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Motivation and frustration of horses and mules: behavioral and physiological differences

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Frustration responses of animals when environmental resources are present, but inaccessible may help to understand their motivation, i.e. the importance to access these resources. However, neither motivation nor frustration have been investigated in mules. Here, we investigated whether horses and mules are motivated to access a biologically relevant resource and whether they express frustration when their access is blocked. Eight mules and eight horses were tested for 3 days with varying difficulty degrees requiring physical effort to cross a barrier and access feed. The maximum effort was made on day 3 (blocked barrier). The animals were filmed during the tests and their stress levels were evaluated. Only mules exhibited significantly more behaviors associated with motivation when the barrier was blocked. However, this test situation caused both mules and horses to express behaviors associated with frustration, whereas only horses expressed a greater variation in the cortisol level. Thus, only mules are motivated to access feed, but both of them exhibit frustration when unable to access such resources, which has important welfare and management applications.

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Preference tests are an approach based on the concept that preference responses of animals should be satisfied to ensure better welfare conditions for them [Dawkins 2006, 2008]. However, it is also important to identify how motivated an animal is to access specific preferred resources [Duncan 2006]. This may facilitate better selection of major resources for animals considering their preferences [Fraser and Matthews 1997]. Several studies have been carried out to evaluate motivation responses of animals in terms of effort made to access different environmental resources or conditions [Albentosa and Cooper 2005, Asher *et al*. 2009, Houpt 2012, Hovland *et al.* 2006, Mason *et al.* 2001, Matthews and Ladewig 1994, Patterson-Kane *et al.* 2011, Sherwin 2004]. However, to the best of our knowledge there are only a few studies in horses [Elia *et al*. 2010, Houpt 2012, Sondergaard *et al.* 2011] and no studies in its hybrid mules (*Eqqus caballus* × *Eqqus asinus*).

When an important environmental resource is present but inaccessible, it is possible that an animal would express some kind of a frustration behavior, because it is motivated to reach such a preferred condition. In fact, minks secreted high levels of cortisol, which was interpreted as a frustration response, when their access to a resource that they were mostly motivated to reach (a water pool) was blocked [Mason *et al*. 2001]. Thus, evaluating frustration response is a complementary approach that helps to better determine the importance of specific resources for animals.

Equids are intensively used for several economically important activities and thus are frequently maintained under restricted and artificial conditions, which can easily impair their welfare. Thus, evaluating the motivation and frustration behaviors of these animals to access resources is an important approach to improve their welfare, especially considering that they express behaviors that can be interpreted as frustration [Lesimple *et al*. 2012, Ninomiya *et al*. 2004].

In this study we evaluated whether horses and mules are motivated to access a biologically relevant resource and whether, once they are unable to access such a resource, they express responses indicative of frustration. Additionally, we also hypothesized that there are differences in motivation and frustration responses between horses and mules, as it is known that there are natural differences between the parental species and their hybrids [Burden and Thiemann 2015].

Material and methods

Animals and housing

All experimental procedures were performed according to the ethical principles for animal tests and were approved by the Ethics Committee on the Use of Animals (CEUA) of the University of Veterinary Medicine and Animal Science (FMVZ), UNESP, Botucatu, SP (Brazil). Protocol number: #134-135/2015.

The experiment was conducted in an experimental farm, located in Botucatu, SP (Brazil), under the geographic coordinates 22°49'56.19" S, 48°25'11.89"W, and 577 m a.s.l. We tested 16 equids (8 mules and 8 horses) mainly housed in an extensive system containing a native grass pasture, a natural watercourse, and mineral salt supplementation (*ad libitum)*. The mule group was composed of both males and females aged between 2 and 14 years (mean 9.12±3.75), weighing 340-400 kg (mean = 415.0±17.72 kg). The horse group comprised only mares, aged between 8 and 16 (mean = 9.75±3.28) years, weighing 350-450 kg (mean = 378.75±22.32 kg). These mares were the only equines available for experimentation at the time of this study and this group, similar to the mule group, comprised individuals living together for a long time (at least 8 years), born and raised on a farm. This indicated that there was already a clearly defined hierarchy among individuals in both groups, which could be disregarded as another variable in our study.

Moreover, these animals were sporadically supplemented with a commercial grain ration throughout their life – the resource further used for motivation tests, mainly during drought periods or after riding (animals were sporadically used for light riding around the farm). Both mules and horses were already accustomed to the grain ration, feeding management and other handling procedures that were used in our tests. However, to prevent influences on motivation responses of the animals, during the experimentation period the mules and horses did not receive feed before the tests.

General procedures

To evaluate the effort and frustration responses of horses and mules, the animals were subjected to a motivation test for 3 consecutive days with progressively more physical effort required from the individuals to push a barrier and access a biologically relevant resource each day. Thus, we used a strategy of gradually blocking the barrier that allows access to grain feed near a co-specific individual over the test period. This type of test is commonly used to infer the motivation of animals to access a desirable resource [Olczak *et al.* 2018, Bujis *et al*. 2001]. We also evaluated the variations in stress responses in individuals during the effort tests by measuring their cortisol levels in blood samples, as stress responses may also indicate frustration in animals [Mason *et al*. 2001].

Apparatus and testing area

We performed the experimental procedures in a compartmentalized corral with two open paddocks and an interconnected barn. For the experimental routine the horses and mules were maintained in paddock 1 (located 30 m away from the barn). Before starting the motivation test the selected equines were haltered and their blood samples were collected (basal cortisol). The animals were then led to paddock 2 and released from the halter in front of a closed wooden gate that divided the barn entrance. This wooden gate was used as a barrier in the motivation test.

Pilot test

To determine the intermediary difficulty degree (day 2) we performed a pilot test before the main experiment, where we added additional weight to the barrier until it reached the maximum weight of 138 kg (corresponding to a 200% increase in relation to the initial weight of the barrier). This final weight of the barrier was sufficient to create at least some degree of difficulty for the animals to cross it (practical observations) and at the same time did not put the whole structure at risk.

This pilot test was performed over 4 consecutive days with the same animals that were subsequently used in the experiment. During the pilot test the animals received grain feed at different times, ranging from 0 to 30 min after crossing the barrier. This procedure was performed to prevent animals from getting used to receiving feed as soon as they passed the barrier. This is because during the first two test days, although the animals had up to 30 min to pass the barrier, they could do it within 30 min, but they received the feed only 30 min after the start of the test (see: experimental design). Thus, such a procedure during the pilot test helped to avoid undesired effects of possible frustration not related to the actual effort of the animal. Moreover, as a successful conditioning procedure requires consistent training with an immediate reward after the expression of the desired behavior, the learning response of animals associated with the effort was prevented by the nonconsecutive tests and variable schedules of rewarding [Baragli *et al.* 2015, Foster 2017]. Additionally, because the animals always received the feed, we prevented the influence of possible frustration due to the absence of resources.

Moreover, considering that blood samples were used in our experiment to measure cortisol levels, during this pilot test animals were also habituated to the procedures related to the blood collection. Thus, in our tests all the animals were already habituated regarding this management. No traumas or fear associated with blood collection that could interfere with cortisol parameters were observed during our experiments.

Experimental design

The mule and horse groups were tested independently for 3 consecutive days, always in the morning period. On each of these consecutive test days the order, in which horses or mules were used in the experimental test area was randomized by drawing. Moreover, to increase the motivational response of the animals a co-specific individual was placed near the grain feed during all tests [Krueger and Heinze 2008], as these animals were accustomed to feeding together. The choice of the co-specific individuals used (1 for the horse group and 1 for the mule group) was based on previous observations of the hierarchical relationship among individuals of both groups. We used the leader of each group (these leaders were not dominant); they are animals that guide the group for resource achievement [Kang and Lee 2016]. These leaders were positioned backward and at a distance of 16 m from the barrier where the test was conducted (the other extremity of the paddock). These precautions were taken to prevent the inhibition of motivation responses of the test animals toward the feed by the dominance behaviors of the co-specifics. The leader animals were not used as test animals at any time.

On test day 1 the gate was maintained unlocked without the addition of any weight (control: 46 kg, day 1). On test day 2 the gate was maintained unlocked, but its weight was 200% of the original weight (138 kg, day 2). The addition of weight on day 2 was obtained by troughs and clay bricks of up to 200% of the original weight of the gate. On test day 3 the gate was locked with a steel chain (blocked barrier, day 3). The maximum degree of difficulty corresponded to the blocked barrier. This allowed us to evaluate the maximum effort that individuals were willing to spend to access resources.

Data collection

We determined a 30-min period for the effort tests as the level of cortisol in blood samples peaked after approximately 30 min from the moment a stressor appears or starts to manifest itself [Proops *et al.* 2009]. During this period in each test the animals were filmed and the frequency of their behavioral expressions indicative of effort (pushing the gate with the chest or neck – Burden and Thiemann, 2015, Miller 2001]), attention (both ears positioned simultaneously forward – Chamove *et al*. 2002, Kaiser *et al*. 2006, Young *et al*. 2012]) and frustration (repeated forward and backward motions of the body, both ears positioned simultaneously backward and nodding – Urden and Thiemann 2015, Lesimple *et al*. 2012]) were recorded at 30-s intervals for further analysis. The same observer registered all behaviors (Tab. 1).

Table 1. Detailed descriptions of registered behaviours indicative of motivation (effor t and attention) or frustration

Behaviours		Description
Effort	pushing the gate with the chest or neck	Head up, ears moving back and forth and muscles of the face contracted, the animal repeatedly pushes the barrier with its neck and chest
Attention	both ears positioned simultaneously forward	Alert posture with both ears directed to the attention target
Frustration	repeated forward and backward motion of the body	With both ears facing back and low head position (chest level or below), the animal moves its body forward and backward, eventually pushing the gate
	both ears positioned simultaneously backwards	Standing awake posture with both ears dir ected backwards for a long time
	nodding	With both ears directed backwards and visible mouth contraction the animal shakes its head vertically 3 or more times consecutively

During the first 2 days of the test (unlocked gate) the individuals that managed to cross the barrier in less than 30 min were removed from the penthouse (Fig. 1) before gaining access to the grain feed. These individuals remained in a separate paddock until the 30-min test period had elapsed. On day 3 of the test, as the gate was locked, the animals remained in the effort test area (paddock 2) until the 30-min test period had elapsed. After this period blood samples were collected on all test days and the animal gained access to the grain feed near the co-specific. When the individual did not manage to cross the gate after the 30-min period, the reward was not offered and the blood sample was promptly collected.

It is important to mention here that before the experiment the procedures related to feeding the animals in the barn were daily used in the natural management routine related to animal feeding. Thus, the tested individuals were already habituated to receive feed one by one inside the barn. Moreover, after each test the individual was immediately returned to its group and, as the test area (paddock 2) was not visually blocked during our experiments, the tested individual was able to see the whole group during the tests. These procedures were applied to avoid any kind of separation anxiety of the animals being individually tested apart from its group, which could have interfered with our results. None of the tested animals expressed conflicting behaviors when leaving the group and neither tried to return to it when they were alone for testing.

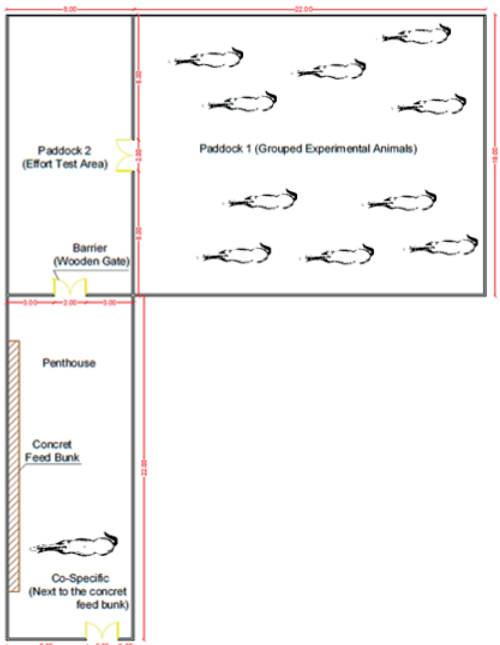


Fig. 1. Upper view scheme of the compartmentalized corral with opened paddocks and an interconnected penthouse for feeding managements and experimental procedures.

To evaluate the plasma cortisol level in horses and mules before and after the effort tests, blood samples were collected from the jugular vein using 25 mm × 8 mm BD Vacutainer® needles and 4-mL Vacutainer® BD tubes with EDTA for storage. This procedure was conducted in paddock 1. The test tubes with blood samples were stored under refrigeration (-18°C) and were centrifuged at 3000 rpm for 10-20 min.

Subsequently, the plasma was pipetted into 1.5-mL Eppendorf (JProlab®) tubes used as duplicates and then stored in freezers (©Beckman Coulter, 2000-2020). All samples were then sent to the Animal Reproduction Laboratory of FMVZ – Unesp, Botucatu, where they were analyzed using the radioimmunoassay technique.

Statistical analysis

The occurrence rate of each motivation or frustration behavior was compared between the test days (no effort, day 1; intermediary effort, day 2; and maximum effort, day 3) for each group (horses and mules) and between groups for each test day to better evaluate the differences between horses and mules. For comparisons among the test days in the case of horses the data corresponding to the types of behavior involving both ears positioned forward and nodding presented normal (Kolmogorov-Smirnov test, $P>0.05$) and homoscedastic distributions (Levene test, $P>0.05$) and therefore were compared using repeated measures ANOVA, with Tukey's test as the post-hoc test. The data for the other types of behaviors for both horses and mules presented non-normal (Kolmogorov-Smirnov, $P<0.05$) and/or heteroscedastic distributions (Levene test, $P<0.05$); therefore, they were compared using a corresponding test for non-parametric data, that is Friedman test, with Dunn's test as the post-hoc test. When Dunn's test was too rigid for Friedman's test (in other words, when statistical significance was found for Friedman's test, but not for Dunn's test), we applied Tukey's test as a post-hoc test.

Moreover, for comparisons between the animal groups the data corresponding to ears forward, ears backward, moving the body backward and forward, nodding and forcing the barrier with the chest, all on day 3, and forcing the barrier with the neck on all the test days presented normal (Kolmogorov-Smirnov test, $P>0.05$) and homoscedastic distributions (Levene test, $P>0.05$) and were then compared using Student's independent t-test. On the other test days for the same behaviors, as data were not normally distributed (Kolmogorov-Smirnov test, $P<0.05$) or were heterocedastic (Levene test, $P<0.05$), they were analyzed by the Mann-Whitney test.

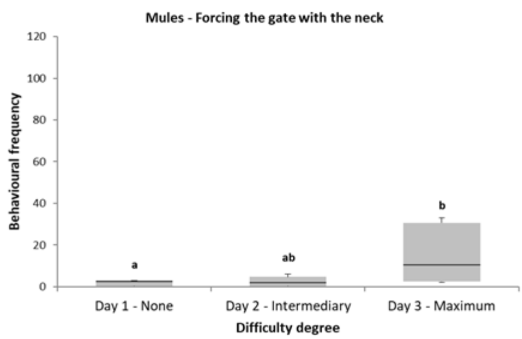
Cortisol levels were normally (Kolmogorov-Smirnov test, $P>0.05$) and homoscedastically distributed (Levene test, $P>0.05$), both considering comparisons between the test days and between groups. Thus, the variation between baseline and post-effort cortisol levels was compared between different test days for horses and mules separately using repeated measures ANOVA and between horses and mules by Student's independent t-test (t) for each test day. In addition, to assess whether baseline cortisol levels were similar among the test days, the data were also compared by repeated measures ANOVA over test days for each species. For these analyses we used Tukey's test as the post-hoc test. For all the statistical tests the level of significance was set to 0.05. The package program of Statistica 7.0 was used to analyze all data.

Results and discussion

Behaviors associated with motivation (effort and attention)

Regarding the physical effort made to access the feed near the co-specific, mules more significantly forced the barrier with the neck when it was locked in relation to the control test (p = 0.02; median, minimum and maximum effort with the neck, respectively: day 1 = 2.5, 0.0 and 3.0; day 2 = 2.0, 0.0 and 6.0; day 3 = 10.5, 2.0 and 99.0; day 3 compared with day 1, Fig. 2a). In addition, under the same test conditions the mules also positioned ears forward more often than in the control test (p = 0.03; median, minimum and maximum for positioning ears forward, respectively: day 1 = 4.0, 1.0 and 92.0; day 2 = 7.0, 0.0 and 43.0; day 3 = 265.5, 35.0 and 805.0; day 3 in relation to day 1, Fig. 2b). In contrast, horses did not present any significant difference between the test days in relation to the behavior involving forcing the barrier with the neck (p = 0.24; data presented as median, minimum and maximum: day 1 = 3.0, 0 and 7.0, day 2 = 3.5, 0 and 10.0, day 3 = 21.5, 0 and 41.0). The horses also did not express

(a)



(b)

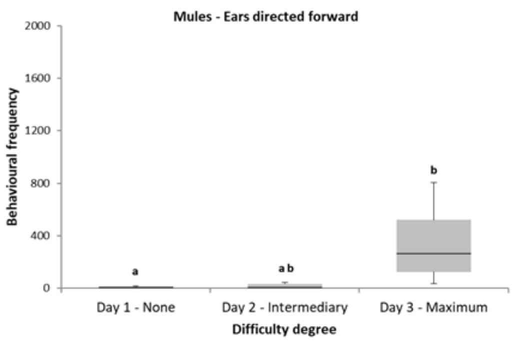


Fig. 2. Behaviors associated with the motivation of mules (effort and attention). (a) – behavioral frequencies of forcing the gate with the neck in mules compared between different difficult effort degrees imposed to access feed ration. (P<0.05). (b) – behavioral frequencies of ears positioned forward in mules compared between different difficult effort degrees imposed to access feed ration (P<0.05). Different lowercase letters indicate significant differences of applied effort between different difficulty degrees.

a significant difference in relation to the behavior of ears positioned forward between the test days (p = 0.62; data presented as mean±SD): day 1 = 224.25±382.09, day 2 = 141.50±187.9, day 3 = 251.0±169.58).

When directly comparing mules and horses, there was no difference between the test days for the behavior of forcing the barrier with the neck (day 1: p = 0.26; day 2: p = 0.28; day 3: p = 0.71) or chest (day 1: null data; day 2: p = 0.67, day 3: p = 0.41). Moreover, no difference was found between horses and mules for the behavior of ears positioned forward (day 1: p = 0.09; day 2: p = 0.1; day 3: p = 0.5).

Behaviors associated with frustration

Both mules (p<0.001; presented as median, minimum and maximum, respectively: day 1 = 0.0, 0.0 and 0.0; day 2 = 0.0, 0.0 and 0.0; day 3 = 4.5, 1.0 and 43.0) and horses (p = 0.02; presented as median, minimum and maximum, respectively: day 1 = 0.0, 0.0 and 1.0; day 2 = 0.0, 0.0 and 3.0; day 3 = 6.5, 0.0 and 17.0) expressed behavior that involved moving the body forward and backward more frequently when the barrier

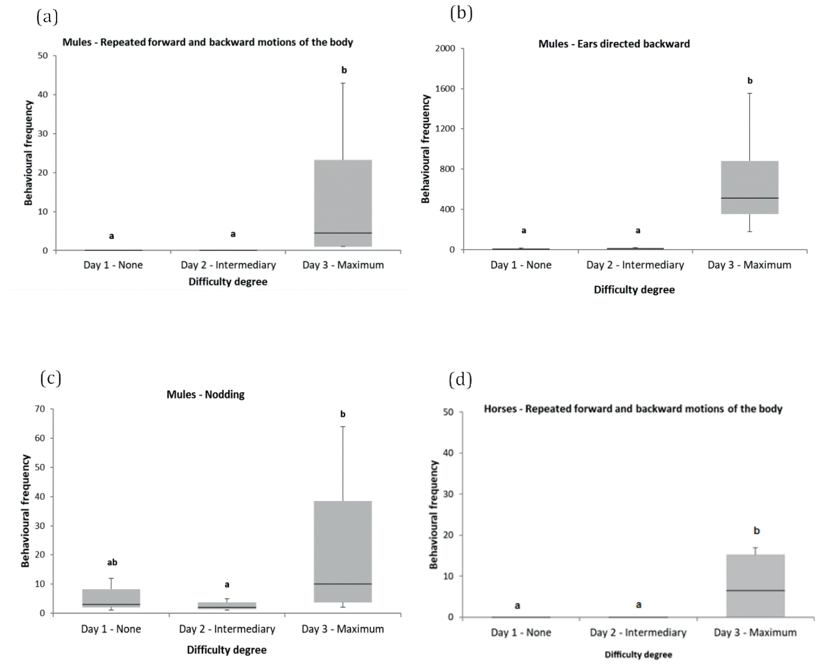


Fig. 3. Behaviors associated with frustration. (a) – behavioral frequencies of mules repeating forward and backward motions of the body compared between different difficult effort degrees imposed to access ration (P<0.05). (b) – behavioral frequencies of ears positioned backward in mules compared between different difficult effort degrees imposed to access ration (P<0.05). (c) – behavioral frequencies of nodding in mules compared among different difficult effort degrees imposed to access ration (P<0.05). (d) – behavioural frequencies of horses repeating forward and backward motions of the body compared among different difficult effort degrees imposed to access ration (Friedman test; P<0.05; Fr = 7.63). Different lowercase letters indicate significant differences of the applied effort among different difficulty degrees.

was locked (day 3; Fig. 3a and Fig. 3d). In addition, mules more often expressed behaviors that involved ears positioned backward (p = 0.00; presented as median, minimum and maximum, respectively: day 1 = 8.5, 5.0 and 174.0; day 2 = 9.0, 2.0 and 129.0; day 3 = 360.5, 75.0 and 828.0; Fig. 3b) and nodding (p = 0.01; presented as median, minimum and maximum, respectively: day 1 = 3.0, 1.0 and 12.0; day 2 = 2.0, 1.0 and 5.0; day 3 = 10.2, 2.0 and 64.0; Fig. 3c). These behavioral responses were not observed in horses (p = 0.24; behavioral data of ears positioned backward presented as median, minimum and maximum for each test day, respectively: day 1 = 9.0, 2.0 and 167.0; day 2 = 21.0, 2.0 and 167.0; day 3 = 254.5, 16.0 and 479.0; p = 0.94; data for the nodding behavior presented as mean±SD: day 1 = 24.6±29.5, day 2 = 23.4±30.6, day 3 = 20.2±16.2).

When directly comparing mules and horses there was no difference between the test days for the behavior moving the body forward and backward (day 1: p = 0.67; day 2: p = 0.67; day 3: p = 0.41 – Fig. 4a). However, for the behavior of ears pointed backward, mules presented it significantly more when the barrier was blocked (day 1: p = 0.15, day 2: p = 0.4, day 3: p = 0.023). For the behavior of nodding, horses

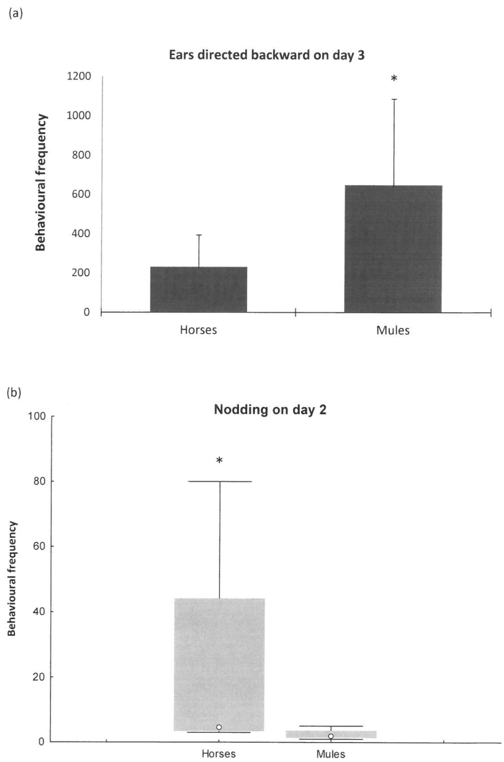


Fig. 4. Behavioral frequency of ears directed backward comparing horses and mules on day 3 (p = 0.023) and behavioral frequency of nodding when comparing horses and mules on day 2 (p = 0.01).

expressed it significantly more than mules when the barrier was 200% heavier (day 1: p = 0.1; day 2: p = 0.01, day 3: p = 0.99 – Fig. 4b).

Cortisol level

Neither mules nor horses expressed intense movement during the trials. Thus, the cortisol level should not have been influenced by exercise, as low and moderate intensities of physical exercise reportedly did not significantly affect the cortisol level in horses [Kang and Lee 2016, Linden *et al.* 1991].

Mules did not present a significant difference in the baseline (pre-effort) cortisol levels between the test days (p = 0.06; mean±SD, respectively: day 1 = 41.51±16.58; day 2 = 27.42±11.30; day 3 = 25.89±11.8 – Fig. 5a). However, horses had a significantly lower baseline level of cortisol on the last day of the test (p<0.001; mean±SD, day 1 = 36.78±6.12; day 2 = 33.09±6.78; day 3 = 20.81±4.96; day 3, locked gate – Fig. 5b). Considering the variation in the post-effort cortisol level in relation to the baseline cortisol level, mules did not present a significant difference between the test days (p = 0.71; mean±SD, respectively: day 1 = -4.35±18.26; day 2 = 0.57±11.46; day 3 = -6.54±14.14 – Fig. 6a). However, horses expressed a significantly greater variation between these levels on the last day of the test (p<0.001; mean and SD, respectively:

a

b

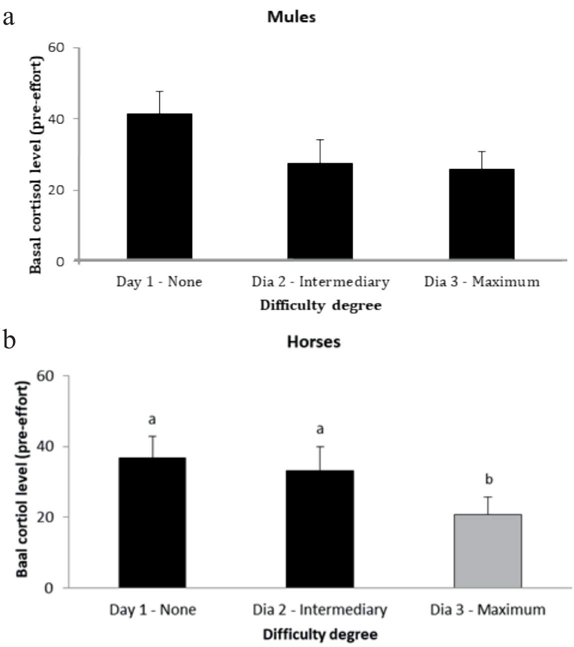


Fig. 5. Variation in basal cortisol levels between different days of the effort test. (a) – mules and (b) – horses. Different lowercase letters indicate significant differences (P<0.05). Note that mules did not express any significant difference over the test days, whereas horses expressed a reduced level of basal cortisol on the last test day.

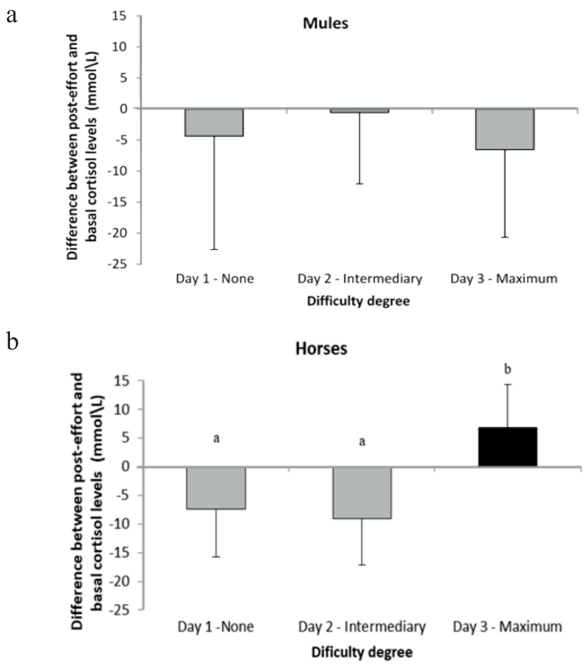


Fig. 6. Variation in the difference between post-effort and basal cortisol levels between different days of the effort test. (a) – mules and (b) – horses. Different lowercase letters indicate significant differences (P<0.05). Note that mules did not express any significant difference over the test days, whereas horses expressed an increased difference in cortisol levels on the last test day.

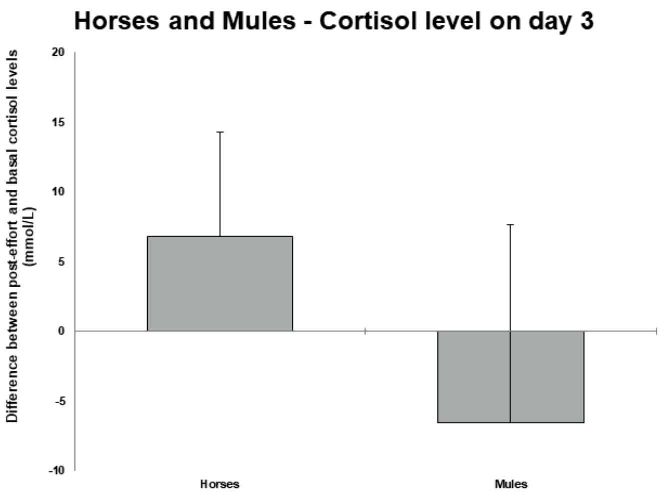


Fig. 7. Variation in the difference between post-effort and basal cortisol levels on day 3 when comparing horses and mules. * indicates a significant difference (P = 0.03).

day 1 = -7.46±8.26; day 2 = -9.11±8.05; day 3 = 6.77±7.51; day 3, locked gate – Fig. 6b).

When directly comparing mules and horses in terms of variation in the post-effort cortisol level in relation to the baseline cortisol level, there was no difference on day one or two (mean and standard deviation for horses and mules, respectively: day 1: -7.46±8.26 and - 4.35±18.26; day 2: -9.11±8.05 and -0.57±11.46). On the other hand, on day 3 horses presented a greater difference between post-effort and basal cortisol levels compared to mules (mean and SD for horses and mules, respectively: 6.77±7.51 and -6.54±14.14 – Fig. 7). Behaviors that did not differ significantly are detailed in Table 2.

Table 2. Detailed results for behaviors that did not differ significantly between horses and mules during the test days

Behaviours	Group	Day 1	Day 2	Day 3
Ears direct foward	horses	median = 61.5; min-max = 1-1102	median = 75.5; min-max = 4-543	mean±SD: 230.62±163.05
	mules	median = 4; min-max = 1-92	median = 7; min-max = 0-43	mean±SD: 648.25±436.89
		P = 0.09; U = 16	P = 0.1; U = 16.5	P = 0.5; t = -0.7
Forcing the barrier with the neck	horses	mean ±SD: 3.0±2.39	mean±SD: 4.25±3.80	mean±SD: 18.25±16.55
	mules	mean±SD: 1.87±1.35	mean±SD: 2.5±2.32	mean±SD: 23.12±32.59
		P = 0.26; t = 1.16	P = 0.28; t = 1.11	P = 0.71; t = -0.38
Forcing the barrier with the chest	horses	median = 0; min-max = 0-0	median = 0; min-max = 0-0	mean±SD: 0.62±0
	mules	median = 0; min-max = 0-0	median = 0; min-max = 0-3	mean±SD: 2.25±5.25
		-	P = 0.67; U =28	P = 0.41; t = -0.85
Ears directed backward	horses	median = 9; min-max = 2-167	median = 21; min-max = 2-79	mean±SD: 230.62±163.05
	mules	median = 3; min-max = 0-509	median = 7.5; min-max = 1-241	mean±SD: 648.25±436.89
		P = 0.15; U = 18.5	P = 0.4; U = 24	P = 0.023; t = -2.5
Nodding	horses	median = 10.5; min-max = 2-72	median = 4.5; min-max = 3-80	mean±SD: 20.25±16.16
	mules	median = 0; min-max = 0-0	median = 0; min-max = 0-0	mean±SD: 12.25±15.31
		P = 0.1; U = 16.5	P = 0.01; U =8.5	P = 0.99; t = -0.01
Moving the body backward and foward	horses	median = 61.5; min-max = 1-1102	median = 61.5; min-max = 1-1102	mean±SD: 230.62±163.05
	mules	median = 4; min-max = 1-92	median = 4; min-max = 1-92	mean±SD: 648.25±436.89
		P = 0.67; U = 28	P = 0.67; U =28	P = 0.41; t = -0.85

While mules demonstrate motivation by paying attention and by applying physical effort to access a relevant resource, horses do not express these motivational responses. However, not only mules, but also horses express frustration when they are unable to access the relevant resources. Mules express such a response through associated behavioral changes observed here as a higher incidence of repeated forward and backward motions of the body, nodding and ears positioned backward. On the contrary, horses express such frustration responses with minimal behavioral alterations. However, important physiological stress responses were not observed in mules, that is, a higher variation in the cortisol level when prevented from accessing the resource.

Mules are motivated to access a biologically relevant resource (grain ration), whereas horses do not present this response. When the wooden gate that provided access to the grain ration was blocked, only the mules pushed the gate with their necks and positioned the ears forward. This finding indicates that these animals make physical effort and display more attention when trying to access the resource at the maximum level of difficulty, whereas horses do not. To the best of our knowledge, this is the first study to demonstrate motivation to access resources in mules. In addition, blocking access to a resource is often used to determine the maximum effort that an animal is motivated to spend [Mason *et al.* 2001] and this was observed here when we blocked the gate. Thus, the fact that horses did not spend effort and did not express attention behaviors more frequently when the gate was blocked indicates that these animals are not motivated to reach a resource in situations, where access to such a resource is associated with great difficulty. Indeed, horses expressed no motivation to access feed even when the only thing needed to reach the resource was to repeat certain behavioral patterns [Olczak *et al.* 2018]. These findings indicate that mules are more persistent than horses. This can be attributed to cognitive differences between mules and horses that might be reflected in different motivational responses, as mules performed better than horses in cognitive tests in a previous study [Proops *et al.* 2009]. Another possibility is that mules and horses have different ways of coping with difficulty in accessing resources [Proops *et al.* 2009, Burden and Thiemann 2015]. However, additional studies are required to clarify this issue.

Here, in addition to motivation responses mules also demonstrated frustration when they were unable to access relevant resources. Based on our results, when trying to cross a blocked barrier to access feed near a co-specific, mules frequently expressed behaviors that can be considered indicative of frustration responses [Lesimple *et al*. 2012, Ninomiya *et al*. 2004), such as moving the body forward and backward repeatedly, positioning ears backward and nodding. Moreover, mules also positioned ears backward more frequently than horses when the barrier was blocked. These findings corroborate the idea that these animals may, in fact, become frustrated when they cannot access some relevant resources in the environment, as proposed by Hansen and Jensen [2006] for minks.

On the contrary, horses displayed minimal behavioral changes that can be considered indicative of frustration. Although the nodding behavior was significantly more frequent in horses than in mules on day 2, by observing the frequency of such behavior in horses it was constantly expressed by such animals during all the test days. This was not the case for the mules, which expressed it significantly only when the barrier was blocked (day 3). Thus, in the case of horses the nodding behavior seems to be better related to an anticipation of the arrival of feed [Cooper *et al.* 2000] as a pre-feeding stereotypical behavior instead of a frustration behavior.

However, when the gate was blocked, horses expressed a greater variation in post-effort compared to baseline cortisol levels, indicating a physiological stress response in situations where the resource is inaccessible. This response was reinforced by the fact that horses also presented a greater difference between post-effort and basal cortisol levels than mules when the barrier was blocked. In this particular case it can be assumed that the stress response also indicates frustration [Mason *et al*. 2001]. Thus, although horses and mules express their internal states in different ways, they are frustrated when they are unable to access a relevant resource. These differences between the hybrid and its parental species can be explained by the fact that horses exhibit high cortisol levels even under conditions of low stress [Kedzierski *et al*. 2014], which indicates that they are sensitive animals. Moreover, mules are known for their rigidity [McLean *et al*. 2019] and perseverance [Osthaus *et al*. 2012]; therefore, they may demonstrate greater resistance, expressed with lower variations in cortisol levels.

Here, on test day 3 (blocked barrier) the basal cortisol levels in horses were lower than on the other test days; it may be assumed that the difference between post-effort and basal cortisol levels on day 3 was only a consequence of such lower basal levels of cortisol. However, this should not have been the case here, as the cortisol levels detected after the effort test were evaluated only in comparison with the baseline levels presented on each test day, and not independently. In this context, such a significant variation indicates a considerable increase in cortisol levels, which suggests a stress response in situations where access to a relevant resource is blocked. Therefore, this lower level of basal cortisol in horses on test day 3 did not negate the observed results regarding the significant variation in cortisol levels. Another possibility is that the lower level of basal cortisol on test day 3 reflects the process of habituation of the animals to the test conditions, a response not observed in mules. In fact, horses have the capacity to quickly habituate to different environmental stimuli [Miller 2001]. This fact is also consistent with the idea that there are differences between the parental species and their hybrids [Burden and Thiemann 2015].

It is important to mention that we used the same individuals in the pilot test and the experiment. This may have influenced the motivation and frustration responses of animals evaluated here, such as conditioning of the equids to the test conditions. However, because the pilot test was conducted on non-consecutive days and included a variable time schedule of animals receiving feed, the influences of previous experiences in these animals related to the findings discussed here might have been minimized. Furthermore, this study lays a foundation for further investigations especially in mules, because to the best of our knowledge no other study has evaluated the motivation or frustration of mules to obtain a biologically relevant resource. Thus, future studies can generalize the findings of this study.

Thus, although only mules are motivated (expressing behaviors indicative of effort and attention) to access a biological relevant resource, both horses and mules are frustrated when they cannot access such resource. In addition, while behavioral changes are the basis for the expression of this frustration response in mules, physiological changes associated with stress responses are more relevant for horses. Thus, for equine management it is important to maintain a routine feeding practice (work plan) that avoids possible frustration and stress of animals when perceiving the feed that is not yet available, which in turn would impair the welfare of such animals.

The authors declare no conflict of interest.

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