Applied Animal Behaviour Science 254 (2022) 105707

Contents lists available at ScienceDirect

Applied Animal Behaviour Science

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

Acute changes in oxytocin predict behavioral responses to foundation training in horses

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ARTICLE INFO

Keywords:
Horse
Training
Oxytocin
Cortisol
Behavior
Stress

ABSTRACT

Ensuring horse welfare is a central aim in equestrian activities. Training is an important context for welfare, as horses form long-lasting representations of people and actions at a young age. However, only a few studies have addressed horses' emotional responses during early training with humans. In this study, we followed N = 19 young horses, including naïve yearlings and more experienced two- to three-year-olds, through five foundation training sessions over nine months. Our goal was to combine physiological and behavioral measures to assess emotional responses to early foundation training. Specifically, we measured salivary oxytocin (sOXT) in N = 100 samples and salivary cortisol (sCORT) in N = 96 samples before and after training sessions. We also recoded behavioral responses during training. Changes in sOXT during training predicted individual variation in behavioral responses: Horses who showed more affiliative human-directed behaviors during training had increases in sOXT, while horses who showed more behavioral indicators of discomfort during training had decreases in sOXT. Salivary cortisol was not related to individual behavioral responses, but experienced horses had lower sCORT concentrations both before and after training, and all horses showed decreases in sCORT and in behaviors indicative of fear or discomfort as training progressed. In addition, sCORT increased during longer training sessions, consistent with the established role of cortisol in responding to physical stressors. We conclude that individual variation in positive or negative behavioral responses to foundation training corresponds with acute changes in sOXT concentrations in young horses, suggesting that sOXT may be useful as a non-invasive indicator of emotional responses in young horses.

1. Introduction

Horses used in sports or recreational activities require systematic training to be safe to handle and to learn the various tasks required of them. Horses are typically subjected to their task specific training from three years of age onwards, preceded by foundation training in which horses learn basic responses before being ridden (McGreevy et al., 2018). Young horses may experience considerable stress during training, as measured by changes in behavior and physiology (e.g., Schmidt et al., 2010a; Kędzierski et al., 2012). There is also concern that some training and restraint practices may cause physical injuries and discomfort (McLean and McGreevy, 2010). Therefore, increasing attention is being given to training practices and their effects on horses' emotional well-being (McGreevy and McLean, 2007; King et al., 2018; ISES, 2018). While emotions cannot be directly assessed in animals, there have been major advances in recent years in methods for indirect assessment of emotions, based on identifying short-term changes in behavior, physiology and/or cognition in response to biologically-relevant stimuli that help individuals to avoid harmful situations and to seek out positive and rewarding experiences (reviewed in: Mendl et al., 2010; Paul and Mendl, 2018).

As training lays the base for the horses' experiential knowledge of interactions with people, promoting positive emotions and reducing negative emotions during training is highly relevant (Sankey et al.,

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https://doi.org/10.1016/j.applanim.2022.105707
Received 26 April 2022; Received in revised form 9 July 2022; Accepted 12 July 2022
Available online 14 July 2022

2010a; 2010b). However, it is still relatively unclear how various training practices influence horses' emotions and welfare, and how this is related to horses' age and previous experience with humans. Handling at a very young age has been proposed to affect horses' later emotional expression and trainability. Neonatal handling may decrease emotionality and facilitate learning (Heird et al., 1986; Henry et al., 2009), although the effects may be only temporary (Lansade et al., 2005), and may result in too-familiar attitudes towards humans, which may in turn cause a lack of motivation later in life (Pereira-Figueiredo et al., 2017). Some studies show that "sympathetic" (or: "freestyle") training, which proceeds stepwise and is adjusted to individual progress, is less stressful than standard training in 2- to 3-year-olds (Visser et al., 2009; Kędzierski et al., 2012) and results in improved relationships with people (Fureix et al., 2009). Yet, the classical European training program has been reported to cause only mild elevation stress in 3-year-olds, as measured by cortisol and heart rate, with the exception of first mounting, which is a significant stressor (Schmidt et al., 2010a). There is also debate over the application of "natural horsemanship" practices, which aim to adopt horses' natural social behavior to the context of training with humans (McGreevy et al., 2009). Some studies suggest that application of natural horsemanship methods results in accelerated learning and lower heart rates than conventional training (Fowler et al., 2012), but others have argued that these methods increase stress and fear and involve erroneous assumptions about dominance (Fenner et al., 2018; McGreevy et al., 2009). It has also been suggested that ultimately, horses' emotional responses to the applied training depend more on the trainer's skills than on the method as such (Rozempolska-Rucińska et al., 2013).

In addition to the aforementioned forms of handling and training, horses typically go through foundation training that takes place after weaning, prior to the initiation of formal training to learn more specific skills. The timing and methods of foundation training are less formalized and less often studied, but typically they involve a mixture of sensitizing and desensitizing practices (McGreevy and McLean, 2007). The skills being trained include basic handling, standing, forward walking, being led and allowing hoof care and grooming, and possibly also more discipline-specific aspects. However, only a few studies have assessed emotional responses of horses under 3 years of age in foundation training. Thus far, studies have shown that housing and the use of bit affect emotional responses to training in young horses. Pasture-housed yearling Arabians showed calmer responses to foundation training compared to stalled yearlings (Rivera et al., 2002); group-housed foals learned faster than single-housed foals in foundation training from 6 months to 2 years of age (Søndergaard and Ladewig, 2004); and bitted two-year-olds exhibited more distress behaviors than those wearing an unbitted bridle (Quick and Warren-Smith, 2009). However, we know much less about the factors that influence positive emotional responses during early training practices, a pattern which is also evident in applied animal science research more generally (Kremer et al., 2020).

Identifying behavioral and physiological correlates that are associated with positive emotional responses is critical for promoting positive welfare in horses during training, and in domesticated animals more generally (Boissy et al., 2007). Behaviorally, positive emotions in a horse are recognized by relaxed eyes, extended upper lip, and backward pointing, unpinned ears in the nose line (Lansade et al., 2018). Positive behavior towards humans includes approaching, nudging, and nibbling (Sankey et al., 2010b). Regarding physiological correlates, there is increasing interest in the potential for increases in the neuropeptide hormone oxytocin to serve as an indicator of positive valence (Rault et al., 2017). Oxytocin is synthesized in the hypothalamus and can be released centrally and peripherally, in a coordinated or independent manner, in response to species-specific salient social stimuli (reviewed in Valstad et al., 2017). In domesticated animals, affiliative interactions with humans have been linked to increases in oxytocin concentrations either centrally (pigs: Rault, 2016) or peripherally (dogs: Romero et al., 2014; MacLean et al., 2017). The few prior studies assessing oxytocin in horses in relation to behavior have relied on blood sampling. Plasma oxytocin levels were positively associated with docility and friendliness and tended towards a negative association with fearfulness in adult horses (Lee and Yoon, 2021; Kim et al., 2021). Another study reported, counter-intuitively, that gentle grooming was related to decreases in plasma oxytocin concentrations in horses, while standard grooming did not influence oxytocin (Lansade et al., 2018). Finally, being involved in equine assisted therapy did not cause changes in horse plasma oxytocin concentrations (Malinowski et al., 2018).

To obtain a more complete picture of emotional responses of horses during training, one should address possible negative emotions and effects of stressors as well. Several behavioral indicators of acute stress have been identified in horses, such as head tossing and shaking, head upping, neck tension, tail swishing, stepping, and bucking (König von Borstel et al., 2017; Visser et al., 2009; Quick and Warren-Smith, 2009; Rietmann et al., 2004). Stressful situations can also provoke aggressive behaviour such as kicking and biting (Wulf et al., 2013). Physiological correlates of stress may include changes in cardiac function and/or hormone concentrations (reviewed in König von Borstel et al., 2017; Hall et al., 2018). Previous research has established that salivary and plasma cortisol concentrations reflect changes in heart rate in young (Schmidt et al., 2010a) and adult horses (Rietmann et al., 2004; Becker-Birck et al., 2013, Schmidt et al., 2010b). However, other studies comparing cortisol and behavioral indicators of stress have found somewhat contradictory results (Rivera et al., 2002; Ellis et al., 2014; Perez-Manrique et al., 2021), which may in part be due to the potential for elevations in cortisol in response to a range of arousing physical as well as psychological stimuli, regardless of valence (reviewed in Hall et al., 2018, König von Borstel et al., 2017).

In this study, we assessed physiological and behavioral responses to foundation training in young horses. The subjects were yearlings that were completely naïve to handling, and 2- and 3-year-olds that had some, albeit still limited, previous training experience. We measured salivary concentrations of oxytocin and cortisol in horses both before and after training. Methods to measure salivary oxytocin (sOXT) are relatively new compared to the more established methods to measure salivary cortisol (sCORT), and valid concerns still exist, especially regarding the mode of transfer of oxytocin from blood to saliva (Gröschl, 2009). However biological and laboratory validations of sOXT have been conducted in several species (e.g., dogs: MacLean et al., 2018; pigs: López-Arjona et al., 2020; Moscovice et al., 2022; humans: de Jong et al., 2015), and sOXT is increasingly favored as a non-invasive alternative to more established methods that rely on brain CSF or blood sampling (MacLean et al., 2019). We also measured a range of behaviors that have been linked to positive and negative emotions in other studies, as described above (Table 1).

If training was rewarding, we expected horses to show increases in affiliative behavior towards their trainers, potentially linked to increases in sOXT, over the course of the training sessions. We also predicted that within a training session, sOXT would increase in horses who showed more positive behaviors towards trainers. We further predicted that as training progressed, horses would show decreases in negatively-valenced behaviors, and corresponding decreases in sCORT in later training sessions. We also expected that horses who showed more negatively-valenced behavioral responses within a training session would exhibit increases in sCORT within that session (e.g. Aurich et al., 2015). Since training sessions varied in duration and longer training sessions also required more physical activity, we controlled for any effects of duration of training on sCORT in all models, as a way to account for influences of physical activity more generally on cortisol responses. Since previous studies have reported contradictory results on sex differences in responses to training (Aurich et al., 2015; Schmidt et al., 2010a), we included sex as a predictor in the models but made no directional predictions.

Table 1
Ethogram used for behavioral analyses during horse training sessions.

Grouping for composite measures	Behaviour	Coded as	Description
Explosive	Jump	event	Jumping in any direction[3]
	Buck	event	Hind legs tossed up at the same time[3,4]
	Lunge	event	Sudden acceleration forward or sideways[3]
	Overrun	event	Pushing past the trainer with physical contact[3]
Fearful	Rearing	event	Front legs are lifted from the ground, head up[3]
	Fearful snort	event	Snorting with tense position[5]
	Freeze	state	Afraid to move forward, tense position[5]
	Startle	event	Sudden tension of muscles, possible flight[5]
Affiliative contact	Affection	event	Touching trainer with muzzle[2]
	Exploration	event	Touching trainer or object with lips and/or teeth[2]
	Nibble	event	Fumbling the trainer with lips and pushing with muzzle[6]
Not grouped	Head toss	event	Tossing head sideways or upwards[2,4]
	Head up	state	Head tense and higher than withers[1,3,4]

[1]Visser et al., 2009; [2]Wulf et al., 2013; [3]Rietmann et al., 2004; [4]Quick & Warren-Smith, 2009; [5]Lansade et al., 2008; [6]Sankey et al., 2010a

2. Methods

2.1. Subjects and housing

Subjects were one-year-old naïve (n = 11) and two- to three-year old more experienced (n = 8) horses (n = 9 fillies, n = 9 colts, n = 1 gelding; Finnhorses and warmbloods, Table S1), group housed in an open shed stable at a breeding farm in Southern Finland. Two horses (FR and FU) died after the second or third training session due to an injury and illness, respectively, and one horse (FM) was dropped out of the study by the owner after the second training session. One horse (MA) failed to give the saliva samples on the first sampling round. As a result, we obtained only partial data from these horses. The rest of the horses participated in the full study. Most subjects had been born at the farm and all were maintained in identical conditions. Prior to weaning, the foals had lived in groups consisting of mares with dependent offspring. After weaning all subjects lived continuously in social groups of 6–14 individuals throughout the year. In the colder half of the year, from October to April, groups were housed in an open shed stable with semi-heated covered indoor spaces, peat and straw bedding and free access to the fields. Water and hay were provided ad libitum and oat and mineral supplements were given daily. From early May until late September the horses lived on large natural outdoor paddocks with streams, woodland and grassy growth, and had no visual access to neighboring groups. They did not receive any training during this time.

2.2. Training

The naïve horses (yearlings) were not accustomed to handling, as it had been applied scarcely during their first year of life. They had been haltered a few times prior to the study but were not yet desensitized to a halter and had never been led. The experienced horses (two- to three-year-olds) were familiar with the halter and bridle and had practiced long reigning and lunging. They had received the year before the same training that was given to the yearlings during our study.

We did not enforce a controlled training regime, as we aimed to study early training as it typically proceeds on this farm. The benefit of this approach is that we could assess the effects of the most typical practice, rather than enforcing a training system that is not typically applied. The trainers were n = 12 experienced farm staff and other individuals who regularly trained young and adult horses. All horses were trained by several (2−4) trainers, often within the same training session. There was no systematic rotation of the trainers or horses. Although causing variability, this also minimized possible influences of differences among the trainers on the horses' behavior or hormone responses. Trainers were not aware of the study hypotheses or the ethogram that was used.

The training progressed gradually and included a mixture of standard European foundation training and more individually paced, sympathetic/freestyle training (sensu Visser et al., 2009 and Kędzierski et al., 2012), which was consistent across all subjects. It relied on standard negative reinforcement of pressure-release but included also frequent positive reinforcement with verbal praise and gentle scratching as rewards. In negative reinforcement, the trainer removes an outcome that the horse wants to avoid to increase the occurrence of a desired behavior. In the pressure-release technique the negative outcome is pressure applied by the trainer and, upon the correct response, released. Positive reinforcement, in turn, means addition of an outcome the horse values to increase the occurrence of a desired behavior (McGreevy et al., 2018). The general training regime was the same across all horses within each age group: the naïve horses (yearlings) received training on accepting a lead rope, standing, walking with a trainer and, on the latter three training sessions, trotting, long-reining, bit in mouth, and carriage training. The experienced horses (two- and three-year-olds) received lunging, riding and carriage training, which often lasted for longer and required more physical exertion. The pace of training followed the idea of sympathetic training in that the trainers accounted for the horses' individual skill and confidence at each phase when determining the progression of the training. There were therefore differences among the subjects in the number of training sessions received and in the particular tasks trained within a session, which unfortunately could not be controlled for.

The subjects received 11–17 training sessions applied in two periods in 2020: Period 1 from March– April and Period 2 from October–December. Between these periods the horses had a five-month-long summer pasture season during which no handling or training was applied. Two authors (TN and VR) video-recorded five training sessions of each subject: two in Period 1 and three in Period 2 (Fig. 1). The fifth recorded training session was timed at the point when the subject was able to complete the required tasks. Between these five sessions, the horses received some additional training sessions (1−3) that were not recorded. Therefore, while we cannot assess the precise learning progress with recorded data from each training session, we can address the general behavioral and physiological patterns over the course of training, as well as changes in individual behavior and hormones within each of the five recorded training sessions. The sessions were recorded in full length and varied in duration from ca. 5–30 min (median = 11 min). When the horses were trained in a manege or arena, the researcher recorded behavior from a fixed spot; when training occurred outside, footage was taken while walking beside or behind the trainer and the horse.

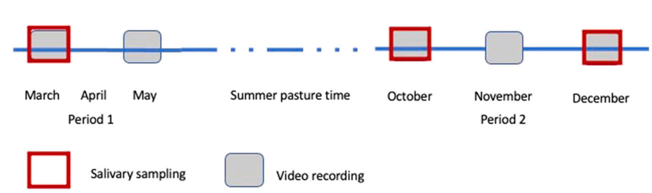

Fig. 1. The schedule of the video-recorded training sessions and associated saliva sampling. Between each recorded training session, the subjects received 1–3 unrecorded training sessions.

2.3. Hormone sampling and measurement

Saliva samples were collected from subjects immediately before and after the first, third and fifth recorded training session (Fig. 1). The goals were to assess short-term changes in sCORT and sOXT concentrations during the training, and to gain insights into general patterns of hormone reactivity across training sessions in more and less experienced horses. By using a within-subject sampling paradigm, we minimized effects of variation in timing of sampling, individual differences, and any uncontrollable prior events on hormone concentrations (Bohák et al., 2013). In addition, all samples were collected by one researcher (TN), who also spent time habituating the horses to her presence prior to collecting samples for the study.

For the pre-training sampling, the horses were walked into a traditional box stable from the open shed stable in pairs or small groups where they were allowed time to adjust. When calm, horses were placed in single-horse stalls with auditory and visual, but not physical contact with other horses. Horses did not receive food during the adjustment time or training, and no food was available during sampling, to minimize any potential interference with sampling. Prior to collecting the pre-training saliva sample, horses were kept in the stalls for 15 min, to ensure that no food had been consumed for > 15 min before sampling. Sampling was identical for all subjects in all sampling rounds with one exception: the first pre-training sample of yearlings was taken while they were still in the shed stable, as walking them to the unfamiliar box-stable might have been stressful.

After the pre-training sample was taken, the horse was tacked and taken out for the training session. Training ended when the horse was returned to the box stable, at which point the post-training sample was taken. The saliva sampling was conducted without restraining the horses and was well accepted by all horses, who did not show any signs of distress during sampling (see example video in Supplementary Materials). We recorded the time of the sampling and the duration of the training. If the training lasted for less than 10 min, we waited until 15 min has passed from the onset of the training to ensure sufficient time for excretion of hormones into saliva. Previous research indicates that the release rate of cortisol into saliva depends on the duration of exercise and is detectable within 15 min from the onset of a stressor, with the peak occurring at 15–30 min following the stressor (Kędzierski et al., 2013; Peeters et al., 2011). In dogs (MacLean et al., 2017) and pigs (Lürzel et al., 2020), increases in sOXT are observed within 10–15 min following affiliative interactions with humans.

Saliva was collected using synthetic SalivaBio childrens swabs (Salimetrics©, PA, USA). The swabs are designed to collect passive drool from humans and have been used in validations of salivary oxytocin in a range of animal species (e.g. dogs: MacLean et al., 2018, pigs, cattle and goats: Lürzel et al., 2020). The swab was taped onto a plastic rod, inserted into the mouth of the horse and placed gently beside or under the tongue for 1–2 min until saturated. Saturated swabs were immediately placed into a polypropylene tube and stored in dry ice until transfer to a − 80 °C freezer. Between one to six months after collection, samples were shipped on dry ice to the Research Institute for Farm Animal Biology (FBN, Dummerstorf, DE), where they were analyzed in the Institute of Behavioral Physiology. Samples were thawed and centrifuged, and the eluent was aliquoted into eppendorf tubes and stored at − 80 °C until analysis. Cortisol was run in a commercially available enzyme-immunoassay (Demeditec Diagnostics®, GmbH, Kiel, Germany). The standard curve ranges from 0.1 to 30 ng mL^{-1} and assay sensitivity is 0.019 ng mL^{-1}. After thawing, samples were spun at 2500 g for 5 min, diluted 1:1 with assay buffer and 50 μl of supernatant were run in duplicate. We performed analytical validations of parallelism, accuracy/recovery and reproducibility. To test for parallelism, we estimated and compared the slopes of the fitted lines between the cortisol standard curve and a serial dilution of pooled horse saliva, using the 'lstrends' and 'pairs' functions in the package 'lsmeans' (Lenth, 2016). The test revealed no differences in the slopes (t-test, n = 5–6, df = 7, t ratio = −1.159, p = 0.285, see Supplementary Fig. S1a). Recovery of sCORT in three different pooled samples of known concentrations, each spiked with cortisol standards at two different concentrations (1.7 ng mL^{-1} and 30 ng mL^{-1}), was 106.4 (± 10.5)% (n = 6 spiked samples). To determine reproducibility, we measured intra- and inter-assay correlations of variation (cvs) in low (2 ng mL^{-1}) and high (5 ng mL^{-1}) pooled horse saliva samples that were stored in multiple aliquots and run on each plate in replicates. The intra-assay cvs for low- and high-concentration controls were 4.2% and 3.8% respectively (n = 10 samples). The inter-assay cvs for low- and high-concentration controls were 13.5% and 11.1% (n = 6 samples).

Prior to sOXT measurement, we performed a solid phase extraction (SPE) following a protocol modified from Lürzel and colleagues (2020). Samples were thawed on ice and centrifuged at 4 °C (5 min, 2,000g). We mixed 250 μl of saliva with equal parts 0.1% trifluoroacetic acid (TFA)/ water, vortexed, and then centrifuged at 4 °C (15 min, 17,000g). We loaded the supernatant on 1 mL, 30 mg sorbent, HLB cartridges (Oasis Prime®, cat no. 186008055, Waters Corporation, MA, USA), primed with 1 mL 99% acetonitrile (ACN), followed by 3 mL 0.1% TFA/water. Samples were washed with 3 mL 0.1% TFA/water and eluted with 3 mL 36% ACN/ 0.1% TFA. Eluents were evaporated using a Vacuum concentrator (SpeedVac® SC210, Thermo Fisher Scientific, MA, USA), and frozen at − 80 °C until measurement. We measured sOXT using a commercially available enzyme immuno-assay (Cayman Chemical®, kit no. Cay500440, MI, USA) that has been validated for sOXT in several species (dogs: MacLean et al., 2018, farm animals: Lürzel et al., 2020). The standard curve ranges from 5.9 to 750 pg mL^{-1} and assay sensitivity is 20 pg mL^{-1}. Prior to measurement, we reconstituted samples in 250 μl assay buffer, and then followed the kit instructions. We performed analytical validations to confirm that the assay measures the majority of oxytocin present and is suitable for measuring oxytocin in horse saliva. Tests of parallelism revealed no differences in the slopes between the standard curve and a serial dilution of pooled horse saliva (t-test, n = 4–5, df = 5, t ratio = 0.649, p = 0.545, see Supplementary Fig. S1b). Recovery of sOXT in three different pooled samples of known concentrations, each spiked with oxytocin standard at two different concentrations (375 pg mL^{-1}, 187.5 pg mL^{-1}) was 99.9 ± 6.9% (n = 6 spiked samples). To determine reproducibility, we measured intra- and inter-assay cvs of pooled low (30 pg mL^{-1}) and high (150 pg mL^{-1}) horse saliva samples that were stored in multiple aliquots, extracted along with test samples and run in replicates on plates. The intra-assay cvs for low- and high-concentration controls were 6.2% and 5.4% (n = 11 samples) and the inter-assay cvs were 14.6% and 12.8% (n = 6 samples).

2.4. Behavior coding

The coding of videos was done with Boris (version 7.10.2). We coded several behaviors from the training session videos as indicators of positive (affiliative contact) and negative (explosive, fearful, head toss, head up) affective responses based on earlier research (Table 1). When the horse was not completely visible, no video was scored and we subtracted the not-visible time from the total duration. Behaviors that were functionally similar based on earlier behavioral studies (references in Table 1) were combined into composite scores by summing the event behaviors and, for duration behaviors, first scaling and then summing them.

2.5. Statistical analyses

Statistical analyses were performed in R (v 4.0.5 R Core Team, 2021). We tested for repeated measures correlations between each horses' cortisol and oxytocin concentrations either before or after training using the package 'rmcorr' (Bakdash and Marusich, 2020). We built linear mixed models (LMMs) and generalized linear mixed models (GLMMs) using the package 'lme4' (Bates et al., 2015). We first tested whether changes in sOXT or sCORT were related to horses' experience level or their progression with training. Since we were particularly interested in the effects of training on changes in hormones, we included the sample context (pre- or post- training) as an additional predictor, and tested for a three-way interaction of sample context, experience and training session, since we expected that more experienced horses would acclimate to repeated training more rapidly, as measured by decreased sCORT responses and/or increases in sOXT responses to later stages of training. We also tested whether changes in hormones from pre- to post-training were influenced by the duration of training or subject sex. We included the time of each sample collection as an additional control predictor.

We then tested whether changes in behavior during training were related to each subject's characteristics, particularly their level of experience and their training progression across the five training sessions, as well as an interaction between experience and training session. We tested whether the two uncategorized behaviors, head up and head toss, were correlated. We treated rare behavioral categories (explosive and fear behaviors) as binomial data and modeled their likelihood of occurrence using GLMMs with a binomial error distribution. For the other behaviors (affiliative contact and head-toss), we used LMMs with gaussian error distributions to model their frequencies of occurrence. For each behavioral model, we included subject sex as a control predictor, and we included the duration of training as an offset term. We also included random intercepts of subject.

Finally, we tested whether changes in hormone concentrations during each training session were predicted by the occurrence (for exploding and fear behaviors) or the frequency (for affiliative contact and head-toss) of distinct behavioral indicators, to provide further insights into the usefulness of using sOXT and sCORT as indicators of emotional responses in horses. To test for a relationship between specific behaviors and changes in hormone concentrations, we tested whether hormone concentrations were influenced by an interaction between each behavioral measure and the hormone sampling context (pre- or post-training). We included the time of sample collection as a control predictor, and the duration of training as an offset-term. For all models predicting hormone concentrations, we included random intercepts for subject and for the paired hormone samples from each subject. To avoid over-fitting the data, and to keep the type I error rate at a nominal 5% (Schielzeth and Forstmeier, 2009), we also included all theoretically identifiable random slopes (see Supplementary Materials). We log-transformed hormone concentrations to approximate normal distributions, and we z-transformed continuous predictors, to facilitate interpretability of the results. We confirmed that models were stable by comparing the model estimates derived from the full data set with those obtained from a model with each subject excluded one by one. We tested for variance inflation using the 'vif' function in the package 'car' (Fox and Weisberg, 2019) and found no evidence for collinearity of the predictors (VIFs < 1.44). For the two binomial models, dispersion parameters were between 0.56 and 0.88, indicating no evidence of over-dispersion. We used likelihood ratio tests (Dobson, 2002) to compare each model to a reduced model excluding the test predictors, and present results of models that differed significantly from the reduced model. We tested for significance levels of the fixed effect predictors using t-tests with the Satterthwaite approximation (Luke, 2017) in the function lmer of the package lmerTest (v 3.1–3, Kuznetsova et al., 2017) and a model fitted with restricted maximum likelihood. To determine overall effects of experience level and training session, we further compared the full model with a reduced model excluding the specific factor of interest. Unless otherwise noted, results are presented as mean (± SD) and a *P*-value < 0.05 was considered significant.

2.6. Ethics

The study was evaluated and granted permission by the Board of Ethical Evaluation of the University of Helsinki (decision 5/2020) and complied with the ethical guidelines of the International Society for Applied Ethology (Sherwin et al., 2003). All horse owners gave informed consent and were free to withdraw from the study at any time. All data was held according to the EU General Data Protection Regulation Act 12–14 (2016/679). The individual behavioral and hormonal means are provided in the supplementary materials; full raw data is available upon request.

3. Results

3.1. Changes in hormones related to experience and training progression

Mean sOXT concentrations were similar before and after training sessions (117.33 ± 57.25 pg mL^{-1} vs. 111.06 ± 59.93 pg mL^{-1}; see Supplementary Materials Table S2). The full model predicting changes in sOXT during training differed significantly from a reduced model with horse experience and training session excluded (likelihood ratio test, chi-square = 19.85, df = 10, p = 0.03). The interaction terms were not significant and were removed from the model. In the final model, there was a significant effect of training session on overall sOXT before and after training ($F_{2,28} = 7.52$, p = 0.002), due to peaks in sOXT in experienced and naïve horses mid-way through training (see Table 2 and Fig. 2). In addition, males had lower sOXT concentrations than females ($F_{1,64} = 12.45$, p < 0.001, see Table 2). Horse experience level had no effect on sOXT concentrations (see Table 2).

Mean sCORT concentrations were 2.54 (± 1.40) ng mL^{-1} before and 3.30 (± 2.62) ng mL^{-1} after training (see Supplementary Materials, Table S2). There was no correlation between each subject's sCORT and sOXT concentrations in pre- (r_{rm} = −0.06, df = 27, p = 0.75) or post-training (r_{rm} = −0.07, df = 28, p = 0.73) samples. The full sCORT model differed from a reduced model excluding the effects of experience and training session (full-null model comparisons with likelihood ratio tests, chi-square = 18.93, df = 10, p = 0.041). The three-way and several two-way interactions were not significant and were removed from the model. In the final model, the only significant predictor of changes in sCORT during training was training duration. Horses who had longer training sessions also had greater increases in sCORT from pre- to post-training samples ($F_{1,52} = 13.46$, p < 0.001, see Table 3 and Fig. 3). In addition, experienced horses had lower sCORT concentrations overall (both before and after training, $F_{1,22} = 8.39$, p = 0.008, see Table 3 and Fig. 4). There was also a significant main effect of training session on overall sCORT concentrations ($F_{2,23} = 4.27$, p = 0.03), mainly due to lower concentrations on the last training session, for both experienced and naïve horses (see Table 3 and Fig. 4).

3.2. Behavioral responses related to experience and training progression

Over the course of the five training sessions, we analyzed the expression of three functional behavioral categories referring to Affiliative contact, Fear and Explosive behavior (see Table 1). Two additional uncategorized behaviors, head toss and head up, were positively

Table 2
Model estimates of predictors influencing horse salivary OXT concentrations (log transformed) during training. Significant predictors are indicated in bold.

Predictor	Estimate	Std. Error	*t* value	*p* value
(Intercept)	4.71	0.09		
experienced†	-0.00	0.10	-0.04	0.970
training (3)‡	0.31	0.11	2.75	**0.010**
training (5)‡	0.07	0.10	0.66	**0.520**
sex (male)§	-0.26	0.07	-3.53	**< 0.001**
sample context (post-training)	-0.07	0.06	-1.13	0.270
log training duration	0.02	0.06	0.37	0.720
time of sample collection	-0.01	0.03	-0.26	0.800

†,‡,§ In comparison with reference level: †naïve horses, ‡training session 1, §females

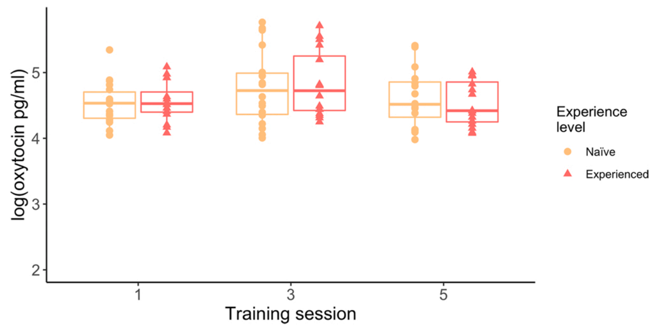

Fig. 2. Salivary OXT concentrations in naïve and experienced horses as training progressed. Regardless of experience, horses had higher sOXT concentrations mid-way through training, during Training session 3. For full model results see Table 2.

Table 3
Model estimates of predictors influencing sCORT concentrations (log transformed) in horses during training sessions. Significant main effects are indicated in bold.

Predictor	Estimate	Std. Error	*t* value	*p* value
(Intercept)	1.17	0.14		
experienced[†]	-0.37	0.13	-2.90	**0.008**
training (3)[‡]	0.04	0.13	0.29	**0.776**
training (5)[‡]	-0.27	0.14	-1.97	**0.068**
sex (male)[§]	-0.19	0.11	-1.63	0.111
sample context (post-training)[¶]	0.19	0.09		
log training duration[¶]	-0.05	0.07		
time of sample collection	-0.08	0.06	-1.50	0.151
context(post-training):log training duration	0.29	0.08	3.67	**0.001**

[†,‡,§]In comparison with reference level: [†]naïve horses, [‡]training session 1, [§]females
[¶]Significance tests of main effects not indicated due to limited interpretability

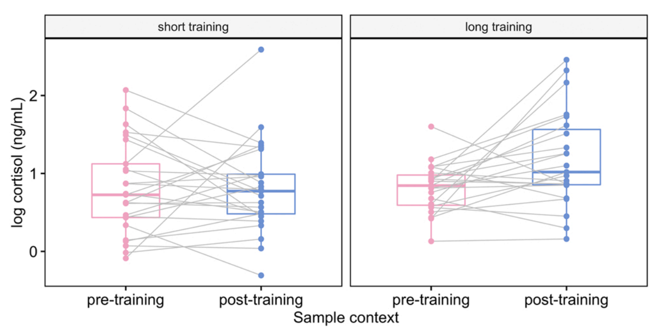

Fig. 3. Changes in sCORT concentrations during training in horses who received shorter or longer training sessions than on average. Note that training duration was analyzed as actual duration in the models; the categorization is included here for illustration purposes only.

correlated (Pearson, $r = 0.47$, $t = 4.92$, $df = 85$, $p < 0.001$) so we excluded head up from further analyses and analyzed head toss as an indicator of mild discomfort or stress (Wulf et al., 2013; Quick and Warren-Smith, 2009).

The full models containing experience level and test session explained more of the variation in each behavioral measure than reduced models that did not include the test predictors (full-null model comparisons with likelihood ratio tests, affiliative contact: chi-sq $= 39.15$, $df = 9$, $p < 0.001$, head toss: chi-sq $= 46.42$, $df = 9$, $p < 0.001$, fear: chi-sq $= 32.61$, $df = 9$, $p < 0.001$, explosive behavior: chi-sq $= 23.61$, $df = 9$, $p < 0.001$). However, only head toss was influenced by an interaction between experience and session ($F_{4,69} = 6.58$, $p < 0.001$, see Table 4a), such that experienced horses showed consistent decreases in head tossing over sessions, while inexperienced horses did not (see Fig. 5). Regardless of experience, horses showed decreases in fearful behavior ($F_9 = 29.94$, $p < 0.001$, see Table 4b) and explosive behavior ($F_4 = 22.60$, $p < 0.001$, see Table 4c) across sessions. However, affiliative contact also decreased over training sessions for all horses ($F_{4,69} = 8.45$, $p < 0.001$, see Table 4d). There were no sex differences in any of the behavioral responses (see Table 4a-d), although males tended to show more explosive behavior than females (est $\pm$ SE$= 1.46 \pm 0.80$, $p = 0.07$, see Table 4c).

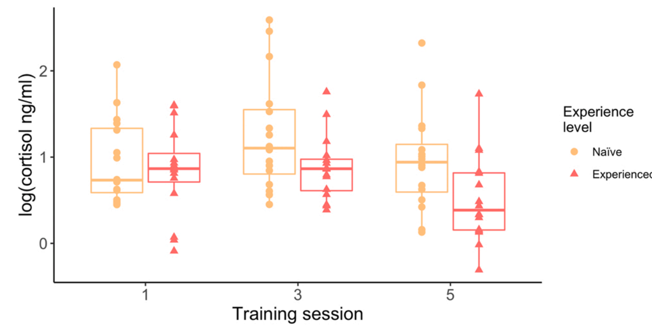

Fig. 4. Salivary cortisol concentrations in naïve and experienced horses as training progressed. Experience and training had independent effects on sCORT concentrations. For full model results see Table 3.

Table 4a
Model estimates of predictors influencing head tossing during training. Significant main effects are indicated in bold.

Predictor	Estimate	Std. Error	*t* value	*p* value
(Intercept)	4.37	0.44		
experienced[†]	1.22	0.59		
training (2)[†]	-1.69	0.46		
training (3)[†]	-0.34	0.49		
training (4)[†]	-1.51	0.51		
training (5)[†]	-0.13	0.51		
sex (male)[‡]	0.50	0.40	1.24	0.23
experienced:training (2)	0.04	0.71	0.05	**0.96**
experienced:training (3)	-2.06	0.73	-2.82	**0.01**
experienced:training (4)	-1.32	0.74	-1.77	**0.08**
experienced:training (5)	-3.11	0.74	-4.18	**< 0.001**

[†]Significance tests of main effects not indicated due to limited interpretability
[‡]In comparison with females

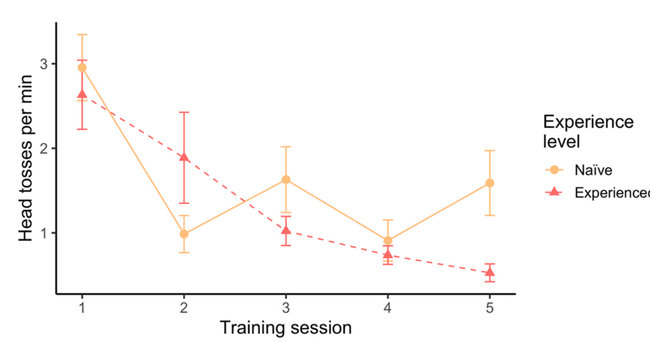

Fig. 5. Mean ($\pm$ SE) number of head tosses per minute by experienced and naïve horses across five training sessions. For full model results refer to Table 4a.

Table 4b
Model estimates of predictors influencing fear behaviors during training. Significant main effects are indicated in bold.

Predictor	Estimate	Std. Error	*z* value	*p* value
(Intercept)	2.26	1.19		
experienced†	1.91	1.17	1.63	0.1
training (2)‡	-2.91	1.13	-2.56	**0.01**
training (3)‡	-4.65	1.15	-4.05	**< 0.001**
training (4)‡	-4.26	1.14	-3.73	**< 0.001**
training (5)‡	-5.15	1.17	-4.41	**< 0.001**
sex (male)§	1.23	1.16	1.06	0.29

†,‡,§ In comparison with reference level: †naïve horses, ‡training session 1, §females

Table 4c
Model estimates of predictors influencing explosive behaviors during training. Significant main effects are indicated in bold.

Predictor	Estimate	Std. Error	*z* value	*p* value
(Intercept)	0.50	0.80		
experienced†	1.06	0.79	1.33	0.18
training (2)‡	-1.87	0.86	-2.18	**0.03**
training (3)‡	-2.35	0.82	-2.87	**0.004**
training (4)‡	-3.90	0.98	-4.00	**< 0.001**
training (5)‡	-3.07	0.87	-3.51	**< 0.001**
sex (male)§	1.46	0.80	1.83	0.068

†,‡,§ In comparison with reference level: †naïve horses, ‡training session 1, §females

Table 4d
Model estimates of predictors influencing affiliative contact with humans during training. Significant main effects are indicated in bold.

Predictor	Estimate	Std. Error	*t* value	*p* value
(Intercept)	2.15	0.29		
experienced†	-0.43	0.30	-1.41	0.17
training (2)‡	-0.25	0.21	-1.18	**0.24**
training (3)‡	-0.72	0.22	-3.22	**< 0.001**
training (4)‡	-0.96	0.23	-4.22	**< 0.001**
training (5)‡	-1.11	0.23	-4.89	**< 0.001**
sex (male)§	0.23	0.30	0.76	0.46

†,‡,§ In comparison with reference level: †naïve horses, ‡training session 1, §females

3.3. *Influence of behavioral responses to training on changes in hormones*

The full model relating changes in sOXT concentrations during training to behavioral measures differed from the reduced model with the behaviors excluded (likelihood ratio test, chi-square = 28.02, df = 9, $p < 0.001$). Several behavioral measures contributed significantly to explaining acute changes in sOXT during training. As predicted, horses who had more affiliative contact with trainers during a training session had increases in sOXT concentrations during training (est ± SE = 0.10 ± 0.05, p = 0.039, see Table 5). Interestingly, acute changes in sOXT were also strongly inversely related to behaviors associated with negative affect. Specifically, horses who showed explosive behavior during training had decreases in sOXT from pre- to post-training samples (est ± SE = −0.30 ± 0.10, p = 0.003, see Table 5 and Fig. 6). In addition, horses who showed fear behaviors during training tended to have decreases in sOXT concentrations after training (est ± SE = −0.19 ± 0.10, p = 0.06, see Table 5).

The full model relating changes in sCORT concentrations during training sessions to behavioral measures differed from a reduced model with the behaviors excluded (full-null model comparison, likelihood ratio test, chi-square = 17.39, df = 9, p = 0.043). Unlike the sOXT results, there were no significant effects of any behavioral measures on changes in sCORT during training, although there was a tendency for horses who exhibited more head tossing during a training session to have increased post-training sCORT (est ± SE = 0.18 ± 0.09, p = 0.057, see Supplementary Table S3).

Table 5
Model estimates of predictors influencing changes in salivary oxytocin concentrations (log transformed) from pre- to post-training samples. Significant main effects are indicated in bold.

Predictor	Estimate	Std. Error	*t* value	*p* value
(Intercept)	4.67	0.14		
sample context (post-training)†	0.11	0.09		
positive contact†	0.12	0.08		
explode behavior (yes)†	0.50	0.17		
fear behavior (yes)†	-0.11	0.17		
head toss†	-0.35	0.09		
time of sample collection	0.18	0.09	1.95	0.066
context (post-training):positive contact	0.10	0.05	2.12	**0.039**
context (post-training):explosive behavior (yes)	-0.30	0.10	-3.09	**0.003**
context (post-training):fear behavior (yes)	-0.19	0.10	-1.94	0.058
context (post-training):head toss	0.02	0.05	0.38	0.704

†Significance tests of main effects not indicated due to limited interpretability.

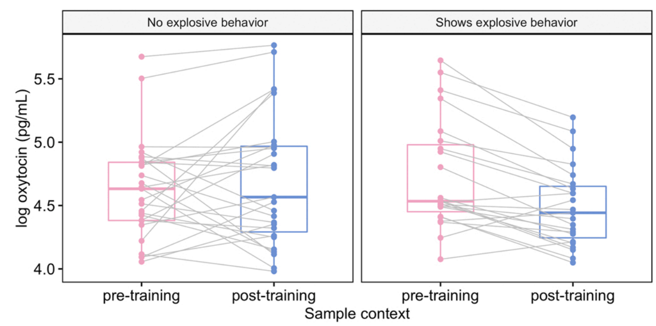

Fig. 6. Changes in sOXT concentrations in horses who exhibited explosive behavior during training, and those who did not. For full model results see Table 5.

4. Discussion

We assessed behavioral and non-invasive physiological indicators of emotional responses in young horses during opportunistically recorded foundation training. We specifically focused on sOXT, which to our knowledge has not been measured previously in horses, as a potential indicator of positive emotions. In addition, we measured sCORT and assessed the relationships between these hormones, horse experience and training progression, and behavioral expression during training.

We found that acute changes in sOXT during training were predicted by individual behavioral responses, although effects were strongest for behavioral indicators of negative emotions. Specifically, horses that showed explosive behavior during training had decreases in sOXT. Explosive behaviors indicate horse's discomfort and attempts to avoid a situation (Rietmann et al., 2004; Quick and Warren-Smith, 2009). Fear behaviors tended to show the same pattern. These results suggest that sOXT decreases when horses experience mental or physical discomfort. In addition, horses who had more positive contact with the trainer exhibited increases in sOXT during the training. These results are consistent with evidence that horse friendliness and trainability positively predict plasma oxytocin concentrations (Lee and Yoon, 2021; Kim et al., 2021). In addition, results support evidence for increases in sOXT associated with affiliative human interactions in domesticated animals more generally (pigs: Lürzel et al., 2020; dogs: MacLean et al., 2017). The finding that positive contact behaviors did not increase over the course of training is not necessarily inconsistent with this, given that the training became progressively more demanding over the course of training, which may have reduced possibilities for affiliative behaviors. Although this is the first study to assess sOXT in horses, results are supportive of further research into the use of sOXT as a non-invasive indicator of short-term changes in emotions in horses.

Both pre- and post-training levels of sOXT were higher on the third training session in comparison with the other sessions. However, we have no obvious, biologically meaningful explanation for this statistically significant finding. The third recorded training session took place within days of bringing the horses back to the open-shed stable after the long summer pasture season. It is possible that some aspect of returning to the stable and being housed in different social groups after the summer season may have influenced sOXT concentrations. It would be interesting to collect a larger number of sOXT samples from these horses under varied housing conditions and social contexts, to examine whether specific differences in the husbandry conditions between seasons may be reflected in sOXT concentrations.

In line with our general predictions that young horses would adjust to training, all horses showed decreases in behavioral indicators of fear, explosive behaviors and head-tossing from their first to last training sessions during the study period. Our interpretation is supported by the decrease in overall sCORT across training sessions in both naïve and experienced horses. Further, in support of our predictions, sCORT was higher in samples taken both before and after training in naïve yearlings compared with the more experienced two- and three-year-olds. This suggests that yearlings may have had increased arousal already when being brought for tacking to the box-stable, which was an unfamiliar place to them, and that their sCORT may have remained high during training, in comparison with more experienced horses, due to greater psychological stress. We also found that sCORT increased, as predicted, during longer training sessions, which usually included more strenuous exercise. This suggests that short-term changes in peripheral cortisol during training may have been triggered primarily by physical arousal and exercise (Ferlazzo et al., 2020), while heightened sCORT both before and after training may reflect greater psychological stress. Indeed, the behavioral indicators of negative emotions within each training session did not directly predict changes in sCORT during the session, which lends further support for short-term increases in cortisol reflecting energetic, rather than psychological, stress. Our results emphasize the importance of controlling for differences in energetic activity when attempting to investigate the link between peripheral cortisol and psychological stress.

An important caveat of the study is that the training regime was not standardized to follow a predetermined pace and order, and that multiple trainers were often involved in a training session. A more strictly standardized system would have allowed us to specify the exact effects of each training task as well as trainer identity on hormones and behavior. However, as the training was paced individually according to each horses' level of development, it closely resembles the 'sympathetic training', which is shown to be supportive of positive emotional experiences in young horses (Visser et al., 2009; Kędzierski et al., 2012; Fureix et al., 2009). The training method may have contributed to the horses' positive emotional responses and, conversely, minimal stress during the training sessions.

Another caveat is that we were not able to include measures of facial expressions as indicators of emotions due to the face being out of sight during much of the video recordings. Horses have highly expressive faces (Wathan et al., 2015), and many studies on horse emotions assess facial expressions (Lansade et al., 2018; Trösch et al., 2020). Integrating measures of facial expression with other behavioral and physiological responses may provide further insights into emotional responses during training. In addition, personality is likely to have influenced the horses' responses. Adult and young horses have stable inter-individual differences in responses to novelty and threat, isolation, and humans (Perez-Manrique et al., 2021; Rankins and Wickens, 2020). Personality is known to influences stress responses (Hausberger et al., 2019) and learning (LeScolan et al., 1997; Lansade et al., 2017; but see Christensen et al., 2012) in horses, so individual differences probably also contributed to the variability observed in our study. Future research should incorporate personality trait scores in targeted test paradigms with emotional responses to foundation training to gain further insights into the factors underlying stable inter-individual variation in responses (Duberstein and Gilkeson, 2010).

5. Conclusions

Our results indicate that salivary oxytocin can be measured non-invasively in young horses and may be useful as an indicator of young horses' acute emotional responses during foundation training. Salivary oxytocin did not reflect horses' previous training experience, nor did it show clear patterns of changes across the training period. Rather, short-term decreases in oxytocin during training corresponded with behavioral indicators of negative emotional responses, while short-term increases in oxytocin corresponded with behavioral indicators of positive emotional responses. We also found that although early foundation training was somewhat stressful for young horses, the applied training methods facilitated positive experiences. During this study, all horses exhibited reductions in cortisol and in negative behaviors during later training sessions at the end of the nine-month training period, compared to earlier training sessions. Interestingly, acute cortisol reactivity during training was not related to horse experience level or to any of the behavioral indicators, but rather reflected differences in training duration and likely energetic expenditure. The combined results suggest that sOXT and sCORT, together with appropriate behavioral measures, may be useful as non-invasive indicators of more immediate and long-term emotional responses to training in young horses.

Author contributions

SEK and LM conceived and funded the study, TN and VR collected the data, LM conducted laboratory analyses, LM and TN conducted statistical analyses, SEK and LM wrote the manuscript and the revision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We are grateful to the Kylämäki Farm and the horse owners for permission to conduct the study and the farm personnel for assisting at every stage, and to Tero Pohjolainen and Anders Ingman Stable and Irina Kokko at Kulta-ahon Ratsutila. We thank Océane Liehrmann for assistance in data collection. We are grateful to Birgit Sobczak and Petra Müntzel of the FBN Institute for Behavioral Physiology, for their assistance with hormone analyses. Funding: This work was supported by Kone Foundation [to SEK, grant number 201806482] and by the FBN.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.applanim.2022.105707.

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