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Physiological (heart rate and cortisol concentration in saliva) and behavioural responses in horses to four reactivity tests compared to the trainer's opinion about the horses' temperament

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Physiological (heart rate and cortisol concentration in saliva) and behavioural responses in horses to four reactivity tests compared to the trainer's opinion about the horses' temperament

Fysiologiska (hjärtfrekvens och kortisolkoncentration i saliven) och beteendemässiga reaktioner hos hästar i fyra reaktivitetstester jämfört med tränarens uppfattning om hästarnas temperament

Hanna Selinus

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Table of content

Abstract

When performing studies that test the reactions of horses in different situations, important information may be available about the horse's reactivity level. With information from these tests, accidents and dangerous situations may be prevented. When the behaviours from tests are compared with race performance it may be possible to evaluate if some behaviours are more desirable at the race track than others. The aim of this study was to see if behavioural responses correlated with physiological parameters (heart rate, cortisol), the trainer's opinions and actual race performance. Furthermore, it was investigated whether there were any significant differences in physiological and behavioural responses of horses between the first and the second day of testing.

The reactions of 16, two-year old Swedish Standardbred trotters were tested in four different behaviour tests. The tests included: 1) novel objects (blue rubber ball and bridge), 2) novel smell, 3) sudden sound and 4) isolation from other horses. The horses experienced all four tests during one day before and once after a 3-day confinement in boxes using the same experimental procedures. Behavioural reactions to the different stimuli and to social isolation were recorded (e.g. move feet, touch object or defecate) and heart rate was measured throughout testing. Three samples of saliva were taken each day (control sample before the tests, after the reactivity test and after isolation) to measure the concentration of cortisol. Furthermore, the horses' trainer filled in a form where he graded different temperamental traits of the horses.

The results indicated that the behaviours were not affected by the 3-day stall confinement or repetition of tests since there were no significant differences between days. The heart rate was lower day two compared with day one, which may be due to that the horses were habituated to the tests on day two. The cortisol level was higher on day two, indicating that the horses were more stressed compared to day one. Some behaviours from the tests had a strong correlation with the trainer's opinion of the horses' temperament. This included the behaviour 'alert' recorded during the tests that had a strong correlation with the trainer's opinion of the horse being 'afraid' and 'spooky'. The latter behaviours also correlated with higher winning percentage or place percentage, indicating that these behaviours could be more favorable on the race track than during daily handling and training.

Sammanfattning

Att studera beteenden på hästar i olika situationer och jämföra med relevant litteratur kan ge viktig information hur individer reagerar i olika situationer. Med kunskap från beteendetester kan olyckor och farliga situationer förebyggas genom att i vissa fall kunna förutse reaktioner. När beteenden i tester jämförs med prestationer på tävlingsbanan kan det också vara möjligt att se om vissa beteenden är mer önskvärda när det kommer till att prestera bra.

Denna studie syftade till att se hur 16 stycken tvååriga travare reagerade på okända föremål (blå gummiboll och bro), okänd lukt, plötsligt ljud och när de blev isolerade från andra hästar. Testet genomfördes på samma sätt under två dagar med tre dagars boxvila mellan. Fyra olika beteendetester genomfördes och pulsen mättes hela tiden. Kortisolprover togs från saliven vid tre tillfällen (ett kontrolltest innan beteendetesterna, ett prov efter reaktivitetstestet samt ett efter isolering). Hästarnas tränare fick fylla i ett formulär där han graderade olika egenskaper hos hästarna.

Resultaten visade att det inte fanns några signifikanta skillnader i beteendefrekvenser mellan dagarna, vilket tyder på att hästarnas beteende inte påverkas av tre dagars boxvila eller av att testen upprepades. Hästarnas puls var lägre dag två, vilket kan bero på att de dag två var mer vana vid miljön och testerna. Kortisolnivån var högre dag två, vilket indikerar på att hästarna kan ha varit mer stressade dag två jämfört med dag ett. Vissa av beteendena hade en stark korrelation med tränarens uppfattning om hästarna temperament. Ett fåtal av dessa temperamentsegenskaper korrelerade också med en högre vinstprocent eller platsprocent. Detta tyder på att vissa beteenden skulle kunna vara mer önskvärda på tävlingsbanan.

1. Introduction

Horses are prey animals and flight is their main defense mechanism (Spier et al., 2004). Even though they have been selected for breeding by humans and specific traits such as ease of handling have been favored, horses' instinctive response to danger is to flee. Since horses are social animals and prefer to live in herds, situations that include isolation from conspecifics and confrontation with scary objects can affect the behaviour and trigger a flight response. Horses have been domesticated for a long time, but there has been no selection for a horse that can spend most of its time inside a very limited area. Nevertheless many horses often spend much of their time confined in stalls without the possibility to move freely in outdoor paddocks.

If horses are selected mostly based on their performance and less on temperamental traits, this may lead to more reactive horses that could be difficult to handle. Because of this, it is important to know more about different horses' temperament. The more knowledge about individual differences in temperament, the greater is the chance to minimize potentially dangerous situations and accidents. Traditionally English thoroughbreds have been selected for speed and "fighting spirit" (Arnason & Dale Van Vleck, 2000). The English thoroughbred is, maybe because of this, said to be quite reactive and more nervous, whereas the Swedish Standardbred trotter is said to have a more balanced temperament (Lloyd et al., 2007; ATG, 2014). The breeding goals for the Standardbred trotter is to produce a healthy horse that is easy to handle and has a distinct winning touch. This makes it interesting to study Standardbred trotters in behaviour tests and compare results to studies performed with English thoroughbreds since both breeds are bred for high speed.

Behavioural tests are an important way to get information about how horses react in different situations, such as when coming in contact with new objects. In a study by Lansade et al. (2007) reactivity was tested in Welsh ponies and Arabian horses with an age of 8 months and then again at 1.5 and 2.5 years of age. The authors tested the existence of the trait 'gregariousness' (social reactivity) in different test situations; during isolation and separation, and attraction towards and passing other horses. Different behavioural parameters were recorded during all four tests. Behavioural indicators, for example for anxiety, can be neighing, defecating, raising the head when walking towards a novel object or circling in the box when being isolated. The study showed that behaviour tests performed when horses are young can predict how the same horses would react in certain situations at an older age. This is because behavioural reactions in relation to the trait gregariousness were consistant across situations and years.

Another way to analyze reactions of horses to the same situation is to measure physiological parameters such as heart rate (HR) and cortisol. This can assist in the interpretation of behavioural responses and even small fluctuations in behaviour that are otherwise not possible to notice, may be detected. Measurements of HR and cortisol are objective ways to detect differences and correlations between and within horses (Tarrant & Grandin, 2000). The concentration of cortisol in blood and saliva is a known indicator of stress (Sjaastad et al., 2003). This can be a temporary increased level of cortisol or prolonged periods of increased levels due to severe chronic stress. It is though important to keep in mind that the quantity of cortisol in saliva is less than in plasma (Möstl & Palme, 2002).

A lot of behavioural studies concerning reactivity in horses are available, but most are focused on riding horses such as the Swedish-, Dutch- or Danish Warmblood horse (Visser et al., 2010; Visser et al., 2001; Winther Christensen et al., 2005) or the Arabian horse (Heleski et al., 2008; Lansade et al., 2007). Behavioural studies using horses bred for racing are rare, especially studies where Standardbred trotters were tested. For example, Larose et al. (2006) studied the reactivity of Standardbreds in a novel object test. Another study was performed by Lee Butler (2009) who studied stereotypic behaviours in Standardbreds while they were fed twice daily with concentrate feed. Studies of

Standardbreds could be very interesting since they are bred for alertness and fast reactions as well as for high speed.

To evaluate a behavioural test, it may be advantageous to be able to compare the results from the test with the opinions of a person who knows the horses well and who has a long experience of handling and training horses. Therefore, in the present study, the horse-trainer filled out a form where he could grade different temperamental traits for each horse. The aim was to study how well the trainer's opinion of the horses' temperament and willingness to run corresponds with results from the behavioural tests and their performance on the race-track. Momozawa et al. (2003) concluded that a survey is an effective way to estimate temperamental traits in horses.

The reactions of 16, 2-year old Swedish Standardbred trotters towards novel objects (rubber ball, bridge), novel smell (pig odour), and during isolation from conspecifics and sudden noise were tested twice, once before and once after a 3-day confinement in boxes (with 45 minutes of exercise per day). The overall aim of this study was to investigate whether behavioural responses correlate with physiological parameters (heart rate, cortisol), the trainer's opinions and actual race performance. The aim was also to compare the two test days and see if there were any significant differences between days.

This study is a sidetrack from another more extended study evaluating health, training response and performance in 1.5-3 year old Standardbred racehorses fed a forage only diet.

2. Literature review

2.1. Behavioural tests

Behavioural tests are important sources of information. By documentation of responses from horses to different test stimuli one may predict their reactions in certain situations, thereby help decrease the risk of accidents when interacting with horses. In behavioural tests, the horse's approach to different situations is observed and recorded (Hausberger et al., 2007). There are a few behavioural studies done on Standardbreds. One of them is from Larose et al. (2006) who studied Standardbreds in a novel object test and found that the more emotional the horse seemed to be, the higher percentage it seemed to look at the novel object with the left eye. They also found that the age of the horses didn´t seem to effect the results.

There are a lot of behavioural tests available in different species and breeds (Ijichi et al., 2013; König von Borstel et al., 2011; Lansade et al., 2007; Forkman et al., 2007). With a behavioural test it is possible to compare the reactions from different horses towards the same stimuli using the same ethogram.

Fear is an emotion that can be triggered by exposing individuals to situations that involve pain or stress. Fear-reactivity is, according to König von Borstel et al. (2011), stable across time and situations and one of the most important components of temperament when studying behaviours in a novel object test. A temperament trait is according to Lansade et al. (2008a) a behavioural tendency present early in life which is relatively stable across various kinds of situations and over the course of time. Neophobic reactions, i.e. fear of novel things seem to be more influenced by genetic factors while reactivity to social separation is more influenced by environmental factors (Hausberger et al., 2004).

Lansade et al. (2008b) studied sensory sensitivity as part of the horse's temperament. This reflects how much the horse reacts to auditory, olfactory, tactile, gustatory and visual stimuli. The reactivity to these five sensory stimuli was tested in two identical tests with an interval of five months. They found that all the sensory sensitivities were stable across time. Lansade et al. (2008b) suggested that by identifying how sensitive horses are for these kinds of different stimuli training and handling could be adjusted.

Isolation

Harewood et al. (2005) concluded that first time stabling induced some stress even though HR and cortisol concentration were not significantly higher compared to being on pasture. A group of fillies was studied 24 hours on pasture and then 24 hours during individual housing indoors. When comparing the two different housing systems, Harewood et al. (2005) found that some stress-related behaviours were seen more frequently indoor. These were, for example, throwing head, pawing, frequent vocalizations and moving. Lansade et al. (2007) studied how 110 horses reacted in four specific situations, including separation and isolation. They wanted to see the existence of the trait gregariousness. The separation test was divided into two phases, during phase one the test horse could see two other horses, and during phase two these horses were removed from the test horse. The test horse was then observed for 90 seconds while being alone and 12 behaviours were recorded. Some of the horses were tested in their boxes and some in a pen. In the isolation test, the test horse was removed from the other horses, isolated and observed for five minutes. Eleven behaviours were recorded. Lansade et al. (2007) found that the neighing behaviour correlated with some other behaviours such as defecation, locomotion and vigilant behaviour. The neighing behaviour also showed a quite strong stability over time. However, the boxes were of a smaller size than the pen and therefore restricted the movements more whereas the pen was bigger in size and allowed the expression of more/other behaviours.

In the same study, horses were also tested at different ages (between eight months and 2.5 years) to see if there was an effect of age. Lansade et al. (2007) found that, for example, the frequency of neighing decreased with age. According to the authors this could be due to maturation of the horses and habituation to the tests over time (Lansade et al., 2007).

In another study, 36 mares were isolated in stalls and nine behaviours were recorded (Mal et al., 1990). The authors concluded the mares were more affected by being socially isolated than just being confined while allowing social contact with neighbouring horses (Mal et al., 1990). The effects of long or short-term isolation can be increased heart rate and higher concentration of cortisol in plasma as well as higher frequency of defecation and vocalization (Harewood & McGowan, 2005).

Smell

Horses are sensitive to smells and olfaction is an important sense when it comes to the recognition of conspecifics or the rejection of “new” food or water (Saslow, 2002). How the horse reacts to new smells can be of great importance, especially if it triggers fear responses. Christensen et al. (2005) studied horses’ reactions to a novel visual, auditory and olfactory stimulus. In the olfactory test (smell), eucalyptus oil was added to the edges and inside a feeding container. This container contained feed the horses were used to. The authors found, that compared with a control situation, the eucalyptus oil reduced eating time and increased investigation time. In particular, the behaviours “investigate food” and “sniff food” had a higher frequency compared with the control situation. The horses also had an increased frequency of eating bouts and they became more watchful of their surroundings (Christensen *et al.*, 2005). The authors did not find an increased HR due to the olfactory stimulus. This result is consistent with Herskin et al. (2003), who studied cattle. Herskin et al. (2003) concluded that cattle that were offered their usual food but with 80 drops of eucalyptus oil had no increase in HR. Therefore, they suggested that novel food was not a fear-inducing stimulus (Herskin et al., 2003).

Lansade et al. (2008b) did an olfactory test with 26 Welsh ponies. Under the opening of a 2 cm hole in a feed trough, a Petri dish was attached, containing a compress either soaked in cinnamon liquid or lavender. The test lasted for two minutes. They recorded how many seconds of the two minutes the pony spent with the nose near the smell zone (the Petri dish). The authors tested five different concentrations of cinnamon and lavender (1-5 ml), cinnamon during 5 days and a week later lavender. The authors found that only the highest concentrations of lavendel and cinnamon interested the ponies. But since the ponies were exposed to the highest concentrations first, it is possible that they lost interest for the lower concentrations because of habituation to the smell (Lansade et al., 2008b).

Strong smells and pheromones may induce the flehmen behaviour when the horses lift their upper lip. Pheromones are air born chemical substances emitted into the air and perceived by other horses using the flehmen behaviour. Even foals have been shown to use the flehmen behaviour when trying to sense some substances, possibly pheromones (Crowell-Davis & Houpt, 1985). Pheromones may affect the behaviour of the horse. In a study by Falewee et al. (2006) two groups of horses were tested in a fear-eliciting situation where one of the group had been treated with pheromones (equine appeasing pheromone). The results showed that horses treated with the pheromones showed less behaviours associated with fear and a lower heart rate (Falewee et al., 2006). Pheromones can also act as stress inducing. An animal that is frightened and maybe does not want to enter a specific room can secrete fear pheromones and make other animals refuse entering the room as well (Grandin, 1993).

Sound/hearing

Horses have a wide range of hearing from 55 Hz to ca 25 kHz. Compared to other mammals horses have a good low-frequency hearing (Heffner & Heffner, 1983). Christensen et al. (2005) investigated horses' response to an auditory (sound) stimulus and how that affected the eating behaviour. The auditory test comprised of a sound (white noise) from a cd player placed behind a feed container. Eating time was reduced compared with a control test when no sound occurred. The behaviours "alert food", "investigate food" and "backing away from the sound" had a higher frequency than during the control situation. The auditory stimulus also resulted in an increased HR. There were negative correlations between the HR and time spent eating (Christensen et al., 2005).

A sound test was also performed by Lansade et al. (2008b). The ponies were exposed to a short sound, six meters away. This trial was divided into two parts. The sound during the first part, i.e. the first five days, comprised of a beep and during the second part (a week later) a shrill beep. The ponies were tested in pairs and with five sound intensities during these days. The reaction to the sound was recorded on a scale from 0 to 3. The result showed that a sound with high intensity resulted in a greater response, i.e. increased movement of the ears/head (Lansade et al., 2008b).

2.2. Heart rate

The heart rate (HR) is primarily regulated by the parasympathetic nervous system and the sympathetic nervous system. Excitement or fear trigger the sympathetic system and increase the HR (Sjaastad et al., 2003). It is important for horses used for sports and recreation, to learn and remember new tasks, but a frightened horse can have difficulties with this. The HR of horses when they are exposed to stressful environments can predict the performance of horses (Christensen et al., 2013). Visser et al. (2002) studied 41 young horses to evaluate the effect of training on HR and whether HR could predict temperament. The horses were divided into two groups where one group was trained from six months and the other group was not. The test included a novel object and walking over a bridge. The authors found that there were significant differences in HR and heart rate variability (HRV) between individuals when facing the novel object and the bridge and HR was higher for the untrained horses. They concluded that HR and HRV are useful when comparing temperament between individuals (Visser et al., 2002).

The rider or handler can influence and control the horse's reaction in a test situation, but according to König von Borstel et al. (2011) not even a good rider can hide all reactions to, for example, a novel object. In this study by König von Borstel et al. (2011) the horses' heart rate seemed to be quite unaffected by the handler or rider, whereas HRV seemed to be under strong influence by the rider or handler. The authors concluded that the HR and HRV are variables that can facilitate the evaluation of a horse's temperament whether it is during riding or handling. It is especially applicable when evaluating the behavioural trait reactivity in the horse. Leading resulted, with few exceptions, also in the least behavioural reactions in this study, while riding resulted in a medium or strong reaction. Both leading and riding were done while walking to minimize the effect of physical activity on HR. This is consistent with Visser et al. (2002) that concluded that the increase in mean HR when exposed to a novel object was rather due to emotional reactions than physical activity. König von Borstel et al. (2001) also concluded that the horses' reactions were stronger to a moving stimulus approaching from behind than a stationary visual or a tactile stimulus. The moving stimulus consisted of a bag with cans falling to the floor. The visual stimulus was a red rubber ball and the tactile was a brown floor mat the horses had to cross (König von Borstel et al., 2001). However, in another study by Keeling et al. (2009) the authors found that an increase in HR in the human led to increased HR in the horse. This was true for both leading and riding.

There have been several conclusions drawn about how HR is affected by stable confinement. Rivera et al. (2002) studied three groups of horses; horses on pasture with training, horses in stable with training and a control group with no training (half of them on pasture and half in stable). HR was recorded one hour before training, horses on pasture were led to the stable where the recording of HR took place. Rivera et al. (2002) found no significant differences in HR between horses on pastures and stabled horses. Neither did Harewood et al. (2005) who compared behavioural indices, HR and cortisol concentration in the saliva between horses on pasture and horses stabled for the first time. However, they found a slightly lower mean HR (even if not significant) on horses stabled indoor compared to outdoor stabling.

2.3. Cortisol

Stress can increase the secretion of cortisol in the blood. It stimulates the degradation of protein and fat and thus makes more energy sources available. It is important for an animal to enable it to cope with stress and it has an anti-inflammatory effect (Sjaastad et al., 2003). It is also possible to measure cortisol level in the saliva. Measurement of saliva cortisol levels have earlier been used to measure horses' reactions to stall confinement compared with when kept outdoors. Harewood et al. (2005) could not find any significant difference in saliva cortisol levels between horses that were outdoor and horses that had been stabled for 24 hours. Harewood and McGowan (2005) studied the response to isolation in young horses by looking at behaviour, heart rate and cortisol concentration in saliva. They found no significant increase of cortisol concentration due to isolation. This is in contradiction to Houpt et al. (2001) who found an increased concentration of cortisol after short-term isolation and confinement. Horses have a normal concentration variation in the daily cortisol cycle. For the untrained horses the concentration seems to peak in the morning and has a lower concentration in the evening, though there are individual differences as well (Irvine & Alexander, 1994). A novel environment seems to affect the cortisol level. In the study by Irvine and Alexander (1994) the mean level was higher for horses in the novel environment than in a familiar.

3. Material and method

3.1. Horses and management

A total of 16, 2-year-old Standardbred trotters, all geldings, were used in this study. The horses came from four different breeders and had been kept at the study site Wången (National Center for Education in Trotting) since they were 1.5 years old. All horses were regularly turned out together in a paddock (~20000m^2) with access to shelters, feed and water for 8 h/day during 4 days/week and for 24 h/day during 3 days/week. Inside, horses were stabled in boxes measuring 9 m² each and contained sawdust as litter. Horses were fed a forage only diet *ad libitum* and had been fed according to this for more than one year before the study started. This was defined as always having at least 2 kg of haylage left in boxes. When kept inside, the horses received haylage in a large crib placed on the floor. The nutrient content in the haylage can be found in table 1. The haylage was mainly consisting of Meadow fescue, Timothy and Ryegrass. The diet was complemented with a commercial mineral- and vitamin supplement and some pelleted Lucerne soaked in water prior to feeding. Horses were trained since the age of 1.5 years old with the goal to race at three years old.

Table 1. Nutrient content in haylage fed to the horses during the study

Content and nutritional value	Per kg feed	Per kg DM	Unit
Dry matter (DM)	67		%
Crude protein	90	134	g
Digestible crude protein	63	94	g
Metabolizable energy	7,0	10,4	MJ
NDF	349	522	g
Calcium	2,9	4,4	g
Phosphorus	1,6	2,3	g
Magnesium	1,4	2,1	g
Potassium	13,4	20,0	g
Sulphur	1,3	1,9	g
Calcium/Phosphorus (Ca/P)		1,9	ratio

3.2. Test description

The behavioural test was performed during two days in December when the horses were two years old. Between the two test days, all horses were kept in their home boxes for three consecutive days with 45 min per day of either exercise at the race track or in a walker as part of their regular training routines.

All 16 horses were tested on both days, and the horses were tested in the same test order both days. However, the order of horses was chosen randomly. The test was divided into four parts as follows: reactivity test (foreign object/walking over bridge), isolation test, noise test and smell test.

The horses were familiar with the test areas during the reactivity (room) and smell test (home box), but not familiar with the box during the isolation and sound test. Furthermore, the bridge (scale) was novel although horses were habituated to another scale with different design.

The horses were led by the same handler during all tests and were treated equally as far as possible. All tests were documented on film. Heart rate was measured for the horses all the time (Polar CS 600X, Polar, Finland). Saliva samples were taken on three occasions to analyse the cortisol level.

3.2.1. Reactivity test - foreign object and walking over bridge

The design of the reactivity test room is shown in figure 1. The horses were led from the home box into the room where a foreign object was placed on the floor (a blue rubber ball, ~70cm diameter). They were walked pass it and then stopped approximately two meters past the ball, the rear end turned towards it. The ball was then rolled from behind the horse by a person, passing about one meter from the horse on its left side. The ball went into an opaque barrier to keep it still and out of sight. When the horse wasn’t able to see or hear the ball anymore it was asked to move forward and cross a bridge (scale) positioned in the other end of the room (figure 1). The handler did not try to pull the lead rope or talk to the horse when trying to cross the bridge. The test lasted about one minute and the behaviours were recorded according to the ethogram in table 2.

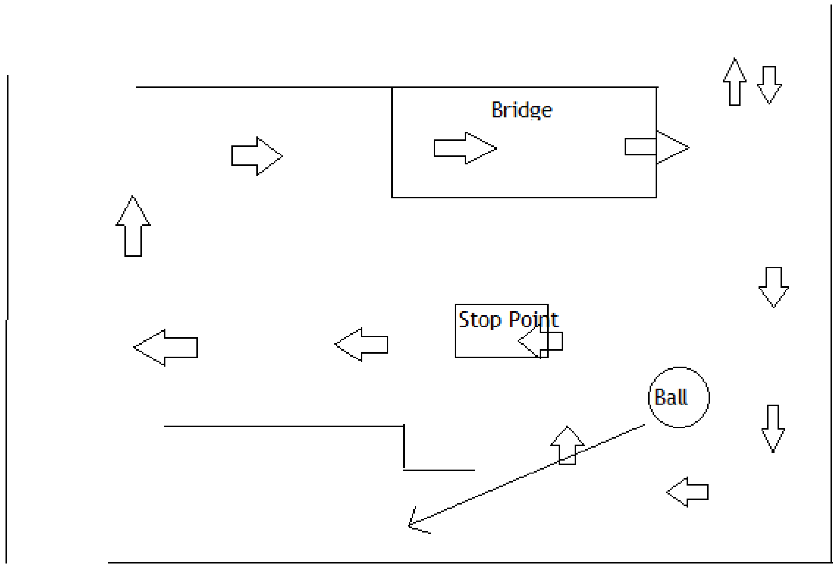

Figure 1. Design of the room for the reactivity test (not drawn to scale).

3.4. Isolation test

After the reactivity test, horses were led to a stable path, where a saliva sample was taken. After this the horses were led into a box situated in a different stable building and left there alone for six minutes. The isolation box (9 m^2, saw dust bedding) was located in an empty stable with restricted view to surroundings, but not totally sound proof. Some hay was offered in the box during the test. After the isolation test ended, horses were led back to their home-box and another saliva sample was collected.

3.5. Sound test

After five minutes of isolation the horse was exposed to a sudden sound (a vuvuzela), lasting for one second. The sound was made from an opposite box in the same stable, but out of sight of the horse. After this, the horse was left in the box for another minute and then brought back to its home box. The reaction to this sound and five seconds after were recorded according to the ethogram (table 2 and 3.

3.6. Smell test

A towel with pig smell was placed in the crib along with some feed (Lucerne, which the horses were used to eat). The horses were left in the box for one minute and the behaviour was recorded. This included how many feeding bouts the horses had, how long time it took from being left until the horse started to eat, if it didn’t want to eat at all etc. At day two, the towel was placed in the crib without pig smell as a control of the horses’ general feeding behaviour.

3.7. Saliva sampling

Saliva samples to analyse cortisol levels were taken from each horse at three different times both days. First, a control sample was taken in the box before any of the tests were performed. The second sample after the reactivity test and the last one after the isolation test had been performed. Saliva samples were taken using cotton swabs in the horse's mouth. The swabs were then put in small plastic tubes and stored in the freezer (-21 °C). The samples were analysed with Elisa essay (IBL. Hamburg, Germany).

3.8. Heart rate

Heart rate was measured continuously during all tests with a HR monitor (Polar CS 600X, Polar, Finland). The device was applied to the horse before the behaviour test started, but after the first saliva sample was taken. The HR monitor was then removed after the last behaviour test and saliva sample. Sequences of the HR were picked from all the different tests and analysed. The HR curve from each horse was examined and missing values where the HR monitor had not worked properly were excluded. It was noted what time each test began which made it possible to pick valid sequences from every test. Each sequence lasted for 10 seconds and 1-4 sequences were picked. One sequence was picked from "entering the room" and "crossing scale", and two from "passing the ball" and "moving the ball". Longer tests resulted in a larger number of sequences. Furthermore, four sequences were picked from the isolation test due to the longer duration of the test (several minutes) and to get results from different time points during the isolation. The sound test contained two frequencies, one exactly when the horse was exposed to the sound and then a sequence ten seconds after. From the smell test, one sequence was picked. From all these sequences, mean values were calculated and maximum values were picked out.

3.9. Survey

The trainer of the horses filled in a form for every horse where he had to grade different personality traits on a linear scale. This scale ranged from 0 to 10, where 0 corresponded to 'applies not at all' and 10 was totally applicable. If the trainer experienced a horse for example as being extremely friendly towards other horses it got 10 on this trait.

3.10. Analyses of behaviour

All tests were filmed and behaviour was recorded from video recordings as frequencies. The reactivity test was split into four parts. These parts were: 1) entering the room, 2) passing the ball, 3) moving the ball and 4) walking over the bridge. This was done in order to facilitate the comparison between different horses.

The films were analysed and behaviours were recorded according to the ethogram definitions in table 3. Seven films were lost due to technical problems when filming (five films from the isolation test, one from the smell test and one from the foreign object and walking over the bridge test).

Table 2. Behaviours recorded in the different tests

	Reactivity test				Isolation test	Sound test	Smell test
	Enter room	Passing ball	Moving ball	Bridge			
Ear flick	x	x	x	x		x	
Ears forward	x	x	x	x		x	
Move ear						x	
Alert	x	x					
Stop	x	x		x			
Moves head/neck	x	x		x	x	x	x
Accelerate	x	x		x			
Jerk		x	x			x	
Lower head		x		x			
Sniffs the object		x		x			
Neighs		x			x		
Blows	x	x	x	x	x		x
Hesitates		x		x			
Turns head towards the ball			x				
Move feet			x		x	x	x
Focussing on other things	x	x	x	x			
Raise the head				x		x	
Shift weight backwards				x			
Moving backwards				x			
Touches the crib							x
Shakes feed							x
Pushes the food (without eating)							x
Head in crib (without eating)							x
Sniffs other than feed							x
Numbers of eating bouts							x
Seconds to first chewing							x
Defecate	x	x			x		
Circling					x		
Shakes the head							x
Investigate/sniff walls/interior					x		
Bites the interior					x		
Ingest forage					x		

Table 3. Ethogram with the definition of the different behaviours

Behaviour	Description
Move feet	One or more steps in any direction or just a stamp (Not when it is supposed to walk)
Move backwards	Moving feet backwards away from the object
Shift weight backwards	Shift body weight backwards without moving feet
Accelerate	Sudden and fast forward movement
Circle	Walking more than half a circle around the perimeter of the box/stall
Jerk	Short, sudden movement with the body without moving feet
Lower head	Walking with lowered head and focussing on object
Hesitate	Slows down towards the object
Stop	Stopping with all legs remaining stationary
Move head/neck	Head/neck moves to the left/right or upwards
Raise head	Head and neck tense and turned upwards
Ear flick	Rapid rotation of one or both ears without moving the head
Ears forward	Ears and head oriented towards the object, neck not elevated
Alert	Head and ears oriented towards the object with elevated neck
Focus on other things	Focussing on other things than the object, ears and head oriented towards other things than the object
Shake head	Fast movement of head back and forth or sideways
Touch object	Muzzle positioned near the object, may or may not be in contact with object
Move object	Muzzle in contact with object and the object moves
Shake feed	Fast movement of head back and forth with feed in mouth
Push feed	Pushes the feed with the muzzle around in the crib (without eating)
Head in crib	Head is positioned in the crib but the horse is not ingesting feed
Sniff other than feed	Muzzle positioned near other things than the feed, may or may not be in contact with the object (walls etc.)
Investigate/sniff floor/walls interior	Muzzle positioned near the floor/walls/interior, may or may not be in contact with floor/walls/interior
Bite/push interior	Teeth or body in contact with the interior
Number of eating bouts	Quantity of feed intakes
Seconds to first chewing	Number of seconds between the starting point and when feed is ingested
Neigh	Vocalisation, long, high-pitched sound
Blow	Exhalation of air through the nostrils
Defecate	Expelling of faecal material
Ingest forage	Take forage in mouth and chewing

3.11. Statistical analyses

The statistical analysis was performed with SAS (Statistical Analysis System, Cary USA, version 9.3). The models used were GLIMMIX procedure to analyse behaviour and the trainer's forms. To analyze differences in HR and cortisol between test days, the Generalized Linear Model (GLM) was used with Tukey's test to adjust for multiple comparisons. To analyze correlations between parameters, a Pearson correlation test was performed.

Results for behaviour, HR and cortisol are presented as Least Square Means and standard error (SE).

Some of the behaviours had too low frequencies to perform an analysis. Behaviours with a frequency lower than three were therefore not analyzed. The level of significance was P=0.05.

Data for the horses' race performances were collected from the Swedish Trotting Association. All 15 horses that had passed the qualifying race were included in the statistical analyses. One horse died before it had a successful qualifying race, this horse was not included in the analyses of race results.

4. Results

4.1. Behaviour on different test days

None of the behaviours in the reactivity test differed significantly between days (table 4, 5 and 6). The behaviour "move head and neck" had a tendency to have a higher mean day 2 (P=0.086).

Table 4. Mean (± SE) during the reactivity test (ball) day 1 and day 2 and level of significance for differences between days. Means too low to be analyzable are marked with a dash

Behaviour	Day 1	Day 2	P-value
Ear forward	0.1 ± 0.1	0.4 ± 0.2	0.899
Ear flick	3.0 ± 0.6	4.9 ± 0.8	0.478
Move head and neck	0.1 ± 0.1	0.5 ± 0.2	0.086
Lower head	0.4 ± 0.1	0.3 ± 0.1	0.904
Alert	0.1 ± 0.1	0.1 ± 0.1	0.543
Sniff object	0.3 ± 0.1	0.2 ± 0.1	0.939
Focus on other things	0.5 ± 0.2	0.2 ± 0.1	0.603
Move feet	-	-	-

Table 5. Mean (± SE) during the reactivity test (moving ball) day 1 and day 2 and level of significance for differences between days. Means too low to be analyzable are marked with a dash

Behaviour	Day 1	Day 2	P-value
Ear forward	1.1 ± 0.3	1.3 ± 0.3	1.000
Ear flick	2.9 ± 0.6	4.3 ± 0.7	0.760
Focus on other things	1.1 ± 0.2	0.6 ± 0.2	0.564
Jerk	0.4 ± 0.1	0.2 ± 0.1	0.288
Blows	0.2 ± 0.1	0.4 ± 0.2	0.759
Move feet	-	-	-

Table 6. Mean (± SE) during the reactivity test (bridge) day 1 and day 2 and level of significance for differences between days. Means too low to be analyzable are marked with a dash

Behaviour	Day 1	Day 2	P-value
Ear forward	1.0 ± 0.2	0.4 ± 0.3	0.686
Ear flick	5.7 ± 0.9	5.6 ± 0.9	1.000
Move head and neck	0.1 ± 0.1	0.3 ± 0.1	0.434
Lower head	0.5 ± 0.1	0.4 ± 0.1	0.984
Raise head	0.4 ± 0.2	0.1 ± 0.1	0.280
Sniff object	0.2 ± 0.1	0.1 ± 0.1	0.974
Stops	-	-	-
Accelerate	0.2 ± 0.1	0.1 ± 0.1	0.589

In the isolation test, none of the behaviours differed significantly between days (table 7). The behaviour "investigate/sniff interior/walls/floor" had a tendency to be significantly higher day 1.

Table 7. Mean (± SE) during the isolation test day 1 and day 2 and level of significance for differences between days. Means too low to be analyzable are marked with a dash

Behaviour	Day 1	Day 2	P value
Move head and neck	49.6 ± 3.5	44.0 ± 2.7	0.613
Blows	7.9 ± 1.7	6.6 ± 1.3	0.843
Move feet	-	-	-
Circling	-	-	-
Investigate/sniff interior/walls/floor	6.3 ± 0.9	4.6 ± 0.6	0.058
Push/bite interior	-	-	-
Neigh	-	-	-
Defecate	1.3 ± 0.4	1.0 ± 0.3	0.512
Ingest forage	-	-	-

None of the behaviour in the sound tests differed significantly between days (table 8).

Table 8. Means (± SE) during sound test day 1 and day 2 and level of significance for differences between days

Behaviour	Day 1	Day 2	P-value
Ear flick	0.7 ± 0.23	1.7 ± 0.4	0.669
Ear forward	0.6 ± 0.23	1.0 ± 0.3	0.990
Move ear	3.1 ± 0.59	3.4 ± 0.5	0.759
Move head and neck	1.0 ± 0.40	1.1 ± 0.3	1.000
Raise head	0.2 ± 0.11	0.4 ± 0.2	0.568
Jerk	0.5 ± 0.17	0.9 ± 0.2	0.434
Move feet	3.8 ± 0.80	4.1 ± 0.8	0.400

The behaviours “head in crib but not eating” and “seconds to first chewing” had a significant lower mean day 2 (table 9).

Table 9. Mean *(± SE) during smell test day 1 and day 2 and level of significance for differences between days. Means too low to be analyzable are marked with a dash*

Behaviour	Day 1	Day 2	P-value
Move head and neck	4.5 ± 0.7	3.3 ± 0.6	0.719
Shake head	-	-	-
Move feet	-	-	-
Touch crib	-	-	-
Shake feed	-	-	-
Sniff other than feed	-	-	-
Head in crib but not eating	0.7 ± 0.2	0.1 ± 0.1	0.036
Number of eating bouts	0.9 ± 0.2	1.0 ± 0.2	0.793
Seconds to first chewing	27.2 ± 8.8	14.4 ± 6.4	0.011

4.2 Heart rate

Reactivity test

The mean HR (in bpm) in the reactivity test was significantly higher day 1 (79 bpm) compared with day 2 (65 bpm, P=0.0061). The maximum HR in the reactivity test was also significantly higher day 1 (82 bpm) compared with day 2 (68 bpm, P=0.0087) (figure 2).

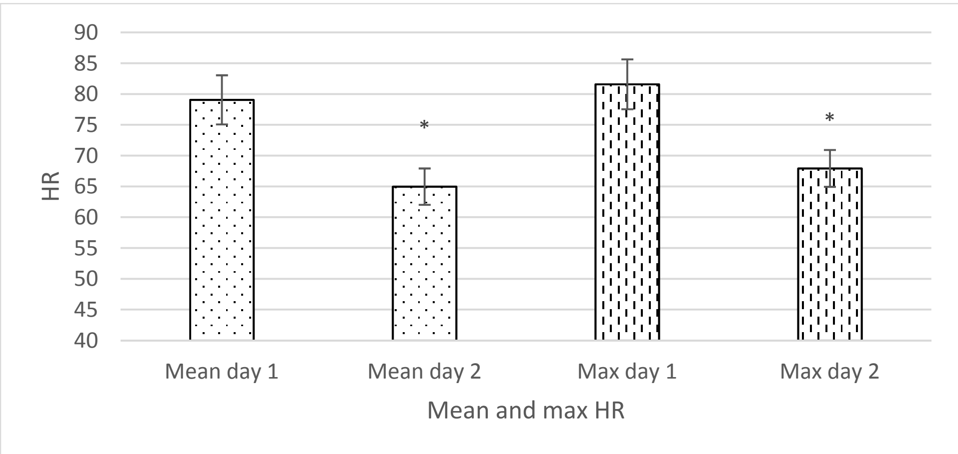

*Figure 2. Mean and maximum HR (in bpm) with standard error in reactivity test day 1 and 2.*Indicates a significant difference between day 1 and day 2.*

The differences between tests regarding HR were not significantly different with one exception. During the smell test, horses had a significantly lower HR compared to all the other tests (table 10).

Table 10. Mean HR (± SE) and significance for differences between tests

Test	HR (bpm)	HR Test:	P-value
Reactivity	74.6 ± 6.3	Smell: 47.7 ± 6.7	0.0158
Isolation	78.6 ± 1.6	Smell: 51.7 ± 1.9	<0.0001
Sound	75.1 ± 2.4	Smell:51.8 ± 2.6	<0.0001

Isolation test

The difference between days in mean HR after one minute of isolation (figure 3) was significant. A difference between day 1 and 2 in Maximum HR at the same occasion on the other hand was not significant in the Tukeys' test but in the Anova-table (GLM-procedure, P=0.0426).
The mean HR after two minutes of isolation was significantly higher day 1 compared with day 2, and the maximum HR was also significantly higher day 1 compared with day 2 (figure 3).

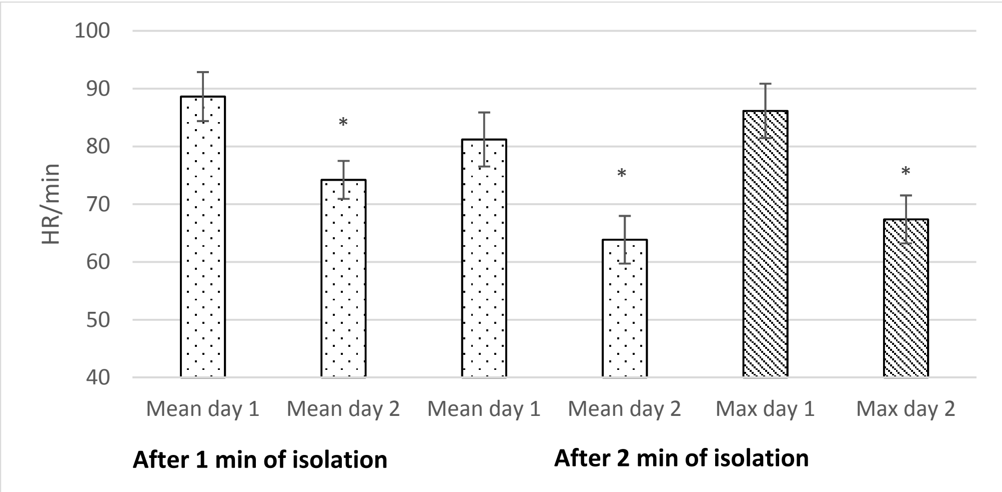

*Figure 3. Mean and maximum HR (bpm) with standard error after one and two minutes of isolation day 1 and 2. *Indicates a significant difference between day 1 and day 2.*

Sound and smell test

None of the tested variables for HR in the sound and smell test had significant differences between days.

4.3. Cortisol

The cortisol in the saliva in the control sample differed between horses (Figure 4). Half the horses had a higher cortisol level day 1 compared to day 2 and the other half the other way around. The differences between days in the control sample were not significant (day 1: 3.12±0.28, day 2: 2.96±0.28, P>0.05). However, the differences between horses were significant (P<0.05).

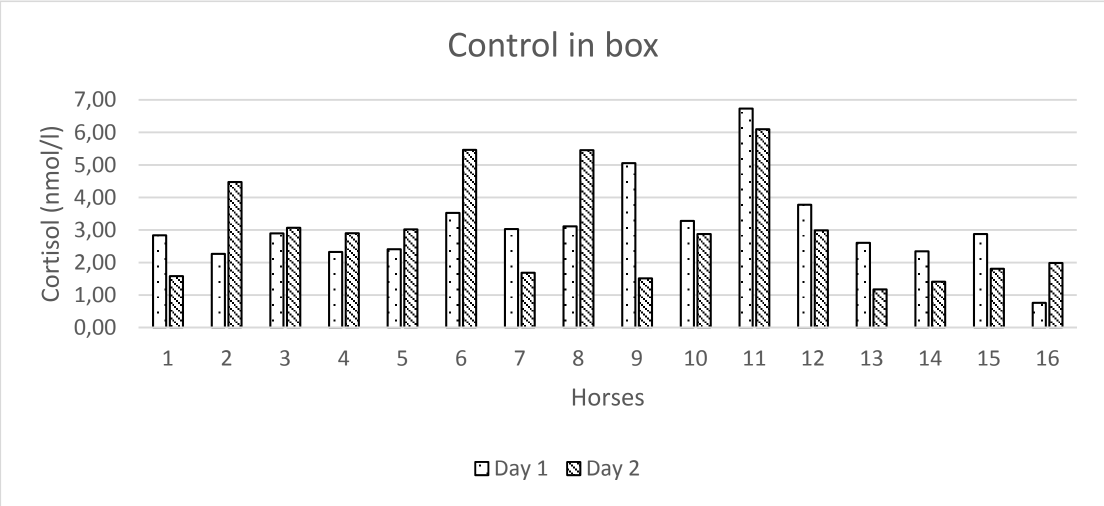

Figure 4. Cortisol level in saliva. Control sample of 16 horses taken in the box, comparison between day 1 and day 2.

The level of cortisol in the saliva was significantly higher day 2 when taking the sample after the reactivity test (day 1: 1.55±0.16, day 2: 2.66±0.16, P=0.0002) (figure 5). The differences between horses were not significant, but had a tendency to be (P=0.059). The cortisol level was also significantly higher day 2 compared to day 1 when comparing the samples taken after the reactivity test and after the isolation test (0.0002) (figure 5 & 6).

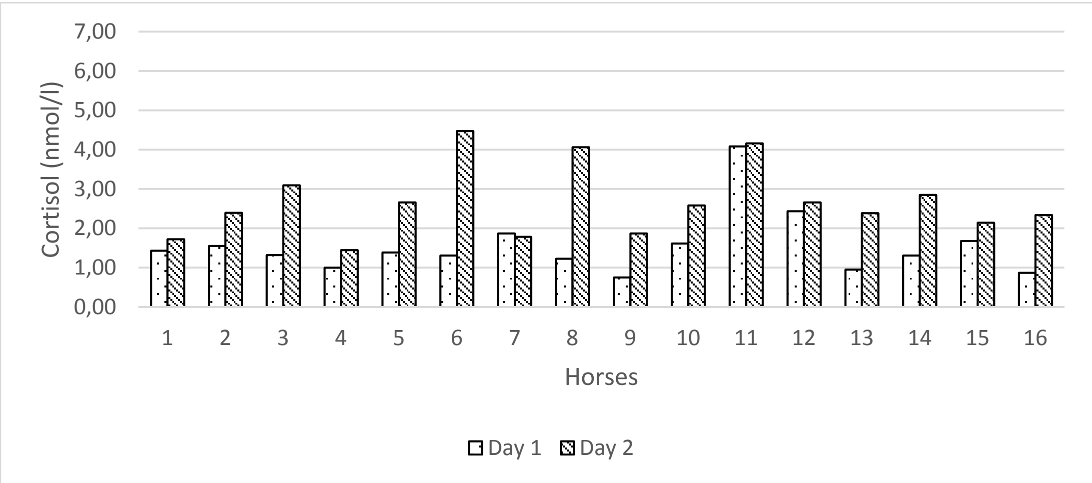

Figure 5. Cortisol level in saliva. Comparison between day 1 and day 2, sample taken in stable aisle after reactivity test.

Figure 6. Cortisol level in saliva. Comparison between day 1 and day 2, sample taken in home box after isolation test.

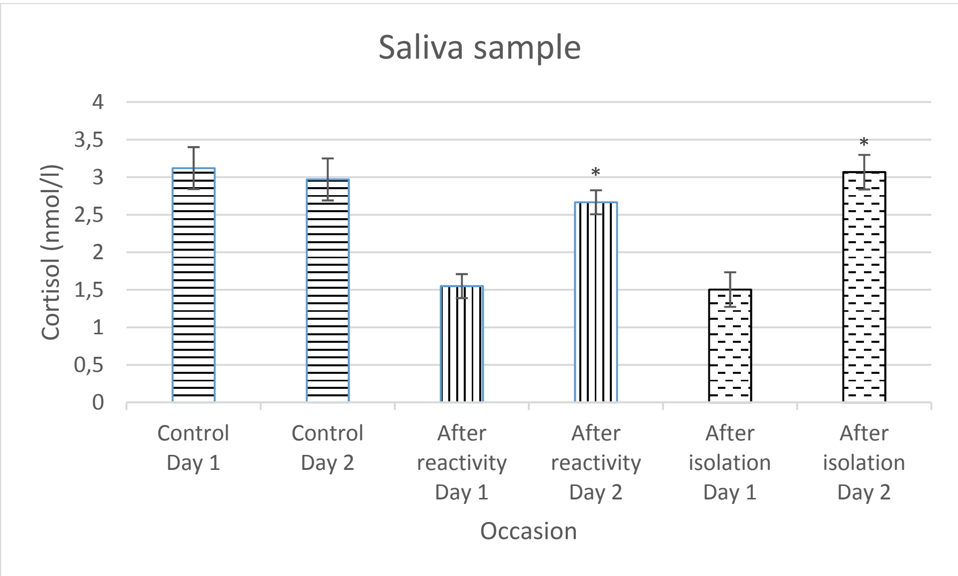

*Figure 7. Cortisol level in saliva with standard error day 1 and day 2. Comparison between control, after reactivity test and after isolation test. *Indicates a significant difference between day 1 and day 2.*

The level of cortisol was significant higher day 2 compared with day 1 when taking the sample after the isolation test (day 1: 1.50±0.23, day 2: 3.07±0.23, p=0.0002) (figure 7). The differences between horses were not significant (P>0.05).

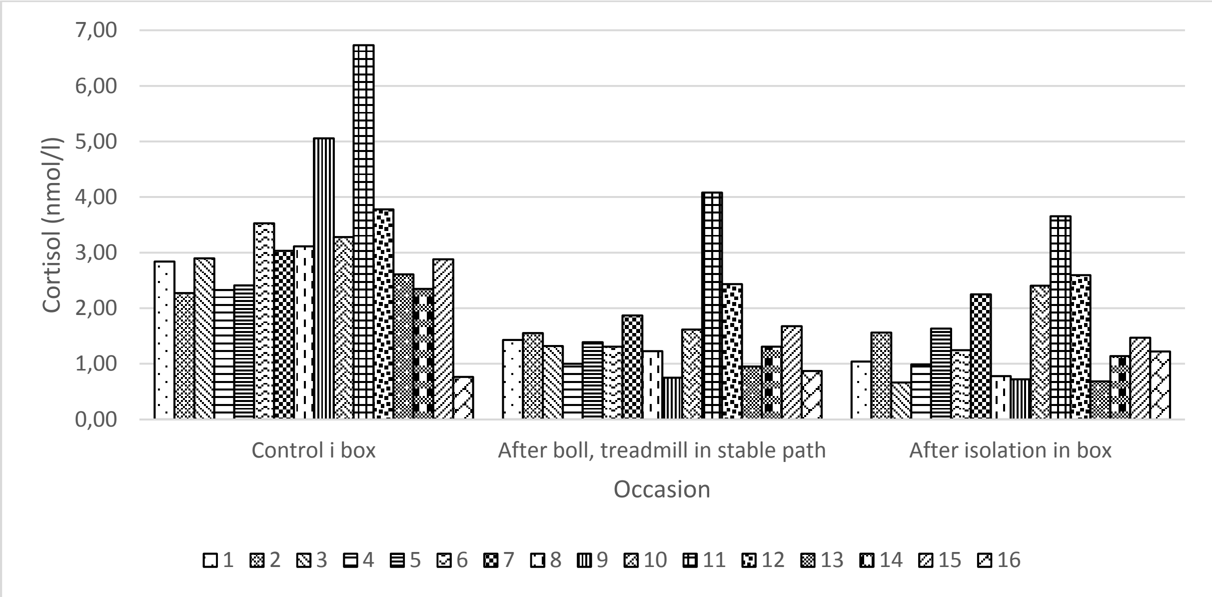

Figure 8. Level of cortisol in saliva for the different horses day 1.

The cortisol levels differed between individuals and horses with a higher level when taking the control sample also had a higher level when taking samples after the tests. Overall, the levels were higher after the isolation test compared with after the reactivity test. This difference was not significant, but had tended to be (day 1:2.66±0.28, day 2: p=0.063). The individual cortisol level after the isolation test differed significantly between 0.68 and 3.66 day 1 and 1.58 and 5.62 day 2 (figure 8 & 9).

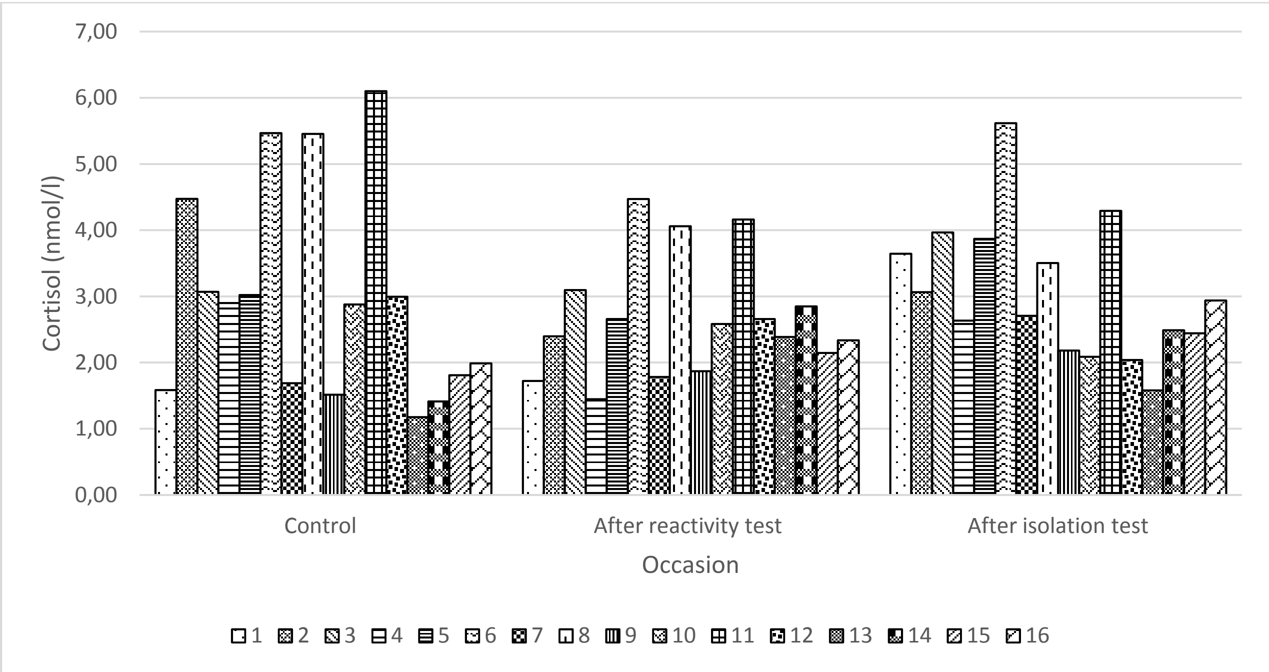

Figure 9. Level of cortisol in saliva for the different horses day 2.

4.4. Correlations between behaviours, HR and cortisol

Behaviours reactivity test versus trainer information

Table 11 shows the behaviours with a significant strong correlation (P<0.05, r>0.8) between behaviour, and temperament assessed by the trainer, HR and cortisol level.

None of the behaviours in the trainer form had a strong correlation with heart rate or cortisol during tests.

A few behaviours had a significant but a modest or weak tendency to correlate This were for example the trainers' opinion of a calm horse, that had a negative modest correlation with "shake head" (P=0.047). Trainers' opinion of "friendly towards other horses" had a modest (-0.599) negative correlation with the cortisol level after the reactivity test (P=0.014).

Table 11. Correlations between different recorded behaviours, behaviours from the trainer forms and p-values.

Parameter 1	**Parameter 2**	**Correlation coefficient**	**P-value**
Ear flick	Raise head	0.864	<0.0001
Ear flick	Stops	0.914	<0.0001
Ear flick	Weigh back	0.904	<0.0001
Ear flick	Move backwards	0.932	<0.0001
Ear flick	Mean HR	0.928	0.0003
Ear forward	Raise head	0.903	<0.0001
Ear forward	Stops	0.867	<0.0001
Ear forward	Weigh back	0.883	<0.0001
Ear forward	Move backwards	0.816	<0.0001
Raise head	Stops	0.913	<0.0001
Raise head	Weigh back	0.965	<0.0001
Raise head	Move backwards	0.863	<0.0001
Sniff object	Stops	0.815	<0.0001
Sniff object	Move back	0.836	<0.0001
Stops	Weigh back	0.950	<0.0001
Stops	Move backwards	0.979	<0.0001
Move backwards	Weigh back	0.941	<0.0001
Behaviour in reactivity test	**Temperament assessed by trainer**	**Correlation**	**P-value**
Alert	Afraid	0.905	0.0020
Alert	Curious	0.928	0.0009
Alert	Spooky	0.910	0.0017
Alert	Sound sensitive	0.856	0.0067
Alert	Lazy	0.898	0.0025
Investigate/sniff floor/walls/interior	Spooky	0.852	0.0312
Investigate/sniff floor/walls/interior	Controllable when driving	-0.906	0.0128
Bite/push interior	Friendly towards other horses	-0.826	0.0428
Neigh	Unpredictable	-0.924	0.0085
Defecate	Easy to learn	0.844	0.0346
Ingest forage	Easy to learn	-0.864	0.0266
Ingest forage	Cooperative	-0.836	0.0380
Ingest forage	Lazy	-0.852	0.0313

Behaviours isolation, sound and smell test

None of the behaviours recorded in the isolation test, sound test or smell test correlated with temperament assessed by the trainer.

Correlation between horses' competition results and trainer information

The behaviour afraid (according to the trainer) correlated with a higher place % (r=0.603, P=0.017) and win % (r=0.526, P=0.044). The place % also correlated with "alert" (r=0.569, P=0.027), "spooky" (r=0.550, P=0.033) and "sensitive for sounds" (r=0.613, P=0.015).

5. Discussion

Some behaviours had a strong correlation with other behaviours shown in the same test. This means that when the horse expresses a certain behaviour it may be more or less likely to express another behaviour simultaneously (depending if it is a positive or negative correlation value). For example, when the horse moved the ears a lot it was also more likely to stop, move backwards or raise the head. Behaviours also correlated with the trainers' opinion about the horse's temperament. When evaluating these correlations, the behaviour "alert" seemed to have a positive correlation with the trainer's opinion that the horse is prone to be "afraid", "curious", "spooky", "sound sensitive" and "lazy". All of these traits seem logic with an exception of "lazy". It seems a little bit strange that a horse that is alert may also act lazy when being on the race track. The behaviour "investigate floor/interior/wall" had a positive correlation with the trainer's opinion of "spooky" and a very strong negative correlation with "controllable at runtime". It seems that horses that are suspicious of the surroundings are also the ones that are a bit nervous of new environments and objects and may not be that easy to drive.

However, quite few of the trainer's opinion of the horses correlated with the competition results. Still, the trainers' opinion of a horse that is afraid correlated with higher win %. Furthermore, higher place % correlated with the opinion of the traits alert, spooky and sensitive for sounds. This indicates that a more reactive horse is more successful on the racing track, even though one of the breeding goals for the breed is to have a horse that is easy to handle. When the competition results and the behaviours during the tests were evaluated, it was noted that horses with at least one win seem to be more alert, unpredictable and moody. This is quite interesting, since these are behaviours that could correspond to a reactive horse and that is something that may not be desirable when it comes to handling the horse. However, according to some trainers it is an advantage with a reactive horse in relation to good performance on the race track. This is consistent with the findings of the present study. Still, more conclusions could be drawn with a larger study and with using horses that have competed more, as the competition results from this study are limited.

Some of the behaviours were not performed more than a few times which made it difficult to analyze them. If the tests would have been performed more times or during a longer period of time, this could have increased the means. However, this was a fairly small scale study that had to be performed in two days. Furthermore, with longer tests the horses would have been even more habituated and that may have influenced the results as well. None of the behaviours, with an exception for two behaviours in the smell test, differed significantly between days. However, since no control group was available it is hard to make conclusions if the 3-day confinement affected the horses' behaviours at all during the tests. It would have been interesting to have a control group that was regularly turned out in paddocks during these three days to compare the frequencies of behaviour.

The statistics for the behaviours are presented with mean values. This turned out not to be ideal since a behaviour either happened or not (the result is 0 or 1). Therefore it would have been better to present the values as number of observations in a given time interval. This is something to consider in future studies.

Even though the reactions were not significantly different between test days, the handler leading the horses indicated that many of the horses were much more active day 2 between the test stations. When leading horses between the different tests the second day, the majority of the horses were experienced to be quite spooky and some were even hard to control. However, all this was not notable during the test procedures. It is possible that the handler also affected the horse, but according to König von Borstel et al. (2011) are horses' reactions in a behaviour test moderately correlated to the reactions for the same horses when tested without a human handler. Since the handler aimed not to interfere with the horses, this influence may be negligent. Since the test order was kept the same it is also possible that the previous test could have affected the subsequent test. This was something not taken into account in the statistical analyzes.

In the smell test, the horses had a significant longer time before starting to eat day 1 compared to day 2, which indicates that the pig smell did affect the eating behaviour. This finding is consistent with the study by Christensen et al. (2005).

Between the two test days, horses were kept inside for 23h/day which is contradictive to their ordinary routines and may have affected the expression of behaviours on day 2. Harewood et al. (2005) found that some stress-related behaviours were seen more in horses stabled in individual boxes indoors. However, these horses were stabled for the first time, and it would be interesting to see the same study applied to horses used to be kept indoors. Another reflection is if it is the indoor versus outdoor environment or the group versus individual housing that affects the stress level most. Horses are social animals and restricted contact to other conspecifics may affect them as much as indoor housing.

During the tests there were occasionally different distractions for the horses. A few times a person accidently walked into the test room and this may have influenced the results. This is also true for the isolation test where the only window in the box was covered during day 1, but in the beginning of the second day it was open and the horses could see other people and conspecifics outside. This can be seen in the films as horses turning their heads towards the person walking into the test room and spending more time at the window looking out outside.

Overall, the HR was significantly higher day 1 than day 2. This may be explained by the new objects and situations the horses were exposed to. Thus, the horses were probably habituated day 2 and it was maybe shown by a lower HR. Visser et al. (2002) concluded that horses in training had a lower HR when facing novel objects. Maybe this could have affected the different HR day 1 and 2 as well. In this study, mean and maximum HR were used, however it is also possible to use heart rate variability (HRV) as a measurement. Visser et al. (2002) measured both HR and HRV when studying horses' reactions to a novel object. Fear is known to increase HR which decreases HRV. If HRV had been used as a parameter in this study it may have been possible to detect even more differences between tests and days. HR had no significant difference between tests except between the smell test and the other tests. This may partly be explained by the fact that the smell test took place in the horses' home box while in the other tests the horses were less familiar with the environment.

The results of the cortisol level in saliva day 2 were more widespread than day 1 as figure 9 shows. The higher concentration of cortisol on day 2 compared to day 1 contradicts the results of the HR. Higher cortisol levels could indicate higher level of stress or excitement, even though this was not reflected in the HR.. However, the cortisol level was not significantly higher day 2 in the control sample, but in the samples taken after the reactivity and isolation test. This may indicate that the reactions to the tests were stronger day 2. It is a normal concentration variation in the daily cortisol cycle, with a lower level in the morning. This could have affected the results since the horses performed the tests during different time of the day.

The horses in this study were two years old and according to Lansade et al. (2007) the horses may habituate faster when they are older. In the study by Lansade et al. (2007) the horses were between 8 months and 2.5 years. Therefore, it would be interesting to do the same study with younger horses and repeat the test at two years of age. The horses in the study by Lansade et al. (2007) were not in training and had limited human contact which may of course have affected the results. Since they concluded that the trait “gregariousness” decreases with age, some traits should maybe be tested at a younger age.

Overall, compared to other studies of young horses in novel object tests (e.g. Visser et al., 2001; Visser et al., 2003; Winther Christensen et al., 2005) the frequency of flight related behaviours in our study seemed to be low. This may be due to several factors such as the temperament of this breed, training and handling experience and perhaps also the diet. The horses in this study have also been trained from an early age compared to most other horses used in behavioural studies which may also have affected the results. The present study stands out since all the horses were fed a forage only diet instead of a diet rich in concentrates which is the most common feeding strategy for race horses (Jansson and Harris, 2013). It is believed among trainers that a high amount of concentrates may increase the alertness of horses. One factor believed to cause this is the use of starch rich concentrates, commonly used to cover the high energy requirements of race horses (MacLeay et al, 1999). It is the most common practice in Sweden and in race horse management all over the world to use high amounts of concentrate in the diet (Williamson et al., 2011; Southwood et al., 1993) and it is believed among trainers that replacing the concentrates with forage could alter the horse’s temperament as well as its performance. This may explain why the means of flight-related behaviours were low, even though the horses’ had been confined in the stable between test day one and two.

6. Conclusion

Since some of the behavioural responses had strong correlations with the trainer’s opinion of the horse this kind of knowledge could be valuable when evaluating behavioural tests. Due to the moderate correlation between some temperamental traits and win % or place %, some behaviours related to high reactivity seem to be favorable on the race track and may predict racing success. There are individual differences in cortisol, but still the level seems to respond to the tests in the same way, i.e. the horses, with few exceptions, have a higher cortisol level day 2 compared with day 1. Due to this, cortisol concentration in saliva may be used as an indicator of stress even if it should be carefully analyzed. The higher HR day 1 may reflect that habituation occurred day 2, as the horses may have recognized the tests and felt more confident which resulted in a drop in HR. The mean of the recorded behaviours compared to other studies of the same kind seems to be quite low, this may be due the diet, training, early management or choice of breed. The use of behavioural tests needs further evaluation but could potentially be a tool in the future to assess desirable temperamental traits in Standardbred horses.

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