Journal of Veterinary Behavior 76 (2024) 25–33

Contents lists available at ScienceDirect

Journal of Veterinary Behavior

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

Effects of chiropractic manipulations on behavioral and physiological parameters in healthy horses: A preliminary investigation

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ARTICLE INFO

Keywords:
Complementary medicine
Alternative medicine
Equine
Welfare
Behavior
Animal-based measures

ABSTRACT

Human chiropractic is a well-established, evidence-based complementary medicine. Contrariwise, there is a lack of research on its impact on horses. This study aimed to evaluate the effects of chiropractic treatment on healthy horses by measuring behavior, maximum temperature of the eyes (MaxTE), salivary cortisol (SC), and heart rate variability (HRV). Eight mares were selected and exposed to a 15-day habituation period. Then, they were randomly assigned to a grooming (GROOM) or chiropractic (CHIRO) session, performed by the same qualified chiropractor. The experiment was designed as a cross-over study and repeated after a 10-day washout (i.e., Week 1 and Week 2). During-CHIRO, the chiropractor treated and recorded joint hypomobility. During-GROOM, the mares were subjected to a standardized grooming session. MaxTE and SC were obtained before (T0), immediately after (T1), and one day after (T24) both sessions. In addition, before (Pre), during (During), and after (Post) both sessions, the mares were video recorded, and heart rate for HRV analysis was taken. Videos were analyzed using a specific ethogram and a Behavioral Discomfort Score (BDS) was obtained. Results showed that the mares manifested temporary signs of discomfort During-CHIRO, highlighted by an increase of 'avoidance movements' ($p < 0.001$) and 'alert' ($p = 0.038$). This was confirmed by an increase in BDS During-CHIRO ($p = 0.044$). No sympathetic shift was shown by MaxTE, SC, and HRV. A significant increase in the standard deviation of RR intervals (SDNN) suggested a parasympathetic shift in Post-CHIRO ($p = 0.040$). Joint hypomobility tended to decrease between the first and second CHIRO ($p = 0.09$). Our results showed that equine chiropractic could be performed by a qualified veterinary chiropractor, leading to transient discomfort behavior, not accompanied by acute stress response. On the contrary, there seems to be a subsequent relaxation, demonstrated by an autonomic nervous system shift toward the parasympathetic branch. Consequently, chiropractic manipulation could be considered as an integrative treatment to improve the horses' welfare.

Introduction

Chiropractic is an established human healthcare profession in the field of musculoskeletal health (Gevers-Montoro et al., 2021). In human medicine, chiropractic is the largest and best-recognized complementary and alternative medicine (Meeker and Haldeman, 2002), being licensed and regulated in many countries throughout the world (Chapman-Smith, 2000). Along with human chiropractic, also veterinary chiropractic has developed and has been mainly applied to cats, dogs, and horses (Taylor and Romano, 1999). Equine chiropractic was mainly used to treat disorders such as lameness, neck or back pain, poor sports performance, and trauma (Haussler et al., 1999; Haussler and Erb,

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https://doi.org/10.1016/j.jveb.2024.10.006
Received 4 June 2024; Received in revised form 12 October 2024; Accepted 26 October 2024
Available online 2 November 2024

2003; Gomez Alvarez et al., 2008). Nevertheless, research on equine chiropractic is limited and does not reach the level of knowledge achieved in humans (Langstone et al., 2015; Acutt et al., 2019). Some studies have documented the positive effects of chiropractic techniques on equine spinal mobility and pain reduction (Haussler et al., 2010, 2020) and according to them, chiropractic often improved the horses' health and welfare. However, in humans, even if beneficial, in 30% of cases chiropractic manipulation was shown to be painful and to cause adverse reactions, such as headaches (Hurwitz et al., 2004; Hurwitz et al., 2005). To the authors' knowledge, no studies investigated the adverse effects on horses.

With these premises, the authors hypothesized that chiropractic manipulations may cause transient discomfort and distress during the manipulation, but relaxation and improvement in joint hypomobilities afterward. Hence, this study aimed to provide scientific evidence of any effects of chiropractic on healthy horses (*Equus caballus*) by collecting maximum temperature of the eyes (MaxTE), salivary cortisol (SC), behavior, and Heart Rate Variability (HRV) before, during, and after the chiropractic treatment, as these measures have already been proven to be objective in identifying stress and pain in horses (Valera et al., 2012; Reid et al., 2017).

Materials and methods

The protocol was approved by the Committee on Animal Welfare and Ethics of the University of Bologna (Approval number 75573).

Animal selection

Eight healthy mares, not used for sport, recruited from the research herd of the University of Bologna, were selected for inclusion in the study. The study was conducted outside the breeding season, and the mares did not show any oestrus-related behavioral and physiological signs. The mares were subjected to a prior psycho-physical examination, to exclude behavioral (e.g., abnormal repetitive behaviors (ARBs), signs of aggression) and physical problems. The physical examination included a 5-minute electrocardiogram (ECG; Televet 100, Engel Engineering GmbH, Heusenstamm, Germany) to exclude the presence of arrhythmias, and a lameness assessment using the American Association of Equine Practitioners (AAEP) Lameness Scale (Anonymous, 1991) to exclude severe lameness (grade 3 or above). The clinical data of the eight selected mares are shown in Table S1. The husbandry system of the mares was not modified during the study. The mares were kept in individual loose boxes in a fully enclosed stable during the night and they were led to a large paddock where they were kept in group during the day (on average 6–8 h/day). They were fed lucerne hay three times a day covering their nutritional requirement, water was offered *ad libitum* in the paddock and in the box. They were retired harness racing mares and they were not not undergoing any exercise routine. They were just kept outdoor and used for veterinary teaching purpose.

Experimental protocol

Habituation phase

The mares were subjected to a 15-day habituation phase to minimize the stress response associated with the experimental process. They were subjected for 15–20 min daily to the procedures described below. During days 1–5, the mares were habituated to being touched and groomed in each part of the body, including the legs and the head. The brush used was always the same (length of 20 cm, plastic bristles of 5 cm and wooden back) throughout the habituation and experimental phase. They were introduced to and allowed to interact with the polystyrene blocks used by the chiropractor to perform the manipulation, first in an external area (Figure 1) and subsequently inside the large stall (5 m x 4 m) where the experimental phase would have taken place. These green polystyrene blocks (1 m x 0.6 m x 0.8 m) were used as supports to reach certain areas to be manipulated. Specifically, the chiropractor stepped on the blocks to better reach spines, necks and heads, so that the proper pressure could be applied during manipulation. After that, during days 6–10, they were then exposed to and habituated to standard saliva collection procedures (see "Experimental phase" for more details on the procedure). They were also habituated to an HR monitor belt (Polar M460; Polar Electro Oy, Kempele, Finland) and to the presence of the thermal imaging camera (FLIR E76 24°, FLIR Systems AB, Danderyd, Sweden) and the operator taking the images in their box. Finally, during days 11–15 all the procedures above described were performed together in sequence, including moving the mares from their box to the experimental area. The handler and the operators present in this phase were the same present during the experimental phase.

Experimental phase

After 15 days, all the mares proved to be relaxed and comfortable with the procedures, so the experimental phase started. This phase was conducted over two weeks (i.e., Week 1 and Week 2), in a cross-over design. During Week 1, the eight mares were randomly assigned to either the grooming session (GROOM), considered as a control, or chiropractic treatment (CHIRO), both lasting 20 min and carried out by the same operator, i.e., a qualified veterinary chiropractor (Figure 2). The CHIRO was standard for all subjects and was structured in 2 stages. The first stage consisted of the diagnostic assessment of joint range of motion, necessary to identify the reduction in joint mobility of each motor unit (Taylor and Romano, 1999). The second stage was represented by therapeutic chiropractic manipulation to restore normal joint mobility, so that any finding of altered joint mobility was immediately followed by chiropractic manipulations if deemed appropriate. The treatment started at the sacroiliac area and then moved cranially to the lumbar, thoracic, and cervical areas. It continued at the level of the fore and hind limbs. At the end of each CHIRO, the chiropractor summed up in a report the joint hypomobilities detected and manipulated. From this report, the type and number of hypomobilities were determined for each motor unit manipulated. The total sum of the hypomobilities was then calculated.

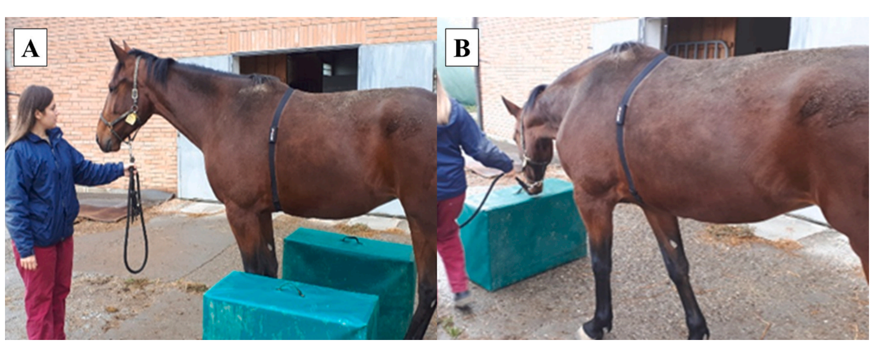

Fig. 1. Habituation phase. The moment in which the mare was habituated (A) and could interact (B) with the polystyrene blocks used for chiropractic manipulations.

Fig. 2. Experimental phase. A) chiropractic treatment (CHIRO). B) grooming session (GROOM).

During GROOM, the chiropractor touched the same body areas in the same order, but only brushed them and did not perform any manipulation or hypomobility assessment. This experimental scheme was repeated at Week 2 after a 10-day washout.

During the experimental phase, maximum temperature of the eyes (MaxTE), salivary cortisol (SC), behavior, and Heart Rate Variability (HRV) were obtained from the mares. Experimental procedures were conducted in the order described below. Firstly, IRT images of the left and right eye region were obtained before (T0), immediately after (T1), and one day after (T24) GROOM and CHIRO with the FLIR E76 thermal imaging camera. The camera was positioned at 90° to the sagittal plane and at a distance of approximately 1 m from the horse. The resolution of the camera was 160 × 120 pixels, with a thermic sensitivity of < 0.06 °C and an accuracy of ± 2 °C. Environmental temperature (°C) and humidity (%), taken with a weather tracker (Kestrel 4000 Pocket Weather Tracker, Nielsen-Kellerman Co., Boothwyn, PA, USA), were set on the camera before acquiring the images.

The next step was collecting salivary samples with a standard procedure that consisted of placing a cotton swab or gauze held by an arterial clamp at the side of the horse's mouth and having the horse chew on it for about 20 s (Bohák et al., 2013). Afterward, the gauze was squeezed with the help of a sterile syringe into Eppendorf test tubes that were immediately frozen and stored at −80° C.

After that, the mares were video recorded, using a focal animal sampling continuous recording method (Altmann, 1974). Videos were recorded by an operator using a portable video camera (HDR-CX405, Sony, Tokyo, Japan). They lasted 20 min and were obtained before (Pre), during (During), and after (Post) GROOM and CHIRO. The Pre and Post videos were recorded while the mares were free in their stalls, while the During videos were recorded while the mares were in the experimental area (i.e., the large stall). At the same time points (i.e., Pre, During, and Post) heart rate was recorded for 20 min with the Polar belt placed around the mares' thorax. Figure 3 describes the complete timeline of the procedures performed in the study. All experimental procedures were conducted with maximum care to minimize possible distress during the handling.

IRT images of the eyes

The IRT images of both eyes were analyzed using FLIR Tools® software (FLIR Systems AB, Danderyd, Sweden), as already reported in the literature (Menchetti et al., 2021) and the MaxTE (°C) of the ocular area was extrapolated. When it was not possible to extrapolate the MaxTE, a missing value (n.a.) was reported.

Salivary Cortisol (SC)

Samples of saliva were unfrozen and SC concentration (Nmol/L) was determined by radioimmunoassay (RIA) following the method described by Tamanini et al. (1983). The cortisol RIA was performed using an antiserum to cortisol-21-hemisuccinate-BSA, raised in a rabbit, at a working dilution of 1:20000 and [3 H]-cortisol (amount 30 pg/tube vial) as tracer. Validation parameters of the analysis were: sensitivity 0.26 pg/mg; intra-assay variability 6.8%; inter-assay variability 9.3%.

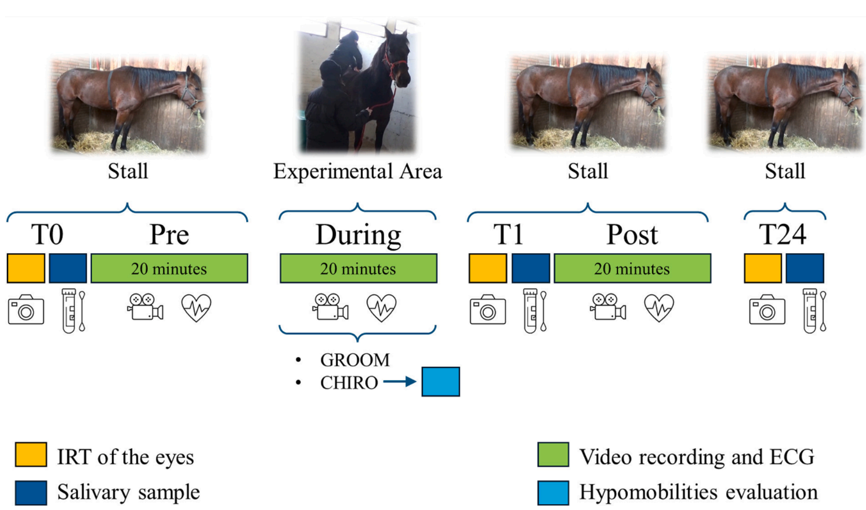

Fig. 3. Timeline explaining the procedures performed during the study for GROOM and CHIRO.

Behavioral parameters and Behavioral Discomfort Score (BDS)

To analyze the mares' behaviors, an ethogram was developed by the research team on purpose for this study (Table 1). The video recordings were analyzed by the same operator using Solomon Coder (version: beta 19.08.02). The behavioral events were expressed as frequency (numbers/20 min), while the behavioral states as duration (seconds/20 min). As reported in Table 1, according to the time point (i.e., Pre, During, and Post), some behaviors were not detected, so could not be performed by the mares in that context. In particular, the ethogram of the Pre and Post time points did not include the behaviors 'avoidance movements', 'interaction with handler', 'interaction with chiropractor', and 'playing with objects', and the ethogram of the During did not include the behaviors 'eating', and 'drinking'.

In addition, in order to compare a possible discomfort experience of the mares across the different phases (Pre, During and Post) a Behavioral Discomfort Score (BDS) was also calculated for each video. The BDS included the discomfort-related behaviors of 'attempt to bite or biting, 'attempt to kick or kicking', 'foot stamping', 'head shaking', 'head surveying', 'licking/chewing', 'lips movements', 'pawing', 'pulling back', 'sclera', 'squealing', and 'tail swishing'. Scores were assigned with a one-zero sampling method (i.e., occurrence or non-occurrence of each behavior) (Altmann, 1974). The individual scores of the aforementioned behaviors were then summed to obtain the final BDS. The BDS could range from 0 = no discomfort to 12 = maximum discomfort.

Heart Rate Variability (HRV)

The Polar data was uploaded into the Polar Flow software and RR intervals (i.e., the time elapsed between two successive R-peaks of the QRS complex on the electrocardiogram (Lanfranchi et al., 2011)) exported as a txt file. RR intervals were analyzed with Kubios HRV Standard program (Kubios version 3.5.0. for Windows; Kubios Oy, Finland). From each recording a central time window of 10 min was selected for the analysis. For each analyzed window, heart rate and time and frequency domain parameters were extrapolated (Shaffer and Ginsberg, 2017):

- mean heart rate (meanHR, in bpm)
- mean of RR intervals (meanRR, in ms)
- standard deviation of RR intervals (SDNN, in ms)
- root mean square of successive RR differences (RMSSD, in ms)
- absolute power of the low-frequency band (0.07–0.6 Hz) (LF absolute power, in ms^2)
- absolute power of the high-frequency band (0.01–0.07 Hz) (HF absolute power, in ms^2)
- ratio of LF-to-HF power (LF/HF, in %)

Statistical analysis

Descriptive statistics were performed, reporting median values and interquartile range (i.e., IQ1-IQ3). Since MaxTE, SC, behaviors, and HRV were not expected to differ between Week 1 and Week 2, the effect of the week (i.e., Week 1 *vs* Week 2) was tested using Generalized Linear Mixed Models (GLMM) and Linear Mixed Models (LMM). As no significant effect was found, it was decided to combine the two weeks of observation in subsequent analyses. On the contrary, joint hypomobilities data were not combined as authors wanted to test the effect of the week on each motor unit (cervical, thoracic, lumbar, sacro-pelvic, and limbs) and on the total sum of hypomobilities, using GLMM.

Then, as the time point Pre (for behaviors and HRV) and T0 (for MaxTE and SC) could constitute a basal level, the effect of GROOM/CHIRO within Pre/T0 was tested using GLMM and LMM. As expected, no significant effect was found, thus Pre-GROOM/Pre-CHIRO and T0-GROOM/T0-CHIRO data were merged into a single category renamed Pre-BASAL/T0-BASAL.

Table 1
Developed and applied ethogram, divided by events (numbers/20 min) and states (seconds/20 min). The column 'Time point' indicates at which experimental time point the behavior was considered and analyzed in the videos.

Behavior	Time point	Description
Behavioral events		
Attempt to bite or biting	Pre, During and Post	The horse puts the ears flat back, and bites or tries to bite the chiropractor or the handler (adapted from Riva et al., 2022)
Attempt to kick or kicking	Pre, During and Post	One or both hind legs lift off the ground and rapidly extend backward, the forelegs support the weight of the body and the neck is often lowered (Mcdonnell and Haviland, 1995).
Avoidance movements	During	Avoiding the massage/grooming by evasive steps to the side/backward/forwards or turning the head and neck to the right or to the left to avoid the chiropractor (adapted from Riva et al., 2022)
Body shaking	Pre, During and Post	Rapid, rhythmic rotation of the head, neck, and upper body along the long axis while standing with feet planted (Mcdonnell, 2003)
Defecation	Pre, During and Post	Elimination of feces (Siniscalchi et al., 2015)
Flehmen	Pre, During and Post	Head elevated and neck extended, with the eyes rolled back, the ears rotated to the side, and the upper lip everted exposing the upper incisors and adjacent gums. The head may roll to one side or from side to side (Mcdonnell, 2003)
Foot stamping	Pre, During and Post	Sharply strike the ground with a hoof by flexing and raising and then rapidly lowering a fore or hind leg (Mcdonnell, 2003)
Head shaking	Pre, During and Post	The horse shakes its head (Padalino et al., 2018)
Head surveying	Pre, During and Post	Head scanning through forty-five degrees or more, ears pricked up pointing toward the stimulus and stationary for < 5 s (adapted from Padalino and Raidal, 2020)
Licking/chewing	Pre, During and Post	Opening of mouth with extension and retraction of tongue, lip smacking without tongue extension, lateral jaw movements involving partial opening of lips (Mcgreevy, 2004)
Lips movements	Pre, During and Post	Upper or low lip movements
Pawing	Pre, During and Post	One front leg is lifted from the ground slightly, then extended quickly in a forward direction, followed by movement backward, dragging the toe against the ground in a digging motion (Mcdonnell and Haviland, 1995)
Pulling back	Pre, During and Post	The horse tries to elude the handler's control by pulling back with its head (Padalino et al., 2018)
Sclera	Pre, During and Post	Exposure of the white sclera around the eye (Thorbergson et al., 2016)
Self-grooming	Pre, During and Post	Nibbling, biting, licking, or rubbing a part of the body (Mcdonnell, 2003)
Sighing	Pre, During and Post	Emitting an audible long exhalation following a deep inhalation (Torcivia and Mcdonnell, 2021)
Sniffing	Pre, During and Post	The horse sniffs around, it sniffs some areas of the box (adapted from Padalino and Raidal, 2020)
Snorting	Pre, During and Post	Emitting an audible sudden forced exhalation through the nares (Torcivia and Mcdonnell, 2021)
Squealing	Pre, During and Post	Emitting a short, sharp, high-pitched vocalization (Torcivia and Mcdonnell, 2021)
Tail swishing	Pre, During and Post	The horse swishes his tail rapidly (Riva et al., 2022)
Turning head	Pre, During and Post	The horse turns its head and neck to the right or left (Padalino et al., 2018)
Urination	Pre, During and Post	Elimination of urine (Siniscalchi et al., 2015)

(*continued on next page*)

Table 1 (*continued*)

Behavior	Time point	Description
Wood chewing	Pre, During and Post	Chewing and/or ingesting wooden objects such as fences or stall construction materials (Mcdonnell, 2003)
Yawning	Pre, During and Post	An involuntary sequence consisting of mouth opening, deep inspiration, brief apnea, and slow expiration (Waring, 2003)
Behavioral states		
Alert	Pre, During and Post	Rigid stance with the neck elevated (45° or more) for ≥ 5 s. The ears are held stiffly upright and oriented toward the stimulus (forward or backward), and the nostrils may be slightly dilated (Ransom and Cade, 2009)
Drinking	Pre and Post	Ingestion of water
Eating	Pre and Post	Ingestion of food
Interaction with handler	During	The horse reaches the handler with the head and interacts with him (sniffing, licking, rubbing, bumping)
Interaction with chiropractor	During	The horse reaches the chiropractor with the head and interacts with him (sniffing, licking, rubbing, bumping) (Mcbride et al., 2004)
Playing with objects	During	The horse uses its mouth to grab its own attached lead rope and chew it, or uses lips and teeth to grasp the green polystyrene blocks and pick them up, or licks/bites the blocks or other objects (adapted from Heleski et al., 2002)
Resting	Pre, During and Post	Standing inactive in a relaxed posture, the head and neck are at the withers level or lower and often bearing weight on three legs (one hind leg slightly flexed). The muscles relax, the ears rotate laterally, the eyelids and lips can get droopy. Eyes may be partly or nearly closed (Fureix et al., 2012, Mcdonnell, 2003)
Standing	Pre, During and Post	The horse stands still (Fureix et al., 2011), the weight is carried on four legs and the neck is between 45° and the withers level
Tripodal	Pre, During and Post	The horse is standing on 3 limbs without moving in any direction (Pierard et al., 2019). The head and the neck are higher than the withers level

For MaxTE the possible differences between the left and right eye were tested with LMM and, as no significant differences were found, the data of the left and right eye were merged.

For all the regression models performed, the mares' ID was considered as a random factor to account for repeated measures. Tukey test was applied for post hoc comparisons. The results were reported as Estimated Mean (EM), Standard Error (SE), 95% Confidence Interval (CI), and model p-value. The significance was set at p-values $\leqslant 0.05$. Trends toward significance were set at p-values > 0.05 and < 0.10. The statistical analysis was conducted in R environment, using a combination of functions within the packages 'lme4', 'lmerTest', 'glmmTMB', 'emmeans' and 'car'.

MaxTE, SC, and HRV analysis

For MaxTE, SC, and HRV the effect of time was tested independently on GROOM/CHIRO using LMM. Hence, two models were built; one for GROOM (T0-BASAL *vs.* T1-GROOM *vs.* T24-GROOM for MaxTE and SC; Pre-BASAL *vs.* During-GROOM *vs.* Post-GROOM for HRV) and one for CHIRO (T0-BASAL *vs.* T1-CHIRO *vs.* T24-CHIRO for MaxTE and SC; Pre-BASAL *vs.* During-CHIRO *vs.* Post-CHIRO for HRV). Moreover, LMM were used to test the effect of GROOM/CHIRO at T1 for MaxTE and SC (T1-GROOM *vs.* T1-CHIRO) and at During for HRV (During-GROOM *vs.* During-CHIRO).

Behaviors and BDS

Behaviors with a median equal to 0 were excluded from the subsequent statistical analysis. As some behaviors could not be manifested in Pre and Post compared to During (e.g., 'interaction with chiropractor' only possible at During), it was not possible to test the effect of time in a single model. Therefore, the authors decided to compare Pre *vs.* Post and to test the effect of GROOM/CHIRO within During.

Hence, the effect of phase, defined as the combination of the time point and GROOM/CHIRO, on Pre and Post (Pre-BASAL *vs.* Post-GROOM *vs.* Post-CHIRO) was tested. Moreover, the effect of GROOM/CHIRO at During was tested (During-GROOM *vs.* During-CHIRO). In particular, for the behavioral states LMM were used, while for the behavioral events GLMM were used.

For BDS, the effect of phase (Pre-BASAL *vs.* During-GROOM *vs.* During-CHIRO *vs.* Post-GROOM *vs.* Post-CHIRO) was tested using GLMM.

Results

MaxTE, SC and HRV

Descriptive statistics of the MaxTE, SC, and HRV parameters are shown in Supplementary Table S2, Table S3, and Table S4, respectively.

The LMM for MaxTE and SC showed no significant effect of time and GROOM/CHIRO.

The LMM for HRV parameters investigating the effect of time on GROOM showed no significant differences. The LMM investigating the effect of time on CHIRO showed significant differences for the SDNN parameter (model p-value = 0.04), which was lower During-CHIRO (EM: 281.00, SE: ± 114.00, CI: 24.20–537.00) compared with Post-CHIRO (EM: 453.00, SE: ± 114.00, CI: 196.00–709.00). Moreover, the parameter RMSSD tended toward a significant increase (model p-value = 0.07) Post-CHIRO (EM: 652.00, SE: ± 186.00, CI: 229.67–1073.00) compared to During-CHIRO (EM: 418.00, SE: ± 186.00, CI: −3.90–840.00).

Furthermore, the LMM for HRV parameters showed no significant effect of GROOM/CHIRO at During.

Behaviors and BDS

Effect of During GROOM/CHIRO

Descriptive statistics of the states and events observed During-GROOM and During-CHIRO is shown in Supplementary Table S5. The behavior 'attempt to bite or biting' was only shown During-CHIRO by only one mare, whereas the behavior 'attempt to kick or kicking' was only shown During-CHIRO by two mares. Table 2 shows the results obtained from GLMM and LMM testing the effect of GROOM/CHIRO at During.

Significant p-values are shown in bold. The frequency of 'avoidance movements' and the duration of 'alert' were higher During-CHIRO in comparison with During-GROOM. Contrariwise, the frequency of 'sniffing', 'snorting', and 'turning head', and the duration of 'resting', and 'standing' were higher During-GROOM compared to During-CHIRO.

Effect of Pre and Post on the phase

Descriptive statistics of the behavioral states and events observed at Pre-BASAL, Post-GROOM, and Post-CHIRO are shown in Supplementary Table S6. Table 3 shows the results obtained from GLMM and LMM testing the effect of phase at Pre and Post.

The frequency of 'licking/chewing' was higher in Pre-BASAL compared to Post-GROOM and Post-CHIRO. Moreover, the same parameter was higher in Post-GROOM compared to Post-CHIRO.

The frequency of 'head shaking', 'self-grooming', and 'sniffing' was higher in Pre-BASAL when compared with Post-GROOM and Post-CHIRO. The frequency of 'turning head' was higher in the Pre-BASAL and Post-GROOM compared to Post-CHIRO.

The duration of 'drinking', 'eating', and 'standing' was higher in

Table 2
Results from GLMM and LMM testing the effect of GROOM/CHIRO on the behavioral parameters at During. For each parameter, Estimated Mean (EM), Standard Error (SE), 95% Confidence Interval (CI), and model p-value are reported. Behavioral events are reported as numbers/minutes, while behavioral states are reported as seconds/minutes.

Behavior	During-GROOM (EM ± SE (CI))	During-CHIRO (EM ± SE (CI))	p-value
Behavioral events			
Avoidance movements	2.45 ± 0.19 (2.08–2.82)	3.11 ± 0.18 (2.75–3.47)	**< 0.001**
Foot stamping	0.34 ± 0.55 (−0.74–1.43)	0.49 ± 0.55 (−0.59–1.58)	0.365
Head surveying	2.40 ± 0.12 (2.17–2.63)	2.46 ± 0.11 (2.23–2.68)	0.570
Licking/chewing	2.57 ± 0.16 (2.25–2.80)	2.65 ± 0.16 (2.34–2.96)	0.384
Lips movements	−0.21 ± 0.80 (−1.79–1.37)	−0.08 ± 0.80 (−1.66–1.50)	0.473
Pulling back	0.76 ± 0.42 (−0.06–1.58)	0.83 ± 0.42 (0.01–1.65)	0.703
Sniffing	2.84 ± 0.16 (2.54–3.15)	2.52 ± 0.16 (2.21–2.83)	**< 0.001**
Snorting	0.60 ± 0.26 (0.09–1.11)	−0.19 ± 0.32 (−0.82–0.44)	**0.011**
Turning head	3.86 ± 0.10 (3.66–4.06)	3.56 ± 0.10 (3.35–3.77)	**< 0.001**
Behavioral states			
Alert	452.00 ± 76.40 (278.00–626.00)	561.00 ± 76.40 (388.00–735.00)	**0.038**
Interaction with handler	71.20 ±19.10 (28.10–114.00)	76.90 ± 19.10 (33.80–120.00)	0.677
Interaction with chiropractor	19.00 ± 3.91 (10.45–27.50)	13.80 ± 3.91 (5.29–22.40)	0.184
Playing with objects	13.40 ± 7.55 (−3.70–30.50)	13.60 ± 7.55 (−3.51–30.70)	0.971
Resting	74.90 ± 12.10 (49.49–100.00)	26.60 ± 12.10 (1.13–52.00)	**0.004**
Standing	614.00 ± 82.10 (422.00–805.00)	550.00 ± 82.10 (358.00–741.00)	**0.043**
Tripodal	40.30 ± 21.40 (−5.65–86.20)	41.50 ± 21.40 (−4.44–87.40)	0.960

Post-GROOM and Post-CHIRO compared to Pre-BASAL.

BDS

Descriptive statistics of the BDS at the different phases is shown in Supplementary Table S7.

BDS was higher During-CHIRO compared to Pre-BASAL (p-value = 0.044), Post-GROOM (p-value = 0.007) and Post-CHIRO (p-value <0.001) (Figure 4).

Joint hypomobilities

Table 4 shows the descriptive statistics of the number of joint hypomobilities for the 8 mares found at each motor unit and in total at Week 1 and Week 2.

The GLMM investigating the effect of the Week (Week 1 *vs* Week 2) on CHIRO showed no significant differences. However, the data suggest that treatment and assessment longer than Week 2 (p=0.09) may be needed to assess improvement.

Discussion

This study used objective indicators to assess the effects of chiropractic treatment on 8 healthy mares. The first hypothesis was partially confirmed, with the results showing that the chiropractic treatment caused transient discomfort behaviors in the mares. However, this discomfort was not supported by any noticeable shift of the autonomic nervous system toward the sympathetic branch, as salivary cortisol, eye temperature, and heart rate variability did not vary. The second hypothesis, that there was an improvement in health and relaxation after treatment, was partially verified. The relaxation was significantly shown with differences in behaviors such as 'self-grooming', 'turning head', 'eating', and 'drinking' after both GROOM and CHIRO. Moreover, the increase in SDNN showed a shift toward the parasympathetic nervous system after CHIRO. Despite this, the number of hypomobilities registered by the chiropractor between the first and second CHIRO did not significantly decrease, suggesting that a single manipulation is not sufficient to restore the body's equilibrium. It is worth highlighting that these results were obtained with the mares accustomed to the environment and the tools, and with the treatment being performed by a qualified veterinary chiropractor.

Discomfort behaviors of 'avoidance movements' and 'alert' were more frequent During-CHIRO compared to During-GROOM. The 'avoidance movements' represent the animal's attempt to move away from an acute dangerous stimulus. In prey animals like horses, flight and avoidance responses are considered stress and fear reactions (Goodwin, 2007), manifested in response to stimuli perceived as threatening (Waring, 2003). These reactions are more strongly expressed in response to novel stimuli (Scopa et al., 2018; Felici et al., 2023). In this study, mares were previously habituated to the environments and the tools used, making chiropractic manipulation the only new stimulus provided. Therefore, it is possible to affirm that the avoidance movements expressed were exclusively in response to manipulations and not related to environmental conditions. This is in line with what was found by Mcbride et al., 2004, which showed that horses tend to enact avoidance and retraction behaviors when they do not like the manipulation

Table 3
Results from GLMM and LMM testing the effect of phase on the behavioral parameters at Pre-BASAL, Post-GROOM, and Post-CHIRO. For each parameter, Estimated Mean (EM), Standard Error (SE), 95% Confidence Interval (CI), and p-value of the model are reported. Behavioral events are reported as numbers/minutes, while behavioral states are reported as seconds/minutes.

Behavior	Pre-BASAL (EM ± SE (CI))	Post-GROOM (EM ± SE (CI))	Post-CHIRO (EM ± SE (CI))	p-value
Behavioral events				
Body shaking	0.13 ± 0.41 (−0.67–0.94)	−0.55 ± 0.57 (−1.66–0.57)	−0.84 ± 0.59 (−2.00–0.32)	0.289
Head shaking	0.96 ± 0.26 (0.45–1.47)[a]	0.37 ± 0.31 (−0.23–0.97)[b]	0.41 ± 0.30 (−0.19–1.00)[b]	**0.002**
Head surveying	1.99 ± 0.15 (1.70–2.28)	1.92 ± 0.16 (1.59–2.24)	1.87 ± 0.17 (1.55–2.20)	0.548
Licking/chewing	2.22 ± 0.15 (1.93–2.50)[a]	1.07 ± 0.20 (0.68–1.45)[b]	0.41 ± 0.24 (−0.05–0.88)[c]	**< 0.001**
Self-grooming	1.13 ± 0.26 (0.61–1.65)[a]	0.07 ± 0.33 (−0.57–0.72)[b]	0.16 ± 0.32 (−0.47–0.80)[b]	**< 0.001**
Sniffing	2.26 ± 0.20 (1.88–2.64)[a]	1.35 ± 0.22 (0.92–1.79)[b]	1.08 ± 0.23 (0.62–1.53)[b]	**< 0.001**
Snorting	0.94 ± 0.17 (0.60–1.28)	1.08 ± 0.20 (0.69–1.47)	0.93 ± 0.20 (0.53–1.33)	0.695
Turning head	3.24 ± 0.13 (2.96–3.50)[a]	3.25 ± 0.14 (2.98–3.53)[a]	2.97 ± 0.14 (2.69–3.25)[b]	**< 0.001**
Behavioral states				
Alert	188.50 ± 34.30 (114.00–263.00)	106.10 ± 42.00 (19.60–193.00)	91.10 ± 42.00 (4.60–178.00)	**0.031**
Drinking	3.99 ± 1.96 (−0.06–8.04)[a]	16.32 ± 2.75 (10.81–21.84)[b]	13.14 ± 2.75 (7.62–18.65)[b]	**< 0.001**
Eating	515.00 ± 46.00 (420.00–610.00)[a]	919.00 ± 65.10 (788.00–1049.00)[b]	944.00 ± 65.10 (813.00–1075.00)[b]	**< 0.001**
Standing	726.00 ± 34.90 (654.00–798.00)[a]	945.00 ± 49.40 (846.00–1044.00)[b]	961.00 ± 49.40 (862.00–1060.00)[b]	**< 0.001**

Significant p-values are shown in bold. Values with different superscripts differ significantly (a, b, c = p ≤ 0.05).

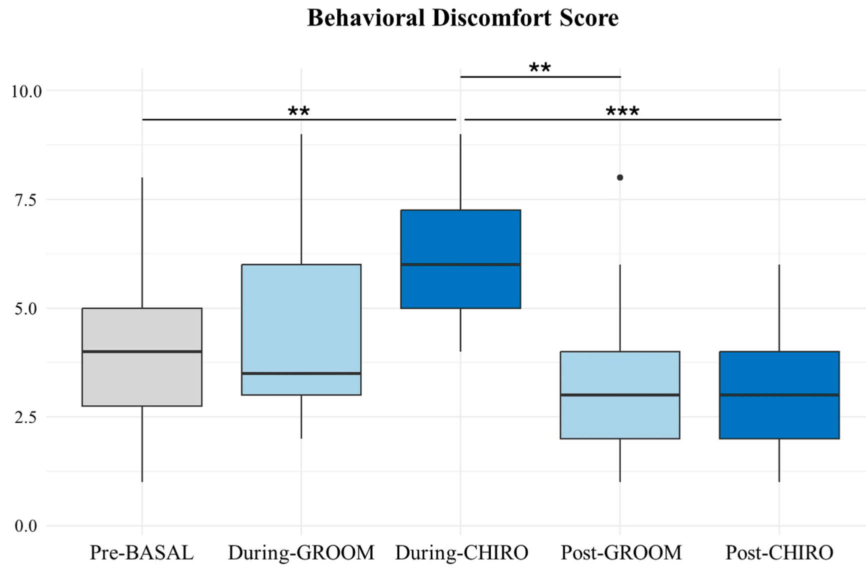

Fig. 4. Box-and-Whisker plot of Behavioral Discomfort Score (BDS) at the different phases. Each boxplot shows the median (band near the middle of the box) and interquartile ranges (bottom and top of the box); dots show outliers. The presence of *** indicates a significant difference <0.001; The presence of ** indicates a significant difference <0.05.

Table 4
Descriptive statistics reporting median and IQR interval (IQ1-IQ3) of the hypomobilities at Week 1 and Week 2 of CHIRO. Hypomobilities are reported for each motor unit and the total sum.

Number of hypomobilities	Week 1	Week 2
Cervical area	5.00 (4.50–5.25)	3.50 (3.00–4.00)
Thoracic area	3.50 (2.75–5.00)	3.50 (2.75–4.25)
Lumbar area	2.00 (1.00–2.25)	1.50 (0.75–2.00)
Sacroiliac area	1.50 (0.75–2.00)	1.00 (0.75–1.00)
Limbs	3.50 (3.00–5.00)	4.00 (3.00–5.25)
Total	14.5 (12.50–20.75)	12.50 (11.75–16.00)

performed. In line with what has been discussed above, the state 'alert' was more expressed during chiropractic treatment probably because the mares appeared to be more attentive to what was happening, which was possibly causing them some discomfort. This state of hypervigilance of the organism is described as an alarm reaction (Ransom and Cade, 2009), representing an internal state of anxiety that allows horses to avoid danger (Panksepp, 2011; Sylvers et al., 2011). Because of this alert state, it is also understandable why exploratory behaviors such as 'turning head' and 'sniffing', were more expressed During-GROOM than During-CHIRO. Furthermore, another possible interpretation of the increased frequency of 'turning head' During-GROOM is related to the animal's attempt to interact with the chiropractor. This behavior could be interpreted as an attempt to interact with humans in the form of allogrooming (Mcdonnell, 2003). It has been shown that if the horse likes the stimulation, in this case provided by grooming, it will try to interact more often with those providing it (Mcbride et al., 2004).

Finally, contrary to what was expected, the event 'snorting' was more frequently registered During-GROOM. This loud exhalation emitted from the nostrils (Torcivia and Mcdonnell, 2021) was commonly considered a sign of restlessness and frustration (Waring, 2003). However, it has recently been described as a possible indicator of positive emotions (Stomp et al., 2018). Such an interpretation could be consistent with the other results found, indicating that horses were relaxed During-GROOM and hypervigilant and potentially stressed During-CHIRO.

During-CHIRO the aggressive behaviors of 'attempt to bite or biting' and 'attempt to kick or kicking' were observed only in two mares. Therefore, it is possible to affirm that these mares may have been particularly annoyed by the chiropractic manipulations, due perhaps to the stimulation of painful areas that may have induced an aggressive behavioral response, as previously studied (Mcbride et al., 2004). The fact that the mares never expressed those behaviors During-GROOM is a further confirmation that, probably, the manipulations applied During-CHIRO may induce a temporary reaction of discomfort or possibly even pain if sore areas are touched.

A general relaxation was shown after both Post-GROOM and Post-CHIRO compared to Pre-BASAL. However, unlike what was hypothesized there is no evidence that this relaxation is higher in Post-CHIRO compared to Post-GROOM. In fact, after both sessions, a reduction in the expression of stress and discomfort behaviors, such as 'licking/chewing' and 'head shaking' was observed, suggesting, a greater relaxation of the mares in the Post phases than in the Pre-BASAL. The behaviour 'turning head' was significantly lower in both Post-CHIRO and Post-GROOM compared to Pre-BASAL, possibly because in the Post phases the mares were more relaxed and spent more time performing other behaviors such as eating. In addition, it is possible to indirectly state that mares, which had shown discomfort During-CHIRO, no longer showed them in the Post phase, suggesting, as hypothesized, that discomfort was only transient and had no short-term repercussions. Following the above discussion, also the results of the variation of the BDS confirmed the temporary discomfort reaction of the mares During-CHIRO, followed by a significant reduction in the scores in the time Post-CHIRO. These results are promising, and provide a harmonious picture between them. However, it is important to consider that any behavioral response, although extremely useful in investigations like this, is still a partially subjective response. Behaviors can be influenced by a variety of factors, including the animal's past experiences (Yarnell et al., 2015; Squibb et al., 2018), temperament (Ellis et al., 2014), and age (Baragli et al., 2014). Moreover, the use of a combined absence-presence (0−1) score such as the BDS may have partially reduced individual differences in the expression of discomfort. In particular, a mare showing the same behavior several times has been scored lower than a mare showing different behaviors but only a single time. It is for this reason that in this study, behavioral analysis was performed along with other objective parameters of acute stress response, such as heart rate variability, salivary cortisol, and eye temperature.

MaxTE, SC, and HRV parameters did not vary demonstrating that chiropractic treatment did not cause any shift in the autonomic nervous system. Therefore, it is possible to state that, although mares manifested some signs of discomfort, chiropractic treatment was well tolerated by these subjects. In addition, variations of HRV and temperature of the eye have been correlated with emotional arousal, especially fear (Visser et al., 2002; Dai et al., 2015). Thus, it can be said that the manipulated mares in this study did not experience fear, probably due to the habituation period performed, which allowed them to familiarise themselves with the environment and tools used.

It is interesting to note that there was a significant increase in SDNN in the Post-CHIRO compared to During-CHIRO, indicating a predominance of parasympathetic nervous system post treatment. This difference was not seen in Post-GROOM, highlighting that only the chiropractic manipulation relaxed the mares afterward. Although the SDNN is considered a mixed parameter (Shaffer and Ginsberg, 2017) indicating both sympathetic and parasympathetic activity, in this context it is more likely to represent the latter. In fact, the RMSSD, a parameter more influenced by the parasympathetic nervous system than SDNN (Malik et al., 1996; Shaffer and Ginsberg, 2017), showed the same pattern.

Finally, although it was not the main objective of the study, the analysis of joint hypomobilities after the first treatment did not show any significant decrease in them, but only a downward trend was observed. This lack of significance, however, is probably due to other considerations unrelated to the efficiency of the chiropractic treatment. Primarily, one treatment alone is not exhaustive in restoring the body's balance; it is necessary to carry out a series of treatments to be able to see clear results. In addition, the mares in this study were healthy, hence it is more difficult to correct the minimal hypomobilities that were found than in animals with a definite musculoskeletal pathology. Nevertheless, the results are promising as they show a trend that would have probably been confirmed after the second treatment. Unfortunately, no assessment of joint hypomobilities was done after the second treatment and our results may be affected by the light lameness presented by four mares

Although this study provides interesting evidence, our results must be interpreted with caution because of some limitations. Firstly, the heterogeneity of the locations where the measurements were taken required the use of two separate ethograms, which did not allow the comparison of the During with the Pre and Post for each behavioral state and event. Secondly, the videos were recorded by an operator and not by environmental cameras. Although the operator did not interfere with the mares, the presence of an operator has been shown to cause the horse to partially mask discomfort behaviors (Torcivia and Mcdonnell, 2021). Future studies should use remotely operated environmental cameras mounted in an elevated position within the stable. This will reduce interference with the expression of certain behaviors and provide a better overall view. Notwithstanding these limitations, this pilot study was able to demonstrate that equine chiropractic treatment can cause some transient discomfort but is safe to be practiced by qualified veterinary chiropractors. In future studies, these results should be confirmed and improved using a larger sample size.

Conclusions

This preliminary study showed that chiropractic treatment is well tolerated by the equine species. The manipulation led the mares to manifest some discomfort behaviors but it did not induce any acute stress response, on the contrary promoting a relaxed state after the treatment. On the basis of one treatment only, no significant reduction in the mares' joint hypomobilities was seen, but only a decreasing trend, confirming that a single treatment is not sufficient to rebalance the organism. Based on these assumptions, it can be stated that equine chiropractic can be considered a safe treatment for healthy subjects. Further studies are needed to evaluate the risks and benefits of chiropractic treatment on horses subjected to musculoskeletal disorders.

Ethical approval

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Committee on Animal Welfare and Ethics of the University of Bologna (Approval number 75573).

Authorship statement

The idea for the paper was conceived by F. Freccero, M. Zappaterra, P.A. Accorsi and B. Padalino. The experiments were designed by F. Freccero, M. Zappaterra, I. Arena, P.A. Accorsi and B. Padalino. The experiments were performed by B. Benedetti, F.Freccero, M. Piscopiello, M. Felici, A. Mannini, M. Zappaterra, M.G. Angeloni, I. Arena and B. Padalino. The data were analyzed by B. Benedetti, M. Piscopiello, M. Felici, A. Mannini, M. Zappaterra, M.G. Angeloni, I. Arena, G. Marliani and B. Padalino. The paper was written by B.Benedetti, F.Freccero, M. Piscopiello, M. Felici, A. Mannini, M. Zappaterra, M.G. Angeloni, I. Arena, P.A. Accorsi, G. Marliani and B. Padalino.

CRediT authorship contribution statement

Aurora Mannini: Writing – original draft, Investigation. **Martina Zappaterra:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Manuela Piscopiello:** Writing – original draft, Investigation, Formal analysis. **Martina Felici:** Writing – original draft, Investigation, Formal analysis. **Maria Gaia Angeloni:** Writing – original draft, Investigation, Formal analysis. **Ilaria Arena:** Writing – review & editing, Methodology, Investigation. **Pier Attilio Accorsi:** Writing – review & editing, Methodology, Conceptualization. **Beatrice Benedetti:** Writing – original draft, Project administration, Investigation, Formal analysis. **Francesca Freccero:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Conceptualization. **Giovanna Marliani:** Writing – original draft. **Barbara Padalino:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

The authors are grateful to Professor Gaetano Mari, Professor Leonardo Nanni Costa, and Doctor Beatrice Mislei for their help in recruiting mares and providing us with locations for the experiment.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jveb.2024.10.006.

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