in response to this pain (cortisol). HGS is the most easily accessible method of quantifying pain expression in an applied context as it requires no specialist equipment and is not time-consuming (Dalla Costa et al., 2014). Therefore, it is crucial to know whether HGS is both resilient to the confounding effects of personality and sensitive to the underlying affective state of the animal.

4.4. Aim 4 HGS as an indicator of individual affective responses to pain

HGS did not correlate with IRT at four hours post-castration. Conceptually, a positive association would be expected; however, the meaningful effect of Veterinarian and, to a lesser degree, Home Farm on IRT could not be accounted for as a potential confounding factor in the analysis of the relationship between HGS and IRT due to a reduced sample size. Therefore, while our data does not support HGS reflecting underlying affective state as measured by IRT, this should be considered conservatively due to analytical limitations. However, the Short-Form Glasgow Composite Measure Pain Scale (Reid et al., 2013) also did not correlate with IRT changes in dog's post-castration (Lush and Ijichi, 2018). While IRT is consistently shown to respond to pain in non-human animals, it is yet to be shown to relate to behavioural indicators of pain. In contrast, HGS is associated with salivary cortisol at four hours post-castration, suggesting that it may be a suitable indicator of individual affective states in response to pain. This support previous research (Dalla Costa et al., 2014; Dalla Costa et al., 2016) validating this tool to determine pain in response to castration and laminitis and suggests that it can be used to gain valuable insight into individual responses to painful stimuli without being confounded by differences in pain expression that result from personality. This promising early finding should be accepted with caution and will require further research with larger sample sizes, ideally in one location using complementary physiological measures of arousal, including the addition of heart rate variability ideally (Rietmann et al., 2004; Shafford et al., 2005).

5. Conclusion

IRT, Cortisol, HGS and Maintenance behaviour all showed significant changes from baseline in response to castration, with behavioural disruption lasting for three days. This indicates a significant welfare challenge of standard castration and indicates that increased pain relief may be warranted. Physiological and behavioural responses to castration varied but were not consistent individual differences, with the exception of Maintenance Behaviour. Veterinarian performing the procedure affected IRT and Maintenance behaviour, potentially reflecting the importance of clinician's approach. Personality explained differences in cortisol responses with Neuroticism reducing, and Extroversion magnifying, the magnitude of response to castration. While previous studies show that indicators of animal pain relying on gross, overt, behaviour patterns are confounded by personality, here HGS was not, suggesting that this pain indicator based on more subtle, involuntary changes to facial expression may be more resilient to individual differences in pain expression. Crucially, it appears to reflect underlying affective pain states as it was associated with cortisol. Therefore, it is potentially an important tool in sensitive recognition of pain at an individual level. These results are very promising but should be interpreted with caution. Further research utilising larger samples, fully controlled castration methods, a range of species and a wider range of physiological measures including heart rate variability should be done to determine both the effect of baseline welfare on pain resilience and the sensitivity of facial expressions of pain as an indicator of suffering during painful experiences.

Data availability

Original data and R code can be accessed at the following repository https://uclandata.uclan.ac.uk/id/eprint/604

CRediT authorship contribution statement

Sabrina Briefer-Freymond: Writing – review & editing, Resources, Methodology, Investigation, Funding acquisition. **Carrie Ijichi:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Emanuela Dalla Costa:** Writing – review & editing, Methodology, Investigation, Funding acquisition. **Bruckmaier Rupert M.:** Resources, Formal analysis. **Aurelie Jolivald:** Writing – review & editing, Formal analysis. **Maria Giorgia Riva:** Writing – review & editing, Investigation. **Elie Atallah:** Writing – review & editing, Investigation.

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Declaration of Competing Interest

The authors have no competing interests to declare.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tvjl.2025.106538.

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