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RESEARCH

Behavioral and physiological responses of weaned foals treated with equine appeasing pheromone: A double-blinded, placebo-controlled, randomized trial

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KEYWORDS:

foal;
weaning;
stress;
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serum cortisol;
cytokines

Abstract Weaning, particularly the widespread practice of abrupt separation of the mare and foal, has been shown to be a stressful event for horses. Physiological changes in foals measured after weaning include increased blood cortisol concentrations and a subsequent decrease in cell-mediated immune responses. In the randomized, double-blinded, placebo-controlled trial reported here, we assessed the effect of an equine appeasing pheromone (EAP; Modipher EQ, E.A.P. Mist [Pherosynthese s.n.c., Le Rieu Neuf, Saint-Saturnin-les-Apt, France]) as an aid for reducing the behavioral and physiological signs of stress during weaning. Fourteen quarter horse foals were separated from their dam (equid mother) between 105 and 146 days of age, in age-matched pairs, and placed in 3.66 × 3.66 m stalls (one treated and one control foal in each stall). Treated foals received the synthetic analogue of the EAP by intranasal wipe 30 minutes before separation and twice daily thereafter for 48 hours. Control foals received placebo by intranasal wipe on the same schedule. The foals were continuously video-taped for 48 hours postweaning to monitor behavioral responses. Blood samples were drawn 24 hours pretreatment and 24 and 48 hours postweaning for evaluation of physiological indicators of stress (serum cortisol) and cytokines as stress-related and immune-mediated response parameters. Interestingly, although behavioral and serum cortisol measures were similar between groups, treatment with EAP had a significant ($P < 0.05$) effect on interleukin 6 and transforming growth factor β , whereas monocyte opiate receptor 1 was significantly upregulated in both groups independent of treatment when compared with baseline values. Although the link between EAP treatment and activation of the measured cytokines remains unexplained, our findings suggest immune-related gene transcription focused on the acute causes of stress in a time frame shortly after weaning.

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Introduction

The many stressful effects of weaning in horses, including maternal deprivation as well as changes in housing, feeding, and social structure, have been well documented

(McCall et al., 1987; Malinowski et al., 1990; Heleski et al., 2002; McGee and Smith, 2004; Moons et al., 2005). Behavioral, physiological, and immune response indicators have been used as measures of weaning stress in foals (McCall et al., 1985; McCall et al., 1987; Malinowski et al., 1990; Heleski et al., 2002; Moons et al., 2005). Weaning method, type of feeding, and environment in which weaning occurs have been studied as modulators of the intensity of foals' stress responses (McCall et al., 1987; Malinowski et al., 1990; Hoffman et al., 1995; Holland et al., 1996; McGee and Smith, 2004; Moons et al., 2005; Waran et al., 2008).

Pheromones are chemicals produced by specific regions of epithelial cells that produce behavioral and physiological effects when perceived in the environment by other animals, usually but not exclusively conspecifics (Mills, 2005). Events or states modulated by pheromones include copulation, aggression, dam-infant bonding, familial recognition, appeasement, and synchronization of estrous (Bigiani et al., 2005). It has been hypothesized that pheromones stimulate the specialized chemoreceptive cells in the vomeronasal organ (VNO), an organ located at the base of the nasal cavity and anatomically separated from the main olfactory bulb. Nevertheless, studies suggest that humans although having no VNO can still perceive certain pheromones; for example, women living in close proximity seem to synchronize menstrual cycles (Stern and McClintock, 1998). Comparing gene responses for pheromones in old world and new world monkeys, it was suggested that a particular habitat selection may affect the evolution of pheromone perception (Webb et al., 2004). Horses, similar to other placental mammals, have a VNO, which opens to the intranasal cavity; hence, intranasal application is thought to be effective. The receptor cells of the VNO project their axons to the accessory olfactory tract and then to the amygdala through the limbic system, which controls emotions. Thus, pheromones do not require complex processing at higher levels of the brain, and the pheromones might affect the behavior of an individual unconsciously (Stern and McClintock, 1998; Bigiani et al., 2005; Mills, 2005).

It is hypothesized that equine pheromones have a calming or reassuring (appeasing) effect and are produced near mammary glands of dams after parturition (Gaultier et al., 2005; Mills, 2005). 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 (Pageat and Gaultier, 2003). 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 (Sheppard and Mills, 2003; Gaultier et al., 2005; Mills, 2005; Estelles and Mills, 2006) and in cats (Griffith et al., 2000; Mills, 2005). Pheromones could have their effects

altered by environment or context, and their structure and function are far more complex as recently suggested. This could explain the findings that 11 of 14 reviewed reports provided insufficient or lack of support for effectiveness of pheromones for the treatment of undesirable behavior in cats and dogs (Frank et al., 2010). One equine study using an equine appeasing pheromone (EAP; Modipher EQ, E.A.P. Mist [Pherosynthese s.n.c., Le Rieu Neuf, Saint-Saturnin-les-Apt, France]) suggested a reduction in fear responses in adult horses subjected to a number of stressful events when compared with controls (Falewee et al., 2006). Positive effects with the use of EAP on multiple short-term separations of foal and mare were recently reported (Van Sommeren and Van Dierendonck, 2010).

To the authors' knowledge, none of the aforementioned studies assessed behavioral, stress, and immune response indicators on the effects of weaning.

Modulation of the neuroendocrine-immune system is a central component of the biological stress response and has been studied in other species including humans (Bartlett et al., 1993; Stefano et al., 2004; Bittman et al., 2005). Limited research has been published on the effect of stress on the equine immune system; however, the effect of different management practices on the hypothalamic-pituitary-adrenal (HPA) axis has been reported elsewhere (Stephens, 1980; Dantzer and Mormède, 1983; Plotsky, 2000). Equine cortisol concentrations increase with stress-causing factors, such as colic, fractures, transportation, and exercise (Stull et al., 2004; Hinchcliff et al., 2005). Exercise-induced stress can increase serum cortisol concentration and consequently alter the responsiveness of equine mononucleocytes (Kurcz et al., 1988). Furthermore, there is evidence that stressful events such as weaning elevate plasma cortisol in foals (Malinowski et al., 1990).

Differences in gene expression of only a few key stress-related molecules may be important determinants of individual responsiveness to stress (Kabbaj et al., 2000; Bittman et al., 2005). Stress research has shown that the neuroendocrine-immune system communicates multidirectionally in both humans and animals (Kabbaj and Akil, 2001). Three different cytokines (interleukin 6 [IL-6], monocyte opiate receptor type 1 [Mor-1], and transforming growth factor β [TGF- β]) were chosen for analysis based on differences in gene expression after stress induction or stress amelioration, as documented in previous studies. These 3 cytokines were found to be altered in individuals by music listening (stress amelioration), whereas controls did not exhibit these changes (Stefano et al., 2004; Bittman et al., 2005). Classical music also seems to have a relaxation effect on dogs in shelters shown by more time spend resting and less time spend standing (Wells et al., 2002).

In this study, we aimed to test a stress amelioration method by using an intranasal wipe containing a synthetic equine pheromone (EAP) analogue in foals. The correlation between stress reduction and impact on cytokine expression is supported by scientific reports in humans but has not yet

been tested in horses during weaning (Stefano et al., 2004; Bittman et al., 2005). The categories of cytokines chosen for this study play an integral role in immune and inflammatory processes and differentiated cell function. Gene expression targets selected for this study included TGF- β , which inhibits inflammatory response and has a wide variety of immune functions; IL-6, which acts as a differentiation factor for B cells and mediates proinflammatory responses (Horohov, 2003; Quinlivan et al., 2007); and Mor-1, which, when bound by endogenous opiates, mediates analgesic responses, counteracts stress-induced pain, and is upregulated by IL-6.

As herd animals, many adult horses and foals show signs of distress when separated from a group; thus, treatment methods that relieve separation-related distress at weaning or later in life merit investigation. In this double-blinded randomized trial, we evaluated the effect of a commercially available analogue of an EAP (Pherosynthèse) on stress-related behaviors and physiological parameters (serum cortisol values) and immune response indicators (blood cytokine values) associated with weaning in pair-housed quarter horse foals. We hypothesized that pair-housed newly weaned quarter horse foals treated intranasal with synthetic pheromones would display fewer stress-related behaviors, as well as displaying selected stress-related behaviors and stress-related physiological changes for a shorter period when compared with placebo-treated foals. We further hypothesized that exposure to EAP could alter immune response shown by measuring cytokines in abruptly weaned and therefore stressed foals.

Materials and methods

Animals

The study protocol was approved by the University of California, Davis Institutional Animal Care and Use Committee. Sixteen foals (10 fillies, 6 colts) born and raised in the United States at the Center for Equine Health (CEH), University of California, Davis, were eligible for inclusion in the study. At the time of the study, all mares and foals at the CEH were handled on a regular basis by the staff and, thus, these were accustomed to human contact and handling. Pilot observations were made on the first weaned foal pair (2 colts) to test the camera settings and the ease of the product application, as well as to familiarize the observers (J.M.B., R.D.) with the ethogram, shown in Table 1, before the remaining 14 foals were formally assessed. The observations of the pilot pair were excluded from the analysis.

The 14 foals (10 fillies, 4 colts) included in this study were weaned between 105 and 146 days of age at weights between 185 and 200 kg. The mares and their foals were kept in groups (2-4 mare/foal pairs) in 0.8 hectare (2 acre) pastures before weaning. Twenty-four hours before

weaning (T0), 2 age-matched foals from the same pasture were brought into the main barn at the CEH together with their dam. Each mare and foal pair was then housed in a 3.66×3.66 m holding stall with access to a 3.66×7.6 m outdoor paddock area adjacent to the foal that they were to be paired with. In the paddock area, visual and physical contact between the pairs was possible through the dividing panels. Each stall consisted of 1.5 m tall solid wood walls with 91 cm of vertical pipe on top of the wood. The bedding in each stall consisted of dust-free pine wood shavings, and the paddock area was decomposed granite. The horses were fed ad libitum alfalfa and oat hay, with free access to water by means of automatic water bowls to which all foals were accustomed to.

Each weaning stall was identical to, and directly across from, the holding stalls. No other horses were allowed in adjacent stalls during the study. Foals of each pair were randomly allocated to treatment (A) or control group (B), so that there was 1 control and 1 treatment foal in each stall. The times of procedures, treatment, and blood collections are summarized in Figure 1.

Treatment

On the day of weaning, EAP or placebo (both provided by Pherosynthèse s.n.c., Le Rieu Neuf, Saint-Saturnin-les-Apt, France; placebo containing the same ingredients without the active pheromone ingredient) was applied to the foals in the form of an intranasal wipe (5×6 cm moist towelette) by a handler (J.M.B. or R.D.) in a double-blinded fashion. The moist wipes were company supplied and individually packaged in light protected and sealed wraps. They were labeled either "A" (0.1% EAP buffered with water, glycerin, and preservatives, pH 6.7) or "B" (0% EAP with water, glycerin, and preservatives, pH 6.7) for the treatment and control groups, respectively. However, whether A or B actually contained the EAP solution was only revealed by the company to the researchers after all data were extracted from the videos, and this information was sent with the data for statistical evaluation. Both treatments were transparent, odorless, and undistinguishable and considered safe for horses and humans. The treatment procedure consisted of wiping the inside of both nostrils with the moist towelette. Each researcher wore latex gloves that were changed between foals. Treatment order was randomized each time by flipping a coin. The interaction for treatment of both foals lasted less than 1 minute each time. The wipes were administered to each foal in the holding stall, with the dam present, 30 minutes before separation (first treatment application [TA1]). During this period, there was no human interaction with the horses. Thirty minutes after intranasal wipe application, both foals were led with their dam from the holding stall into the weaning stall. Each mare was led by a handler, and the foals followed their dam freely into the weaning stall.

Table 1 Ethogram for behavior observations^a in a study of equine appeasing pheromone treated and placebo-treated foals

Behavior	Observations
Locomotor movements	
Standing	Standing still, no feet moving
Moving	Taking a step, walking, trotting, or backing (cantering or running was not observed)
Maintenance behavior	
Eating	Muzzle low to the ground, lips searching for food, chewing movements of the jaw
Drinking	Muzzle lowered into water bowl
Urinating	
Defecating	
Escape behavior	
Pawing	Repeatedly using one foot in quick, scraping motion on footing or wall
Kicking, striking stall wall	Impact of front or hind hoof on stall wall or door
Jumping into wall	Jump or slam the body against the stall wall or door
Positive foal-to-foal interactions	
Showing friendly behavior toward the other horse	Social facilitative interactions such as nuzzling
Non-nutritional suckling	Reaching with muzzle toward other foal's groin area
Mutual grooming	Standing head to tail, usually, and using the teeth firmly and repetitiously to manipulate, typically, the crest area of the neck and withers
Negative foal-to-foal interactions	
Showing agonistic behavior toward the other horse	Nipping, biting interactions with or without aggressive body language, raising of hind leg or kicking, with or without contact with the other foal
Behaviors	
Vocalizing	Whinny, squeal, or nicker
Lying sternal	Lying in contact with ground, on sternum, or on side
Lying lateral	
Rolling	Lying down then rolling onto one side or onto back

^aModified from Heleski et al. 2002.

The mares were then removed by the same handlers from their foals and brought back into the original pasture approximately 0.8 km away from the weaning barn. Because there was no physical, visual, or auditory contact between the weaned foals and their dam, the foals were considered abruptly weaned from their dam (weaning). Videotaping started immediately after entering the weaning

stall. Intranasal wipes were reapplied in the same fashion and in random order within group A or B to each foal again 12 hours (second application, TA2) and 24 hours (third application, TA3) postweaning. Alfalfa, oat hay, and water were available ad libitum during this time.

Behavioral observations

Recording was performed with a video camera (JVC TK-1270U) and a direct TV/VCR system (Toshiba 13" Color Television 13A26; Mitsubishi Video recorder HS-U776). Six 8h VHS tapes (Video Cassette, Maxell, Standard Grade, 8h, T-160) were continuously recorded per weaning pair for 48 hours, with tapes changed at 8 AM, 4 PM, and 12 AM, respectively. All tapes were collected first and reviewed at the end of the study. Each tape was labeled with the name of the foal and its signalment for recognition but not as with the treatment group. Evaluation of the tapes was performed by the same 2 observers (J.M.B. and R.D.) who were experienced in collecting equine behavioral data. The behavior patterns were quantified by measuring time with a stopwatch and marking an individual behavior chart

Figure 1 Timeline for procedures, treatment, and blood collection in a double-blinded, placebo controlled trial of the effects of equine appeasing pheromone (EAP).

for each animal. The 2 observers (J.B.E. and R.D.) were observing simultaneously and collecting data from the tapes. Each foal was assigned to one observer randomly by pulling a name out of a hat. At the time of data extractions from the video, the researchers were not aware whether the foal belonged to group A or B as the observation was done by foal name only. And although the same 2 researchers (J.B.E. and R.D.) were doing the treatment several months prior as were doing all video observations, they did not remember the foals at the time of data extraction. In addition, at that time, it was still unknown to the researchers whether A or B actually contained the EAP or placebo solution. This fact was revealed to the researchers by the company after all data were collected. To assess interobserver reliability, simultaneous observations in a crossover design using the pilot weaning pair and 3 randomly chosen foal pairs were performed.

A total of 48 hours of taped behavior were available; however, for this study, selected time points were analyzed. Several continuous 30-minute video samples were evaluated and counted for frequency or duration of each behavioral category (Figure 1; time: T1, T2, T3, and T4). Categorical behaviors were combined (Table 1) as previously described (Heleski et al., 2002). Only vocalization was recorded in real time 30 minutes before and 2 hours after separation and 30 minutes after wipes application (TA). The weaned foals were not able to see but could potentially hear the observer outside the stall.

Duration of locomotor movements was measured with a stopwatch; movement around the stall was calculated by the total time of measuring time increments minus the time recorded for standing still. The selection of the duration of observations was based on the time passed after weaning (T1, T2, T3, and T4). The only human contact with the foals in their stalls during the 48-hour study period was during stall cleaning once a day, feeding twice a day by the barn personnel, and wipe application every 12 hours by the researchers (TA1, TA2, and TA3).

The first observation period took place in the 30 minutes immediately after weaning (T1: 0-30 minutes), the second observation period was at 90 minutes to 2 hours after weaning (T2: 90-120 minutes), the third and fourth observations took place before and after treatment at 24 hours postweaning (T3: 23.5-24 hours = 30 minutes before the third application of the wipe, T4: 24-24.5 hours = 30 minutes after the third application [TA3] of the wipe). Vocalizations were evaluated 30 minutes before and after the second and third TAs (± 30 minutes of TA2 and TA3). Overall, a total of 14 hours of observations for 14 foals were evaluated by 2 researchers.

Blood collection

Blood samples were drawn for baseline evaluation of serum cortisol and 3 immune system markers (TGF- β ,

IL-6, and Mor-1) from all foals in pasture, 24 hours before moving them to the barn with their dams. These foals had been raised and handled since birth by the CEH staff; therefore, catching, haltering, and blood collection were done with minimal restraint and elicited no observable stress response. Two additional blood samples were collected 24 and 48 hours postweaning. Samples were collected at 09:00 hours.

Cortisol measurements

Blood was collected in sterile glass tubes without additives, and samples were kept cooled and spun within a few minutes after collection at 4000 rpm for 5 minutes to collect serum that was stored at -15°C until the end of the study. Serum cortisol was measured at the Veterinary Medical Teaching Hospital Chemistry Laboratory at the School of Veterinary Medicine, University of California, Davis, using a radioimmunoassay "Coat-a-Count" (Diagnostic Products Corporation, Los Angeles, CA).

Gene transcription analysis by real-time PCR for immune system markers

Blood was collected in ABI Tempus tubes (Applied Biosystems, Foster City, CA) at the same time as serum samples were collected (-24 , 24, 48). These tubes were immediately vigorously mixed, and the sample in these specific tubes can then be stored at room temperature. RNA was then extracted on a 6100 Nucleic Acid PrepStation (Applied Biosystems) within 24 hours of collection. Complementary DNA (cDNA) was synthesized from DNase I digested total RNA using 100 units of SuperScript III (Invitrogen Corp, Carlsbad, CA), 600 ng random hexadeoxyribonucleotide (pd(N)6) primers (random hexamer primer) 10 U RNaseOut (RNase inhibitor), and 1 mM deoxynucleotide triphosphates (all Invitrogen) in a final volume of 40 μL . The reverse transcription (RT) reaction proceeded for 120 minutes at 50°C . After addition of 60 μL of water, the reaction was terminated by heating for 5 minutes to 95°C and cooling on ice. Non-RT controls were run to confirm elimination of genomic DNA background. Each polymerase chain reaction (PCR) contained 20x primer and probes for the respective TaqMan system with a final concentration of 400 nM for each primer and 80 nM for the TaqMan probe and commercially available PCR mastermix containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl_2 , 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase uracil-*N*-glycosylase per reaction, and 5 μL of the diluted cDNA sample in a final volume of 12 μL . The samples were placed in 384-well plates and amplified in an automated fluorometer (ABI PRISM 7900 HTA FAST, ABI, Foster City, CA). ABI's standard amplification conditions were used: 2 minutes at 50°C , 10 minutes at 95°C , 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C . Fluorescent signals were collected during the annealing temperature, and cycle threshold

Table 2 Sequence of PCR primers and TaqMan probes used in a study of equine appeasing pheromone treated and placebo-treated foals

Target	Primer	Sequence (5'→3')	Length	Probe	Probe sequence (5'→3')	Accession no.
GAPDH	eGAPDH. 74f	AAGTGGATATTGTCGCCATCAAT	88	eGAPDH. 108p	ACCTCAACTACATGGTCTACA-TGTTTCAGTATGATTCC	AF097179
	eGAPDH. 161r	AACCTGCCATGGGTGGAATC				
IL-6	eIL6-478f	GTAACCACCCCTGACCCAAC	82	eIL6-510p	CCTGCTGGCTAAGCTGCATTCCACAGA	AF005227
	eIL6-559r	TTGTTGTGTTCTTCAGCCACTCA				
MOR-1	eMOR1-1359f	CTCCACGGCCAATACAGTGG	79	eMOR1-1382p	TCTGCTCCAGATTTTCTAGC-TGATGGTTAGTCC	XM_003364576
	eMOR1-1438	CCACTTAGGGCAACGGAGC				
TGF-β	eTGFb-293f	GCCTGAGGCGGACTACTACA	92	eTGFb-331p	ATAGATTTCGTTTTCTTTTC-CACCATTAGCA	AJ519535
	eTGFb-384r	TGAGCCGGTCTCCACAGTCT				

PCR, polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-6, interleukin 6; MOR-1, monocyte opiate receptor type 1; TGF-β, transforming growth factor β; FAM, 6-carboxyfluorescein (6-FAM); TAMRA, tetramethylrhodamine.

TaqMan probes: 5' 6-FAM label; 3' TAMRA label.

(CT) values (point at which the fluorescence crosses the threshold) were extracted with a threshold of 0.04 and baseline values of 3–15. For stronger signals, the baseline was adjusted manually to 3–10.

All sequence information for the proposed target genes is available in the GenBank (NCBI). Oligonucleotide sequences for the real-time PCR assays are shown in Table 2. Real-time PCR primers and probes were designed using PrimerExpress v1.5 (Applied Biosystems) and validated according to ABI published guidelines (User Bulletin no. 2, Applied Biosystems). Quantitation of gene transcription was determined using the comparative CT method by using a housekeeping gene. To determine the most stably transcribed housekeeping gene in whole blood under current experimental conditions, a housekeeping gene validation experiment comparing equine glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and 18S rRNA (Assay ID Hs99999901_s1, Applied Biosystems), equine beta-2-microglobulin (*B2M*, AY124653) and equine beta actin (*ACTB*, AF035774) was run on a representative number of samples. The most stably transcribed housekeeping gene was used to normalize the target gene CT values (GAPDH).

Statistical analysis

Baseline comparisons (T_0) of cortisol and behavioral measurements were done by paired *t* tests ($n = 7$ foal pairs) to determine whether randomization had produced comparable groups before treatment. A generalized linear mixed-effects (GLM) model was used to evaluate whether EAP treatment (fixed factor) affected any of the behavioral responses over time (repeated-measures factor). Foal, nested within foal pair, was included as a random effect in the model. Log transformation of behavioral responses was done for all variables, except walking, where it was not necessary to ensure that assumptions of normally distributed data were met. For the evaluation of the effects of EAP treatment on immune system markers and serum cortisol, transformation of data was not necessary. The GLM models for these analyses were simplified by considering

the change in value between baseline and the subsequent measurement times as the outcome. This precluded the need to assess time as a fixed effect in the model. Interobserver reliability of behavioral measurements was evaluated by calculating the difference of the observer scores for each behavioral response and testing whether the mean difference for each score was zero. Statistical tests were 2-tailed with a *P* value of <0.05 considered significant. Statistical analyses were done using Stata version 10 (StataCorp, College Station, TX).

Results

Fourteen quarter horse foals were pair weaned, and the individual foals were randomly assigned to either treatment or placebo. Decision to wean at the CEH is based on date of birth; thus, pairings were matched as closely as possible by age and not by gender. There were 4 filly–filly pairs, 1 colt–colt pair, and 2 filly–colt pairs. The maximum difference in age was 19 days. The weight of the foals at weaning was similar between groups ($P > 0.05$) and measured between 185 and 200 kg. Therefore, the 2 groups were comparable with respect to gender, age, and weight.

Behavior

The application protocol with the intranasal wipe was well tolerated by all foals during the entire length of the study. Each foal tolerated the repeated application with minimal restraint; one handler could easily apply the intranasal wipe.

There was no evidence of differences between observers ($P > 0.18$ for all comparisons) in the recording of 10 behaviors (allogroom, bite, drink, eat, escape, kick, paw ground, paw wall, self-groom, and urinate). In addition, the recorded measurements for 7 behaviors (defecate, vocalize, suck, serious kick, serious bite, lying sternal, and lying lateral) were all 0 for both observers; and hence, no statistical comparison was possible.

Table 3 Mean ($\pm$ standard deviation) behaviors observed in EAP treated and placebo treated foals over time

Time period	T1	T2	T3	T4	P value for between-group comparison	P value for all foals over time
Treatment						
Vocalizations (n)					0.646	<0.0001*
EAP	33.3 $\pm$ 17.4	12.1 $\pm$ 4.8	2.1 $\pm$ 2.1	2.6 $\pm$ 2.6		
Placebo	27.3 $\pm$ 15.0	10.0 $\pm$ 5.9	2.4 $\pm$ 2.7	2.9 $\pm$ 5.0		
Walking in stall (min)					0.788	0.041*
EAP	11.7 $\pm$ 4.8	6.7 $\pm$ 4.7	9.7 $\pm$ 6.1	10.3 $\pm$ 5.8		
Placebo	11.0 $\pm$ 2.8	6.7 $\pm$ 3.4	9.0 $\pm$ 4.0	10.0 $\pm$ 3.5		
Pawing (n)					0.164	0.001*
EAP	8.1 $\pm$ 7.2	3.7 $\pm$ 6.0	1.1 $\pm$ 2.3	3.4 $\pm$ 4.6		
Placebo	7.0 $\pm$ 9.8	1.3 $\pm$ 2.0	0.3 $\pm$ 0.5	0.6 $\pm$ 0.5		
Escape behavior (n)					0.520	0.011*
EAP	1.4 $\pm$ 2.6	0.3 $\pm$ 0.5	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0		
Placebo	1.4 $\pm$ 3.4	0.9 $\pm$ 2.3	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0		
Eating bouts (n)					0.845	0.009*
EAP	6.1 $\pm$ 3.0	2.7 $\pm$ 2.0	10.0 $\pm$ 8.6	12.4 $\pm$ 10.2		
Placebo	6.3 $\pm$ 6.3	6.9 $\pm$ 7.1	9.9 $\pm$ 10.3	13.4 $\pm$ 10.0		
Drinking bouts (n)					0.366	0.026*
EAP	1.7 $\pm$ 2.1	1.6 $\pm$ 1.7	1.1 $\pm$ 1.1	2.0 $\pm$ 2.1		
Placebo	3.6 $\pm$ 3.2	0.9 $\pm$ 1.5	1.4 $\pm$ 1.5	3.4 $\pm$ 1.7		
Positive foal-to-foal interaction					0.392	0.114
EAP	2.7 $\pm$ 3.7	2.4 $\pm$ 1.8	4.3 $\pm$ 3.5	1.6 $\pm$ 1.3		
Placebo	2.0 $\pm$ 2.8	2.0 $\pm$ 1.9	4.6 $\pm$ 3.9	5.4 $\pm$ 3.7		
Agonistic foal-to-foal interaction					0.608	0.030*
EAP	12.4 $\pm$ 24.3	3.1 $\pm$ 4.3	2.3 $\pm$ 3.7	3.9 $\pm$ 5.9		
Placebo	6.4 $\pm$ 7.7	2.6 $\pm$ 4.8	2.0 $\pm$ 2.0	1.1 $\pm$ 1.6		

EAP, equine appeasing pheromone.

*Significant at $P < 0.05$.

Treatment with EAP did not significantly ($P > 0.16$ for all comparisons) affect vocalization, eating and drinking bouts, locomotor behavior such as pawing, walking, escape behavior and contact between the foals, and either agonistic or positive interactions. However, there was a statistically significant effect of time ($P < 0.04$ for all comparisons) in the following behaviors independent of treatment: vocalization, defecation, eating and drinking, pawing, walking, escape behavior, and agonistic interactions (Table 3).

Vocalization

All weanlings in both treatment groups vocalized more frequently in the first half an hour after separation from their dams than during at any other time point after weaning. Two hours after weaning, the vocalization was decreased by approximately 50%. It continued to decrease until 12 hours postweaning and then stayed consistent for the rest of the 48-hour study period (Table 3).

Locomotor movements

Total time spent moving ranged from 7 to 19 minutes (mean $\pm$ standard deviation [SD] = 12 $\pm$ 5) and from 8 to 15 minutes (mean $\pm$ SD = 11 $\pm$ 3) during T1 (first half hour) in the treatment and placebo groups, respectively.

The total time spent moving in T2 ranged from 1 to 12 minutes (mean $\pm$ SD = 8 $\pm$ 5) and from 1 to 11 minutes (mean $\pm$ SD = 8 $\pm$ 3) in the treatment and placebo groups, respectively (Table 3).

Foal-to-foal interactions

The highest number of agonistic interactions between the weaned foals was observed during T1 (range, 0-67). One individual animal (no. 12) showed a higher number of agonistic interactions (67) during T1 when compared with all other foals. The foal no. 11 that was housed with no. 12 did not return any agonistic behaviors (2 during T1 and 0 thereafter). Agonistic interactions were not affected by treatment ($P = 0.608$); however, they decreased over time ($P = 0.03$) in all foals.

Immune-physiological measurements

Cortisol

Serum cortisol values measured in weaned quarter horse foals 24 hours after weaning increased significantly ($P = 0.006$) in both groups of weaned foals independent of treatment. After 48 hours postweaning, serum cortisol values were within normal limits and reflected values

Table 4 Mean ($\pm$ standard deviation) serum cortisol concentrations (ng/mL) of EAP treated and placebo-treated foals over time^a

Treatment	At weaning	24 h postweaning	48 h postweaning
EAP	21.7 $\pm$ 8.3	37.6 $\pm$ 13.3	32.4 $\pm$ 10.8
Placebo	24.4 $\pm$ 8.7	35.4 $\pm$ 7.0	25.7 $\pm$ 4.5

EAP, equine appeasing pheromone.

^aReference range for adult horses/foals: 48–62 ng/mL (Stull and Rodiek, 1988).

compared with preweaning ($P = 0.076$) for all foals. Interestingly, the cortisol values of all foals at all measured time points remained within normal limits based on published reference values (Stull and Rodiek, 1988). Results are summarized in Table 4.

Gene transcription of cytokines

Gene transcription of 3 different cytokine genes (IL-6, TGF- β , and Mor-1) thought to respond to stress inducing or stress amelioration processes was quantified at 2 different time points (T-24, T48). Because of the small number of foals, the values before weaning and treatment were combined for EAP and placebo-treated foals into T0 to increase statistical power. These values were then compared with the values of treatment and control groups at 48 hours postweaning. Our findings were 2-fold. First, with respect to IL-6 ($P = 0.019$) and TGF- β ($P = 0.018$) transcription, mononuclear cells showed statistically significant increases in subjects with EAP treatment compared with the control group. Mean IL-6 (5.71 ± 6.05) and mean TGF- β (1.67 ± 0.67) transcription measurements for 48 hours postweaning were significantly increased when compared with the preweaning status. Second, Mor-1 transcription was significantly higher increased 48 hours postweaning when compare to gene transcription pre-weaning. In all study subjects independent of treatment (EAP treated: $P = 0.009$; placebo treated: $P = 0.003$) (Figure 2).

Discussion

Behavior

In this study, significant temporal changes were observed in the behavior of abruptly weaned quarter horse foals. Over time foals exhibited decreasing vocalization, walking, defecation, escape behavior, pawing, and agonistic foal-to-foal interactions with increases in eating and drinking bouts. These data supported previously reported behavioral and physiological data in which abruptly weaned foals exhibited signs of distress that decreased over time (Houpt, 1984; McCall et al., 1985, 1987; Hoffman et al., 1995; Heleski et al., 2002; Houpt, 2002;

Figure 2 Relative transcription (n-fold differences, y-axis) of IL6, Mor1 and TGFb in EAP treated and control horses. Significant transcriptional increases were found for IL6 ($P = 0.019$) and TGF β ($P = 0.018$) in subjects with EAP treatment compared to the control group. Mean IL6 (5.71 ± 6.05) and mean TGF β (1.67 ± 0.67) measurements for 48 h post weaning were significantly increased when compared to the pre-weaning status. Second, Mor1 transcription was significantly up-regulated 48 h post weaning in all study subjects independent of treatment (EAP treated: $P = 0.009$; Placebo treated: $P = 0.003$).

Moons et al., 2005; Nicol et al., 2005). There was, however, no significant difference between foals treated with EAP and the pair-weaned control foal in measures of behavior or cortisol.

Different weaning methods and the important effects they have on the foal's behavior are discussed in the literature (Houpt, 2005). Currently, the 2 most frequent weaning practices are abruptly separating mare and foal on a given day or gradual separation (McCall et al., 1985, 1987). The most stressful weaning methods are those that are abrupt and that isolate the foal from all social contacts. When physically separated from the dam, foals showed fear-related behaviors, such as vocalization and increased locomotion, which are associated with increased cortisol secretion (Nicol et al., 2005). Weaning in free-ranging situations is a gradual process where suckling occurs for 35–40 weeks, ceasing when the mare approaches delivery of her next foal. If not pregnant, a mare may allow her yearling to continue nursing (Duncan et al., 1984). As a foal ages, the mare–foal bond weakens, leading to increased mare-to-foal distance and decreased nursing frequency. At 5 months of age, foals still spend about 52% of their time in close proximity to their dam even if they have achieved adult grazing levels (Barber and Crowell-Davis, 1994). Weaning from the dam as a food source occurs before weaning from the dam as a social companion (Houpt, 2005).

The foals in this study were weaned at a young age (mean = 119 days) when compared with natural settings (Houpt, 2005); however, the selected age coincides with the general weaning practice under commercial management conditions (Waran et al., 2008). The group of foals used in this study showed immediate increases in vocalization and locomotor activity on removal of their dams, then

appeared to adjust to their new living situation with minimal stress responses after the first 24 hours. This finding is supported by an increase in cortisol values, however, not above reference values throughout the study period. Factors contributing to rapid adjustment were likely the familiarity foals had from being pastured together in the previous months, the 24-hour period foals spent with their dams becoming accustomed to the stalls just before weaning, and being paired rather than isolated. The most stressful time seemed to be the first 24 hours, as evidenced by a statistical increase in cortisol values from baseline. The weaning effects in our study were considered to be 3-fold; separation of each foal from its dam, separation from the rest of the band in pasture, and confinement to a stall. The study design tried to minimize these effects by weaning foals in pairs known to each other from pasture and confining the foals for 24 hours before weaning with the mares in similar stalls.

In some studies, the high number of foals jumping at walls and doors during weaning, including some injury (McGee and Smith, 2004), caused considerable concern for foals' welfare. The foals in this study did show some striking at the wall and rearing up with their front legs onto the wall in the first 2 hours after separation, but this decreased to 0 in both groups for the remainder of the study. None of the foals were injured. However, this observation confirms the aversion and stress experienced with confinement and the strong need of a foal to reunite with the dam or the herd. Abrupt social isolation could lead to serious consequences for a herd animal. This shows the importance of providing a safe environment for weaning foals, such as high solid walls and soft footing.

A high frequency of pawing during the first 30 minutes postweaning also appeared to be motivated by frustration or escape attempts. Pawing can be indicative of food-seeking behavior, moving displacement behavior, conflict, aggression, frustration, stereotypic and escape behaviors (McGreevy, 2004). Because roughage was freely available at all times throughout the study period, food-seeking behavior was unlikely.

Consistent with findings in prior studies, lying down was not observed (McCall et al., 1985; Moons et al., 2005; Nicol and Badnell-Waters, 2005). This indicates that resting behavior occurred in standing position during the day, reflecting a high activity or an arousal level. However, during the night when the videotapes were changed, the researchers observed many of the foals resting lying down in recumbent position.

Gender differences in weaning stress have been suggested (McCall et al., 1985; Moons et al., 2005) but were not found in this study. The small number of horses used in our study and the distribution of genders between the different groups may have contributed to these conflicting results. For this study, 10 fillies and 4 colts were available; this did not allow for statistical power of analysis of gender differences in behavior or effect to EAP.

Pheromones

Pheromones mainly perceived by the VNO may elicit behavioral or physiological changes in some species. The synthetically produced EAP could have a stress-reducing effect on frequency and duration of unrest behaviors in separated foals (Van Sommeren and Van Dierendonck, 2010) and fear- and anxiety-related behaviors in puppies (Denenberg and Landsberg, 2008) as well as in social separation or isolation (Gaultier et al., 2005). However, we did find neither evidence of decrease in anxiety-related behaviors immediately after weaning in foals nor differences in serum cortisol between treatment groups. Our results supported previously conducted experiments that found no appreciable appeasing or soothing effects of EAP during gentling of foals and yearlings (Riley et al., 2002). The lack of calming behavior found with the synthetic formulation of EAP in our study might also suggest that the product might not be biologically active in the foal at weaning age as its function might be to guide the newborn foal to the teat by olfaction. A synthetically produced analogue of a substance produced near the mammary gland of mares or dogs after parturition might not be acting as a calming pheromone later in life. Furthermore, concerns were published about the lack of evidence that the compound of the synthetic analogue of the EAP functions as an appeasing or calming pheromone, and the authors' are not aware of scientific proof that such a pheromone even exists (Dodman, 2008). Similarly, it has been concluded in a study after reviewing several canine and feline pheromone product reports that the results did not yield sufficient support of the effectiveness of pheromones for the treatment of unwanted behavior in dogs and cats (Frank et al., 2010). Pheromones are species-specific chemicals that regulate innate social behavior, and it could be concluded that the synthetically produced pheromone product used as an intranasal wipe might in fact not have any or just little antianxiety or anticalming effects in the foal. In addition, the original product known as "Modipher EQ" was originally designed as a "mist" to be sprayed into horse nostrils. Because of the resistance that most horses show when a spray is applied to the inside of the nose, we requested to have the product application changed to an intranasal wipe for ease of application similar to the cotton glove application of Riley et al. (2002). This research group similar to our finding did not report a positive effect on handling with this form of application of EAP. It is possible that the synthetically produced product does not stimulate the VNO or not in the same way as the natural pheromone or a mist would. The impregnated wipe containing a 0.1% solution may not reach its effect because of lower than needed concentration to achieve sufficient anti-anxiety responses. It has not been proven if the natural pheromone at the mammary gland of the mare indeed has an appeasing or pacifying effect on the foal, or if the foal needs to experience suckling to benefit from any putative calming effect. A synthetically produced

solution might not be able to reproduce the effect of the natural pheromone. If suckling is needed then separating the foal from the mare will cause stress-related behavior independent of treatment, just as found in our study.

All the foals in our study demonstrated low levels of stress indicated by serum cortisol values remaining within reference range throughout the study (Stull and Rodiek, 1988). A crossover effect of EAP from the treated foal to control foal in each pair is possible as they shared one stall and had physical contact. This might explain the relatively homogeneous decrease in stress-related behaviors after 24 hours. In a study in which EAP was applied during multiple short-term separations to both mares and foals, mares or foals, or neither (controls), mare/foal pairs in which just one was treated showed a similar behavioral response as those mare/foal pairs in which both were treated (personal communication, M. van Dierendonck, 2010), supporting the crossover theory. Our study might have been able to reveal a similar effect with a different study design; however, a much larger number of foals would have been required, which unfortunately was not available to us at that time. We would have preferably divided the foals in 3 groups of treatment combinations for weaning: (1) placebo-placebo; (2) treatment-treatment, and (3) treatment-placebo, and the results of such a design could have shown an effect of a possible "cross-contamination" effect.

Cortisol values

Interestingly, we found that all serum cortisol values were within reported reference values for normal equine serum cortisol concentration (65 ± 17 ng/mL) (Stull and Rodiek, 1988; Hoffman et al., 1995) and those previously reported in weaned foals (McCall et al., 1987; Malinowski et al., 1990) at all 3 time points of this study. Cortisol is secreted by the adrenal glands with a circadian rhythm having its highest values in the morning in diurnal animals such as horses (Stull and Rodiek, 1988; Cavallone et al., 2002). Each blood collection was performed at 9 AM and therefore should have been at the peak value (Lebelt et al., 1996). The serum cortisol concentrations for all foals in this study increased significantly 24 hours after weaning when compared with baseline but nonetheless stayed within normal values, with no significant difference between treatment groups. By 48 hours postweaning, the control group had almost reached baseline values, whereas the treatment group was still slightly but not significantly elevated. These findings indicate an overall low level of stress-related activation of the HPA axis in these foals during the 48 hours of weaning period.

The lack of significant difference between treatment groups could reflect variability of cortisol secretion within and between animals, the low number of subjects in each group, limited stress as a result of social pairing and familiarization with the facility and handling before separation from their dams, or a crossover effect of housing a treated

with an untreated foal in the same stall. Increased salivary and plasma cortisol concentrations in singly and paired foals up to 40 hours postweaning were previously reported results (Malinowski et al., 1990; Moons et al., 2005).

The possibility of an inadvertent effect of indirect exchange on the untreated foal, which could potentially negate differences between the groups, was ruled out. The researcher wore and exchanged gloves after each treatment and the foals were not observed to have nasal contact immediately after application. However, an improved study design with larger number of foals divided into 3 different treatment groups (placebo-placebo; treatment-treatment, and treatment-placebo) would be needed to answer the questions of any possible "cross-contamination" effect and increased cortisol levels because of weaning and dam/foal separation.

Our observation is consistent with previously reported data that weaned foals with social contact displayed fewest stress responses (Waran et al., 2008). McCall et al. (1987) found increased cortisol concentrations 2 days postweaning in foals subjected to total separation, with values decreasing to normal by 9 days postweaning. Although some studies have demonstrated significant increases in fecal, plasma, saliva, or serum total cortisol after separation or weaning (McCall et al., 1987; Malinowski et al., 1990; Heleski et al., 2002; Moons et al., 2005), our findings are in agreement with studies of who detected no changes in plasma cortisol concentrations after weaning (Houpt, 1984). It has been previously concluded, that a positive correlation of adrenocorticotrophic hormone response and behavior score in weanlings reinforces the acceptance of both assessments as valid indicators of stress (Hoffman et al., 1995).

Of note was 1 foal, no. 12, that showed a high number of agonistic interactions with his partner, no. 11. Serum cortisol concentrations of both foals increased from 24 and 26 ng/mL, respectively, to 41 and 40 ng/mL, respectively; then both decreased by 48 hours back to 20 and 24 ng/mL, suggesting relatively low stress levels for both foals (aggressor and recipient). We concluded that serum cortisol values or the timing of collection may not have been a good indicator for a stress response for the observed agonistic encounters or for evaluating a treatment effect in this pairing.

Breed has been suggested to affect stress behavior during paired weaning, showing that pony foals seemed less stressed than thoroughbreds when weaned in pairs because of more aggression between the thoroughbred weanlings (Houpt, 2005). The quarter horses in this study exhibited overall few aggressive interfoal interactions, again indicating minimal stress levels similar to pair-weaned ponies. This could have been because of the foals being familiar with each other from being pasture housed before weaning.

Immune response

Cytokines (IL-6 and TGF- β) for this study were chosen based on their integral role in immune and inflammatory

processes. Gene expression was also selected for stress-related and stress-ameliorating processes (Mor-1). These choices were based on the established link between weaning and increased stress responses, leading to effects on the immune response secondarily. The calming or appeasing effect of EAP on stress-related pathways and modulating effect on disturbed immune responses during weaning were to be tested in this study.

There is limited research on the effect of weaning stress on the immune system of foals. Our findings regarding the alteration in immune response are in agreement with previous research that demonstrated an effect of weaning on cell-mediated immunity in horses. Malinowski et al. (1990) reported lower lymphocyte proliferation response based on weaning protocol. Measuring proinflammatory cytokines with methods such as real-time qRT-PCR (Taqman) has been validated (von Rechenberg et al., 2001). We found immune-mediated responses in the absence of elevated serum cortisol concentrations, as has been reported by others in foals (Malinowski et al., 1990) and humans (Stefano et al., 2004). We attribute the measured immune response in the absence of an increased cortisol response to the high sensitivity of this qRT-PCR assay.

The so-called type 1 cytokines that include the tumor necrosis factor (TNF) are involved in the initiation and regulation of cell-mediated immune responses. IL-6 and TNF have also been identified for their role as effector molecules in inflammatory responses. These proinflammatory cytokines have profound biological effects such as neutrophil recruitment and immune activation (Horohov, 2003). IL-6 is also involved in B cell differentiation and induces cytotoxic T lymphocytes and many acute phase proteins (Simpson et al., 1997). Currently, the link between EAP treatment and activation of the measured cytokines remains an unexplained observation that warrants further investigation. In our study, immune-related gene transcription was determined in a time frame shortly after weaning and focused more on the acute causes of stress of separation and new environment. Although chronic stress is immunosuppressive (Mench et al., 1986), it is also known that acute stress has a profound immune-enhancing effect (Dhabhar, 2002) as evidenced by the significant upregulation of these 2 genes in this study. Our findings suggest that EAP had either a direct stimulating effect on IL-6 and TGF- β transcription or ameliorated effects suppressing transcription of pathways to which IL-6 and TGF- β belong. Combined with the conclusions of Stefano et al. (2004) where music listening induced a stress ameliorating effect and as a result significantly lowered the proinflammatory cytokine IL-6, we conclude that EAP in our study did not exert a stress reducing or calming effect.

In contrast, Mor-1 showed increased relative expression in weaned foals independent of treatment. Human music listeners showed a significant increase of these receptors in peripheral blood mononuclear cells, suggesting increased Mor-1 expression in response to increase opiate production

as a means of stress amelioration (Stefano et al., 2004). Our findings in weaned foals do not necessarily contradict the findings in humans; opiate production in our foals may have been stimulated by the weaning stress as evidenced by the behavior scores. As a response, opiates would have mediated an analgesic response to counteract the weaning stress-induced pain. Mor-1 expression seems to be increased related to weaning stress in general and was not modified by EAP application.

The differences between baseline and the 48-hour time point could be explained by changes in the environment, feeding, or foal developmental factors during weaning. A better understanding of the interactions particularly within the context of weaning and the specific immunological responses could be achieved in follow-up studies over a longer time range and with a larger number of foals.

The combined effects of the small sample size and large individual variation in responses might have contributed to the different findings regarding the measured cytokines in this study. Hence, we cannot support our hypothesis that EAP led to amelioration of stress signs in behavior or serum cortisol as well as selected cytokines. IL-6 and TNF- β as proinflammatory cytokines might be involved in the supportive immune responses during the weaning process. The finding of increased expression of opiate receptors during the immediate postweaning period, independent of treatment, warrants further studies in the context of stereotypic behaviors such as cribbing and their development during weaning (Dodman et al., 1987; Waters et al., 2002).

Evaluating transcriptional activity of an individual horse's stress induction or amelioration is a difficult task due to, in part, subtle regulation of transcriptional elements of hormones and cytokines. We have demonstrated the feasibility and potential of this approach to measure immune- and stress-related responses during weaning. The molecular mechanisms by which weaning stress alters an immune response and any potential ramification merits further investigation with larger numbers of animals and an extended panel of transcriptional targets potentially involve in neuroendocrine and immune systems.

Conclusion

Fourteen quarter horse foals, aged 105-146 days, were abruptly weaned and pair housed, and 1 member of each pair was treated with EAP. All foals showed significant decrease in vocalization, walking, defecation, escape behavior, pawing and agonistic foal-to-foal interaction, and an increase in eating and drinking bouts in the first 48 hours. Although weaning increased serum cortisol concentration, there was no significant effect of EAP treatment on cortisol values or on stress-related behavioral responses. However, EAP-treated foals showed a significantly greater increase from baseline values in 2 of the 3 selected immune system markers (IL-6 and TGF- β) than control foals. The effect of immune markers suggests that Mor-1 expression seems to

be increased related to weaning stress in general and was not modified by EAP application. Although the link between EAP treatment and activation of the measured cytokines remains unexplained, these findings suggest immune-related gene transcription as a sensitive indicator of stress during weaning.

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