Social buffering in horses (Equus caballus): Influence of context, companion familiarity and companion habituation

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Abstract

Social buffering occurs when the presence of one animal attenuates another’s stress response during a stressful event and/or helps the subject to recover more quickly after a stressful event. Inconsistent previous results might reflect previously unrecognised contextual influences, such as the nature of the stimulus presented or social factors. We addressed these issues in a two-part study of horses paired with familiar (16 subjects) or unfamiliar (16 subjects) companions. Each subject performed 4 tests in a counterbalanced order: novel object test (static ball) – alone or with companion; and umbrella opening test – alone or with companion. Social buffering was significantly influenced by the nature of the stimulus presented, but not by companion’s habituation status or familiarity. Importantly, the stimulus used produced differential effects on behavioural and physiological measures of buffering. A companion significantly reduced behavioural response (reactivity) in the novel object test but not in the umbrella test. However, heart rate recovered more quickly for subjects with a companion in the umbrella test but not in the novel object test. We propose that circumstances which permit greater contextual processing may facilitate demonstration of behavioural effects of social buffering, whereas buffering in response to startling events may be manifest only during post-event physiological recovery.

1. Introduction

The concept of social support applies to a diversity of situations whereby an individual subject benefits, physically or emotionally, from the presence or behaviour of a companion. There are two predominant, but not mutually exclusive, models relating to social support, and these make slightly different predictions. The first “direct effects” model predicts that companions will provide beneficial effects to the subject, regardless of whether the subject is stressed or not [1]. For example, in wild chimpanzees, a close social companion, i.e. a companion with whom the subject exchanges high rates of affiliative and cooperative behaviours, can help to reduce the activation of a subject’s hypothalamic-pituitary-adrenocortical (HPA) axis within a stressful context (intergroup encounters) but also throughout daily life (everyday affiliation) [2]. The second “social buffering” model focusses on discrete contexts only where the subject experiences a negative or stressful life event, and the companion has the potential to attenuate the stress response in one or more ways in these circumstances. The latter is the focus of the current paper.

There are many examples of social buffering in the literature where conspecific animals have been shown to reduce the subject’s stress response during a stressful event (reviewed by [3, 4, 5]). For example, marmosets exposed to novelty show fewer behavioural signs of distress and agitation, and do not have significantly increased levels of urinary cortisol if accompanied by a companion [6]. The effects of social buffering can be further distinguished according to whether the companion is present during or after the stressful event experienced by the subject. In both cases, the presence of a companion seems to have a positive impact on the subject’s response. For example, the presence of a mother hen can reduce behavioural signs of stress when her chicks receive an air puff [7]. In a similar way, rats housed with a
littermate after experiencing social defeat show more exploratory behaviour in an elevated plus maze test than those housed alone [8].

The qualitative properties of stressful stimuli that have been used to examine social buffering vary from novel objects or environments to the appearance of a sudden stimulus, white noise, electric shocks, social defeat and social separation and/or isolation (reviewed by [5]). Similarly, measures of social buffering are very diverse, and include the return of corticosteroid levels to baseline, heart rate, body temperature and Fos gene expression and/or behavioural measures of vocalization, escape attempts, immobility, defecation rate and motivation to seek social contact depending on the study (reviewed by [3, 5]). This diversity in both treatment and measurement parameters has resulted in studies with very different aims and objectives (from assessing the social effects of anxiolytic drugs [9] to developing animal models of PTSD [10]) but such diversity also hinders the development of a coherent understanding of important sources of variability in the social buffering process.

This is especially apparent when trying to draw conclusions about the impact of familiarity and/or companion’s arousal state on social buffering. Indeed, contradictory results about the role of companion familiarity abound, with some studies showing familiar animals are better buffers (guinea pigs: [11, 12]; marmoset: [13]; rat: [14, 15]; Siberian dwarf hamster: [16, 17]), others that unfamiliar animals are better ([18, 19]) and others no effect (cattle: [20]; rat: [21]). Moreover, many researchers have demonstrated that the arousal status of the companion can affect its efficiency as a social buffer (cattle: [22]; chicken: [7]; rat: [23, 24]). Companions that have previously experienced or that anticipate the arrival of a stressor do not appear to be good social buffers in cattle [22] or rats [23, 24]. However, in horses [25] and mice [26], if companion arousal is reduced by prior habituation to a novel but harmless stimulus then this can increase the companion’s effectiveness as a social buffer. We consider that it is likely that there are some general principles governing the social buffering process but that these are currently masked by diversity (of species, stimuli and measures taken), confounding (e.g. no standard definitions or measures of subject-partner association level) and gaps in knowledge (e.g. effect of individuals’ personality on both companion’s effectiveness and subject’s responses). For example, individual studies of social buffering effects have all focussed on one stressor at a time, so it is not possible to known from any individual study whether the impact of the companion on the subject’s stress response is context-dependent. To our knowledge, only one study has compared the impact of different stressors [27]. In this rare example, Takeda et al. (2003) [27] examined the responses of groups of heifers to three different tests: a novel object test (a red paper doll as strange object), a surprise test (a tin bucket that contain weights was dropped to produce a loud sound) and a conflict test (feed bin containing food pellets covered with wire mesh). Conspecific familiarity affected the behavioural responses in the novelty test and surprise test, and the physiological responses in the surprise test and conflict test. Takeda et al. (2003) [27] also noted, in the surprise test and conflict test, an interaction between familiarity and group size with a greater effect on cardiac responses, when the subject was in a group of 5 familiar individuals. However, in this study the heifers were never alone, but in groups of either 2 or 5, and so this study examines group size effects rather than factors affecting the occurrence of social buffering.
Our aim is to contribute to the development of a general model of social buffering by investigating, for the first time, whether the effectiveness of factors such as companion arousal and familiarity are influenced by stimulus context. We used horses as a study species because of their complex social system [28, 29] and increasing evidence of the sophistication of their social cognition. Horses are, for example, able to exchange information through subtle communication ([30]; reviewed by [31]) and can perceive the emotional state of their conspecifics [32]. We also (and unusually within the field) used both behavioural and physiological measures to assess the extent of social buffering.

In a set of sequential studies involving two different groups of subjects, we tested if a companion can attenuate the subject's stress response. In the first study, we also tested whether the impact of companion arousal on the subject's stress responses was context-dependent by exposing subjects to two distinct stressful events while using pairs of subjects with a high level of consistent familiarity. The hypotheses were that the companion will help to reduce the behavioural and physiological responses across different stressful contexts, and that a habituated companion will be more efficient at buffering the subject's stress responses than a naïve one. We then evaluated in a second study whether similar effects of companion arousal and the nature of the stressful event were obtained when individuals were not familiar with their companions. The hypothesis was that a familiar companion would be more effective in reducing behavioural and physiological responses than an unfamiliar companion.

2. Results

2.1. Study 1: Impact of familiar companion

The median value of companion's reactivity score in the novel object test was 0.5 (Quartile 1 (Q1) = 0; Quartile 3 (Q3) = 1) for habituated companions versus 1 (Q1 = 0; Q3 = 2.5) for naïve companions. These values were not significantly different (Mann-Whitney test: \( W=23.5, n1=8, n2=8, P = 0.372 \)). In the umbrella test, the median value of companion's reactivity score was significantly lower at 1 (Q1 = 1; Q3 = 1.5) for habituated companions compared to 2 (Q1 = 2; Q3 = 3) for naïve companions (Mann-Whitney test: \( W=4, n1=8, n2=7, P = 0.004 \)).

Only during the novel object test, did any subjects investigated the ball, and these numbers were very low (2 in the alone condition and 2 others in the condition with the companion).

2.1.1. Influence of context

Reactivity scores were analysed for 16 pairs for the novel object test. In the novel object test, subjects in pairs had significantly lower reactivity scores than those alone (Cumulative link mixed models (CLMM): estimate ± S.E.: −2.12 ± 0.88, \( P = 0.016 \)) (see Figure 1a).

The mean value of the subject's pre-test heart rate measurement before the experiment was 42.29 (SD = 4.58) and significantly increased to 48.33 (SD = 12.54) during the novel object test (Wilcoxon tests: \( V = 6, P = 0.004 \)).
The analyses of heart rate recovery were run on 12 pairs in the novel object test. The difference in time to recover obtained from the subjects in pairs and when alone was not significantly different (Linear mixed effect models (LMM): t-value, df: −1.336, 11, P = 0.208) (see Figure 1b).

The companion’s reactivity score did not have an impact on the subject’s reactivity score in the novel object test (Cumulative link model (CLM): estimate ± S.E.: 0.31 ± 0.51, P = 0.535).

Reactivity scores were analysed for 14 pairs for the umbrella test. In this test, considering only subjects who showed a reaction, the reactivity score for the subjects in pairs was not significantly lower than that obtained from those alone (CLMM: estimate ± S.E.: −1.14 ± 0.78, P = 0.145) (see Figure 2a).

The mean value of the subject’s pre-test heart rate measurement before the experiment was 41.50 (SD = 3.66) and significantly increased to 50.68 (SD = 8.66) during the umbrella test (Wilcoxon tests: V = 7, n = 13, p = 0.004).

The data on heart rate recovery were analysed for 14 pairs for the umbrella test. The presence of a companion was associated with quicker heart rate recovery (LMM: t-value, df: −2.319, 13, P = 0.037) (see Figure 2b).

The reactivity score of the companion did not have an impact on the subject’s reactivity score in the umbrella test by pair (CLM: estimate ± S.E.: −0.37 ± 0.77, P = 0.623).

### 2.1.2. Impact of companion habituation

The difference in reactivity scores and heart rate recovery were not significantly different between subjects with habituated companions and subjects with naïve companions regardless of the test (Reactivity score (RS), CLM: Novel object test (NO), estimate ± S.E.: 0.36 ± 0.95, P = 0.705; Umbrella test (UM), estimate ± S.E.: 0.20 ± 1.36, P = 0.881; Heart rate recovery (HRR), Linear effect model (LM): NO, t-value, df: −1.027, 10, P = 0.329; UM, t-value, df: 0.216, 12, P = 0.833).

### 2.2. Study 2: Impact of unfamiliar companion

The median value of companion’s reactivity score in the novel object test was 0 (Quartile 1 (Q1) = 0; Quartile 3 (Q3) = 0) for habituated companions versus 0 (Q1 = 0; Q3 = 1) for naïve companions. These values were not significantly different (Mann-Whitney test: W=25, n1=8, n2=8, P = 0.369).

In the umbrella test, the median value of companion’s reactivity score was significantly lower at 1 (Q1 = 0.5; Q3 = 3) for habituated companions versus 4 (Q1 = 2.5; Q3 = 4) for naïve companions (Mann-Whitney test: W=11, n1=8, n2=7, P = 0.047).
Only one subject investigated the umbrella during the umbrella test with a companion. Also, it has been observed that during the tests 11 pairs had exchanged agonistic behaviour such as head threat when they ate, i.e. one of the horses turned its heard toward the other with the ears pinned back.

2.2.1. Influence of context

For the novel object test, the analysis of reactivity scores was run on 12 pairs. The subject’s reactivity score in the novel object test for paired subjects was significantly lower than when alone (CLMM: estimate ± S.E.: −2.87 ± 1.19, P = 0.015) (see Figure 3a).

The mean value of the subject’s pre-test heart rate measurement before the experiment was 48.34 (SD = 4.73) versus 50.53 (SD = 10.52) for the novel object test. These values are not significantly different (Wilcoxon tests: V = 56, n = 16, p = 0.552).

The data on heart rate recovery were analysed for 11 pairs in the novel object test. The time to recover for the subjects in pairs was not significantly different to those alone (LMM: t-value, df: −1.209, 10, P = 0.254) (see Figure 3b).

In the novel object test pairs, the companion’s reactivity score did not have an impact on the subject’s reactivity score (CLM: estimate ± S.E.: 0.07 ± 0.52, P = 0.885).

In the umbrella test, the reactivity scores were analysed for 15 pairs. The reactivity score for the subjects in pairs was not significantly lower than that obtained from those alone (CLMM: estimate ± S.E.: −1.08 ± 0.96, P = 0.259) (see Figure 4a).

The mean value of the subject’s heart rate pre-test measurement before the experiment was significantly lower at 48.34 (SD = 4.73) versus 71.54 (SD = 33.04) during the umbrella test (Wilcoxon tests: V = 28.5, n = 16, p = 0.044).

The data on heart rate recovery were analysed for 15 pairs for the umbrella test. Subjects in pairs had significantly lower recover times compared than those alone (LMM: t-value, df: −2.229, 14, P = 0.043) (see Figure 4b).

The subject’s reactivity score was not impacted by the reactivity score of the companion in the umbrella test (CLM: estimate ± S.E.: −0.68 ± 0.46, P = 0.137).

2.2.2. Impact of companion habituation

Regardless of the test, there were no significant differences in reactivity scores and heart rate recovery, between subjects with a habituated companion and subjects with a naïve companion (RS, CLM: NO, estimate ± S.E.: 0.37 ± 1.08, P = 0.731; UM, estimate ± S.E.: 1.92 ± 1.42, P = 0.174; HRR, LM: NO, t-value, df: −1.477, 9, P = 0.173; UM, t-value, df: 1.287, 13, P = 0.221).
3. Discussion

Among the many examples of social buffering [3, 4, 5], there is inconsistency in the conclusions drawn about general effects [33]. These relate to variability associated with stimuli and measurements assessed; our work allows the development of a framework for rationalising these inconsistencies, with the nature of the stressor producing a differential effect on behavioural and physiological measures of buffering but the characteristics of the companion such as its arousal status or familiarity having little effect in our model species. The companion significantly reduced the subjects' behavioural response (reactivity) in the novel object test but not in the umbrella test; the converse was found in relation to heart rate recovery time. This interaction between stimulus type and behavioural versus physiological buffering effect has not been noted before. Moreover, the consistency of the effects in the two studies show the robustness of the phenomenon.

The differential impact of the companion might relate to the emotional quality and intensity of the response provoked by the two stimuli. Like others, we found a greater reaction to an umbrella than to a ball (or ball-like stimulus such as a cone e.g. [34, 35]). Although theoretically, this might simply relate to the umbrella being larger, for example, Bulens et al. (2015) [35] suggested that the large size of an opened umbrella makes it difficult to ignore compared to the cone and plastic ball used in their test. However, stimulus size alone does not seem to be a full explanation as horses have been observed to react similarly to a stationery umbrella and a stationery but larger plastic tarp (5m x 6m) [36]. Another possibility, evident in our study, is that stimulus movement played an important role on the subsequent response: the umbrella was a novel, suddenly changing stimulus which led to a rapid change in the environment at a given distance from the subjects, unlike the ball, which subjects could observe initially from their own preferred distance. The sudden change provoked a more dramatic reaction (flight response) in our subjects, consistent with other findings that horses accept a novel unchanging stimulus more easily than a novel suddenly changing stimulus [37]. The suddenness of the change reduced the degree of control that subjects had over the umbrella stimulus. Having an effective active response in the presence of an aversive stimulus has repeatedly been shown to reduce the physiological and behavioural stress response (reviewed by [38] as well as the degree of aversion experienced [39]. The differential ability of the subject to control the level of exposure to these stimuli may influence the quality of emotional responses.

Fear is associated with threat avoidance with Fight-Flight-Freeze behaviours occurring as natural responses to a direct and real threat (reviewed by [40]). Startle movements in particular involve the rapid contraction of the muscles and thus facilitate effective fear responses [41]. In these potentially dangerous situations individuals will not seek further information about the situation but will directly react [42]. In contrast, anxiety is associated with evaluation of risk related to a potential (uncertain) threat. In this state, the animal displays increased attention and investigatory behaviours, such as smelling and licking in order to learn more about the situation with inhibition of many other behaviours (reviewed by [40, 43]). This additional cortical processing of information allows the subject to have a less reflexive response to the situation [42]. The uncertainty associated with anxiety can result in various forms of
motivational conflict (approach-avoidance; approach-approach; avoidance-avoidance - [44]). This could be an adaptive strategy as the uncertain animal gains an advantage from gathering further information, it is therefore more likely to be influenced by social cues, unlike fear, where there are clear proximate personal strategic goals, which are not likely to be influenced by the lack of response of others.

Another possibility is that in the umbrella test, the immediacy and mental focus of the startle response may not be susceptible to social buffering as the individual did not have time to process the relevant social information. However, after moving away from the umbrella, when only the open umbrella remains, it might be that the companion can act as a social buffer. In this situation, it would be predicted that the companion would not reduce the subject’s behavioural responses but it may help the subject to recover after the stressful event, which may only be manifest physiologically (e.g. in heart rate). In addition, it can be argued that the intensity of the emotional response (or general arousal) rather than its quality also differentiates the two conditions. Thus the opening of the umbrella may have increased heart rate to a greater peak, thus permitting a reduction by the companion, which is not the case with the novel object test. In our studies, the mean heart rate obtained during the 5 minutes of test in the novel object test was not significantly different from the baseline. The same result was found in Study 1 for the umbrella test, contrary to Study 2 in which the mean heart rate was significantly different from the baseline. A similar result to our Study 2 was found by Takeda et al. (2003) [27] with Japanese Black Heifers, where a weak (non-significant) effect was reported for a strange object on mean heart rate compared with a marked increase in heart rate during a surprise test (bucket dropped). However, as already noted, their study examined group size effects and not social buffering effects since there was no condition where individuals were alone. This argument would explain the lack of physiological social buffering effect in the ball, novel object effect. The more gradual mental processing [45] may facilitate greater social buffering of the behavioural response in these circumstances.

Thus we postulate that the ball caused a state of lower level increased arousal and possibly anxiety, while the sudden opening of the umbrella resulted in a fear state with a discernible startle response [45]. The differing social buffering effects that we noted with the two stimuli can be explained by the fact that the animal needs time to process relevant information such as a companion’s behaviours, however during sudden changes this mental process is focused on the immediate, rapid response that might be necessary to protect the individual from harm.

The arousal status of the companion (i.e. companion’s habituation or not to the stimuli) did not affect the subject’s reaction and recovery time in either of our studies. This is not surprising for the novel stimulus test, given that the behavioural response of habituated and naïve companions was similar. Only 4 horses from the first study and 2 from the second study produced a reaction score higher than that allowed for a habituated companion (and one of these horses in each study was a habituated companion). However, in the sudden stimulus test, the behavioural response was different between the two types of companion, with habituated companions showing less reaction than naïve companions. Indeed, throughout our studies, companion responses had no measurable impact on subjects’ reactions. This finding is in contrast to some others who have reported that companions are more effective social buffers if their
arousal is reduced by prior habituation to a novel but harmless stimulus [25, 26]. Christensen et al. (2008) [25] found that a habituated demonstrator horse reduced the fear reactions of unrelated young horses facing an opening umbrella. This difference to our own results might be due to our horses being older and thus potentially more generally habituated to sudden changes in the environment [46, 47]. As the behavioural response is not always correlated with the physiological response [48, 49], it would be useful, in future, to record the behavioural and the physiological responses of the companion as well as the focal subjects in order to have greater insight into their arousal state (reviewed by [50]).

Familiarity between the subject and companion also had no effect on the subject's stress responses. In the literature, the role of companion familiarity varies with factors such as the nature of the stressor. Some [20] have found that after social isolation, reunion with any conspecific (familiar or unfamiliar) helped to reduce behavioural responses; while others [27] found that a familiar companion was a more efficient buffer of the behavioural response during a novelty test and surprise test, and the physiological response during a surprise test and conflict test. The importance of familiarity between individuals seems to vary with age, potentially being less important with older subjects [5], and might also vary with species [5].

It is important to highlight that the familiarity of a subject is not the same as its relationship. Familiarity implies recognition of an individual, i.e. an individual is able to identify another whom it has previously met based on distinctive characteristics [51, 52]; but does not imply any form of social bond. We suggest future studies dealing with the impact of familiarity on social buffering should also consider this issue of relationship. For example, Sanchez et al. (2015) [53] found that in rhesus monkeys maltreating mothers (i.e. those engaged in physical abuse and a high rate of infant rejection) were a less effective buffer of the cortisol response of their infant during a novel stress test compared to non-abusive mothers.

In conclusion, we propose that social buffering of behaviour has adaptive value in contexts where mental processing permits a subject to acknowledge the presence and behaviour of a companion and to use this to mediate its own level of anxiety and investigation behaviour. By contrast, social buffering of behaviour is unlikely to occur during sudden startle responses where an immediate reflexive response should prevail. In these contexts, signs of reduced behavioural response will not be observed but social buffering of physiological recovery may be apparent once the immediate threat is over.

4. Methods

Ethics statement

This research was carried out in compliance with the ARRIVE guidelines. The delegated authority of the University of Lincoln Research Ethics Committee approved this research (CosREC433) and all methods were carried out in accordance with the university Research Ethics Policy and with the ethical guidelines of ISAE. Written informed consent was obtained from the owner of the horses.
4.1. Study animals

The first study took place between May and June 2018 in the riding school of Haras de Jardy (Versailles, France). 32 horses (16 pairs of familiar horses; pairs were housed together in stables within a large building and had lived together for over 1 year) of various breeds, aged 6 to 24 years old (mean +/- SD: 13.2 years, +/- 4.1) representing 10 geldings and 22 females which belong to a busy riding school environment. Each pair (n=16) consisted of a focal subject and a companion and the roles were not reversed during the experiment.

The second study was undertaken at the same location as the previous experiment, from April to June 2019. 32 horses of different breeds, consisting of 16 pairs of unfamiliar horses (unfamiliar companions lived at the riding school, but were housed in a separate building from the subjects) were used, aged 4 to 23 years old (mean +/- SD: 14.2 years, +/- 4.6) representing 19 geldings and 13 females. Among the 32 horses, 7 were used in both studies and played the role of habituated companion on both occasions. As in the first study, each pair (n=16) consisted of a focal subject and a companion. Within a pair the roles were not reversed during the experiment.

4.2. Experimental set-up

A rectangular test arena (6m x 3m) with a starting area (3mx3m) [54] was built with wooden poles (see Figure S1 in the electronic supplementary material). This test arena contained 2 buckets of food to avoid competition within pairs [55], separated by 1m (each bucket contains 600g of pellets) with free access during the test. The distance between the buckets and the novel object or the umbrella was 1m.

4.3. Test stimuli

Two different types of novel stimuli were presented in a counterbalanced order to control for prior exposure effects. One was a novel unchanging stimulus (75cm black and white striped ball, “novel object test”), the other a novel suddenly changing stimulus (opening of a blue and white 120cm umbrella, “umbrella test”). These different stimuli were presented in front of the horses by an experimenter [as per 56].

4.4. General habituation to test environment

Each horse was habituated individually to the test arena prior to the experiment to reduce the risk of stress due to a new environment, using the same principles of habituation described by Christensen et al. (2008) [25]. The horses were considered acclimatised and therefore ready for use when they met the following criteria: the horses entered the arena voluntarily and walked directly to the bucket to eat for at least 90s out of a total of 120s. During this habituation process, the novel object was absent, the
umbrella was closed and the person who would open the umbrella stood still behind it to habituate the horse to their presence. The umbrella and the person, who would open the umbrella, were always present during all experiments (novel object and umbrella test). It was the same person throughout the tests.

The allocation of experimental roles (subject or companion) within a pair was done by measuring the time taken by individuals to go to the bucket and eat during the habituation period and by the number of habituations achieved per horse [25]. The horse which required fewer trials was selected as the companion, with the other horse being the subject. If the two horses required the same number of trials, then the fastest responding horse within the trials was made the companion: it was decided that a faster-responding horse spending less time examining its environment would be more confident and therefore potentially a better social buffer.

4.5. Habituation of habituated companion to the different stimuli

In the first study, half of the companions (n=8) were habituated to the test stimuli (novel object test and umbrella test), they are referred to as habituated companions. The other eight companions were not habituated to the test stimulus, they are referred to as naïve (control) companions.

The eight habituated companions were regarded as habituated when they met the predefined criteria of habituation during the stimulus exposure. To meet these criteria the habituated companion had to show little (reactivity score of 1) or no behavioural reaction (reactivity score of 0) to the stimulus – ball and an increase in heart rate that did not exceed 20 beats/min during the stimulus exposure [25]. Following Christensen et al. (2008) [25], companions underwent up to 13 habituation trials. If companions had not reached the habituation criteria after the 13th trial, they would have been dropped from the study. To ensure that the companion remained habituated, the companion did the habituation again to the stimulus (ball or umbrella) one last time less than 10 minutes before performing the test with the subject.

In the second study, the same eight habituated companions from the first study were scheduled for use, however one horse had to be replaced because it had been retired. These companions did further habituation sessions to the test stimuli (novel object test and umbrella test) as part of this study, following the same habituation procedure as before (based on Christensen et al., (2008) [25]).

4.6. Recordings

The heart rate was only recorded for the subjects. All subjects were habituated to a Polar Equine H7 heart rate monitor, and heart rate data analysed with the software Kubios HRV 3.0.2. The settings of the heart rate monitor were such that the sampling occurred 1/second and the sensor always sent the current heart rate, not the rolling average. The week before the tests, the subject’s heart rate was measured for two minutes in the test arena with the same conditions as in the test (i.e. when the subject was eating from
the bucket) but without being exposed to any test stimuli (i.e. pre-test heart rate) [57, 58]. Heart rate recovery (HRR) was measured from the time it took for the subject’s heart rate to return to within 15% of its pre-test heart rate value (bpm) after being exposed to the test stimulus [32, 59]. The threshold for determining recovery of pre-test heart rate value plus 15% was chosen as it represents a substantial degree of decline and accommodates individual variability in baseline values based on the pre-test heart rate value; as a result, most horses recovered during the allocated time (5 minutes). If a horse escaped from the test arena in response to the presentation of the stimulus during the test, it was attributed the upper censored maximum value of 300 seconds. The heart rate data was checked for artefacts. In the analysis, all recordings with more than 5% heart beat errors were excluded (3 in the novel object test alone and 2 in the umbrella test alone for the first study) [59].

Behaviours were recorded by two cameras during testing which had been present throughout habituation.

The behavioural responses of both subjects and companion horses to the novel object (ball) (NO) and the opening of the umbrella (UM) were evaluated by assigning a score (ranked in order of intensity: 0 = no reaction, 1 = head up, 2 = alert, 3 = away, 4 = flight and 5 = escape), called reactivity score (RS) (see Table S1 for behavioural description in electronic supplementary material) [25]. Two evaluators separately watched and rated the same videos (see the electronic supplementary material).

4.7. Experimental procedure

In order to determine if the companion could help to reduce the subject’s stress response, the subject was tested alone and with its companion for each stimulus.

The tests took place between 8am and 1pm. Once the focal subject with or without its companion had been released into the test arena, the experimenters waited until the focal subject had eaten with its head in the bucket for 3 seconds before introducing the novel object or opening the umbrella in the arena [60], the test then ran for a further 5 minutes. If the companion was present, it was next to the subject when the stimulus was applied. The two horses were free to interact and to eat in the buckets in the test arena (see Figure 5). At the end of the 5 minutes, the horses were taken back to their housing.

To control for order effects, the 16 pairs were randomly allocated into 4 groups; each group contained 2 pairs of subjects with habituated companions and 2 pairs of subjects with naïve companions. These 4 groups performed the tests in a different order to control for order effects.

Each subject performed 4 tests: novel object test (ball) – alone or with companion; and umbrella test – alone or with companion. Each horse did only one test per week.

Figure 5: Subject (brown horse) during the umbrella test with its companion (white horse)

4.8. Data analysis
The software R (version 3.5.2) was used for all analyses.

For each study, the data from one pair was removed because the companion stayed in the starting area during the umbrella test. The companion was therefore not considered able to play the role of social buffer. Only the data obtained in the novel object test alone and by pair were kept in the analyses.

First we established that there was no order effect on either the behavioural or physiological responses of subjects (see the electronic supplementary material).

In order to verify if the habituation of the companion had an impact on their responses, the companions’ reactivity score was compared between habituated and naïve individuals across the two different situations (Mann-Whitney test). In order to compare the median values of the pre-test heart rate with the heart rate obtained when the subject was alone in the novel object and umbrella test, a Wilcoxon test was used. This determined if the horse’s heart rate was significantly higher when the stimulus was presented.

For the next stage of analysis, since our focus was on factors affecting the occurrence of social buffering, all subjects with a reactivity score of 0 when they performed the test alone were excluded from the behavioural analyses as there was no potential to show a social buffering effect on their responses. Subjects who did not show a change in heart rate (obtained 0 sec recovery time) when they were alone were excluded from the physiological analyses. Exclusion for one of the analyses did not necessitate exclusion from other analyses. To examine if the companion reduced the behavioural and/or physiological responses of subjects during stressful situations, the reactivity score and the heart rate recovery were compared between when the subject was alone or with a companion for each test. Cumulative link mixed models (CLMM) using the adaptive Gauss-Hermite quadrature (AGQ) (with the R-package \textit{ordinal}) were used for the data on reactivity score and linear mixed effect models (LMM) in the R-package \textit{lmerTest} used for the data on heart rate recovery, with companion (absent vs. present) as a fixed factor and the identity of the subjects as a random factor.

Finally, to determine if a habituated companion had a different effect to a naïve companion, the difference in reactivity score (between when the subject was alone or with a companion) and heart rate recovery time (between when the subject is alone or with a companion) were compared as a function of the types of companion (naïve vs. habituated) for each test. The data on reactivity score were analysed with a cumulative link model (CLM) and the data of heart rate recovery with a linear effects model (LM). Similar models were used for each dependent variable, with type of companion (habituated vs. naïve) as fixed factors. In addition, companion reactivity score was added as a fixed factor in the CLM of reactivity score.

The significance of the results was assessed at a threshold of $p < 0.05$.

\textbf{Declarations}

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Authors’ contributions

C.R.B, T.R., C.N. and D.M. conceived and designed the experiments, and wrote the paper. C.R.B. performed the experiments, analysed and interpreted the data with the support of the other authors. All authors gave final approval for publication and agree to be held accountable for the work performed.

Competing interests

The authors declare no competing interests.

References

1. Cohen, S., & Wills, T. A. (1985). Stress, social support, and the buffering hypothesis. *Psychological Bulletin, 98*(2), 310.

2. Wittig, R. M., Crockford, C., Weltring, A., Langergraber, K. E., Deschner, T., & Zuberbühler, K. (2016). Social support reduces stress hormone levels in wild chimpanzees across stressful events and everyday affiliations. *Nature Communications, 7*, 13361. (doi:10.1038/ncomms13361)

3. Kikusui, T., Winslow, J. T., & Mori, Y. (2006). Social buffering: Relief from stress and anxiety. *Philosophical Transactions of the Royal Society of London.Series B, Biological Sciences, 361*(1476), 2215-2228. (doi:10.1098/rstb.2006.1941)

4. Hennessy, M. B., Kaiser, S., & Sachser, N. (2009). Social buffering of the stress response: Diversity, mechanisms, and functions. *Frontiers in Neuroendocrinology, 30*(4), 470-482. (doi:10.1016/j.yfrne.2009.06.001)

5. Rault, J. (2012). Friends with benefits: Social support and its relevance for farm animal welfare. *Applied Animal Behaviour Science, 136*(1), 1-14. (doi:10.1016/j.applanim.2011.10.002)

6. Smith, T. E., McGreer-Whitworth, B., & French, J. A. (1998). Close proximity of the heterosexual partner reduces the physiological and behavioral consequences of novel-cage housing in black tufted-ear marmosets (callithrix kuhlii). *Hormones and Behavior, 34*(3), 211-222. (doi:10.1006/hbeh.1998.1469)

7. Edgar, J., Held, S., Paul, E., Pettersson, I., Price, R. I., & Nicol, C. (2015). Social buffering in a bird. *Animal Behaviour, 105*, 11-19. (doi:10.1016/j.anbehav.2015.04.007)

8. Nakayasu, T., & Ishii, K. (2008). Effects of pair-housing after social defeat experience on elevated plus-maze behavior in rats. *Behavioural Processes, 78*(3), 477-480.

9. Berardi, A., Trezza, V., Palmery, M., Trabace, L., Cuomo, V., & Campolongo, P. (2014). An updated animal model capturing both the cognitive and emotional features of post-traumatic stress disorder (PTSD). *Frontiers in Behavioral Neuroscience, 8*, 142. (doi: 10.3389/fnbeh.2014.00142)

10. Ben-Ami Bartal, I., Shan, H., Molasky, N. M., Murray, T. M., Williams, J. Z., Decety, J., & Mason, P. (2016). Anxiolytic treatment impairs helping behavior in rats. *Frontiers in Psychology, 7*, 850.
11. Graves, F. C., & Hennessy, M. B. (2000). Comparison of the effects of the mother and an unfamiliar adult female on cortisol and behavioral responses of pre- and postweaning guinea pigs. *Developmental Psychobiology, 36*(2), 91-100. (doi:10.1002/(SICI)1098-2302(200003)36:2<91::AID-DEV1>3.0.CO;2-1)

12. Hennessy, M. B., Maken, D. S., & Graves, F. C. (2002). Presence of mother and unfamiliar female alters levels of testosterone, progesterone, cortisol, adrenocorticotropin, and behavior in maturing guinea pigs. *Hormones and Behavior, 42*(1), 42-52. (doi:10.1006/hbeh.2002.1794)

13. Rukstalis, M., & French, J. A. (2005). Vocal buffering of the stress response: Exposure to conspecific vocalizations moderates urinary cortisol excretion in isolated marmosets. *Hormones and Behavior, 47*(1), 1-7. (doi:10.1016/j.yhbeh.2004.09.004)

14. Terranova, M. L., Cirulli, F., & Laviola, G. (1999). Behavioral and hormonal effects of partner familiarity in periadolescent rat pairs upon novelty exposure. *Psychoneuroendocrinology, 24*(6), 639-656. (doi:10.1016/S0306-4530(99)00019-0)

15. Kiyokawa, Y., Honda, A., Takeuchi, Y., & Mori, Y. (2014). A familiar conspecific is more effective than an unfamiliar conspecific for social buffering of conditioned fear responses in male rats. *Behavioural Brain Research, 267*, 189-193. (doi:10.1016/j.bbr.2014.03.043)

16. Castro, W. L. R., & Matt, K. S. (1997). The importance of social condition in the hormonal and behavioral responses to an acute social stressor in the male siberian dwarf hamster (phodopus sungorus). *Hormones and Behavior, 32*(3), 209-216. (doi:10.1006/hbeh.1997.1423)

17. Castro, W. L. R., & Matt, K. S. (1997). Neuroendocrine correlates of separation stress in the siberian dwarf hamster (phodopus sungorus). *Physiology & Behavior, 61*(4), 477-484. (doi:10.1016/S0031-9384(96)00456-8)

18. Armario, A., Luna, G., & Balasch, J. (1983). The effect of conspecifics on corticoadrenal response of rats to a novel environment. *Behavioral and Neural Biology, 37*(2), 332-337. (doi:10.1016/S0163-1047(83)91425-5)

19. Armario, A., Ortiz, R., & Balasch, J. (1983). Corticoadrenal and behavioral response to open field in pairs of male rats either familiar or non-familiar to each other. *Experientia, 39*(11), 1316-1317. (doi:10.1007/BF01990391)

20. Boissy, A., & Le Neindre, P. (1997). Behavioral, cardiac and cortisol responses to brief peer separation and reunion in cattle. *Physiology & Behavior, 61*(5), 693-699. (doi:10.1016/S0031-9384(96)00521-5)

21. Cirulli, F., Terranova, M. L., & Laviola, G. (1996). Affiliation in periadolescent rats: Behavioral and corticosterone response to social reunion with familiar or unfamiliar partners. *Pharmacology Biochemistry and Behavior, 54*(1), 99-105. (doi:10.1016/0091-3057(95)02169-8)

22. Boissy, A., Terlouw, C., & Le Neindre, P. (1998). Presence of cues from stressed conspecifics increases reactivity to aversive events in cattle: Evidence for the existence of alarm substances in urine. *Physiology & Behavior, 63*(4), 489-495. (doi:10.1016/S0031-9384(97)00466-6)
23. Davitz, J. R., & Mason, D. J. (1955). Socially facilitated reduction of a fear response in rats. *Journal of Comparative and Physiological Psychology, 48*(3), 149-151. (doi:10.1037/h0046411)

24. Kiyokawa, Y., Kikusui, T., Takeuchi, Y., & Mori, Y. (2004). Partner's stress status influences social buffering effects in rats. *Behavioral Neuroscience, 118*(4), 798-804. (doi:10.1037/0735-7044.118.4.798)

25. Christensen, J. W., Malmkvist, J., Nielsen, B. L., & Keeling, L. (2008). Effects of a calm companion on fear reactions in naive test horses. *Equine Veterinary Journal, 40*(1), 46-50. (doi:10.2746/042516408X245171)

26. Klein, B., Bautze, V., Maier, A., Deussing, J., Breer, H., & Strotmann, J. (2015). Activation of the mouse odorant receptor 37 subsystem coincides with a reduction of novel environment-induced activity within the paraventricular nucleus of the hypothalamus. *European Journal of Neuroscience, 41*(6), 793-801. (doi:10.1111/ejn.12838)

27. Takeda, K., Sato, S., & Sugawara, K. (2003). Familiarity and group size affect emotional stress in japanese black heifers. *Applied Animal Behaviour Science, 82*(1), 1-11. (doi:10.1016/S0168-1591(03)00039-X)

28. Feh, C. (2005). Relationships and communication in socially natural herds. In: Mills D., McDonnell S. (Eds) The domestic horse, the evolution, development and management of its behaviour. Cambridge University Press, Cambridge, pp 83-93.

29. Cozzi, A., Sighieri, C., Gazzano, A., Nicol, C. J., & Baragli, P. (2010). Post-conflict friendly reunion in a permanent group of horses (equus caballus). *Behavioural Processes, 85*(2), 185-190. (doi:10.1016/j.beproc.2010.07.007)

30. Wathan, J., & McComb, K. (2014). The eyes and ears are visual indicators of attention in domestic horses. *Current Biology, 24*(15), R677-R679. (doi:10.1016/j.cub.2014.06.023)

31. Fureix, C., Bourjade, M., Henry, S., Sankey, C., & Hausberger, M. (2012). Exploring aggression regulation in managed groups of horses equus caballus. *Applied Animal Behaviour Science, 138*(3), 216-228. (doi:10.1016/j.applanim.2012.02.009)

32. Wathan, J., Proops, L., Grounds, K., & McComb, K. (2016). Horses discriminate between facial expressions of conspecifics. *Scientific Reports, 6*, 38322. (doi:10.1038/srep38322)

33. Nakayasu, T., & Kato, K. (2011). Is full physical contact necessary for buffering effects of pair housing on social stress in rats? *Behavioural Processes, 86*(2), 230-235. (doi:10.1016/j.beproc.2010.12.002)

34. Anderson, M. K., Friend, T. H., Evans, J. W., & Bushong, D. M. (1999). Behavioral assessment of horses in therapeutic riding programs. *Applied Animal Behaviour Science, 63*(1), 11-24. (doi:10.1016/S0168-1591(98)00237-8)

35. Bulens, A., Sterken, H., Van Beirendonck, S., Van Thielen, J., & Driessen, B. (2015). The use of different objects during a novel object test in stabled horses. *Journal of Veterinary Behavior: Clinical Applications and Research, 10*(1), 54-58. (doi:10.1016/j.jveb.2014.09.002)
36. Leiner, L., & Fendt, M. (2011). Behavioural fear and heart rate responses of horses after exposure to novel objects: Effects of habituation. *Applied Animal Behaviour Science, 131*(3), 104-109. (doi:10.1016/j.applanim.2011.02.004)

37. Górecka-Bruzda, A., Jastrzębska, E., Sosnowska, Z., Jaworski, Z., Jeziorski, T., & Chruszczewski, M. H. (2011). Reactivity to humans and fearfulness tests: Field validation in polish cold blood horses. *Applied Animal Behaviour Science, 133*(3), 207-215. (doi:10.1016/j.applanim.2011.05.011)

38. Bassett, L., & Buchanan-Smith, H. M. (2007). Effects of predictability on the welfare of captive animals. *Applied Animal Behaviour Science, 102*(3-4), 223-245. (doi:10.1016/j.applanim.2006.05.029)

39. Browne, W. J., Caplen, G., Statham, P., & Nicol, C. J. (2011). Mild environmental aversion is detected by a discrete-choice preference testing method but not by a free-access method. *Applied Animal Behaviour Science, 134*(3-4), 152-163. (doi:10.1016/j.applanim.2011.07.004)

40. McNaughton, N., & Corr, P. J. (2008). The neuropsychology of fear and anxiety: A foundation for Reinforcement Sensitivity Theory. In P. J. Corr (Eds.) The reinforcement sensitivity theory of personality. Cambridge University Press, pp 44–94.

41. Grillon, C., & Baas, J. (2003). A review of the modulation of the startle reflex by affective states and its application in psychiatry. *Clinical Neurophysiology, 114*(9), 1557-1579. (doi:10.1016/S1388-2457(03)00202-5)

42. LeDoux, J. (1998). The emotional brain: The mysterious underpinnings of emotional life. (Eds.) Simon and Schuster.

43. Mills, D.S., Ricci-Bonot, C., Hall, S.S. (2019). Mental health issues in the horse. In: McMillan, F.D. (Eds.) Mental Health and Well-being in Animals. CABI, pp 242-256.

44. Aldao, A., & Wisco, B. E. (2015). Motivational conflict influences the timing of emotions and their regulation. *Motivation and Emotion, 39*(6), 943-952. (doi:10.1007/s11031-015-9496-8)

45. Lansade, L., Bouissou, M., & Boivin, X. (2007). Temperament in preweanling horses: Development of reactions to humans and novelty, and startle responses. *Developmental Psychobiology, 49*(5), 501-513. (doi:10.1002/dev.20233)

46. Popescu, S., & Diugan, E. (2013). The relationship between behavioral and other welfare indicators of working horses. *Journal of Equine Veterinary Science, 33*(1), 1-12. (doi:10.1016/j.jjevs.2012.04.001)

47. Baragli, P., Vitale, V., Banti, L., & Sighieri, C. (2014). Effect of aging on behavioural and physiological responses to a stressful stimulus in horses (equus caballus). *Behaviour, 151*(11), 1513-1533. (doi:10.1163/1568539X-00003197)

48. Yarnell, K., Hall, C., & Billett, E. (2013). An assessment of the aversive nature of an animal management procedure (clipping) using behavioral and physiological measures. *Physiology & Behavior, 118*, 32-39. (doi:10.1016/j.physbeh.2013.05.013)

49. Squibb, K., Griffin, K., Favier, R., & Ijichi, C. (2018). Poker face: Discrepancies in behaviour and affective states in horses during stressful handling procedures. *Applied Animal Behaviour Science, 202*, 34-38. (doi:10.1016/j.applanim.2018.02.003)
50. Hall, C., Randle, H., Pearson, G., Preshaw, L., & Waran, N. (2018). Assessing equine emotional state. *Applied Animal Behaviour Science, 205*, 183-193. (doi:10.1016/j.applanim.2018.03.006)

51. Gheusi, G., Bluthé, R., Goodall, G., & Dantzer, R. (1994). Social and individual recognition in rodents: Methodological aspects and neurobiological bases. *Behavioural Processes, 33*(1-2), 59-87. (doi:10.1016/0376-6357(94)90060-4)

52. Dale, J., Lank, D. B., & Reeve, H. K. (2001). Signaling individual identity versus quality: A model and case studies with ruffs, queleas, and house finches. *The American Naturalist, 158*(1), 75-86. (doi:10.1086/320861)

53. Sanchez, M. M., McCormack, K. M., & Howell, B. R. (2015). Social buffering of stress responses in nonhuman primates: Maternal regulation of the development of emotional regulatory brain circuits. *Social Neuroscience, 10*(5), 512-526.

54. Lansade, L., Bouissou, M., & Erhard, H. W. (2008). Fearfulness in horses: A temperament trait stable across time and situations. *Applied Animal Behaviour Science, 115*(3), 182-200. (doi:10.1016/j.applanim.2008.06.011)

55. Rørvang, M. V., Ahrendt, L. P., & Christensen, J. W. (2015). A trained demonstrator has a calming effect on naïve horses when crossing a novel surface. *Applied Animal Behaviour Science, 171*, 117-120. (doi:10.1016/j.applanim.2015.08.008)

56. Visser, E. K., Ellis, A. D., & Van Reenen, C. G. (2008). The effect of two different housing conditions on the welfare of young horses stabled for the first time. *Applied Animal Behaviour Science, 114*(3), 521-533. (doi:10.1016/j.applanim.2008.03.003)

57. Visser, E., Van Reenen, C., Van der Werf, J., Schilder, M., Knaap, J., Barneveld, A., & Blokhuis, H. (2002). Heart rate and heart rate variability during a novel object test and a handling test in young horses. *Physiology & Behavior, 76*(2), 289-296.

58. Christensen, J. W., Ahrendt, L. P., Lintrup, R., Gaillard, C., Palme, R., & Malmkvist, J. (2012). Does learning performance in horses relate to fearfulness, baseline stress hormone, and social rank? *Applied Animal Behaviour Science, 140*(1-2), 44-52.

59. Smith, A. V., Proops, L., Grounds, K., Wathan, J., & McComb, K. (2016). Functionally relevant responses to human facial expressions of emotion in the domestic horse (equus caballus). *Biology Letters, 12*(2), 20150907. (doi:10.1098/rsbl.2015.0907)

60. Lansade, L., & Simon, F. (2010). Horses’ learning performances are under the influence of several temperamental dimensions. *Applied Animal Behaviour Science, 125*(1), 30-37. (doi:10.1016/j.applanim.2010.02.010)