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Behavioral and Physiological Responses to Stabling in Naive Horses

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REFEREED

SUMMARY

The purpose of this study was to investigate the response of horses to confinement and isolation in a stable (indoor individual housing) for the first time using behavioral indices, heart rate, and salivary cortisol concentration. Six naive 2-year-old Australian Stock Horse fillies were examined at 4-hour intervals over 24 hours in an outdoor group paddock followed by 24 hours in indoor individual housing. Behavioral observations and scores and heart rates were recorded and saliva samples were taken at each interval. During stabling, all horses became agitated and demonstrated increased vocalization and movement. Behavioral scores were significantly higher in the indoor individual housing ($P < .001$). No significant difference in heart rates between the two environments was detected. Mean salivary cortisol did not increase significantly (2 ng/mL $\pm$ 1.4 ng/mL in outdoor group paddock vs 2.5 mL $\pm$ 1.2 ng/mL in indoor individual housing). No diurnal rhythm in salivary cortisol was evident in either the outdoor group paddock or indoor individual housing. The results of this study highlight that a combination of behavioral and physiological measures allow better understanding of stress, where one measurement may be misleading. First time stabling of horses elicited marked behavioral responses indicative of stress that were not reflected in increased heart rates or salivary cortisol concentrations. The lack of a diurnal cortisol rhythm and the comparatively high basal cortisol concentrations found in the outdoor group paddock environment may imply that the fillies were already stressed; therefore, stabling did not cause further aberrations detectable by salivary cortisol analysis.

INTRODUCTION

Confinement stress and environmental preferences have been studied in a variety of farm animals, however relatively little attention has been paid to the effects of confinement and isolation on the horse.[1] It is perhaps due to the commonly held belief that horses enjoy a superior standard of care that they have been largely overlooked in stress research.[2] While horses are generally housed and managed under what would be considered superior conditions to farm animals, it is debatable whether such conditions are optimal for the horse. It is unlikely that common housing and management practices used in the equine industry such as box stall confinement, limited and controlled exercise, and highly concentrated feeding regimens are optimal for a social herd animal that is naturally free-roaming and pasture-grazing.[3]

The effects of long- or even short-term isolation and confinement include distress, demonstrated by abnormal behavior, increased heart rate, vocalization, defecation, and feeding disturbances.[4,5] In sheep, pigs, and cattle, isolation stress results in increased plasma cortisol concentrations and disrupted behavior.[6-8] However, research on isolation and confinement stress in horses has been limited, with varying results; some horses showing increased and others showing decreased plasma cortisol concentrations.[1] Further, a study on equine isolation and confinement stress found that plasma cortisol changes did not always reflect the animal's behavioral response.[9]

Although the stress response is an extremely complex phenomenon that can involve the activation of several different biological responses, the measurement of plasma cortisol has been the cornerstone of stress research for many years, as it is relatively easy to assess and is released in response to a wide range of stressors.[10-12] Cortisol is synthesized and secreted from the adrenal glands into the circulating blood where it is bound rapidly to carrier proteins such as corticosteroid-binding globulin (CBG), albumin, and erythrocytes.[13] Of the cortisol released, 2% to 15% is unbound or "free," and it is this free portion that can cross into cells and exert its biological effect.[14] Due to the

highly lipophilic nature of the hormone, "free" cortisol can be found in all body fluids including cerebral spinal fluid, urine, sweat, semen, and saliva.[14]

The invasive sampling procedures required for plasma cortisol assessment are often counterproductive in stress research, as the procedure of venipuncture itself can lead to significant hypothalamic-pituitary-adrenal activation.[13,15] More recently, the advent of biochemical assays capable of measuring cortisol reliably in the lower nanomolar range have enabled the noninvasive assessment of "free" cortisol in saliva.[13] Since bound and unbound cortisol can be measured in the blood but only "free" cortisol appears in saliva, the assessment of salivary cortisol also provides a direct measure of the biologically active portion of the hormone.[13,16]

Measurement of cortisol concentrations in bodily fluids alone cannot prove or exclude the presence of stress, but in combination with other observations such as behavioral and cardiovascular changes, it can provide important evidence of a stress response.[17] The purpose of this study was to assess in the young horse the stress response elicited by the confinement and isolation of first time stabling by using behavioral indices, heart rate, and salivary cortisol concentrations.

MATERIALS AND METHODS

Animals and Experimental Design

Six unbroken, 2-year-old Australian Stock horse fillies were recruited for the study. All fillies were clinically healthy, of a similar genetic background, and had been raised under similar environmental conditions and management practices. None had been stabled prior to the experiment, but each had been handled and taught to lead. A repeated measure design was used where each horse served as its own control. Saliva samples, heart rate, and behavioral observations were taken and recorded at four-hour intervals over a 48-hour period.

During the first 24 hours, horses were housed in a large outdoor paddock as a group (outdoor group paddock). The horses had been in the paddock in the two weeks prior to the experiment. The paddock measured 30 m × 35 m and contained a large hay feeder, a small automatic water dispenser, and a 3 m × 4 m shelter. The horses had views of nearby paddocks housing stallions, mares, and foals. At night, a nearby road light dimly illuminated the paddock. The horses had ad libitum access to lucerne hay and water throughout the period spent in the outdoor group paddock. Samples taken during the period in the outdoor group paddock served as the controls for the study.

Immediately following the final sampling in the outdoor group paddock, the horses were randomly assigned to individual stables (indoor individual housing), for the indoor individual housing samples. The horses were housed in relative isolation being at least 10 m away from each other at all times. All horses had visual contact with at least one other conspecific during the stress period. Each stable measured 5 m × 5 m and had lights, a small automatic water dispenser, and sawdust bedding. The lights in the stables remained on throughout the 24-hour period. During the stabled period the horses were fed approximately 2 kg of lucerne hay after each sampling with the aim of reducing the feed content of the saliva being sampled. Although feeding intervals were altered during the time in the stable, the diet of the horses remained identical to that in the outdoor group paddock environment.

Experimental procedures which involved the use of horses were approved by the University of Queensland Animal Ethics Committee and complied with the Australian Code of Practice for the care and use of animals for scientific purposes.

Sampling and Observations

All samples were collected at the same times in the outdoor group paddock and indoor individual housings to enable comparison between the outdoor group paddock and indoor individual housing measures without interference from diurnal rhythms. It took no longer than 5 minutes to take each horse's heart rate and saliva sample, assign them a score for their behavior, and note any other behavioral observations. At each collection period in both the outdoor group paddock and indoor individual housings, all sampling was completed within 30 minutes.

A behavioral score was assigned to the horses at each sampling period (Table 1). Observations of general behavior were also noted for each horse at each sampling period and the frequency of display of behaviors indicative of stress was recorded for each horse in both the outdoor group paddock and indoor individual housings (Table 2). The horse's heart rate was taken using a stethoscope just behind the horse's left elbow.

Saliva sampling involved insertion of 10 cotton wool balls, encased in a mesh material, into the horse's mouth over the tongue at the diastema. The ends of the mesh were tied to either side of the halter making it possible to leave the swab in the horse's mouth for a few minutes to facilitate maximum absorption of saliva with minimal interference from the sampler.

Once saturated, the swabs were untied from the halter and placed back into 20 mL conical tubes, sealed, and placed on ice. The samples were stored on ice for a maximum of 48 hours before centrifugation and freezing. Samples were centrifuged in the tubes at 2500 rpm for 5 minutes to separate the saliva from the swabs. To prevent

Table 1	Criteria for behavior categories in six Stock horse fillies during saliva sampling procedure in outdoor group paddock and indoor individual housing environments
0	Standing, relaxed (resting a hoof, ears to the side)
1	Standing, alert (ears forward, four hooves on ground)
2	Standing, mouthing (horse making attempts to remove swab from mouth)
3	Standing, throwing head and mouthing
4	Moving and throwing head (pawing, striking)

Table 2	Description of stress-related behaviors recorded before and during stabling of six naive fillies
Behavior	**Description of behavior**
Pawing	Foreleg extended anteriorly then drawn back, dragging ventral toe of hoof along ground repetitively
Throwing head	Moving head up and down rapidly and repetitively
Vocalization/whinny	High-pitched sound emitted from mouth
Sweating	Presence of sweat anywhere on body
Pacing	Repetitive walking of the horse at gread speed around its stable with little regard to obstacles that may be in the way of its chosen path
Aggressive behavior	Any combination of: ears flattened posteriorly, kicking with hind legs, striking with forelegs, showing of teeth
Nervous behavior	Any combination of: quivering, attempts to flee, ears moving rapidly in all directions

reabsorption during centrifugation, the swab was elevated in the tube by plastic mesh. The saliva that collected in the tip of the tube was pipetted off into 1.5-mL eppendorf tubes. Any extra saliva was then squeezed out of the swabs by placing them in 20-mL syringes and applying pressure. This saliva was also added to the sample in the relevant eppendorf tube, which was then sealed. Samples were stored in a conventional freezer at –20° C to prevent mould formation.[13]

Cortisol Assay

ELISA analysis of the samples was performed using a direct immunoenzymatic determination kit (Salivary Cortisol ELISA kit, Diametra, Bioclone, NSW; Cat. No. DKO 020). The kit had a detectible range of 0 ng/mL to 100 ng/mL and a sensitivity of 0.5 ng/mL. The intra-assay and inter-assay variation was 5.8% and 3.2% respectively, and the mean recovery was 103% ± 4.2%.[18]

Kit instructions were followed, and final analysis was conducted using a Beckman microplates reader set to 450 nm. As well as the normal samples that were run in duplicate, 4 extra tests were run on a single sample (making a total of 6 samples) to determine the repeatability of the ELISA. The reliability of the standards was also assessed by diluting the 100 ng/mL standard with the 0 ng/mL standard to give concentrations of 12.5, 25, 50, and 100 ng/mL.

A series of samples made from the dilution of one sample with predicted high concentrations of cortisol was analyzed to assess the parallelism between sample and control values. The "high cortisol" sample was diluted by adding saliva that had been stripped of its steroid content to give a series of 0, 25%, 50%, and 100% "high cortisol" saliva. The procedure for the removal of the steroid content of the saliva involved adding 10 mg of activated charcoal to the sample, which was then mixed and heated in a water bath until it reached 40° C. The sample was then centrifuged for 3 minutes at high speed to separate the charcoal from the saliva. The supernatant was subsequently pipetted off and centrifuged again to ensure all charcoal was removed from the stripped saliva sample.

Data Analysis

Data analysis was performed using SAS version 6.12 and Microsoft Excel version 2000. The behavioral scores from the outdoor group paddock and indoor individual housings were compared using a ∂^2 test. Heart rates recorded from the outdoor group paddock and indoor individual housings were compared using a paired t-test. A repeated measures analysis of variance F-test was performed to compare salivary cortisol concentrations in samples from horses housed in the outdoor group paddock and indoor individual housings. The same method was used to determine if any diurnal rhythm in cortisol concentrations was evident in the outdoor group paddock and indoor individual housings.

The coefficient of variation was calculated to assess the repeatability of the ELISA. The reliability of the standards in the ELISA test was evaluated through the mean absolute error. Parallelism between the controls and the samples was determined graphically.

RESULTS

General behavior patterns exhibited by horses in the outdoor group paddock environment were similar; most horses appeared relaxed and stood quietly during the sampling period. However, once the horses were moved into the indoor individual housing environment, general behavior observed became more variable. All horses appeared to become agitated and vocalize frequently. A

	Outdoor group paddock environment			**Indoor individual housing**		
Horse	**Cortisol (ng/mL)**	**Behavioral observations**	**Observed frequency of stress-related behavior**	**Cortisol (ng/mL)**	**Behavioral observations**	**Observed frequency of stress-related behavior**
1	1.8	Accepted swab well, stood quietly, mouthed the swab	0	2.3	Moving, throwing head, pawing occasionally, frequent vocalizations	7
2	2.1	Resisted swab insertion, stood, mouthed	1	2.9	Resisted swab insertion, throwing head, pawing, frequent vocalizations	8
3	3.6	Relaxed, standing, chewing occasionally	0	3.0	Sweaty, moving, throwing head, chewing swab	6
4	2.4	Relaxed, standing, mouthing occasionally	0	2.7	Standing quietly, some chewing	4
5	1.5	Reactive to swab and stethoscope, nervous	2	2.0	Highly reactive in stable, pacing, frequent vocalizations, sweaty, calmed when samples were taken	9
6	0.8	Relaxed, standing, occasional chewing on swab	0	1.9	Throwing head, moving, pawing, chewing swab, aggressive	7

Table 3 Frequency and description of behavior and mean salivary cortisol concentrations of six fillies during 24 hours in a group paddock and 24 hours in an indoor individual housing environment

high frequency of stress-related behaviors was observed in the indoor individual housing (Table 3).

Behavioral scores were significantly higher whilst in the indoor individual housing compared to the outdoor group paddock environment ($P < .001$) (Figure 1). Despite the behavioral changes indicative of stress during the indoor individual housing period, heart rate was not higher when horses were stabled (mean heart rate 40.9 bpm ± 1.8 bpm in the indoor individual housing vs 42.1 bpm ± 2.0 bpm in the outdoor group paddock). In fact, heart rate was significantly higher at 5 pm in horses in the outdoor group paddock environment ($P = .03$), and mean heart rate dropped slightly but not significantly from 47.3 bpm to 41.3 bpm when the horses were moved into the indoor individual housing.

Repeatability of the ELISA test was high with a coefficient of variation of 2.55%. The reliability of the standards was also high with a mean absolute error of 6.67 ng/mL. On visual appraisal there was good parallelism between the equine salivary sample and standard. A high degree of variability in salivary cortisol concentrations was found between individual horses ($P = .005$) (Table 3), and first time stabling had no effect on salivary cortisol concentration. Mean salivary cortisol concentration during the outdoor group paddock period was 2.0 ng/mL ± 1.4 ng/mL and during the stabling period was 2.5 ng/mL +/– 1.2 ng/mL; no differences were detected between sampled obtained from horses in outdoor group paddock and indoor individual housings at any individual sampling period (Figure 2). Retrospective power analysis revealed that the power to detect a difference of 0.5 ng/mL in this study was only 0.31. No evidence of a diurnal rhythm was found in either the outdoor group paddock or indoor individual housing samples (Figure 3). Although a significant difference was found between the times of 9 pm and 5 am ($P = .0402$), no significant difference was found between the sample times overall.

DISCUSSION

Marked changes in the behaviors displayed by the fillies in the indoor individual housing clearly indicate that first time stabling results in the initiation of a stress response. Similarly, Bagshaw et al[5] and Alexander and Irvine[9] found that horses subjected to isolation stress showed significantly increased vocalizations, defecation, and movement than stabled horses with visual contact between conspecifics.

The mean heart rate of the fillies did not show the increase in response to stabling that has been reported by other studies.[5] In the present study, mean heart rate dropped slightly but not significantly from 47.3 bpm to 41.3 bpm when the horses were moved into the indoor individual housing. While these rates are within the normal resting range of between 25 bpm to 50 bpm for horses, they are above the 35 bpm average.[19] Elevated baseline heart rates have been found in dogs subjected to handling procedures, and it is possible that the unbroken fillies were stimulated by the handling involved in the sampling procedures but became habituated to the process as the
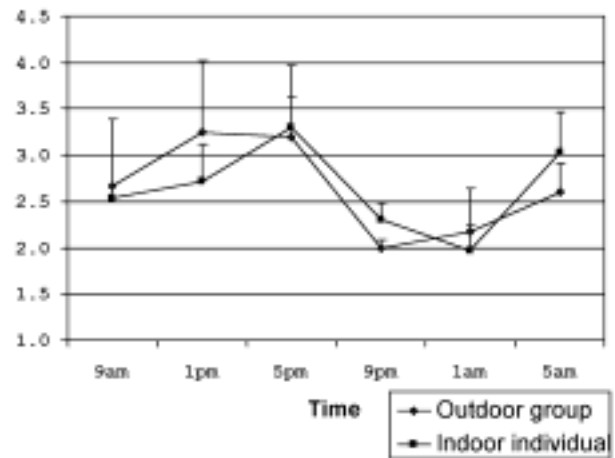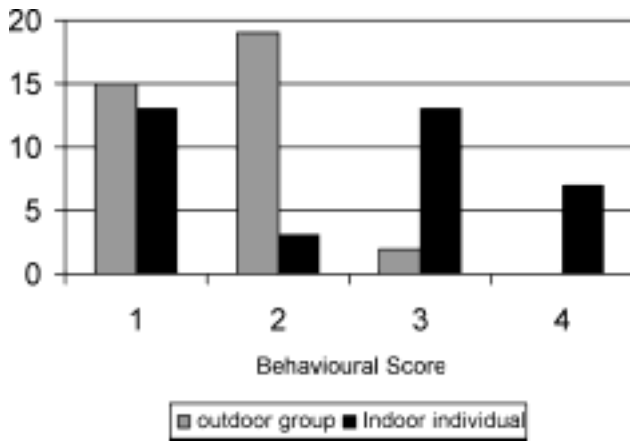
Figure 1. The frequency of observation of behaviors of assigned categories observed in 6 fillies during saliva sampling over a 24 hour period in an outdoor group paddock and a 24 hour period in an indoor individual housing. The frequency represents the sum for all 6 fillies of observations of each behavioral category (Tables 1 and 2)

Figure 2. Comparisons between the mean salivary cortisol concentrations of 6 fillies housed for 24 hours in an outdoor group paddock and 24 hours in an indoor individual housing (bars represent standard errors)
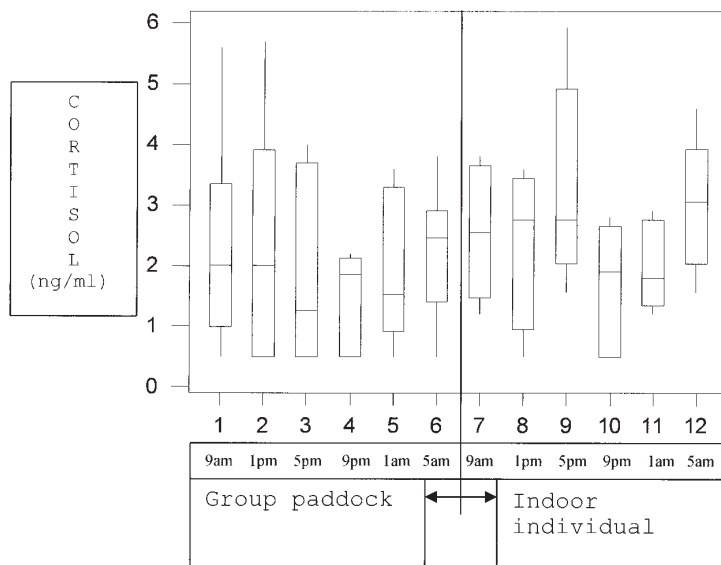

Figure 3. Box and whisker plot of the diurnal variations in mean salivary cortisol concentration (ng/mL) present in 6 fillies over 24 hours in an outdoor group paddock and 24 hours in an indoor individual housing

trial continued.[20] However, it is unlikely that this explains the unexpected drop in the heart rates observed, as the fillies had all been extensively handled since they were foals. In a study conducted by Bagshaw et al,[5] the highest heart rates were recorded 5 minutes after initial isolation of the horses. One of the limitations of the present study was that heart rates were taken at four-hour intervals, and it is possible that initial peaks in heart rate following stabling were undetected. It is not known why a significant difference was detected between the 5 pm heart rates.

While the current study did not show a significant increase in salivary cortisol with stabling and isolation, there

was a small increase of 0.5 ng/mL, and 5 of the 6 subjects had higher mean cortisol concentrations in the indoor individual housing than the outdoor group paddock. Previous studies on horses have had conflicting results. Mal et al[1] reported no significant differences between the plasma cortisol concentrations of horses placed in pasture, confinement, and isolation environments. In a study conducted by Alexander et al,[9] a decrease in plasma cortisol concentrations was reported in 2 out of 5 horses following isolation. More recently, a study on the effects of long-term isolation and confinement of mares reported significant increases in plasma cortisol following initial confinement.[22] Studies on the confinement and transportation of sheep[21] and cattle[16] have reported significant increases in salivary cortisol concentrations in the ranges of 7.2 ng/mL and 1.9 ng/mL, respectively. The current study design had sufficient power to detect a mean difference of 1 ng/mL at 90% power and significance level of .05. It may be possible that handled horses do not show the same magnitude of salivary cortisol change as other species, or that individual variation is greater. However, from the values in the literature from other species, this study should have had sufficient power to detect a significant change in salivary cortisol concentration.

The increase in cortisol concentrations did not correspond to the scale of the behavioral responses, and a high degree of variability in salivary cortisol concentration was found between individual horses. One of the fillies displayed a decrease in salivary cortisol concentration when stabled and, of the remaining five horses, only one showed an increase in free cortisol of greater than 50%. In pigs and horses an increase in free cortiocosteroids of 50% or more, which is sufficient to produce changes in secondary metabolism, is considered evidence of a real risk to welfare.[6,23] As only one horse displayed cortisol elevations in this range, it could be concluded that first time stabling is not a significant welfare concern for most horses with the assumption that the horses in the present study did not have elevated salivary cortisol concentrations prior to stabling.

Average equine baseline salivary cortisol concentrations were found to range between 0.5 ng/mL and 1 ng/mL in a study conducted by van der Kolk et al,[24] whereas Pell and McGreevy[25] found higher levels of between 1.5 ng/mL to 3.5ng/mL. While the current study's results, which ranged between 0.8 ng/mL and 3.6 ng/mL, agreed more closely with those of Pell and McGreevy,[25] it should be noted that Pell and McGreevy found high salivary cortisol values in conjunction with low plasma cortisol levels. This discrepancy of Pell and McGreevy[25] may be due to the stimulation of saliva production with acetic acid soaked swabs; low pH samples have been found to provide falsely high values in humans.[13] While many factors affect baseline cortisol concentrations including age,

breed, and past experiences, the high baseline values obtained in the present study suggest that the horses may have already had elevated basal cortisol concentrations. It has been suggested that animals with chronically elevated serum cortisol concentration may have a reduced ability to respond to further stressors via increases in cortisol concentrations.[6] This may explain the failure of the fillies to respond to stabling with an increase in free cortisol concentration, despite clear behavioral signs of stress.

In rats, it has been shown that handling alone is stressful enough to increase plasma cortisol concentration.[15] Similarly in sheep, baseline concentrations of salivary cortisol were significantly elevated in untrained sheep when handled as opposed to previously handled animals.[21] The fillies used in the study were unaccustomed to a bit and had no prior experience of the salivary sampling techniques used. It is therefore possible that the handling procedures and introduction of saliva sampling techniques were sufficient to cause an increase in their basal cortisol concentrations. No significant diurnal variation in salivary cortisol levels was found in the present study, in either the outdoor group paddock or indoor individual housings. Previous studies of diurnal cortisol rhythms in horses have produced variable results. Some report peak levels between 6 am and 9 am and trough levels between 7 pm and 11 pm, while others report that diurnal changes in cortisol concentrations do not or only occasionally occur in horses.[24, 26-28] In the only study known to assess the diurnal cortisol rhythm in both equine plasma and saliva samples, a diurnal rhythm was only evident in the results obtained from the plasma samples.[24] Such variability in results can be attributed to the inherent nature of cortisol secretion, which has been shown to be affected by many day to day variations including stress.[29] Exposure to an artificially extended or altered photoperiod has been shown to disrupt the diurnal rhythm in cortisol secretion, which occurs in horses.[9,30] It is therefore possible that the presence of a road light near the outdoor group paddock also contributed to the disruption of the cortisol rhythm in the fillies.[29] Sexual excitement has been shown to increase cortisol levels in stallions, and it is possible that the close proximity of a stallion to the fillies during the breeding season may have disrupted basal cortisol levels.[31]

In studies on squirrel monkeys it has been found that housing conditions were among the most important factors that influenced the quality and reproducibility of hormonal assessments.[32] Similar observations have also been made in horses. In a study conducted by Irvine and Alexander,[26] untrained thoroughbred mares in their home paddock and trained geldings in a stabled environment expressed a diurnal rhythm; however, untrained mares

placed in a large barn with dim lighting and untrained mares placed in a small yard for the first time did not express a rhythm. In this study, the diurnal rhythm of the horses was possibly disrupted in the outdoor group paddock environment. Even though the fillies in the current study were placed in the paddock two weeks prior to the experiment to habituate them to their surroundings, it is possible that they had not re-established a normal diurnal rhythm by the time sampling occurred.

CONCLUSION

The isolation and confinement associated with first time stabling was sufficient to initiate a stress response as indicated by behavioral changes in naïve horses. Although this was not reflected by a subsequent increase in salivary cortisol, it is possible that the fillies had previously elevated cortisol concentrations and were unable to respond to further stressors. The results of this study highlight that a combination of behavioral and physiological measures allow better understanding of stress, where one measurement may be misleading.

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