Journal of Veterinary Behavior 31 (2019) 28–35

Contents lists available at ScienceDirect

Journal of Veterinary Behavior

journal homepage: www.journalvetbehavior.com

Equine Research

A test of the effects of the equine maternal pheromone on the clinical and ethological parameters of equines undergoing hoof trimming

Renata Alves de Paula [a], Amanda Sarita Cruz Aleixo [a], Leticia Peternelli da Silva [b], Marina Cecília Grandi [b], Miriam Harumi Tsunemi [c], Maria Lucia Gomes Lourenço [a,*], Simone Biagio Chiacchio [a]

[a] Department of Veterinary Clinics, São Paulo State University (Unesp), School of Veterinary Medicine and Animal Science, Botucatu, São Paulo, Brazil
[b] Department of Animal Health, School of Veterinary Medicine and Animal Science, UNIMAR, Marília, São Paulo, Brazil
[c] Department of Biostathistics, São Paulo State University (UNESP), Institute of Biosciences, Botucatu, São Paulo, Brazil

ARTICLE INFO

Article history:
Received 29 June 2018
Received in revised form
18 January 2019
Accepted 28 January 2019
Available online 4 February 2019

Keywords:
animal well-being
HRV
behavior
pheromone therapy
hoof trimming

ABSTRACT

"Pheromonal therapy" has been promoted as a promising alternative therapy to improve the human-animal relationship and to reduce behavioral reactions to stressful stimuli. This placebo-controlled double blind study evaluated the use of a synthetic equine maternal pheromone (EMP) in animals undergoing hoof trimming for effects on behaviors and autonomous nervous system responses. Twenty foals (14 females and 6 males) with an average age of 24 months were divided into two experimental groups (A and B); one group receiving treatment with EMP and the other receiving placebo (excipient without the active ingredient). The parameters analyzed were heart rate (HR), respiratory rate, blood sugar levels, heart rate variability and behavioral reactions. There were no statistically significant differences in the HR, respiratory rate, and blood sugar levels when comparing the EMP and placebo groups. The heart rate variability indexes (minimum HR, average HR, maximum HR, NN, SDNN, RMSSD, and PNN50) also were not statistically significantly different when comparing the groups before, during, and after trimming. We also found no behavioral changes during the procedure of hoof trimming associated with the use of synthetic pheromonal analogues. This study provided no evidence for the effectiveness of synthetic pheromonal analogues to aid in the process of hoof trimming.

Introduction

Interest in animal well-being has been on the rise both in Brazil and around the world as several sectors of society have become more organized to demand better conditions in animal husbandry (Gontijo et al., 2014). These conversations have been, in part, focused on equestrian activities, supporting the use of a scientific approach to determine situations in which animal quality of life and well-being may improve.

Since the time humanity has first interfered in the biology and nature of equines, some methods have been introduced in the handling of these animals, including intensive hoof care. Hoof trimming is a procedure that should be conducted in stabled horses, which require daily care to maintain foot health and balance (Sampaio et al., 2014).

Equines show varying temperaments, which can be reflected in response to human handling. If the response is fear, it can lead to behavioral and physiological changes. New situations, intense stimuli, and sudden actions may cause fear in the animal. These behavioral responses or changes range from low reactivity and meekness to fear, escape attempts, or aggression (Calviello, 2013). When these situations become traumatic and stressful, the well-being of the animal has apparently been compromised (Heleski et al., 2002).

Stress may lead to increases in heart rate (HR) because of the activation of the sympathetic nervous system and the release of catecholamines. The discovery of the relationship with the autonomic nervous system (ANS) led to studies regarding the increase in sympathetic activity and the resulting decreases in parasympathetic activity, with heart rate variability (HRV) representing the most promising marker for ANS activity (Ohmura and Jones,

* Address for reprint requests and correspondence: Maria Lucia Gomes Lourenço, Department of Veterinary Clinics, School of Veterinary Medicine and Animal Science, UNESP, Botucatu, São Paulo, Brazil. Tel: 55 14 3880-2044; fax: 55 14 3815-2343.
E-mail address: maria-lucia.lourenco@unesp.br (M.L.G. Lourenço).

https://doi.org/10.1016/j.jveb.2019.01.005
1558-7878/

2017). However, the evaluation of responses to stress is better conducted by comparing behaviors and physiological measurements (Cruz-Aleixo et al., 2017).

Neuroleptic, sedative, and tranquilizing substances are often administered to equines to facilitate certain types of handling, but they often lead to side effects such as hematological and biochemical alterations, which compromise homeostasis. For this reason, some studies have been conducted regarding the use of pheromone therapy to calm the animals and reduce fear and anxiety without causing any adverse effects (Falewee et al., 2006). This intervention can be administrated by owners (Mills, 2005).

Pheromones are chemicals produced by specific regions of epithelial cells that produce behavioral and physiological effects when perceived in the environment by other animals. Events or states modulated by pheromones include copulation, aggression, dam–infant bonding, familial recognition, appeasement, and synchronization of estrous (Berger et al., 2013). Pheromones stimulate the specialized chemoreceptive cells in the vomeronasal organ, an organ located at the base of the nasal cavity and anatomically separated from the main olfactory bulb (Trotier, 2011).

Horses, similar to other placental mammals, have a vomeronasal organ, which opens to the intranasal cavity; hence, intranasal application is thought to be effective. It is hypothesized that equine pheromones have a calming or reassuring (appeasing) effect and are produced near mammary glands of dams after parturition (Berger et al., 2013).

Pheromones secreted by the sebaceous glands of the sulcus between the mammary glands isolated from pigs, cats, and dogs are thought to have an appeasing or reassuring effect on young and adult animals (Chamero et al., 2007). Such a substance produced near the mammary gland could be speculated to not only send calming signals but also guide the newborn with olfactory signals to the teats to facilitate suckling. Synthetic analogues of appeasing pheromones have been used therapeutically for undesirable behaviors in dogs, including stress-related behavior of puppies facing a novel environment after adoption (Stowers and Marton, 2005).

True pheromones are known to initiate and control behavior, but synthetic analogues have methodological limitations that make it inherently difficult to determine any true effectiveness (Hermiston et al., 2018). Therefore, we conducted a double blind study and to determine if the use of equine maternal pheromone (EMP) might have a positive effect on the human-animal relationship and equine well-being. We assessed clinical, laboratory and behavioral parameters.

Table 1
Demographics of population sample

Name	Horse ID number	Sex	Age (months)
Flag	B3	F	12
Calendar	B1	M	16
Ditty	A2	F	26
Corisco	A3	M	24
Costelinha	A10	F	18
Formozo	A1	M	28
Iberian	B7	F	25
Ibitiuva	B4	F	27
Beautiful island	A9	F	28
Imagination	B5	F	25
Imola	A4	F	26
Empire	B9	M	27
Indian	A7	M	26
Intrigue	A8	F	28
Iraq	B6	M	26
Ireland	A5	F	14
Isa	B10	F	26
Italy	A6	F	26
Little neck	B8	F	28
Unnamed	B2	F	24

Figure 1. Horse corral before procedures.

Materials and methods

Study design

The study was conducted as a placebo-controlled, double-blinded trial. The treatment received by each animal was revealed on the end of the experiment; animals in group A received the placebo, and animals in group B received treatment with EMP.

To measure the effects of the EMP and the placebo, 20 animals were evaluated regarding their physiological parameters (HR, RR, blood glucose levels, and HRV) and their behavior.

Experiment location

The study was conducted at two stud farms in the midwestern region of the state of São Paulo, Brazil. The first, identified as *Fazenda Santa Fé*, is located in the city of Areiópolis, SP, Brazil (latitude 22°40′05″ S and longitude 48°39′54″ W), and the second, identified as *Haras Josilmar*, is located in the city of Bauru, SP, Brazil (latitude 22°18′55″ S and longitude 49°3′41″ W). The study was approved by the Ethics Commission for Animal Experimentation (CEUA, *Comissão de Ética no Uso de Animais*) at the School of Veterinary Medicine and Animal Sciences (FMVZ–UNESP, Botucatu, Brazil) under protocol no. n°0214/2016 after written consent by the animal owners.

Figure 2. The foal in stocks.

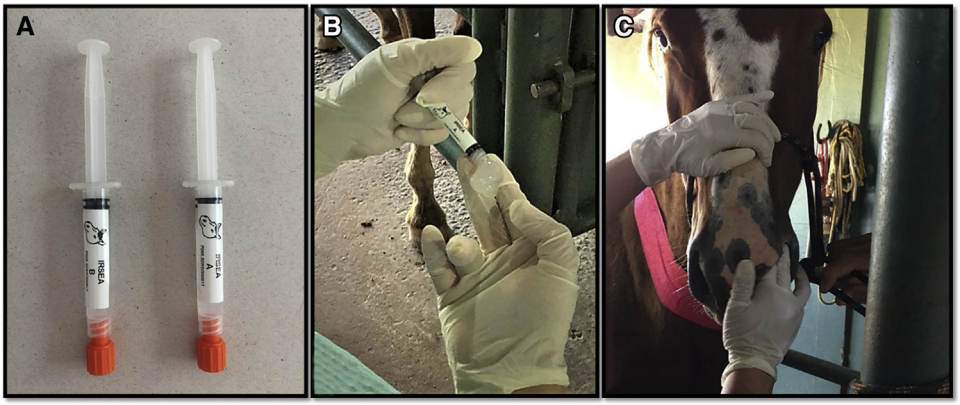

Figure 3. Identified syringes (A and B) (A), the product being prepared (B) and application by the examiner in the outer edges of the animal's nostril (C).

Study subjects

The study included 20 Mangalarga Paulista horses, 14 females and 6 males, with an average age of 24 months (Table 1). The inclusion criteria for the study were animals that were healthy on a physical examination, no history of infectious or contagious diseases in the previous 30 days, regular vaccination and deworming, age between 12 and 28 months, uninitiated taming process (e.g., no previous handling), and no previous hoof trimming.

All animals came from similar husbandry systems: free-roaming in Tifton fields with water and mineral salts *ad libitum*. For this study, the foals were gathered in a handling corral (Figure 1), subdued and led to a horse stock for the procedures to begin (Figure 2) where they were kept in stalls.

Synthetic pheromone

The manufacturer provided the product in syringes identified as A or B on the outside Figure 3). One group contained a synthetic equivalent of the equine maternal pheromone (1%) and an excipient (glycerol, methyl hexadecanoate, 2-tert-butyl-cresol 99%), whereas the other (placebo) contained only the excipient (glycerol, methyl hexadecanoate, 2-tert-butyl-cresol 99%) without the active ingredient. The researchers did not know which syringe contained what until the end of the experiment.

The carton contained 2.5 ml of a gel, which was distributed over the examiner's index finger and applied over the outer edge of the animal's nostril, one unit for each nostril. The examiners used gloves for each application, discarding them afterward as per the manufacturer's instructions (Figure 3).

Clinical parameters

The study assessed the heart rate (HR) and respiratory rate (RR) of the animals through a conventional clinical examination with the aid of a stethoscope. Values between 32 and 44 beats per minute for the HR and between 8 and 15 breaths per minute for the RR were considered normal (Cunningham, 2008). Blood glucose levels were determined with the aid of a portable device (Accu-Chek® Performa—Roche), using specific bands, after collection through jugular venipuncture with 5 mL syringes and 19.5 G needles. Glucose values were considered normal between 75 and 115 mg/dL (Kaneko et al., 2008). These parameters were evaluated at two times: M1—20 minutes before the application of the EMP; and M2—immediately after the trimming procedure. We did not measure during the procedure so as not to compromise the time of the hoof trimming because the same per animal was compiled.

Heart rate variability (Holter)

The electrocardiographic recording happened after the application of a Holter device (Cardio Light, Cardios, ANVISA, São Paulo, Brazil) (Figure 4). The area was epilated and cleaned with 70% alcohol before the electrodes were secured with the aid of glue (Super Bonder®- Loctite).

The positive electrodes (red and black) were positioned on the animal's left side, with the red electrode placed at the withers and the black electrode in the ventral region of the thorax, in the fifth intercostal space behind the olecranon. The negative electrode (white) and the ground electrode (green) were positioned in the middle third of the seventh intercostal space on the right thorax,

Figure 4. Holter Equipment—Cables, electrodes, SD card, and protective cover.

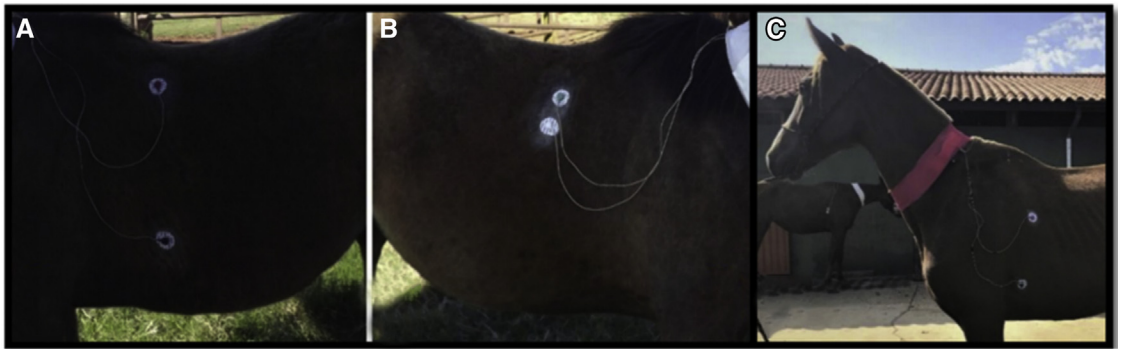

Figure 5. Position of the electrodes in the animal left (A) and right (B) sides; Holter device attached to the animal's neck (C).

Index	Unit	Definition
NN	Ms	Mean of all RR intervals
SDNN	Ms	Standard deviation of all RR intervals
SDNNi	Ms	Mean of the standard deviations of RR intervals calculated over 5-second segments
SDANN	Ms	Standard deviation of the average RR intervals calculated over 5-second segments
RMSSD	Ms	Square root of the mean of the squares of the successive differences between adjacent RR intervals
pNN50	%	Proportion of successive differences between RR intervals that differ by more than 50 ms

Chart 1. Time-domain HRV indexes.

with the green electrode positioned slightly closer than the white electrode (Figure 5).

The recorder was then secured at the animal's neck with an elastic band to protect the equipment (Figure 5). The data collected was stored in an electromagnetic card (SD card) (Figure 4).

The time-domain HRV indexes are shown in Chart 1 and were analyzed in all animals through computer-based decoding of the Holter electrocardiographic monitoring employing the software CardionetClient® at three distinct times: 20 minutes before, during (20 minutes), and after the hoof trimming procedure (20 minutes).

Experimental design

After the clinical examination and blood glucose level test, the Holter device was placed on the animal and the electrocardiographic recording initiated. After recording for 20 minutes, the product was administered. The first animal, selected by lottery, was given the contents of syringe A (Group A), and the second animal was given the contents of syringe B (Group B) and so on until all animals received the product. All applications were conducted by the same person.

Figure 6. Equipment used in the hoof trimming procedure (pincer, rasp, and hoof knife) and trimming being conducted.

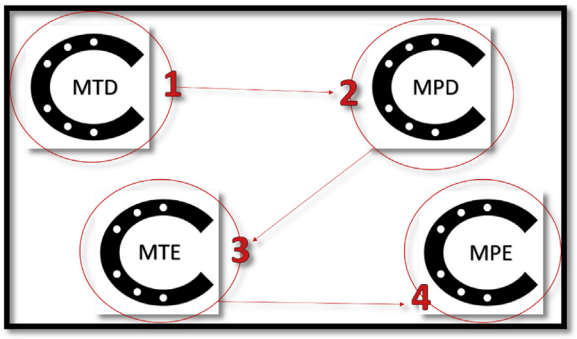

Figure 7. Sequence established for the hoof trimming procedure: (1) right thoracic limb (MTD); (2) right pelvic limb (MPD); (3) left thoracic limb (MTE); (4) left pelvic limb (MPE).

A 20-minute wait period was observed after application (as per the product's minimum action time), during which the animals were not in contact with humans or other animals.

Hoof trimming

To carry out the hoof trimming, the animals were removed from the stalls, and the procedure was performed in an external area. A preventive hoof trimming procedure was then conducted on the animals employing a hoof pick, a hoof knife, a rasp, and hoof nippers (Figure 6). The sequence of this procedure consisted in cleaning the hooves with a hoof pick to remove dirt and debris from the bottom of the hoof. The hoof knife was then used to carefully remove excess material from the sole, the central groove of the frog, and the white line. The hoof wall was then trimmed with the nippers and leveled to the sole with a rasp. The procedure was finalized by rounding the hoof with the rasp.

The procedure was conducted by the same person and followed the same limb order in all animals: right thoracic limb (RTL), right pelvic limb (RPL), left thoracic limb (LTL), and left pelvic limb (LPL) (Figure 7). After concluding the procedure, the clinical examination and the blood glucose level test were repeated. After another 20-minute wait period, the cardiac monitoring device was removed, with a total recording time of approximately 20 minutes per animal.

Behavioral parameters

The trimming procedures were recorded with a digital camera (iPhone 7 – Full HD 1080p–Apple), and two observers identified, noted the type of reaction that the animals displayed during hoof trimming as: walking backward, running away, walking sideways, hitting, kicking, stepping, throwing their weight on the limb on which the hoof trimming was being performed, putting the tail between the legs and prancing. These behaviors were not previously standardized. As observers visualize them, they document the types of behaviors presented and then quantify the presentations.

The entire sequence of the experiment is represented in Figure 8.

Statistical analysis

Variable distribution was evaluated through Kolmogorov-Smirnov's normality tests. Comparisons between the groups employed the t-test for independent samples. All data were checked for normality using Kolmogorov-Smirnov tests. The significance level was set a priori at $P < 0.05$, and all statistical analysis was performed using a program SAS (Statistical Analysis Software, version 9.4).

Results

Clinical examination

The data obtained through conventional clinical examinations did not reveal any statistically significant difference, between the two groups, the EMP and placebo groups for the HR, RR, and blood glucose level parameters when comparing moments M1 and M2, as described in Table 2.

Heart rate variability

There was a problem with the HRV data recording of four animals (three in control group and one on treatment group), which were excluded from the study.

In the Holter examination, the minimum HR in the placebo group (45.00 ± 10.28), minimum HR in the EMP group (41.20 ± 7.89), average HR in the placebo group (63.60 ± 25.16), average HR in the EMP group (64.80 ± 22.89), maximum HR in the placebo group (112.40 ± 31.44), and maximum HR in the EMP group (122.30 ± 47.60) were not statistically significantly differences ($P > 0.05$) between the three analyzed moments (before, during, and after hoof trimming) (Table 3).

Likewise, the remaining HRV indexes NN (mean of all RR intervals), SDNN (standard deviation of all RR intervals), RMSSD (square root of the mean of the squares of the successive differences between adjacent RR intervals), and PNN50 (proportion of successive differences between RR intervals that differ by more than 50 ms) also were not statistically significantly differences between each group at the three analyzed moments: before (M1), during (M2), and after (M3) hoof trimming. These data are shown in Table 4.

Behavioral reactions

Although the final results of the observations showed a greater number of reactions in the animals of the placebo group, there was

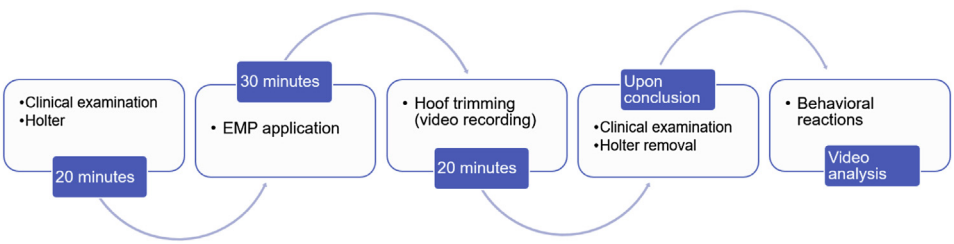

Figure 8. Overview of the experiment sequence for the study.

Table 2
Mean (standard deviation) and *P* values for HR, RR, and blood glucose level measurements obtained through clinical examination of equines undergoing hoof trimming with previous treatment with EMP and placebo

Variable	Placebo	EMP	*P* value
HR M1	56.40 (12.99)	64.80 (14.34)	0.187
HR M2	57.60 (16.78)	55.60 (14.78)	0.781
HR_dif_M2-1	1.20	−9.20	0.211
RR M1	31.20 (11.12)	31.60 (9.13)	0.931
RR M2	34.00 (7.36)	36.40 (14.87)	0.655
RR_dif_M2-1	2.80	4.80	0.777
Blood glucose M1	99.60 (14.65)	102.90 (14.93)	0.624
Blood glucose M2	99.70 (15.17)	95.00 (11.04)	0.438
Blood glucose _dif_M2-1	0.10	−7.90	0.255

HR, heart rate; RR, respiratory rate.
Comparisons between the groups through the t-test for independent samples.

no significant difference in our study between the treated animals and the control group during trimming.

Discussion

In this study, the HR of the foals in both the EMP and placebo groups started above the reference values described for the species in the clinical examination conducted at rest, before handling to secure the Holter device and before the trimming procedure. This indicates that the animals were already in an alert state, which may be explained by the fact that they had been restrained. An animal may undergo physiological changes when feeling insecure in a particular situation, which highlights the importance of studying parameters such as HR, which is an expression of emotional activation (Visser et al., 2008).

The HR in the EMP group was higher at M1 (64.80 ± 14.34) and then decreased at M2 (55.60 ± 14.78), a difference of −9.20 beats per minute, and in the placebo group behaved in the opposite way, but there was no statistical difference between groups for HR (Figure 9). Some studies report that differences exceeding 6 beats per minute from the measurement taken at rest may indicate stress (Craig and Nunan, 1998). Increases in the HR are the result of reduced vagal activity and increased sympathetic activity or, in some cases, a combination of concomitant changes in both branches of the ANS (Borell et al., 2007).

When comparing the blood glucose levels of each group between moments M1 and M2, the differences observed were not statistically different. As was the case with the HR, the EMC presented higher values before the trimming procedure than afterward, with a difference of −7.90 mg/dL (Table 2). In the placebo group, the blood glucose levels remained similar at M1 and M2.

The levels of glucose in the blood in stress situations are related to an increase in the levels of cortisol because when there is a stress stimulus, the hypothalamus synthesizes the corticotropin-releasing hormone (CRH), which induces the hypophysis into producing the adrenocorticotropic hormone (ACTH). The ATCH, in turn, runs through the bloodstream to the adrenal glands, stimulating the synthesis of cortisol, which is known as the "stress hormone." At the same time, the sympathetic nervous system is activated, leading to the release of catecholamines (noradrenaline and adrenaline), leading to an increase in glycogenolysis (Nascimento and Barros, 2008).

Table 3
Mean (standard deviation) and *P* values for minimum HR, average HR, and maximum HR obtained through Holter examination of equines undergoing hoof trimming with previous treatment with EMP and placebo

Variable	Placebo	EMP	*P* value
Min HR	45.00 (10.28)	41.20 (7.89)	0.366
Avg HR	63.60 (25.16)	64.80 (22.89)	0.912
Max HR	112.40 (31.44)	122.30 (47.60)	0.590

Avg HR, average heart rate; Min HR, minimum heart rate; Max HR: maximum heart rate.
Comparisons between the groups through the t-test for independent samples.

Table 4
Heart rate variability indexes (mean ± standard deviation) obtained through Holter examination in the EMP and placebo groups before (M1), during (M2), and after (M3) hoof trimming

Parameter	EMP group	Placebo	*P* value
NN (ms)_M1	752.26 ± 192.50	740.89 ± 262.67	0.921
NN (ms)_M2	859.03 ± 290.11	721.75 ± 196.08	0.277
NN (ms)_M3	731.12 ± 240.47	751.73 ± 427.94	0.918
SDNN (ms)_M1	207.32 ± 110.76	184.82 ± 63.68	0.623
SDNN (ms)_M2	247.97 ± 142.43	229.98 ± 123.86	0.786
SDNN (ms)_M3	155.81 ± 122.59	177.47 ± 107.52	0.731
RMSSD (ms)_M1	260.90 ± 210.56	171.15 ± 129.27	0.322
RMSSD (ms)_M2	204.66 ± 148.19	237.15 ± 229.24	0.730
RMSSD (ms)_M3	237.13 ± 243.03	158.32 ± 151.32	0.449
PNN50_M1	56.67 ± 32.42	44.04 ± 25.66	0.347
PNN50_M2	50.08 ± 21.33	42.82 ± 29.19	0.533
PNN50_M3	49.63 ± 36.76	39.87 ± 32.08	0.535

NN, Interval between two consecutive R waves, or mean NN for all the recording; pNN50, proportion of differences exceeding 50 ms between adjacent normal RR intervals in a 24-hour examination; rMSSD, Square root of the mean of the squares of the successive differences between adjacent normal RR intervals in the entire examination; SDNN, standard deviation of all normal RR intervals in the examination.
Statistical analysis: Comparisons between the groups through the t-test for independent samples.

The analysis of the indexes obtained through the Holter examination revealed the minimum, average, and maximum HR was high in both groups, but it is known that, in some cases, the HR may rise quickly to over 100 bpm, especially in cases involving sudden fear, excitation, or before physical exercise (Clayton, 1991; Evans, 1994).

The remaining indexes reflect the variability in the cardiac activity and are influenced by sympathetic and parasympathetic activity (Borell et al., 2007). The index SDNN is obtained from long recordings and represents both sympathetic and parasympathetic activity, but it does not allow the distinction of whether the HRV changes are caused by increases in the sympathetic tonus or reductions in the vagal tonus. On the other hand, the indexes RMSSD and pNN50 represent parasympathetic activity, given that they are calculated from the analysis of adjacent RR intervals (Vanderlei et al., 2009). High HRV is a sign of good adaptation, characterizing a healthy individual with efficient autonomic mechanisms, whereas low variability often is an indicator of abnormal and insufficient ANS adaptation, implying the presence of some physiological "malfunction" in the individual (Abreu, 2012). A

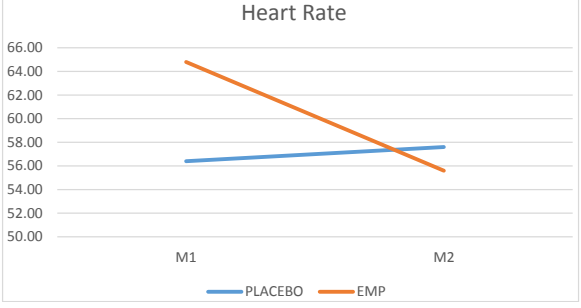

Figure 9. Behavior of the HR in placebo and EMP groups before (M1) and after (M2) the hoof trimming procedure.

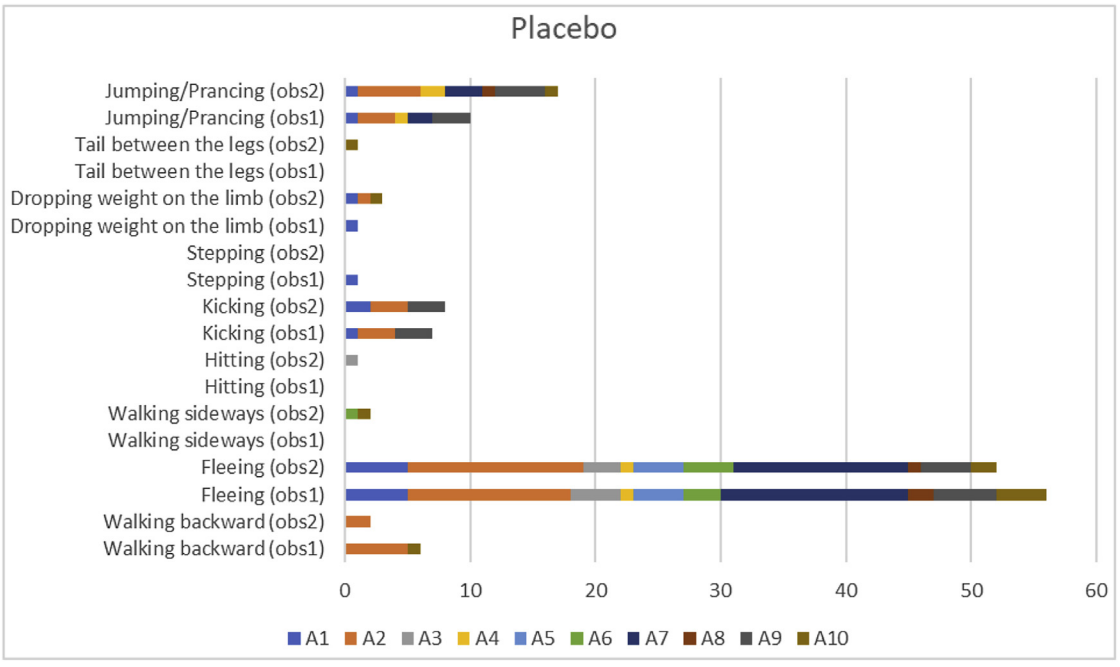

Figure 10. Quantification of the behavioral reactions given by the animals in the placebo group as registered by Observer 1 (Obs1) and Observer 2 (Obs2).

problem in the data recording of four animals during the procedure compromised the statistical analysis because the number of samples analyzed in this study may have been a relevant factor. Evaluation of a response to stress should be conducted using both physiological and behavioral measurements (Broom, 1991; Mason and Mendl, 1993).

The statistical analysis revealed no significant differences regarding behavior. Both groups had fleeing as the most common reaction in this situation, as shown in Figures 10 and 11.

Equines have high learning ability, and this has a substantial effect of handling and training in the initial stages. The more conditioned the animal is to a particular situation, the easier handling will be if the first instance was free of fear. However, positive reinforcement facilitates understanding, leading to positive behaviors and reactions during the subsequent handling (Mengoli et al., 2014). In this study, the animals were young and untamed, with most accepting handling only with a halter. Considering that most animals had never undergone hoof trimming and, therefore, did not go through the learning procedure, the expected result was a high reactivity level in all animals because the procedure would be interpreted as a threat, especially during restraint.

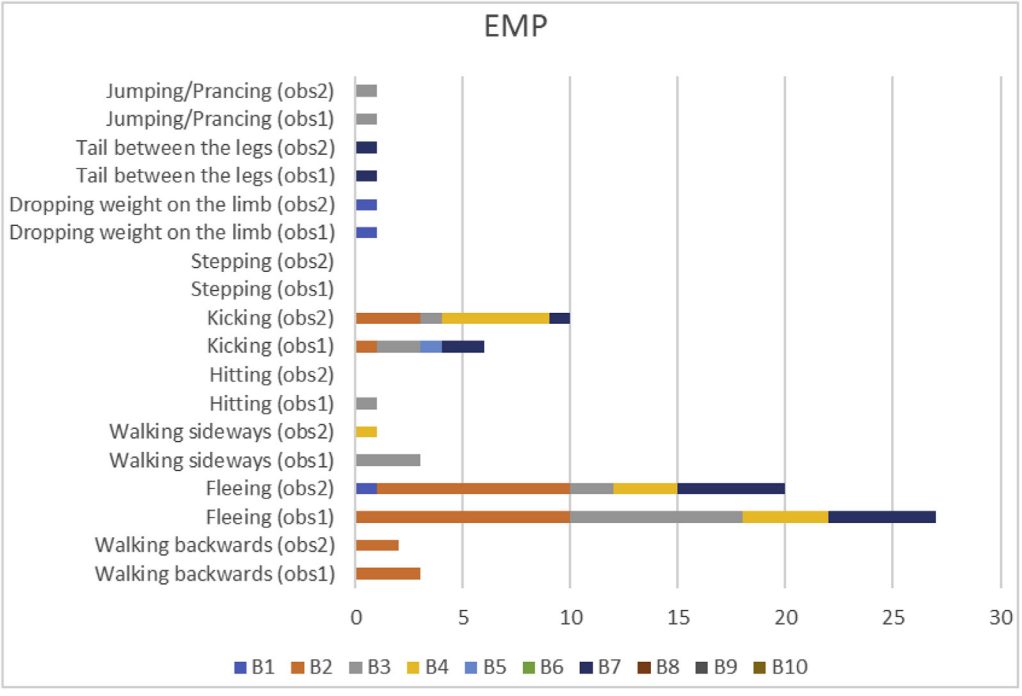

Figure 11. Quantification of the behavioral reactions given by the animals in the EMP group as registered by Observer 1 (Obs1) and Observer 2 (Obs2).

Conclusion

In our study, we did not observe behavioral or physiological changes during the procedure of hoof trimming that recommended the use of synthetic pheromones. There was no statistical difference between the treatment (pheromonal analogue) and placebo in this blinded study. Before recommending the use of synthetic pheromones for hoof trimming, controlled, blinded studies demonstrating clinical efficacy would need to be published.

Acknowledgments

The concept for the article was conceived by Renata Alves de Paula. The experiment was designed and conducted by all the authors. The data were analyzed by Miriam Harumi Tsunemi. The article was written by all authors.

Conflict of interest

The authors declare they have no conflicting interests.

References

Abreu, L.C., 2012. Heart rate variability as a functional marker of development. J. Hum. Growth. Dev. 22, 279–282.
Berger, J.M., Spier, S.J., Davies, R., Gardner, I.A., Leutenegger, C.M., Bain, M., 2013. Behavioral and physiological responses of weaned foals treated with equine appeasing pheromone: a double-blinded, placebo-controlled, randomized trial. J. Vet. Behav.: Clin. Appl. Res. 8, 265–277.
Borell, E., Langbein, J., Després, G., Hansen, S., Leterrier, C., Marchant-Forde, J., Marchant-Forde, R., Minero, M., Mohr, E., Prunier, A., Valance, D., Veissier, I., 2007. Heart rate variability as a measure of autonomic regulation of cardiac activity for assessing stress and welfare in farm animals — a review'. Physiol. Behav. 92, 293–316.
Broom, D.M., 1991. Animal welfare: concepts and measurement. J. Anim. Sci. 69, 4167–4175.
Calviello, R.F., 2013. Evaluation of the Reactivity of Horses during Handling and Presence of Unknown Stimulus. Dissertation presented to the Faculty of Animal Science and Food Engineering of the University of São Paulo, Pirassununga.
Chamero, P., Marton, T.F., Logan, D.W., Flanagan, K., Cruz, J.R., Saghatelian, A., Cravatt, B.F., Stowers, L., 2007. Identification of protein pheromones that promote aggressive behaviour. Nature 450, 899–903.
Clayton, H.M., 1991. Conditioning Sport Horses. Sport Horse Publications, Mason, p. 242.
Craig, N., Nunan, M., 1998. Entrenamiento del ritmo cardiaco para caballos. Performance Matters, Adelaida, Australia Sur.
Cruz-Aleixo, A.S., Alfonso, A., Oba, E., Ferreira de Souza, F., Salgueiro Cruz, R.K., Fillippi, M.G., Chiacchio, S.B., Tsunemi, M., Gomes Lourenço, M.L., 2017. Scaling relationships among heart rate, electrocardiography parameters, and body weight. Top. Companion Anim. Med. 32, 66–71.
Cunningham, J.G., 2008. Treaty of veterinary physiology. Guanabara Koogan, Rio de Janeiro, RJ, p. 710.
Evans, D.L., 1994. The cardiovascular system: anatomy, physiology, and adaptations to exercise and traninig. In: Hodgson, D.R., Rose, R.J. (Eds.), The Athletic Horse. W. B. Saunders Company, Philadelphia, pp. 129–144.
Falewee, C., Gaultier, E., Lafont, C., Bougrat, L., Pageat, P., 2006. Effect of a synthetic equine maternal pheromone during a controlled fear-eliciting situation. Appl. Anim. Behav. Sci. 101, 144–153.
Gontijo, L.D., Cassou, F., Michelotto Junior, P.V., Alves, G.E.S., Bringel, B., Ribeiro, R.M., Lago, L.A., Faleiros, R.R., 2014. Well-being in equine policing in Curitiba/PR: clinical, ethological and circadian cortisol rhythm indicators. Rural Sci. 44, 1272–1276.
Heleski, C.R., Shelle, A.C., Nielsen, B.D., Zanella, A.J., 2002. Influence of housing on weanling horse behavior and subsequent welfare. Appl. Anim. Behav. Sci. 78, 291–302.
Hermiston, C., Montrose, V.T., Taylor, S., 2018. The effects of dog-appeasing pheromone spray upon canine vocalizations and stress-related behaviors in a rescue shelter. J. Vet. Behav.: Clin. Appl. Res. 26, 11–16.
Kaneko, J.J., Harvey, J.W., Bruss, M.L., 2008. Clinical Biochemistry of Domestic Animals, 6.ed. Academic Press, New York, p. 916.
Mason, G., Mendl, M., 1993. Why is there no simple way for measuring animal welfare? Anim. Welf. 2, 301–319.
Mengoli, M., Pageat, P., Lafont-Lecuelle, C., Monneret, P., Giacalone, A., Siguieri, C., Cozzi, A., 2014. Influence of emotional balance during a learning and recall test in horses (*Equus caballus*). Behav. Processes 106, 141–150.
Mills, D., 2005. Pheromonatherapy: theory and applications. Practice 27, 368–373.
Nascimento, M.F., Barros, J.A., 2008. Effects of physical conditioning on resting heart rate and its variability in sedentary male and physical exercise subjects. Braz. J. Exerc. Presc. Physiol. 2, 209–220.
Ohmura, H., Jones, J.H., 2017. Changes in heart rate and heart rate variability as a function of age in Thoroughbred horses. J. Equine Vet. Sci. 28, 99–103.
Sampaio, B.F.B., Shiroma, M.Y.M., Bertozzo, B.R., Costa e Silva, E.V., Zúccari, C.E.S.N., 2014. Equilibrio del casco equino. REDVET. Rev. Electrón. Vet, Málaga, Andaluzia.
Stowers, L., Marton, T.F., 2005. What is a pheromone? Mammalian pheromones reconsidered. Neuron 46, 699–702.
Trotier, D., 2011. Vomeronasal organ and human pheromones. Eur. Ann. Otorhinolaryngol. Head Neck Dis. 128, 184–190.
Vanderlei, L.C.M., Pastre, C.M., Hoshi, R.A., Carvalho, T.D., Godoy, M.F., 2009. Basics of heart rate variability and its clinical applicability. Braz. J. Cardiovasc. Surg. 24 (2), 205–217.
Visser, E.K., Ellis, A.D., Van Reenen, C.G., 2008. The effect of two different housing conditions on the welfare of young horses stabled for the first time. Appl. Anim. Behav. Sci. 114, 521–533.