Fear in Horses

Responses to Novelty and Habituation

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Thesis (Licentiate)

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Abstract

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Fear is generally considered to be an undesirable emotional state that may reduce welfare, growth and reproductive performance in animals. Fear in horses is additionally problematic, because fear reactions can cause serious injury to both horse and human. Horses are primarily used for sports and leisure for a large number of children and young women. Unfortunately, horse riding ranks as one of the most dangerous sports in terms of the number and seriousness of accidents, and the ability of a horse to habituate to a range of otherwise frightening stimuli greatly increases safety in the horse-human relationship. However, there is a lack of research on fear reactions and no published research on basic habituation processes in horses. This licentiate project aimed to investigate the types of fear responses horses show towards novel stimuli acting on different senses, and to study how horses learn to be confident with an otherwise frightening stimulus using classical learning theory techniques. The experiments were conducted on two different groups of naïve stallions (n=24 and n=27). The first experiment showed that horses responded differently towards an olfactory stimulus compared to auditory and visual stimuli. The heart rate responses correlated between tests and probably reflected a non-differentiated activation of the sympathetic nervous system, while the behavioural responses were linked to the type of stimulus. The second experiment showed that gradual habituation was the most effective training method for horses to learn to react calmly to an otherwise frightening stimulus, compared to classic habituation and associative learning. Heart rate data revealed that horses may show physiological responses even when their behavioural response towards the stimulus has ceased. Choice of training method is likely to be especially important for the most fearful horses.

Keywords: Horses, behaviour, heart rate, fear, novelty, habituation.

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Appendix

Papers I-II

The present thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

Paper I. Christensen, J.W., Keeling L.J., Nielsen, B.L., 2005. Responses of horses to novel visual, olfactory and auditory stimuli. Appl. Anim. Behav. Sci. 93, 53-65. (Journal Impact Factor: 1.40)

Paper II. Christensen, J.W., Rundgren, M., Olsson, K., 2005. Training methods for horses: Habituation to a frightening stimulus (submitted to Equine Vet J). (Journal Impact Factor: 1.44)

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Introduction

Fear can have negative effects on welfare, health and reproduction in animals (Boissy, 1995). In horses fear is additionally problematic, because fear reactions can cause serious injury to both horse and human. Investigations have shown that the major cause of horse-human accidents is unexpected fear reactions in the horse (Keeling *et al*., 1999). However, basic fear responses and habituation processes have not yet been scientifically investigated in horses. The ability of a horse to habituate to a range of otherwise frightening stimuli greatly increases safety in the horse-human relationship, and finding appropriate methods for reducing fear in horses has important practical applications. This licentiate project investigates how horses respond to novel stimuli, acting on their different senses, as well as how horses learn to be confident with an otherwise frightening stimulus.

The concept of fear

Fear can be regarded as a “state of the brain or the neuroendocrine system arising under certain conditions and eventuating in certain forms of behaviour” (Gray, 1987). Stimuli, which members of a species will avoid, work to prevent, or flee from can be categorised as fear-releasing. Since states of fear do not lead to an obvious behavioural expression in all cases, both behavioural and physiological measures should be considered when assessing the state of fear (Manteca & Deag, 1993).

According to Moberg (1985) “the central nervous system assesses whether a stimulus or a group of stimuli represents a significant challenge to the animal. If the stimulus is perceived as threatening, three general types of biological responses are available: behavioural, autonomic, and neuroendocrine”. This assessment of stimuli may differ between individuals, and the predisposition for assessment of stimuli may be termed fearfulness. Jones *et al*. (1997) defined fearfulness as “an underlying behavioural characteristic” defined as “propensity to be easily frightened by diverse alarming stimuli”. Boissy (1995) defined fearfulness as a “basic psychological characteristic of the individual that predisposes it to perceive and react in a similar manner to a wide range of potentially frightening events.”

The elicitation of fear at the right moment, *e.g.* when an animal encounters a predator, is needed for rapid evasive action in which adrenal hormones play a crucial role (Korte, 2001). The body’s immediate physiological reaction is characterised by activation of the sympathetic system, which speeds things up and gets the body ready for action. The fight or flight reaction of the sympathetic nervous system is started by stimulation of the hypothalamus which sends out signals that are transmitted via the reticular formation in the brain stem into the spinal cord to cause sympathetic discharge. This immediately results in a number of physiological changes, which lead to a greater physical and mental ability so that the animal can perform more strenuous physical activity than would otherwise be possible. Sympathetic stimulation increases both the rate and force of contraction of the heart, as well as the arterial blood pressure. Blood flow is redirected, because the muscles in the blood vessels supplying the gut constrict so that less blood goes there and more blood is directed to the skeletal muscles and the brain. The glycolysis in both liver and muscle increases and blood glucose levels rise (Guyton & Hall, 1997). The transmitter at the neuromuscular junction is noradrenaline, a close relative of the hormone adrenaline, which is the chief neurotransmitter in the sympathetic system. These hormones prepare the body for bursts of physical exercise, for example when about to take flight from a predator.

The sympathetic system is counteracted and modulated by the parasympathetic system, where the actions are principally opposite to those of the sympathetic system (Guyton & Hall, 1997). The parasympathetic system becomes active when the body is engaged in processes relating to general body maintenance, such as eating and ingesting food. It slows the heart and respiration rates, and increases the blood supply to the gut. The normal state of rest is characterised by a predominant parasympathetic nervous activity. In stressful situations, the parasympathetic nervous activity will decrease in favour of a higher sympathetic activity, enabling the animal to react in a biological appropriate manner, for example with flight.

The secondary physiological reaction to danger is characterised by the release of corticosteroids from the adrenal cortex. In response to external or internal stimuli the hypothalamus produces corticotrophin-releasing factor (CRF). CRF stimulates the pituitary gland to secrete adrenocorticotropic hormone (ACTH), which in turn activates the adrenal cortex to release corticosteroids (*e.g.* cortisol), which facilitates energy availability over prolonged periods. The reaction of the hypothalamic-pituitary-adrenal-cortical system takes a few minutes and is somewhat slower than the sympathetic response, which happens in a matter of seconds (Guyton & Hall, 1997). Circulating corticosteroids reach a peak some minutes after the stressful event (Korte, 2001).

After 'fight or flight' responses, corticosteroids are required to re-establish homeostasis via feedback mechanisms. The animal needs to consolidate its memories of the predator's appearance, location, smell, and sound, because such information may predict the occurrence and nature of the next encounter, thereby maximising the likelihood of survival. Corticosteroids act to facilitate behavioural adaptation via their effect on the consolidation and potentiation of fear or the facilitation of avoidance extinction, *i.e.* habituation (Korte, 2001).

Fear has definite survival value in wild animals. The life expectancy of an animal is obviously increased if it can react to avoid sources of danger. In this context fear related behaviour is adaptive. In contrast, excessive fear may be regarded as abnormal behaviour, defined as behaviour unable to attribute positively to the survival of an offspring (Malmkvist, 2001). Excessive fear can lead to psychopathology and mental suffering as well as physical damage (Korte, 2001), and fear is generally considered to be an undesirable emotional state that may reduce welfare, growth and reproductive performance in farm and zoo animals. Boissy (1995) states that studies aimed at reducing fear in animals reared in captivity are of ethical significance due to the potential impact on animal welfare.

Fear has been investigated in many species (reviewed by Boissy, 1995), but there is a lack of research on fear in horses. Several studies have investigated temperamental traits (*e.g.* Scolan *et al*., 1997; Visser *et al*., 2001; Seaman *et al*., 2002), reactivity (*e.g.* McCann *et al*., 1988; Lansade *et al*., 2005; McCall *et al*., 2005), or emotionality (*e.g.* Wolff *et al*., 1997) in horses, but these have mainly been concerned with the consistency of performance in different test situations, while none have dealt with basic fear reactions, nor investigating which situations and which stimuli are perceived as frightening.

Although it is biologically relevant to respond to frightening stimuli, it is also highly relevant to suppress non-functional behaviour, *i.e.* it is adaptive to learn not to respond to non-threatening stimuli. Investigation of fear responses in horses therefore inevitably involves investigations of learning.

Learning in horses

Learning may be defined as "the process of adaptive changes in individual behaviour as a result of experience" (Thorpe, 1963). If animals are to learn to change their behaviour to meet a new situation anything new happening in their environment will have to be taken note of and its importance assessed. However, if a novel stimulus has no consequences, *i.e.* there is no reinforcement, a repeated stimulation will cause the stimulus to capture less and less of the animal's attention and it will eventually ignore the stimulus. This waning of responsiveness is termed habituation. Reduced responsiveness may also be caused by motivational changes, muscular fatigue as well as sensory adaptation. Manning & Dawkins (1998) defined habituation as "a persistent waning of responsiveness which is a property of the central nervous system and not the sense organs". Habituation can be regarded as a simple form of learning because it involves reduction of a response which is already there. In contrast, associative learning is about acquiring new responses. After some repetitions of an event followed by the same consequences, a long-term association is built up between the event and its result and the animal's response changes accordingly (Manning & Dawkins, 1998). In classical or Pavlovian conditioning, an environmental event or stimulus is followed predictably by some other events. A second type of associative learning is instrumental or operant conditioning, where the first event is a response made by the animal and the second event is the associated reinforcing consequence. Horses are well able to form associations where the relationship between two events guides behavioural change (Nicol, 2005). Mackenzie *et al*. (1987) and Gough (1999) studied the use of food as a conditioned stimulus to reduce the reactions of horses to clipping. However, these studies were performed on a very limited number of animals, lacking a control group, and Nicol (2005) argues that more controlled studies of application of learning theory to a practical problem are desperately needed.

An adaptive feature of learning is that animals are able to generalize many of the properties of the stimuli involved in the expression of a classical or instrumental response. Rather than focusing on some small and unique feature of a given stimulus, animals are able to form associations with a broader range of stimulus features than were present during training (Nicol, 2005).

There has been considerable scientific interest in learning in a wide range of animals *e.g.* rats, pigeons, and primates, while there is no published research on such basic learning abilities as habituation and sensitisation in horses, and very little research on basic associative learning processes (see review by Nicol 2002). This is surprising given the importance of these processes in every elementary training programme. However, there has been a scientific interest in horse learning in more complex situations (*e.g.* Sappington & Goldman, 1994; Flannery, 1997; Hanggi, 1999, 2003; McCall *et al*., 2003), on the effects of handling or training schedule on learning (Heird *et al*., 1986b; Mal *et al*., 1994; Kusunose & Yamanobe, 2002; Lansade *et al*., 2005), and on consistency of performance in learning situations (*e.g.* Heird *et al*., 1986a; Sappington *et al*., 1997; Visser *et al*., 2003). Low correlations between learning performance in different tests suggest that other characteristics, such as fearfulness and motivation, may be the factors which govern success or failure in learning performance tests (Nicol, 2002). For instance Heird *et al*. (1986b) found that less emotional horses tended to perform better in a discrimination task and ultimately achieved a higher level of performance. Better learning by horses that are naturally calm may be due to reduced interference with the learning process (Nicol, 2002).

Aims

The project consists of two parts; the first part investigates the types of fear responses horses show towards novel stimuli acting on different senses (Paper I), whereas the second part focuses on how horses learn to be confident with an otherwise frightening stimulus (Paper II).

Paper I. The aim of this study was to investigate i) whether horses show different behavioural responses to novel visual, olfactory and auditory stimuli under standardised conditions; ii) whether behavioural responses reflect heart rate responses; iii) whether responses correlate between tests.

Paper II. The aim of this study was to investigate which of three different training methods, based on learning theory, was the most effective for horses to learn to act calmly in an otherwise frightening situation; i) classic habituation, ii) gradual habituation, and iii) associative learning. The study further aims to investigate whether behavioural and heart rate responses correlate during the training sessions.

Summary of materials and methods

The response of a particular horse in a given test situation is highly dependent on previous experiences, and the use of naïve individuals is therefore necessary to ensure the scientific quality of the experiments. Also, the social nature of horses contributes significantly to their responses in different test situations, and it is necessary that the animals are habituated to the test conditions prior to the experiment, and especially to being socially isolated.

I was kindly allowed to use two groups of young stallions from a large stud during 2003 and 2004. The two experiments in the present thesis were carried out in the same test arena, on two different groups of 2-year old stallions (2003: n=29; 2004: n=39), all of which were relatively unhandled and had experienced the same housing conditions prior to the experiments. The majority of the stallions were born at the stud; others were purchased after weaning at six months of age. All the stallions were kept on pasture with the dam before weaning and were subsequently housed in large groups in straw-bedded boxes with access to outdoor areas during the winter. The horses received a minimum of handling, only for necessary veterinary or farrier treatment. During the summer (May - October) the stallions were pastured in a large enclosure (30 ha) with hills, natural vegetation and access to an inlet, which also served as their water source. The horses received no additional feed or minerals during the summer period.

Within the 30-ha enclosure, a smaller capture enclosure (1-ha) contained a fenced waiting area (50 m^2). Next to the waiting area a start box (2.5 m^2) and a test arena (10 m in diameter) were constructed out of straw bales (1.2 x 1.2 x 2.4 m^3) in two layers, making the height of the walls of the arena 2.4 m (Paper I and II, Fig. 1). The set-up enabled the horses to hear, but not see their group mates during the tests. The arena was equipped with a feed container with a mixture of alfalfa and the horses' usual winter feed (oat, barley, soybeans, minerals and molasses).

Prior to the experiments, the stallions were habituated to separation from the group and to receiving a food reward inside the arena in a gradual, step-wise approach (Paper I, Table 1). This preparation of the animals prior to the experiment was rather time consuming, since some horses showed strong aversive reactions towards the initial handling and fitting of halters. Additionally, the fact that the horses were kept in a large (30 ha) and long (1 km) enclosure made the capturing of the group difficult. During the first summer, we walked the enclosure with a feed container in order to attract the group of stallions to our capture enclosure. However, this procedure turned out to be quite dangerous, so during 2004 we changed our capture procedure and used a 4x4 truck, with a second person feeding the horses on the truck body, whereas a third person followed behind the horses in order to make all horses keep up with the group. Once inside the capture enclosure we identified and caught the horses that were to be tested and led them to the fenced waiting area. Horses sometimes escaped from the waiting area, and on very few occasions, the entire group was frightened by incidental happenings in the environment (*e.g.* a passing helicopter), broke through the fence of the capture enclosure and escaped. After such random events we did not try to recapture the horses and the experiment was postponed until the next day. During 2003, the stallion group escaped from the pasture several times, and we had to suspend our experiment until the stallions had calmed down. Although the use of naïve, unhandled horses strengthens the quality of the experiment it is also a significant challenge for everybody engaged in the experiments. For future experiments, I recommend that sufficient time is allocated to the initial handling of the animals, as well as to unexpected incidents. It is also very important that all experimental staff has experience with horses and is able to read the signals of the horses before they panic.

We used the highest possible number of horses within each group to fit the experimental design (2003: 24 of 29 horses; 2004: 36 of 39 horses). A few horses were left out due to apparent illness, or lameness; otherwise the capturing was random. During 2004, we first carried out an experiment with social transmission of fear responses in horses, which will be reported in my Ph.D. thesis. The experiment included training some horses to be confident with a frightening stimulus. These horses were considered to be treated differently from the rest of the group, so Experiment II in the present thesis was carried out on only n=27 horses.

The choice of test stimuli was based on reactions of horses in pilot studies, and the test stimuli are discussed further in the discussion section. Detailed descriptions of the test designs are given in the respective papers.

Paper I

The horses (n=24) were exposed to three 2-minute tests in a balanced design where, in addition to the feed container, a visual, an auditory, or an olfactory stimulus was presented. Prior to the visual test, a 75 cm high, orange traffic cone with two reflective bands was placed 1 m in front of the feed container, forcing the horse to pass the stimulus in order to approach the food. During the auditory test a novel sound (white noise, 10-20.000 Hz, 60 dBA) was played from a CD player, hidden behind the feed container. For the olfactory test, eucalyptus oil was applied to the edges and the inside of an identical feed container, which was used for this test only. The horses were tested in two blocks of 12 horses. Between test days the horses were exposed to the usual arena without novel stimuli (control). Heart rate and behavioural responses were registered.

Paper II

The horses (n=27) were trained according to three different methods, based on classical learning theory. In the first method (Classic habituation), the horses (n=9) were exposed repeatedly to the full stimulus (a moving, white nylon bag, 1.2 x 0.75 m) until they met a predefined habituation criterion. In the second method (Gradual habituation), the horses (n=9) were introduced gradually to the moving stimulus and were habituated to each of three steps, before the full stimulus was applied. In the third method (Associative learning), the horses (n=9) were trained to feed on the bag, *i.e.* to associate the stimulus with a positive reward, before they were exposed to the full stimulus. Five training sessions of 3 minutes each were allowed per horse per day. Immediate behavioural reaction, latency to return to the feed container, and heart rate responses were registered.

Summary of results

Paper I. Responses of horses to novel visual, olfactory and auditory stimuli

- All novel stimuli resulted in reduced feeding time, compared to the control situation.
- The visual and auditory test stimulus elicited significantly increased heart rate (HR) in the horses compared to their response to the control situation, whereas there was no increase in HR response to the olfactory stimulus.
- There was no difference in locomotion activity in the various test situations and generally very little locomotion activity during the tests.
- During the olfactory test the horses had an increased number of eating bouts and became more vigilant towards their surroundings.
- During the visual and auditory tests, more time was spent alert towards the stimulus.
- Significant, negative correlations were found between time spent eating and HR in the auditory and visual tests, whereas there was no correlation in the control and olfactory test.
- The heart rate responses correlated between tests and reflect a non-differentiated activation of the sympathetic nervous system, while the behavioural responses were linked to the type of stimulus.

Publications

- Christensen, J.W., Keeling, J., Nielsen, B.L., 2005. Responses of horses to novel visual, auditory and olfactory stimuli. Appl. Anim. Behav. Sci. 93, 53-65.
- Christensen, J.W., Keeling, L., Nielsen, B.L., Rundgren, T.M.K., 2004. Do horses generalise their responses to novel stimuli? Proc. 38th Int. Congr. of the ISAE, Helsinki, Finland, p. 115.
- Christensen, J.W., 2005. Heste er ikke bange for ukendte lugte. Månedsmagasinet HEST 5, Maj 2005, 44-45.

Paper II. Training methods for horses: Habituation to a frightening stimulus

- Horses that were habituated gradually showed fewer flight responses totally and needed fewer training sessions to learn to be confident with the test stimulus.
- All horses trained gradually eventually habituated to the test stimulus, whereas a few horses on the other treatments did not.

- Heart rate and behavioural responses correlated well during the first training session.
- Variations in HR existed between horses even when the behavioural response had ceased.
- There were strong correlations between the initial response of a horse and the number of required training sessions, except for horses trained gradually.

Publications

- Christensen, J.W., Rundgren, M., Olsson, K., 2005. Training methods for horses: Habituation to a frightening stimulus (submitted to Equine Vet J.).
- Christensen, J.W., Olsson, K., Rundgren, T.M.K., Keeling, L., 2005. Learning performance in relation to fear in young horses. Proc. EAAP, Uppsala, Sweden, June. Book of abstracts 11, 333.
- Christensen, J.W., 2005. Træningsmetoder - hvordan vænnes hesten lettest til nye situationer? Appendix: "Opdræt af ungheste - godt management", April 1st 2005. Danish Institute of Agricultural Sciences, Report 219, 15-16.
- Christensen, J.W., 2005. Risikoen for rideulykker kan nedsættes med træning. Månedsmagasinet HEST 5, Maj 2005, 36-41.
- Christensen, J.W., 2005. Gradvis tilvænning - den hurtigste måde. Månedsmagasinet HEST 5, Maj 2005, 42-43.

Discussion

Detailed discussion of the results can be found in the respective papers. In the following I discuss general results, methodological considerations as well as practical applications.

General results

Exposure to a suddenly moving test stimulus caused more arousal behaviour and higher heart rate responses (Paper II), compared to horses exposed to stationary novelty in the same test conditions (Paper I). Likewise, it has been shown in lambs that responses to suddenness are stronger than responses to novelty *per se* (Désiré *et al*., 2003), probably due to similarities with moving predators. This is in accordance with the finding that horses react to any new movement in their environment, regardless of the intensity (Paper II), *i.e.* it is biologically relevant to react rapidly to suddenness. We also found that horses are likely to use their visual and auditory senses for immediate detection and orientation towards danger, while olfactory cues may cause the animal to become more vigilant towards its surroundings (Paper I).

Interpretation of responses in fear tests requires that the animal is motivated to explore or approach a test object (novelty tests), or return to a point of interest (surprise tests), for instance through the use of positive reinforcement (*e.g.* food or social partners), thereby creating a motivational conflict between avoiding the novelty and approaching the reward. In our experiments, we used food as a reinforcer; the use of which can be criticised due to the difficulty of controlling feeding motivation in the animals. However, in the present study the horses were of similar age, breed and exercise level, and as they were pastured, their food resources were unlimited. Therefore I find it reasonable to assume that the horses were equally motivated to eat. Indeed we found that time spent eating (Paper I), and latency to return to the feed container (Paper II) correlated very well with physiological responses within tests, indicating that the use of positive reinforcement can be a valuable indicator of reactions in fear tests. Discrepancies between other studies of horse reactivity may partly be due to the lack of reinforcement during novelty tests (*e.g.* Scolan *et al*., 1997; Wolff *et al*., 1997; Visser *et al*., 2001; Seaman *et al*., 2002). An animal which does not approach a novel object during a test may not do so either because it is fearful, or simply because it has no reason to approach and explore. In contrast to carnivores, where investigation is an important part of food localisation, horses are less dependent on exploration in their foraging and they instinctively avoid novelty. This pleads for the use of positive reinforcement in novelty tests for horses.

In Experiment I we found that heart rate responses correlated between test situations and probably reflect the immediate physiological reaction to perceived danger, characterised by activation of the sympathetic system, while the behavioural responses were linked to the type of stimulus. This result is in perfect agreement with recently published results by McCall *et al*. (2005) who compared four methods of ranking horses based on reactivity and found that mean heart rates correlated between tests, while behavioural measures ranked horses differently. These results may be a further explanation of the lack of consistency of behavioural reactions in previous reactivity tests (Scolan *et al*., 1997; Wolff *et al*., 1997; Visser *et al*., 2001; Seaman *et al*., 2002).

In Experiment I, all horses approached and ate from the feed container within the test time of 2 min, although they either had to pass the visual stimulus, or approach the unknown sound or smell. In Experiment II, the horses on the associative learning method needed several training sessions to learn to feed from the test stimulus. This may have been caused either by a stronger fear-eliciting strength of the white nylon bag, compared to the novel stimuli in Experiment I, or that the horses had to learn a new behaviour; to feed on the bag, rather than continue feeding from the feed container, which they were trained to do. The mere presence of the well-known feed container may act to calm the horses. Time taken to learn to feed from the test stimulus during the associative learning in Experiment II meant that this training method was considerably more time consuming, compared to the other training methods. However, it is unknown whether associative learning may be more resistant to extinction, and future studies should focus on this aspect in horses.

Methodological considerations

We chose to use 'unnatural' test stimuli due to difficulties of mimicking natural stimuli. Predator urine can probably be applied in odour experiments whereas it is less obvious whether recordings of predator roar or howl contain the right frequencies, and whether a visual image of a predator has the right appearance. The novel stimuli in Experiment I were chosen in accordance with results in pilot studies; we wanted stimuli which were sufficiently strong to elicit responses in the majority of the test animals, but not to be so frightening that a large part of the animals would not approach the feed container within a relatively short test time. In pilot studies horses did not react to artificially produced boar smell, whereas they did react to eucalyptus oil. Drops of eucalyptus oil have previously been used in novelty tests for animals (*e.g.* Hutson *et al*., 1993; Herskin *et al*., 2003). Likewise, white noise is commonly applied in auditory experiments (*e.g.* Talling *et al*., 1995; Malmkvist *et al*., 2004). The traffic cone was chosen as a visual stimulus, because riding horses are likely to encounter such stimuli at some point in their lives.

Comparison of responses to different types of stimuli requires that the stimuli are equally fear-eliciting. However, the strength of a visual stimulus cannot be equated to the strength of an auditory or olfactory stimulus, and a study of the specific responses horses show towards a particular type of stimuli requires exposure to a range of different stimuli. However, the selected test stimuli all reduced eating time to the same amount, indicating a similar fear-eliciting strength. The finding that responses to visual and auditory stimuli were similar, whereas responses to the olfactory stimulus differed, calls for further research into the importance of olfactory cues to horses. Such research should include exposure to biologically relevant smells, *e.g.* predator odour.

In Experiment II, we exposed the animals to a combination of novelty and suddenness. We wanted to make sure that the test stimulus elicited sufficiently strong responses to be able to see a decrease during the subsequent training sessions. On the other hand, we had to take care that the stimulus was not so strong as to make the most fearful horses panic, *i.e.* the stimulus should not be too mild for the most calm horses and not too frightening for the most fearful horses. The choice of test stimulus was made more difficult by horses varying widely in their reactions; even similarly housed and aged horses as in the present experiment, *e.g.* some horses on each treatment did not react to the test stimulus at all whereas others showed major reactions and did not habituate within the course of the experiment. Nevertheless, the selected stimulus elicited appropriate responses in most horses and a sudden movement in a nylon or plastic bag is likely to be an appropriate stimulus in fear tests for horses, which can be easily applied and standardised under a wide range of conditions.

Considering the large variation in responses even in these groups of similarly aged and housed stallions, there is a need for a large number of animals in experiments concerning fear and learning. This is often difficult to achieve in horse research, because horses are expensive animals and scientists typically have to borrow horses from private owners, which may cause limitations to the experiment. In the reported experiments we were lucky to have access to relatively unique horse groups.

Other studies on fearfulness or reactivity in animals have often included measurements of HPA axis activity, such as a change in plasma cortisol concentration, in response to an acute stressor (*e.g.* Rivera *et al*., 2002; Van Reenen *et al*., 2005), because these are less dependent on behavioural reactions (locomotion, flight responses) compared to heart rate data. However, obtaining blood samples from relatively unhandled horses can be a challenge and may jeopardise the safety of the handlers as well as the horses' willingness to be caught and take part in the rest of the experiment. Another method of obtaining physiological data, which are less affected by locomotion, is the use of data on heart rate variability (HRV; *e.g.* Visser *et al*., 2002; Bachmann *et al*., 2003; Rietmann *et al*., 2004). HRV is an established parameter to quantify the state of the autonomic nervous system, reflecting the sympatho-vagal balance (Bachmann *et al*., 2003). Relations between heart rate variability measures and behavioural responses will be studied in my Ph.D.

Practical applications

It has previously been hypothesised that certain smells (chemicals) can cause horses to be fearful (*e.g.* Mills & Nankervis, 1999). However, it is likely that horses are not innately fearful of certain smells; rather they have learned to associate some smells with fear or alarm. Riders often report that horses are afraid of passing pig farms due to the smell. Our pilot studies showed that horses did not react to artificially produced boar smell, and it is likely that horses learn to associate the smell of pigs with the sound of screaming pigs, which may have startled the horse when passing the farm in the past. Rather than worrying about unknown smells, I recommend that riders are aware of associations that horses may make to a novel smell, *e.g.* if the rider is nervous that the horse will react to the smell, this nervousness is likely to affect the horse.

Horses instinctively avoid entering dark narrow areas, such as the interior of a horse trailer. To perform such tasks, the modern horse must learn to suppress many of its natural instincts as well as learn to discriminate and to respond appropriately to a wide variety of stimuli. The ability to learn and to respond appropriately to different stimuli usually directly influences the horse's usefulness and monetary value to humans (McCall, 1990; Cooper, 1998), and there are several good reasons to avoid the most fearful horses. Firstly, fearful horses can be a significant safety risk for both horse and rider. Also, fearful horses are more difficult to handle and thus more time consuming, and research indicates that they may have a poorer learning ability (Fiske & Potter, 1979; Heird *et al*., 1986b), possibly due to increased interference with the learning process. Additionally, very fearful horses may be costly due to more veterinary treatment, partly because of injuries and partly because there is higher risk of diseases since prolonged stress can depress immune system function (Manning & Dawkins, 1998).

Horses, however, are well able to habituate to a range of different unnatural situations, and they generally have a good learning ability (Nicol, 2002). The ability of a horse to habituate to a range of fear-eliciting situations can reduce the risk of accidents, and safety training should – and can easily – be applied at public riding schools. Our results show that the choice of training method is important mainly for the most fearful horses. In police horse training, they aim at never provoking the unwanted behavioural responses (*e.g.* the flight response) and thereby avoid the risk of the horse getting a bad experience during training. This procedure corresponds well to the gradual habituation training applied in the present project, which showed to be the most gentle and effective training method for horses in frightening situations.

Conclusions

Paper I

- Horses showed similar responses towards a novel visual and auditory stimulus, whereas responses to a novel olfactory stimulus differed.
- Behavioural responses reflected heart rate responses.
- Heart rate responses correlated between tests and reflected a non-differentiated activation of the sympathetic nervous system, while the behavioural responses were linked to the type of stimulus.

Paper II

- Horses were more easily trained to be confident with an otherwise frightening stimulus if trained using a gradual habituation procedure.
- Behavioural and heart rate responses correlated well during the first training session.
- Although the behavioural reactions towards a repeated stimulus had ceased, variations in heart rate responses persisted.
- Choice of training method is likely to be especially important for more fearful horses.

Thoughts for future studies

To my knowledge this is the first scientific study of basic fear responses and habituation processes in horses. A number of highly relevant research questions arose during the study, including whether horses learn from the responses of other horses by social transmission of behaviour. Although previous studies have failed to demonstrate social learning in horses in more complex learning situations (Baer *et al*., 1983; Baker & Crawford, 1986; Nicol, 1995; Lindberg *et al*., 1999), social influence is very likely for responses in simpler, biologically relevant situations, such as responses to fear-eliciting stimuli.

Another area of research is whether habituation is stimulus specific, or whether horses are able to generalise across fear-eliciting situations, so that once habituated to a range of fear-eliciting stimuli a horse is less likely to respond fearfully to any novel stimulus, *i.e.* its general fearfulness is reduced. Generalisation of behavioural responses across several different situations has been demonstrated in other species (*e.g.* Boissy & Bouissou, 1995; Malmkvist & Hansen, 2002), whereas results on horses have been less convincing (*e.g.* Scolan *et al*., 1997; Wolff *et al*., 1997).

Research on other species has shown that enrichment of the animals' environment can reduce fear responses (Jones & Waddington, 1992; Meehan & Mench, 2002). It would be relevant to investigate the effects of introducing different fear-eliciting objects in the home environment on horse reactivity.

Bachmann *et al*. (2003) found that crib-biting horses had a lower basal parasympathetic activity and suggest that stereotyping horses are more stress sensitive and less flexible when coping with stress than non-stereotyping horses. Minero *et al*. (1999) found that the overall mean heart rate was higher in crib-biting horses in a fear-eliciting situation. Further investigations into the fear responses of stereotyping and non-stereotyping horses could help throw light on the possible differences in response to challenging events.

Some of these questions will be addressed in my Ph.D. project.

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Responses of horses to novel visual, olfactory and auditory stimuli

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Abstract

Responses of horses towards fear-eliciting stimuli can have important consequences for both human and horse safety. This experiment was designed to investigate behavioural and heart rate (HR) responses of horses to novel visual, auditory and olfactory stimuli. Twenty-four 2-year-old, previously unhandled, stallions were habituated to receive a food reward from a container in a test arena. Each horse was exposed to three 2 min tests in a balanced design where in addition to the feed container, either a traffic cone (visual test), white noise (auditory test) or eucalyptus oil applied to the inside of the container (olfactory test) were used as the novel stimuli. Compared to the control, less time was spent eating during all tests. There was no difference in locomotion activity in the different test situations, but presentation of the novel visual and auditory stimuli elicited significantly increased HR responses in the horses, compared to their response to the arena without novel stimuli (control), whereas there was no increase in HR response to the olfactory stimulus. However, during the olfactory test, the horses had an increased number of eating bouts and became more vigilant towards their surroundings, whereas during the visual and auditory tests, more time was spent alert towards the stimulus. The horses also took significantly more steps backwards in response to the auditory test. The heart rate responses correlated between tests and reflect a non-differentiated

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activation of the sympathetic nervous system, while the behavioural responses were linked to the type of stimulus.

Keywords: Horses; Novelty responses; Fear; Behaviour; Heart rate

1. Introduction

The appropriate response of a horse towards a potentially dangerous stimulus has been important to its survival through millions of years in the wild and domestic horses respond to perceived threats and novelty in much the same way as their wild ancestors. For instance, horses tend to react with a rapid flight response when alarmed and to avoid potentially fear-eliciting situations in general, e.g. they do not approach threatening stimuli and they tend to respond nervously to novelty in a known environment. Responses towards novelty have often been used in tests of fearfulness or emotionality in animals (Gray, 1987; Boissy, 1995). Novel object tests have been applied to a wide variety of animal species from rodents, to poultry, fish, carnivores and prey animals (e.g. Hemsworth et al., 1996; Malmkvist and Hansen, 2002; Meehan and Mench, 2002; King et al., 2003; Sneddon et al., 2003). Like other prey species, the horse's sensory systems have adapted to facilitate early detection of danger (Saslow, 2002). Horses probably use a combination of visual, auditory and possibly olfactory cues for detection of danger. Currently, we have little knowledge of how horses respond to potentially fear-eliciting situations, which act on their different senses.

In contrast to predators, where exploration is an important part of localising food, the survival of many prey species in their natural environment is less dependent on the tendency of the animal to explore. Responses in novel object tests, therefore, may reflect exploratory motivation, fearfulness or simply that the animal is not interested in the object. In order to be able to interpret responses, it is required that the animal under study is motivated to approach the test object, for instance through the use of positive reinforcement (e.g. food or social partners), thereby creating a motivational conflict between avoiding the novelty and approaching the reward (e.g. Boissy and Bouissou, 1995; Désiré et al., 2003).

In prey species, it is especially likely that responses to suddenness are stronger than responses to novelty per se, due to similarities with moving predators. In this experiment, we separate novelty from suddenness, focussing only on the effects of novelty in a known environment. This is in contrast to previous studies of novelty responses in horses, which have included exposure to a combination of novelty and suddenness (e.g. Visser et al., 2001, 2002; Momozawa et al., 2003).

It is hypothesised that exposure to novelty causes the emotional state fear, which may be reflected in changes in behaviour, an increase in heart rate and neuroendocrine changes. The body has two principally different pathways of reaction to perceived danger: the immediate reaction of the sympathetic nervous system and the slower endocrine secretion of cortisol (Guyton and Hall, 1997). Sympathetic stimulation increases both the rate and force of contraction of the heart, preparing the organism for flight. Although behaviour and heart rate responses are often linked, they may also occur separately. The aim of this study was to investigate this interrelationship in different test situations. The present experiment was designed to explore: (i) whether horses show different behavioural responses to novel visual, olfactory and auditory stimuli under standardised conditions; (ii) whether behavioural responses reflect heart rate responses; (iii) whether behavioural and heart rate responses are correlated between tests.

2. Materials and methods

2.1. Animals and housing

A total of 24, 2-year-old Danish Warmblood (*Equus caballus*) stallions from a large stud were used in this study. Three breeding stallions sired the colts, of which the majority were born at the stud, others were purchased after weaning at six months of age. All colts were kept on pasture with the dam before weaning and were subsequently housed in large groups in straw-bedded boxes with access to outdoor areas during the winter. The colts received a minimum of handling, only for necessary veterinary or farrier treatment. During the summer (May–October), the colts were pastured in a large enclosure (30 ha) with hills, natural vegetation and access to an inlet, which also served as their water source. The horses received no additional feed or minerals during the summer period.

2.2. Experimental design

Within the 30 ha enclosure, a smaller capture enclosure (1 ha) contained a fenced waiting area (50 m^2). Next to the waiting area, a start box (2.5 m^2) and a test arena (10 m in diameter) were constructed out of straw bales (1.2 m $\times$ 1.2 m $\times$ 2.4 m) in two layers, making the height of the walls of the arena 2.4 m (Fig. 1). The set-up enabled the horses to hear, but not see their group mates during the tests. The arena was equipped with a feed container, placed opposite the entrance, with a mixture of alfalfa and the horses' usual winter feed (oat, barley, soybeans, minerals and molasses). The ground in the arena was covered with a thin layer of wood shavings.

2.2.1. Habituation

Prior to the experiment, the stallions were habituated to being isolated and receiving a food reward inside the arena in a gradual, step-wise approach (Table 1). Most horses (75%) passed directly through the three habituation steps, whereas six horses needed more than one trial on one or more of the steps (up to four trials on a step). When a horse met the habituation criteria, it was not exposed further to the test arena until the rest of the horses were habituated. The day prior to a test, all horses were again exposed to the arena, ensuring that all horses fulfilled the habituation criteria and to standardise the time interval between last exposure to the arena and the test.

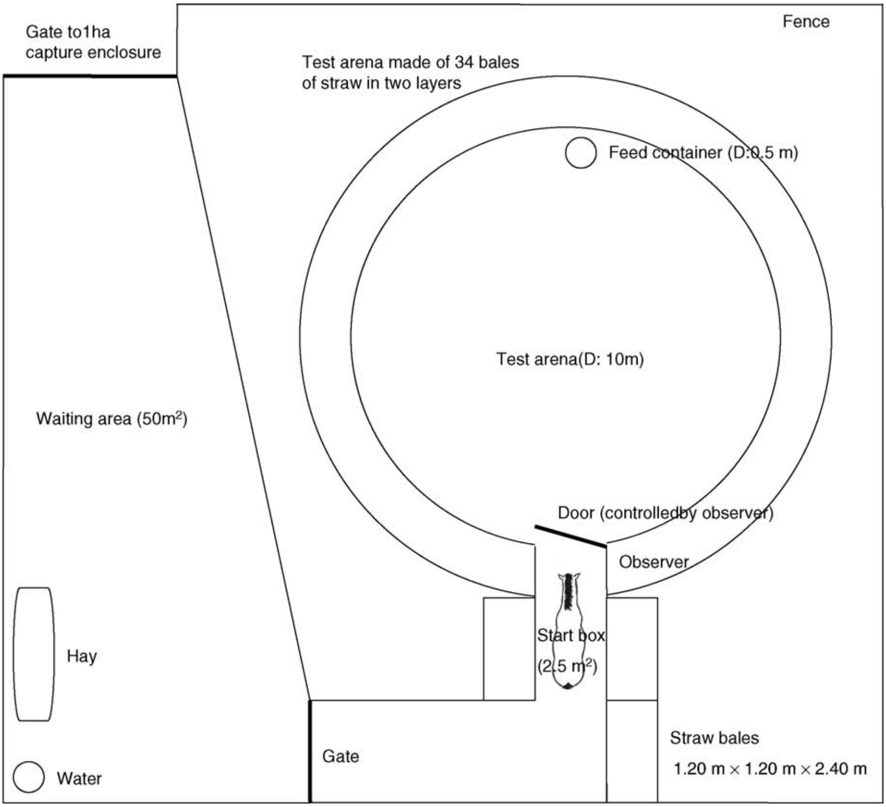

Fig. 1. Diagram of the test arena, start box and waiting area.

2.2.2. *Tests*

The horses were exposed to three 2 min tests in a balanced design where, in addition to the feed container, a visual, an auditory or an olfactory stimulus was presented. Prior to the visual test, a 75 cm high, orange traffic cone with two reflective bands was placed 1 m in front of the feed container, forcing the horse to pass the stimulus in order to approach the food. During the auditory test, a novel sound (white noise, 10–20,000 Hz, 60 dBA) was played from a CD player, hidden behind the feed container. For the olfactory test, eucalyptus oil was applied to the edges and the inside of a similar feed container, which was used for this test only. The horses were tested in two blocks of 12 horses. Between test days, the horses were exposed to the usual arena without novel stimuli (control). The experiment was carried out in August and September 2003, during which the average temperature was approximately 20 °C. Testing on days with heavy rain, wind or other extreme weather conditions was avoided.

The behavioural variables described in Table 2 were recorded using a handheld computer (Workabout, PSION PLC, UK). The observer sat quietly on top of the straw wall next to the start box during all exposures. Subsequently, the data were transferred

Table 1
Habituation procedure

	Training session	Procedure	Note
1.	Identification and 3-step introduction to arena	The horse is caught, identified using ID cards and fitted with a coloured ring in the mane for recognition The handler leads the horse directly to the feed container inside the arena and offers it feed. The horse is led around in the arena once and is offered feed again before returning to visual contact with the other horses. The horse is led to the arena again, the observer closes the door and the same procedure as above is carried out. During the third entry, the horse is allowed to run loose for 2 min, while the handler stands by the feed container. The horse is caught and led to the feed container, if not already there, whereupon it returns to the group	A varying number of horses (2–12) were introduced daily due to their varying willingness to be caught and led
2.	Habituation, step 1	The horse is led to the feed container inside the arena, the door is closed, the handler releases the horse and stands by the feed container for 2 min. The horse is caught and returns to visual contact with the group	A step is passed when the horse stands and eats from the feed container for a period of 30 s during the 2 min exposure. Upon passing a step, the horse carries on to the next step, whereas a failed horse carries on with the same step, until the criterion is reached. A maximum of five trials were allowed per horse per day. On the next day, a horse started on the last passed step
3.	Habituation, step 2	The horse is led to the feed container inside the arena, the door is closed, the handler releases the horse and leaves the arena for 2 min. The handler returns, the horse is caught and returns to visual contact with the group	
4.	Habituation, step 3	The horse is led to the start box in front of the arena while the arena door is closed. The handler releases the horse, the observer opens the door and the horse is free to enter the arena. When inside, the arena door is closed and the horse is left alone inside the arena for 2 min. The handler enters, the horse is caught and returns to the group.	If no eating occurred during the 2 min, the handler led the horse to the feed container and offered it feed before leaving the arena. An observer was always present on top of the arena wall next to the door, habituating the horses to the presence of an observer

Table 2
Ethogram of recorded behaviours

Behaviour	Definition
Duration (s)	
Stand	Standing relaxed, head and neck relaxed
Walk	Walking, energetically or relaxed
Canter/trot	Cantering or trotting
Alert food	Vigilant with elevated neck, with or without tail elevation, head and ears oriented towards food container/novel stimulus, standing or walking
Alert other	As above, but with head and ears oriented in other directions
Investigate food	Neck horizontal or lower, head and ears oriented towards food container/novel stimulus, standing or walking
Investigate other	As above, but with head and ears oriented in other directions, includes touching and manipulating arena walls or ground
Investigate cone	As above, but with head and ears oriented towards the cone
Touch cone	Touching or manipulating the cone
Sniff food	Head within 1 m of food container, neck horizontal or lower, clear exhalations from nostrils
Eat food	Chewing the food; the head may be lifted from the food container for short periods while chewing continues
Frequency	
Eat bout	Eating after pauses of more than 5 s were recorded as a new bout
Nicker	Low amplitude call of long duration
Whinny	High amplitude call of long duration that fluctuates in frequency
Snort	Short powerful exhalations from nostrils
Back	Stepping backwards (minimum two steps)
Flehmen	Head elevated and neck extended, upper lip curled (olfactory investigation)
Paw bout	Striking the ground or air with a forelimb, pawing after pauses of more than 5 s were recorded as a new bout
Defecation	Elimination of faeces
Urination	Elimination of urine

from the PSION to a PC, using the software PSION Manager, Version 1.1. Heart rate (HR) was recorded with Polar Vantage (Polar Electro OY, Kemple, Finland), which consisted of an electrode belt with a built-in transmitter and a wristwatch receiver. Water and exploratory gel were used to optimise the contact between electrode and skin. The HR monitoring equipment was fitted on the horse in the waiting area prior to testing and the receiver stored data from the transmitter (every 5 s). Subsequently, data were downloaded via a Polar Interface to a PC, using the software Polar Precision Performance^{TM} SW 4.

2.2.3. Test procedure

The 12 horses of a particular block were caught and led into the waiting area prior to testing and stayed there until all horses had been tested. Hay and water were available in the area. The rest of the group was kept inside the 1 ha capture enclosure next to the waiting area, ensuring proximity of the entire group during the tests. The test horse was fitted with HR equipment and led to the start box by a handler with whom the horses became familiar during the initial habituation training. After approximately 1 min, the arena door opened, allowing the horse to enter the arena. After the test, the horse was caught by the handler and led back to the waiting area where the HR equipment was removed. After each test, defecations were removed from the arena and extra feed added to the container, if necessary. The test order was the same during all test and control days.

2.3. Data analysis

Latencies, frequencies and time spent on different behaviours were calculated in SAS 8.0 (http://www.sas.com/). Behaviours, which were observed in less than three horses, were excluded from further analysis, i.e. defecation, urination, flehmen and all vocalisations (nicker, whinny and snort). The order in which the horses received the tests was balanced between horses and block and was not considered further in the analysis.

Preliminary tests showed that there was no effect of horse on the initial HR (before the tests), probably due to the similar age, breed and exercise level of the horses, making it unnecessary to correct for individual differences in initial HR. Thus, the analysis was carried out on data for average HR (reflecting the shape of the HR curve; HR_avg) and maximum HR (reflecting the immediate response of a horse towards the test stimulus; HR_max) during the tests. Likewise, preliminary analysis showed that there were no significant differences in behaviour and HR responses between the control days, indicating that there was no carry-over effect from the different test situations and no trend to increasing or decreasing HR during the course of the experiment. Thus, an average for each horse from all control exposures was used as control data in the analysis. The HR data were analysed using Mixed Models in SAS estimating degrees of freedom using Satterthwaite's formula (Littell et al., 1996) with test ($n = 4$), sire ($n = 3$) and their interaction as fixed effects and animal within block within test as a random effect. The model was reduced if terms were not significant ($P > 0.05$). The response variables were HR_avg and HR_max.

Due to skewed distributions and non-constant variances of the behaviour data, these were analysed for effect of test, block and sire separately by Friedman Repeated Measures Analysis of Variance on Ranks, using SigmaStat 3.0 (http://www.spss.com/). All horses, except one horse in the auditory test, approached and ate from the feed container within the test period of 2 min. However, this horse did approach and eat from the container before the handler entered the arena after the end of the test period. It was, therefore, considered unnecessary to treat the latency data as censored values. One horse reared during the olfactory test, which was its first test and the test was stopped due to risk of injury. The horse had previously reacted with this type of behaviour during the initial handling, but did not respond with rearing during the habituation, nor in any of the subsequent tests. However, the Repeated Measures ANOVA on Ranks does not allow for missing data in a balanced design and the horse had to be deleted from the analysis. Thus, this part of the analysis was carried out on $n = 23$ horses.

Correlations between variables within tests and correlations between tests were carried out by Spearman rank-order correlations (coefficients denoted as r_s). Technical problems with the heart rate equipment caused a loss of data in the first block and the correlations between tests were thus based upon smaller sample sizes ($n = 16$, 13 and 11). Due to the low occurrence of many behavioural variables, some related behaviours were grouped (all behaviours related to focussing on 'other' or 'food'; Table 3).

Table 3
Number of animals (out of 23) showing the behaviour in each test and median [25, 75% quartiles]

Behaviour	Control	Visual	Olfactory	Auditory	Chi-square	P-value
Stand	9	1	4	3		
(s)	0 [0, 0.4]	0 [0, 0]	0 [0, 0]	0 [0, 0]	6.33	0.097*
Walk	23	23	23	21		
(s)	8 [7, 9]	6 [3, 9]	8 [7, 9]	7 [4, 9]	9.95	0.019*
Canter/trot	3	4	4	4		
(s)	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]	1.52	0.677
Alert food	1	9	1	13		
(s)	0 [0, 0]	0 [0, 5]	0 [0, 0]	2 [0, 10]	21.25	<0.001**
Alert other	6	8	10	8		
(s)	0 [0, 0]	0 [0, 4]	0 [0, 7]	0 [0, 3]	5.45	0.142
Investigate food	2	6	11	14		
(s)	0 [0, 0]	0 [0, 2]	0 [0, 6]	2 [0, 5]	19.24	<0.001**
Investigate other	13	5	10	7		
(s)	0 [0, 1]	0 [0, 0]	0 [0, 5]	0 [0, 3]	3.79	0.285
Investigate cone		21				
(s)	–	6 [2, 13]	–	–	–	–
Touch cone		3				
(s)	–	0 [0, 0]	–	–	–	–
Sniff food	2	0	10	2		
(s)	0 [0, 0]	0 [0, 0]	0 [0, 6]	0 [0, 0]	20.73	<0.001**
Eat food	23	23	23	22		
(s)	105 [103, 107]	97 [85, 104]	92 [76, 105]	95 [75, 105]	20.85	<0.001**
Eat bout	23	23	23	22		
(freq)	1 [1, 2]	2 [2, 3]	3 [2, 5]	2 [1, 3]	34.75	<0.001**
Back	0	0	0	6		
(freq)	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0.8]	18.00	<0.001**
Paw bout	11	8	10	6		
(freq)	0 [0, 0.6]	0 [0, 1]	0 [0, 2]	0 [0, 1]	1.84	0.606
Latency to enter arena	23	23	23	23		
(s)	3 [2, 5]	3 [2, 5]	2 [2, 5]	2 [2, 4]	5.45	0.142
Latency to eat	23	23	23	22		
(s)	10 [9, 11]	12 [10, 20]	10 [8, 10]	12 [10, 17]	19.51	<0.001**
Focus food[a]	2	22	12	18		
(s)	0 [0, 0]	9 [4, 20]	2 [0, 8]	9 [2, 15]	37.14	<0.001**
Focus other[b]	17	10	18	11		
(s)	1 [0, 2]	0 [0, 7]	5 [1, 11]	0 [0, 5]	8.87	0.031*

Test statistics for the comparison between tests are given in the two last columns.
[a] Time spent focussing on the food/test stimulus (alert food + investigate food + investigate cone).
[b] Time spent focussing on other (alert other + investigate other).

3. Results

3.1. Responses to the different test stimuli

All test stimuli resulted in reduced eating time and increased investigation (investigate food, cone or other features of the arena) compared to the control situation. The number of animals, which showed the different behaviours in the respective test situations, medians (25, 75% quartiles) and the test statistics are shown in Table 3. The latency to eat was

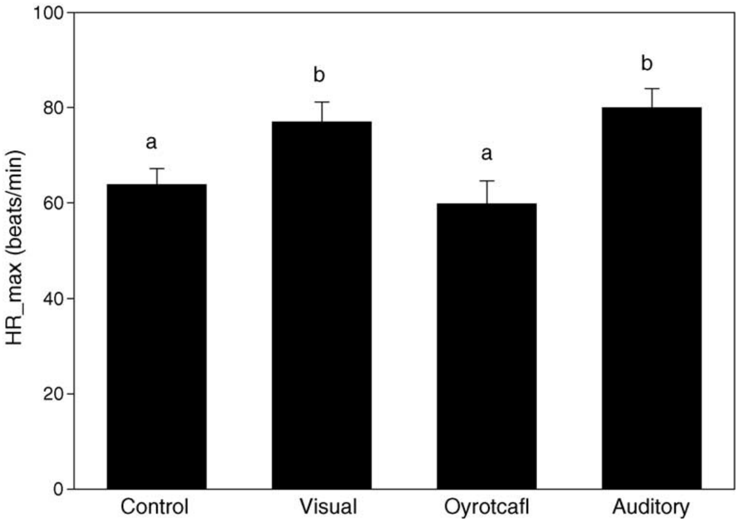

Fig. 2. Maximum heart rate responses (mean ± S.E.) during the tests. Different letters indicate significant difference at $P < 0.05$.

significantly increased for the visual and auditory tests, whereas more disrupted feeding (an increased number of eating bouts) was shown in the olfactory test. Likewise, sniffing the food and focussing on other features in the arena was primarily shown during the olfactory test, whereas the horses spent more time focussing on the food/stimulus in the visual and auditory tests. Backing away from the test stimulus was only shown in the auditory test. In general, very little locomotion was shown by the horses during the tests.

Exposure to the novel visual and auditory stimuli elicited significantly increased HR responses in the horses compared to their response to the control arena, whereas there was no increase in HR to the olfactory stimulus. The maximum HR responses during exposure to the control arena and the three test stimuli are shown in Fig. 2 ($F_{3,65} = 5.73$, $P = 0.002$). The average HR responses to the different test situations gave a similar picture (control: 52.26 ± 2.05, visual: 57.49 ± 2.34, olfactory: 51.02 ± 2.72, auditory: 62.22 ± 2.28; $F_{3,70} = 4.84$, $P = 0.004$).

There were no significant differences between the test days in HR or behavioural response, indicating that the horses did not habituate nor sensitize to being tested. There was no effect of sire in any variables in any tests.

3.2. Correlations between behaviour and heart rate responses

Time spent eating was negatively correlated with all other variables, i.e. the more time a horse spent eating, the less it responded to the test stimulus. Thus, time spent eating was used as a reference variable to study the interrelationship between behaviour and HR responses. There were significant, negative correlations between time spent eating and HR in the visual and the auditory tests (HR_avg: visual: −0.49, $P = 0.032$; auditory: −0.51, $P = 0.022$; HR_max: visual: −0.41, $P = 0.076$; auditory: −0.46, $P = 0.041$), whereas there was no correlation in the control and olfactory tests.

3.3. Correlations between test situations

Horses, which responded with a high HR in the auditory test, also had a high HR in the olfactory and the visual test, i.e. there were significant, positive correlations between HR responses in the olfactory and auditory tests (HR_max: $r_s = 0.66$, $P = 0.013$ and HR_avg: $r_s = 0.72$, $P = 0.005$) and between the visual and auditory tests (HR_max: $r_s = 0.55$, $P = 0.026$ and HR_avg: $r_s = 0.48$, $P = 0.056$). However, there was no correlation in HR between the visual and olfactory tests. Also, there were no significant correlations between the test situations for any behavioural variables.

4. Discussion

4.1. Responses to the different test stimuli

The test stimuli elicited different behavioural responses in the horses and the heart rate increased in response to the visual and auditory stimuli, but not to the olfactory stimulus. Apart from a significantly reduced eating time in all test situations compared to the control situation, it is noteworthy that the behavioural responses to the novel visual and auditory stimuli were similar, whereas the responses towards the novel olfactory stimulus differed. The visual and auditory stimuli elicited increased latencies to eat and more time spent alert towards or investigating the stimulus. During the olfactory test, the horses spent more time sniffing and focussing on other things in the arena and they showed more disrupted feeding. Boissy (1995) suggested that transitions between behaviours can be induced by conflicts between emotional states and motivations and thereby are expressions of fear. In the present study, we found significantly increased heart rate responses to the visual and auditory stimuli, whereas the heart rate did not increase during exposure to the olfactory stimulus. Herskin et al. (2003) also found behavioural but no heart rate responses in cattle towards different types of novel food, including drops of eucalyptus oil added to their usual food. The horses in our study showed very little locomotion activity in all tests; thus, the difference in heart rate responses cannot be attributed to differences in physical activity. The question is why horses only show behavioural and not heart rate responses to an unknown smell and whether this applies to this particular smell only or whether it is adaptive? An increase in heart rate is the body's physiological response to localised danger, preparing the animal for flight. Since smell travels slowly in air, there may be no sense in running away from an unknown smell because the source of the smell may have moved before the animal perceives the smell. However, unknown smells may act to make the animal more vigilant towards the surroundings. In our study, increased vigilance was identified by a significant increase in the number of eating bouts. Vigilance may thus be a measure of fearfulness as suggested by Welp et al. (2004), who studied fear in dairy cattle and found alterations in vigilance according to their degree of fearfulness. Terlouw et al. (1998) investigated responses of cattle to odours of urine and blood from conspecifics and faeces from carnivores and found that the odours induced heightened vigilance, e.g. increased sniffing, but that the odours did not interfere with the expression of feeding motivation. These results correspond to the responses of horses towards a novel smell in the present experiment. On the contrary, it is biologically relevant to be prepared to run away from an unknown sound or visual stimulus and maybe these senses are primarily used for immediate predator detection. Thus, it seems that horses respond to unknown visual and auditory stimuli by both behavioural and heart rate changes, whereas an unknown olfactory stimulus elicits behavioural changes only in terms of increased vigilance towards the surrounding environment. Boissy (1995, 1998) discussed the concepts of fear and anxiety and suggested that the perception of actual danger causes the emotional state fear, whereas potential danger causes the emotional state anxiety. The responses of the horses in the visual and the auditory tests probably reflect the fact that in these tests, the horses were able to localise the stimulus, inducing behaviours to avoid the stimulus (“fear”), whereas the olfactory test induced an expectation of danger (“anxiety”). Further experiments, in which horses are exposed to more biologically relevant smells, e.g. predator odour, are necessary in order to investigate the interrelationship between behavioural and heart rate responses to novel olfactory stimuli.

Backing away from the stimulus was only shown in the auditory test, indicating that this type of behaviour may be an innate response to an unknown sound. However, differences in responses between the test stimuli may not only be attributed to the type of stimulus. Responses may also be linked to the fear-eliciting strength of the test stimulus, which cannot be compared in the present study. The strength of a visual stimulus cannot be equated to the strength of an auditory or olfactory stimulus. However, in the present study, the total eating time was reduced to the same amount in all test situations. This equal eating time indicates a relatively similar strength of the stimuli. Further experiments in which horses are exposed to same type of stimulus but with different fear-eliciting properties would be necessary in order to link specific responses to the activation of different senses. While this may be achieved by studying responses to the same sound at different intensities, it is not so obvious whether a larger visual object is more frightening than a small or a stronger smell more frightening than a weak one.

There were no indications of carry-over effects, which probably relates to the fact that all horses managed to eat within the duration of the tests. The infrequent occurrence of some behaviours, e.g. defecations, vocalisations and the flehmen response, may be a consequence of these behaviours relating to different situations. Defecations and whinnying were shown during the initial habituation, indicating that once habituated to social isolation, these behaviours cease and do not reoccur even when horses are exposed to novelty in a known environment. Our results would also suggest that flehmen behaviour, which is typically shown by stallions when investigating urine, is probably associated more with sexual behaviour rather than the smell of novelty.

4.2. Correlations between behaviour and heart rate responses

The total eating time was found to reflect the reactions of the horses in all test situations in that the more a horse responded to the test stimulus, the greater the reduction in total eating time. The total eating time also reflected the heart rate response in the visual and auditory tests, but not in the control and olfactory tests. The results indicate that an interrelationship between behavioural and heart rate responses exists, given that the heart rate responses are sufficiently strong. This is in agreement with other studies on horses (e.g. Jezierski and Górecka, 1999; Lansade et al., 2003).

4.3. Correlations between test situations

There were no significant correlations in any behavioural variables between the tests, which is most likely due to the stimuli eliciting different and not very strong behavioural responses. Previous studies of consistency of behavioural variables across test situations in horses have shown varying results (e.g. Scolan et al., 1997; Wolff et al., 1997; Visser et al., 2001; Seaman et al., 2002). Discrepancies between studies may relate to the variation in tests to which the horses are exposed, sometimes mixing social and non-social situations. In other species, generalisation of behavioural responses across several different situations has been demonstrated (e.g. mink: Malmkvist and Hansen, 2002; cattle: Boissy and Bouissou, 1995; dogs: Goddard and Beilharz, 1984).

The strong correlation between heart rate responses in the olfactory and auditory tests may reflect the way in which these stimuli were presented as they were probably perceived only as the horses approached the food. This is in contrast to the visual stimulus, which could be easily seen from the start box when the door opened. Considering the small sample sizes, due to loss of heart rate data, as well as the fact that the horses were very similar in their responses, which gives only little variation, it is very likely that even stronger correlations in heart rate responses between different test situations exist. Other studies on horses have also shown correlations in heart rate responses between different tests (Visser et al., 2002). The heart rate correlations across situations indicate that heart rate responses simply reflect a non-differentiated activation of the sympathetic nervous system, whereas the behavioural responses are linked to the type of stimulus.

In conclusion, the responses of the horses to the novel olfactory stimulus differed from those to the visual and auditory stimuli, since the horses showed behavioural responses without a corresponding increase in heart rate towards the novel smell. Heart rate responses correlated between test situations, while the behavioural response was linked to the type of stimulus.

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Training methods for horses: Habituation to a frightening stimulus

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Summary

Responses of horses in frightening situations are important to both horse and human safety. While considerable scientific interest has been shown to the development of reactivity tests, little effort has been dedicated to the development of appropriate training methods for reducing fearfulness. The process, where young horses learn not to react to otherwise frightening stimuli, was studied using 27, 2-year-old, naïve Danish Warmblod stallions. The horses were trained according to three different methods, based on classical learning theory. In the first method (Classic habituation), the horses (n=9) were exposed repeatedly to the full stimulus (a moving, white nylon bag, 1.2 x 0.75 m) until they met a predefined habituation criterion. In the second method (Gradual habituation), the horses (n=9) were introduced gradually to the moving stimulus and were habituated to each step, before the full stimulus was applied. In the third method (Associative learning), the horses (n=9) were trained to feed from the bag, i.e. to associate the stimulus with a positive reward, before they were exposed to the full stimulus. Five training sessions of 3 minutes were allowed per horse per day. Heart rate and behavioural responses were registered. There was no significant difference between methods in behavioural or heart rate responses during the first training session. However, horses that were habituated gradually showed fewer flight responses totally and needed fewer training sessions to learn to react calmly to test stimulus. Variations in heart rate persisted even when the behavioural responses had ceased. We conclude that gradual habituation is the most effective training method for horses in frightening situations. Further research is needed in order to establish the role of positive reinforcement, such as food, in the training of horses.

Keywords: Horse, behaviour, fear, habituation, learning, training.

Introduction

Horses are primarily used for sports and leisure for a large number of children and young women. Unfortunately, horse riding ranks as one of the most dangerous sports in terms of the number and seriousness of accidents, and investigations have shown that unexpected flight responses are a major cause of accidents (Keeling *et al.* 1999). The ability of a horse to habituate to a range of otherwise frightening stimuli greatly increases safety in the horse-human relationship. Development of effective methods for reducing fear in horses therefore has important practical applications for safety of both horse and handler. While considerable scientific interest has been shown in the development of reactivity tests (e.g. Wolff *et al.* 1997; Visser *et al.* 2001; Seaman *et al.* 2002; McCall *et al.* 2005), very little effort has been dedicated to the development of appropriate training methods for reducing fearfulness in horses.

Habituation is the waning of responsiveness towards a repeated stimulus and is probably one of the most common types of learning in horses (Cooper, 1998). Habituation can be regarded as a simple form of learning because it involves reduction of a response which is already there. Associative learning, on the other hand, is about acquiring new responses. After some repetitions of an event followed by the same consequences, a long-term association is built up between the event and its result and the animal's behaviour changes accordingly. The best known example of associative learning is Pavlov's dogs, which learned to associate the sound of a bell with food. Pavlov also found that dogs were able to generalize their responses to include stimuli similar to the conditioned one (Manning and Dawkins 1998). There has been considerable scientific interest in animal learning in a wide range of animals e.g. rats, pigeons and primates, while there is no published research on such basic learning abilities as habituation and sensitisation in horses, and very little research on basic associative learning processes (see review by Nicol 2002). This is surprising given the importance of these processes in every elementary training programme, and Nicol (2005) states that more studies of the application of learning theory to practical problems are desperately needed.

In this study we trained horses according to three different methods; 1) classic habituation, 2) gradual habituation and 3) associative learning in order to investigate which of the three methods is most efficient for training horses to react calmly to an otherwise frightening stimulus. We hypothesise that horses are able to generalize about the test stimulus so that once familiar with the test stimulus in one situation, the stimulus will appear less frightening, i.e. elicit a reduced response, even when presented differently. Based on this assumption, we further hypothesise that alternative methods, such as gradual habituation and associative learning, will be more efficient, compared to the more simple type of learning in a classic habituation approach.

Recent studies have shown that behavioural reactions combined with physiological data are reliable measurements of horse reactivity (Christensen *et al.* 2005; McCall *et al.* 2005). In this study we use heart rate data, combined with two behavioural scores (immediate reaction to the test stimulus as well as latency to return to a feed container) to determine reactions of the horses during the training sessions. Equine reactions may be influenced substantially by human handlers, and we therefore developed training methods without human handling.

The aim of this study was to investigate which of three different training methods, based on learning theory, was the most effective for horses to learn to react calmly in an otherwise frightening situation.

Materials and Methods

Animals

A total of 27, 2-year old Danish Warmblod stallions from a large stud were used in this study. All colts were kept on pasture with the dam before weaning and were subsequently housed in large groups in straw-bedded boxes with access to outdoor areas during the winter. The colts had received a minimum of handling, only for necessary veterinary or farrier treatment. During the summer (May - October) the colts were pastured in a large enclosure (30 ha). Prior to the experiment, the stallions were habituated to wearing halters, heart rate monitoring equipment, to being socially isolated and to feeding from the container inside the test arena. Before the present experiment the horses took part in a different research project, investigating social transmission in horses, during which they all had similar experiences with the test arena. The horses were assigned to one of three treatments (training methods) based upon their reactions during the initial handling and in the first experiment, balancing the number of highly reactive horses and calm horses on each treatment. One horse became lame during the experiment and had to be excluded from the study, i.e. the data analysis is carried out on n=26 horses.

Test arena

Within the 30-ha summer enclosure, a smaller capture enclosure (1-ha) contained a fenced waiting area (50 m^2). Next to the waiting area a start box (2.5 m^2) and a test arena (10 m in diameter) was constructed out of straw bales (1.2 x 1.2 x 2.4 m^3) in two layers, making the height of the walls of the arena 2.4 m (Fig. 1). The set-up enabled the horses to hear, but not see their group mates during the tests. A feed container, placed in the middle of the arena, contained a mixture of alfalfa and the horses' usual winter feed (oat, barley, soybean oil meal, minerals and molasses).

Test stimulus and training methods

The test stimulus was a white nylon bag (1.2 x 0.75 m) which was pulled from outside the arena from a flat, folded to an unfolded, upright position by use of a string. The test stimulus was applied when the horse had been feeding for at least 30 sec. The horses were trained according to three different methods (Table 1). In the first method (**Classic habituation; MET1**), the horses (n=9) were exposed repeatedly to the full stimulus until they met the predefined habituation criterion (see below). In the second method (**Gradual habituation; MET2**), the stimulus was divided into several less frightening steps, i.e. the intensity of the movement was lower during the first steps, and the horses (n=9) were habituated to each step, before the full stimulus was applied. In the third method (**Associative learning; MET3**), the horses (n=8) were trained to feed from the bag, i.e. to associate the stimulus with a positive reward, before they were exposed to the full stimulus.

An equal number of horses on each method were trained during a test day. The study conforms to the 'Guidelines for Ethical Treatment of Animals in Applied Animal Behaviour and Welfare Research' prepared by the Ethics Board of the International Society of Applied Ethology (www.applied-ethology.org).

Recordings

Behavioural reaction to the test stimulus (Table 2) and latency to return to the food were registered using a handheld computer (Workabout, PSION PLC, UK). The observer sat quietly on top of the straw wall next to the start box, and the horses were used to the presence of the observer. Heart rate (HR) was recorded with Polar Vantage (Polar Electro OY, Kemple, Finland), which consisted of an electrode belt with a built-in transmitter and a wristwatch receiver. Water and gel were used to optimise the contact between electrode and skin. The HR monitoring equipment was fitted on the horse in the waiting area prior to testing, and the receiver stored data from the transmitter (every 5 sec). Subsequently, data were downloaded via a Polar Interface to a PC, using the software Polar Precision Performance^{TM} SW 4.

Habituation criteria

The habituation criterion was met when the horses showed only 'head up' or no behavioural response to the test stimulus (Table 2). For MET3 the first step was passed when the horse fed from the bag for at least one minute during the 3-minute training session. Horses which did not habituate within 20 training sessions were excluded from further training.

Data analysis

Data on the number of required training sessions were registered as the number of extra sessions a horse needed in order to meet the habituation criterion, i.e. a horse on MET1 which did not react to the test stimulus at the first presentation was scored as receiving 0 training sessions. A horse on MET2 or MET3, which passed directly through the three different steps corresponding to their training method, was also scored as receiving 0 training sessions. In case a horse needed e.g. two extra sessions in order to learn to feed from the bag in MET3, and passed directly through the other two steps, the horse was scored as receiving two training sessions, and so forth.

The number of horses on each training method was balanced between test days, and test day is not considered further in the analysis. Preliminary tests showed that there was no effect of horse on the initial HR (before the training sessions), probably due to the similar age, breed, and exercise level of the horses, making it unnecessary to correct for individual differences in initial HR. Thus, the analysis was carried out on data for average HR (HR_avg) and maximum HR (reflecting the immediate response of a horse towards the test stimulus; HR_max).

The number of required training sessions was analysed in Kaplan-Meier Survival Analysis: Log-Rank test, considering censored values, in the statistical program SigmaStat 3.0 (www.spss.com). The analysis was carried out on 'number of training sessions + 1', to include also horses with zero extra training sessions. Behavioural scores, latencies, and heart rate data (HR_max and HR_avg) were analysed for effect of training method by One Way Analysis of Variance (ANOVA; SigmaStat 3.0). Correlations were analysed using Spearman Rank-Order correlations (SigmaStat 3.0). A significance level of $P<0.05$ was used throughout.

Results

Number of required training sessions

Four of the 26 horses (15.4%) did not habituate within the course of the experiment (MET1: 1 horse (11.1%); MET3: 3 horses (37.5%)). Horses which were trained according to MET2 (Gradual habituation) needed fewer training sessions (Required training sessions (mean ±se): MET2: 2.4 ±0.7, MET1: 4.6 ±2.0, MET3: 12.0 ±2.8; $U_2=9.16$, $P=0.01$; Fig. 2) to learn to react calmly to the test stimulus.

Reactions during the first training session

Surprisingly, there was no significant difference between horses on MET1 and horses on MET2 in their behavioural reaction, latency to return to the food, HR_max, or HR_avg during the first training session, i.e. the horses did not react more to the full stimulus (MET1) than to the slower, less moving test stimulus (MET2) during the very first presentation. MET3 differed from the other two methods during the first training session in that the horses only had to learn to feed from the bag and they were not exposed to a sudden stimulus. Although insignificant, their HR responses suggest that this situation may be even more frightening than exposure to a sudden stimulus (HR_avg (bpm, mean±se): MET3: 89.3 ±9.9, MET1: 75.6 ±8.6, MET2: 71.0 ±6.2, n.s.; HR_max: MET3: 117.1 ±12.1, MET1: 95.7 ±10.7, MET2: 86.9 ±8.3, n.s.).

Reactions upon presentation of the full stimulus

There was a significant difference between the horses' reaction to the first presentation of the full stimulus in that horses on MET1 received a higher reactivity score (Fig. 3), a longer latency to return to the food (seconds (mean±se);

MET1: 21.7 ±7.7; MET2: 4.4 ±1.1; MET3: 3.6 ±1.0, F_2=3.88, P=0.038), and showed a higher HR response (e.g. HR_max: MET1: 95.7 ±10.7, MET2: 68.1 ±3.0, MET3: 66.4 ±6.4, F_2=4.52, P=0.024) than did horses on MET2 and MET3. There was no difference between horses on MET2 and MET3.

Correlations between HR and behavioural responses

There were significant correlations between the horses' HR response and their behavioural response (score and latency) during the first training session (Table 3), i.e. horses with a high HR also received a higher behavioural score and took longer to return to the food.

Correlations between HR and number of required training sessions

There was a strong correlation between HR responses during the first training session and the total number of required training sessions, i.e. horses which were highly reactive during the first training session needed more training sessions to learn to react calmly to the stimulus (all horses, r_s=0.73, P<0.001). However, looking at the correlations separately for each training method it appears that while the correlation remains strong for horses on MET1 (r_s=0.86, P<0.001) and MET3 (r_s=0.90, P<0.001), it is insignificant for horses on MET2 (r_s=0.62, n.s.).

Variations in HR at point of habituation

There were large variations between horses in their HR response, even when the behavioural response towards the test stimulus had ceased, but the variation did not differ between the methods (max_HR range (bpm): MET1: 60-105, MET2: 59-77, MET3: 50-85).

Discussion

Our results show that gradual habituation was the most effective and gentle training method for horses in order to learn to react calmly in an otherwise frightening situation. In addition, all horses on this method eventually habituated to the frightening stimulus, whereas some horses on the other two methods did not habituate within the experimental period. Associative learning appeared to be the least appropriate training method. In this method we encouraged the horses to explore the test stimulus by the use of food. Although food is a very strong motivator and is the most commonly used reinforcer in experimental work on equine learning (Nicol 2002), our results imply that the use of food in order to train horses not to be fearful is inefficient.

Feeding from frightening objects is often applied in police horse training where it appears to be useful (pers. com. UC Carlsson-Lindkvist, responsible for education of Swedish police horses). However, the police horses are typically left with a task in an enclosure, e.g. food is placed in the middle of a large piece of plastic and the horse needs to step on the plastic in order to get to the food. In this way the horse is able to train itself and has plenty of time to solve the problem (obtain the food). Since the high number of required training sessions for horses on MET3 in the present experiment were caused by the time taken to learn to feed from the test stimulus, the training method may prove useful in other circumstances when horses are able to train alone. Gough (1999) succeeded in reducing fear responses towards a clipper by allowing the horses to listen to the sound of the clipper while feeding during 2 weeks. However, the study was carried out on a small number of animals and there was a lack of control, meaning that the reduction in response may be caused by a general habituation. Further research is necessary in order to identify possible benefits of the use of positive reinforcement and associative learning in relation to fear reactions in horses.

In the present experiment, food was used in all methods to estimate the responses of the horses in terms of their latency to return to the feed container after being exposed to the test stimulus. The latency shows when the horse has calmed sufficiently to start feeding again and there were strong correlations between behavioural (latency and score) and HR responses of the horses during the first training session. The strong correlation between heart rate responses during the first training session and the total number of required training sessions shows that the immediate response of a horse in a frightening situation may be used to predict its trainability. However, the result that the correlation was insignificant for horses trained gradually implies that a more gentle training method is beneficial for the more emotional horses. In combination with the result that all horses passed on this method whereas some horses never habituated on the other two methods, our study indicates that the choice of training method is likely to be especially important for the more reactive horses. In fact, the horses which did not habituate seemed to sensitize, i.e. they became more and more reactive, to the test stimulus. The risk of developing phobias in horses further emphasizes the importance of choosing appropriate and gentle training methods for the most fearful horses.

In this experiment we based the habituation criterion on behavioural measures, since a reduction in behavioural response typically is the end goal for horse trainers. However, the analysis of the heart rate data revealed large individual variations between horses, which met the behavioural habituation criterion. Thus some horses may still be frightened by the test stimulus, even when the behavioural reaction has ceased. Previous experiments have shown that more emotional horses are poorer learners and are less trainable for riding (Heird *et al.* 1986), and it may therefore be beneficial to promote the use of heart rate data as a quick, easy and non-invasive tool in the daily training of horses.

Surprisingly, there were no significant differences between the methods in heart rate and latency to return to the food during the first training session in the present experiment, i.e. the horses did not react more to the full stimulus (Classic habituation) than to the slower, less moving test stimulus (Gradual habituation) during the very first presentation. This is in accordance to results on lambs where behaviour towards a novel object was independent of the speed of appearance (Désiré *et al.* 2004). The results probably reflect the biological relevance of prey species reacting to any unknown movement in the environment, regardless of the intensity. Despite the lack of difference during the first training session, the weaker and gradually increasing movement intensity of the test stimulus in MET2 had a positive effect on the speed of learning in the test horses, which was due to the fact that the horses reacted less during subsequent exposures.

Results from the first presentation of the full stimulus showed that horses on MET2 and MET3 had learned to generalize about the test stimulus from previous presentations and reacted less even when the stimulus was presented differently, i.e. they reacted less during the first presentation of the full stimulus, compared to horses on MET1. The similar responses towards the full stimulus in horses on MET2 and MET3 indicate that any difference between these methods has disappeared for horses that do get this far in the training program. It should be noted though that horses may become used to being tested, rather than learn to generalize about the test stimulus. However, the test horses already had experience with the test arena and test situations prior to the present study. In fear tests, there is also a risk of the animals becoming sensitized, because they know that something is going to happen in the test arena. While a few horses appeared to sensitize on MET1 and MET3, all horses on MET2 habituated to the test stimulus and they needed fewer training sessions in total, supporting that a gradual approach is more easily learned by the animals.

Conclusion

We conclude that gradual habituation is the most effective and gentle training method for horses in frightening situations.

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Table 1. Training methods

<table>
<tr><th>Training method</th><th>Procedure</th><th>Comment</th></tr>
<tr><td>MET1: Classic habituation</td><td>Step 1: The horse is exposed to the full stimulus while feeding from the feed container in the middle of the test arena (i.e. the bag is lifted from a flat folded to an upright position at full speed).</td><td rowspan="3">Prior to a training session, the test horse is fitted with a halter and HR equipment and led to the start box where it is released. The session starts when the arena door is opened by the observer and the horse is free to enter the arena. The horse is allowed to feed for at least 30 sec. before the test stimulus is applied.
If the horse does not return to the feed container within the test time of 3 min. it is led to the container by the handler and offered feed before leaving the arena.
Each horse receives a maximum of five training sessions per day. Between each training session the horse returns to visual contact with the group mates in the capture enclosure, while the arena is prepared for the next training session (e.g. placing the test stimulus in the start position, removal of defecations, and adding extra feed if necessary).
Whenever a horse meets the habituation criterion (see text) it carries on with the next step until it is habituated to the full stimulus. A horse, which needs more than five training sessions, starts on the last passed step on the subsequent training day.

MET3, step 1: If the horse does not feed within the test time of 3 min. it is led to the bag by the handler and offered feed before leaving the arena.</td></tr>
<tr><td>MET2: Gradual habituation</td><td>Step 1: As in MET1, but with the bag lifted only halfway up at half speed.
Step 2: The bag is lifted all the way up at half speed.
Step 3. The horse is exposed to the full stimulus (as in MET1).</td></tr>
<tr><td>MET3: Associative learning</td><td>Step 1: A similar bag is placed with the test stimulus and feed is spread on the bag, while the usual feed container is removed from the arena. The horse is allowed to feed from the bag during the entire test time.
Step 2: The horse is exposed to the full stimulus, while feeding from the other bag below the test stimulus.
Step 3: The horse is exposed to the full stimulus while feeding from the usual feed container in the middle of the test arena (as in MET1).</td></tr>
</table>

Table 2. Ethogram of behavioural reactions

Reaction	Description
1. None[1]	The horse does not react to the test stimulus and chewing is not interrupted
2. Head up[1]	The horse raises the head from the feed container and chewing is interrupted, but it is not alert and does not move away from the feed container
3. Alert	The horses stands vigilant with elevated neck, with or without tail elevation, head and ears oriented towards test stimulus, chewing is interrupted and the horse may move up to two steps away from the feed container
4. Away	The horse moves three or more steps backwards or sideways from the feed container in response to the test stimulus, typically followed by alertness
5. Flight	The horse turns/jumps away from the feed container in a sudden movement, typically followed by trotting/galloping, alertness and possibly snorting

[1]Reaction allowed in habituation criterion

Table 3. Correlations between HR and behavioural responses during first training session

	MET1 (Classic hab.)	**MET2 (Gradual hab.)**	**MET3 (Ass. learning)**
Max_HR vs. React. score	R_s=0.83; P=0.002	R_s=0.80; P=0.006	Data not analysed*
Max_HR vs. Latency	R_s=0.71; P=0.025	R_s=0.63; P=0.058	
Latency vs. React. score	R_s=0.78; P=0.009	R_s=0.82; P=0.004	

* The horses did not receive a reactivity score during the first step in MET3. Besides, 7 of 8 horses were censored, i.e. did not approach the bag with food within the test time.

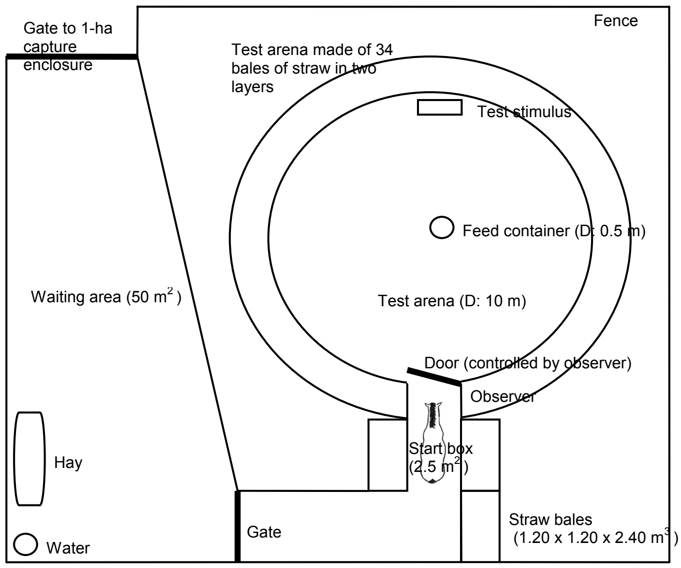

Figure 1. Test arena.

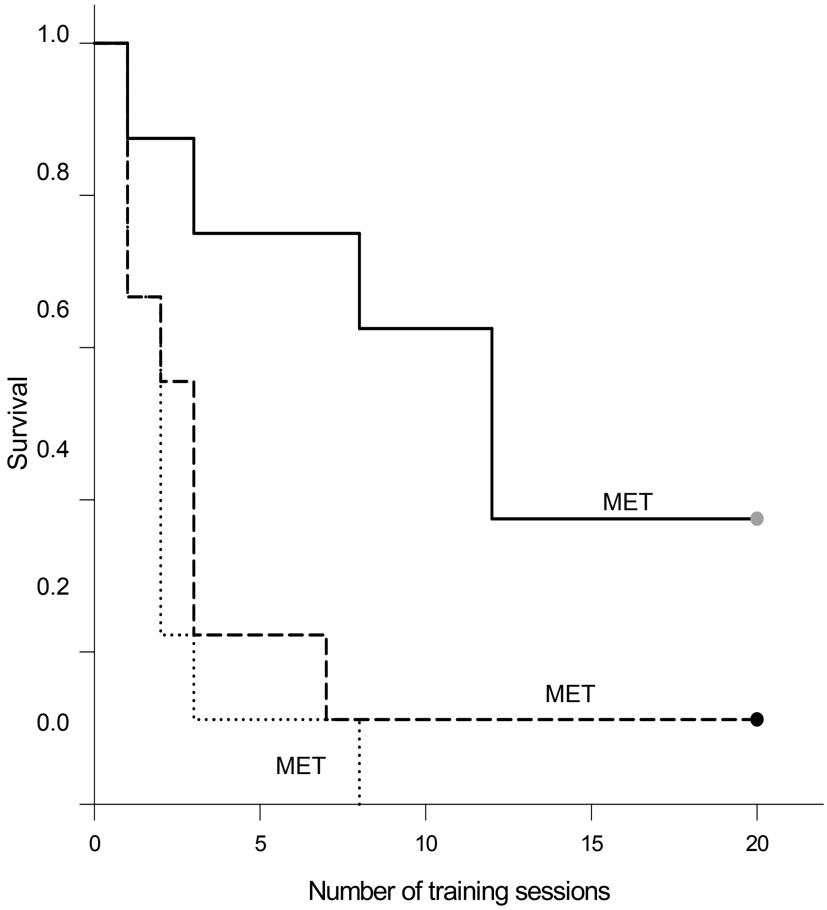

Fig.2. Required training sessions: Kaplan-Meier Survivor functions for each training method.

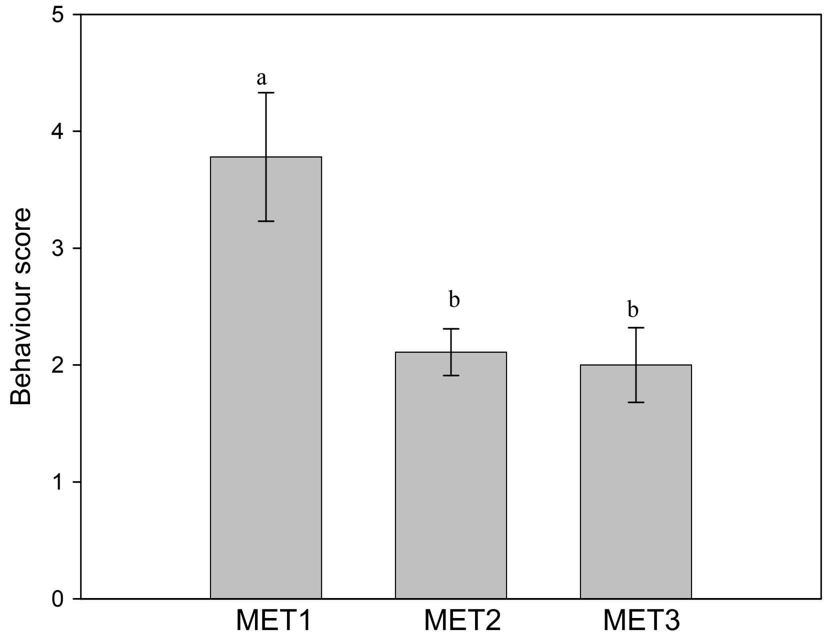

Fig. 3. Behavioural reaction to first presentation of full stimulus. Bars with different letters differ at P<0.05.