Effects of handling on fear reactions in young horses

Effekten af håndtering på unge hestes frygtreaktioner

M.Sc. thesis by

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Aarhus University
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Cover illustrations

Front: Novel object test (left) and handling fear test (right)
Photos by Maria Vilain Rørvang

Preface

This thesis of 60 ECTS points is written as the completion of my Master of Science in Agrobiology, Department of Animal Science, Faculty of Science and Technology, Aarhus University.

The thesis contains a main part and an appendix. The main part is based on an experiment conducted during the autumn of 2012 at Research Centre AU-Foulum. The appendix is a 2 ECTS written assignment titled "Using heart rate and heart rate variability as indicators of the stress response in horses", which was prepared in connection with the PhD course "Interpretation of animal stress responses", Aarhus University, January 2013.

I would like to thank my supervisors Janne Winther Christensen and Jan Ladewig for their guidance and inspiration throughout the process. Especially thanks to Janne Winther Christensen, for entrusting me to work with the horses at Research Centre AU-Foulum. I have learnt so much!

A big thank to the horse owners for allowing me to use their horses. It would not have been possible without you. Also thanks to Dansk Islandshesteforening for their support, to Salvana for providing vitamins, and to Dangro Nordic providing the test feed.

I would also like to thank Maria Vilain Rørvang and Line Peerstrup Ahrendt for the many hours we spent in the stable when conducting experiments or preoccupied with theoretical discussions. Thank you for your support and valuable comments along the way. Also thanks to Emeline Cornet and Henrik Krogh Andersen for helping out with the experiment.

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Aarhus, September 2013

Anna Feldberg Marsbøll

Contents

Summary

Fearfulness is in horses a temperamental trait important to human safety, horse welfare and the usability of the horse. The inclusion of fear tests in horse breeding evaluations in order to assess fearfulness is of increased interest, and it has been debated whether or not to use fear tests involving human handling. As horses are kept under many different management systems, there may be considerable differences in their previous experiences with handling. It is therefore relevant to investigate how differences in previous handling experience may affect the response of horses in fear tests, and whether the responses differ between tests with or without human handling involved.

This study investigated the effect of a standardized handling procedure on the response of 3-year-old Icelandic horses (n=24). Handled horses (n=12) were trained according to a standardized handling procedure and the rest of the horses were untrained controls (n=12). The horses behavioural and heart rate responses towards static novelty in a novel object test (NOT) and a handling fear test (HFT) were measured. The HFT was conducted both with an unknown handler (HFT-unknown) and with the same person who trained the horses during the handling procedure (HFT-known).

There was no effect of the standardized handling procedure on the horses' behavioural or heart rate response in the NOT or in the HFT-unknown. However, in the HFT-known, there was a difference in reluctance behaviour, with handled horses showing less reluctant behaviour than the control horses.

Previous handling may therefore affect the behavioural fear response of horses when handled by their usual handler. Despite the findings of other studies, this study suggests that the effect does not apply to unknown handlers. Hence, if fear tests are to be included in practical breeding evaluations, the use of handling by the horses' usual handler in the tests may impair the results. However, measurements of heart rate were less affected by the handling than the behavioural variables and thus may be a more reliable indicator of fear.

Keywords: Behaviour; Fear; Fearfulness; Handling; Heart rate; Horse

Sammendrag (Danish Summary)

Frygtsomhed er hos heste en temperamentsegenskab med betydning for menneskers sikkerhed samt hestens velfærd og brugbarhed. Inddragelsen af frygttests i avlsvurderingen af heste er af stigende interesse, og det har været debatteret, hvorvidt der skal benyttes frygttests, som involverer menneskelig håndtering. Da heste holdes under mange forskellige management-systemer, kan der være betydelige forskelle i deres forudgående erfaring med håndtering. Det er derfor relevant at undersøge, hvordan forskelle i erfaring med håndtering påvirker hestes reaktioner i frygttests, og om reaktionerne er forskellige i tests henholdsvis med eller uden menneskelig håndtering.

Dette studie undersøgte effekten af en standardiseret håndteringsprocedure på frygtresponsen hos 3-årige islandske heste (n=24). De håndterede heste (n=12) blev trænet ud fra en standardiseret håndteringsprocedure, mens resten af hestene var utrænede kontroller (n=12). Hestenes adfærdsmæssige og hjerterate respons i en novel objekt test (NOT) og en håndterings frygt test (HFT) blev undersøgt. Sidstnævnte blev gennemført med både en ukendt person (HFT-unknown) og med den samme person, som trænede hestene i håndteringsproceduren (HFT-known).

Der var ingen effekt af den standardiserede håndteringsprocedure på hestenes adfærdsmæssige eller hjerteraterespons i NOT eller HFT-unknown. Dog var der i HFT-known en forskel i modstandsadfærd, hvor de håndterede heste udviste mindre modstand end kontrol hestene.

Forudgående håndtering kan således påvirke den adfærdsmæssige respons, når hestene håndteres af deres sædvanlige træner. Men på trods af andre studiers resultater, er der i dette studie ingen indikationer på, at dette også gælder, når hestene håndteres af en ukendt person. Derfor, hvis frygttests skal inkluderes i avlsvurderingen af heste, kan resultaterne blive påvirket, hvis hestene håndteres af deres sædvanlige træner. Dog ser det ud til, at hjerterate er mindre påvirket af håndtering end de adfærdsmæssige variable, og hjerterate kan derfor være en mere pålidelig frygtindikator.

Nøgleord: Adfærd; Frygt; Frygtsomhed; Hest; Hjerte rate; Håndtering;

1. Introduction

Assessment of fearfulness, with the aim of genetically selecting for reduced fearfulness in order to improve animal welfare, is receiving increasing attention in various species (e.g. Boissy et al., 2005). In horses, assessments of fearfulness is relevant to both welfare and safety, as it may be used to optimize the matching of horses with specific humans, types of work or housing conditions, and to genetically select for the desired degree of fearfulness (König von Borstel, 2013). In most horse breeding associations, fearfulness is only assessed indirectly at evaluations as one component of broader terms like "spirit" (Icelandic horses) or "rideability" (Danish warmblood) and several horse breeding associations wish to move from the current scoring toward a more objective assessment. The inclusion of fear tests (or variants of fear tests) developed for scientific purposes, in horse breeding evaluations, is therefore of increased interest.

This thesis aims to extend the knowledge of what affects fear reactions in horses. The focus is on the effect of previous handling and handling during fear tests, in order to provide information that may be used in the development and implementation of fear tests into horse breeding evaluations. The introduction gives an overview of the concepts and definitions in relation to fear, fearfulness and fear response (section 1.1.) and how these relate to horses (section 1.2.). Thereafter, the experimental and practical assessment of fearfulness in horses is addressed (section 1.3.), and, finally, the aim and hypothesis of the study are presented (section 1.4.). A more detailed description of the physiological fear response is presented in Appendix A.

1.1. The concepts of fear and fearfulness

Fear is an emotional state induced by the perception of a frightening stimulus (Gray, 1987). When a stimulus is perceived as frightening, a behavioural and a physiological response is initiated, which enables the animal to perform an appropriate reaction in order to increase its chances of survival. In wild animals, fear has a definite survival value, as, responding to danger increases the life expectancy of the animal. Fear and fear related responses are therefore adaptive (Boissy, 1995). In domestic animals the threshold for experiencing fear has been elevated, but they still show the same kind of fear responses as their wild relatives once the threshold has been reached (Price, 1999). As fear in domestic animals may have a negative effect on health, welfare and productivity it is generally considered an unwanted emotional state (Boissy, 1995).

The behavioural response to a fear eliciting stimulus may be active or passive. Active responses include defence (attack, threat) and avoidance (flight, hiding, escape), and passive responses include movement inhibition (freezing, tonic immobility). The behavioural response may involve expressive movements as head or tail postures and the animal may also vocalise or release pheromones (Boissy, 1995). The two main physiological responses are the activations of the sympathetic nervous system (SNS) and the hyphothalamus-pitutiary-adrenocortical (HPA) axis. The SNS is activated by stimulation of hypothalamus, and stimulates the release of the catecholamines noradrenalin and adrenalin. Noradrenalin is primarily released from sympathetic nerve endings and adrenalin is released from the medulla of the adrenal gland directly to the blood (Sapolsky, 2002). The HPA-axis is activated by stimulation of hypothalamus, and stimulates the release of corticosteroids (cortisol in cattle, pig, mink and horses and corticosterone in birds and rodents) from the cortex of the adrenal gland (Morméde et al., 2007). The overall effect of the physiological fear response is to enable the animal to perform an appropriate behavioural reaction to the fear eliciting stimuli. Therefore, both metabolic and cardiovascular changes occur in order to mobilize energy and deliver it rapidly to where it is needed the most (Sapolsky, 2002). These changes are mainly caused by the activation of the SNS and the HPA-axis. The released catecholamines causes heart rate and blood pressure to increase, and blood is directed away from processes, which are not immediately necessary, to the skeletal muscles and the brain (Sapolsky, 2002). The catecholamines also increase the availability of energy substrates by inhibiting insulin release and increasing glycogenolysis, gluconeogenesis and lipolysis (Greco and Stabenfeldt, 2007; Sapolsky, 2002). The released glucocorticoids increase gluconeogenesis but also have a permissive effect on the metabolic and cardiovascular changes induced by the catecolamines (Greco and Stabenfeldt, 2007; Sapolsky et al., 2000).

The response to a frightening stimulus may vary between individuals due to differences in temperament (Boissy, 1995). This difference may be termed fearfulness and was defined by Boissy (1995) as a basic temperamental trait "defining the general susceptibility of an individual to react to a variety of potentially threatening situations". The fearfulness of an individual is modulated during development by interaction between the genetic background and environmental factors, mainly in the prenatal and early postnatal period, and is found to be relatively stable across time and situations (Boissy, 1998). But, although there is some stability in individual fear responses, the response to any frightening stimulus is also affected by the individuals' previous experiences and its motivational and neuroendocrine state. Environmental characteristics, as the social context and the characteristics of the stimuli may also affect its response (Boissy, 1995). Various factors therefore interact in shaping the fear response of an animal.

1.2. Fear and fearfulness in horses

Horses are prey animals and primarily react actively with avoidance or flight to fear eliciting stimuli like unfamiliar objects or sudden events (Waring, 2003). Besides its possible welfare consequences, fear in horses is also relevant to human safety. The number of accidents related to riding is located in the top three of all registered sport related accidents in Denmark (The Injury Register, 2010), and the horse being frightened is one of the major causes of horse related accidents (Hawson et al., 2010; Keeling et al., 1999). Fear may also impair learning and in a recent study, fearfulness was found to reduce the performance of horses in a negatively reinforced learning test performed in a novel environment, suggesting that fear of stimuli in the novel environment overshadowed the signals from the trainer (Christensen et al., 2012). Fear is therefore generally unwanted in horses, as it may affect both human safety, horse welfare and the usability of the horse. Fear may, however, also positively influence learning in horses and fear induced before a positively reinforced instrumental task was in another recent study found to improve the horses' acquisition of the task (Valenchon et al., 2013). Fearfulness has also been found to enhance the performance in an active avoidance test, which may have been due to the more fearful horses perceiving the given stimulation as more frightening and hence learning the task faster (Lansade and Simon, 2010).

Fearfulness is founded in horses up until the age of about 8 months (Lansade et al., 2008a). The prenatal and early postnatal environment has been found to effect the modulation of fearfulness in other species. For instance, Meaney (2001) found that the time the mother rat spent grooming her pups affected the pups' reaction to acute stress and novelty, as pups with high frequency grooming mothers had a lower response to acute stress and showed more explorative behaviour and had shorter latency to eat in a novel environment. In horses, Henry et al. (2005) found that daily handling of mares in front of the foals on day 1-5 post-partum had a positive effect on the reactions of the foal towards humans at least until one year of age. However, not much is known about the influence of other environmental factors on the modulation of fearfulness in horses, and an ongoing study is investigating how the modulation of fearfulness in foals may be influenced by social transmission (Aarhus University, n.d.).

The genetics of temperament have been investigated in various species, and through experiments with directional selection, and by estimating heritabilities, parental effects and breed differences, it has been found that it is possible to select on the basis of fearfulness in several animal species, including ruminant livestock and mink (Boissy et al., 2005; Malmkvist and Hansen, 2002). It has not been possible to perform directional selection experiments based on fearfulness in horses. However, studies investigating fear in horses have found an effect of the parents on the behavioural responses (Hausberger et al., 2004; Wolff et al., 1997). Another study, investigating the heritability of horses' fear responses, found a heritability of the behavioural responses ranging from 0.24 to 0.42 (Hausberger et al., 2007), which is in the range of what have been found in other species (e.g. Boissy et al., 2005). In a more recent study on 90 warmblood riding horses, it has also been shown that horses of dressage lines have more pronounced reactions to a fear eliciting stimulus than horses of mixed or show-jumping lines (König von Borstel et al., 2010). Together these studies indicate a genetic basis for fearfulness in horses.

Several factors have been shown to affect the response of horses to a frightening stimulus. The horses' previous experiences with the stimulus may affect its response, as it may have habituated or sensitized to the stimulus on a prior occasion. It may also be classically conditioned to associate the stimulus with a positive or a negative event, or be operantly conditioned to perform a certain behavioural response to the stimulus (Nicol, 2005). Also, the horses' previous experiences with other stimuli may affect its response, as horses may generalise between objects (Christensen et al., 2011). The social context in which the horse experiences the stimuli may also affect its response. Horses are social animals and the social transmission of fear responses in wild horses have an adaptive value. In a study by Christensen et al. (2008a) it was shown that a calm companion reduced the fear responses of naive horses. There was also evidence of the calm companion being affected by the fear response of the naive horse (Christensen, 2007). Humans may also affect the response of horses and it has been shown that a nervous human leading or riding a horse may increase the horses' heart rate (Keeling et al., 2009).

1.3. Assessments of fearfulness in horses

The scientific literature offers several methodologies for investigating fear and fearfulness in various species (e.g. Archer, 1973; Forkman et al., 2007). Fear tests used in horse research include a range of tests (e.g. arena test, novel object, handling test) in which the horses' behavioural (e.g. posture, movement, vocalisation) and/or physiological (e.g. heart rate, heart rate variability, salivary cortisol, plasma cortisol) responses are measured (Forkman et al., 2007; Hausberger and Richard-Yris, 2005). The design of the tests and the measured parameters vary depending on the objective of the investigation as there is no "gold standard" for the execution of

fear tests in horses. However, as an animals' experience of fear is not necessarily linked to its expression of fear, it is generally recommended to use both physiological and behavioural measures (Mancteca and Deag, 1993).
Fear tests in horses have, among others, been used to investigate horses' basic fear responses, including the response towards different types of stimuli (Christensen et al., 2005), habituation (Christensen, 2013; Christensen et al., 2006; Leiner and Fendt, 2011) and object recognition and generalisation (Christensen et al., 2008b; 2011). They have also been used to investigate fearfulness as a temperamental trait, and the experimental assessments of fearfulness in horses using fear tests have been found to be relatively stable over time (Lansade et al., 2008a; Visser et al., 2001; 2002) and situations (Lansade et al., 2008a; Wolff et al., 1997) with no differences between sexes (Visser et al., 2002; Wolff et al., 1997). Both physiological and behavioural parameters have been used and studies have found correlations between the behavioural and the physiological response towards a fear eliciting stimulus in tests (Christensen et al., 2012; Leiner and Fendt, 2011; Visser et al., 2003). The assessments of fearfulness in tests have also been found to correlate with the assessment by riding teachers (Le Scolan et al., 1997) and riders (Visser et al., 2003).

For fear tests (or variants of fear tests) developed for scientific purposes to be useful in field conditions, they should be valid, repeatable and feasible also when used in less standardized conditions (Górecka-Bruzda et al., 2011). Recent studies have investigated fear tests when used in field conditions on 53 Polish cold blood horses (Górecka-Bruzda et al., 2011), on more than 2000 Franches-Montagnes horses (Burger et al., 2007) or on 224 mainly German warmblood riding horses (König von Borstel et al., 2012). During the tests, horses were presented with different stimuli (e.g. static or sudden novelty) while ridden (König von Borstel et al., 2012), handled, ridden or driven (Burger et al., 2007) or free in a box (Górecka-Bruzda et al., 2011). The horses' response was evaluated by judges either on a scale ranging from no reaction to strong reaction (Burger et al., 2007; König von Borstel et al., 2012) or by measuring the latency to resume eating (Górecka-Bruzda et al., 2011). From these studies it was concluded that the assessment of fearfulness in field conditions using fear tests are feasible (Burger et al., 2007; Górecka-Bruzda et al., 2011; König von Borstel et al., 2012). The tests were also found to be repeatable over time (Burger et al., 2007; König von Borstel et al., 2012) and to have a predictive validity, as the results of the tests correlated with the caretakers assessment of the horses' fearfulness (Górecka-Bruzda et al., 2011). The use of fear tests in field conditions therefore show promising results.

As horses are kept under many different environmental and management systems, there may be considerable differences in their experience with e.g. social isolation, humans, handling and novelty. This may impair the validity of the fearfulness assessed in tests in field conditions, as the fear response of the horses may be affected by their previous experiences, cf. section 1.2. Two studies have found an effect of previous long-term handling on horses' fear response (Søndergaard and Halokoh, 2003; Visser et al., 2002). One study used 40 Danish warmblood horses (Søndergaard and Halokoh, 2003) and the other study 41 Dutch warmblood horses (Visser et al., 2002). Half of the horses in each study were trained from five months of age and onwards in standard handling routines like leading, tying up, lifting feet etc. (Søndergaard and Halokoh, 2003) or free jumping and exercise in rotary exerciser (Visser et al., 2002) and were tested when they were at about one and two years of age (Søndergaard and Halokoh, 2003; Visser et al., 2002). In both studies, handled horses had a lower increase in heart rate in a subsequent novel arena test (Søndergaard and Halokoh, 2003) and a novel object test (Visser et al., 2002) compared to control horses, indicating that the long-term handling procedure reduced the horses fear response. However, this effect could also be caused by the handled horses being more habituated to social isolation in an arena (Søndergaard and Halokoh, 2003; Visser et al., 2002). In the study by Visser et al. (2002) the handled horses were also habituated to jumps and a rotary exerciser, which may have lowered the response towards the novel object, as the horses may have generalised between them (Christensen et al., 2011). Also, the handled horses having an improved physical condition from the training may have had an effect (Visser et al., 2002). Hence it is possible that the difference between the response of handled and non-handled horses was not caused by the handling procedure itself but instead by other differences in their experiences. In this study, is it therefore hypothesised that a short-term handling procedure will not affect the behavioural or physiological response of horses in fear tests without human handling.

Previous handling may, however, affect the behavioural response when the horse is handled, due to overshadowing. Overshadowing occurs when one stimuli is stronger than the other and therefore overshadows the weaker one (McLean, 2008). Therefore, a human encouraging a horse to perform an exercise, while the horse is subjected to a frightening stimulus, may change the motivation of the horse (e.g. the horse is motivated to express a flight response, but this is overshadowed by the motivation to continue walking as signalled by the human). For this to happen, the human signal needs to be trained to the extent that it overshadows the fear eliciting stimuli (McLean, 2008). Previous handling would therefore be expected to increase the motivation to respond to the handlers' signals and therefore to a higher degree overshadow the motivation to respond to the fear eliciting stimuli. In the study by Visser et al. (2002) there was no effect of the previous handling on the horses' heart rate response to passing a bridge while handled. Whether the horses' behavioural response was affected by the previous handling was not reported (Visser et al., 2002). However, as the handling procedure did not directly involve human handling, it would not be expected to affect the horses' motivations in a fear test involving human handling. In another study, comparing the behavioural response of 98 breeding stallion in nine different sites, Hausberger et al. (2004) found differences between the sites in the stallions' behavioural response to passing a bridge while handled. As the sites did not differ in housing, feeding or activity (breeding), it may have been differences in the handling of the horses at the sites, which caused this difference. It is, however, not clear what the difference in handling may have been. In this study, it is therefore hypothesised that a short-term handling procedure will affect the behavioural response of horses in fear tests involving handling, due to the horses' increased motivation to respond to the handlers' signals. However, heart rate is not hypothesised to be affected, as the horses are expected to be equally frightened.

It has been debated whether or not to use fear tests involving humans in horse breeding evaluations. Proponents of using tests involving human handling or riding argue that humans should be in the tests in order to increase the applied value and the practicability of the tests. However, opponents emphasise that any additional variables that may influence the horses' reactions in the tests should be removed (König von Borstel et al., 2011). It is therefore relevant to investigate how differences in previous handling may affect the fear response of horses in tests and if the responses differ between tests with and without handling.

1.4. Aims and hypotheses

This study aims to investigate if the responses of horses in fear tests are affected by a short-term standardized handling procedure and if there is a difference between responses in tests involving handling and tests that do not involve handling. The study was conducted on a group of young Icelandic horses. Half of the horses were trained according to a standardized handling procedure. The horses were subsequently tested in two types of fear tests; a novel object test (NOT) and a handling fear test (HFT). The HFT was conducted with both a known (HFT-known) and an unknown (HFT-unknown) handler in order to separate the effect of knowing the handler.

The main hypotheses were 1) that there would be no difference between the fear responses of horses subjected to a standardized handling procedure and untrained control horses in a NOT, 2) that horses subjected to a standardized handling procedure would show less behavioural fear responses than control horses in a HFT, and 3) that there would be no difference between the heart rate responses of horses subjected to a standardized handling procedure and control horses in a HFT.

2. Materials and Method

2.1. Animals and management

The experiment was conducted in September and October 2012 at Research Centre AU-Foulum. Twenty-four 3-year-old Icelandic horses (12 mares and 12 geldings) borrowed from 14 private studs (1-5 horses per stud) were used. All the horses were raised in loose housing systems and had been handled in connection with routine management procedures at the studs. The horses were kept at Research Centre AU-Foulum during the experimental period. Mares and geldings were kept in two separate herds. Each herd was pastured 24 h/day on a five ha grass covered field, with free access to hay (available in a feeding house; 2 x 3 m, 14 feeding places), water and a shed (6 x 5 m). Vitamins and minerals were provided daily when handled for the experiments. Horses were wearing a halter with a rope attached when handled and it was the same female handler, who handled the horses throughout the experimental period. All handling involving leading was conducted using lead pressure and negative reinforcement.

2.2. Experimental design

An overview of the experimental period is presented in figure 1. At arrival, the horses were assigned to one of two groups, balanced according to sex and stud, and the groups were randomly assigned as either handling (n=12) or control (n=12) group. When all the horses were habituated to the initial handling and the test procedure (section 2.4.), they were initially tested in two fear tests, one NOT and one HFT-known, in which their behavioural and heart rate responses towards two different novel objects were recorded. The two tests were carried out in continuation of each other using a standardized test procedure (section 2.5.).

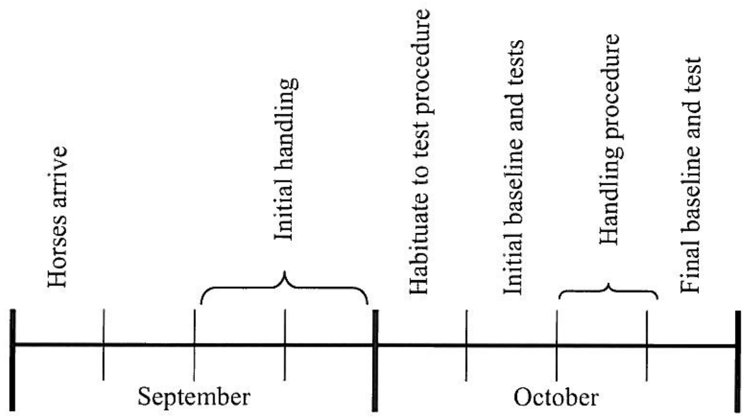

Figure 1
Overview of the experimental period.

The results of the initial tests were used to investigate whether the treatment groups were balanced in relation to the horses fear response, and were also used to asses if the novel objects induced a response with sufficient variation. After the initial tests, horses in the handling group were trained according to a standardized handling procedure whereas horses in the control group remained untrained (section 2.6.). The horses were finally tested in three fear tests, one NOT, one HFT-known and one HFT-unknown, in which their behavioural and heart rate responses towards three different new novel objects were recorded. The three tests were carried out in continuation of each other using the same test procedure as in the initial tests (section 2.5.). Two days prior to both initial and final testing, a NOT and a HFT baseline were conducted, in which the horses' responses with no objects present were recorded. This enabled the measurement of the horses' baseline responses and was also used to ensure that horses were sufficiently habituated to the test procedure in order to avoid reactions to the test procedure itself.

2.3. Experimental environment

Handling and tests were carried out in a rectangular arena (18 x 20 m) inside a stable building located next to the fields. The arena was bounded by the outer walls, straw bales (1.2 x 1.2 x 2.4 m) in two layers (total height: 2.40 m) or box elements (height: 2.40 m) (Figure 2). A corridor (2 x 4.5 m) led into the arena through the arena door and was used as the start position of the horses during tests (start position, X in figure 2). Two boxes (4.5 x 4.5 m) could be accessed from the corridor. Horses were placed in one box when fitting heart rate equipment and when waiting during tests (waiting box, A in figure 2). One or more companion horses were always present in the other box (companion box, B in figure 2). There were visual contact between horses in the companion box and horses in the corridor and waiting box while the arena was visually separated from the boxes and the corridor. This enabled horses in the arena to hear but not see the companion horses.

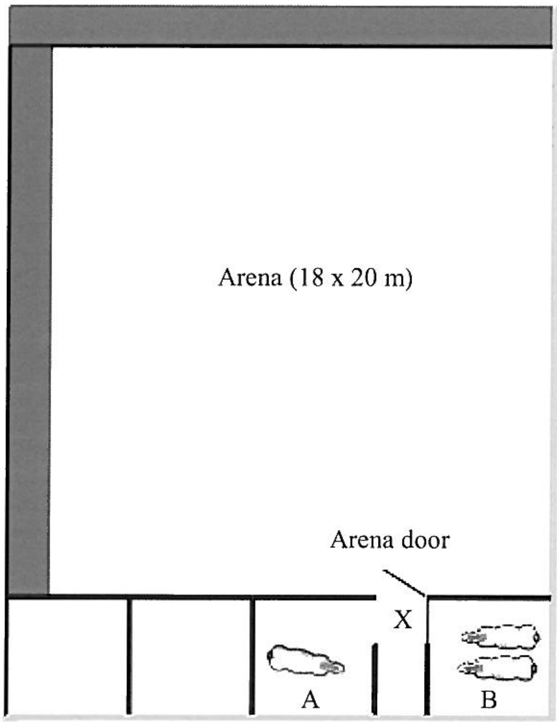

Figure 2
Overview of the arena, corridor and boxes.
A: waiting box (4.5 x 4.5 m), B: companion box (4.5 x 4.5 m), X: start position in the corridor (2 x 4.5 m). Light grey: outer walls, dark grey: straw bales (1.2 x 1.2 x 2.4 m) in two layers (total height: 2.4 m), black: box elements (height: 2.4m).

2.4. Initial handling and habituation to test procedure

One week upon arrival, all horses were gradually habituated to being caught and led, to the test environment, to wearing an elastic girth with heart rate equipment, and to eating from the feed container (Ø: 50 cm, height: 30 cm), containing cracked corn mixed with molasses (Videbæk Horsespeed, Dangro Nordic A/S, Videbæk, Denmark) and alfalfa mixed with soybean oil (Videbæk Lucerne-Topmix Light, Dangro Nordic A/S, Videbæk, Denmark). The horses were subsequently habituated to the test procedure, which included leading and eating from the feed container in the arena (position of feed container is the same as in figure 3A and C) using a step-wise approach (table 1). When a horse met the habituation criterion for one stage, it immediately proceeded with the next stage, whereas a failed horse proceeded with the same stage until the criterion was met or until a maximum five trails on the same stage per day. On the following day, a horse started on the last passed stage.

Table 1
Habituation to the test procedure.

Stage	Description	Habituation criterion
1	The horse is placed in the start position with the arena door closed. When the arena door is opened the horse is led one round in the arena before returning to the waiting box. The arena door is left open while the horse is in the arena.	Stage 1 and 2: The horse voluntary enters the arena and walks in the direction given by the handler without stopping or trying to free itself.
2	As in stage 1, but with the arena door closing behind the horse when entering the arena.	
3	The horse is placed in the start position with the arena door open. The horse is led to the feed container inside the arena and the arena door is closed behind it. The horse is offered to eat for 40 s before it is led back to the waiting box.	Stage 3, 4, 5 and 6: The horse voluntary enters the arena, walks directly to the feed container and eats at least 30 out of 40 s. If no eating occurred the handler led the horse to the feed container and offered it feed before leaving the arena.
4	The horse is placed in the start position with the arena door closed. When the arena door is opened, the horse is led to the feed container inside the arena and the arena door is closed behind it. The handler releases the horse and stands by the feed container for 40 s before catching the horse and leading it back to the waiting box.	
5	As in stage 4, but with the handler walking to stand next to the arena door inside the arena after releasing the horse.	
6	The horse is placed in the start position with the arena door closed. When the door is opened the horse is led forward and released. As the horse enters the arena the door is closed behind it. 40 s after the horse starts eating it is caught and led back to the waiting box.	
7	As in stage 6, but with the horse being caught and led back to the waiting box 120 s after the horse is released.	Stage 7 and 8: The horse voluntary enters the arena, walks directly to the feed container and eats at least 90 out of 120 s. If no eating occurred the handler led the horse to the feed container and offered it feed before leaving the arena.
8	The horse is placed in the start position with the arena door closed. When the arena door is opened the horse is led to the feed container and given the opportunity to eat for 15 s before it is led back to the start position. When the door is opened the horse is led forward and released. As the horse enters the arena the door is closed behind it. 120 s after the horse is released it is caught and led back to the waiting box.	

When a horse met the criterion for stage 7 it was not exposed to stage 8 before the rest of the horses also met the criterion for stage 7. The same observer was always present next to the arena door inside the arena, habituating the horses to her presence. Most horses (87.5%) passed the seven habituation stages on the first day, while three horses needed an additional day before passing all seven stages. Most horses (91.7%) also passed stage 8 on the first day they were exposed to it. Two horses needed an additional day before passing stage 8. The initial handling and habituation to the test procedure lasted three weeks.

2.5. Test procedure

The procedures for baselines and tests were the same, except there were no objects present during baselines, and that the baselines for the HFTs were only conducted with the known handler. The habituation criterions for the NOT and HFT baselines were the same as for stage 1 and 2 and stage 7 and 8, respectively, in the habituation to the test procedure (table 1). Horses that did not meet the habituation criterion for either the NOT or the HFT baselines were included in the rest of the experiment on equal basis with the other horses but were excluded from the analysis of that test.

In the NOTs, the test horse was first led from the waiting box to the feed container in the arena, given the opportunity to eat for 15 s and led back to the waiting box. A novel object was placed next to the feed container. The horse was placed in the start position and the arena door was opened in a 45° angle by the observer. The handler led the horse forward and released it as it entered the arena. The test time started when the horses head had passed the door, i.e. when the object became visible to the horse. When the horse had entered the arena the arena door was closed behind it. When the 120 s test time was up the horse was caught and led back to the waiting box. In the final NOT, horses that had not approached the feed container within the 120 s test time, was given up to 300 s to approach the feed container, in order to obtain real latencies and to avoid censored values. In the HFTs, the test horse was first led one round in the empty arena and back to the waiting box. Two novel objects were placed in the arena. The horse was placed in the start position and the arena door was opened in a 45° angle by the observer. The handler led the horse into the arena and encouraged it to pass between the two objects using rope lead pressure. The handler was not allowed to touch or talk to the horse. The test time started when the horses head had passed the door, i.e. when the objects became visible to the horse. After the horse had passed the objects or after 300 s the horse was led back to the waiting box. In the initial HFT, the known handler led the horse. In the final tests, one HFT was first completed with an unknown, male handler followed by a HFT with the known handler. Defecations were removed from the arena after each test and feed was added to the container if necessary. All tests were carried out between 09:00 and 16:00.

The objects used in the tests differed in size, shape and colour in order to avoid generalisation between test situations (Christensen et al., 2011). In the initial and the final NOT, one novel object was placed next to the feed container in the test arena. In the initial NOT, the object was a grey cylinder (Ø: 30 cm, height: 35 cm) with a pink ball (Ø: 50 cm) placed on top of it (figure 3, A) and in the final NOT, the object was a multicoloured open umbrella (Ø: 125 cm) (figure 3, C). In the HFTs, two novel objects were placed with two meters distance. In the initial HFT-known, each object consisted of a tripod made out of white fencing posts (height: 100 cm) wrapped with red and white plastic band (figure 3, B). As the tripods did not induce a response with sufficient variation more complex objects were used in the final handling tests. Also the distance from the arena door to the objects was increased. In the final HFT-unknown, each object consisted of a white sheet (75 x 125 cm) lying on the arena floor with two different size boxes on top, one wrapped in green paper (h x w x l: 38 x 38 x 38 cm) and one wrapped in pink paper (h x w x l: 38 x 38 x 56 cm). The latter was placed in extension of the first (figure 3, D). In the final HFT-known each object consisted of a black plastic bag (w x l: 70 x 100 cm) filled with straw lying on the arena floor and a black plastic bag (w x l: 70 x 100 cm) covering a tripod made out of white fencing posts (height: 100 cm) placed in extension of it (figure 3, E).

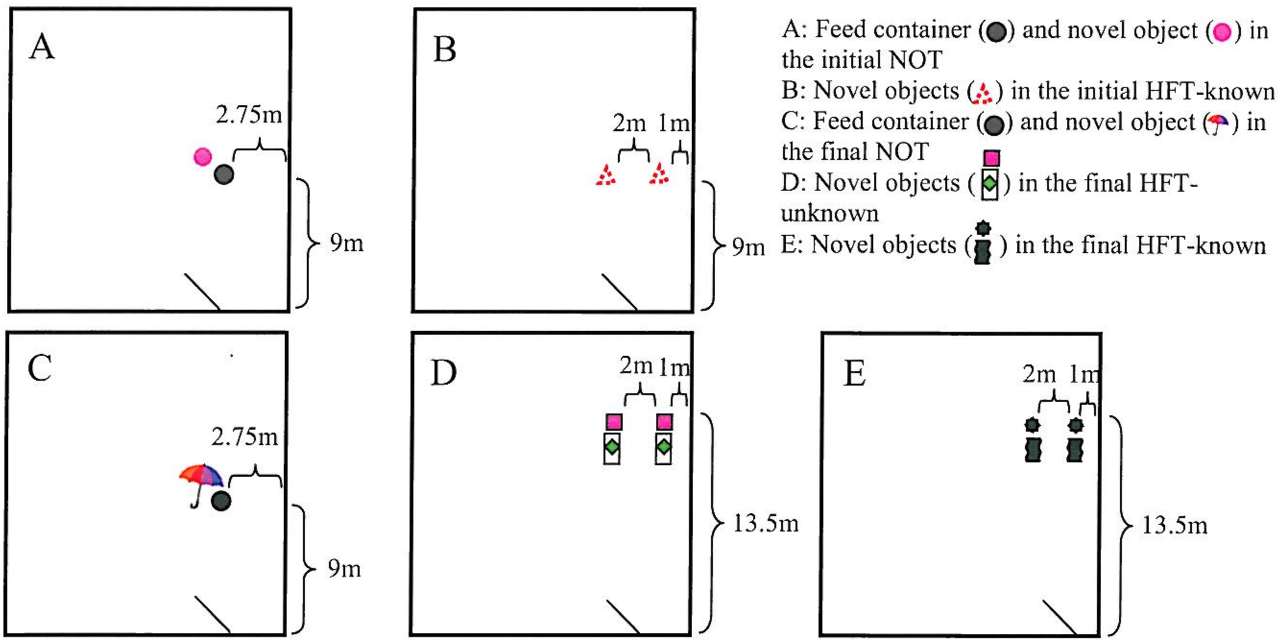

Figure 3
Position of feed container and novel objects in the novel object tests (NOT), handling fear test with unknown handler (HFT-unknown) and handling fears test with known handler (HFT-known).

2.6. Handling procedure

All the horses were alone in the arena with the handler for one 10 min session per training day. Horses in the control group were free in the arena for 10 min while horses in the handling group were trained for 10 min. The two groups were therefore equally exposed to the arena and the handler. Horses in the handling group were trained according to a standardized handling procedure to perform five different exercises using negative reinforcement (table 2). If a horse did not respond to a given signal during training, the signal was increased, e.g. by applying more pressure into the rope, until the horse responded. The horses' response was hereafter shaped to meet the criterion. Whether a horse met the criterion for each exercise was subjectively evaluated by the handler. The exercises were trained in a fixed order and when a horse met the criterion for one exercise, the trainer carried on with the next exercise. All sessions began with the first exercise, irrespective of the previous performance of the horse. It was intended to train the horses in a minimum of seven sessions, and when a horse completed all five exercises, or at the earliest after seven sessions, it would not be trained again, until the rest of the horses had also completed all the exercises. Instead the horse would be free in the arena for one 10 min session per training day. After all the handling horses had completed all five exercises, an additional training session would be conducted in order to standardize the interval between the last time the horses were trained and the final tests. However, the first handling horses completed all five exercises in session 3 and all the horses completed all five exercises in session 7 (section 3.2). The horses were therefore in the arena in a total of eight sessions, including the additional session.

Table 2
The exercises of the standardized handling procedure.

Exercise	Description	Criterion
1: Start and stop walking	The horse is trained to start and stop walking on signal from the trainer (lead pressure in the anterior/posterior direction)	Three successive correct start and stop responses to a light lead pressure
2: Walk backwards	The horse is trained to walk three steps backwards on signal from the trainer (lead pressure in the posterior direction)	Three successive correct responses to a light lead pressure
3: Walk in figures of eight	The horse is trained to be led in figures of eight (diameter: 5m) on signal from the trainer (lead pressure in the wanted direction)	Three successive repetitions using only light lead pressure
4: Walk sideways	The horse is trained to walk at least three steps sideways by crossing both front and hind legs on signal from the trainer (light whip tapping on the side)	One correct response to a light whip tapping on each side
5: Lunge in walk	The horse is trained to be lunged in walk on both sides (diameter: 5m), using a whip to accelerate the horse and pressure on the rope to slow down the horse	Three successive rounds on each side using only light signals to accelerate and decelerate the horse

2.7. Data collection

All baselines and tests were recorded on video for later determination of latencies and analysis of behaviour. In the NOTs, the latency to eat and the behaviour during the 120 s test session was determined (table 3). In the HFTs, the latency to pass the objects and the behaviour until passage of the objects and was determined (table 4). In the NOT and HFT baselines, only the latency to eat and the latency to pass the position of the objects in the tests, were determined. For all behavioural durations, it is required that the behaviour is shown in minimum 0.5 s.

Heart rate was recorded with Polar Equine RS800 CX, which consisted of two electrodes, a transmitter and a wristwatch receiver. The heart rate monitoring equipment was fitted on the horse prior to testing. One electrode was positioned on the right side of the withers and the other behind the front leg on the left side of the body. An elastic girth kept the electrodes in position and water and gel were used to enhance conduction between electrode and skin. The transmitter and the receiver were attached to the elastic girth. In the initial baselines and tests 5 s recordings were used. In the final baselines and tests R-R recordings was used in order to obtain more accurate recordings. Data were downloaded from the receiver to a PC, using the software Polar ProTrainer 5, Equine Edition.

Table 3

Ethogram of behaviours recorded in the novel object tests (modified from Christensen et al., 2005; 2008b; 2011). Durations and latencies are measured in seconds and frequencies in number of displays.

Behaviour	Description
Latency	
Latency to eat	Latency until the horse eats from the feed container.
Duration	
Eat [a]	Standing by the feed container chewing food; including lifting head while chewing continues.
Alert [a]	Vigilant with elevated neck, head and ears oriented towards novel object.
Invest [a]	Neck horizontal or lower, head and ears oriented towards novel object. Includes sniffing, touching and manipulation of novel object
Other behaviours [a]	Behaviours not included in the above, e.g. focussing on other stimuli, such as sounds from outside the arena or not having focus on an object.
Walk [b]	Moving forward in walk. Requires that more than one leg is moved.
Run [b]	Moving forward in any gait but walk. Requires that more than one leg is moved.
Stand [b]	Standing still with four feet in the ground. Movement of one leg is not an interrupted stand.
Other movements [b]	Movements not included in the above, e.g. turning, jumping, backwards or sideways. Requires that more than one leg is moved.
Frequency	
Defecation	Elimination of faeces
Snort	Short powerful exhalation from nostrils
Paw bout	Striking the ground or air with forelimb; pawing after pauses of more than 5s was recorded as a new bout.

[a] Behaviour characterizing the orientation of the horse, [b] behaviour characterizing the movement of the horse.

Table 4
Ethogram of behaviours recorded in the handling fear tests (modified from Visser et al., 2001; 2003, Wolff et al., 1997). Durations and latencies are measured in seconds.

Behaviour	Description
Latency	
Latency to pass objects	Latency until the horse has passed between the objects. The objects are considered passed when all four legs has passed the objects.
Duration	
Forward	Moving forward in walk in the direction given by the handler. Requires that more than one leg is moved.
Stand [a]	Standing still with four feet in the ground at any given time. Movement of one leg is not an interrupted stand.
Away [a]	Moving in another direction than the one given by the handler. Requires that more than one leg is moved.
Other [a]	Other movements, where the horse is neither moving forward in walk in the direction given by the handler nor standing still or moving away. Requires that more than one leg is moved.
Forward flight	Moving in front of the handler while passing the objects.

[a] Behaviour, characterized by the horse being reluctant to move in the direction given by the handler.

Artefacts in the data were corrected using the error correction function in the program. Heart rate recordings were initiated prior to testing but for the analysis only recordings from baselines or tests were used. In the NOT-baselines and NOTs, average heart rate (HR-avg) and maximum heart rate (HR-max) during the 120 s test session, measured in beats per minute (bpm), were determined for each horse. HR-avg was not considered informative in the HFT-baselines and HFTs as the duration of the test sessions varied. Only HR-max was therefore determined for each horse in the HFT-baselines and HFTs.

2.8. Data analysis

As pawing and forward flight was only shown by a few horses and there were no snorting and no defecations, these data were not included in the analysis. The movements run, walk and other movements in the NOTs were analysed as one variable; duration of movements, and the reluctant behaviours stand, away and other in the HFTs were analysed as one variable; duration of reluctance behaviour. Their opposites, which were stand in the NOTs and forward in the HFTs, were also not included in the analysis. Also, since all horses which began to eat in the NOT continued to eat until the test time was up, latency to eat and duration of eating was each other's opposite. Duration of eating was therefore not included in the analysis. An overview of the response variables used in the analysis is presented in table 5.

Table 5
The response variables.

Response variables	unit
Novel object test	
HR-avg	bpm
HR-max	bpm
Latency to eat	s
Alert	s
Invest	s
Other behaviours	s
Movements	s
Handling fear test	
HR-max	bpm
Latency to pass objects	s
Reluctance behaviour	s

Unfortunately, not all data were available from all horses. One heart rate file was lost in the initial NOT, along with three files in the final NOT-baseline and two files in the final HFT-baseline, due to a high number of errors. Also, one control horse did not meet the habituation criterion in the initial NOT-baseline and data from this horse in the NOTs was therefore excluded from the analysis. Not all horses ate within the test time or the extended test time in the NOT. In the initial NOT the latency to eat was censored for seven horses and set to 120 s and in the final NOT the latency to eat was censored for two horses and set to 300 s. In addition, one horse did not pass the objects within the 300 s test time in the HFT-unknown. The latency for this horse was censored and set to 300 s.

Data were analysed using R (version 2.15.2; www.R-project.org). Distributions were evaluated by visualisation and the Shapiro-Wilks Normality test. P-values in all statistical analysis were evaluated at a 5% significance level.
The effect of the novel objects on the horses' responses in the initial and in the final tests were analysed by comparing the latencies and heart rate responses in the baselines with the latencies and heart rate responses in tests. When data was normally distributed, the paired t-test was applied. If the assumption of a normal distribution was not met, the matched-pairs Wilcoxon test was applied. Censored data was included in the analysis with the censored value as the latency value.
The difference between handled and control horses in the initial and in the final tests were analysed by comparing the responses of horses in the handling group with the responses of horses in the control group. When data was normally distributed the Welch two-sample t-test

was applied. When the assumption of a normal distribution was not met the two-sample Wilcoxon test was applied. Latencies containing censored data were analysed using survival analysis. The differences between the two treatment groups in latency to eat or pass the objects was analysed using the Log-rank test, with latency to eat or pass the objects as response variable and treatment as explanatory variable.

Correlations between the behavioural and heart rate responses within each test and the correlations between the responses in the NOTs and the HFTs were analysed using Spearman´s rank correlation. Censored data was included in the analysis with the censored value as the latency value.

3. Results

3.1. Initial baselines and tests

In the initial NOT there was a significant increase compared to the baseline measurements in latency to eat (Matched-pairs Wilcoxon test: (s) median [25;75% quartile], Baseline: 8.0 [7.0;8,0] vs. Test: 14.0 [10.0;120.0], n=23, P<0.001) and HR-max (Paired t-test: (bpm) mean ± s.e, Baseline: 80.2 ± 2.0 vs. Test: 98.4 ± 5.7, n=22, P=0.007). There was also a tendency towards an increase in HR-avg but the increase was not significant (Matched-pairs Wilcoxon test: (bpm) median [25;75% quartile], Baseline: 63.0 [61.0;69.0] vs. Test: 72.5 [60.0;78.3], n=22, P=0.10). In the initial HFT-known, latency to pass the objects was significantly increased compared to the baseline measurement (Matched-pairs Wilcoxon test: (s) median [25;75% quartile], Baseline: 10.0 [9.8;10.3] vs. Test: 12.0 [11.0;14.3], n=24, P<0.001), while there was no difference in HR-max (Paired t-test: (bpm) mean ± s.e, Baseline: 78.5 ± 1.4 vs. Test: 79.4 ± 2.0, n=24, P= 0.69).

There was no difference between any of the behavioural or heart rate variables of horses in the handling group and horses in the control group in the initial tests (P>0.05, table 6).

Table 6
Responses of handling and control horses in the initial novel object test (NOT) and handling fear test with known handler (HFT-known).

	Variables	Handling	n	Control	n	P-value
NOT	HR-avg (bpm)	72.1 ± 5.2	11	75.0 ± 5.4	11	0.702 [a]
	HR-max (bpm)	92.9 ± 8.6	11	103.9 ± 7.4	11	0.344 [a]
	Latency to eat (s)	12.5 [9.75;64.5] (3)	12	14.0 [10.0;Inf] (4)	11	0.628 [c]
	Alert (s)	6.8 [5.0;18.9]	12	7.0 [6.0;33.3]	11	0.280 [b]
	Invest (s)	5.8 [3.4;10.1]	12	4.0 [3.3;7.5]	11	0.642 [b]
	Other behaviours (s)	0.0 [0.0;2.4]	12	0.0 [0.0;35.0]	11	0.294 [b]
	Movements (s)	11.8 [9.0;32.8]	12	12.5 [10.0;36.75]	11	0.664 [b]
HFT-known	HR-max (bpm)	79.7 ± 3.1	12	79.2 ± 2.7	12	0.903 [a]
	Latency to pass objects (s)	11.5 [11.0;12.3]	12	12.0 [10.8;15.3]	12	0.860 [b]
	Reluctance behaviour (s)	0.00 [0.0;0.5]	12	0.3 [0.0;2.5]	12	0.420 [b]

[a] Welch two-sample t-test (mean ± s.e.), [b] Two-sample Wilcoxon test (median [25;75%-quartile]), [c] Log-rank test (median [25;75%-quartile](number of censored values).

3.2. Handling procedure

All the horses were in the arena in eight sessions. The average number of exercises completed by the handling horses in each training session increased with session number. In session 7, all the handling horses completed all five exercises. Also in the additional session (session 8), all the horses completed all five exercises (figure 4).

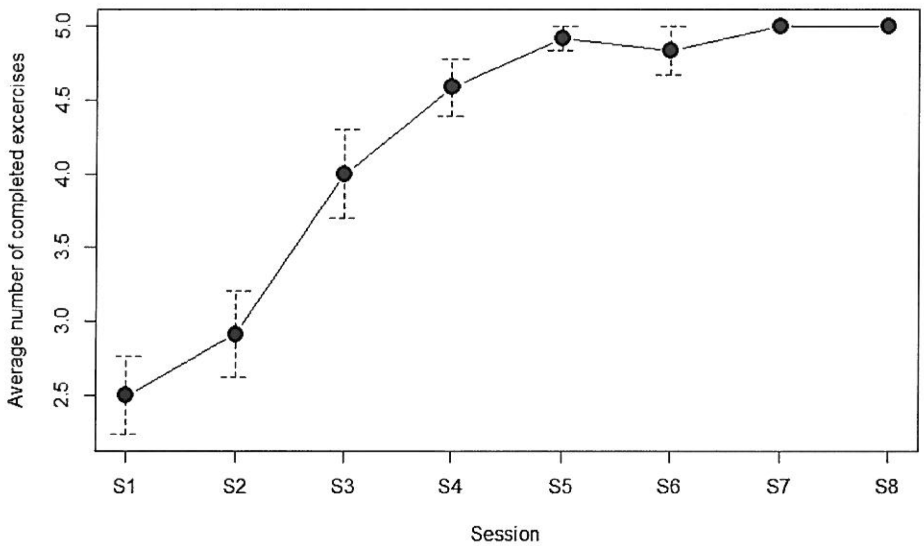

Figure 4
Number of exercises completed by the handling horses in each training session (mean ± s.e.).

3.3. Final baselines and tests

In the final NOT there was a significant increase in latency to eat (Matched-pairs Wilcoxon test:: (s) median [25;75% quartile], Baseline: 8.0 [7.5;8.5] vs. Test: 13.5 [12.8;14.0], n=23, P<0.001), HR-avg (Paired t-test: (bpm) mean ± s.e, Baseline: 51.6 ± 1.2 vs. Test: 64.13 ± 2.6, n=20, P<0.001) and HR-max (Paired t-test: (bpm) mean ± s.e, Baseline: 74.8 ± 2.4 vs. Test: 93.4 ± 3.0, n=20, P<0.001) compared to the baseline measurements. Also in the final HFT-unknown and the final HFT-known was there a significant increase compared to the baseline measurements in latency to pass the objects (HFT-unknown: Matched-pairs Wilcoxon test: (s) median [25;75% quartile], Baseline: 13.5 [12.8;14.0] vs. Test: 18.0 [15.0;25.0], n=24, P<0.001, HFT-known: Matched-pairs Wilcoxon test: (s) median [25;75% quartile], Baseline: 13.5 [12.8;14.0] vs. Test: 13.0 [12.8;18.0], n=24, P=0.035) and HR-max (HFT-unknown: Paired t-test: (bpm) mean ± s.e, Baseline: 71.2 ± 2.6 vs. Test: 95.3 ± 3.2, n=22, P<0.001, HFT-known: Paired t-test: (bpm) mean ± s.e, Baseline: 71.2 ± 2.6 vs. Test: 80.8 ± 2.4, n=22, P=0.012).

The handling procedure had no effect on the responses of the horses in the final NOT or the final HFT-unknown, as there were no difference between the behavioural or heart rate responses of horses in the handling group and horses in the control group in these tests (Table 7). But in the final HFT-known there was a significant difference between horses in the handling group and horses in the control group in the duration of reluctance behaviour (Two-sample Wilcoxon test: P=0.031, table 7), as handled horses showed less reluctance behaviour than control horses (figure 5), while there was no difference between horses in the handling group and horses in the control group in latency to pass objects (Two-sample Wilcoxon test: P=0.66, table 7) or HR-max (Welch two-sample t-test: P=0.14, table 7).

Table 7
Responses of handling and control horses in the final novel object test (NOT), handling fear tests with unknown handler (HFT-unknown) and handling fear test with known handler (HFT-known).

	Variables	Handling	n	Control	n	P-value
NOT	HR-avg (bpm)s	61.7 ± 3.1	12	66.8 ± 4.3	11	0.341 [a]
	HR-max (bpm)	91.3 ± 4.3	12	95.7 ± 4.2	11	0.473 [a]
	Latency to eat (s)	19.5 [12.5;117] (2)	12	30.0 [13.0; 79.0]	11	0.584 [c]
	Alert (s)	9.5 [4.5;22.6]	12	17.0 [8.0;26.5]	11	0.388 [b]
	Invest (s)	8.0 [3.4;11.1]	12	6.0 [4.3;17.5]	11	0.497 [b]
	Other behaviours (s)	0.0 [0.0;53.1]	12	3.0 [0.0;22.3]	11	0.974 [b]
	Movements (s)	18.5 [11.0;50.3]	12	29.5 [13.0;59.5]	11	0.735 [b]
HFT-unknown	HR-max (bpm)	92.4 ± 3.6	12	98.1 ±5.2	12	0.384 [a]
	Latency to pass objects (s)	21.0 [16.0;25] (1)	12	16.5 [15.0;34.3]	12	0.773 [c]
	Reluctance behaviour (s)	2.5 [0.0;6.1]	12	1.5 [0.0;15.6]	12	0.976 [b]
HFT-known	HR-max (bpm)	77.2 ± 2.5	12	84.4 ± 3.9	12	0.136 [a]
	Latency to pass objects (s)	13.0 [13.0;14.3]	12	15.5 [12.0;24.3]	12	0.658 [b]
	Reluctance behaviour (s)	0.0 [0.0;0.0]	12	0.75 [0.0;10.4]	12	0.031 [b]

[a] Welch two-sample t-test (mean ± s.e.), [b] Two-sample Wilcoxon test (median [25;75%-quartile]), [c] Log-rank test (median [25;75%-quartile](number of censored values).

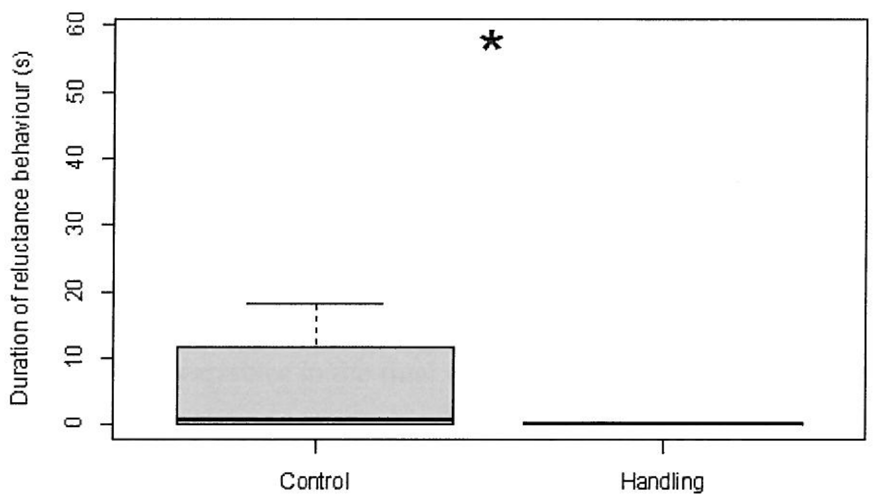

Figure 5
Duration of reluctance behaviour (s, median [25;75%-quartile]) for handling and control horses in the final handling fear test with known handler.

3.4. Correlations

The correlations from the initial and the final tests were similar, but as the responses in the final tests were more pronounced and with a larger variation than in the initial tests and no data was lost in the final tests, only correlations from the final tests are shown below.

In the final NOT, all the behavioural variables except invest correlated strongly with the heart rate variables (table 8). The heart rate variables were positively correlated with latency to eat and the duration of alert, other behaviours and movements. In the HFT-unknown, both latency to pass the objects and reluctance behaviour correlated positively with HR-max (table 9). However, in the HFT-known, latency to pass the objects did not correlate with HR-max and reluctance behaviour only showed a tendency towards a positive correlation with HR-max (table 9).

Table 8
Correlations between the behavioural and heart rate variables in the final novel object test (n=23).

Variables	HR-avg (bpm)		HR-max (bpm)	
	r	P-value	r	P-value
Latency to eat (s)	0.71	<0.001	0.61	0.002
Alert (s)	0.72	<0.001	0.65	<0.001
Invest (s)	0.14	0.530	-0.02	0.967
Other behaviours (s)	0.73	<0.001	0.63	0.001
Movements (s)	0.74	<0.001	0.64	<0.001

Table 9
Correlations between the behavioural and heart rate variables in the final handling fear test with unknown handler (HFT-unknown) and the final handling fear test with known handler (HFT-known) (n=24).

	Variables	HR-max (bpm)	
		r	P-value
HFT-unknown	Latency to pass objects (s)	0.69	<0.001
	Reluctance behaviour (s)	0.66	<0.001
HFT-known	Latency to pass objects (s)	0.25	0.245
	Reluctance behaviour (s)	0.38	0.065

Almost all the variables in the final NOT showed strong positive correlations with the variables latency to pass the objects, reluctance behaviour and HR-max in the final HFT-unknown. However, HR-avg in the final NOT only showed a tendency towards a positive correlation with reluctance behaviour in the final HFT-unknown, and the variable invest was not correlated with any of the variables in the final HFT-unknown (table 10). Strong positive correlations were also found between the most of the variables in the final NOT and HR-max in the final HFT-known. However, latency to eat and other behaviours in the final NOT only showed a tendency towards positive correlations with HR-max in the final HFT-known and the variable invest in the final NOT was not correlated with HR-max in the final HFT-known. Also were there no correlations between any of the variables in the final NOT and the behavioural variables latency to pass the objects and reluctance behaviour in the final HFT-known (table 11).

Table 10
Correlations between responses in the final novel object test (NOT) and the final handling fear test with unknown handler (HFT-unknown) (n=23).

Variables		HFT-unknown					
		Latency to pass objects (s)		HR-max (bpm)		Reluctance behaviour (s)	
		R	P-value	R	P-value	R	P-value
NOT	HR-avg (bpm)	0.47	0.024	0.67	<0.001	0.40	0.061
	HR-max (bpm)	0.51	0.013	0.60	0.003	0.43	0.038
	Latency to eat (s)	0.52	0.011	0.51	0.012	0.50	0.016
	Alert (s)	0.54	0.008	0.52	0.011	0.49	0.018
	Invest (s)	0.06	0.784	0.24	0.269	0.06	0.777
	Other behaviours (s)	0.49	0.017	0.52	0.011	0.44	0.035
	Movements (s)	0.51	0.013	0.55	0.006	0.45	0.033

Table 11
Correlations between responses in the final novel object test (NOT) and the final handling fear test with known handler (HFT-known) (n=23).

Variables		HFT-known					
		Latency to pass objects (s)		HR-max (bpm)		Reluctance behaviour (s)	
		R	P-value	R	P-value	R	P-value
NOT	HR-avg (bpm)	0.08	0.727	0.67	<0.001	0.17	0.427
	HR-max (bpm)	0.14	0.518	0.50	0.016	0.17	0.443
	Latency to eat (s)	0.06	0.783	0.36	0.095	0.10	0.652
	Alert (s)	0.21	0.348	0.42	0.048	0.22	0.310
	Invest (s)	-0.15	0.503	0.34	0.113	0.26	0.235
	Other behaviours (s)	0.00	0.986	0.37	0.086	0.06	0.790
	Movements (s)	0.11	0.613	0.41	0.050	0.19	0.383

4. Discussion

There was no difference between the behavioural or physiological response of handled and control horses in the initial NOT or in the initial HFT. This suggests that the treatment groups were appropriately balanced and no adjustments of the groups were necessary. There was also no difference between the response of handled and control horses in the final NOT or in the final HFT-unknown. However, handled horses showed less behavioural fear responses than control horses in the final HFT-known, where they were handled by the same person, who trained the horses during the handling procedure. The results suggest that differences in previous handling experience may affect the behavioural responses of horses in tests involving handling by their usual handler.

4.1. Effects of the handling procedure on the responses in the final tests

Handled horses was hypothesised to show less behavioural fear response than control horses in the final HFTs, as the handling procedure was expected to increase the horses motivation to respond to the handlers' signals and therefore to a higher degree overshadow the horses motivation to respond to the fear eliciting stimuli (Mclean, 2008). This was, however, only the case in the final HFT-known and only for reluctance behaviour. That handled and control horses did not differ in latency to pass the objects may be due to the objects not inducing a response with sufficient variance. As there was no difference in heart rate between handled and control horses, it is suggested that they were equally frightened by the stimuli.

That the differences between handled and control horses was not apparent in the final HFT-unknown, may have been caused by the known and unknown handler not using the exact same signals, even if the two handlers were instructed to use the same signals. The horses were thereby not given the same signals as they had been taught to respond to in the handling procedure and therefore did not respond to the unknown handler. It may also be possible that the signals were not consolidated enough for the handled horses to generalise them to another handler. In a study on 16 Anglo-Arabian and French Saddlebred horses of 2 years of age, Sankey et al. (2011) found that horses taught to stand still when given a voice command also stood still when given the command by an unknown handler of the opposite sex. It was thereby shown that horses are capable of generalising what they have learnt from one handler to another. In the study by Sankey et al. (2011) horses were trained to perform one specific exercise and were tested under the same conditions, as they were thought to perform the exercise. In the present study, testing was different from the training situation and the horses were taught to perform several exercises. The horses may therefore have been less likely to generalise and a handling procedure of a longer duration might have increased the consolidation of the signals. Differences in the relationship with the known handler may also have had an effect. A study investigating human-animal-relationship (HAR) have found that cows may perceive a veterinary procedure as less frightening if a handler is interacting positively with the cows during the procedure. Also, cows that have previously experienced positive interactions with the handler were less frightened than control cows (Waiblinger et al., 2004). In the present study, horses in the handling group were forced to interact with the handler while horses in the control group were not. The difference in experiences with the known handler may have affected the horses' response during the final HFT-known, but not in the final HFT-unknown, as no horses had a relationship with the unknown handler. What the differences in the horses' relationship with the known handler may consist of is not known, but if differences in HAR affect horses' response in handling tests with known handlers, this should be investigated further. Additionally, horses' previous experiences with one human may be generalised to other humans, as it was shown by Hausberger and Muller (2002) in a study on 224 horses from the same site, where horses managed by the same caretaker tended to show similar reactions to an unfamiliar person.

Hence, despite the findings in other studies, there are no indications in the present study, that differences in horses' previous experiences with handling will affect their response in a handling fear tests with an unknown handler. However, when horses are handled by their usual handler, their response appears to be affected.

4.2. Correlations

4.2.1. Correlations between HR and behaviour

The novel objects elicited significant behavioural and physiological responses in all tests compared to the baseline tests. As the behavioural (except the variable invest) and heart rate variables in the NOT correlated, it is indicated that they are all useful as measures of the fear response in this test. The correlations were in the same range as in other studies using static novelty (e.g. Christensen et al., 2012). In the present study, the variable invest did not correlate with any of the HR variables. It has been suggested that alertness may be primarily related to an animal's first exposures to a novel stimulus, while investigation may be related to the process where the animal becomes increasingly familiar with the stimulus (Christensen, 2013). The variable invest may therefore not be useful as a measure of fear during an initial exposure to a stimulus in a NOT.

In the HFT-unknown, the behavioural and heart rate variables were also correlated. This is in accordance with the findings of a study by Leiner and Fendt (2011), who found significant correlations between the behavioural fear response and the heart rate of 18 German warmblood horses of 2½ year of age when led toward a novel object. These horses had almost no previous experience with handling, and were, therefore, equally naive regarding handling. The handling during the test may therefore have been perceived as equally frightening and the test may therefore also have been a test of the horses' fear of handling. Additionally, in a study by Visser et al. (2003), where 18 Swedish warmblood horses of varying age were tested in a handling bridge test, heart rate correlated to resistance behaviour and standing still. The horses were all from the same site and were used for education and competitions. It is, however, not apparent if the horses had any previous experiences with the handler. In contrast were there only tendencies towards correlations between the behavioural and heart rate responses in the HFT-known in this study. This may be due to the handling during the HFT-known affecting the handled horses' behavioural response differently than the control horses.

4.2.2 Correlations between tests

The responses in the NOT, except the variable invest, and the responses in the HFT-unknown were correlated. This indicates that the two tests measure the same underlying trait. This is in accordance with a study by Wolff et al. (1997) on 24 French Saddlebred horses of 1-3 years of age, which were all from the same farm and used to being handled, where the behaviour during a novel object test correlated with the time used to cross a bridge while handled. Also, in another study on 65 Hanoverian riding horses of 3-19 years of age, König von Borstel et al. (2011) found that horses' behavioural reactions towards the same novel objects, when free running, handled or ridden, was correlated. However, in the present study, the responses in the NOT only correlated with heart rate and not with the behavioural responses in the HFT-known. It is, therefore, indicated, that the heart rate variables in the two tests measure the same underlying trait, while the behavioural variables do not. In the study by König von Borstel et al. (2011), the heart rate variables showed the highest repeatabilities over the tests, and the behavioural variables the lowest. In a study where the response of 24 Danish warmblood horses of 2 years of age towards novel, olfactory and auditory stimuli were measured, the heart rate response was found to correlate between test situations, while the behavioural response was linked to the type of stimulus (Christensen et al., 2005). In another study on 40 horses of different age and breed, the heart rate response towards novel stimulus and isolation ranked the horses similarly, while the behavioural response did not (McCall et al., 2006). It has therefore been suggested that heart rate is a more reliable indicator of the fear response than behavioural variables (König von Borstel et al., 2011). This is supported by the results in the present study, as the heart rate measurements seemed less affected by the handling by the known handler than the behavioural variables.

4.3. Methodological considerations

4.3.1. Motivations in tests

There has to be a motivational conflict between avoiding and approaching the test stimulus, in order to interpret horses' response in fear tests, as avoiding a stimulus may not necessarily mean that the horse is frightened of it (Christensen, 2007). In the NOT, horses were motivated to approach the novel objects due to the presence of feed using the same approach as in other studies (e.g. Christensen, 2013; Christensen et al., 2012). The feeding motivation of horses may differ, but as the horses used in this study were of the same breed, age and exercise level, and were kept on pasture, it was assumed that they were equally motivated to feed. However, if fear tests are to be applied at breeding evaluations, the feed motivations of the subject horses may be more variable and it might be beneficial to avoid the use of feed in tests.

In the HFTs, horses were motivated to pass between the novel objects due to the handlers forward signalling. As the aim was to investigate the effect of handling on horses fear reactions, differences in the subject horses' previous experiences with handling before the experiment may impair the results. Attempts were, therefore, made to standardise the horses' previous experiences with handling by using young horses of the same age and by subjecting them to the same initial handling before testing. The exercises in the handling procedure are commonly used in basic horse training and were trained using negative reinforcement. This procedure was chosen, as practical horse training is typically based on this technique (McLean, 2005). The aim of the handling procedure was not to train the horses to perform a range of exercises, but to increase the consolidation of the forward signal. Not all the exercises directly involved training walking forward, but when training the rest of the exercises, the forward signal was used (e.g. to position the horse in the desired position) and therefore indirectly trained.

4.3.2. Test stimuli

The test stimuli used in this study were objects of different size, shape and colour. It was decided to apply the same kind of stimuli (static novelty) in all tests, as studies have indicated that horses' behavioural response may be linked to which sense the stimuli relates to (Christensen et al., 2005; Lansade et al., 2008b). The selected stimuli were aimed to be strong enough to elicit a response in the majority of the horses but not so strong that the animals would not approach the feed container or pass the objects within the test time. In the initial NOT, the novel object increased latency to eat and HR-max compared to the baseline measurements, but the stimulus was too mild to significantly increase HR-avg in the two-minute test period. The latency to pass the objects in the initial HFT-known was increased compared to the baseline measurements, but the stimulus was too mild to significantly increase HR-max. The limited responses in the initial tests indicate the difficulty in selecting a stimulus that induces a response with sufficient response and variation. Increasing the strength of the stimuli in the final NOT and HFTs was therefore attempted. As a result, all latencies and heart rate variables in the final tests were significantly increased compared to the baseline measurements.

Previous studies have recommended using sudden stimuli in order to ensure a sufficient response, as the behavioural and physiological response of horses to suddenness have been found to be larger than the response to static novelty (Christensen, 2007). Also, when using static objects in fear tests, there is a risk of the horses' response being affected by their previous experiences with similar objects (section 1.2.), while horses' response towards sudden stimuli is expected to be less affected by their previous experiences. However, suddenness was deselected in this study, due to the difficulty in standardising the application of a sudden stimulus and that the perception of a sudden stimulus may, to a higher degree than the perception of a static stimulus, be influenced by the orientation of the horse (e.g. if the horse is looking in the other direction when the sudden stimuli is applied). In addition, as human handling was involved, the use of suddenness may have impaired human safety during the tests, due to the increased response of the horses.

4.3.3. Physiological and behavioural variables

Heart rate has been validated as a reliable and non-invasive measure of the physiological response in horses during exposure to a fear eliciting stimulus (e.g. McCall et al., 2006). Heart rate variability (HRV) was initially also intended as a physiological variable, but, as described in appendix A, HRV is not expected to provide any information not already given by heart rate to stressors of a short duration, as used in this study. Additionally, the variable duration of the HFTs and the relatively short duration of the test sessions may impair HRV analysis as HRV is affected by the duration of the recordings, and it is generally recommended to use recordings of minimum 5 minutes (Von Borell et al., 2007). The use of other physiological measures, such as salivary and plasma cortisol, was deselected due to both economic and practical reasons. Also, it was not expected that they would provide any information not already given by heart rate.

It was emphasised that the behavioural measurements should be as objective as possible. Grading of the horse's response on a predefined scale was therefore deselected, and the measurements included latencies, durations and frequencies. The behavioural measurements used in the NOTs have been found useful in assessing fearfulness in other studies (Christensen, 2013; Christensen et al., 2005; 2008b; 2011), and were focused on the horses' posture and orientation. The recorded behaviours in the HFT were different from the ones in the NOT, as it was expected that the handling would restrict and thereby alter the horses' head movements. The choice made was, therefore, to focus at the horses' reluctance towards passing between the objects, with a focus on the direction of the horses' movements. A similar approach has been used in other studies (e.g. Visser et al., 2001; 2003; Wolff et al., 1997).

4.4. Perspectives

4.4.1. Including fear tests in horse breeding evaluations

Assessments of fearfulness may be used to select horses for breeding or for specific humans, works or housing conditions (König von Borstel, 2013). If selecting horses for breeding based on their assessed fearfulness, it is important that the assessed fearfulness has an inheritable variance. The emphasis is therefore on revealing the horses' genotype, and the less the assessment is affected by the horses previous experiences the better. The present study therefore supports the use of fear tests not involving handling by the horses' usual handler.

But if fear tests are to be included in horse breeding evaluations, horse owners may start habituating their horses to a variety of fear eliciting stimuli before the evaluation (Burger et al., 2007). However, even if horses are capable of object recognition and generalisation, habituation to some stimuli may not necessarily decrease the response towards other stimuli (Christensen et al., 2008b; 2011). The assessment of performance traits such as conformation and jumping ability are also affected by the horses' previous experiences and are deliberately trained before breeding evaluations. And as the heritability of fearfulness assessed in fear tests is in the range of other traits assessed at breeding evaluations, it is expected to be possible to use fearfulness assessed in fear tests to select horses for breeding (Hausberger et al., 2007).

Fearfulness may be correlated to other traits due to pleiotropy or genetic linkage (Rauw et al., 1998). This has, among others, been observed in silver foxes, where animals were selected over a 20 year's period based on their fear response to humans. Over generations, the animals' response changed from aggression and fear to contact seeking, and correlated responses such as morphological changes in tail position and coat colour patterns were observed (Belyaev, 1979). There has also been evidence of such correlations in horses, and in a study on 90 Warmblood riding horses it was found that horses with a higher genetic potential for show jumping, showed less behavioural response to a fear eliciting stimuli (König von Borstel et al., 2010). Also, in a recent study on 27 Icelandic horses of varying age it was found that horses with the silver coat colour mutation are more cautious in novel situations than horses without the mutation, which was suggested to be caused by the silver mutation being genetically linked to a mutation causing an eye defect syndrome (Brunberg et al., 2013). Estimation of breeding values using multivariate BLUP (best linear unbiased prediction) is increasingly used in horse breeding and correlations between traits may increase the accuracy of the estimated breeding values for the traits involved, especially for traits with a low heritability or when dealing with traits where indicators are missing or that are difficult to measure (Mrode, 2005). But as selecting for one trait may cause an indirect selection for other correlated traits, it is important to include temperamental traits such as fearfulness in both breeding goals and breeding evaluations, in order to avoid or minimize the possible undesirable side effects of the selection for other correlated traits on temperament (Rauw et al., 1998).

However, if using assessments of fearfulness to select horses for specific purposes (e.g. for therapeutic riding or riding schools), the horses phenotype is more important than its genotype. Tests of fearfulness conducted in order to select horses for specific purposes should therefore to a higher degree reflect the practical use of the horse, and the use of fear tests involving various kinds of human handling and situations may be useful in this context.

Also, when dealing with horses in practice, the horses' ability to habituate to a fear eliciting stimulus and its ability to learn not to express a behavioural response to a fear eliciting stimulus while ridden or handled, are also factors related to fear and fearfulness, which may affect the usability and the welfare of the horse. Hence, whilst this study was focused on horse's basic response to fear eliciting stimuli, would it be interesting also to include relevant aspects of horses' learning ability in future research in horses' fear reactions.

4.4.2. Horse training

Studies have investigated how to train horses not to be frightened of fear eliciting stimuli using different methods (e.g. Christensen et al., 2006; 2008a). However, horses may not necessarily generalise between the fear eliciting stimuli used during training and other stimuli (Christensen et al., 2008b; 2011). It may, therefore, be beneficial to be able to affect the basic response of horses to novel stimuli when interacting with the horse during handling and riding (Christensen, 2007) and the results of the present study suggests that training a horse in basic handling routines may decrease the behavioural response in a fear eliciting situation when the horse is handled by its usual handler. The horse may still be frightened, but the behavioural response is altered, which may be due to the horses' motivation to respond to the handlers' signal overshadowing the

horses' motivation to respond to the fear eliciting stimuli.
Mclean (2008) suggested that overshadowing the horses' motivation to respond to a fear eliciting stimuli, e.g. by making the horse perform a previously trained exercise, may be used as a method to habituate horses to fear eliciting stimuli like shoeing or electric clippers. This is supported by a recent habituation study on 22 Danish warmblood horses of 2-3 years of age, where horses that were negatively reinforced by a handler to approach a collection of novel objects, habituated to the objects (Christensen, 2013). However, horses that were negatively reinforced to approach the novel objects had an increased heart rate compared to horses that were free to explore the objects, and may therefore have perceived the situation as more frightening (Christensen, 2013). Also, preventing horses from showing a fear response using handling, may, if not carefully managed, impose a safety risk to the handler as the handler may be unable to control the horses. In addition, when physically restricting a horse, the risk of flooding and thereby inducing learnt helplessness is increased (Hall et al., 2008).
Previous training of horses in order to enhance overshadowing in a fear eliciting situation may, therefore, assist in increasing safety, as it may alter the horses' basic response to a fear eliciting stimulus while handled or ridden. However, when training a horse not to be frightened by specific stimuli, overshadowing may not always be an appropriate method due to both safety and horse welfare and the use of other training methods or combinations of methods may be more ideal.

5. Conclusion

Based on the results of the present study it is concluded that differences in previous handling does not affect the behavioural or the physiological fear response of horses in a novel object test. However, previous handling may affect the behavioural response of horses in handling fear tests, when handled by the same person, who was responsible for the previous handling of the horse, while heart rate was unaffected of the previous handling. There were no indications of previous handling affecting the fear response of horses when handled by an unknown handler. The results therefore imply that if fear tests are to be included in breeding evaluations, the use of handling in the tests may impair the results, if the handler is known to the horse. However, measurements of heart rate were less affected by the handling than the behavioural variables and thus may be a more reliable indicator of fear.

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Appendix A

This appendix is a 2 ECTS written assignment, prepared in connection with the PhD course "Interpretation of animal stress responses", Aarhus University, January 2013. The assignment was approved in July 2013, and since then only minor adjustments have been made.

Using heart rate and heart rate variability as indicators of the stress response in horses
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A stressor is a real or a potential threat against homeostasis, and the stress response is the adaptations of the body to reestablish the balance (Sapolsky, 2002). Measurements of heart rate (HR) and heart rate variability (HRV) can be used as stress response indicators. The aims of this assignment are to describe how measurements of HR and HRV can be used as indicators of the stress response, and to discuss when it is relevant to apply measurements of HR and HRV in horses. In order to do this, the physiological stress response and how it affects HR and HRV is described. The use of HR and HRV in horse research is analysed, and the relevance of using HR and HRV as stress indicators in horses is discussed.

The physiological stress response

The stress response consists of a behavioral and a physiological response, which enables the animal to perform an appropriate reaction in order to increase its chances of survival. The main physiological responses are the activation of the sympathetic nervous system (SNS) and the hyphothalamus-pitutiary-adrenocortical (HPA) axis. In response to a stressor, SNS activity increases while the activity of the parasympathetic nervous system (PNS) decreases. The SNS is activated by stimulation of hypothalamus, which sends out signals that are transmitted via the brain steam and the spinal cord. This stimulates the release of the catecholamines noradrenalin and adrenalin. Noradrenalin is primarily released from sympathetic nerve endings, and to a smaller extent from the medulla of the adrenal gland directly to the blood. Adrenalin is released together with noradrenalin in the medulla of the adrenal gland, directly to the blood (Sapolsky, 2002). The HPA-axis is activated by stimulation of hypothalamus. This simulates the secretion of corticotrophin-releasing hormone (CRH) and vasopressin (APV) from the hypothalamus. CRH and AVP then stimulate the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland, after which ACTH stimulates the release of corticosteroids from the

cortex of the adrenal gland. The released corticosteroids are cortisol in horses, cattle, sheep, pig and mink and corticosterone in birds and laboratory rodents (Mormède et al., 2007). The sympathetic stress response takes place within seconds after the perception of a stress inducing stimuli. The endocrine response is slower than the sympathetic response and the level of corticosteroids in the blood reaches its maximum after a few minutes (Korte, 2001).
The overall effect of the physiological stress response is to enable the animal to perform an appropriate behavioral reaction to a stress eliciting stimuli. Therefore, both metabolic and cardiovascular changes occur in order to mobilize energy and deliver it rapidly to where it is needed (Sapolsky, 2002). These changes are mainly caused by the activation of the SNS and the HPA-axis. The released catecholamines causes heart rate and blood pressure to increase and blood is redirected away from processes that are unnecessary in the moment, e.g. digestion in the gastrointestinal tract, to skeletal muscles and the brain through vasoconstriction or vasodilation of blood vessels. The catecholamines also increase glycogenolysis, gluconeogenesis and lipolysis, and the availability of energy substrates therefore increases (Sapolsky, 2002). Insulin release is inhibited (Greco and Stabenfeldt, 2007) while glucagon release from the pancreas is stimulated (Jones et al., 2012). As insulin stimulates storage of energy substrates (Greco and Stabenfeldt, 2007) and glucagon increase glycogenolysis and gluconeogenesis, the availability of energy substrates is further increased (Jones et al., 2012). The released glucocorticoids increase gluconeogenesis but also have a permissive effect on metabolism, as their presence is required for the glycogenolytic and gluconeogenic effects induced by the catecholamines and glucagon (Sapolsky et al., 2000, Greco and Stabenfeldt, 2007). Similarly, have glucocorticoids been found to have a permissive effect on the cardiovascular effect of the catecholamines (Sapolsky et al., 2000). The physiological stress response may also induce changes to immune function, reproduction and pain sensitivity (Sapolsky, 2002).

Heart rate and heart rate variability

Every heart beat is initiated by an action potential that arises spontaneously in one of the pacemakers cells in the sinoatrial node (SN). Once formed, the action potential propagates across the left and right atria, causing both atria to contract. Subsequently the action potential reaches the ventricles via the atrioventricular node (AN), the AV bundle and the bundle branches, causing both ventricles to contract (Stephenson, 2007).
The rapidity of the pacemaker cells to reach the threshold for depolarisation affects HR, and is modulated by the regulatory activity of the branches of the autonomic nervous system (ANS). When PNS neurons are stimulated they release acetylcholine at the SN. This activates the

muscarinic cholinergic receptors on the cell membranes of the pacemaker cells, which slows down the ion channel changes that are responsible for the pacemaker cells spontaneous depolarisation. The pacemaker cells, therefore, reach threshold more slowly, the interval between heartbeats (inter-beat-interval, IBI) increases and HR decreases. When SNS neurons are stimulated they release noradrenalin at the SN. Noradrenalin activates β-adrenergic receptors at the cell membranes of pacemaker cells, which speeds up the ion channel changes that are responsible for the spontaneous depolarisation of the pacemaker cells. Because the pacemaker cells reach threshold more quickly, the IBI decreases and HR increases (Stephenson, 2007). As a consequence of the ongoing regulatory activity of the ANS, HR is never constant but varies from beat to beat. The variation over time of the interval between consecutive heart beats is the heart rate variability (HRV) (von Borell et al., 2007).

By analysing HRV, it is possible to assess the activity of the ANS. HRV analysis is based on the fact that HR, at any point in time, is the net result of the activity of the PNS and SNS, and that the modulations induced by the SNS and PNS occur at different frequencies. The modulations induced by PNS occur within 5 s whereas modulations induced by the SNS occur more slowly, with a maximum response after 20-30 s. This difference is mainly due to the relatively slow exocytotic release of noradrenalin from sympathetic nerve endings and a secondary messenger (adenylyl cyclase) beeing involved in SNS modulation (von Borell et al., 2007). The maximum frequencies of SNS modulation are, therefore, lower than the frequencies of PNS modulation. SNS activity is, thus, associated with the low frequency (LF) range of modulation frequencies while PNS activity is associated with the high frequency (HF) range. This difference in frequencies makes it possible to separate the modulations caused by SNS and PNS activity (Acharya et al., 2006).

HRV is most commonly analysed using time domain analysis or frequency domain analysis. In both methods the interval between heart beats (IBI) are first determined. In time domain analysis, various aspects of the statistical variability in the IBI data series are calculated (von Borell et al., 2007). According to von Borell et al. (2007), the most informative parameter is the RMSSD (ms; root mean square of successive differences between normal heart beats) which estimates the high frequency beat to beat variations that reflects PNS activity. In frequency domain analysis the IBI data is transformed into a spectrum of frequencies (power spectrum). The power spectrum shows two major peaks. One is mainly due to the SNS activity (low frequency component (LF)), and the other reflects the PNS activity (high frequency component

(HF)). The LF/HF ratio is, therefore, an indicator of the balance between the SNS and the PNS activity (von Borell et al., 2007).

Measurements of heart rate and heart rate variability in horses

Measurements of HR and HRV in horses is non invasive and easy to perform. Several studies have used HR as an indicator of the stress response in horses and found significant correlations between the horses behavioural and heart rate responses towards different stressors of relatively short duration (Christensen, 2013; Christensen et al., 2005; 2006; 2012; Leiner and Fendt, 2011). HR is therefore considered a useful measure of the stress response in horses towards stressors of relatively short duration. While HR provides information of the net result of the PNS and SNS activity, HRV analysis may provide more detailed information about the ANS activity. HRV may therefore be useful as a stress indicator, as stress induced changes in ANS activity may occur in the absence of changes in HR (von Borell et al., 2007).

In a study by Visser et al. (2002), the HR and HRV response of horses in a novel object test and a handling test was investigated. In both tests HR increased and RMSSD decreased compared to the baseline measurements, indicating a reduced PNS activity. Also the HR and HRV responses were significantly correlated, indicating that they are both indicators of the stress response (Visser et al., 2002). However, as both HR and HRV in horses are affected by physical activity and the baseline was measured while the horses were standing still, the changes in HR and HRV may be caused by the increased physical activity during the tests (Voss et al., 2000; Physick-Sheard et al., 2000). In another study by Rietmann et al. (2004a), it was shown, that 3 min forced backwards walking without any previous training significantly increased HR and LF/HF ratio compared to measures when the horses were walking forward. After training the horses to walk backwards, HR and LF/HF ratio during 3 min forced backwards walking was significantly lower. This therefore indicates, that the second exposure to the backwards stressor was perceived at less stressful, which was supported by the reduced behavioural reactions of the horses during the second exposure (Rietmann et al., 2004a). The effect of transport on HR and HRV in horses has also been investigated. In three studies investigating the effect of transport on both experienced and transport naive horses, it was found that HR was increased during transport. Also HRV was affected as RMSSD was decreased during transport, and a reduced PNS activity was therefore indicated (Schmidt et al., 2010abc). It was not possible to assess directly, if the changes in HR and HRV was caused by the physical activity of the horses during transport. But as the changes in HR and HRV in transport naive horses decreased with repeated transport, it was suggested that the horses perceived transport a less stressful with increasing experience (Schmidt et al.,

2010c). Hence, it was indicated, that transport may be perceived as stressful in horses. The HR and HRV responses of horses towards an acute occurrence of pain have also been investigated. In horses admitted to hospital due to acute suffering from laminitis, it was found that HR and HF/LF ratio decreased after treatment with non-steroidal anti-inflammatory drugs, indicating a reduced stress response as the condition of the horses improved (Rietmann et al., 2004b). There may, however, also have been an effect of the novelty of the surroundings at the hospital, which was not controlled for in this study. Together, these studies suggest that HR and HRV are useful as indicators of the stress response towards stressors of relatively short duration in horses. HRV, though, did not provide any information not already given by the HR in the studies.
However, horses' basal HRV have been found to provide information not given by the HR. In a study by Bachmann et al. (2003) it was found, that crib-biting horses at rest had a lower HF (reflecting PNS activity) and a higher LF (reflecting SNS) than control horses. When exposed to a stressor, HR and arousal behaviour increased for both groups while crib-biting decreased. Control horses also showed a decreased HF and an increased LF. In the crib-biting horses this response was not significant and it was concluded that the lower basal PNS activity may indicate a reduced ability to respond to stress in the crib-biting horses (Bachmann et al., 2003).

The relevance of measuring heart rate and heart rate variability in horses

HR is considered a useful measure of the stress response in horses towards stressors of relatively short duration. However, as described above, there are no indications of HRV providing any information not already given by HR when measuring the responses of horses towards stressors of relatively short duration. This is in accordance with other studies in humans (Delaney and Brodie, 2000), quails (Valance et al., 2008) and calves (Mohr et al., 2002). In these studies, both HR and HRV were affected simultaneously by the applied stressors (humans: physiological stress, quails: tonic immobility induced by human handling, calves: disease, heat and insects). Therefore, it does not seem relevant to measure the HRV response of horses towards stressors of relative short duration, as HRV is not expected to provide any information not already given by HR. However, measurements of basal HRV may be used to assess the susceptibility of horses to respond to a stressful situation. It has also previously been suggested that a low PNS activity is associated with a reduced flexibly and ability to respond to stress (Porges, 1995). Measures of basal HRV might therefore be useful in objectively assessing the temperament of horses. Measurements of basal HRV may also be used to assess the response of horses towards chronic stressors, as HRV may be able to detect changes in basal ANS activity not shown by HR. In a study by Hagen et al. (2005), the milking system was shown to have an effect on HRV in cows during lying down, as cows in an automatic milking system had a lower activity of the PNS and higher activity of the SNS than cows in a herringbone milking parlour. This difference was not shown by the HR, as HR did not differ between milking systems (Hagen et al., 2005). If similar effects are found in horses, there might be a perspective in applying measurements of basal HRV to assess the response of horses to chronic stressors.

It is therefore relevant to further investigate the use of HRV measurements in horses. Based on the literature presented in this assignment, the emphasis should be on basal HRV in relation to chronic stressors, as there are no indications of measures of HRV providing any information not already given by HR in response to stressors of relatively short duration. Measures of basal HRV may also be used to objectively assess the horses' temperament.

Conclusion

While HR provides information of the net result of the PNS and SNS activity, HRV analysis may provide more detailed information about the ANS activity. As stress induced changes in ANS activity may occur in the absence of changes in HR, HRV may be useful as a stress indicator in addition to HR. While HR is considered useful as an indicator of the response of horses to stressors of relatively short duration, HRV does not seem to provide any information not already given by the HR. The might, however, be a perspective in measuring basal HRV in response to chronic stressors. Also, measurements of HRV have been found useful in assessing the susceptibility of horses to respond to stressful situations.

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