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Behavioral and hormonal effects of two weaning situations in trotter foals

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Highlights

- Foals in both studs ate less and vocalized more on the day they were weaned.
- In Stud 1 the decrease in eating lasted up to 7 days after weaning.
- Foals weaned younger had higher hair cortisol concentrations post weaning.
- Abrupt weaning appeared to be stressful for the foals in both situations.

Abstract

Weaning is recognized as being a stressful event for foals. The outcome of this weaning stress can depend upon the procedure that was used. The aim of the present study was to investigate the behavioral and hormonal effects of two different weaning situations in Standardbred trotter foals born in Italy. In situation 1 (10 foals), weaning was performed at around 5 months of age, two foals/week, and foals were handled regularly after weaning in stud 1 (S1). In situation 2 (12 foals) weaning was performed at around 7 months of age, all foals on the same day, and there was little handling after weaning in Stud 2 (S2). Behavioral observations and saliva and hair sampling for RIA cortisol levels assessment were done on day -7, day 0 (weaning day), day 7 and day 30.

Behavioral data were analyzed using Generalized Linear Mixed Models, Friedman ANOVAs, U-Mann Whitney tests. Cortisol data were analyzed using repeated measure ANOVAs. Spearman correlations were used to assess correlations. Foals in both studs vocalized (all Bonferroni adjusted $p<0.05$) significantly more on the day they had been weaned than on any other day. They also showed a decrease in time spent eating on D0 supporting that weaning was a stressful event for them. The decrease in eating behavior was present also on D7 for S1, but not for S2 foals (S1: Bonferroni adjusted $p<0.05$ for contrasts between D0 and D-7/D30; S2: Bonferroni adjusted $p<0.05$ for all contrasts between days). Saliva cortisol concentrations differed significantly only depending on the covariate "age at the beginning of the experiment" (AB) and sex of the foal, whereas hair cortisol varied depending the day and the interaction day*AB and day*stud (all $p<0.01$), with S1 foals having higher hair concentrations than S2 foals on the seventh day after weaning. Results suggest that the weaning situation in stud 1 (which included being weaned two at a time at 5 months of age) could be more stressful for foals than that experienced in stud 2 (which included being weaned all together at 7 months of age). As the weaning situations evaluated in the present experiment were compound stimuli, it would be interesting to confirm them in an experiment in which all features of the compound situation are teased apart (including possible confounding variables, such as stud) and foals are randomly allocated into experimental groups.

Keywords

Horse, weaning, foal behavior, foal age, cortisol

Introduction

Weaning in foals has been the focus of much scientific interest (e.g., McCall et al., 1985; Malinowski et al., 1990; Hoffman et al., 1995; Apter and Householder, 1996; Rogers et al., 2004; Moons et al., 2005; Erber et al., 2011; Henry et al., 2012; Berger et al., 2013; Qureshi et al., 2013;

Dubcova et al., 2015; Gorecka-Bruzda et al, 2015; Xiao et al., 2015; Merkies et al., 2016; Bruschetta et al., 2017; Wulf et al., 2018; Henry et al., 2020). In horses, artificial weaning takes place earlier and more abruptly than natural weaning (Waran et al., 2008) and it is largely recognized as being a stressful event for foals (e.g., Apter and Householder, 1996; Heleski et al., 2002), which can have long term effects (Heleski et al., 2002; Nicol et al., 2005; Parker et al., 2008).

Artificial weaning procedures can vary in many respects, such as: age of the foal, diet, individual vs pair/group weaning, abrupt vs different forms of gradual weaning, relocation schedule, environment of the foal (stall vs pasture), presence of unrelated adult horses with the weaned foals, etc (e.g., Apter and Householder, 1996; Waran et al., 2008; Erber et al., 2011). Weaning procedures are termed abrupt when there is a complete and long-lasting termination of the mother-young relationship (Waran et al., 2008). Gradual weaning is a less defined entity, including procedures in which foals are allowed to retain olfactory, visual and auditory access to the mare, but are prevented from sucking, procedures in which foals are subjected to increasing periods of separation over several days, and procedures in which the mares are removed one at a time from the group (Apter and Householder, 1996; Waran et al., 2008). There is a consensus in the scientific literature that artificial weaning is a very stressful event for foals, however it happens (Waran et al., 2008). However, the results of several studies have shown that the specific characteristics of each weaning procedure can contribute to increase or decrease the stressful effect of weaning *per se* (Waran et al., 2008). For example, individual weaning (i.e., the foal is housed alone after weaning) has been found to be usually more stressful than when the weaned foal remains in the company of at least another foal (Waring et al., 2008), although Apter and Householder (1996) report stall weaning of foals in pairs possibly being more stressful than stall weaning of individual foals. Abrupt weaning has been found to be more stressful than the form of gradual weaning in which foals are allowed to retain olfactory, visual and auditory access to the mare (e.g., Merkies et al., 2016; Bruschetta et al., 2017; Lansade et al., 2018). Abrupt weaning has been found to be more stressful also than the form of gradual weaning in which mares are removed one at a time (Waran et al., 2008). On the contrary, studies on effects of repeated separations, in which the mother-foal dyad is separated and then reunited a number of times before long-lasting termination of the relationship, have given controversial results. For example, Moons et al. (2005) found higher morning salivary cortisol concentrations, but lower locomotion, after weaning in previously separated foals as compared to the control group. This notwithstanding, most authors deem them to have a sensitizing effect (Waran et al., 2008; Eber et al., 2012).

The presence of unrelated adult horses post weaning has been found to decrease the stress associated with abrupt weaning (Eber et al., 2012), which is likely to be the case also when a related horse is involved, but the latter situation, to our knowledge, has not been investigated yet.

Therefore, the aim of the present study was to investigate the behavioral and hormonal effects on trotter foals of two different weaning situations, in which two studs used two weaning procedures/methods, one for each stud. The two weaning procedures/methods differed in foal age at weaning, in number of foals weaned per day, in number of stabling relocations and in handling regimen of the foals. It is important to note that, as it was not possible to allocate foals of both studs randomly to the two weaning procedures/methods, the present study investigates the effects not of the two weaning procedures/methods, but of the two compound weaning situations, each resulting by the interaction of the weaning procedure with the stud in which such procedure was applied.

The tested hypothesis was that there would be differences between the foals in the two studs in behavioral and/or endocrine parameters suggestive of stress. In particular, we predicted that stress-related changes would be shown more intensely by the foals in the situation in which they were weaned at an earlier age, two at a time, handled and relocated more often, and housed in stud 1.

Animals, Materials and Methods

Animals, management and study design

The study took place in Northern Italy, between September and December 2016, approximately in a contemporary way in the two studs, which had the same climate conditions, and were only 10 Km apart, and at the same altitude compared to sea level. The weather was quite similar on all the days of observation (i.e., no rain, strong winds, extremes of temperature, et cetera). Twenty-two Standardbred trotter foals born in Italy (termed simply "trotter foals" from now on), housed in two different studs (i.e., S1 and S2), participated in the present study. In both studs, on the day of weaning, mares with their foals were led to a stable (two mother-foal dyads per stall) and then the mothers were led away from the stall one at a time, leaving the foals inside. Relevant characteristics of the foals and the weaning procedures used by the studs are detailed in table 1. The main differences in weaning procedure were:

1. the age at which foals are weaned, with S1 foals being weaned earlier than S2 ones (S1: median 5.39 months old, range 4.14-6.14; S2: median 7.14 months old, range 6.64-7.64; U-Mann Whitney test, $Z=-3.92$, $p<0.001$).
2. the fact that foals are weaned two at a time in S1 and all at the same time in S2,
3. the absence, apart from basic medical procedures and trimming of hooves, of regular post-weaning handling in S2 foals, who had been regularly handled before weaning,
4. the fact that foals in S1 were relocated to a different, bigger, paddock two weeks after weaning whereas S2 foals stayed in the same paddock (for details on the size of the paddocks, please see Table 1).

It is important to keep in mind, as already mentioned, that we could not allocate foals of both studs randomly to the two weaning procedures, therefore the present study investigates the effects of two compound weaning situations, each resulting by the interaction of the weaning procedure*the stud in which such procedure was applied. As the stud and the weaning situation cannot be teased apart, for ease of reading, we will refer only to “stud” (S) in the following sections, because the “stud” encompasses all the variables included in the compounded “weaning situation”.

Behavioral observations and saliva and hair (mane) sampling were performed on day -7 (seven days before weaning, D-7), day 0 (the day in which weaning took place; D0), day 7 (seven days after weaning, D7) and on day 30 (30 days after weaning, D30). Saliva sampling was performed approximately at the same hour on all the days and in both studs in order to take in account its daily fluctuations (Bohák et al., 2013). In general, salivary cortisol represents the free (active) fraction of circulating cortisol, even though the free cortisol increase in the saliva is lower than in blood (Schwinn et al., 2018). Free cortisol crosses the salivary gland epithelium by passive diffusion until the equilibrium between blood and saliva is established. In the horse, salivary cortisol usually peaks approximately 10 minutes later than blood cortisol after ACTH stimulation or exposure to an acute stress and a high correlation is found between the hormone concentrations in the two matrices (Peeters et al., 2011). As weaning has been reported to be a significant stressor for foals (Waran et al., 2008), saliva cortisol was used in the present study, instead of serum, in order to minimize invasive sampling procedures.

Hair cortisol was assessed in order to assess long term stress response (Comin et al., 2012; Russell et al., 2012; Duran et al., 2017). Blood cortisol can be incorporated in growing hair, and cortisol concentrations in hair are representative of cumulative cortisol exposure over a period of several weeks or months. In foals between birth and 60 days of age, hair cortisol can give a retrospective picture of the HPA axis activation, as hair cortisol is detectable from birth until 2 months of age, decreases with age, but displays a great variability between individuals (Comin et al., 2012). However, several factors can affect hair cortisol concentrations and bias the results: hair growth rate, hair color, dirtiness, hair location, sampling method (clipping vs. plucking) and the possible local cortisol production in hair follicles can affect cortisol concentrations.

Behavioral data

Foals were video-recorded for two 10 minutes' periods each day of observation using six video cameras (Panasonic Lumix DMC-TZ18; GoPro Hero 4 Silver video cameras). Videos recorded on D-7, D7 and D30 were obtained when foals were in paddocks. It is worth noting that foals had been released into their post-weaning paddock on D6. On D0, video-recordings were performed when foals were inside the stalls, just after separation from the mother. In S1, the first period of video-recording for each pair of foals on day 0 began when the second mare was led away from the weaning stall. In S1, where foals were weaned two every week, foals on their weaning day (D0) were always video-recorded before foals who had already been weaned (and were therefore on their D7 or on their D30) or were still with their mother (i.e., on their D-7). In S2, where all foals were weaned on the same day (D0 for everyone) all foals were video-recorded simultaneously when the last mother was led away. The timeline of the experiment is shown in Figure 1.

The same observer watched all the video and recorded the behavioral data using continuous focal animal rules (Martin and Bateson, 2007, p. 51). The working ethogram and the analyzed variables are detailed in table 2. Some short behavioral patterns were treated as events so that only their frequency (but not their duration) was calculated and analyzed. It is important to note that differentiating fighting (included in agonistic behavior) from play fighting (included in affiliative behavior) is not always easy in foals. In the present study, we classified the behavior as play when there was less tension of the muscles of the head, the ears were not tightly pinned back, the behavior was less intense and caused no damage, and when there was a measure of reciprocity (e.g., one foal was the actor for a few acts, then was the receiver of the initiative of the other foal for some other acts and then was again the actor). However, due to aforementioned difficulty in classification, results concerning these two behaviors should be interpreted with some caution.

Saliva and hair cortisol concentrations

Cortisol concentrations were measured in saliva and hair samples collected in the morning. On D0, sampling took place after the behavioral observations, about 60 minutes after actual mother-foal separation. On D-7, D7, and D30 samples were collected before the behavioral observations. People performing the sampling wore latex gloves in order to avoid sample contamination. The mothers of the foals were also sampled when they were together with the foals on D-7, for a parallel study (Falomo et al., 2020).

Saliva was collected using a cotton swab collection device (Salivette®, Sarstedt, UK) fixed on a thin plastic band. The swab was inserted in the buccal vestibule or under the tongue and kept there for approximately 30 seconds until it was soaked with saliva. The swabs were stored at 4°C until centrifuged (5000g for 10 minutes). The recovered saliva was then stored at -20°C until analysis.

The hair was cut from the mane, near the withers, as near the skin as possible, for a length of 3 cm along the neck, always in the same place. Samples were immediately stored at 4°C, transported to the lab within 2 hours and stored at -20°C until analysis.

Cortisol extraction was performed from minced hair (50 mg) with 5 ml methanol (Carlo Erba, Rodano, MI, Italy). Extraction vials were incubated at 50°C with gentle shaking for 18 h. The vial content was filtered to separate the liquid phase containing the hormone, which was evaporated to dryness under nitrogen at 37°C. Dry extracts were dissolved in 0.5 ml of assay buffer (phosphate buffered saline, 0.1 % BSA, pH 7.4) (Accorsi et al., 2008).

Cortisol concentrations were measured in saliva samples (0.1 ml), and hair extracts (0.2 ml) by a microtiter solid-phase radioimmunoassay (Posic et al., 2017). The sensitivity of the assay, defined as the dose of hormone at 90% binding (B/B0), was 3 pg/well for saliva samples, and 0.21 ng/g for hair extracts. Intra-assay and inter-assay coefficients of variation were 5.0% and 7.1%, respectively. Samples were assayed in duplicate.

Statistical analyses

As one major problem in analyzing behavioral data is to account for the time during which animals are out of sight (OOS), a preliminary analysis was performed to assess if OOS recordings were homogeneous across studs and days of observations. The OOS percentage was calculated as the ratio between the total duration of the OOS episodes and total recording time. Friedman ANOVA was used to investigate differences across days within each stud and U-Mann Whitney to compare the two studs on each day. Bonferroni correction for multiple comparisons was applied: as there were four days, implying six possible pair-wise comparisons between days, results with $p < 0.05/6$ (i.e., $p < 0.0083$) were considered an indication of a significantly difference. The time in which the foals were recorded as OOS differed across days in S1 (Chi square (N = 10, df = 3) = 19.19 $p < 0.001$), being higher on D0 than on any other day (all $p<0.0083$). No such difference was seen in S2. OOS differed between studs on day 0 (Z=3.39; $p < 0.001$), with S1 foals being more out of sight than S2 ones. As the places in which the two groups of foals were video-recorded differed, and out of sight periods were likely to be mainly influenced by logistic constraints, subsequent analyses were performed removing periods when animals were OOS from the sample and reducing the total sampling period accordingly (Lehner, 1996, p. 193). Therefore, the relative duration of each behavior was calculated as the ratio between the duration of all episodes in a given day and the duration of the time the foal was visible (i.e., total observation time minus out of sight time) in the same day. Frequencies were calculated as number of recorded episodes of a given behavior in a given day divided by the time during which the foal was visible on the same day.

The obtained data were analyzed using different statistical methods depending on their distribution. Durations of eating, locomotion, inactive standing/passive exploration, and frequencies of affiliative and agonistic behavior among foals, defecation, eating, locomotion, self-grooming, inactive standing/passive exploration, and urination were analyzed using a Generalized Linear Mixed Model. The abovementioned analysis investigated the effects of Age of the foal at the Beginning of the experiment (AB), Stud, Day, sex of the foal, the interactions day*AB and Day*Stud, day*sex of the foal, and included contrasts across days and between studs and sexes (all corrected for multiple comparisons). Foal within stud was random effect. It is important to note that AB of each foal was equal to his/her age at weaning minus seven days.

For the other behaviors, Friedman ANOVA was used to investigate whether the data differed for each stud across days and Wilcoxon rank tests, with Bonferroni correction (i.e., alpha set as =0.0083), used for pairwise contrasts. U-Mann Whitney was used to compare the two studs on each day to compare the behavior of colts to that of fillies, using Bonferroni correction if more than one day was concerned. For behaviors expressed only on one day (e.g., interactions with the dam, which was only possible on day -7), no correction was applied because only one comparison was made.

Cortisol concentration data were log transformed (Log_{10}+1) and then analyzed using Levene test and a repeated measure ANOVA. The latter evaluated the effects of Age of the foal at the Beginning of the experiment (AB), Stud, Day, sex of the foal, and the interactions day*AB and Day*Stud, day*sex of the foal. Corrections for multiple comparisons were applied in order to avoid large and preventable Type I errors.

Possible correlations among variables were investigated by the Spearman correlation test. For all analyses alpha was set as <0.05. Friedman ANOVAs, U-Mann Whitney tests, Wiloxon tests and Spearman correlations were done using the Statistica software (Statistica ver. 13, Statsoft, Hamburg, Germany), whereas the Generalized Linear Mixed Models were performed with SPSS software (SPSS ver. 24, IBM, Armonk, NY, USA).

Results

*Effects of the foal age at the beginning of the experiment, the day, the stud, sex of the foal, and day*stud interaction on eating, locomotion, inactive standing/passive exploration durations, and on affiliative and agonistic interactions between/among foals, defecating, eating, locomotion, self-grooming, inactive standing/passive exploration, and urinating frequencies*

The results of the Generalized Linear Mixed Models are detailed in Table 3. Eating was influenced by the interaction day*stud both in relative duration and in frequency: S1 foals spent significantly less time eating in both D0 and D7 than on D-7 and D30 (D0 vs D-7 $t=-4.9$; D0 vs D30 $t=5.1$; D7 vs D-7 $t=-4.3$; D7 vs D30 $t=-4.4$, all $p<0.001$), whereas S2 foals only on D0 (D0 vs D-7 $t=-6.8$; D0 vs D7 $t=-7.0$; D0 vs D30 $t=-8.7$, all $p<0.001$). In S1, frequency of eating episodes was significantly higher on D0 than on any other day (D0 vs D-7 $t=3.3$; D0 vs D7 $t=3.7$; D0 vs D30 $t=3.8$, all $p<0.01$), whereas no such difference was found in S2. Eating duration was higher in S2 than in S1 on D7 (S2 vs S1 $t=3.1$, $p<0.01$), whereas eating frequency was higher in S1 than in S2 in all the days except D30 (S2 vs S1 D-7 $t=-3.8$, $p<0.001$; D0 and D7 $t=-2.1$, $p<0.05$). The frequency of agonistic interactions between/among foals was higher in S2 on D0 than on any other day (D0 vs D-7 $t=9.1$; D0 vs D7 $t=8.6$; D0 vs D30 $t=8.9$, all $p<0.001$), whereas no such difference was found in S1. This notwithstanding in D0 the frequency of agonistic interactions between/among foals was higher in S1 than in S2 ($t=29.7$, $p<0.001$). Locomotion relative duration in S1 was higher on D7 than on any other day (D7 vs D-7 $t=6.3$, $p<0.001$; D7 vs D0 $t=3.2$, $p<0.01$; D7 vs D30 $t=6.2$, $p<0.001$), and it was significantly higher on D0 than on D-7 and on D30 (D0 vs D-7 $t=5.2$; D0 vs D30 $t=4.9$, both $p<0\text{-}001$). In S2, it was higher on D0 than on D-7 and D30 (D0 vs D-7 $t=3.0$; D0 vs D30 $t=2.8$, both $p<0.05$). Locomotion frequency was higher in D0 than in any other day in both studs (S1 D0 vs D-7 $t=5.8$; D0 vs D7 $t=5.1$; D0 vs D30 $t=5.7$, all $p=0.001$; S2 D0 vs D-7 $t=5.0$; D0 vs D7 $t=4.7$; D0 vs D30 $t=4.7$, all $p<0.001$), and was higher in D7 than D-7 and D30 only in S1 (D7 vs D-7 $t=3.5$, $t<0.01$; D7 vs D30 $t=2.9$, $p<0.05$). Locomotion duration was higher in S1 than in S2 on any of the days (S1 vs S2, D-7 $t=3.2$, $p<0.01$; D0 $t=3.6$, $p=0.001$; D7 $t=5.5$, $t<0.001$; D30 $t=3.0$, $t<0.01$), whereas its frequency on D-7, D7 and D30 (S1 vs S2, D-7 $t=2.8$, $p<0.01$; D7 $t=3.9$, $p<0.001$; D30 $t=2.1$, $p<0.05$). Frequency of affiliative interactions between/among between foals peaked at day 7 in S1 (D7 vs D-7 $t=3.3$, $p=0.007$; D7 vs D0 $t=4.3$, $p<0.001$; D7 vs D30 $t=4.0$, $p=0.001$), whereas no differences were found in S2. On D7 S1 foals showed more affiliative interactions between/among them than S2 ones (S1 vs S2 $t=3.2$, $p=0.002$). Colts showed more affiliative interactions than fillies on D0 (Colts vs Fillies $t=2.3$, $p=0.02$). Defecation, inactive standing/passive exploration's (both duration and frequency), self-grooming and trends were less clear.

Other differences in behavior due to day or stud or sex of the foal

All the other behaviors that significantly differed among days and/or between studs, are detailed in Table 4. Interactions between dam and foal were only present on D-7, as the two were then separated from D0 onward in both studs, so these behaviors are not included in Table 4. Affiliative interactions between mother and foal on D-7 were significantly higher for S1 (i.e., younger) foals than for S2 ones ($Z=2.34$, $p=0.019$, Bonferroni correction not applied because comparison made only for one day, D-7), and were never recorded in the latter.

In both studs there were differences across days in vocalizing (S1: chi-square=27.3, S2: chi-square=30.0, both $p<0.001$). Foals vocalized most often on D0 than on any other day (all $p<0.0083$, i.e., Bonferroni adjusted $p<0.05$), with no differences between studs. Other differences across days were found only in one of the studs. For example, pawing frequency was different across days in S1 (chi-square=24.0, $p<0.001$), whereas no such difference was found in S2. It is interesting to note that, although the pair-wise differences in pawing between D0 and any other day in S1 were not statistically significant (all $Z=2.52$, $p=0.012$) once the Bonferroni correction was applied, pawing was never expressed in any other day than D0. On D0, S1 foals pawed significantly more often than S2 ones ($Z=2.34$, $p=0.019$, Bonferroni correction not applied because comparison made only for one day, D0). Drinking (duration and frequency) significantly varied across days in S1 (both chi-square=18.0, $p<0.001$), where it occurred only in D7, but not in S2 (where it was expressed only by one foal in D0), whereas the opposite was true for resting (duration and frequency, both chi-square=12.0, $p<0.01$), which was expressed only by four S2 foals on D7. On D7 drinking (duration and frequency) was higher in S1 than in S2 ($Z=2.34$, $p=0.019$; as the behavior was expressed only in two days, Bonferroni correction was applied and the threshold for p was set at 0.05/2=0.025).

Also the duration of agonistic interactions between/among foals varied significantly across days only in S2 (chi-square=21.0, $p<0.001$). No differences were found between colts and fillies.

Cortisol concentrations

Saliva and hair cortisol concentrations' observed weighted means±SE are shown in Fig. 2. Saliva cortisol concentrations were significantly affected by the covariate AB ($F=16.05$, $p<0.001$; higher concentrations in younger foals), and by sex of the foal ($F=15.61$, $p<0.001$; fillies>colts). Hair cortisol concentrations were significantly affected by D ($F=4.94$, $p<0.01$), and the interactions D*AB ($F=4.88$, $p<0.01$) and D*S ($F=3.47$, $p<0.05$, with D-7 and D7 in S1 differing from D30 in S2).

Correlations

The behavioral variables correlating with saliva and/or hair cortisol concentrations or the behavioral and endocrine variables correlating with age of the foal (when observed/sampled) are shown in Table 5. No items reached a high degree of correlation (i.e., a $R\geq0.70$ or $R\leq-0.70$; Mukaka, 2012).

Discussion

The present study evaluated the effects on trotter foals' behavior and cortisol levels of two different weaning situations, in which two weaning methods/procedures (Table 1), typically used in the Italian trotters' industry and differing in age of the foal, number of foals weaned per day, extent of post-weaning handling and number of foals' relocations, were each performed in a different breeding facility. Both the investigated weaning situations could be defined as "abrupt" weaning because there was a complete and long-lasting termination of the mother-young relationship (Waran et al., 2008). In agreement with the literature (Waran et al., 2008), also in the present study, both weaning situations appeared to be stressful for foals, although some behavioral changes appeared to somewhat differ in their time pattern between the two studs. In detail, in both studs, foals spent less time eating just after being separated from their mothers than in the other days they were observed, but this decrease extended to seven days after weaning only in S1. A decrease in eating behavior was found in weaned foals also by Henry et al. (2012) and by Wulf et al. (2018) and was interpreted as a sign of stress. Other behaviors, which have been found to increase after weaning, and are therefore interpreted as signs of weaning stress in foals, are changes in activity and locomotion and vocalizations (Henry et al., 2012). In the present study, the frequency of vocalizations was also highest on the day of separation than in any other day in both studs. Vocalizations were interpreted as a sign of distress in foals separated from their mothers also in Budzynska and Krupa (2012). In the present study, locomotion frequency and relative duration were higher when foals had just been separated from their mothers (D0) than before weaning and at 30 days after weaning in both studs. In S1, however, the increase in locomotion lasted longer: locomotion relative duration peaked seven days after weaning, and locomotion frequency seven days after weaning was still higher than before weaning and at 30 days after weaning. The foals in S2, who were around 7 months old, showed a higher frequency in agonistic behavior on day 0 than in any other day. The increase in agonistic behavior between/among S2 foals agrees with the increase of social biting found in 7 months old just-weaned foals by Xiao and collaborators (2015), and, also in their study, younger (i.e., 6 months old) foals did not show such difference. The difference was not present in older (i.e., 8 months old) foals, either (Xiao et al., 2015). The other differences were less easy to interpret. For OOS and behaviors differing only in D0 in both studs, the fact that the foals were video-recorded in loose stalls on D0 and at paddock on all the other days could have contributed to the difference.

As found in the parallel study (Falomo et al., 2020), also the mothers of the foals involved in the present study showed stress-suggesting behavioral changes when separated from their foals. Said changes were more evident in the mares in S2 who had spent more time with their foal before separation, but who were stabled indoors for two days after weaning, whereas in the present study the foals in S1 appeared more affected by the situation. Although it could seem that the weaning situation in S1 favors mares and the one in S2 favors foals, the differences found in mares are likely to be the result of the mares' more restrictive post weaning housing: in the stud in which foals were weaned later, mares were kept indoors in individual box stalls for two days after separation from their foals (Falomo et al., 2020). It is important to note, that although it is tempting to attribute the differences in response between S1 and S2 foals to age at weaning, because that variable presents a great discrepancy form what happens in natural conditions (Waran et al., 2008), and the discrepancy is greater the younger the foal is weaned, the weaning situations compared in the present study are compounded variables, which include, but are not limited to, age at weaning.

In the present study there was no significant effect of day and/or stud and/or interaction day*stud on salivary cortisol. This disagrees with what found by Wulf and collaborators (2018), who found a significant increase in salivary cortisol concentrations after weaning, with no difference between colts and fillies. A possible explanation could be that weaning was not a significant stressor for the foals in the present study. Some concerns about the representativeness of salivary cortisol may arise in the non-stimulated state. Indeed, in species such as the bovine a low correlation between salivary and blood cortisol were observed during routinely management procedures, suggesting that only acute and intense stress or pain can affect salivary cortisol, while mild or chronic stress and circadian variation can only be observed in plasma (Schwinn et al., 2016). However, this explanation is unlikely to be true due to the behavioral findings in the present study and those in the scientific literature especially when abrupt weaning is concerned (e.g., Waran et al., 2008, Merkies et al., 2016; Bruschetta et al., 2017; Lansade et al., 2018). Moreover, in the present study, the significant effect of age at the beginning of the experiment (which corresponds to age at weaning minus 7 days for all foals) suggests that younger foals could have perceived the abrupt weaning situation as more stressful than older foals. This hypothesis could be supported by the increase of pawing found at weaning only in the foals in S1, who were younger than S2 ones. Also hair cortisol varied depending on the interaction between day and age of the foal (at the beginning of the experiment), although the trend was not easy to interpret. However, as specified in the section about

Animals, Materials and Methods, several factors can affect hair cortisol concentrations, so that the lack of standardization of the methodology is a critical issue that limits the exploitation in the field of this methodology for the assessment of stress and welfare (de Almeida et al., 2019; Lesimple, 2020). In our opinion, although worth measuring, one can confer biological significance to hair cortisol only if a great increase over the basal concentrations or large differences among experimental groups can be observed, which does not appear to be the case in the present study.

Although the possibility of weaning at different ages has already been described (Apter and Householder, 1996), to our knowledge, there is only one published study that focusses solely the effects of the age at which weaning takes place (minimizing possible confounding due to other aspects of the weaning method) on foal behavior at weaning and after weaning (Xiao et al. 2015). However, the abovementioned paper (Xiao et al. 2015), the independent variable (i.e., age at weaning) was investigated in the context of the traditional and most common weaning method used in China, therefore the ages included in the study were 6, 7 and 8 months, whereas in Italy it is customary to wean foals also younger than 6 months (from approximately 4.5 months of age), so there is no publish data (to our knowledge) about the effects of weaning foals at 5 months of age to compare the findings of the present study to. Apart from the paper of Xiao and collaborators (2015), only a field report, to our knowledge, describes a difference in behavior among foals weaned at different ages, finding that foals weaned at 2 months of age tended to rank lower than those weaned at 5 months of age when the two groups were mixed at one year of age (McCall, 1979). All the other investigated protocols differed instead (also) in variables such as post-weaning housing, individual vs pair/group weaning, gradual vs abrupt weaning, presence of adults, etc. (Waran et al., 2008; Erber et al., 2011).

Also in the present study, the investigated variable was a compounded weaning situation (i.e., a situation in which a certain, already compounded, weaning procedure was applied in one stud was compared to another situation in which another, equally compounded, weaning procedure was applied in another stud), so effect of age at weaning could not be studied *per se.* Although the chosen studs were as similar as possible for type of horses, location, and general management procedures, other eventual variables linked to the facilities themselves might have acted as confounding factors. Moreover, the evaluated weaning procedures were compounded stimuli in themselves differing not only for age of the foal, but also for number of foals weaned and quantity of post-weaning handling and relocations. All this notwithstanding, the results of the present study suggest an urgent need for further studies on the topic. A possible future step would be to run a larger, randomized controlled true experimental study in which with both weaning procedures are applied in one test location, or even a multifocal study in which both weaning procedures are applied in all the locations, increasing external validity. Another step would be to tease apart the various variables included in the weaning procedure, for example running a, possibly multifocal, experimental study in which age at weaning (including the 5 months of age category), is the only independent variable, whereas all the other variables (e.g., number of foals weaned at a time, handling, relocations, facility) are controlled for. In the meanwhile, it was still interesting to know that the compound variable had an effect, as it was a measure of the cumulative effect of the whole situation the foals were subjected to. In this respect, it has to be borne in mind that each of the two procedures is routinely applied as a whole in the trotter horse industry in Italy. Moreover, the present study could be used as a preliminary research which gives the idea of the variability of the behaviors in foals around weaning to be used as a basis for a power analysis to calculate the needed number of replicates in an eventual new randomized study.

Another aspect in which it is difficult to find literature to compare our findings with is the number of foals weaned. To our knowledge, there are no studies comparing foals weaned in pairs to those weaned in groups (i.e., more than two). The literature focused on comparing individual weaned foals to those weaned more than one at a time (i.e., either in pairs or in groups, the latter especially when the procedure of removing the mares from pasture one by one was applied; Waran et al., 2008). Similarly, studies on the effect on different amounts of post-weaning foal handling are also lacking, so it is impossible to interpret the findings of the present study trying to tease apart the different components of the compound stimuli.

The study of McGee and Smith (2004) found that human presence attenuated the signs of stress caused by separation from the dam in pre-weaned foals. As handling includes the presence of a human, the findings of the present study seem to disagree with what found by McGee and Smith (2004). However, their study focused on short term separations during which the foals were accompanied by a human, whereas in the present study the foals were always separated from their mothers, and humans were only present for a small proportion of time.

Another difference in the two investigated situations was that S1 foals were moved from one paddock to another on day 14 post weaning, however, the differences between studs were evident seven days after weaning and not later, so it was unlikely that the paddock change was the cause of such differences. All of the paddocks had grass in them, but the first one in which S1 foals were housed was smaller than the others (and housed less foals at a time).

In spite of certain study design limitations, our findings (both behavioral and physiological) indicated that there is a need to understand the relative effect of the many variables included in the different weaning methods as applied in different studs, especially for those, such as age at weaning, or stabling after weaning which are very different from what happens in more natural conditions.

Conclusions

In both studs, weaning appeared as a stressful event for foals, associated with decreased eating and increased locomotion and vocalizations. The post-weaning decrease in eating lasted longer in the stud in which foals were weaned younger, two at a time and with post weaning handling, suggesting that foals in that stud found the situation more distressing than the foals weaned as a group at 7 months of age with little routine post-weaning handling in the other stud. As Waran et al. (2008) advocated, there is a need for further experimental studies on the effects of weaning and weaning methods on foals, especially those concerning single facets of the procedure investigated as the only independent variable, in order to find what could constitute best practice with respect to foals' welfare in breeding practice.

Authorship declaration

SN, MEF, EG designed the study; MEF, IS performed the experiment; SN, PM analyzed data; SN prepared the manuscript. All authors read the final version of the manuscript and agreed on its submission

Ethical
The study was conducted in accordance with guidelines established by the Italian Animal Welfare Law (DL n. 26 - 4 March 2014, art. 2).

Conflict of Interest

The study was conducted in accordance with guidelines established by the Italian Animal Welfare Law (DL n. 26 - 4 March 2014, art. 2). None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Table 1: characteristics of the two studs

	Stud 1	**Stud 2**
N° of foals participating	10	12
N° colts/fillies	6 colts, 4 fillies	7 colts, 5 fillies
Age of foals at the beginning of the experiment - median (range)*, being significantly different (Z=-3.92, p<0.0001)	5.25 (4 to 6) months	7 (6.5 to 7.5) months
Housing of the dyads pre-weaning	Delivery happens in the stud. Night - from the first day post foal birth: indoor 4m x 4m stall for each dyad (not the same stall every night) Daytime – from the first day post birth: all foal/mares dyads together in a 100m x 100m field provided with hay.	Dyads arrive at the Stud 5-7 days post-delivery. After that Night - from the first day post arrival: indoor 4m x 4m stall for each dyad (the same stall every night). Dyads could be inside the stalls also in case of temperatures being too hot. Daytime – from the first day after arrival: all foal/mares dyads

		together in one of the 500 m x 200 m fields. Field rotation can happen 1 – 2 times a year.
Feeding regime (pre weaning)	Twice a day (hay and concentrates together) in the same trough for mare and foal	Twice a day (hay and concentrates together) in different troughs for the mare and for the foal
Handling (foals) pre-weaning	From the first day of age, fitted halter, lead alongside the mother. No other ad hoc training	From the first day at the stud: they are walked with a halter on alongside the mother who is led by halter and lead): Ad hoc training: to be touched over all parts of the body 2 times a day, to be led by halter and lead, to hooves trimming once a month.
Weaning age and schedule	Between 4 and 6 months, two foals (same age and possibly same sex) weaned each week. Procedure: The first mother/foal dyad is led to the indoor stall 4m x 6 square-meters in which foals were born, then the foal is left there while the mother is led to a paddock (500 m away). Then the same procedure is carried out with the other dyad.	At an age between 6 ½ and 7 ½ months. All foals weaned at the same moment, two per stall. Procedure as for S1
Housing of the foals post weaning	For the first 6 days post-weaning the two foals are in the indoor stall, then they are moved to a 10 m x 30 m paddock (with grass) for another week, then moved again to a 200 m x 200 paddock, with grass. Both paddocks are unknown to the foals. From the second week after weaning, foals are stabled outside both during the day and the night. At the end of the weaning season all the foals are in the same paddock.	For the first 6 days post-weaning: indoor weaning stalls, then they are moved to two adjacent 500 m x 200 m paddocks (with grass), divided for sex. The paddocks are at the opposite side of the facility from the place in which the mares are kept. They stay in the paddocks both day and night.
Feeding regime (post-weaning)	Hay and concentrates twice daily	Hay and cereals twice daily
Handling of the foals post-weaning	First week (foals in stall): handling increases. They are led with lead and halter 3 times a day, first inside the stall and then outside. After that (foals in paddock): handling decreases (foals lead every two weeks plus hoof trimming and vaccinations)	No more handling unless for foot trimming (and medical procedures if needed)

Water	An ad lib water source is always provided in all the housing conditions both pre- and post- weaning.	An ad lib water source is always provided in all the housing conditions both pre- and post- weaning.

Table 2: working ethogram and analyzed variables

Behavior	**Definition**	**Variable**
Affiliative interactions between/among foals	The foal sniffs (i.e., performs an exploratory behavior in which the animal positions his/her nose near an item and inhales and exhales air through the nose in short repetitive manner) or licks (i.e., the foal puts his/her tongue repetitively in contact with a part of the other animal's body) another foal, or stands near him/her. It includes allo-grooming (as defined below). It does not include social play.	Relative duration on visible time + frequency on visible time
Affiliative interactions between foal and mother	The foal sniffs (i.e., performs an exploratory behavior in which the animal positions his/her nose near an item and inhales and exhales air through the nose in short repetitive manner) or licks (i.e., the foal puts his/her tongue repetitively in contact with a part of the mother's body) the dam, or stands near his/her mother and ingests milk from her udder. Includes allo-grooming: the behavior of two horses standing, often parallel and facing opposite directions, and in unison scraping the hide of the other with their upper incisors	Duration on visible time + frequency on visible time
Agonistic interactions between/among foals	The foal walks away from an approaching other foal, or threatens (i.e., shows mimic and postural signs, which convey its readiness to engage in an agonistic confrontation, such as ears flattened) or bites (i.e., the act of "opening and rapid closing of the jaws with the teeth grasping the skin of another animal; lips are retracted and ears pinned" (McDonnel, 2003, p. 134) or kicks (i.e., action in which "one or both hind legs lift off the ground, and rapidly extend backward towards another animal with apparent intent of making contact", McDonnel, 2003, p. 138) the other foal.	Relative duration on visible time + frequency on visible time
Agonistic interactions between foal and mother	The foal walks away from the approaching mother, or threatens (i.e., shows mimic and postural signs, which convey its readiness to engage in an agonistic confrontation, such as ears flattened) or bites (i.e., the act of "opening and rapid closing of the jaws with the teeth grasping the skin of another animal; lips are retracted and ears pinned" (McDonnel, 2003,	Duration on visible time + frequency on visible time

	p. 134) or kicks (i.e., action in which "one or both hind legs lift off the ground, and rapidly extend backward towards another animal with apparent intent of making contact", McDonnel, 2003, p. 138) her.	
Defecating	The act of eliminating feces via the anus	Frequency on visible time
Drinking	The act of ingesting liquids, usually water	Relative duration on visible time + frequency on visible time
Eating	The act of ingesting solid edible material	Relative duration on visible time + frequency on visible time
Escape attempts	The foal walks into the walls of the stall or appears to try to exit the stall via the door or fence.	Frequency on visible time
Inactive standing/passive exploration	The situation in which an animal is standing with paws mainly extended and his/her weigh on at least 3 of his/her hooves. Head and neck are elevated, and gaze and/or ears appear to be directed to some environmental stimuli (McDonnel, 2003). The animal does not move and does not evidently sniff at something.	Relative duration on visible time + frequency on visible time
Locomotion	The foal moves through space by means of a rhythmic activity of his/her four legs, remaining on the same horizontal plane	Relative duration on visible time + frequency on visible time
Pawing	The foal lifts a foreleg off the ground slightly, extends it quickly in a forward direction and performs a backward toe-dragging movement as if digging (McDonnel, 2003)	Frequency on visible time
Rearing	Fore legs are raised and not in contact with the ground while the hooves of the hind legs remain in contact with the ground. The body is in an almost vertical position (McDonnel, 2003). The behavior appears to be part neither of a play sequence nor of a social exchange.	Frequency on visible time
Resting	The situation in which an animal is either standing with paws mainly extended and his/her weigh on 3 of his/her hooves (one of both ears are relaxed and backward, neck lowered, eyelids and lips are relaxed), or recumbent in a sterno-costal position with head not leaning on the ground	Relative duration on visible time + frequency on visible time
Self-grooming	The act of cleaning one's hide by means of licking, nibbling, biting, scratching, or rubbing a part of the body	Relative duration on

		visible time + frequency on visible time
Sleeping/dozing	The situation in which an animal is either standing as in resting but with closed eyes or recumbent in a lateral position or in a sterno-costal one with the head completely on the ground (McDonnel, 2003).	Relative duration on visible time + frequency on visible time
Social play	Activities appearing to have no immediate use or function, involving a sense of pleasure and elements of surprise. These activities are mostly modified versions of serious survival activities distinguished from the "serious" analogues by postures or expressions denoting less serious intent (McDonnell, 2003). The foal is playing with another animal	Relative duration on visible time + frequency on visible time
Solitary play	Activities appearing to have no immediate use or function, involving a sense of pleasure and elements of surprise. These activities are mostly modified versions of serious survival activities distinguished from the "serious" analogues by postures or expressions denoting less serious intent (McDonnell, 2003). The foal is playing alone or with objects	Relative duration on visible time + frequency on visible time
Stereotypies	Invariant repetitive activities with no apparent goal or function	Relative duration on visible time + frequency on visible time
Vocalizing	The foal emits a sound using his/her upper respiratory tract in what appears to be an intentional way	Frequency on visible time
Urinating	The act of voiding urinary bladder content to the exterior	Frequency on visible time
Other behavior	Any other behavioral pattern	Duration on visible time (not analyzed)
Out of sight	Any situation in which the observer cannot see the animal and assess what he/she is doing	Duration on total recording time

Table 3: Frequencies and relative duration of the behavioral variables (median; lower, upper quartile) recorded in the two studs during the experimental period. The fixed effects of foal age at the beginning of the experiment (AB; covariate), sex of the foal (FS), Stud (S), Day (D) and the interactions S*D, AB*D and FS*D are reported (Generalized Mixed Model). In the table, * stands

for p<0.05, ** for p<0.01, *** for p<0.001. Different lowercase superscript letters mean differences among days (the level of significance, adapted for sequential Bonferroni is p<0.05) within stud. Different capital superscript letters mean differences between the two studs (the level of significance, adapted for sequential Bonferroni is p<0.05) within days.

							Fixed Effects (F)					
Behavior	Stud	D-7	D0	D7	D30	AB	FS	S	D	S*D	AB*D	FS*D
Affiliative interactions between/among foals (frequency; n/min)	S1	0.00^{a} (0.00-0.05)	0.00^{a} (0.00-0.06)	$0.15^{b,A}$ (0.05-0.30)	0.00^{a} (0.00-0.05)	0.4	0.5	1.3	2.1	4.3**	2.2	3.2*
	S2	0.00 (0.00-0.00)	0.00 (0.00-0.05)	0.00^{B} (0.00-0.00)	0.00 (0.00-0.00)							
Agonistic interactions between/among foals (frequency; n/min)	S1	0.03 (0.00-0.05)	0.61^{A} (0.18-0.77)	0.00 (0.00-0.05)	0.05 (0.00-0.20)	11.6***	2.6	6.0*	15.2***	9.7***	10.7***	2.0
	S2	0.00^{a} (0.00-0.00)	$0.50^{b,B}$ (0.25-0.80)	0.00^{a} (0.00-0.03)	0.00^{a} (0.00-0.00)							
Defecating (frequency; n/min)	S1	0.0^{a} (0.00-0.00)	0.06^{b} (0.05-0.11)	0.00^{a} (0.00-0.05)	0.00^{a} (0.00-0.00)	1.5	0.8	1.1	4.5**	2.4	3.2*	2.8*
	S2	0.00^{ab} (0.00-0.00)	0.05^{a} (0.03-0.10)	0.00^{ab} (0.00-0.03)	0.00^{b} (0.00-0.00)							
Eating (duration; sec/sec)	S1	75.95^{a} (60.0-95.0)	16.70^{b} (5.45-28.00)	$20.53^{b,A}$ (5.00-30.25)	80.29^{a} (65.82-90.76)	1.7	0.6	0.6	44.2***	7.8***	0.07	0.7
	S2	100.0	13.33	70.96	88.9							

		0^{a} (100.00-100.00)	^{b} (1.50-21.37)	^{a,B} (50.64-98.75)	7^{a} (82.50-98.70)							
Eating (frequency; n/min)	S1	$0.18^{a,A}$ (0.10-0.30)	$0.45^{b,A}$ (0.29-0.65)	$0.15^{a,A}$ (0.05-0.25)	0.20^{a} (0.11-0.20)	0.9	0.7	9.1**	6.9***	5.3**	0.5	1.4
	S2	$0.05^{a,B}$ (0.05-0.05)	$0.23^{ab,B}$ (0.13-0.38)	$0.05^{a,B}$ (0.05-0.08)	0.20^{b} (0.15-0.21)							
Inactive standing/passive exploration (duration; sec/sec)	S1	1.26 (0.00-38.33)	0.72 (0.00-2.63)	20.26 (5.00-32.50)	10.06 (0.00-25.10)	1.1	0.2	0.0	1.8	1.6	0.3	1.7
	S2	0.00 (0.00-0.00)	3.77 (1.25-12.50)	1.72 (0.00-26.54)	10.00 (0.00-15.00)							
Inactive standing/passive exploration (frequency; n/min)	S1	0.03^{a} (0.00-0.10)	$0.05^{ab,A}$ (0.00-0.12)	$0.15^{b,A}$ (0.11-0.25)	0.05^{ab} (0.00-0.20)	5.9*	3.0	1.2	13.1***	4.3**	0.3	1.7
	S2	0.00^{a} (0.00-0.00)	$0.16^{b,B}$ (0.08-0.55)	$0.03^{a,B}$ (0.00-0.06)	0.05^{a} (0.00-0.05)							
Locomotion (duration; sec/sec)	S1	$4.17^{a,A}$ (0.00-15.06)	$25.00^{b,A}$ (13.16-38.60)	$61.45^{c,A}$ (35.00-75.63)	$5.05^{a,A}$ (5.00-11.40)	1.4	12.1***	40.1***	19.3***	8.1***	1.6	2.3
	S2	$0.00^{a,B}$ (0.00-0.00)	$6.04^{b,B}$ (2.50-19.17)	$0.00^{ab,B}$ (0.00-2.53)	$0.83^{a,B}$ (0.43-1.08)							

Locomotion (frequency; n/min)	S1	$0.15^{a,A}$ (0.00-0.25)	1.31^{b} (0.95-1.94)	$0.35^{c,A}$ (0.15-0.47)	$0.18^{a,A}$ (0.10-0.25)	0.7	10.6**	6.6*	22.3***	2.6	0.6	1.8
	S2	$0.00^{a,B}$ (0.00-0.00)	0.80^{b} (0.38-1.63)	$0.00^{a,B}$ (0.00-0.05)	$0.05^{a,B}$ (0.05-0.05)							
Self-grooming (frequency; n/min)	S1	$0.03^{a,A}$ (0.00-0.10)	0.00^{b} (0.00-0.00)	$0.10^{a,A}$ (0.00-0.20)	0.00^{b} (0.00-0.00)	0.7	0.6	5.0*	3.8*	6.0**	0.4	0.4
	S2	0.00^{B} (0.00-0.00)	0.00 (0.00-0.03)	0.00^{B} (0.00-0.00)	0.00 (0.00-0.00)							
Urinating (frequency; n/min)	S1	0.00 (0.00-0.00)	0.00 (0.00-0.06)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.0	0.0	3.3	4.1**	1.5	0.4	0.2
	S2	0.00 (0.00-0.00)	0.00 (0.00-0.05)	0.00 (0.00-0.00)	0.00 (0.00-0.00)							

Table 4. Medians, lower and higher interquartile of the other behavioral variables (frequencies and/or relative durations), which resulted significantly different among days (Friedman rank ANOVA) and/or between studs (U-Mann Whitney) and Friedman related statistics. D stands for Day, S1 for Stud 1; S2 for Stud 2. Different lowercase superscript letters mean differences among days within stud ($p<0.0083$). Different uppercase superscript letters mean differences between S1 and S2 within day ($p<0.05$).

						Friedman ANOVA	
Behavior	Stud	D-7	D0	D7	D30	Chi Square	P
Agonistic interactions between/among foals	S1	0.00 (0.00-0.00)	0.00 (0.0-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	2.0	NS
Duration (sec/sec)	S2	0.00 (0.00-0.00)	0.63 (0.00-2.50)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	21.0	<0.001
Drinking Duration (sec/sec)	S1	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.42[A] (0.00-4.20)	0.00 (0.00-0.00)	18.0	<0.001
	S2	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00[B] (0.00-0.00)	0.00 (0.00-0.00)	3.0	NS
Drinking Frequency (n/min)	S1	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.05[A] (0.00-0.05)	0.00 (0.00-0.00)	18.0	<0.001
	S2	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00[B] (0.00-0.00)	0.00 (0.00-0.00)	3.0	NS
Pawing Frequency (n/min)	S1	0.00 (0.00-0.00)	0.11[A] (0.06-0.17)	0.00 (0.00-0.00)	0.0 (0.00-0.00)	24.0	<0.001
	S2	0.00 (0.00-0.00)	0.00[B] (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	6.0	NS
Resting Duration (sec/sec)	S1	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	No variance	NS
	S2	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-42.50)	0.00 (0.00-0.00)	12.0	<0.01
Resting Frequency (n/min)	S1	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	No variance	NS
	S2	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.05)	0.00 (0.00-0.00)	12.0	<0.01
Vocalizations Frequency (n/min)	S1	0.00[a] (0.00-0.00)	0.90[b] (0.68-1.31)	0.00[a] (0.00-0.10)	0.00[a] (0.00-0.00)	27.3	<0.001
	S2	0.00[a] (0.00-0.00)	0.58[b] (0.30-1.13)	0.00[a] (0.00-0.00)	0.00[a] (0.00-0.00)	30.0	<0.001

Table 5: significant correlations between age of the foal when observed/sampled (months, AF) and behavioral data and saliva (pg/ml, SC) and hair (pg/ml, HC) cortisol concentrations, and behavior, SC and HC, and between SC and HC and behavior.

		valid N	Spearman R	t(N-2)	p value
AF	affiliative interactions between/among foals (frequency)	88	-0.34	-3.38	0.001
	affiliative interactions between mother and foal (frequency)	88	-0.32	-3.12	0.002
	agonistic interactions between/among foals (frequency)	88	-0.30	-2.91	0.005
	defecation (frequency)	88	-0.36	-3.60	0.001
	eating (duration)	88	0.26	2.53	0.013
	locomotion (duration)	88	-0.49	-5.23	0.000
	locomotion (frequency)	88	-0.42	-4.34	0.000
	pawing (frequency)	88	-0.28	-2.72	0.008
	resting (frequency)	88	0.22	2.07	0.042
	HC	88	-0.22	-2.12	0.037
	SC	88	-0.48	-5.07	0.000
HC	eating (frequency)	88	0.33	3.27	0.002
	locomotion (duration)	88	0.22	2.08	0.040
	locomotion (frequency)	88	0.25	2.36	0.020
	play (duration)	88	-0.23	-2.23	0.028
	resting (duration)	88	-0.25	-2.38	0.020
	standing (frequency)	88	-0.25	-2.36	0.020
SC	agonistic interactions between/among foals (frequency)	88	0.30	2.96	0.004
	eating (duration)	88	-0.25	-2.41	0.018
	pawing (frequency)	88	0.30	2.87	0.005
	vocalizations (frequency)	88	0.29	2.86	0.005

Figure 1: timeline - a) overall timeline of the study; b) within day timeline at S1; c) legend.

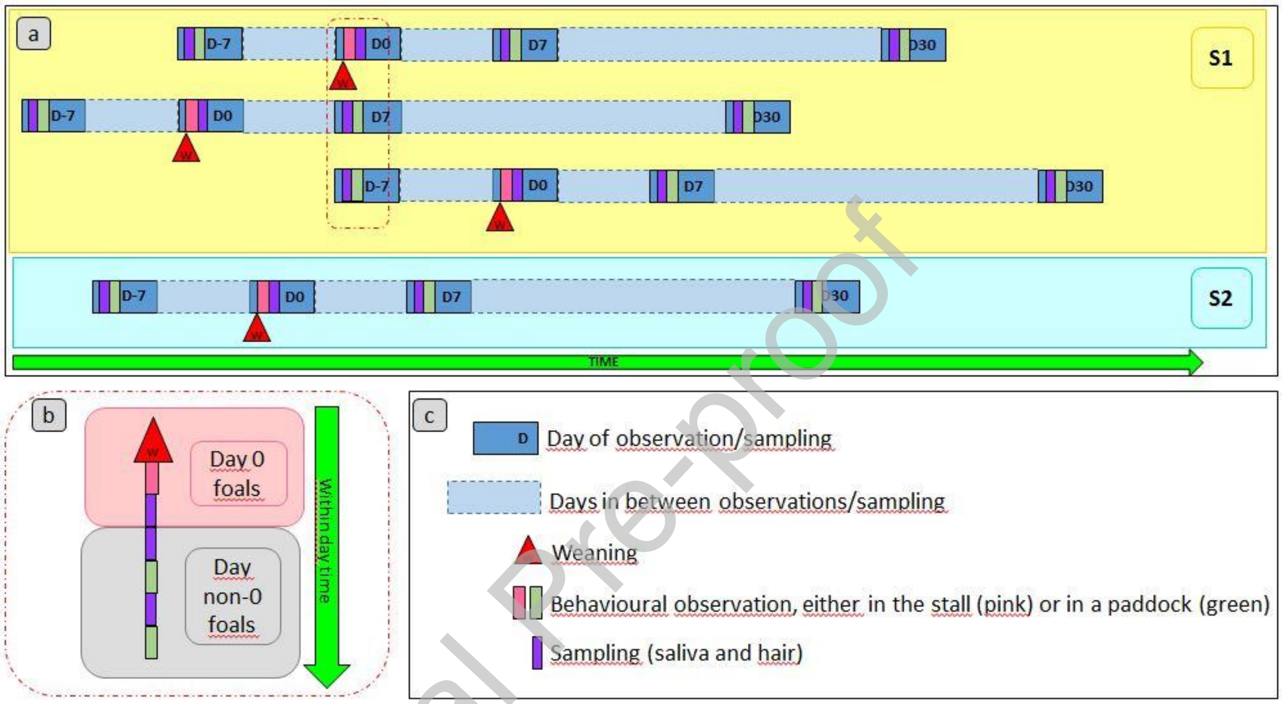
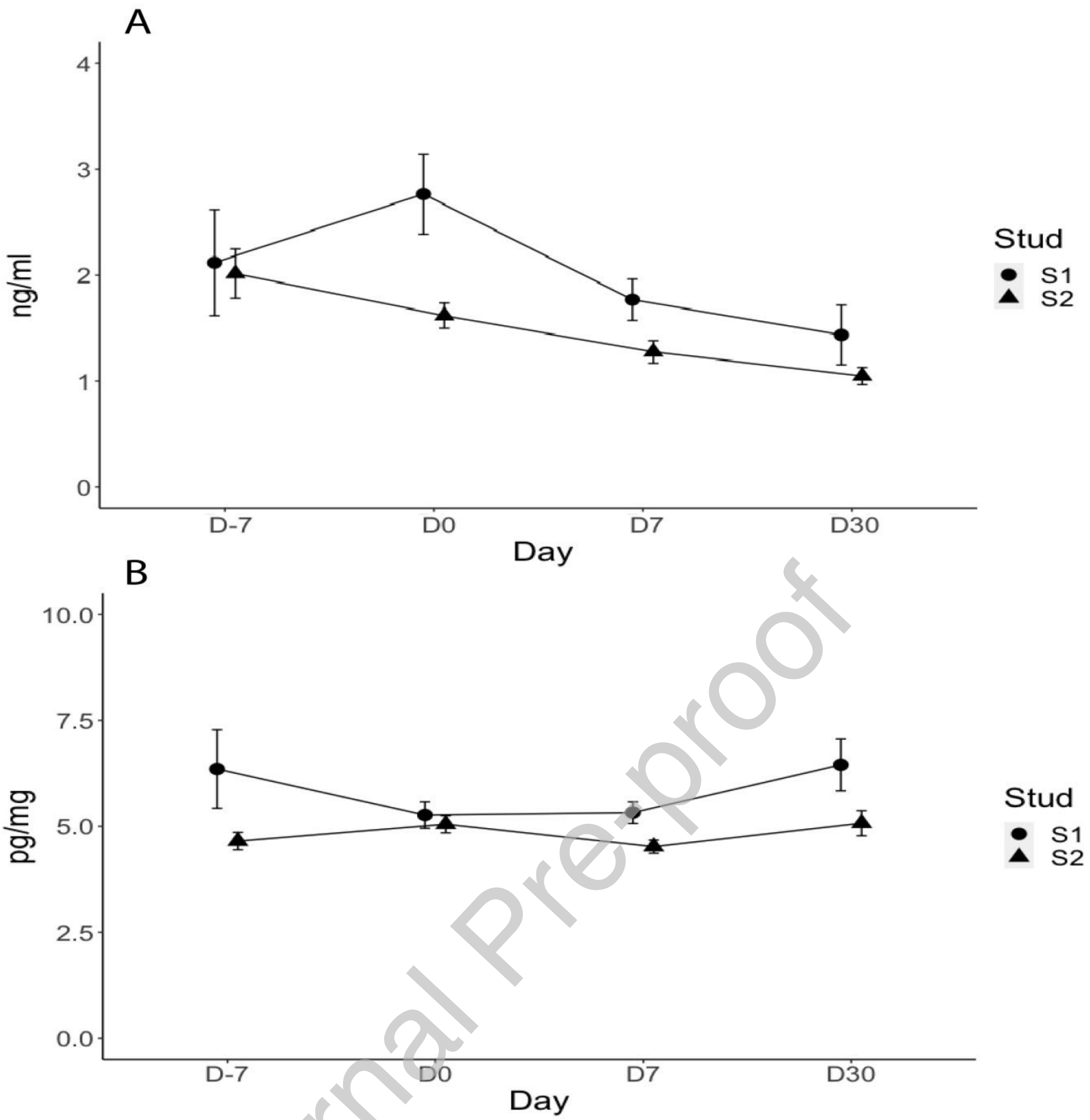


Figure 2: observed weighted means ± SE of cortisol concentrations in foals' saliva (A) and hair (B).


A
ng/ml
4
3
2
1
0
D-7
D0
D7
D30
Day
Stud
S1
S2
B
pg/mg
10.0
7.5
5.0
2.5
0.0
D-7
D0
D7
D30
Day
Stud
S1
S2