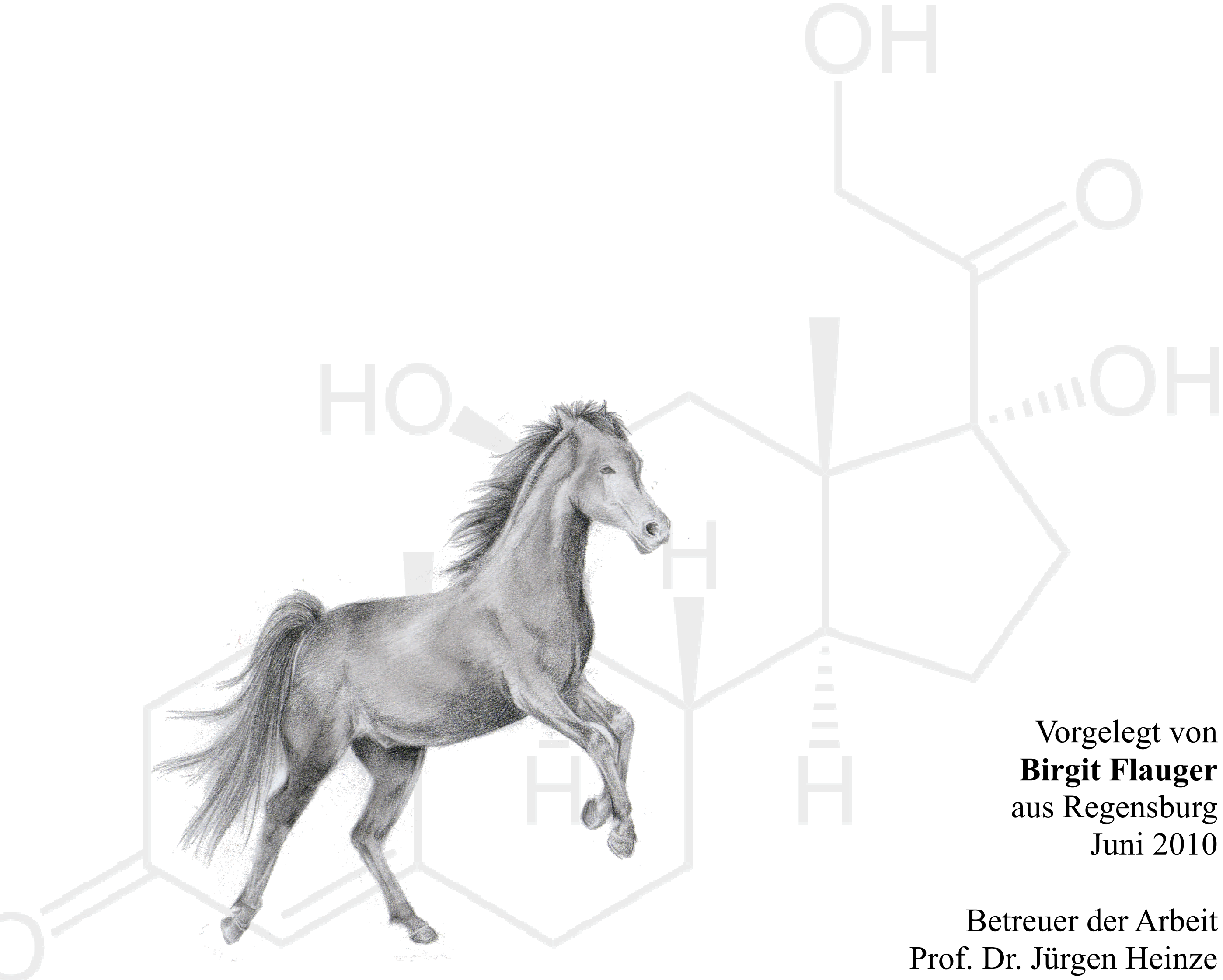
The introduction of horses into new social groups with special regard to their stress level



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Juni 2010

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Dissertation zur Erlangung des Doktorgrades
der Naturwissenschaften (Dr. rer. nat.) der Naturwissenschaftlichen Fakultät III
- Biologie und Vorklinische Medizin - der Universität Regensburg

Promotionsgesuch eingereicht am:		29.06.2010
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HOW IT ALL BEGAN

As it may seem unusual for a biochemist to concentrate on behaviour observations in her PhD thesis, I want to explain how it all began. As a child and teenager I already loved observing animals; for hours on end I was sitting on pastures observing horses and cows. But I was also interested in the processes going on inside a body and inside a cell, interested in the functioning of whole organisms. Therefore I decided to study biochemistry. Again, during my studies I took some unconventional practical courses, e.g. behavioural observations of termites. And then, searching for a topic for my PhD thesis, I seized the chance to start a project with Dr. Konstanze Krueger in which I could combine my two favourite areas: behavioural observations and endocrinological investigations. Moreover, I could work with horses, an animal species about whose behaviour I already knew a good deal, although without a scientific background. We started as a small group (Konstanze and me!) in the department of Prof. Dr. Juergen Heinze, which on the one hand enabled me to contribute and incorporate ideas of my own to the project. On the other hand, it cost a lot of work and time to establish all necessary contacts to the horse and stable owners, and to get permission to observe the horses, as well as to establish national and international contacts and cooperations for the scientific background of my study. For example, I thought I would never get through with the process of obtaining a licence for animal testing; I even got an extra special licence allowing me to collect faecal samples. But now that the work is completed I think it was worth the trouble!

GENERAL INTRODUCTION

The animal species dealt with in this study, the horse, will be introduced through a short overview over its evolutionary history and social structure. After that, I shall briefly discuss my topic in the light of behavioural ecology and point out the behavioural and cognitive capacities of horses. The subsequent paragraph is devoted to a description of the mechanisms and problems going along with stress detection, which play an important role in my thesis. Finally the aims of the project will round up this general introduction.

The horse as model organism

Evolutionary history

In Eocene (55 to 60 million years ago) the first members of the equine family appeared in North America and Europe, though subsequently their evolutionary history centred on North America (Simpson, 1951; Woodburne, 1982). *Hyracotherium* was the first genus, a small, tetradactyl animal about the size of domestic cats. All species were frugivores and foliovores, as indicated by their bunodont (low-crowned) molars (Janis, 1982). The next successful steps in equid evolution were *Mesohippus* and *Miohippus* (Stirton, 1940). Each genus was larger than its ancestors. The animals were now tridactyl and their teeth remained low-crowned, still fitting for browsing, not grazing. With the grassland expansion in the Miocene, the equids became increasingly adapted to select and contend with the highest fibre, lowest protein diet in the grazing community by perfecting cecal (not ruminant) digestion, in conjunction with increased intake and passage rate (Janis, 1976). *Meryhippus* became the first grazer at this time, although not all species were exclusive grass grazers (i.e., using C4 photosynthetic pathway); some species were mixed feeders, and some fed primarily on browse (i.e., C3 pathway) (MacFadden et al., 1999). Originating from *Meryhippus* a variety of descendants appeared. Recent investigators separated the meryhippine complex into two monophyletic clades, namely the tribes Equini and Hipparionini (Waring, 2003). Primitive taxa within the Equini were tridactyl, whereas advanced forms of *Pliohippus*, *Astrohippus*, and *Dinohippus* were monodactyl.

There is consensus that the closest relative of *Equus* is within the Equini, but there is less agreement on the exact ancestral line for *Equus*. Based on mitochondrial DNA analyses, it is suggested that the common ancestor of extant *Equus* species was living approximately 3.9 million years ago and that speciation leading to the lineages of surviving members occurred over the next half million years (George and Ryder, 1986). Members of the genus *Equus* have emigrated in many different directions and at different times in the approximately 4 million years since their first appearance. Each of the many species had their distinct form and distinct habits. Molecular techniques provide some clues, for example, that speciation followed three lineages – zebras, asses, and caballine horses (George and Ryder, 1986). The surviving caballine horses are now reduced to two kinds – the domestic horse and the

Przewalski horse. The domestic horse population of today is a result of the interbreeding of many lines of wild horses from multiple places. According to Zeuner (1961) and Heptner et al. (1966), three lines were present at the beginning of the horse's domestication: a) the Przewalski horse, b) the tarpan, and c) the forest horse. But there still exists controversy which horse type was initially utilised in the domestication process. According to genetic data, horses were domesticated repeatedly from several distinct populations of wild horses (Jansen et al., 2002). Horses are the last animal added to the common livestock (Clutton-Brock, 1981); they were not domesticated until 2500 to 5000 years ago. The domestic horse of today may only partially resemble their wild ancestors in conformation and coloration; yet many traits are little altered by domestication, e.g. domestic horses can still readily adapt to a wild existence. Feral herds show survival traits typical for species that have never been domesticated; they form stable populations and reproduce. The only true extant native horses are the Przewalski horses. Unfortunately, they are declared to be extinct in their native ranges in China, Russia and Mongolia (King and Gurnell, 2005; Moehlmann, 2002). But 11 wild-caught breeding animals survived in zoos from which a new population has been raised.

(Outline of evolutionary history according to Simpson (1951), Berger (1986), MacFadden (1992, 1998) and Waring (2003))

Social organisation in horses

Horses are a highly social species. Given the opportunity, under semi-natural conditions, domestic horses gather in social groups like wild horses. Harem groups usually consist of one to five stallions, several mares and their offspring (Feist and McCullough, 1975; Berger, 1977, Linklater, 2000). Offspring disperses from the natal groups at the age of about three to five years. Mares affiliate to young stallions or other harems, whereas young stallions form bachelor groups. It is still under discussion whether several subgroups form a large structured social unit, called a "herd", and show the same migration patterns within a common home range (Miller, 1979; Duncan, 1992; Feh 2005) or whether a collection of subgroups should rather be termed a "population", which has a synchronous daily and seasonal pattern of movement in response to water, food or climate (Feist and McCullough, 1975; Berger, 1986; Linklater et al., 1999) and which has inter-band hierarchies at resource patches (Miller and Denniston, 1979; Franke Stevens, 1988).

Harem and band stability varies considerably between groups and populations (Berger, 1986; Rubenstein 1986; Rutberg, 1990). Most harem groups have been reported to be relatively stable (Linklater, 2000), but some were described as unstable (Hoffmann, 1983), and mares were seen separated from their groups due to separation or dispersal (Linklater, 1998).The social life of equids can therefore be compared to the fission-fusion model (Dyer, 2000) of other social mammals like apes (Dyer, 2000), elephants (Moss and Poole, 1983) and dolphins (Connor et al., 2000), in which animals frequently split and reunite again. Such complex social systems require horses to memorise and generalize social experiences, distinguish between familiar and unfamiliar, and identify familiar horses, as well as their social status relative to their own group (Krueger and Heinze, 2008).

Questions on the behavioural ecology of introducing horses into new groups

To date, many questions on the endogenous, behavioural and cognitive abilities horses need for living in a fission-fusion social system remain to be answered. For example: Do horses always act aggressively when they meet new group members? Or are there horses which contact each other in a friendly way? Is earlier social experience a relevant influence? What is the effect of introduction techniques on the behaviour of horses? Are horses able to assess their own relative rank position in comparison to the new group members? Is the introduction a stressful event? Are some horses more stressed by additional factors of influence?

These are some of the questions to be asked concerning the topic of this thesis. Behavioural ecology provides a framework for answering these kinds of questions because it combines theories from evolution, ecology and behaviour (Krebs and Davis, 1997). As a fully identified scientific domain behavioural ecology has emerged only 30 - 40 years ago. Since then it has become increasingly interdisciplinary, incorporating approaches from ethology, population biology, genetics, cognition, physiology, anthropology, and neuroscience (Cézilly, 2008).

In behavioural ecology, behaviour is considered to be a set of decisive processes by which an individual adjusts its state and situation according to variations in its environment. Here, decision making simply refers to the fact that an animal is regularly confronted with multiple alternatives, and decision making does not necessarily need elaborate cognitive processes. For example, when a group starts moving, each animal has to make decisions on a cost benefit ratio (i.e. whether to join the group or to cover its own need independently and take the risk of separating from the group). The process can either be based on simple self-organising mechanisms or on cognitive consensus decision making. Nevertheless, as the information processing ability of animals is clearly emerging as an important component of an increasing number of behavioural ecological questions (Giraldeau, 1997), I will continue with a report of studies indicating that horses possess these information processing abilities. Due to their complex social system mentioned earlier on, it can, for example, be expected that they memorise and generalize social experiences, which calls for elaborate cognitive processes.

The behavioural and cognitive capacities of horses

It has been demonstrated that horses are able of discrimination and categorization learning (Hanggi, 1999), of generalising objects, stimuli and persons (e.g. Dougherty and Lewis, 1991; Krueger, 2007), and that they even have long-term memory for categories and concepts, which altogether reflect high-order mental abilities (Hanggi and Ingersoll, 2009). Moreover, Krueger and Heinze (2008) demonstrated that horses are able to differentiate between known and unknown, as well as dominant and subordinate individuals, and know their relative social status. Moreover, Proops et al. (2009) proved horses to be capable of cross-modal individual recognition. In their study, horses showed startling responses when a herd mate, that disappeared behind a barrier did not match a playback call that was recorded from another familiar herd mate.

These social abilities are particularly important for horses that are introduced into new groups. This is why I wanted to further investigate them. The mixing of animals is an event inducing aggression and fights; it therefore represents a conflict situation. As such conflict can reduce the benefits of group life and harm social relationships (Aureli et al., 2002) as well as the individual itself, mechanisms for dealing with conflict situations and the resulting aggressions are likely to exist in social animals. Conflict resolution mechanisms have been observed in a wide range of taxa, e.g. in insects (Heinze, 2010), rooks (Seed et al., 2007), dolphins (Weaver, 2003), and primates (Aureli et al., 2002). The balance in social systems is maintained by a complex system, including mutual surveillance, policing, and punishment on both a chemical and behavioural level (Heinze, 2010) as well as on a cognitive level (Aureli et al., 2002).

Determination of the stress level

In recent years, studies in behavioural ecology have increasingly attempted to incorporate the relationship between hormones and behaviour. This and the fact that stress may severely affect the horse's behaviour and cognition in conflict situations, is the reason why I started my thesis concentrating on the stress level horses might experience during the introduction and comparing it to the observed behaviour.

Stress is a general term used to describe environmental factors sollicitating adaptation mechanisms and the response to these challenges (Mormède et al., 2007). In vertebrates, the main hormones to be synthesised in stressful situations are glucocorticoids and catecholamines. Their increased secretion enhances adaptive physiological responses (Wingfield and Ramenofsky, 1999; Sapolsky et al., 2000). The two main "stress-axes" involved are the autonomic nervous system (Cannon, 1935) and the hypothalamo-pituitary-adrenocortical (Selye, 1936) axes (Fig. 1). Within minutes of the onset of a perceived stressor, the adrenal cortex begins to secrete glucocorticoids, mainly cortisol and corticosterone. Which glucocorticoid is predominantly produced depends mainly on the species. The major glucocorticoid in most primates, carnivores, and ungulates, for example, is cortisol, whereas most rodents, birds, and reptiles excrete corticosterone (Touma and Palme, 2005). In horses the dominant adrenal steroid is cortisol (Bottoms et al., 1972).

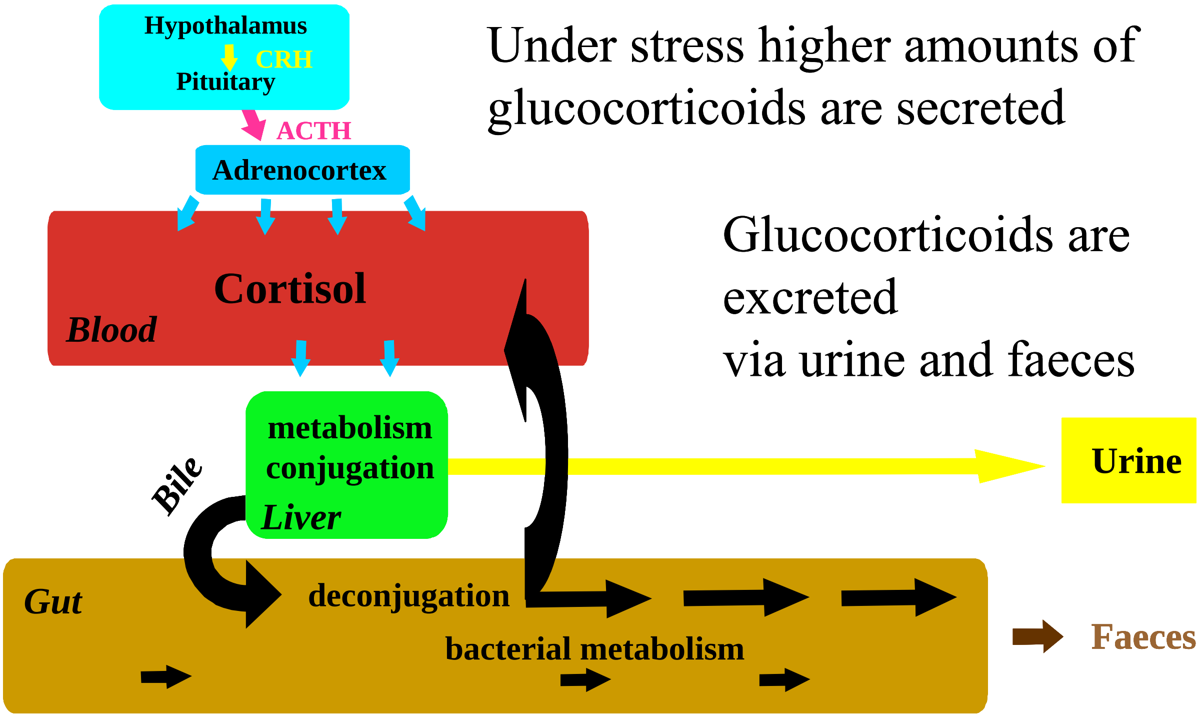

Fig. 1 Metabolism and excretion of glucocorticoids (modified after Möstl and Palme, 2002)

Glucocorticoids, or their metabolites, can be measured in several body fluids or excreta, including plasma, saliva, milk, urine and faeces (e.g. Möstl and Palme, 2002). All methods have both advantages and disadvantages. For sampling blood and saliva it is necessary to catch and handle the animals, which can be a stressor itself and is only possible to a limited extent in free moving animals. Additionally, they show ultradian, diurnal and seasonal rhythms. The cortisol in blood can be divided into a free fraction and a fraction bound to corticoid binding globulins (Matteri et al., 2000), and free cortisol represents the biologically active form (Moons et al., 2002). Stress reduces the binding capacity of the corticosteroid binding globulin (Alexander and Irvine, 1998). Milk, for example, is only available from lactating females. Advantages of faeces as sample material are, for example, that no handling of the animals is necessary, that it is a non-invasive technique and therefore applicable in zoo and wild animals, and that episodic fluctuations are balanced.

Conflicting reports about the correlation of cortisol in equine blood, saliva and faecal samples have been published. Lebelt et al. (1996) reported that salivary and plasma total cortisol in stallions, in response to semen collection, correlate. Van der Kolk et al. (2001) also demonstrated the validity of saliva in assessing adrenal function. But McGreevy and Pell (1998) failed to show a correlation in their control animals. In a study Moons et al. (2002) investigated the correlation between salivary cortisol, free cortisol, plasma total and serum total cortisol. They could show that salivary cortisol correlates better with free cortisol than with plasma and serum total cortisol.

Because metabolism and excretion of steroids via faeces differ significantly between species, and sometimes even between sexes, this non-invasive method must be rigorously validated for each species before application (Palme, 2005) in terms of sample collection, storage, extraction and analysis. Additionally, the percentage of cortisol excretion via faeces appears to differ between species. Even though horses excrete 41% of radioactive cortisol via faeces (Palme et al., 1996) which should therefore be well suited for detecting GCMs in faeces, the extraction procedure for horse faeces described so far (Merl et al., 2000; Gorgasser et al., 2007) is more complicated than in other species. This is due to the fact that the assay used picks up only metabolites which are present in minor amounts, but not the dominant ones. Therefore the potential of the measurement of cortisol metabolites in faeces should be improved.

Aims of thesis

In the main project of my PhD thesis I investigate the behaviour and the stress level of horses during the introduction into new social groups.

When I started my literature survey, I realised conflicting reports about the applicability, the analysis and the evaluation of saliva and faecal samples for measuring the stress level in horses. Therefore, I decided to conduct a stress hormone validation study first (**chapter 1**).

In **chapter 2** I hypothesise that different introduction techniques could have an influence on the behaviour of the horses, mainly on the aggressive interactions. I consider several questions, such as: Are horses able to determine the hierarchy of the new group members when they have the possibility of observing them on a neighbouring paddock before the introduction event? Are they able to define their own relative rank position?

In **chapter 3** I investigate how horses are able to cope with the specific conflict situation of being introduced into a new group. I explicitly ask: Are there differences in experienced stress when they are immediately introduced, introduced after an observation period or introduced together with an integration horse? Does the stress level of the horses during introduction have an influence on the development of social bonds with new group mates?

During the introduction process a multitude of factors can influence the behaviour of the horses and should therefore be taken into consideration. For example, does a horse that stands behind the fence on a neighbouring paddock influence the behaviour of horses? Does the individual recognition play a role during introduction? Therefore I arrange several experiments so as to shed further light on aspects which could affect the introductions.

Social learning could be one of the abilities that horses need for integrating into new groups. In **chapter 4** I discuss several reasons for the failure of former social learning experiments in horses. I suggest that social aspects affecting the behaviour of horses in social learning tasks, e.g. group membership and dominance hierarchies, have been underestimated.

In **chapter 5** some of the possible social effects on a horse’s behaviour are investigated in horses’ feeding decisions. How do the rank, the position and the presence of conspecifics influence the feeding strategy of horses? Is there a difference if the conspecific is tied to a pole behind a fence or absent? This point was especially interesting because in the observation periods before the introduction event the horses were separated by a fence in the same way.

As individual recognition certainly plays an important role during the introduction process, I want to shed further light on individual recognition in horses. In **chapter 6** I address the questions of whether horses are able to recognise their conspecifics' group membership through olfactory perception, and whether they can even determine the particular donor of a faecal sample.

In the final chapter I discuss equine abilities for interspecies interactions. Especially when horses are mixed with conspecifics they have to react to very subtle signs and gestures of the new group members which are often not visible for humans. The experiments in **chapter 7** investigate how horses react to cues given by humans, such as their body posture and gaze consistency. Moreover I investigate if horses even make a difference between familiar and unfamiliar persons.

CHAPTER 1

Simplified method to measure glucocorticoid metabolites in faeces of horses

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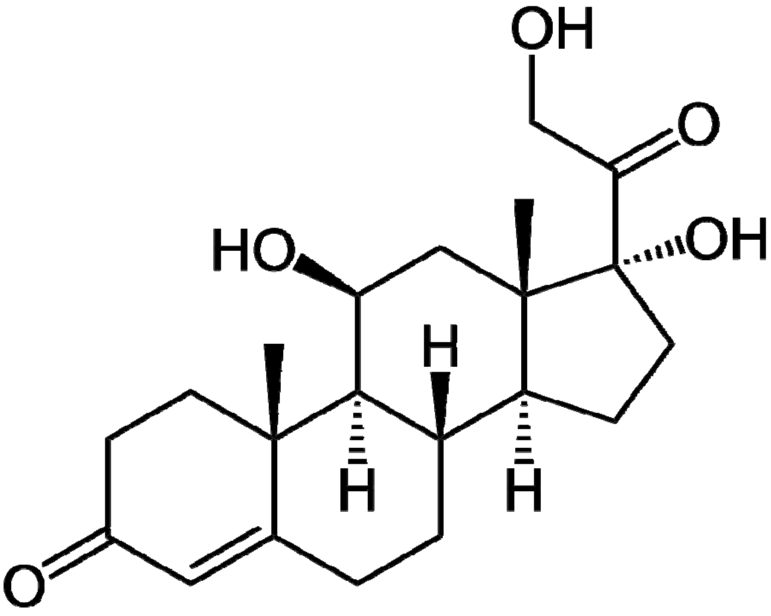

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Published in Veterinary Research Communications (2010) 34, 185-195

Abstract

Glucocorticoids or their metabolites can be measured in several body fluids or excreta, including plasma, saliva, urine and faeces. In recent years the measurement of glucocorticoid metabolites (GCMs) in faeces has gained increasing attention, because of its suitability for wild populations. In horses, however, the group-specific enzyme immunoassay described so far has a limited practicability due to its complex extraction procedure. Therefore, we tested the applicability of other enzyme immunoassays for glucocorticoid metabolites. The present study clearly proved that an enzyme immunoassay (EIA) for 11-oxoaetiocholanolone using 11-oxoaetiocholanolone-17-CMO: BSA (3α,11-oxo-A EIA) as antigen showed high amounts of immunoreactive substances. Therefore it was possible to use just a small amount of the supernatant of a methanolic suspension of faeces. The results correlated well with the already described method for measuring GCMs in horse faeces, i.e. analysing the samples with an EIA after a two step clean up procedure of the samples (Merl et al., 2000). In addition, the 3α,11-oxo-A EIA has the advantage of providing a bigger difference between baseline values and peak values after ACTH stimulation. The new assay increased the accuracy of the test, lowered the expenses per sample, and storing samples at room temperature after collection was less critical than with other assays investigated in our study. This is a big advantage both in the field of wildlife management of equids and in the field of equestrian sports and it shows the importance of choosing an assay which is in good accordance with the metabolites excreted in a given species.

Keywords

ACTH challenge, enzyme immunoassay, stress behaviour, cortisol

Introduction

In vertebrates, the frontline hormones in stressful situations are glucocorticoids and catecholamines. Their increased secretion enhances adaptive physiological responses (Wingfield and Ramenofsky, 1999; Sapolsky et al., 2000). The two main “stress-axes” involved are the autonomic nervous system (Cannon, 1935) and the hypothalamo-pituitary-adrenocortical (Selye, 1936) axes.

Glucocorticoids, or their metabolites, can be measured in several body fluids or excreta, including plasma, saliva, urine and faeces (e.g. Möstl and Palme, 2002). The cortisol in blood can be divided into a free fraction and a fraction bound to corticoid binding globulins (Matteri et al., 2000), free cortisol representing the biologically active form (Moons et al., 2002). Stress reduces the binding capacity of the corticosteroid binding globulin (Alexander and Irvine, 1998). Traditionally, plasma has been used, but sample collection is difficult and stressful for the animals, which may confound the results (Hopster et al., 1999).

Therefore, in recent years the measurement of glucocorticoid metabolites (GCMs) in faeces has gained increasing attention for wild populations (Heistermann et al., 2006), wildlife management, and conservation as well as behavioural biology (Möstl and Palme, 2002; Touma and Palme, 2005), largely because it uses a non-invasive and feedback-free sampling method.

Even though horses excrete 41% of radioactive cortisol via faeces (Palme et al., 1996) which should therefore be well suited for detecting GCMs in faeces, the extraction procedure for horse faeces described so far (Merl et al., 2000; Gorgasser et al., 2007) is more complicated than in other species. This is due to the fact that the assay used picks up only metabolites which are present in minor amounts, but not the dominant ones.

Within the framework of a large project to physiologically validate cortisol and GCM secretion in horses, we tried to find an assay which cross reacts in a higher extent with the GCMs in horse faeces.

Material and Methods

Animals

A total of ten horses (five mares, five stallions) were used for the experiment, eight warmblood horses, one haflinger and one pony, all aged between three and 14 years. The experiment was conducted at the veterinarian department of the Ludwig-Maximilians-University in Munich, where the horses were stabled at least three days prior to the testing. The horses were kept in individual boxes with a bedding of straw and were turned out on paddocks during the day. The daily feed of the horses was composed of hay *ad libitum* and a compound feed twice a day. The animal experiment was permitted by the Bavarian Government (reference number 55.2-1-54-2531-121-07).

ACTH Challenge Test

The horses were tested in three batches consisting of three, three and four horses, respectively. The sampling procedure differed slightly between these experiments because of adjustments to the horses' turn-out times.

The stimulation test was conducted over a period of six days. On day 1 and 2, blood and faecal samples were taken in the morning. On day 3 only faecal samples were taken in the morning, and blood samples soon after inserting a permanent catheter into the vena jugularis. Two hours after the catheterization 0.05 mg/100 kgBM ACTH were injected i.m. (preparation: Synacthen Injektionslösung®, Novartis Pharma GmbH, Nuremberg). Blood samples were taken 30, 60, 90, 120 and 240 min after ACTH application. In the evening of the same day blood and faecal samples were collected again. Thereafter the permanent catheter was removed. On day 4, 5 and 6 blood and faecal samples were taken in the morning and in the evening.

On day 7, after faecal sample collection in the morning, 16 mg/100 kgBM dexamethasone (preparation: Dexamethason-Injektionslösung®, CP-Pharma Handelsges. mbH, Burgdorf) were injected i.m. After 90 min blood samples were taken. On the same day blood and faeces were collected again in the evening. On day 8, 9 and 10 blood and faecal samples were taken in the morning.

Sample Processing

Blood samples were taken with a heparin Monovette® 2ml LH, Sarstedt, and immediately centrifuged for 8 min at 1000 g. The plasma was stored at -20°C. During the 30 min intervals of the ACTH stimulation the blood samples were kept on ice and centrifuged within 2 hours. Plasma cortisol levels were analysed using an EIA as described by Palme and Möstl (1997).

Faecal samples were collected with one-way gloves, stored in glasses and immediately frozen at -20°C, or kept on ice until freezing. For processing faecal samples two different protocols were used and afterwards the samples were analysed by different group-specific EIAs. The first protocol (extraction method described by Merl et al., 2000) is a two step extraction including addition of water/methanol. After centrifugation, the supernatant is diluted with $NaHCO_3$ and reextracted with diethyl ether. The organic solvent has to be evaporated and the residue redissolved in assay buffer before performing the EIA. Using assays which cross reacted with the faecal GCMs of horses in a higher amount than the assay already described; we extracted horse faeces as described for faecal GCM extraction in ruminants (Palme and Möstl, 1997). In brief, 0.5 g faeces plus 1 ml water and 4 ml methanol were vortexed for 30 minutes. The methanolic suspension was centrifuged, a small part of the supernatant diluted in assay buffer and directly analysed by EIA. Further on, we label the use of the diethyl ether extraction protocol with "Extraction", abbreviated "Extr". When no labellings are given we used the simplified method.

Enzyme immunoassays (EIAs)

All enzyme immunoassays used were already published, but not tested in horses. Details about the procedure and the cross-reactivities for the assays are published elsewhere (Palme and Möstl, 1997; Möstl et al., 2002; Ganswindt et al., 2003). Interassay coefficients of variance for the assays in the horses were 7.8% and 20.9% (n = 8) for the EIA measuring blood cortisol, and 13.3% and 15.7% (n = 9) for the 3α,11-oxo-A EIA measuring faecal GCMs. Values represent percentage variance for high and low quality controls.

Details of the assays are shown in Table 1.

Table 1 Characteristics of the three EIAs in use for determining faecal glucocorticoid metabolites

EIA	DOA EIA[a]	3α,11-oxo-A EIA[b]	3α,11β-dihydroxy-A EIA[c]
Antibody against (linked to BSA)	11-oxoaetiocholanolone-3-HS	11-oxoaetiocholanolone-17-CMO	11β-hydroxyaetiocholanolone-17-CMO
Label	11-oxoaetiocholanolone-3-glucorinide[d]	11-oxoaetiocholanolone-17-CMO[e]	11β-hydroxyaetiocholanolone-17-CMO[d]
Standard	11-oxoaetiocholanolone (= 5β-androstane-3α-ol-11,17-dione)	11-oxoaetiocholanolone (= 5β-androstane-3α-ol-11,17-dione)	11β-hydroxyaetiocholanolone (= 5β-androstane-3α,11β-diol-17-one)
Specificity[f]	11,17 DOA[g]	3α,11-oxo-CM[h]	3α,11β-dihydroxy-CM[i]

[a] First described by Palme and Möstl (1997)
[b] First described by Möstl et al. (2002)
[c] First described by Ganswindt et al. (2003)
[d] Coupled with N-biotinyl-1,8-diamino-3,6-dioxaoctane (DADOO-biotin)
[e] Coupled with biotinyl-3,6,9-trioxaundecanediamine (LC-biotin)
[f] Groups of metabolites measured
[g] CMs with 11,17-Dioxoandrostane configuration
[h] CMs with 3α,11oxo configuration
[i] CMs with 3α,11β-dihydroxy configuration

Concentration of immunoreactive metabolites after storage at room temperature

For stability analysis faecal samples were collected and from each sample one portion was frozen immediately whereas the other portions were frozen after storage for 1, 2, 4, 8 and 124 hours at room temperature. The content of GCMs was analysed using an EIA.

High performance liquid chromatography (HPLC)

The supernatant of methanolic suspension of faecal samples was separated by reversed phase high performance liquid chromatography (Novapac C18 column 0.39 x 15 cm, Fa. Waters, Milford, MA, USA). A linear methanol gradient from 50% to 75% in the first 40 min and thereafter 100% methanol up to 55 min were used. Flow rate was 1 ml/min and 3 fractions per minute were collected (95 fractions). Faecal samples of three animals were investigated. Elution positions of reference standards are shown in Fig. 4.

Statistical analysis

Data were analysed with the software package SPSS 15.0 for Windows. All statistical tests were two-tailed, and alpha was set at 0.05. Spearman rank correlations were used for comparing the different extraction methods and EIAs, as well as possible correlation between plasma cortisol and faecal GCMs. The baseline, highest and lowest values were tested for normality with Ks tests. As they do not significantly diverge from normality, we applied a paired t-test for their comparison.

Results

Correlation between different assays

The faecal samples of the ACTH stimulation test were analysed with three different EIAs (DOA EIA, 3α,11-oxo-A EIA and 3α,11β-dihydroxy-A EIA), both using diethyl ether extraction or the supernatant of a methanolic suspension. When using methanolic supernatant, the values from DOA EIA significantly correlated with those of the 3α,11-oxo-A EIA and the 3α,11β-dihydroxy-A EIA (Spearman rank correlation; $n = 43$; 3α,11-oxo-A EIA: $r = 0.678$, $p < 0.001$; 3α,11β-dihydroxy-A EIA: $r = 0.771$, $p < 0.001$). When using extraction, the values from DOA EIA significantly correlated with those of the 3α,11β-dihydroxy-A EIA (Spearman rank correlation; $n = 43$; 3α,11β-dihydroxy-A EIA: $r = 0.466$, $p = 0.002$), but not with the results of the 3α,11-oxo-A EIA (Spearman rank correlation; $n = 43$; 3α,11-oxo-A EIA: $r = 0.109$, $p = 0.488$).

As the data from different assays in general correlated within the same clean-up protocol, we compared data of the three different EIAs from samples treated with methanolic suspension to those from diethyl ether extraction. All assay values for the suspension method correlated highly significantly with values from DOA EIA when extracted with diethyl ether (Fig. 1). But 3α,11β-dihydroxy-A EIA values and DOA EIA values showed a better correlation coefficient than 3α,11-oxo-A EIA values to Extr-DOA EIA values (Spearman rank correlation; $n = 88$; 3α,11β-dihydroxy-A EIA: $r = 0.640$; DOA EIA: $r = 0.628$; 3α,11-oxo-A EIA: $r = 0.432$; all $p < 0.001$).

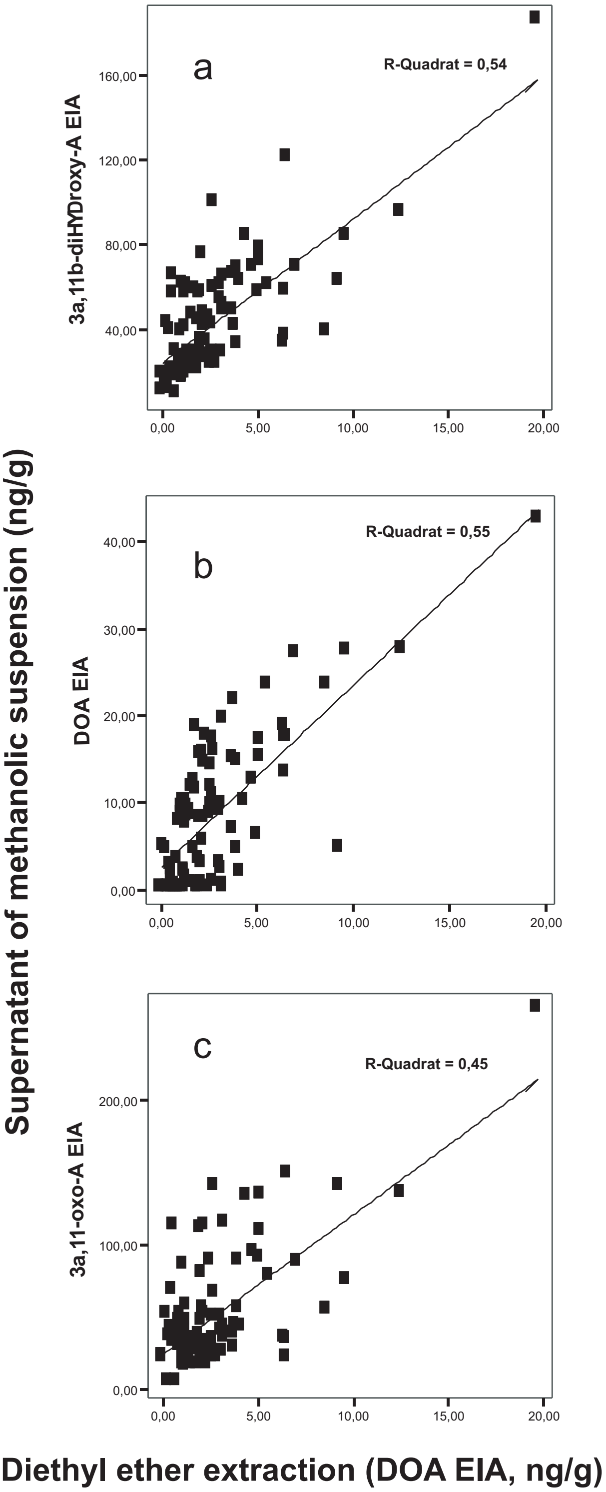

Fig. 1 a-c Correlations between diethyl ether extractions of horse faeces analysed with DOA EIA on the x-axis and supernatants of methanolic suspension of horse faeces analysed with different group-specific EIAs on the particular y-axis. (a) 3α,11β-dihydroxy-A EIA, (b) DOA EIA and (c) 3α,11-oxo-A EIA. Values represent concentrations of faecal GCMs in ng/g

Correlation between mean plasma cortisol and mean faecal GCMs

For a better comparison between plasma cortisol and faecal GCMs we calculated the daily mean values for each parameter in the stimulation test. We compared the faecal GCM mean values from different assays (DOA EIA, 3α,11-oxo-A EIA and 3α,11β-dihydroxy-A EIA) to plasma cortisol mean values. As horses excrete cortisol metabolites in faeces with a delay of about 24 hours (Palme et al., 1996; Möstl et al., 1999), we additionally shifted the correlation calculation between faecal cortisol metabolites and plasma cortisol for 1 and 2 days.

As expected, we could not find correlations between mean values of plasma cortisol to mean values of faecal metabolites for the same day. But for faeces collected one day later the correlation was highly significant for the analysis with 3α,11-oxo-A EIA and Extr-DOA EIA, but slightly weaker for 3α,11β-dihydroxy-A EIA. For the two day shift the correlations diminished again for all three assays. Details of the Spearman rank correlations (Spearman rank correlation coefficient, p-values) are given in Table 2.

Table 2 Correlation between mean plasma cortisol and mean faecal GCMs analysed with three different EIAs (Spearman rank correlations, r = correlation coefficient, p = significance value)

	Mean value of faecal metabolites (ng/g)			
	3α,11-oxo-A EIA	3α,11β-dihydroxy-A EIA	Extr-DOA EIA	
Mean value of plasma cortisol (ng/ml)	r = 0.275 p = 0.037	r = 0.085 p = 0.527	r = 0.359 p = 0.006	Same day (n = 58)
	r = 0.506 **p < 0.001**	r = 0.307 p = 0.027	**r = 0.525** **p < 0.001**	One day shifted (n = 52)
	r = 0.437 p = 0.002	r = 0.389 p = 0.008	r = 0.406 p = 0.005	Two days shifted (n = 46)

Comparison of amplitude range

We calculated the means for the baseline, the highest and the lowest values during the procedure of ACTH stimulation and dexamethasone depression. For each horse the baseline values were averaged from the first three samples before stimulation, whereas the highest values were taken from the peak after ACTH stimulation and the lowest values from the depression after dexamethasone injection. In Fig. 2 faecal GCM concentrations of two horses during the ACTH Challenge Test, measured with 3α,11-oxo-A EIA and Extr-DOA EIA, are depicted.

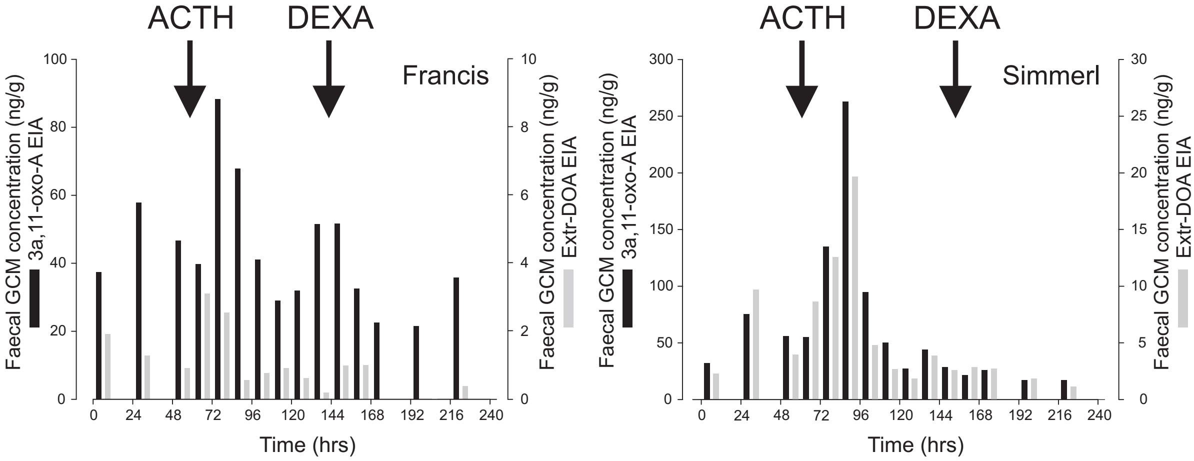

Fig. 2 Faecal GCM concentrations (ng/g) in two horses during the ACTH stimulation and dexamethasone suppression test, analysed using 3α,11-oxo-A EIA and Extr-DOA EIA. Arrows indicate time point of ACTH/dexamethasone application

We compared the assays for their amplitude range. Generally, higher quantities were measured using the 3α,11-oxo-A EIA than using the Extr-DOA EIA (mean baseline values: 3α,11-oxo-A EIA: 49.91 ± 21.13 ng/g; Extr-DOA EIA: 3.48 ± 1.65 ng/g, Table 3). On average the difference between baseline and highest values increased by 55.18 ng/g (110%) for assay 3α,11-oxo-A EIA, which was significant (Paired t-test, see Table 3). The mean increase of 4.76 ng/g (140%) for the assay Extr-DOA EIA values was less significant. For the difference between baseline and lowest values we measured an average decrease of 34.07 ng/g (68%) for the assay 3α,11-oxo-A EIA values, which was highly significant (Paired t-test; see Table 3), and for assay Extr-DOA EIA values the less significant mean decrease of 2.74 ng/g (78%).

Table 3 Comparison of the amplitude range of the ACTH Challenge Test

assay	mean baseline values ± standard deviation (ng/g)	mean highest values ± standard deviation (ng/g)	mean lowest values ± standard deviation (ng/g)	difference baseline - high			difference baseline - low		
					t-test			t-test	
				ng/g (%)	n	p	ng/g (%)	n	p
3α,11-oxo-A EIA	49.91 ± 21.13	105.09 ± 65.21	15.84 ± 9.28	55.18 (110.56)	10	0.016	34.07 (68.26)	10	<0.001
Extr-DOA EIA	3.48 ± 1.65	8.24 ± 6.03	0.74 ± 0.50	4.76 (136.78)	6	0.062	2.74 (78.74)	6	0.005

Stability analysis

For stability analysis we compared the GCM content of immediately frozen faecal samples to those kept at room temperature for 1, 2, 4, 8 and 124 hours. All samples were analysed after methanolic suspension with either assay 3α,11-oxo-A or assay DOA. The values from 3α,11-oxo-A EIA showed little variation and a small standard deviation for up to 8 hours, but for those analysed with DOA EIA a strong variation could be seen (n = 6 for each time point, Fig. 3). In samples, which were stored for 124 hours at room temperature, the GCM concentration strongly declined for assay 3α,11-oxo-A, whereas for assay DOA the values showed a high variability (between 28% and 997%) compared to the particular baseline values from the beginning of the test (Fig. 3, a single outlier at 997% is not depicted in the graph).

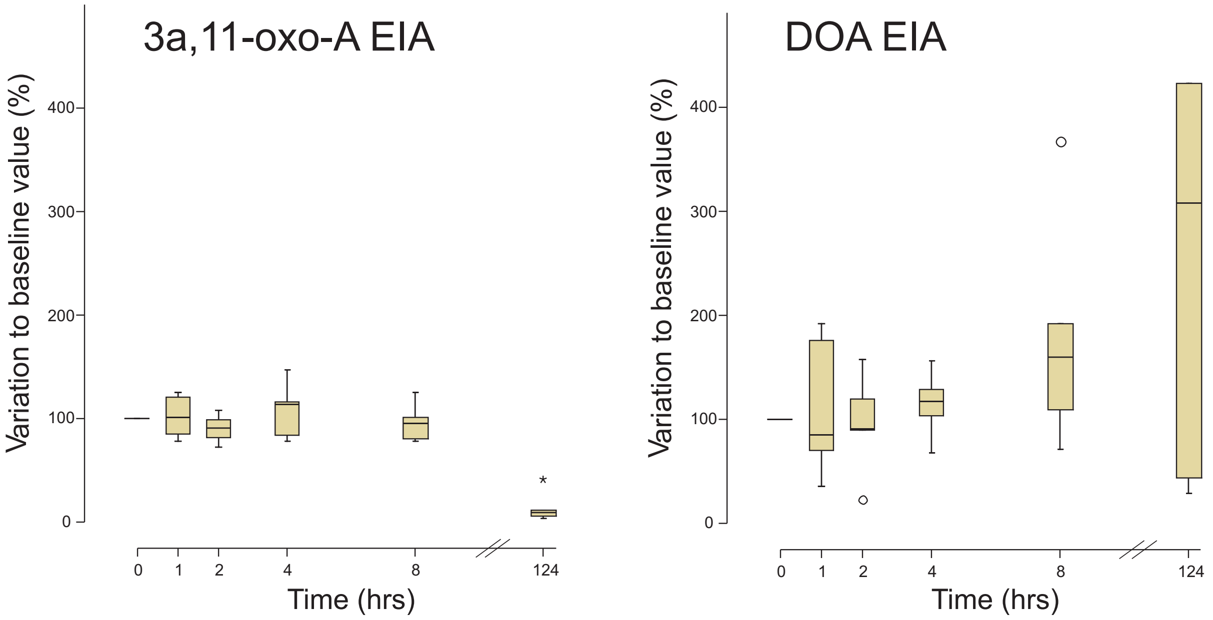

Fig. 3 Boxplot of the variation of GCMs (start time = 100%) after different storage time at room temperature, analysed with 3α,11-oxo-A EIA and DOA EIA, n = 6 per time point. A single outlier (at 997%) for the time point 124 hrs analysed with DOA EIA is not depicted

Immunoreactive metabolites

The immunoreactive substances in the methanolic supernatant of faecal samples were separated by HPLC. The different fractions were analysed with DOA EIA and 3α,11-oxo-A EIA. The DOA EIA showed the dominating peak in fraction 8, whereas the 3α,11-oxo-A EIA showed a peak in fraction 39 and a second peak in fraction 47 (Fig. 4, one faecal sample is depicted as example). Both peaks were much higher than that measured with the DOA EIA. Also some minor peaks were present, amongst others one peak which coeluted like 11-oxoaetiocholanolone (fraction 33).

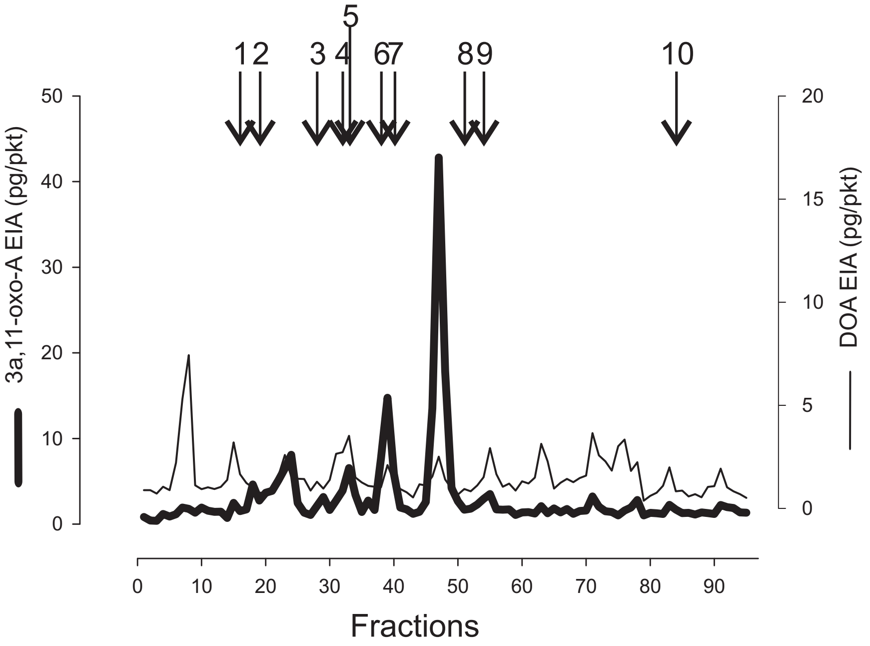

Fig. 4 High performance liquid chromatography (reversed phase) separations of immunoreactive faecal GCMs of a horse. The obtained fractions were analysed with DOA EIA and 3α,11-oxo-A EIA. Note the different scale of the y-axis. Elution positions of reference standards: 1) cortisone (fraction 16), 2) cortisol (19/20), 3) corticosterone (27-30), 4) 11β-hydroxyaetiocholanolone (32), 5) 11-oxoaetiocholanolone (33), 6) tetrahydrocorticosterone (38), 7) androst-4-en-3,17-dion (40), 8) testosterone (51), 9) 17α,20α-dihydroxyprogesterone (54), 10) progesterone (84)

Discussion

The present study clearly proved that the 3α,11-oxo-A EIA using the simplified protocol of methanolic suspension correlates well with the established method for measuring GCMs in horse faeces, i.e. analysing the samples with DOA EIA after extraction (Merl et al., 2000). In addition, the 3α,11-oxo-A EIA has the advantage of providing a bigger difference between baseline values and peak values after ACTH stimulation, cross reacts with at least two other metabolites and in a higher extent than DOA EIA, which is shown by HPLC, and, the detected immunoreactive glucocorticoids are more stable at room temperature. The second EIA (3α,11β-dihydroxy-A) used, also gave good results after applying the methanolic suspension method. It correlates well with the established assay DOA EIA after extraction, but obtains inferior results than the 3α,11-oxo-A EIA concerning the correlation with mean plasma cortisol values.

Additionally, when compared to daily mean values of blood cortisol, daily mean values of faecal GCMs, after methanolic suspension, analysed with 3α,11-oxo-A EIA, produce correlation coefficients which are as good and significant as after extraction analysed with DOA EIA, both around r = 0.5. The correlation is best when faecal samples are taken with one day delay. This is in agreement with Palme et al. (1996) and Möstl et al. (1999) findings of cortisol metabolites in horses being excreted via faeces about 24 hours delayed compared to plasma.

In general, higher values were found with the 3α,11-oxo-A EIA, which can be explained by having a closer look at the HPLC fractions measured with the DOA EIA and 3α,11-oxo-A EIA. Although both assays are designed to detect 11-oxoaetiocholanolone they differ in their linkage of the steroid for antibody production. 11-oxoaetiocholanolone is linked to BSA at position 3 in DOA EIA, and, in 3α,11-oxo-A EIA, to BSA at position 17. Some cross-reactions occur when the structure differences between cross-reacting molecules are located close to the linking position (Niswender and Midgley, 1970; Kohen et al., 1975). As Möstl et al. (2002) already pointed out, the DOA EIA is group-specific concerning the oxo group in position 17, whereas the 3α,11-oxo-A EIA's group specificity concerns the OH-group in position 3. With the 3α,11-oxo-A EIA two peaks were detected in the middle of the chromatogram, which were seen between the elution of tetrahydrocorticosterone and 17α,20α-dihydroxyprogesterone. 3α,11-oxo-A EIA obviously shows cross-reactions with C21 steroids, which are not detected with DOA EIA. Even though we did not conduct a radio metabolism study, it is likely that the detected metabolites are identical or closely related to the dominating GCMs present in horse faeces. These results highlight the importance of choosing an assay which is in good accordance with the metabolites excreted in a given species, and even between closely related species (Bosson et al., 2009).

The accuracy of faecal GCM measurements in general benefits from the cross-reaction characteristics of the 3α,11-oxo-A EIA, resulting in a higher baseline quantity as well as big amplitudes between baseline values and peak values after ACTH administration. The big amplitudes of 3α,11-oxo-A EIA strongly improve the practicability of GCM measurement in horses, because the higher the amplitudes the better small differences can be determined and the assay can be applied to different situations and to animals showing different levels of stress. It has to be mentioned that Extr-DOA values showed a higher increase (in percentage) after ACTH administration than 3α,11-oxo-A values, but this method has not the overall benefit of high baseline quantities.

Another relevant aspect is the modification of GCMs after different storage time at room temperature. The time interval between defecation and freezing appears to be crucial in ruminants. Möstl et al. (1999) demonstrated a significant increase of 45% in horse faecal GCMs after 4 hours when measured with DOA EIA. We measured the stability of GCMs at room temperature with the assays 3α,11-oxo-A and DOA. Obviously, the immunoreactive glucocorticoid values measured with 3α,11-oxo-A EIA are more stable compared to those measured with DOA EIA, showing less variation.

This method is superior to the assay used in horse faeces so far (application of DOA EIA after diethyl ether extraction) concerning the amplitude after ACTH induction and dexamethasone depression, as well as the stability of immunoreactive glucocorticoid metabolites. The simplified method poses big advantages, because it is faster and easier to apply, increases the accuracy and automatisation, but lowers the laboratory expenses (e.g. less consumption of solvent, feasible in simple equipped laboratories without pull-off devices) especially when adopted to huge quantities of faecal samples. The non-invasive sampling method, on the one hand, is particularly suitable when measuring stress in feral or wild horses for wildlife management, as well as conservation and behavioural biology. On the other hand, it is urgently needed for the growing sector of equestrian sports and the popular interest in the horse's welfare. With the simplified method higher amounts of samples can be analysed and the method has a better practicability which is necessary in determining the stress level of horses during sports events, e.g. after the transportation, during the contest itself or during the stay at the competition.

Acknowledgments

We thank Jürgen Heinze for helpful suggestions, Sandra Hake-Paulus, Petra Kölle, Anika Pachten, Bettina Wollanke (LMU Munich) and Hans-Peter Remler (LVFZ Haupt- und Landgestüt Schwaiganger Pferdehaltung) for assistance with conducting the experiment, Alexandra Kuchar and Andreas Trindl for technical assistance in the laboratory, and Henning Thies for language corrections. Horses have been provided by the LMU Munich and the LVFZ Haupt- und Landgestüt Schwaiganger Pferdehaltung. The study was supported by an Excellence grant of the Universität Bayern e.V. and an HWP II grant of the University of Regensburg.

CHAPTER 2

Social interactions among horses (*Equus caballus*): Interrelations between aggression, enclosure sizes and introduction techniques

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Manuscript submitted to Applied Animal Behaviour Science

Abstract

Even though animal welfare organisations propose group housing for horse welfare, many owners stable their horses individually, for fear of aggressive interactions and because of injury risks. In the present study we investigated the effect of enclosure sizes on the behaviour of socially kept domestic horses (*Equus caballus*) during everyday sociality with a focus on aggressive interactions. In addition, aggressive as well as positive social behaviour among the horses was analysed when new group members were introduced. We studied 28 introductions at best practice, with horse groups of different size and composition assigned to three approved methods: (1) Immediate introduction, (2) introduction after an observation period and (3) introduction with "integration horse".

During everyday sociality we found a significant correlation between the enclosure size and the horses' aggressive behaviour ($P = 0.006$) as well as retreat behaviour ($P = 0.003$). Aggressions and retreats diminished with increasing logarithmic enclosure size, but approaches and total interactions were not affected. The curve describing the interrelation between the enclosure size per horse and the expected aggressions per hour took an exponential shape. Starting from a space allowance of 300 m^2 and more per horse, the amount of aggressions per hour approached zero.

Concerning introduction techniques, the introduction with an integration horse led to significantly less total interactions (technique 1 vs 3: $P = 0.013$) and lower levels of aggression (technique 1 vs 3: $P = 0.013$; technique 2 vs 3: $P = 0.015$) than the introduction of single horses, both immediately and after several days of observing the new group.

For the reduction of aggression levels and injury risks in socially kept horses we recommend an enclosure size of at least 300 m^2 per horse, and the introduction of new horses together with an "integration horse".

Keywords

Introduction technique, integration horse, injury risk, aggression, exponential curve, group housing

Introduction

Animals kept under human supervision usually have to deal with limited space. Especially when kept in social groups in unsuitably small enclosures, there may be enhanced aggression levels among the animals because their need for personal space in terms of a minimum distance between conspecifics cannot be satisfied (Wilson, 2000). In fact, several case studies show that decreased space allowance per animal results in higher amounts of aggressive behaviour in cattle (Kondo et al., 1989), pigs (Weng et al., 1998), deer (Li et al., 2007), dama gazelles (Cassinello and Pieters, 2000) and horses (e.g. Skiff, 1982; Hogan et al., 1988; Jørgensen et al., 2009b). In cows, Menke et al. (1999) could show a clear negative coherence between agonistic behaviour and space per animal.

In addition to enclosure size, aggressiveness between social animals can be caused by a multitude of variables, such as group size, group density and enclosure shape (e.g. Christman and Leone, 2007; Leone et al., 2010). Whether and how group size effects aggressiveness is debatable. Estevez et al. (2007) list several studies that challenge the traditional hypothesis of increased group sizes going along with increased aggressions by proving the opposite to be true. In contrast, higher aggression levels were found in large adult cattle groups (Price and Wallach, 1991), but not in calves (Kondo et al., 1989). Aggression in poultry studies is contradictory, as some authors demonstrated higher amounts of aggression (e.g. Al-Rawi and Craig, 1975), while others found less aggression in even larger groups (Hughes et al., 1997; Nicol et al., 1999). Furthermore, Rodenburg and Koene (2007) highlight a main effect of group size on damaging behaviour, fear and stress, rather than on aggression in poultry and pigs. For sheep the aggression level seems to be more sensitive to changes in space allowance than to changes in group size per se (Jørgensen et al., 2009a), and in horses, a study on Arab breeding mares demonstrates rather low aggression levels despite high stocking densities (Benhajali et al., 2008). Finally, primate researchers claimed reduced rates of agonistic behaviour under high-density conditions in chimpanzees to be an inhibition strategy to reduce opportunities for conflict, but only as a short term response (Aureli and de Waal, 1997).

Density is a direct consequence of varying either group size or enclosure size. Their particular contributions to changes in social behaviour are often difficult to determine (Leone et al., 2010). In all the studies mentioned, researchers cannot clearly distinguish group size, enclosure size, or other enclosure attributes as causal variables (Christman and Leone, 2007).

The species under investigation in this study, the horse, is a highly social species. Given the opportunity, under semi-natural conditions, domestic horses gather in social groups like wild horses. Harem groups usually consist of one stallion, several mares and their offspring (Feist and McCullough, 1975; Berger, 1977). Offspring disperses from the groups at the age of about three to five years. Mares affiliate to young stallions or other harems, whereas young stallions form bachelor groups. Obviously domestication did not change the qualitative nature of the horse's social behaviour (Tyler, 1972; Waring, 1983), as social behaviour did not vary between domestic horses that were reared under typical domestic conditions, and non-domestic Przewalski horses (Christensen et al., 2002b). However, domestication may have influenced the quantitative nature of social behaviour. In some studies domestic horses were less aggressive than their non-domestic counterparts (Feh, 1988; Keiper and Receveur, 1992), but not in others (Christensen et al., 2002b).

Under domestic conditions horses are usually kept in so called “fate societies”. They do not have the opportunity to choose their group affiliation themselves. Either they are singly stabled or they are grouped artificially. Both situations can cause problems. Isolation implicates welfare problems for gregarious animals, such as the horse, and lack of social contact may result in redirected behaviour towards less suitable objects (Luescher et al., 1991). In contrast, social housing bears the risk of injury, even though several authors (e.g. Grogan and McDonnell, 2005) found few injuries in group housed horses. Still bite and kick injuries were reported to be more frequent on pasture, although they may occur at any time in horse-to-horse contacts (Derungs et al., 2004). Even though free-living equids rarely hurt each other seriously in real combat (Klingel, 1967; Waring, 1983), vigorous rearing, boxing and chasing does carry a risk of injury. In fact, the occurrence of play fighting is one of the main reasons why domestic horses are kept physically separated (Christensen et al., 2002a).

Animal welfare organisations propose group housing for horse welfare (BMELV, 2009). In this domain veterinarians and scientists need to educate horse and stable owners, as well as constructors of equine facilities on how to reduce injury risks in group management. For generating clear instructions, further studies on the causality of injuries are urgently needed. Jørgensen et al. (2009b) state that gender composition is not decisive for the aggression level, spacing or injuries. However, the horses’ early social experiences, management and space allowance are probably more important for the successful group housing of horses. A stable group hierarchy and a housing system that provides adequate space and that is adapted to horse-specific behaviour are important for preventing kick and bite injuries (Knubben et al., 2008). Fürst et al. (2006) recommend that preventive measures should focus on the disposition of horses within the group, the introduction of new horses to the group, and the design of the housing facility.

The primary aim of this study was to investigate the effect of enclosure size on the social behaviour of horses during everyday sociality with a focus on aggressive interactions. Additionally, we predicted that the more time the horse spends observing the new group before being introduced to it, the less aggression will occur during the introduction process. Thus we compared the amount of interactions among the horses during different introduction techniques. Because studies on common situations are needed, we studied 28 introductions at best practice that were assigned to three approved methods. The groups were of different size and composition.

Material and Methods

Animals

The introductions of 28 horses, aged between 2 and 18 years (Table 1), into twelve different groups were observed between July 2006 and April 2009. The groups comprised three to 20 horses. Two groups consisted of mares and four groups of geldings only, whereas six groups had both mares and geldings. The horses were of different breed, including warmblood horses, quarter horses, trotters, Haflingers and ponies. Most of the horses were used for leisure riding, some for shows or events. They were either kept constantly in open stables or in individual boxes during the night and in their groups on paddocks during the day. Depending on weather conditions they had access to pastures. Sleeping areas included a bedding of straw or wood shavings. For their daily feed the horses received hay twice a day and a compound feed once or twice a day. Additionally they fed on the grass in their pastures. They had free access to water.

Table 1 Information on newcomer horses

Number	Name	Age (years)	Sex	Breed	Introduction technique
1	Grandessa	17	mare	warmblood	immediate
2	Monty	9	gelding	paint	
4	Inka	16	gelding	Lusitano	
5	Sambor	11	gelding	warmblood	
6	SherazII	13	gelding	Kabardian	
11	Diabolo	7	gelding	Friesian	
13	Zlodky	13	gelding	Arab	
20	SherazIII	13	gelding	Kabardian	
9	Rashnu	5	gelding	Friesian	observation period
10	Britta	17	mare	paint	
12	Sissi	15	mare	pony	
14	Sheela	17	mare	thoroughbred	
17	Gaytano	6	gelding	warmblood	
19	Shannon	4	mare	Anglo-Arab	
21	Hexi	8	mare	Arab warmblood mix	
22	Wiebke	18	mare	warmblood	
23	Liesl	15	mare	Haflinger	
24	Mighty	2	gelding	Appaloosa	
25	Robin	16	gelding	Welsh Arab mix	
26	Realla	18	mare	warmblood	
27	Ria	7	mare	Lipizzan	
28	Ali Baba	3	gelding	Arab	
3	Cornelius	17	gelding	warmblood	integration horse
7	Nevada	16	gelding	warmblood	
8	Mirella	7,5	mare	German riding pony	
15	Plainsman	6	gelding	trotter	
16	Toffee	2	mare	quarter trotter mix	
18	Sally	5	mare	quarter mix	

Behaviour observations

The groups were observed five times in total:

a) “pre-control”: before introduction of the new horse, duration 4 hours
b) “introduction”: duration 2 hours
c) “first-control”: six to ten weeks after introduction, duration 2 hours
d) “second-control”: twelve to 20 weeks after introduction, duration 2 hours
e) “one-year-control”: one year after introduction, duration 4 hours

It was impossible to conduct all the control observations for each group, because some newcomers left the groups before all observations had been performed. In other groups two newcomers were introduced within one year. In these cases the second-control observation counts as pre-control for the second introduction. In Table 3 the numbers of control observations are listed.

Continuous all-occurrence sampling (Altmann, 1974) was used, recording the following social behaviour (modified after Feist and McCullough, 1976; McDonnell and Haviland, 1995; McDonnell, 2003):

- Approach: Forward movement towards another horse in a friendly way, which means that the ears are not laid back. Approach may be immediately followed by retreat, no reaction or an approach in return of the other horse.
- Bite: Opening and rapid closing of the jaws with the teeth grasping the flesh of the other horse. The ears are laid back and the lips retracted.
- Threat to bite: Similar to a bite except that no contact is made. The ears are laid back and sometimes the neck is stretched towards the other horse.
- Kick: One or both hind legs lift off the ground and rapidly extend backwards toward another horse, with apparent intent to make contact.
- Threat to kick: Similar to a kick, but without sufficient extension or force to make contact. The hind leg(s) lift slightly off the ground and under the body in tense readiness.
- Chase: One horse pursuing another, in order to displace or direct the movement of the other horse. Usually the chaser has the ears laid back and exposes the teeth. The movement can be either in walk, trot or gallop.
- Retreat: One horse moves away in order to maintain or increase the distance. Normally retreat is a reaction in return to the action of another horse.

The entire social behaviour was categorized in aggressive behaviour (bite, threat to bite, kick, threat to kick and chase), affiliative behaviour (approach) and submissive behaviour (retreat).

Introduction of horses

The introduction techniques for the new horses were chosen by the stable and/or horse owners themselves. We distinguished three introduction techniques:

1. Immediate introduction:

The newcomer horses were either immediately introduced, or they were standing for up to three hours on a neighbouring paddock, or were taken for a ride together with a horse of the new group before being introduced to the new group. They had either no, or very limited time (up to three hours) for observing the new group.

2. Introduction after observation period:

Newcomer horses were allowed to stay on a neighbouring paddock to the new group for several days (1-7 days). They could observe and establish limited sniffing- and tactile contact to new group members.

3. Introduction with "integration horse":

Newcomer horses were allowed to stay on a neighbouring paddock to the new group for several days (1-7 days) accompanied by a new group mate. They could observe and establish limited sniffing- and tactile contact to all the new group members, and associate to one particular group mate. Together with this particular horse they were introduced afterwards.

During the introduction and the following two hours the interactions between the new horse and the group members were video recorded and scored thereafter.

Statistical analysis

Data were analysed with the software package SPSS 15.0 for Windows and the R-project statistical environment (2009). All statistical tests were two-tailed, and alpha was set at 0.05. Multiple testing was corrected with a Sequential Bonferroni Procedure (Holm, 1979).

For adjusting to diverse group sizes and observation periods, the total amount of social interactions per observation was divided by the amount of horses in the group and the time observed. Further on, the mean number of interactions per hour and individual was used for statistical analysis. We tested for normality with a KS test, and as some data were significantly not normally distributed, we continued with applying non-parametric tests. Additionally, because standard deviations were relatively high, we followed the generally accepted procedure to enhance the robustness of the non-parametric tests by applying exact procedures.

Differences in behaviour were assessed using a Generalized Linear Model (GLM) with the newcomer horse itself (1-28), the groups (1-12) and the type of paddock (with/ without grass) as explanatory variables. As they had no significant effect they were removed from the model. When including the type of observation (pre-control, introduction, first-control, second-control, one-year-control) as a factor in the model, we found, after iterations and reordering, a significant difference for the introduction, and therefore proceeded with analysing the introduction separately from the control observations.

The social interactions of the control observations were tested with a Friedman test for their variability and, further on, a Spearman Rank correlation was used to analyse the effect of enclosure size on social interactions.

For analysing the displayed aggressions per horse, per available space, we transformed the enclosure size per horse into exp(enclosure size per horse/-50) and applied a linear model.

In order to test for effects of the introduction technique, a Kruskal-Wallis and a Mann-Whitney U test were applied.

Results

General effects on social interactions

When analysing for effects on the entire aggressive behaviour we found no significant influence of the newcomer horse itself (1-28), the groups (1-12) and the type of paddock (with/without grass) (GLM; N = 122; all P > 0.05).

But significant differences in aggressiveness could be detected between the introduction and the different observation types (GLM; N = 122; pre-control: P = 0.002; first-control: P = 0.047; second-control: P = 0.002; one-year-control: P = 0.003). Therefore we continued to analyse the introduction separately from the control observations.

For the control observations we found a significant effect of the logarithmic enclosure size on aggressive and retreat behaviour (GLM; N = 58; aggression: t = -2.527, P = 0.015; retreat: t = -2.613, P = 0.012; Table 2), but not on approach behaviour or total interactions (GLM; N = 58; approach: t = 1.505, P = 0.138; total interaction: t = -0.643, P = 0.523; Table 2). As the above stated variables had no significant effect we followed the general accepted procedure to omit them and carry on analysing the main data.

Table 2 Generalized linear model for the control observations, showing the influence of several variables on aggressive, approach, retreat behaviour and total social interactions

	Aggression		Approach		Retreat		Total interaction	
	t-value	p-value	t-value	p-value	t-value	p-value	t-value	p-value
Log (enclosure size)	-2.527	**0.015**	1.505	0.138	-2.613	**0.012**	-0.643	0.523
Type of paddock	1.135	0.262	-1.506	0.138	1.115	0.270	0.291	0.772
Group	0.591	0.557	-0.185	0.854	1.219	0.228	1.048	0.300
Newcomer	-0.766	0.447	-0.386	0.701	-0.737	0.464	-1.088	0.282

Effect of enclosure size on social interactions

In Table 3 the mean number of interactions per hour and individual for the different control observations is shown. When comparing the different control observations (i.e.: pre-control, first-control and second-control), we found no differences in the amount of aggressive, approach and retreat behaviour (Friedman test; N = 21; all P > 0.05). The number of all behaviours was slightly higher in the first-control observation, but this was not significant. We additionally compared the observations with all four control observations conducted on the same enclosure size, and again found no significant differences of the amount of aggressive, approach and retreat behaviour (Friedman test; N = 5; all P > 0.05).

This justifies a combined analysis of all control observations for the effect of enclosure size on the social interactions.

Table 3 Amounts of aggressions, approaches and retreats for the different control observations. The mean number of interactions per hour and individual is used for each group

Type of observation	N	Amount of aggressions per hour and individual			Amount of approaches per hour and individual			Amount of retreats per hour and individual		
		Min	Max	Mean ± SD	Min	Max	Mean ± SD	Min	Max	Mean ± SD
Pre-control	30	0.75	11.00	2.38 ± 2.03	0.25	7.17	2.59 ± 1.54	0.80	10.38	2.58 ± 1.95
First-control	24	0.50	11.00	3.15 ± 2.55	1.00	7.17	3.11 ± 1.52	1.13	10.38	3.49 ± 2.35
Second-control	21	0.50	7.08	2.26 ± 1.76	0.25	6.88	2.71 ± 1.57	0.83	6.29	2.55 ± 1.57
One-year-control	9	0.50	3.65	1.76 ± 1.12	0.56	4.25	2.14 ± 1.16	0.83	3.69	2.13 ± 1.30

A significant correlation was found between the logarithmic enclosure size and the amount of aggressive as well as retreat behaviour (Spearman rank correlation; n = 58; aggression: r = -0.353, P = 0.006, Fig. 1; retreat: r = -0.384, P = 0.003), but not between logarithmic enclosure size and approach behaviour or total social interactions (Spearman rank correlation; n = 58; approach: r = 0.012, P = 0.931, Fig. 1; interaction: r = -0.083, P = 0.537). The aggressive and retreat behaviour, but not the approaches and total interactions, decline with the increase of the logarithmic enclosure size.

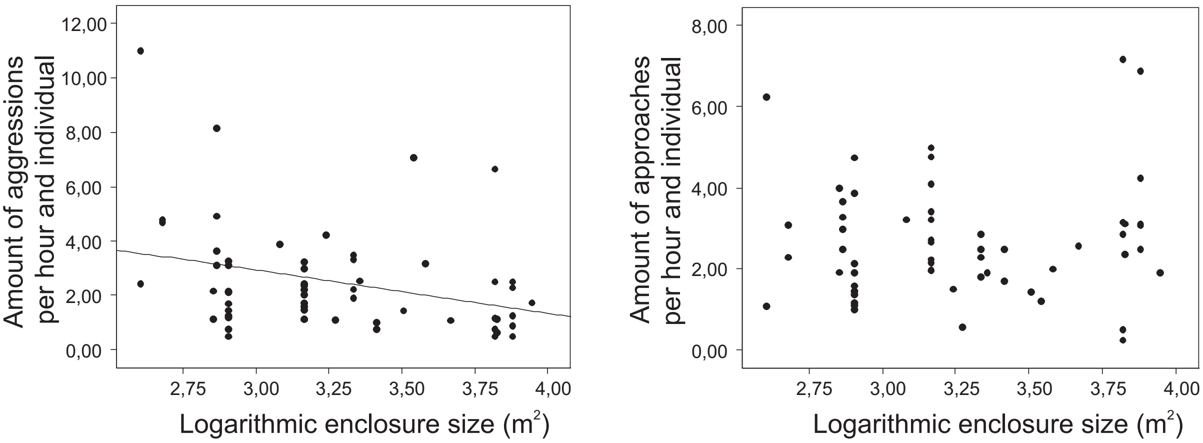

Fig. 1 Spearman Rank Correlation between increasing logarithmic enclosure size and aggressive as well as approach behaviour

Enclosure size per horse and mean aggression per hour

The transformed enclosure size data per horse show significant interrelations with the mean aggressions horses display per hour (LM; n = 45, P < 0.001). The curve features exponential characteristics and three sections can be distinguished (Fig. 2): a section with high aggressiveness (0 m^2 – 150 m^2 enclosure size per horse), a section with intermediate aggressiveness (150 m^2 – 300 m^2 enclosure size per horse), and a section with low aggressiveness (300 m^2 and more enclosure size per horse). Based on the exponential characteristics of the curve, small changes in enclosure sizes between 0 m^2 and 150 m^2 trigger large increases in the aggression level among the horses. In contrast, changes above 300 m^2 enclosure size per horse do not affect the aggressiveness strongly. The aggressions approach zero from an enclosure size of about 300 m^2 up to more.

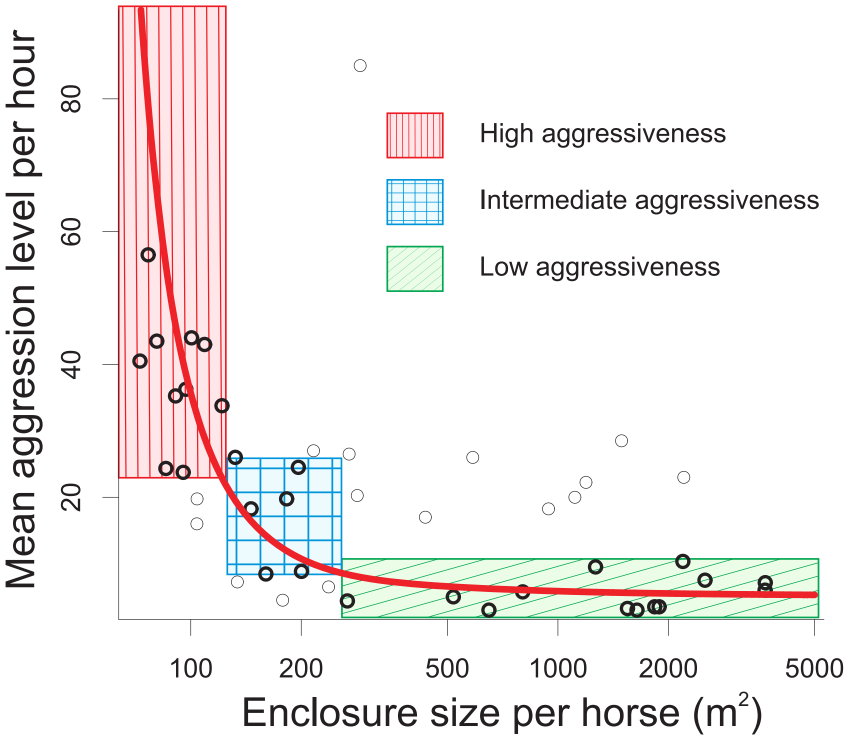

Fig. 2 Relationship between enclosure size per horse and mean aggression per hour. Note the logarithmic scale of the x-axis

Effect of introduction technique on social interactions

When comparing the different introduction techniques for the introduction, significant differences were found for the amount of aggressions, retreats and total interactions (Kruskal-Wallis; df = 2; aggression: $\chi^2 = 7.283$, P = 0.026; retreat: $\chi^2 = 5.978$, P = 0.050; interaction: $\chi^2 = 7.058$, P = 0.029), but not for approaches (Kruskal-Wallis; df = 2; approach: $\chi^2 = 4.113$, P = 0.128). The amount of behaviour is shown in Table 4.

Table 4 Amounts of aggressions, retreats, approaches and total interactions for the different introduction techniques. The mean number of interactions per hour and individual is used for each group

Amount of behaviour per hour and individual	Immediate introduction (n = 8)			Introduction after observation period (n = 14)			Introduction with integration horse (n = 6)		
	Min	Max	Mean ± SD	Min	Max	Mean ± SD	Min	Max	Mean ± SD
Aggression	1.25	8.50	4.31 ± 2.33	1.20	11.70	4.31 ± 2.73	1.05	2.56	1.93 ± 0.63
Approach	1.42	13.00	7.60 ± 3.96	0.50	11.85	5.56 ± 4.06	0.69	6.61	3.02 ± 2.25
Retreat	0.58	9.67	5.51 ± 3.19	1.18	12.00	4.36 ± 3.26	1.41	2.44	1.89 ± 0.38
Interaction	3.58	29.94	19.07 ± 7.95	4.38	28.00	15.22 ± 8.61	3.44	11.06	6.96 ± 2.92

Pairwise comparisons between the situations revealed significantly more aggressive behaviour when horses were integrated with technique 1 and 2 compared to technique 3 (Mann-Whitney U; 1 vs 2: P = 0.868; 1 vs 3: P = 0.013; 2 vs 3: P = 0.015; Fig. 3). The same pattern occurred for retreat behaviour, but the differences failed to be significant after adjusting the significance levels for multiple testing with Sequential Bonferroni Correction. Overall, horses showed higher amounts of total interactions using the introduction technique 1 and 2, compared to situation 3. The difference is significant after Sequential Bonferroni Correction for the comparison between situation 1 and 3 (Mann-Whitney U; 1 vs 2; P = 0.402; 1 vs 3: P = 0.013; 2 vs 3: P = 0.033; Fig. 3).

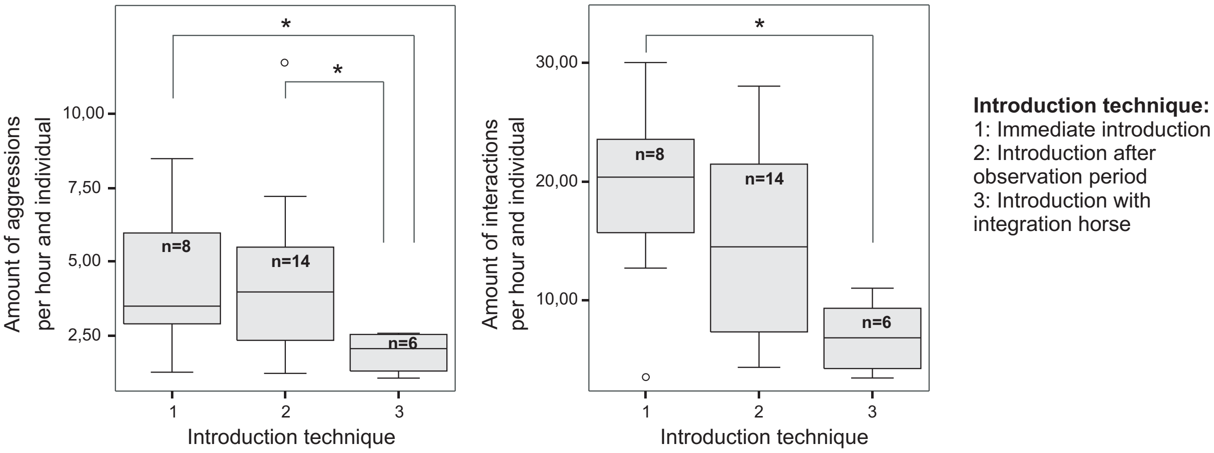

Fig. 3 Boxplot of the amounts of aggressive behaviour and total social interactions for the different introduction techniques

Discussion

In the present study we found a significant correlation between the enclosure size and the horses' aggressive as well as retreat behaviour. With increasing logarithmic enclosure size aggressions and retreats diminished, whereas approaches and total interactions were not affected.

When comparing the control observations that were conducted before the introduction, as well as six weeks, twelve weeks and one year afterwards, the level of aggressive, approach, retreat behaviour and total interactions among the horses of a group remained constant.

Furthermore, particular introduction techniques proved to differ significantly in their effect on the aggressions, retreats and total interactions between horses. In comparison to other introduction techniques, especially the introduction of horses together with a new group mate (so called introduction with "integration horse") led to significantly less increase of total interactions, and resulted in lower aggressiveness. We therefore suggest that introductions with group mates can reduce the injury risk.

During the first weeks the introduced horses quite often tried to stay close to the "integration horse", from which they received protection. In addition, the "integration horse" may have had a calming effect on the group's interactions, which prevented other group members' arousal. The introduction technique together with an integration horse and afterwards successive introduction has already been recommended by Ullstein (1996), Kurtz et al. (2000), Zeitler-Feicht (2001) and the FN (2005).

Furthermore, our data support the findings of Christensen et al. (2002a) that horses need full scale physical contact for the formation of associations. Analogous to their study we did not find a reduction of aggressions and total interactions when horses were kept in a neighbouring box over night or in a neighbouring paddock, in contrast to the situation when they had prior, complete physical contact with one horse. For following this line of reasoning further studies on introducing horses with box neighbours in comparison to "integration horses" are needed. In contrast to our prior hypotheses we could not find a significant difference in the horses' behaviour for introduction techniques 1 and 2. It did not matter whether the newcomer was introduced immediately or after several days of observing the new group from a neighbouring paddock, getting in sniffing contact through the fence and standing in neighbouring boxes to some group mates. Such horse introduction techniques, with a few days of acclimation, are favoured by most horse and stable owners and recommended by several other authors (e.g. Kurtz et al., 2000). The new horses are said to adopt the smell of the new stable and would therefore be chased less during the introduction. They would also be stressed less after getting used to the new surrounding.

There are several possible explanations why the present study cannot support an advantage of this introduction technique. Firstly, horses are highly social animals and may be perfectly able to deal with the situation of meeting other individuals and avoiding conflict (Feh, 2005). If not, they would not have survived. All the horses in our study were socially experienced, as they were kept in groups before. The importance of social experience is supported by Christensen et al. (2002a) findings that group stabled stallions had the tendency to use more mild agonistic interactions than stallions that had been deprived of physical contact. Additionally, habituation to frequent reorganisation of animal groups has been reported, which resulted in reduced agonistic interactions (Kondo et al., 1984; Veissier et al., 2001). Still, Raussi et al. (2005) demonstrate that repeated regrouping of heifers consistently induced agonistic interaction, and Hartmann et al. (2009) found a flexible and modified behaviour in subsequent pairwise horse encounters, depending on the individual horse.

Secondly, as domesticated animals are selected for reduced fear, increased sociability, and reduced anti-predator response (see for review: Jensen, 2006), horses are bred for compatibility and therefore reduced aggressive behaviour. Feh (1988) and Keiper and Receveur (1992) have shown that domestic horses are less aggressive than their non-domestic counterparts.

In contrast to the preceding explanations, the observation period could as well have increased rather than decreased the aggression level. After settling in the new surrounding some horses may have started to protect their new environment, and it seemed as if aggressions dammed up with every day. Separating fences inhibit normal confrontation and result in territorial reactions such as aggressive threats, as it has been reported for dogs frequently (e.g. Mertens and Unshelm, 1996). In fact, horses may not need extended habituation periods for observing and getting in contact with new group members. When they are socially experienced they are able to react to very subtle gesticular signs of other horses and avoid aggressive interaction. A pre-exposition of 5 min in neighbouring boxes has been shown to be sufficient for reducing "contact-aggression" (Hartmann et al., 2009). It may not matter whether horses can observe the group for three hours or several days. Short observation periods may be sufficient.

As stated above, the particular horse groups did not show different amounts of aggressive, approach and retreat behaviour, or total interactions during control observations. All behaviours were slightly, but insignificantly higher for the control observation right after the introduction, which can be attributed to the process of rebuilding the social organisation after the introduction of the new horse. But obviously the newcomers were already accepted in the group and first dominance ranks have been largely established after six weeks, which is supported by the findings of Christensen et al. (2002a). It has been reported that horses usually establish the dominance hierarchy within a few days (Waring, 1983; Tilson et al., 1988), which may be rearranged and finally stabilised in the following weeks.

In the present study a significant relationship between the displayed aggressions per hour and the enclosure size per horse was found. The aggressiveness among horses approaches zero when the enclosure size amounts to 300 m^2 per horse or more. It is noteworthy that this study reveals a recommendable space allowance that is four times as large as mentioned in the guidelines of the BMELV (2009) (i.e. a minimum value of 150 m^2 for two horses, and 40 m^2 for each additional horse). Swedish recommendations state 300 m^2 and Danish recommendations suggest 800 m^2 as individual paddock sizes (see for review: Jørgensen and Bøe, 2007). Nevertheless, we want to emphasize that the value 300 m^2 has to be dealt with caution, as the curve shows exponential characteristics. This clearly demonstrates that an even larger space allowance per horse is desirable in order to diminish aggression levels among horses. We also have to point at the complexity of aggressiveness among horses. Besides aspects of space allowances, individual factors, such as social experience, integration status, age and gender distribution of the group, as well as a multitude of management factors, such as enclosure shape, feeding regimes and others may affect the horses' aggressiveness and call for individual and flexible management strategies for each particular horse group. A minimum enclosure size of 300 m^2 per horse is not a guarantee for low aggression level, but if the enclosure size is smaller, it seems to be even more important to consider other factors of influence.

The fact that approach behaviour and total interactions do not correlate with the available space, in this study, is caused by some particular groups that still showed plenty approach behaviour in large enclosures. The connectedness of these groups may be remarkably well, which results in enhanced friendly contact among the horses. These cases can clearly be distinguished from those with high levels of both non-aggressive and aggressive interactions in small enclosures. The latter may be caused by more frequent transgression of personal space (e.g. Andersen, 1992).

Interestingly, we found correlations between aggression and logarithmic enclosure size only for areas below 10,000 square meters. In larger enclosures horses may simply maintain their personal space and avoid each other. Additionally, larger areas provide opportunities for splitting groups, which is common in Przewalski stallions (Zharkikh and Andersen, 2009), and may lead to reduced aggression among the group members.

It is worth noting that no serious injuries occurred during introductions, except some superficial cuts due to bites, or lesions at the legs. In two cases the introductions had to be terminated after some hours. However the first horse, a mare, received medications prior to the introduction and may have had an anomalous smell. The second horse, a gelding, had problems with a particular horse of the new group, and was successfully introduced into another group thereafter. Generally, all horses are capable of group housing irrespective of their age, breed, sex and type of use, and should be kept in groups in order to increase their welfare (BMELV, 2009). But in reality most horses are kept individually, because of their owner's fear of aggression based injuries. Especially the introduction of horses in new groups arouses severe concerns in horse owners.

Few studies evaluated the introduction/grouping of horses. But in these studies the groups were artificially formed, i.e. groups had only 2-year-old mares (Hartmann et al., 2009), 2-year-old stallions (Christensen et al., 2002a), 1-year-old mares/1-year-old geldings (Vervaecke et al., 2007) and mixed ages of only one sex (Jørgensen et al., 2009b). Evaluating such "matched" groups is helpful for testing specific hypotheses under specific situations and specific surroundings, but does not represent reality and provides only limited information on how to avoid injury risks for introductions at best practice. That is why the present study was conducted on horse groups from different stables, with varying group sizes, different age and sex composition.

The diversity of our study bears several advantages. First of all we had a large sample size. In contrast to the previously published case studies, where only individual animals could be investigated on a meaningful statistical basis, we were able to use group means for comparisons and draw general conclusions. The constraints and possible inaccuracies of using individual animals versus group means for statistics have been discussed by Phillips (1998, 2000). The second advantage is that individual and group variables have not been found to affect the outcome of this study in a multivariate analysis. In fact, we found stable, significant interrelationships between the horses' behaviour and enclosure size as well as introduction techniques despite of possibly confounding factors.

Conclusion

In this study on a variety of management types we found a stable, significant correlation between the available space for horses in group housing and their aggressiveness. Aggressions decreased with increasing logarithmic enclosure size.

We would suggest to introduce new horses in group management together with a new group mate, a so-called “integration horse”. This introduction technique leads to reduced aggressions among the horses. For future studies it would be interesting to investigate the influence of specific characteristics of the integration horse itself, e.g. its age, sex, rank, etc.

Acknowledgments

We thank Jürgen Heinze, Charlotte Hemelrijk and Ulrich Schnitzer for helpful suggestions, Knut Krueger and Klaus Stark for assistance with statistical analysis, and Henning Thies for language corrections. We are grateful to all stable masters and horse owners for allowing us to observe their horses. The study was supported by an Excellence grant of the Universität Bayern e.V. and an HWP II grant of the University of Regensburg.

CHAPTER 3

Cortisol release, social bonds and social rank during the introduction of horses into new groups

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Manuscript to be submitted to Hormones and Behavior

Abstract

As domestic horses are kept in so called “fate societies” they have to deal with frequent mixing. Several studies have evaluated and discussed the aggression level and injury risk during the introduction of horses into new groups, but nothing is known about the endocrine responses and thus if horses experience stress during introduction.

In this study we analysed the efficiency of three approved introduction techniques. We introduced 29 horses into 12 different groups, either immediately (technique I1), after an observation period of the new group for several days (technique I2) or together with an integration horse after several days of observation (technique I3). We focussed on the stress level of the newcomer horses, by measuring faecal GCMs and salivary immunoreactivity, as well as the effects on the social bonds and the rank positions of the horses.

Horses which were immediately introduced did not show elevated faecal GCMs. In contrast, horses which were introduced after an observation period had slightly, but insignificantly, elevated values 2 and 3 days after the introduction (74.80 ± 45.09 ng/g, 67.09 ± 28.63 ng/g). For horses introduced together with an integration horse faecal GCMs were significantly above baseline value already on the day of introduction (79.45 ± 17.98 ng/g) and 1 day thereafter (93.90 ± 27.37 ng/g). Salivary immunoreactivity was generally lower than baseline, only for one sample point it was significantly elevated (1.36 ± 0.29 ng/ml).

Newcomer horses establish a “permanent” rank position soon after the introduction, with nearly no change within the observation period of one year. The social bonds of the horses were unstable.

In general, horses are perfectly able to deal with conflicts when being introduced to new group members. The introduction event itself appears not to be as stressful as previously assumed. We rather suggest that horses experience stress when standing together with an integration horse on a separate paddock and are not able to integrate into the group immediately.

Keywords

Introduction technique, endocrine response, stress, integration horse, social cognition

Introduction

As domestic horses are kept in so called “fate societies” they have to deal with frequently changing groups. However studies on the topic are scarce in horses, in contrast to cattle, where selective introduction into established herds according to age, live weight or production is a frequent and common dairy-management practice that leads to numerous studies on aggression, lying time, body weight or milk yield (e.g. Knierim, 1999; Phillips and Rind, 2001). Some studies in horses have evaluated and discussed the aggression level and injury risk in group mixing, either with matched encounters of the individuals or via questionnaires for stable owners (e.g. Christensen et al., 2002a; Wolfrum, 2007; Hartmann et al., 2009; Jørgensen et al., 2009b). To our knowledge, only two studies focus on the endocrine responses and thus the stress level horses experience during introduction into new groups (Alexander and Irvine, 1998; Lisy, 2009), indicating experienced stress. In contrast, for example, transport stress in horses (Clark et al., 1993; Schmidt et al., 2010a, 2010b) or stress due to exercise is investigated in greater depth (Marc et al., 2000; Gorgasser et al., 2007). If the introduction of horses into new groups is a stressful event, or if they are able to cope with it, is not known as yet. Additionally, effects on the horses’ social bonding and rank positions by the introduction process have hardly been investigated.

The species under investigation in this study, the horse, is a highly social species. Under semi-natural conditions domestic horses gather in social groups, called harems, bands or families, like wild horses. Harem groups usually consist of one to five stallions, several mares and their offspring (Feist and McCullough, 1975; Berger, 1977; Linklater, 2000). Offspring disperses from the natal groups at the age of about three to five years. Mares affiliate to young stallions or other harems, whereas young stallions form bachelor bands. It is still under discussion whether several subgroups form a large structured social unit, called a “herd”, and show the same migration patterns within a common home range (Miller, 1979; Duncan, 1992; Feh, 2005) or whether a collection of subgroups should rather be termed a “population”, which has synchronous daily and seasonal pattern of movement in response to water, food or climate (Feist and McCullough, 1975; Berger, 1986; Linklater et al., 1999) and which has inter-band hierarchies at resource patches (Miller and Denniston, 1979; Franke Stevens, 1988). Harem and band stability varies considerably between groups and populations (Berger, 1986; Rubenstein 1986; Rutberg, 1990). Most harem groups have been reported to be relatively stable (Linklater, 2000), but some were described as unstable (Hoffmann, 1983), and mares were seen separated from their groups due to separation or dispersal (Linklater, 1998).The social system of horses can therefore be compared to fission-fusion models (Dyer, 2000) of other social mammals like apes (Dyer, 2000), elephants (Moss and Poole, 1983) and dolphins (Connor et al., 2000), in which animals frequently split and reunite again. Therefore, the encounter of conspecifics is a common situation in feral horses and a frequent source for conflict.

Domestic horses do not have the opportunity to choose their group affiliation themselves when kept in so called “fate societies” and group composition changes often. The mixing of animals is an event where aggression and fights occur, thus representing a conflict situation. As such conflict can reduce the benefits of group life and harm social relationships (Aureli et al., 2002) cognitive mechanisms for dealing with conflict situations and the resulting aggressions are likely to exist in social animals. For conflict resolution animals need to recognize their group members. Previous studies constituted evidence that horses are able of social cognition, e.g. being able to differentiate between known and unknown, as well as dominant and subordinate individuals (Krueger and Heinze, 2008), as well as social, sexual and individual recognition by means of visual (Grizmek, 1943; Proops et al., 2009) and olfactory perception (Hothersall et al., 2010; Krueger and Flauger, submitted).

If the introduction into new groups were stressful for horses, increased secretion of glucocorticoids and catecholamines for enhancing adaptive physiological responses (Sapolsky et al., 2000; Wingfield and Ramenofsky, 1999) would be expected. Glucocorticoid hormones can be measured in several body fluids or excreta, such as plasma, saliva, urine and faeces. Traditionally, glucocorticoid hormones have been analysed in plasma. In recent years, however, non-invasive techniques such as cortisol analysis in saliva and analysis of cortisol metabolites in faeces have gained increasing attention as they offer the advantage of avoiding stress reactions of the animals when repeatedly venipunctured. The cortisol in blood can be divided into a free fraction and a fraction bound to corticoid binding globulins (Matteri et al., 2000), free cortisol representing the biologically active form (Moons et al., 2002). Salivary cortisol mirrors the unbound, free fraction.

This study analysed the efficiency and stressfulness of three approved introduction techniques into new groups by measuring faecal GCMs and salivary immunoreactivity in newcomer horses. We started by asking how horses will deal with this specific conflict situation. We expected varying levels of experienced stress as a result of the different introduction techniques. Additionally, we hypothesised that the stress levels during the introduction event will correlate with the number of social bonds or the rank position the horses build up after the introduction.

Material and Methods

Animals

The introductions of 29 horses, aged between 2 and 18 years (Table 1), into ten different groups were observed between July 2006 and April 2009. The groups comprised three to 20 horses. Two groups consisted of mares and two groups of geldings only, whereas six groups had both mares and geldings. They were kept in different locations but under comparable conditions in social groups for several years, either constantly in open stables or in individual boxes during the night and in their groups on paddocks during the day. Depending on weather conditions they had access to pastures. Sleeping areas included a bedding of straw or wood shavings. For their daily feed the horses received hay twice a day and a compound feed once or twice a day. Additionally they fed on the grass in their pastures. They had free access to water. The horses were of different breed, including warmblood horses, quarter horses, trotters, Haflingers and ponies. Most of the horses were used for leisure riding, some for shows or events.

Experimental design

Introduction of horses

We distinguished three introduction techniques for the new horses which were chosen by the stable and/or horse owners themselves (Fig. 1):

A. Immediate introduction (technique I1):
   The newcomer horses were either immediately introduced, or they were standing for up to three hours on a neighbouring paddock, or were taken for a ride together with a horse of the new group before being introduced to the new group. They had either no, or very limited time (up to three hours) to observe the new group.
B. Introduction after observation period (technique I2):
   Newcomer horses were allowed to stay on a neighbouring paddock to the new group for several days (1-7 days). They were able to observe and establish limited sniffing- and tactile contact to new group members.

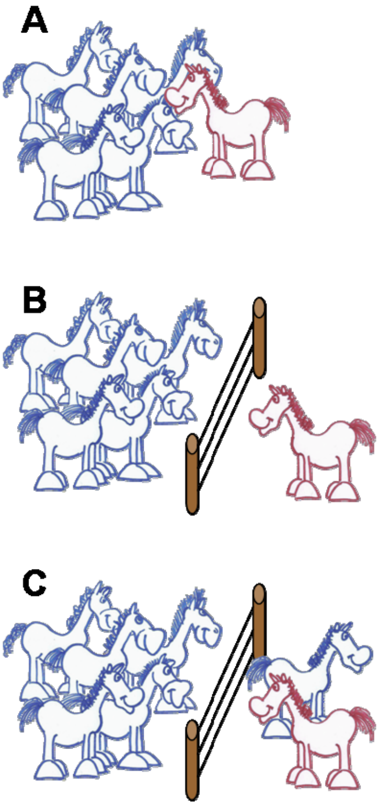


Fig. 1 Sketch of the three applied introduction techniques. A) Immediate introduction, B) Introduction after observation period, C) Introduction with “integration horse”. Drawings: Florian Kolbinger

C. Introduction with "integration horse" (technique I3):

Newcomer horses were allowed to stay on a neighbouring paddock to the new group for several days (1-7 days) accompanied by a new group mate. They were able to observe and establish limited sniffing- and tactile contact to all the new group members, and associate to one particular group mate. Afterwards, they were introduced together with this particular horse.

Behaviour observations and sample points

The groups were observed five times in total:

1. "pre-control": before introduction of the new horse, duration 4 hours
2. "introduction": duration 2 hours
3. "first-control": six to ten weeks after introduction, duration 2 hours
4. "second-control": twelve to 20 weeks after introduction, duration 2 hours
5. "one-year-control": one year after introduction, duration 4 hours

It was impossible to conduct all the control observations for each group, because some newcomers left the groups before all observations had been performed. In other groups two newcomers were introduced within one year. In these cases the second-control observation counts as pre-control for the second introduction.

Sampling started at a defined point in the control observations, when all horses of the group were present. For the introductions the recording began immediately when the newcomer horse had first full physical contact with the group members on the same paddock. It was led to the group by either the horse or the stable owner. Continuous all-occurrence sampling (Altmann, 1974) was used, recording approach, bite, threat to bite, kick, threat to kick, chase and retreat as social behaviours (modified after Feist and McCullough, 1976; McDonnell and Haviland, 1995; McDonnell, 2003).

Saliva and faecal samples were taken once during every control observation. Some horse owners agreed on taking samples only in their presence. As this could not be arranged for all control observations, certain samples are missing. On the day of introduction, saliva was sampled shortly before the grouping of the horses, and half an hour as well as two hours afterwards. Faecal samples were collected on the day of introduction, and one, two and three days after it.

Table 1 Information on newcomer horses

Introduction technique	Age (years, mean ± SD)	Mares	Geldings	Stallions
Immediate	12.8 ± 3.3	1	7	1
Observation period	10.9 ± 6.3	9	5	-
Integration horse	8.9 ± 6.2	3	3	-

Parameters measured

Faecal glucocorticoid metabolites

Faecal samples were collected with one-way gloves, stored in glasses and kept on ice until freezing. The time until freezing did not take longer than 4 hours. For processing faecal samples we extracted horse faeces as described for faecal GCM extraction in ruminants (Palme and Möstl, 1997). In brief, 0.5 g faeces plus 1 ml water and 4 ml methanol were vortexed for 30 minutes. The methanolic suspension was centrifuged, a small part of the supernatant diluted in assay buffer and directly analysed by an enzyme immunoassay directed against 11-oxoaetiocholanolone-17-CMO coupled to bovine serum albumin. Interassay coefficients of variance for the assay were 13.3% and 15.7% (n = 9). Values represent percentage variance for high and low quality controls. The enzyme immunoassay was validated for equine faecal glucocorticoid metabolites with an ACTH Challenge Test (Flauger et al., 2010).

Salivary cortisol immunoreactivity

Saliva samples were taken with an additionally perforated calve sucker in which absorbent gauze (Artiflex soft, 100% polyester) was placed. To standardise the saliva quantity and quality, all horses were given two pieces of sugar before chewing on the sucker for 2-3 min. Immediately after chewing the gauze was taken out of the sucker, put into a freezer bag and kept on ice until it was stored at -20°C. The time until freezing did not take longer than 4 hours. In the lab, after thawing, the gauze was put in a glass tube with a magnetic separator on the bottom. The magnetic separator maintained a cavity between the gauze and the glass tube's bottom for the saliva drainage during centrifugation. After a first centrifugation of 4 min at 1000 g the saliva was separated from the gauze, and the gauze and the magnetic separator were removed. The remaining saliva was again centrifuged for 4 min at 3000 g for separating impureness from the saliva. Thereafter, the supernatant was pipetted in a new tube and again frozen until analysis.

Cortisol immunoreactivity was analysed by a direct enzyme immunoassay without extraction (Palme and Möstl, 1996). The antiserum shows cross-reactivity with cortisone and several corticosterone metabolites. Thus values obtained have to be interpreted as cortisol immunoreactivity (IR). Interassay coefficients of variance for the assay were 7.8% and 20.9% (n = 8) for the EIA measuring blood cortisol. Values represent percentage variance for high and low quality controls.

Social bond and Average Dominance Index

To determine the strength of social relationships, grooming is generally considered a meaningful measure in nonhuman primates (Cords, 1997). However, as grooming does not occur frequently in horses and therefore does not provide a sufficient data set, we calculated a social bond index from approach incidences. Mutual approaches may represent friendly interaction and the desire for closeness to preferred animals. For analysing social bonds, mutual approaches generate an equivalent data frame than those provided by proximity analysis in primates (e.g. Hashimoto et al., 1996; Langergraber et al., 2009) or nearest neighbour analysis in horses (VanDierendonck et al., 1995; Christensen et al., 2002a; Heitor and Vicente, 2010). In order to adjust to diverse group sizes and observation periods, the summed mutual approaches per observation were divided by the number of horses in a group minus one (N – 1) and the time observed. For our analysis we then calculated the social bond index for the introduced horse (sb_intro) and the mean social bond index for the whole group without the introduced horse (sb_group).

Agonistic encounters, such as approach, bite, threat to bite, kick, threat to kick, chase and retreat were observed in the field in order to calculate the dominance relationships among the horses with a modified Average Dominance Index (ADI) method. The ADI is calculated as follows: The dominance index per pair of individuals, w_{ij} is the number of times an individual won against or attacked a certain opponent divided by the total number of agonistic interactions in which the pair was involved with each other, thus $w_{ij} = x_{ij} / (x_{ij} + x_{ji})$. If a pair of individuals was not involved in agonistic interactions with each other, it was excluded from the analysis. The average dominance index of an individual is the average of all its dominance indices with all its interaction partners, thus $1/N\ \Sigma_j\ w_{ij}$. The obtained values are relative ones between 0 and 1, with a higher value indicating a higher dominance in the group (Hemelrijk et al., 2005).

Statistical analysis

Data were analysed with the software package SPSS 17.0 for Windows and the R-project statistical environment (2009). All statistical tests were two-tailed, and alpha was set at 0.05. All data were normally distributed (Kolmogorov-Smirnov test). Faecal GCMs and salivary immunoreactivity were analysed by a One-sample T-test against a reference baseline value. The baseline values originate from a previous validation study in horses and are 50 ng/g for faecal GCMs (Flauger et al., 2010) and 0.82 ng/ml for salivary immunoreactivity (Flauger et al., in preparation), respectively. Differences between the introduction techniques were analysed with an ANOVA. For analysing correlations between control observations as well as correlations between faecal GCMs, salivary immunoreactivity, social bonds and ADI, Pearson Correlation tests were applied.

Results

Faecal glucocorticoid metabolites

Mean GCMs of horses introduced with technique I1 were not significantly higher than baseline values for the different time points of the observations. At the one year control the value was even below baseline (I1; One-year-control: N = 2, 33.57 ± 0.58 ng/g, P = 0.016), however, this may be an artefact due to the low sample size of two horses which were available for this point in time.

For technique I2 higher glucocorticoid metabolites were analysed on day 2 and 3 after the introduction, although the values only tended to be significant (I2: 2 days after introduction: N = 12, 74.80 ± 45.09 ng/g, P = 0.083; 3 days after introduction: N = 12, 67.09 ± 28.63 ng/g, P = 0.063; Fig. 2). In the second-control observation the horses had significantly low levels of GCMs (I2: Second-control: N = 8, 34.70 ± 12.65 ng/g, P = 0.011).

With technique I3 significantly higher mean cortisol metabolites than baseline values could be detected on the day of the introduction and 1 day thereafter (I3; Introduction: N = 5, 79.45 ± 17.98 ng/g, P = 0.022; 1 day after introduction: N = 5, 93.90 ± 27.37 ng/g, P = 0.023; Fig. 2). For day 3 after the introduction faecal cortisol metabolites were still above baseline level even though they were not significant (I3; N = 5, 83.05 ± 30.80 ng/g, P = 0.074; Fig. 2).

Mean GCM values are listed in Table 2. For better visualisation see also Fig. 2.

Table 2 Faecal GCM concentrations (ng/g) of the newcomer horses (Mean ± SD) for the different introduction techniques. Values are compared with a One-sample T-test against a reference baseline value of 50 ng/g. Values that differ significantly from baseline are indicated in bold digits, values showing the tendency to differ are underlined

	Immediate				Observation period				Integration horse			
	N	Mean (ng/g)	SD	P	N	Mean (ng/g)	SD	P	N	Mean (ng/g)	SD	P
Introduction	4	42.01	11.99	0.275	10	48.04	15.06	0.690	**5**	**79.45**	**17.98**	**0.022**
1 day after introduction	7	69.77	44.62	0.286	12	57.89	33.75	0.433	**5**	**93.90**	**27.37**	**0.023**
2 days after introduction	5	49.51	30.91	0.973	12	74.80	45.09	0.083	5	61.72	29.29	0.422
3 days after introduction	6	52.69	20.04	0.756	12	67.09	28.63	0.063	5	83.05	30.80	0.074
First-control	4	73.40	31.48	0.234	8	39.84	27.54	0.331	3	66.73	31.40	0.453
Second-control	2	66.11	39.12	0.664	**8**	**34.70**	**12.65**	**0.011**	4	54.20	7.39	0.338
One-year-control	**2**	**33.57**	**0.58**	**0.016**	3	36.50	10.87	0.164	2	63.39	26.02	0.600

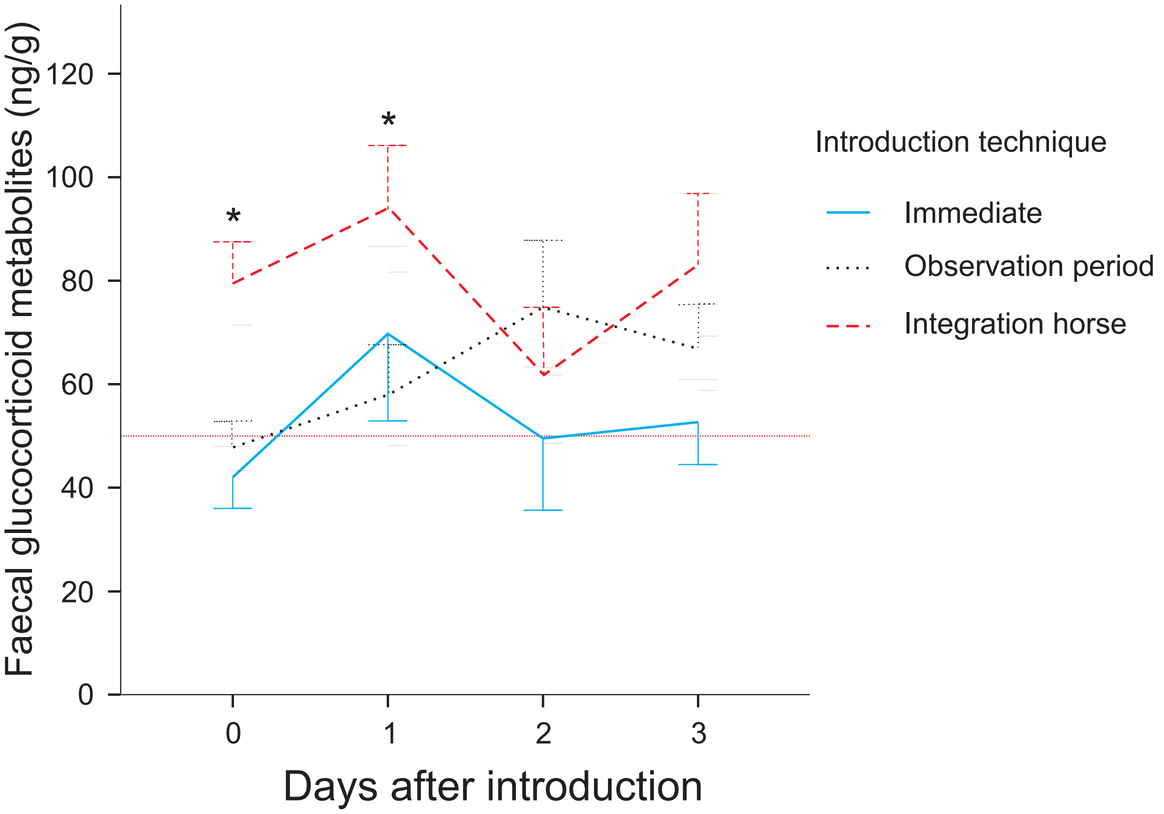


Fig. 2 Faecal GCM concentrations (ng/g) of the newcomer horse on the day of introduction as well as 1, 2 and 3 days afterwards (Mean ± SEM). Error bars are presented either above or below mean values for better visualisation. Values differing significantly from baseline are displayed with asterisk ($P < 0.05$). Dashed line parallel to x-axis indicates baseline value

Salivary cortisol immunoreactivity

For the analysis of salivary cortisol immunoreactivity no clear pattern could be observed.

Horses introduced with technique I1 had significantly low values 2 hours after the introduction as well as in the one-year-control (I1: 2 h after introduction: N = 9, 0.45 ± 0.35 ng/ml, P = 0.013; One-year-control: N = 4, 0.43 ± 0.19 ng/ml, P = 0.027; Fig. 3).

For technique I2, samples from 2 hours after the integration, from the first-control, from the second-control as well as from the one-year-control yielded values significantly below baseline (I2: 2 hours after introduction: N = 14, 0.56 ± 0.33 ng/ml; First-control: N = 11, 0.56 ± 0.29 ng/ml, Second-control: N = 11, 0.45 ± 0.43 ng/ml; One-year-control: N = 4, 0.21 ± 0.15 ng/mg; all $p < 0.05$; Fig. 3).

Horses of technique I3 had significantly low cortisol immunoreactivity in the first-control (I3: First-control: N = 4, 0.44 ± 0.23 ng/ml, P = 0.045). In this group, shortly before the introduction, we measured the only case of significantly elevated mean amounts of cortisol immunoreactivity compared to baseline value (I3: Before introduction: N = 6, 1.36 ± 0.29 ng/ml, P = 0.006; Fig. 3).

Mean salivary cortisol immunoreactivity is depicted in Table 3. For visualisation see also Fig. 3.

Table 3 Salivary immunoreacitvity (ng/ml) of the newcomer horses (Mean ± SD) for the different introduction techniques. Values are compared with a One-sample T-test against a reference baseline value of 0.82 ng/ml. Values that differ significantly from baseline are indicated in bold digits

	Immediate				Observation period				Integration horse			
	N	Mean (ng/ml)	SD	P	N	Mean (ng/ml)	SD	P	N	Mean (ng/ml)	SD	P
Before Introduction	9	1.01	0.76	0.465	13	0.65	0.59	0.306	**6**	**1.36**	**0.29**	**0.006**
½ h after introduction	9	0.93	0.88	0.710	14	0.67	0.70	0.438	6	0.91	0.41	0.622
2 h after introduction	**9**	**0.45**	**0.35**	**0.013**	**14**	**0.56**	**0.33**	**0.010**	6	0.64	0.29	0.178
First control	4	1.15	1.32	0.653	**11**	**0.56**	**0.29**	**0.013**	**4**	**0.44**	**0.23**	**0.045**
Second control	2	0.77	0.47	0.905	**11**	**0.45**	**0.43**	**0.018**	4	0.65	0.33	0.372
One year control	**4**	**0.43**	**0.19**	**0.027**	**4**	**0.21**	**0.15**	**0.004**	2	0.41	0.25	0.258

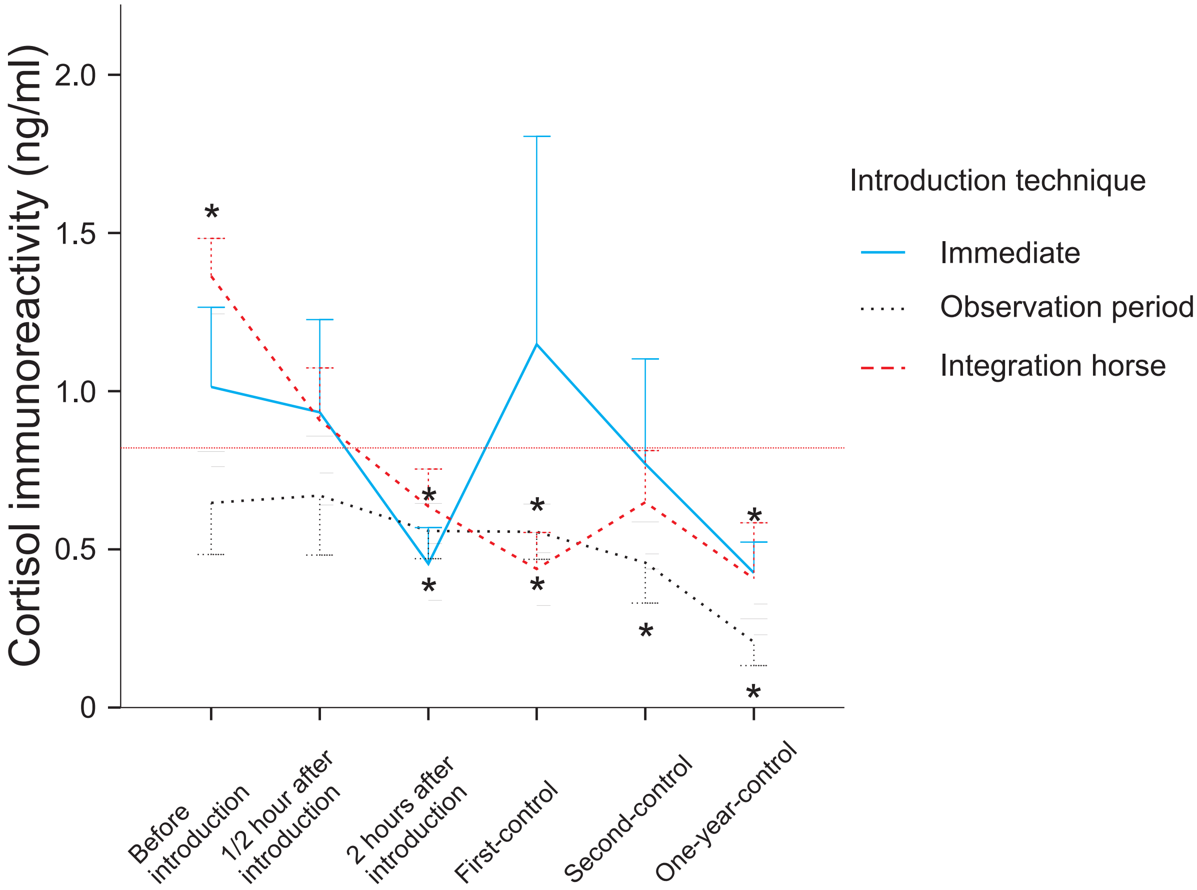


Fig. 3 Salivary glucocorticoid immunoreactivity (ng/ml) of the newcomer horse during and after the introduction (Mean ± SEM). Error bars are presented either above or below mean values for better visualisation. Values differing significantly from baseline are displayed with asterisk (P < 0.05). Dashed line parallel to x-axis indicates baseline value

Social bond index

In general, the introduction techniques had no significant influence on the social bonds of the introduced horses or the mean social bonds among the group members. They had no significant influence on the social bonds of the first-control observation after the introduction (ANOVA; df = 2; N = 25; sb_intro: P > 0.05; sb_group: P > 0.05). Additionally, there was no significant difference of sb_group before the introduction and in the first-control within the different introduction techniques (Paired T-test; Pre-control vs First-control; Immediate: N = 6; Observation period: N = 12, Integration horse: N = 5; all p > 0.05). Therefore we continued with analysing social bonds for the combined three introduction techniques.

The social bonds of newcomer horses do not depend on general bonding situations within their particular groups, because the mean social bonds of the group (sb_group) did not correlate with the individual social bonds of the introduced horses (sb_intro) (Pearson correlation; First-control: N = 25; Second-control: N = 22; One-year-control: N = 9; all p > 0.05).

We did not find a correlation between salivary immunoreactivity and sb_intro nor between faecal glucocorticoid metabolites and sb_intro at different time points (all P > 0.05).

For sb_group there is a tendency for correlations between the first-control, the second-control, and the one-year-control (Person Correlation; First-control/Second-control: N = 22, r = 0.411; P = 0.058; Second-control/One-year-control: N = 9, r = 0.624, P = 0.073), but not for sb_intro (Pearson correlation; First-control/Second control: N = 22, r = 0.197, P = 0.380; Second-control/One-year-control: N = 9, r = 0.445, P = 0.230; Fig. 4). During all the observations the social bonds of the newcomer horses neither augment nor decline, and show no clear picture (Paired T-test; First-control vs Second-control: N = 22, P = 0.263; Second-control vs One-year-control: N = 9, P = 0.515).

Average Dominance Index

We did not find a correlation between salivary immunoreactivity and the ADI nor between faecal glucocorticoid metabolites and the ADI of the introduced horses at different time points (all P > 0.05).

The ADIs of the introduced horses significantly correlated from the first-control, to the second-control, and the one-year-control (Pearson Correlation; First-control/Second-control: N = 22, r = 0.888; P < 0.001; Second-control/One-year-control: N = 9, r = 0.813; P = 0.008; Fig. 4).

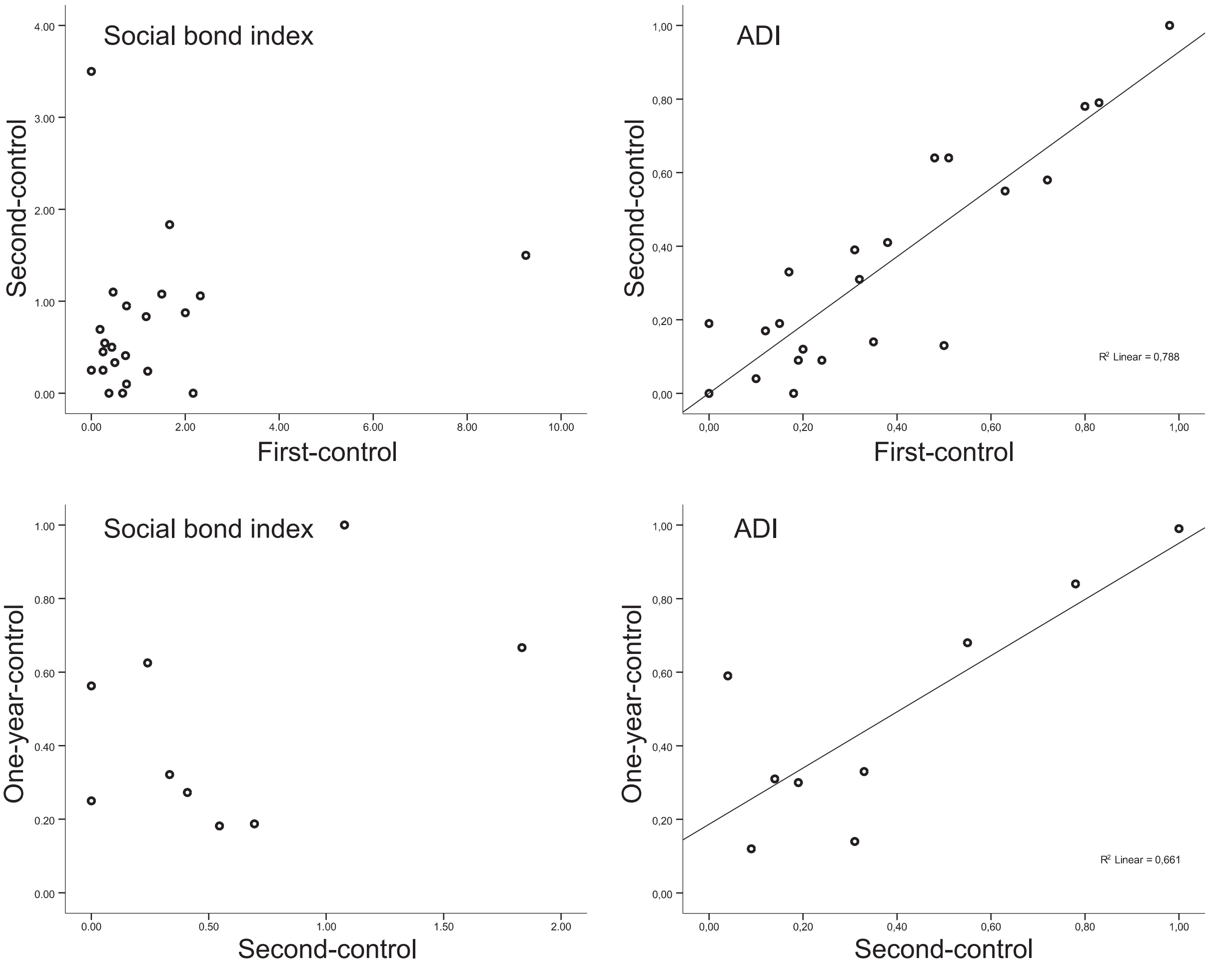


Fig. 4 Relationships between the social bond indices as well as between Average Dominance Indices of the newcomer horses. Contrary to the social bonds (all P > 0.05) the ADIs show significant correlations (Pearson Correlation; First-control/Second-control: N = 22, r = 0.888; P < 0.001; Second-control/One-year-control: N = 9, r = 0.813; P = 0.008)

Discussion

In this study faecal glucocorticoid metabolites and salivary immunoreactivity were analysed in horses during and after the introduction into a new group. The aim of the experiment was to investigate if horses experience different levels of stress when three approved introduction techniques are applied at best practice. The goal was furthermore to examine how they are able to deal with this specific conflict situation. In addition, their level of social bonds and rank position were observed over time.

Faecal glucocorticoid metabolites

Horses had higher faecal GCMs and thus, seemed to be more stressed, when they were introduced together with an integration horse. This result is very interesting as the "integration horse technique" together with successive introduction has been recommended to be most beneficial for the horses' welfare by Ullstein (1996), Kurtz et al. (2000), Zeitler-Feicht (2001) and the FN (2005). We supported these findings in a previous manuscript concerning the level of exchanged interactions among horses. We showed that the introduction of horses together with a new group mate led to a significantly lower increase of total interactions, and resulted in less aggression (Flauger and Krueger, submitted). It may be possible that exactly this low level of stress leads to a positive effect, i.e. that the horses are less aggressive than under normal conditions. But to our knowledge, previous studies did not examine the physiological state of the introduced horses themselves during different introduction techniques.

As horses excrete cortisol metabolites in faeces with a delay of about 24 hours compared to plasma (Palme et al., 1996; Möstl et al., 1999) the elevated levels on the day of the introduction and 1 day later indicate that the horses already experienced stress before the introduction event, i.e. when they were standing together on the same paddock with the integration horse. As faecal GCMs were still higher than baseline level on day 3, the introduction itself also seemed to be stressful.

Horses that were introduced after several days of observation had higher level of GCMs on day 2 and 3 after the introduction, although not significantly. This indicates that some horses experienced low stress levels on day 1 and 2 after the introduction, maybe also on the day of the introduction itself, as the retention time of some horses may be longer than 24 hours. But the introduction event itself appears not to be highly stressful. If the introduction event were very stressful, we would expect elevated GCM levels on day 1 after the introduction and the results should be significant. However, this is not the case in the present study.

In this line of reasoning the values of technique I1 with immediate introduction fit well. Here no values were elevated and the horses appeared not to be stressed at all.

This leads us to the conclusion that horses are not stressed by the introduction into a new group, but rather by the separation from the group on a neighbouring paddock, even when they are accompanied by an integration horse. It is possible that space limitations at small paddocks additionally cause stress, especially when the new horse can not avoid the contact with the integration horse. Alternatively, separation stress from the group may be worse than being able to stand within the group and having the option to self-regulate the distance to other horses, if enclosures are large enough.

The highest mean value of 93.90 ng/g of the introduction with integration horses lies in the range of values measured in other horse studies on experienced stress. After ACTH administration the highest mean values reached 105.09 ng/g (Flauger et al., 2010). The highest values measured 1 day after road transport were 136.2 ng/g for an 8 hour transport and 110.8 ng/g for a 3.5 hour transport (Schmidt et al., 2010b). In a recent study where two horses were introduced together into a new group, after having spent several days in an integration box, these horses only showed little enhancement of faecal GCMs to about 60 ng/g (Lisy, 2009).

Our results are in line with other studies in social species that show increased plasma corticosteroid concentrations during social stress, i.e. when the social hierarchy is disrupted or unstable (baboons: Sapolsky, 1990; pigs: Dalin, 1993; marmosets: Johnson, 1996; guinea pigs: Stefanski and Hendrichs, 1996; horses: Alexander and Irvine, 1998).

Salivary cortisol immunoreactivity

The salivary immunoreactivity data are less clear than the data on faecal GCMs. In our study only mean values of a single sample point (technique I3, sample before the introduction) showed significantly elevated levels of salivary immunoreactivity. The other samples even displayed an overall tendency to be lower than baseline. This indicates that the horses did not experience short term stress, neither during the introduction event itself nor during the control observations afterwards. It may be argued that our reference value was set too high to detect stress. As mentioned in the method section we set the value to 0.82 ng/ml which corresponds to the baseline of an ACTH Challenge Test in horses (Flauger et al., in preparation). This value is perfectly in line with studies by Schmidt et al. (2010a, 2010b) where horses had average cortisol immunoreactivity of 0.38 ng/ml or lower than 1 ng/ml before transport, respectively. Additionally, the highest mean value of 1.36 ng/ml of the introduction is far below the highest values of the ACTH stimulation (7.53 ng/ml) or of transport stress (3.10 ng/ml, 6.5 ng/ml). These findings support our suggestion that the introduction event is not as stressful for the horses as previously assumed. In addition, we could not detect short term stress during the control observations.

Several alternative explanations are at hand. First, saliva may not be the best tool to detect stress in animals in different situations. Although non-invasive techniques such as cortisol analysis in saliva have gained increasing attention in recent years, there are still discussions about its applicability to measure stress. In horses its acceptability ranges from good results after semen collection and transport (Lebelt et al., 1996; Schmidt et al., 2010a; 2010b) to mixed and not suitable results in treadmill exercise and horses showing stereotypical behaviour (Elsaesser et al., 2001; McGreevy and Pell, 1998). Disadvantage of saliva measurements are that large individual variations can occur (Dreschel and Granger, 2009) and that saliva is influenced by circadian rhythm and episodic fluctuations as in blood cortisol (Irvine and Alexander, 1994). A second alternative explanation is that the horses did in fact experience stress during our study, but that it was compensated or influenced, for example by a raised metabolism or food intake. It is worth noting that the horses had an especially low value of salivary cortisol immunoreactivity 2 hours after the introduction, both for technique I1 technique I2. As the newcomer horses are chased and have to run a lot during the first two hours, high cortisol levels may be catabolized and thus be reduced during running. The remaining cortisol levels may be too low to detect stress. Other possibilities are the effect of food contamination in saliva (e.g. humans: Granger et al., 2007; dogs: Dreschel and Granger, 2009). Different stimulants may activate different salivary glands or the saliva rate may influence the detectable cortisol immunoreactivity.

Social Bond Index and Average Dominance Index

The introduction technique did not have an impact on the social bonds of the groups in the first-control observation. In a direct comparison of the pre-control to the first-control none of the three introduction techniques led to a significant change of the social bonds of the groups, neither improvement nor impairment. It is possible that there was an immediate effect for the first few days or weeks, comparable to what has been recorded in cows by Neisen et al. (2009). The authors investigated the dyadic synchronicity and distances of cows as parameter for social relationships and reported that cows were slightly more affected in the first week after the introduction of pairs of heifers than after the introduction of single heifers. But as our first control observation was six to ten weeks after the introduction, this effect may have vanished already.

We did not find any correlations between faecal GCMs or salivary immunoreactivity and the social bonds of the introduced horse. Horses with better social bonds do not experience less or more stress and it is not possible to forecast that horses, which undergo no stress during the introduction event itself integrate better into the group and have better social bonds in the first-control observation. The same is valid for the average dominance index; we did not find any correlation between the stress level and the ADI of the introduced horses. Additionally, it is impossible to make predictions for the rank positions the horse will have later on by means of its stress level during introduction, e.g. less stressed horses won’t necessarily hold a higher rank position later on.

The social bond index can be considered as a parameter to determine the strength of the relationships among the horses of a group. Obviously, the newcomer horses do not engage in stable exchange of affiliative interactions with their group members. Some horses gained social bonds from the first- to the second-control observation, which indicates a better position in the social network and a better integration into the group structure, but lose social bonds afterwards.

It is worth noting that, contrary to the social bond index of the newcomer horses, the ADIs of the introduced horses remain stable in the course of our control observations. While their affiliative relationships were unstable, they had already established their “permanent” rank position a short time after the introduction, and kept it stable from the first- to the second- and one-year-control with no further changes. For the fast and stable realisation of rank positions horses need cognitive abilities such as learning and memory. They need to recognize individuals, track their social status and infer relationships among group members. It has been demonstrated that horses are able of discrimination and categorization learning (Hanggi, 1999), of generalisation (Dougherty and Lewis, 1991; Krueger, 2007), and that they have good long-term memory for categories and concepts: this altogether reflects high-order mental abilities (Hanggi and Ingersoll, 2009). The study by Krueger and Heinze (2008) demonstrated that horses are able of differentiating between known and unknown, as well as dominant and subordinate individuals, and that they know their social rank within their social group. The results of our introduction study indicate that horses even infer the hierarchies of foreign groups very fast and that they rate their own social strength relative to the observed conspecifics. As horses frequently change their groups under natural conditions these abilities may be very useful to avoid conflict situations.

Conclusions

Our findings indicate that horses experienced more stress when they were introduced together with an integration horse than when they were introduced immediately or after observing the group. Thus, it seems that not the introduction event itself is responsible for the elevated faecal GCMs, but the period when the horse stands together with the integration horse on a separate paddock. Measuring faecal GCMs that increase only in response to marked or prolonged cortisol release, proved to be a good tool for detecting stress, in contrast to measuring salivary cortisol immunoreactivity. Moreover, horses seem to be perfectly able to deal with the conflict situation of being introduced to new group members, as they found their “permanent” rank position within a short time after the introduction.

Acknowledgments

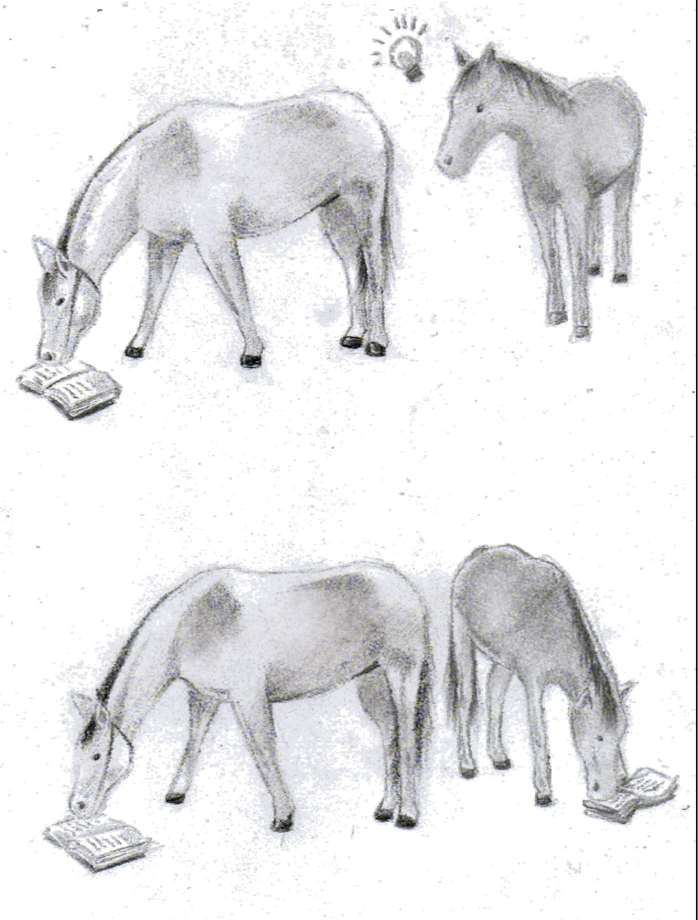
We thank Jürgen Heinze, Erich Möstl and Charlotte Hemelrijk for helpful suggestions, Alexandra Kuchar and Andreas Trindl for technical assistance in the laboratory, and Doris Pfaffinger for language corrections. Special thanks to Erich Möstl for making it possible to analyse the samples at the University of Veterinary Medicine, Vienna. We are grateful to all stable masters and horse owners for allowing us to observe and taking samples of their horses. The study was supported by an Excellence grant of the Universität Bayern e.V. and an HWP II grant of the University of Regensburg.

CHAPTER 4

Commentary

Social learning in horses from a novel perspective

Konstanze Krueger[1]* and Birgit Flauger[1]



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Published in Behavioural Processes (2007) 76, 37-39

Commentary

Horses were domesticated between 2500 and 5000 (Clutton-Brock, 1981) years ago, ever since humans tried to train them for fast and correct responses to human signals to perform special tasks. In this sense it appears to be important to gain more insight in equine learning abilities. Murphy and Arkins (2007) succeeded in writing a comprehensive and very useful review on equine learning behaviour. Animal learning theories in general used to focus on explaining the mechanisms for individual learning. It is only for the last two decades that the social aspects and cognitive abilities of animals have been incorporated into learning research. It is not surprising that most equine learning research concentrates on the mechanisms of individual learning as well, although it is puzzling that there is very little knowledge about horses social learning and their cognitive abilities (Nicol, 2002; Murphy and Arkins, 2007), since horses have been companions to humans for such a long time. May be this can be explained by the fact that it is still under discussion whether animal learning theory is applicable to social learning, or whether different mechanisms are responsible for social and asocial learning (Heyes, 1994; Nicol, 1996).

Murphy and Arkins (2007) as well as Nicol (2002) discuss several studies (Baer et al., 1983; Baker and Crawford, 1986; Clarke et al., 1996; Lindberg et al., 1999) which have been designed to evaluate horses' social learning abilities and which those publications did not demonstrate. These tests and more recent approaches to the subject all used modifications of the same methodology (McLean, 2004).

Nevertheless, the tests of Lindberg et al. (1999) differ fundamentally from those of Baer et al. (1983), Baker and Crawford (1986) and Clarke et al. (1996). The latter tested for a discrimination task between two defined food-buckets, whereas Lindberg et al. (1999) set out to test a goal directed imitation of operating a test apparatus. Therefore, we will not proceed to discuss Lindberg's research any further.

In the research of Baer et al. (1983), Baker and Crawford (1986) and Clarke et al. (1996) food was baited in one of two differential coloured buckets of a learning apparatus from which a demonstrator was trained to discriminate between two buckets. An observer horse watched the demonstrator that only ate from a bucket of one colour randomly placed either on the left or on the right side of the test apparatus. Even though, the authors did not believe that they had demonstrated social learning in horses, a closer analysis of the discrepancies between their experimental set-up and the outcome of their research hint at some alternative explanations.

Baer et al. (1983) found no signs of observational learning in their test series, because observers showed only slightly fewer errors than the control group. Still the data of the first day, which is most interesting in terms of evaluating observational learning effects, was eliminated from the analyses, since the authors felt that the data was affected by extraneous stimuli. A response to the observation of the previous discrimination by the demonstrator was first tested after 24 h.

Baker and Crawford (1986) concluded from the outcome of their tests, that horses might "avoid" the feeding territory of strangers, in this case the demonstrator horse, even though the demonstrator was removed before the observer was tested (*p*-value "avoidance" = 0.01). On their first choice, the horses significantly went to the bucket from which the demonstrator did not feed before. They argued that horses may expect no food to be left in the bucket, where the demonstrator fed. Still they could not prove observational learning because observers and control horses had similar numbers of correct first choices.

This was also demonstrated in Clarke et al. (1996) as well as the tendency for observer horses to "avoid" the bucket the demonstrator had fed from (*p*-value "avoidance" = 0.07). In addition, a strongly significant effect of prior observation on latency to approach the goal area on the first trial could be shown. This suggests that horses learn "something" about the general location of food from the demonstrator's performance. Clarke et al. (1996) argued that local rather than stimulus enhancement mechanisms underlie any effect of observational learning in horses and those horses might be attracted to the general location where they have observed other horses feeding, but not to the same spot.

For a positive proof of social learning matching behaviour, behaviour like that of a demonstrator (Heyes, 1994), was expected. Matching behaviour is indeed the only widely recognized outcome of social learning. Little attention has been paid to the possibility that social learning may have a variety of effects, in addition to the production by the learner or observer of behaviour resembling that of an observed animal or demonstrator. But social learning can also result from socially mediated exposure to a negative relationship involving an aversive stimulus (an avoidance contingency) and thus result in novel non-matching behaviour. Overshadowing by dominance related previous experiences and by procedural feeding experiences could as well influence the outcome of the tests (Heyes, 1994).

As to the argument of Clarke et al. (1996), that horses might rather be affected by local enhancement than by stimulus enhancement, it could be argued that both buckets showed the same cues. The same food was baited in both buckets, Clarke et al. (1996) made sure that both buckets were marked with the same olfactory cues from previous feeding of the demonstrator and the buckets randomly changed positions. This rather hints to a stimulus enhancement mechanism, even more since is usually defined with reference to matching behaviour (Heyes, 1994). But the fact, that horses change their response to the buckets over subsequent trials, and stimulus enhancements should be persistent over all trials, seems to point to the fact that horses actually showed imitation, as it is common in social learning processes.

But what did they actually learn? Clarke et al. (1996) already argued that horses at least learned "something" and they acquired their knowledge socially. So, perhaps we could find a line by discussing social influences on the test horses and their previous feeding experiences.

Murphy and Arkins (2007) felt that the familiarisation process might not have been sufficient for a social learning task. They mention that observational learning may be influenced by a dominance hierarchy, whereby subjects might have more interest in, or possibly be more motivated by, the action of a "respected" conspecific. To solve the problem, they propose the importance of training a dominant type of animal for the demonstrator position. This, actually, is one of the weak points in all three publications (Baer et al., 1983; Baker and Crawford, 1986; Clarke et al., 1996) since there is no hint in either manuscript which kind of dominance status the demonstrator holds.

Murphy and Arkins (2007) propose that it might be important to consider the social background of the horses since research data demonstrate that socially reared and kept horses learn training tasks quicker than those housed in individual stalls (Rivera et al., 2002; Sondergaard and Ladewig, 2004). In addition, it has been demonstrated that socially reared rats had a tendency to imitate the behaviour they had observed, but rats raised in isolation performed the opposite behaviour to that observed, indicating a failure to use a conspecific as a reference point in the task (Reed et al., 1996). In chimpanzees Hare et al. (2000) proved that subordinate chimpanzees only chose food which was hidden behind a small barrier in a manner that the dominant animal could not see it. They even highlighted in a subsequent publication (Hare et al., 2001) that chimpanzees seem to know which particular animal watched the important event, since subordinates went for food when the observing dominant animal was exchanged for a non-observing dominant animal.

Unfortunately no information about how the horses were raised or socialised is given in the papers, with the exception of Baer's et al. (1983) report that their horses were group fed. On the other hand there is no information about whether the demonstrator was still visible to the observer horse after being removed from the test apparatus or not.

While taking advantage of the specific "following behaviour" that horses show towards humans in a riding arena, in a recent research series we investigated whether bystander horses adjust their response to an experimenter according to their own dominance relationship with the horse whose reaction to the experimenter they had observed before. We suggested that horses immediately followed the experimenter after previously watching a dominant horse doing so, but did not follow after observing a subordinate horse or a horse from another social group doing so (Krueger and Heinze, 2008). Additionally, in another test series (Krueger, unpublished data) in which we tried to find out whether horses would follow the gazes of their conspecifics horses paid more attention to dominant horses from their own social group than to subordinate horses or those belonging to other social groups. The social affiliation and the dominance hierarchy seemed to have major influence on the horses' motivation to pay attention to the gazes of conspecifics.

This information encouraged us to start a control trial concerning social aspects of the test-series by Baer et al. (1983), Baker and Crawford (1986) and Clarke et al. (1996). First preliminary data from this series (Krueger and Flauger, 2008) confirm that horses, when they have the opportunity to choose between two feeding sites, do not dare to feed from the same spot as another conspecific, no matter whether this animal is subordinate or dominant, when the conspecific is still feeding. Also they prefer to choose the bucket from which the dominant animal did not feed before when the dominant animal is still present but tied to a post. In contrast, they are unconcerned whether a subordinate animal is still present but tied to a post, because they tend to return to the same bucket no matter which bucket the subordinate had eaten from before. Finally, they stayed with the system of returning to the same feeding spot when the dominant animal was removed from sight. This behaviour is consistent with the observation of Devenport et al. (2005). They declared that after short time intervals horses prefer to return to the same foraging spot unless the spot is cropped or they realise that feed at another spot is plentiful and of a better quality. We could argue that in the case of Baker and Crawford (1986) and Clarke et al. (1996) the decision of the horses to change their normal feeding habits might be influenced by strong mechanisms. Since they changed their behaviour after observation, they must have learned "something" through observational learning. Obviously social effects on horses learning abilities do not always result in matching behaviour.

From this point of view we totally agree with Murphy and Arkins (2007) that the social affiliation and the dominance hierarchy is important in evaluating the reaction of horses to social learning tests. Concerning the test series of Baer et al. (1983), Baker and Crawford (1986) and Clarke et al. (1996) it would also be necessary to know whether the demonstrator was still visible to the observer horses and what kind of previous feeding experiences they were exposed to. Since horses constitute a highly social species much of their cognitive abilities might be connected to social experiences. By incorporating social aspects into learning trials it will be possible to gain insight into horses' social learning abilities. It has also been documented that it is possible to improve horse training tremendously by taking advantage of the horse's highly developed social behaviour (Rivera et al., 2002; Sighieri et al., 2003; Krueger, 2007). Horse training can be gentler to the horse, quicker and more effective by applying social training techniques. Nevertheless it should be taken into account that the effect of social learning could as well result in non-matching behaviour.

Acknowledgements

We would like to thank Katherine Albro Houpt and Elisabeth D'Antoni for helpful comments on an earlier version of the manuscript.

CHAPTER 5

Social feeding decisions in horses *(Equus caballus)*

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Published in Behavioural Processes (2008) 76-83

Abstract

Like many other herbivores, in a natural environment equids feed on rather evenly distributed resources. However, the vegetation in their vast habitats constantly changes. If food is plentiful only little competition occurs over food, and in non-competitive situations domestic horses tend to return to the same feeding site until it is overgrazed. In contrast, they compete over limited food for which the social status of the individuals appears to be important. Especially in ruminants several studies have proved an influence of social organisations, rank, sex and the depletion of feeding sites on the feeding behaviour of individuals. However, it is not yet understood whether and how social aspects affect horses' feeding decisions. Curiosity about the influence of social rank on the horses' feeding decisions between two, equally with high-quality surplus food filled buckets placed in different social feeding conditions, led us to create the test below. The observer horses were alternately tested with a dominant and a subordinate demonstrator placed in one of three different positions. We conclude that domestic horses use social cognition and strategic decision making in order to decide where to feed in a social feeding situation. When possible they tend to return to the same, continuously supplied feeding site and switch to an "avoidance tendency" in the presence of dominant horses or when another horse is already feeding there. Thus, the social rank and the position of conspecifics affect the feeding strategy of horses.

Keywords

Feeding decision, horse, rank, social behaviour

Introduction

This study arose from the urgent need to gain more knowledge about the horses' social feeding decisions. In many tests horses discriminate between different food sources, or the horses' learning behaviour is positively reinforced by food rewards. Especially in the case of social learning tasks, in which animals are tested for their ability to learn specific feeding tasks from their conspecifics (see, for instance, Heyes and Galef, 1996; Nicol, 2006), feeding decisions are influenced by feeding choices of conspecifics. Several studies (Baer et al., 1983; Baker and Crawford, 1986; Clarke et al., 1996; Lindberg et al., 1999; Nicol, 2002) have been designed to prove social learning in feeding situations in horses. But, even though equids are highly social animals, none of the previous tests were able to demonstrate social learning in horses (Nicol, 2002; Krueger and Flauger, 2007). Baker and Crawford (1986), as well as Clarke et al. (1996), concluded from the results of their tests that horses learned something, because of the decrease in latency in approaching the test area after observing a demonstrator feeding. However, they may have "avoided" the feeding territory of the unfamiliar demonstrator (i.e. termed "avoidance hypothesis").

Although, the avoidance hypothesis already suggests, that social cognitive abilities, i.e., the processing, encoding, storage, retrieval, and application of social information, is decisive for the outcome of social feeding tests in horses, knowledge on the topic is very limited (Nicol, 2002). However, in previous studies we showed that horses are capable of social cognition. They memorise and generalize social experiences (Krueger, 2007), and distinguish the social affiliation and the social rank of other horses (Krueger and Heinze, 2008).

For a better understanding of the present study we will proceed with outlining the current state of knowledge on social feeding competitions in grazers and other mammals, as well as on sociality in equids, and finally draw the main aspects for this study.

In general group life in social animals is determined by complex long-term social relationships (Hinde, 1983). The "shareholders" of social interaction may benefit from reduced predation risks, improved defence of resources and communal rearing. On the other hand each of them suffers from increased competition for critical resources to a differential degree (Pusey and Packer, 2003).

In grazers, behaviours which are shown while animals compete over more homogeneously distributed and plentiful resources, so called feeding interactions, occur very rarely (Geist, 1974; Wittenberger, 1981; Wittemyer and Getz, 2007; Fischhoff et al., 2007). However, depletion of food sources causes higher competition (Jarman, 1974; Illius and Gordon, 1987). Analogous, to these findings most competition over food in red deer (*Cervus elaphus*), Roosevelt elk (*Cervus elaphus roosevelti*), and caribou (*Rangifer tarandus*) has been observed in winter when food is scarce (Appleby, 1980; Barrette and Vandal, 1986; Thouless, 1990; Weckerly, 1999). Feeding competition in goats (*Capra hircus*) is influenced by the amount of available food and by the goat's sex, age and rank (Shi and Dunbar, 2006).

However, feeding conspecifics can also serve as reference point where to find preferred food items (Valone, 1989; Valone and Templeton, 2002). In this case, the presence of a foraging animal increases the interest of others in a specific feeding area, which has been termed social or local enhancement (Poysa, 1992; Giraldeau, 1997). It has been shown, that goats (*Capra hircus*) use social information for locating high quality feeding areas after observing others foraging (Shrader et al., 2007). They also raise their intake rate, in terms of feeding bouts per feeding time, in direct relation to the number of increasing competitors (Shrader et al., 2007).

Thus, foraging in groups comprises benefits and costs. Social animals benefit from collective predator protection and social information processing, such as using conspecifics as reference point as described above, but competition over the preferred food items may be costly and even result in serious injuries. Subordinate animals usually pay the highest costs while trying to obtain scarce resources such as food, water, rest places and shelter in the presence of dominant animals (Barton, 1993; Barton and Whiten, 1993). In chimpanzees (Hare et al., 2000), for instance, subordinate animals only choose food that is hidden behind a small barrier in such a way that the dominant animal can not see it. Hare et al. (2001) even highlighted that chimpanzees seem to know which particular animal has watched the crucial event, since subordinates go for food when the observing dominant animal is exchanged for a non-observing dominant animal.

However, it is not yet understood whether and how social aspects affect horses' feeding decisions. Equids live in fission-fusion social systems (Fischhoff et al., 2007) in which the members of social groups frequently disperse and reunite again. Though, social live takes different shapes in equids, for species, which live in wide grasslands, such as the Serengeti Plain of Tanzania (Moehlman, 2002), the valleys of Hustai National Park in Mongolia (King and Gurnell, 2005) and the “Great Basin” in northern America (Berger, 1986), food and water resources are sufficient enough to allow females to feed together and to thus form stable groups, which consist of one or more mares, their offspring and usually one, but occasionally up to five males (i.e. referred to as “harem” or “family”, Tyler, 1972; Berger, 1977; Moehlman, 2002). Surplus stallions gather in separate bachelor bands that differ in size from 2 to approximately 17 horses (Berger, 1977). Many subgroups form a structured social unit, called “herd,” which shows the same migration patterns within a common home range (Miller, 1979; Berger 1986). Horses roam in vast habitats and spend an average of 60% of their time feeding on constantly changing vegetation (Salter and Hudson, 1979; Waring, 2003). They prefer to feed on grasses in areas where preferred food is more plentiful (Salter and Hudson, 1979; Duncan, 1983). In non competitive situations, while horses feed all by themselves with no other horses near by, domestic horses tended to return to the same feeding site until it is overgrazed (Devenport et al., 2005).

When food sources are limited the social status of the individuals appears to be important. In the context of determining dominance relationships among domestic horses, paired feeding tests, an interaction contest over the limited resource “food”, have often been applied (Houpt et al., 1978; Ellard and Crowell-Davis, 1989). This technique is still in use for several species, like monkeys and apes, today (Li et al., 2007), although doubts arose concerning the reliability of dominance hierarchies investigated in the contest over point resources for species, that generally feed on rather homogeneously distributed resources. Ellard and Crowell-Davis (1989) were the first to mention that the results of such a test with draft-horse mares did not match their observations of the dominance hierarchy of the same horses in the field. Accordingly, in recent studies (Berger, 1977; Goldschmidt-Rothschild and Tschanz, 1978; Houpt et al., 1978; Houpt and Wolski, 1980; Ellard and Crowell-Davis, 1989; Linklater and Cameron, 2000; Heitor et al., 2006a, 2006b), dominance relationships in horses have been assessed by using approach-retreat interaction and the direction of threats and submissive gestures (Feist and McCullough, 1976; McDonnell and Haviland, 1995; McDonnell, 2003).

Curiosity about the influence of social rank on the horses' feeding decisions between two, equally with high-quality surplus food filled buckets placed in different social feeding conditions, led us to create the test below. Both feed-buckets were black in colour and marked with olfactory cues from prior feeding of the test horses. The observer horses were alternately tested with a dominant and a subordinate demonstrator, which were determined from dominance relationship data observed in the field. The demonstrator was placed in one of three different positions either defined as i) demonstrator feeding, ii) demonstrator tied up or iii) demonstrator absent. We hypothesised that the decisions of the observer horses would be strongly influenced by the demonstrators' rank in all the three feeding situations.

Materials and Methods

Animals

We investigated the behaviour of 14 horses: 11 standard bred horses and 3 ponies (composed of 12 mares and 2 geldings), all aged between 6 and 30 years. The horses were individually identified by their brands and coloration. For testing social behaviours the social background and the housing conditions of the animals are of importance. Socially kept animals might behave differently from those that are kept individually. The horses that took part in the tests were members of three social groups with 6, 6 and 4 horses, respectively (Table 1). Two horses in group 1 and 2 are genetically related (mother–daughter relationships in both cases).The composition of group 1 has been stable for 6 years. Group 3 was established one year ago, but the members of the group had contact to each other for several years. In group 2, four horses represent the core of this group and have been together for six years; they were joined by two new horses only three months ago. Because of their short time in the group, those two horses were not used in the feeding tests. Nevertheless, to maintain a complete dominance hierarchy, their dominance data has been retained in the dominance tables but is labelled with an asterisk.

The three groups were kept in different types of stables. Horses of groups 1 and 3 were kept in social groups in open stabling, with a bedding of straw, in group 1, and wood shavings, in group 3. They received daily access to their pastures. The horses in group 2 were housed in individual box stalls (sized: 3 m x 4 m, with a bedding of straw) overnight and turned out in a social group in a paddock during the day. The daily feed of the horses was composed of hay twice a day and a compound feed once a day, in groups 1 and 3. Whereas group 2 received hay and a compound feed twice a day. In addition they all had access to grazing while turned out.

Dominance relationships

Before starting the experiments, we determined the dominance relationships among the horses in the field by observing agonistic encounters, such as approaches, retreats, threats to bite or kick, bites, kicks and chases (Feist and McCullough, 1976; McDonnell and Haviland,1995; McDonnell, 2003). For specific sampling of the dominance interaction described above, horses were observed over 6 hours on separate days (at least three different days, with a minimum duration of 30 min. and a maximum of 150 min. each). Observations had to be adjusted to accommodate the horses` commitments as riding horses, but were distributed over daylight period. The interactions of the horses were recorded continuously. For the calculation of the individual dominance scores, we added instances of active antagonism and subtracted cases of retreat (Table 1).

Table 1 Dominance hierarchy of horses

Group	Age	Sex	Breeds	Related	Dom. score
Group 1					
Billy	18	Gelding	Warmblood	No	47
Sara	22	Mare	Haflinger	No	38
Farina	23	Mare	Warmblood	No	18
Peppermint	14	Gelding	Pony	No	4
Anouschka	6	Mare	Haflinger	Daughter	-14
Alexia	20	Mare	Haflinger	Mother	-28
Group 2					
Monty*	9	Gelding	Paint	No	83
Manon	21	Mare	Warmblood	Mother	77
Mahranya	7	Mare	Warmblood	Daughter	69
Lady	16	Mare	Appaloosa	No	3
Daisy	30	Mare	Warmblood	No	-29
Grandessa*	17	Mare	Warmblood	No	-48
Group 3					
Francis	13	Mare	Warmblood	No	34
Traum	14	Mare	Warmblood	No	8
Miss Lala	27	Mare	Pony	No	2
La Belle	14	Mare	Pony	No	-30

* Horses which joined the group only 3 months ago

Experimental set-up feeding test

The experimental area (8m x 8m, separated by fencing, Fig. 1) for groups 1 and 3 was a part of their open stable, and for group 2 it was a part of their riding arena. Two food-buckets (black, 35 cm diameter) were placed in the feeding area at a distance of 3.5 meters apart. To prevent poor performance caused by reinforcer satiation on a single food item (Miyashita et al., 2000) the buckets were constantly filled with three different food items, such as a mixture of compound feed, carrots and apples for groups 1 and 3. Because one horse of group 2 previously showed signs of colic after feeding on apples, horses of this group received the first two food items and bread instead of apples. In addition, large stones were added to the feed buckets to prevent the horses from eating too fast and getting too much feed of high nutritional value. The area opposite the feeding region served as an observation area for an observer horse. The fences next to the buckets could be opened to remove the demonstrator horse.

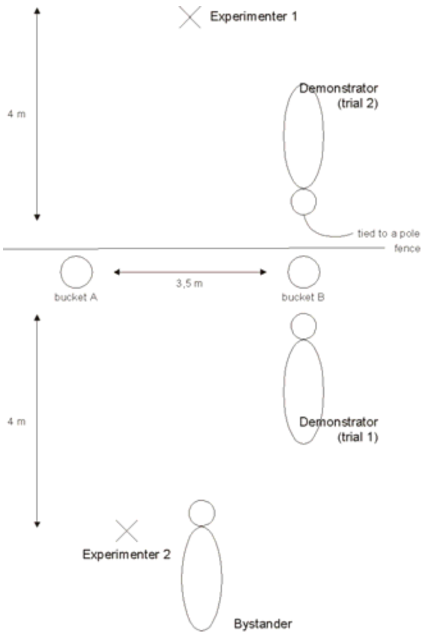

Fig. 1 Experimental set-up feeding test. Buckets A and B always contained food. Trial 1, 4, 9 and 12: demonstrator horse continuously feeds from a specific bucket. Trial 2, 5, 8 and 11: demonstrator horse, tied up to a post beside the bucket it had previously been feeding from for 5 seconds. Trial 3, 6, 7 and 10: demonstrator horse was led out of the observer horse's sight after feeding from a specific bucket for 5 seconds

Experimenter

Three people took part in the study as experimenters: person 1 in the trials of group 1 and 3, person 2 in the trials of all three groups and person 3 only in the trials of group 2. Experimenter 1 handled the demonstrator horses and refilled the food buckets. Experimenter 2 handled the observer horses. In all cases experimenter 1 was unfamiliar to the horses.

Experimental procedure feeding test

Before starting the experiments, all the horses were habituated to the experimental set-up (Fig. 1). All horses were fed from both buckets, and the buckets were continuously refilled to show the horses that there will always be food in both buckets. The experiments were conducted between 9 a.m. and 1 p.m. The normal first feeding time for all groups was at 7 a.m.

For trials 1-6 the respective horses of a group were tested on one test day in the following order: demonstrator feeding, demonstrator tied-up and demonstrator absent. To prevent a serial-order-effect on the rank of the demonstrator horses we conducted each feeding situation with a subordinate and a dominant demonstrator in random order. Two weeks later, the horses of groups 1 and 3 were tested in 6 additional trials in the same manner but in reversed order (trials 7-12, Table 2, Fig. 2 and 3). Unfortunately, the horses of group 2 could not be used for this study any more, because the subordinate demonstrator of this group developed permanent teeth problems, which affect its feeding behaviour. Finally, two months later, horses of groups 1 and 3 were tested in control trials (trials 13-15, Table 2, Fig. 3) similar to trials 4 – 6, because we did not test them in the original trial 6. For the original trial 6, we acted on the assumption that those horses would retain their tendency from trial 5 to return to the same bucket.

Trials 1, 4, 7, 10 and 13: demonstrator feeding

The demonstrator horse was led to a specific bucket in the feeding area by experimenter 1, and released from a lead rope. It immediately ate from the bucket. Experimenter 1 moved 4 meters away and turned her back to the eating demonstrator. The observer horse watched the demonstrator horse eating for 5 seconds and then was released from a leadrope to choose a feed bucket in the feeding area. Experimenter 2, as well, moved 1 additional meter away from the horses and turned her back to them.

Trials 2, 5, 8, 11 and 14: demonstrator tied up

The experimental procedure was similar to the trials “demonstrator feeding” with the exception that the demonstrator horse, after it was allowed to eat for 5 seconds from a specific bucket, was tied up to a post and separated from the bucket it had just fed from by a fence. The observer was released when the demonstrator was already tied up.

Trials 3, 6, 9, 12 and 15: demonstrator absent

Again, the experimental procedure was similar to the trials “demonstrator feeding”, but after eating from the bucket the demonstrator horse was caught by experimenter 1, and led out of sight of the observer horse (i.e. out of the experimental area). Only then was the observer horse released into the feeding area.

Trials 7 -12: trials in reversed order

We conducted an identical experimental procedure as the trials 1-6, but in reversed order, starting with the demonstrator absent (trials 9 and 12), followed by the demonstrator tied up (trials 8 and 11) and the demonstrator already feeding (trials 7 and 10).

Trials 13-15: control for the missing trial 6 in groups 1 and 3

We tested for the missing trial 6 in groups 1 and 3 in terms of creating a test day similar to the one for the original trials 4-6, and therefore, tested horses of group 1 and 3 in all three trials with a subordinate demonstrator a second time in the original order.

In order to standardize the testing for all horses, the order in which the demonstrator horse fed from the buckets in the presence of each observer horse in each trial was as follows: bucket B, A, B, B, A, A. Bucket A was always positioned to the left side and bucket B to the right side of the observers start position (Fig. 1). By leading the demonstrator alternately, but not regularly, to the left and to the right side we tried to prevent a lateralisation bias in the observer horse.

Table 2 Trials

Trial number	Demonstrator rank	Feeding situation	Trial type
1	Dominant	Demonstrator feeding	Original
2	Dominant	Demonstrator tied	Original
3	Dominant	Demonstrator absent	Original
4	Subordinate	Demonstrator feeding	Original
5	Subordinate	Demonstrator tied	Original
6	Subordinate	Demonstrator absent	Original
12	Subordinate	Demonstrator absent	Reverse
11	Subordinate	Demonstrator tied	Reverse
10	Subordinate	Demonstrator feeding	Reverse
9	Dominant	Demonstrator absent	Reverse
8	Dominant	Demonstrator tied	Reverse
7	Dominant	Demonstrator feeding	Reverse
13	Subordinate	Demonstrator feeding	Control
14	Subordinate	Demonstrator tied	Control
15	Subordinate	Demonstrator absent	Control

Statistics and visualisations

Analysis was done with the R statistical environment (2007) and the statistical software SPSS. We applied Generalized Estimating Equations (GEEs), for binomial data, to solve a complex likelihood equation for the influence of the demonstrator position, its rank, and the rank of the observer on the observer horses bucket choice in contrast to the demonstrators' bucket-choice. But for the respective feeding situations we investigated the significance for the probability of the observers to choose the same bucket as the demonstrator. Therefore additional GEEs tested the probability of the observer horses bucket choice ("bucket observer") to be analogous to the response variable ("bucket demonstrator") with the demonstrators rank as an additional explanatory variable. Finally we applied a Chi-Square Test (SPSS) for evidence of the horses' tendency to return to a specific feed-bucket. Tables and Figures were visualised with the R statistical environment (2007).

Results

Feeding test

We analysed the general influence of the demonstrators’ position, their rank and the rank of the observer on the probability of the observer horses’ bucket-choices for the original trials 1-6 and the reversed trials 7-12 (Table 3). The control trials for the situations in which a subordinate demonstrator fed were analysed for an influence of the demonstrators’ position and the rank of the observer (Table 3). Figure 2 illustrates the observers’ bucket choice when confronted with a dominant demonstrator in each of the three possible positions (trials 1-3: original and trials 7-9: reverse), and figure 3 with a subordinate demonstrator in the same three positions (trials 4-6: original, trials 10-12: reverse and trials 13-15: control). The position of the demonstrator (feeding, tied up or absent) significantly influences the observers’ bucket choices in the trials 1-6, in the reversed trials 7 -12 , and the subordinate control trials (all $p < 0.001$, Table 3). The same is true for the rank of the demonstrator in the trial 1-6 ($p = 0.018$, Table 3). However, for the reversed trials 7-12 no significance in the rank of the demonstrator was observed ($p = 0.875$, Table 3). The rank of the observer horses was not significant in any of the cases examined (all $p > 0.05$, Table 3).

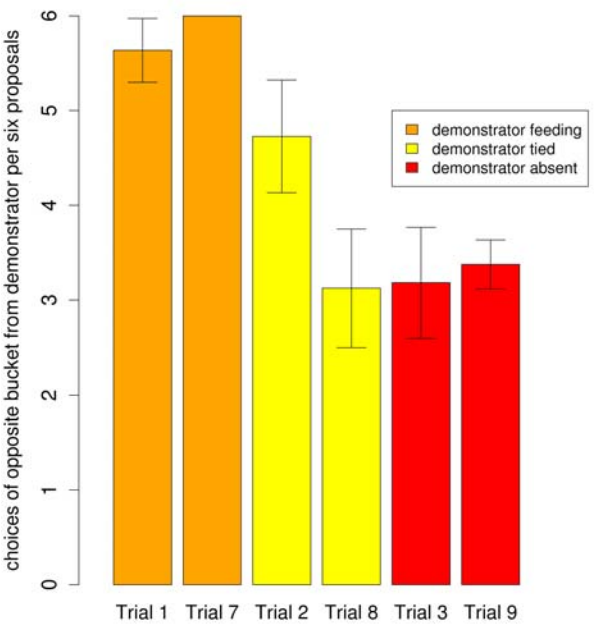

Fig. 2 Feeding test, dominant demonstrator. The observers’ bucket choices contrary to those of a dominant demonstrator significantly differ in the respective feeding situations, such as demonstrator continuously feeding (trials 1 and 7), or tied up (trials 2 and 8) or absent (trials 3 and 9), because the position of the latter significantly influences the observers’ bucket choices in the original trials 1-3 and in the reversed trials 7-9 (all $p < 0.001$, Table 3)

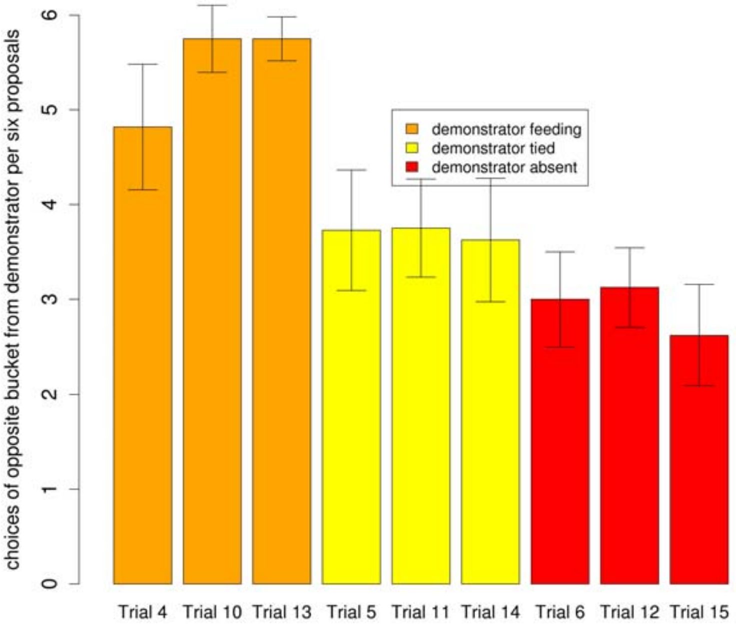

Fig. 3 Feeding test, subordinate demonstrator. The observers' bucket choices contrary to those of a subordinate demonstrator significantly differ while the latter was either continuously feeding (trials 4, 10 and 13), or tied up (trials 5, 11 and 14) or absent (trials 6, 12 and 15), because the position of the demonstrator similarly influences the observers' bucket choices in the original trials 4-6, in the reversed trials 10-12, and the subordinate control trials 13-15 (all $p < 0.001$, Table 3)

For a closer analysis of the observers' bucket choice compared to the demonstrator in the respective feeding conditions we conducted separate GEEs. Because the observers' rank did not have any significant influence on the probability of choosing a specific bucket throughout the test, we excluded "observer rank" from the following formulas but kept demonstrator rank, which proved to be significant in the trials 1 - 6. When the demonstrator was eating, the observer horses generally tended to choose the other bucket (all $p< 0.001$, Table 3). Even though the statistical data for trial 2 and 5 show significance for the observers' choice of the bucket that had not been used by the demonstrator when the demonstrator was tied up (position 2) ($p < 0.001$, Table 3), the visualised data (Fig. 2 and 3) illustrate that this choice was not as consistent as in the trials 1 and 4. In fact, in the analogous but reversed trials 8 and 11 ($p = 0.182$, Table 3) and the control trial 14 ($p = 0.328$, table 3) the choice was not statistically significant. The third position, with the demonstrator absent, also failed to show any significant choice for the other bucket the demonstrator previously ate from (all $p > 0.05$, Table 3). The demonstrators' rank appeared to be of minor importance in the respective feeding situations (p of all tests > 0.05)

Table 3 Results

<table>
<tr><td colspan="8">Generalized Estimating Equations (GEEs)
Formula: choice ~ position demonstrator + rank demonstrator + rank observer
id = nr observer, family = "binomial", corstr = "exchangeable"</td></tr>
<tr><td colspan="4">Impact on choice (N = 348)</td><td colspan="2">Reverse trials (N = 288)</td><td colspan="2">Control trials (N = 144)</td></tr>
<tr><td>Response</td><td>Predictors</td><td>Robust Z</td><td>P-value</td><td>Robust Z</td><td>P-value</td><td>Robust Z</td><td>P-value</td></tr>
<tr><td>Choice observer</td><td>Position demonstrator</td><td>-5.472</td><td>< 0.001</td><td>-7.175</td><td>< 0.001</td><td>-5.975</td><td>< 0.001</td></tr>
<tr><td></td><td>Rank demonstrator</td><td>-2.372</td><td>0.018</td><td>0.157</td><td>0.875</td><td></td><td></td></tr>
<tr><td></td><td>Rank observer</td><td>-2.551</td><td>0.980</td><td>0.048</td><td>0.962</td><td>-0.662</td><td>0.508</td></tr>
<tr><td colspan="8">Formula: bucket demonstrator ~ bucket observer + rank demonstrator
id = nr observer, family = "binomial", corstr = "independence"</td></tr>
<tr><td colspan="4">Demonstrator feeding (N = 132)</td><td colspan="2">Reverse trials (N = 96)</td><td colspan="2">Control trials (N = 48)</td></tr>
<tr><td>Response</td><td>Predictors</td><td>Robust Z</td><td>P-value</td><td>Robust Z</td><td>P-value</td><td>Robust Z</td><td>P-value</td></tr>
<tr><td>Bucket demonstrator</td><td>Bucket observer</td><td>-4.979</td><td>< 0.001</td><td>-3.894</td><td>< 0.001</td><td>-4.907</td><td>< 0.001</td></tr>
<tr><td></td><td>Rank demonstrator</td><td>0.191</td><td>0.849</td><td>0.830</td><td>0.406</td><td></td><td></td></tr>
<tr><td colspan="4">Demonstrator tied (N = 132)</td><td colspan="2">Reverse trials (N = 96)</td><td colspan="2">Control trials (N = 48)</td></tr>
<tr><td>Response</td><td>Predictors</td><td>Robust Z</td><td>P-value</td><td>Robust Z</td><td>P-value</td><td>Robust Z</td><td>P-value</td></tr>
<tr><td>Bucket demonstrator</td><td>Bucket observer</td><td>-3.934</td><td>< 0.001</td><td>-1.334</td><td>0.182</td><td>-0.978</td><td>0.328</td></tr>
<tr><td></td><td>Rank demonstrator</td><td>0.581</td><td>0.561</td><td>-0.479</td><td>0.632</td><td></td><td></td></tr>
<tr><td colspan="4">Demonstrator absent (N = 84)</td><td colspan="2">Reverse trials (N = 96)</td><td colspan="2">Control trials (N = 48)</td></tr>
<tr><td>Response</td><td>Predictors</td><td>Robust Z</td><td>P-value</td><td>Robust Z</td><td>P-value</td><td>Robust Z</td><td>P-value</td></tr>
<tr><td>Bucket demonstrator</td><td>Bucket observer</td><td>-0.499</td><td>0.618</td><td>-1.572</td><td>0.116</td><td>1.008</td><td>0.277</td></tr>
<tr><td></td><td>Rank demonstrator</td><td>0.468</td><td>0.640</td><td>-0.487</td><td>0.626</td><td></td><td></td></tr>
</table>

In general, horses that did not choose the buckets opposite from those from which the demonstrator had previously fed showed a strong tendency to return to the same bucket, i.e. either bucket A or B, in the trials 1-6 (Chi-Square-Test: N = 192, $\chi^2 = 90.750$, df = 1, $p < 0.001$) and in the trials of reversed order (7-12) (Chi-Square-Test: N = 132, $\chi^2 = 39.273$, df = 1, $p < 0.001$). Deviations of only one choice were considered to be due to chance.

Finally, it appeared to be noteworthy that Lady, a member of group 2, received an aggressive action from the dominant demonstrator in trial 1 while trying to feed from the same bucket. In trial 2 and 3 she avoided the buckets from which the same dominant demonstrator had previously fed (Fig. 2).

Discussion

In the present study observer horses were tested for their choice among two food buckets from which a demonstrator horse fed in three respective feeding situations, such as i) demonstrator constantly feeding, ii) demonstrator tied up beside a specific food bucket or iii) demonstrator absent after feeding from a specific food bucket. In each of these social feeding situations the observer horse could decide a) to stay with the tendency of returning to a specific feeding area, or b) to use the feeding conspecific as reference point for the best food source, or finally c) to avoid the feeding territory of its conspecific.

Observer horses obviously avoid feeding from the demonstrators' bucket in direct confrontation, i.e. while the demonstrator is eating. However, for the feeding conditions in which the demonstrator was tied up beside a bucket horses started to develop a strong tendency to return to the same feeding site they chose before, and displayed this tendency, even stronger, when a demonstrator was absent after feeding, similar to what has been reported by Devenport et al. (2005) for non-competitive situations.

Furthermore, the demonstrators' rank did have a significant effect on the observer horses' feeding decisions for the original test (trials 1-6). Nevertheless, the effect was weaker as we expected it to be. It appeared to be strongest for the situation in which the demonstrator was tied beside a bucket, which means that observer horses avoided feeding from a bucket with a dominant demonstrator but not with a subordinate demonstrator tied beside it.

However for the repetitions of the test, during the reversed and the control trials, the influence of the demonstrators' rank diminished. On the one hand, observer horses might simply learn that demonstrator horses will not challenge their feeding decisions in the trials when the latter are tied or absent, no matter what the demonstrators' rank might be. On the other hand, the declining influence of the demonstrators' rank could be due to the fact, that observer horses are repeatedly, positively reinforced from equally filled food-buckets and they simply avoid unnecessary costs for feeding competitions even with subordinate animals. One could also argue, that, prior to the test, domestic horses had learned in their daily routines, or in more natural feeding situations (Salter and Hudson, 1979; Duncan, 1983), that they have to compete for high-quality-food, but, during the repetitions of the test, experience that the preferred food will be available *ad libitum.* The best option to control for these effects in an additional test series would probably emerge from testing the respective feeding situations in random order, such as mixing trials with the demonstrator feeding, tied or absent, as well as the subordinate and dominant demonstrator horses. Another option, of testing horses with only one food source from which the demonstrator feeds, would result in frustration and thus demotivation of the observer horse, and, in addition, would not allow testing for alternative feeding decisions.

Also in social learning tasks observer horses have been tested for feeding decision over equally supplied food sources (Baer et al., 1983; Baker and Crawford, 1986; Clarke et al., 1996; Lindberg et al., 1999), when the demonstrator horse was already out of sight. From the outcome of our tests we conclude that, in this feeding situation, the rank of the demonstrator horse would not affect the choice of the observer horses. Furthermore, we suggest, that the "avoidance hypothesis", which has been proposed in order to explain the feeding choices of observer horses in social learning tasks (Baker and Crawford, 1986; Clarke et al., 1996), can not be supported any longer. In our study we could not find an avoidance tendency for the bucket the demonstrator horse fed from when the latter was absent. Rather a tendency to return to the same feeding site might have biased the social learning tests. Horses appear to stay with this tendency no matter what the demonstrator horses' rank is and whether they are confronted with evenly distributed low-quality food (Devenport et al., 2005) or with limited high quality food. Their motivation to adopt a different feeding strategy from their natural feeding habit might be low in situations where unlimited food rewards are given or food rewards can not be controlled by the dominant animal (Laland, 2004).

Consequently, we agree with Clarke et al. (1996) that the learning effect due to local enhancement, which has been reported to cause a faster approach to the feeding area in the learning situation than in the control trials, is connected to the general feeding location and not to a specific bucket. Furthermore horses did not use social information after observing conspecifics feeding, in terms of using their conspecifics as reference points for where to feed (i.e. through social enhancement, Poysa, 1992; Giraldeau, 1997) or to select a feeding area of higher food-quality (Shrader et al. 2007). Rather than feeding from the same bucket as the demonstrator horses, the observer horses preferred to return to always the same feeding site. This situation might be enhanced by a habituation of the test horses to constantly filled buckets prior to the tests. Horses might not have feared that there is no food left in the buckets, which has been hypothesised by Baker and Crawford (1986).

Furthermore, the fact that horses change their feeding strategies, depending on whether food is limited or plentiful, might explain the contradictory results of dominance evaluations on feeding competition tests over a limited food source to those evaluated from behaviour observations in the field (Houpt et al., 1978; Ellard and Crowell-Davis, 1989; Heitor et al., 2006a, 2006b). In addition, for behavioural observations in the field, competition for all resources is decisive for the construction of dominance hierarchies. Food represents only one of the valued resources, and individuals might cherish resources to a differential degree (Pusey and Packer, 2003).

Finally, we would like to stress the point that the more general lack of avoidance is not due to a cognitive inability to remember where the demonstrator last fed, but rather to a social strategic decision-making process. In some cases, previous feeding experiences affect the observers´ feeding choice, such as in Lady's case, where, after receiving an aggressive action from the dominant demonstrator in trial 1, she did not dare to feed from the same bucket either in the demonstrator's presence or in its absence. Lady, as well as other horses, who avoided feeding from the same buckets as the demonstrators even in their absence, showed an excellent memory for the demonstrators' choices.

Conclusion

In a nutshell, domestic horses use social cognition and strategic decision making in order to decide where to feed in a social feeding situation. Whenever possible they tend to return to the same, continuously supplied feeding site and switch to an "avoidance tendency" in the presence of dominant horses, or when another horse is already eating there. Thus, the social rank and the position of conspecifics affect the feeding strategy of horses.

Acknowledgements

We wish to thank Katherine Albro Houpt for discussions about horses' dominance relationships, Christine Nicol for discussing the scientific background, Juergen Heinze for helpful suggestions, Elisabeth D´Antoni and Jon Seal for language corrections, Knut Krüger for the analysis in "R statistical environment" and all the owners of the horses who allowed us to test their horses or even helped with the tests. We also thank two anonymous referees for all their effort they put into improving an earlier draft of the manuscript. The study was supported by an HWP II grant of the University of Regensburg and an Excellence grant of the Bavarian Government.

CHAPTER 6

Olfactory recognition of individual competitors by means of faeces in horse *(Equus caballus)*

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Manuscript submitted to Animal Cognition

Abstract

Living in complex social systems requires perceptual and cognitive capacities for the recognition of group membership and individual competitors. Olfactory recognition is one means by which this can be achieved. Many animals identify individual proteins in urine, skin secretions, or saliva. Additionally, marking behaviour in several mammals and especially in horses indicate the importance of sniffing conspecifics' faeces for olfactory recognition. To test this hypothesis, we conducted two separate tests: Test one addressed the question of whether horses recognise the group membership of other horses by sniffing their faeces. The horses were presented with four faecal samples: 1) their own, 2) those of other members of their own group, 3) those of unfamiliar mares, and 4) those of unfamiliar geldings. Test two was designed to assess whether horses can identify the group member from whom a faecal sample came. Here, we presented two groups of horses with faecal samples from their group mates in random distribution. As controls for both tests, soil heaps and sheep faecal samples were used. In test one horses distinguished their own from their conspecifics' faeces. In test two, the horses paid most attention to the faeces of the horses from which they received the highest amount of aggressive behaviours. In group 1 the horses` individual sniffing responses were positively correlated with the levels of aggressive behaviour displayed among particular pairs of horses. In group 2, one horse received significantly more sniffing responses and was particularly aggressive towards all the other group mates. Here the sniffing responses received showed a significant linear relationship to the total number of aggressions displayed towards other horses. We therefore suggest that horses of both sexes are capable of distinguishing particular competitors among their group mates by the smell of their faeces.

Keywords

Equids, faecal samples, horse, olfaction, sociality

Introduction

Numerous animal species need to recognize their conspecifics. In dispersed social systems they need to recognize their neighbours, or at least distinguish familiar from unfamiliar individuals for mating and habitat protection. Animals living in social systems require competences for the recognition of conspecifics, the formation of alliances, the discrimination of competitors and hierarchical access to resources (Wilson, 1975). Such recognition has been described as being mediated through auditory, visual, and olfactory perception (Tibbets, 2002; Trillmich, 2006; Ligout and Porter, 2006). Olfactory recognition in particular plays an important role for a variety of social animals such as ants (Dreier et al., 2007), honey bees (Pesenti, 2008), insects in general (Howard and Blomquist, 2005), sticklebacks (Mehlis et al., 2008), ringtailed lemures (Scordato and Drea, 2007), Belding's ground squirrels (Mateo, 2006), rabbits (Patris et al., 2008), mice (Arakawa et al., 2008) and many other social mammals (Eisenberg and Kleiman, 1972).

Analogous to several mammals such as many primate species (Clutton Brock, 1974), elephants (Moss and Poole, 1983), hyenas (Smith et al., 2008), and dolphins (Connor et al., 2000), equids are highly social animals. Behavioural data indicate that horses are capable of social cognition (Krueger and Heinze, 2008), i.e. the processing, encoding, storage, retrieval, and application of social information, an ability which has previously been reported in primates, social birds and social ungulates (Treichler and Van Tilburg, 1996; Veissier et al., 1998; Acuna et al., 2002; Roberts et al., 2003; Paz-y Miño et al., 2004; Allen, 2006; Moses et al., 2006).

Horses live in relatively stable social units, called bands, family groups or harems (Klingel, 1972; Moehlman, 2005; see for review: Linklater, 2000). Harems or bands usually consist of one to five stallions, several mares, and their offspring (Tyler ,1972; Berger, 1977; Moehlman, 2002), and surplus stallions gather in bachelor bands (Berger, 1977). It is still under discussion whether several subgroups form a large structured social unit, called a "herd", and show the same migration patterns within a common home range (Miller, 1979; Duncan, 1992; Feh, 2005) or whether a collection of subgroups should rather be termed a "population", which synchronize daily and seasonal patterns of movements in response to water, food, or climate (Feist and McCullough, 1975; Berger, 1986; Linklater et al., 1999) and show inter-band hierarchies at resource patches (Miller and Denniston, 1979; Franke Stevens, 1988; see for review: Linklater, 2000).

In these herds or populations a considerable number of inter-band movements have been reported, even though horse bands show stable core groups (Linklater et al., 2000; King, 2002; King and Gurnell, 2005). After most offspring have dispersed from their natal groups by 5 years of age (97% of males and 81% of females: Rutberg and Keiper, 1993) adult horses have then frequently been observed to change groups. Male bachelor bands have been described as instable with only few exceptions (Feist and McCullough, 1976; Feh, 1999; Feh, 2001; see for review: Linklater, 2000), all -male bands were observed to be instable in most feral horse populations (Miller, 1981; Berger, 1986; Feh, 2001; see for review: Linklater, 2000), and, finally, some temporary mixed sex peer groups have been observed (Keiper, 1976; Linklater et al., 2000). Several authors also report the dispersal of adult mares from harems (fission: Berger, 1986; Rutberg, 1990; Rutberg and Greenberg, 1990; Linklater and Cameron, 2000) as well as their return (fusion: Goldschmidt-Rothschild and Tschanz, 1978). Between all these groups and populations the harem or band stability varied considerably (Berger, 1986; Rubenstein, 1986; Rutberg, 1990).

Such complex social systems require horses to memorise and generalize social experiences, distinguish between familiar and unfamiliar, and identify familiar horses, as well as their social status relative to their own group (Krueger and Heinze, 2008). Horses exhibited excellent long term memory of memberships of their own group (see for review: Nicol, 2002; Murphy and Arkins, 2007).

Several perceptual methods have been observed for social recognition in horses. They try to stay in contact through auditory cues (Kiley, 1972; Tyler, 1972; Feist and McCullough, 1975; Rubenstein and Hack, 1992; Feh, 2002) and identify their group members by visual (Tyler, 1972; Feist and McCullough, 1975; Feh, 2002) as well as auditory perception (Proops et al., 2009). Additionally, frequent sniffing of conspecifics as well as their faeces indicates the importance of olfaction in the horse's social recognition system (Tyler, 1972; Feist and McCullough, 1975; Marinier et al., 1988; Stahlbaum and Houpt ,1989; Rubenstein and Hack, 1992; Saslow, 2002; Feh, 2005).

However, how horses identify their conspecifics by olfactory means has rarely been investigated under controlled conditions (Saslow, 2002). In many mammals olfactory recognition has been described as being mediated through urine, skin secretions, or saliva (mice: Penn and Potts, 1998; Brennan, 2004; elephants: Bates, 2008; mammals: Lévy et al., 2004; Brennan and Kendrick, 2006). But, interestingly, for marking behaviour in horses faeces and urine appear to be crucial (Kimura, 2001, see for reviews: Eisenberg and Kleiman, 1972; Linklater, 2000; Gosling and Roberts, 2001). Especially faeces evoke strong behavioural reactions in stallions (Marinier et al., 1988; Stahlbaum and Houpt, 1989; Kimura, 2001; King and Gurnell, 2007; see for review Linklater, 2000), and therefore previous studies in horses analyzed stallion responses towards urine and faeces. Stallions were shown to be able to differentiate the sex (Stahlbaum and Houpt, 1989) and the familiarity (Rubenstein and Hack, 1992) of faecal donors, but, for urine samples, neither the sex of the donor (Stahlbaum and Houpt, 1989) nor the oestrus stage of female donors (Marinier et al., 1988; Kimura, 2001) appeared to be identified.

The anatomical requirements for olfactory perception are present in horses. Their noses can move large volumes of air at one breath and trap large numbers of molecules. Additionally, their nostrils are separated and point in different directions, which permits stereo-olfaction for localization (Stoddart, 1980). Furthermore, Lindsay and Burton (1983) documented the existence of a prominent vomeronasal organ in horses which is important for individual olfactory recognition.

For the present study we concentrated on social recognition through olfactory perception in mares and geldings. Given that mares constitute the core of harems, and geldings integrate well into social horse groups, both should be capable of social olfactory perception. Additionally, for geldings the social interest in olfactory cues may outweigh reproductive interests.

Accordingly, we conducted two separate tests. Test one is built on the hypothesis that mares and geldings recognize their conspecifics' group membership through sniffing their faeces (named: Social Test). For this test horses were confronted with their own faecal samples, with those of their group members, and those of unfamiliar female and male horses. Test two approaches the hypothesis that mares and geldings can determine which member of their group was the donor of a faecal sample through olfactory perception (named: Individual Test). Here we confronted horses with faecal samples from their group members in random distribution. As controls for both tests we tested their behaviour towards visually identical samples of soil and individual sheep faeces.

Methods

Animals

We investigated the behaviour of 35 horses, 25 horses for the Social Test and ten for the Individual Test. They comprised 27 warmblood horses and six ponies of mixed breeds, as well as one draught horse, and one thoroughbred horse. There were 23 mares and twelve geldings, and all were aged between four and 27 years. All horses were individually identified by their brands and coloration. Most horses used for the Social Test (N = 20) were kept in individual boxes overnight but turned out in groups numbering from two to eleven horses during the day time. In contrast, one social group for the Social Test (N = 5) and the horses for the Individual Test (N = 10) were kept in open stabling day and night. In two groups two horses were genetically related (mother–daughter relationship). Sleeping areas or box stalls included a bedding of straw or wood shavings. The daily feed of the horses comprised hay twice a day, plus a compound feed three times a day for the boxed horses, and hay twice a day, plus a compound feed once a day for the open stable horses. In addition they had access to grass on their pastures. Horses which were tested among each other were identically housed and fed.

Faecal samples

Prior to testing we picked up approximately two mugs worth of the freshest faecal samples from each of the test horses. We made use of the horses` habit of defecating after being fed, and started collection half an hour after feeding time. The faecal samples were all collected within one hour of each other. They were collected with unused plastic bags or one-way gloves and immediately tightly wrapped up in the bags, to minimise the evaporation of volatile substances and to prevent any possible odour contamination from the collecting person.

Experimental set-up

Tests were conducted in a clean riding area, or in the clean feeding area of the open stable. In both cases faeces have always been immediately cleared away. Horses were confronted with four samples of faeces (six for group 1 of the Individual Test), randomly placed in a line, 1.5 meters apart from each other, and 6 meters away from the starting position (Fig. 1). For the Social Test the faeces were from the test horse itself, from another group member, from an unfamiliar male and an unfamiliar female horse. For the Individual Test, horses were confronted with their own faeces and faeces from each member of their own group.

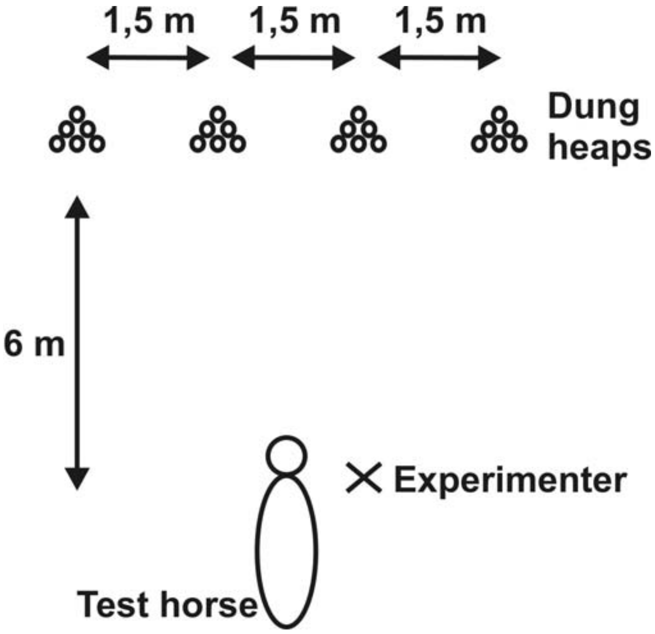

Fig. 1 Experimental set-up Social and Individual Test

Experimental procedure

Prior to the test, all the horses had regular access to the experimental area and were thus well habituated to the surroundings. After the faeces were placed in a line, the horses were tested in random order. Each horse was led to the starting position by experimenter 1. The experimental area was closed. Then experimenter 1 released the horse and left the experimental area. The horses were given a total time of 2 minutes (3 minutes for the six heaps in the Individual Test of group 1) to move freely around in the test area and sniff at all the presented samples in whatever order, and for however long they chose within this total time of 2 (or 3) minutes.

The individual horses differed in their propensity to approach the samples. To ensure that all horses had equal opportunities for sniffing responses and to generate a reliable, comparable data set, it was necessary for experimenter 1 to re-enter the experimental area and lead the horses towards the heaps they had not voluntarily approached, and give them 30 extra seconds to sniff at the respective samples if they chose to. Each of the horses required extra leading at some time during the experimental procedure. On average, the horses needed to be lead to 34% (SD = 18%) of the equine faecal heaps, 23% (SD = 24%) of the soil heaps, and 29% (SD = 21%) of the sheep faeces, which resulted in sniffing responses in 16% (SD = 11%) for equine faecal heaps, 9% (SD = 13%) for the soil heaps, and 22% (SD = 15%) for the sheep faeces. The horses were not forced to sniff at the samples. If they did not choose to sniff after being led to the pile, the behavioural reaction was counted as “0”.

Finally, experimenter 1 led the horse out of the test area. Experimenter 2 was outside the experimental area, and documented the experiment with continuous video recording. She also wrote down the number, location, repetitions and order in which the respective faecal heaps were approached, as well as the sniffing times. The recording of sniffing time started from when the horses lowered their noses to 15 cm or closer to the pile, up until the noses left this range again. The Individual Test was repeated ten times over 21 days to cover the oestrus cycle of the participating mares.

Control samples and control procedure

Two control tests, with soil and with sheep faeces instead of horse faeces, were conducted using the horses from the Individual Test.

To control for a possible visual orientation of the test horses while sniffing at faeces, we conducted a control trial in which we replaced the faecal samples with similar looking soil heaps, which were arranged in the same way as the faecal samples had been before. Thus the test horses were confronted with as many soil samples as there were members in their group.

Additionally, we controlled for other olfactory cues generally present in faecal samples, such as fatty acids, alcohols, aldehydes, phenols, amines and alkanes, and for a possible effect of the samples’ position. To do this we replaced the horses’ faecal samples with samples of fresh sheep faeces, each from different sheep, in similar manner as for the soil samples before.

In both cases quantities of the samples were matched to the horse faecal samples. Therefore, for the sheep, several faecal quantities were taken from the same animal to make up one sample. The control tests’ experimental set-ups and experimental procedures were the same as in the main test. However, in contrast to the main tests, both control situations were only tested once, so the samples remained in a constant position. Therefore, the position of the sheep faecal samples corresponded to the identity of the respective sheep.

Dominance relationships

Before starting the Individual Test, we determined the dominance relationships among the horses in the field by observing agonistic encounters, such as approaches, retreats, threats to bite or kick, bites, kicks and chases (Feist and McCullough, 1976; McDonnell and

Haviland, 1995; McDonnell, 2003). The horses were observed over 6 hours on separate days (at least three different days, with a minimum duration of 30 min. and a maximum of 150 min. each). Observation periods had to be adjusted to accommodate the horses' commitments as riding horses, but were distributed over daylight hours. The interactions of the horses were recorded continuously. For the calculation of the individual dominance scores (Table 1) we used an average dominance index (ADI) method. The ADI is calculated as follows: The dominance index per pair of individuals, w_{ij} is the number of times an individual won against or attacked a certain opponent divided by the total number of agonistic interactions in which the pair was involved with each other, thus $w_{ij} = x_{ij} / (x_{ij} + x_{ji})$. If a pair of individuals was not involved in agonistic interactions with each other, it was excluded from the analysis. The average dominance index of an individual is the average of all its dominance indices with all its interaction partners, thus $1/N \, \Sigma \, j \, w_{ij}$. A higher value indicates a higher dominance in the group (Hemelrijk et al., 2005).

Table 1 Dominance hierarchies for test horses, Individual Test

	Age	Sex	Breed	Related	ADI score	Rank
Group 1						
Billy (Bi)	18	gelding	warmblood	no	0.872	1
Sara (Sa)	22	mare	Haflinger	no	0.732	2
Farina (Fa)	23	mare	warmblood	no	0.684	3
Peppermint (Pe)	14	gelding	pony	no	0.367	4
Alexia (Al)	6	mare	Haflinger	mother	0.204	5
Anouschka (An)	20	mare	Haflinger	daughter	0.186	6
Group 2						
Francis (Fr)	13	mare	warmblood	no	0.895	1
Traum (Tr)	14	mare	warmblood	no	0.635	2
Miss Lala (Mi)	27	mare	pony	no	0.316	3
La Belle (La)	14	mare	pony	no	0.154	4

Statistics

Statistical analysis as well as the depiction of the data was done with the statistical software SPSS 15 and the R-Project statistical environment **(**2010). All tests used were two-tailed. We analysed the frequencies with which horses sniffed the faeces by applying Binomial-Tests (SPSS). For further comparison we equalized the individual habits of the horses by converting the time each horse spent sniffing each pile of faeces to percentages, i.e. we divided each horse's sniffing times on each sample in one trial multiplied by 100% by the total time spent sniffing in this trial. Then we derived the individual percentage of sniffing time the horses spent at each particular donor's faeces by adding the sniffing responses of the ten test days and dividing them by the number of test days (i.e. ten). For the Social Test the KS-tests showed the data to be consistent with a normal distribution. Subsequently, we applied General Linear Models (GLM), for multivariate testing for any possible effect of gender and age, and for the comparison of the main behavioural data sets. Effects on the data of the Individual Test and its two control trials were analysed with Generalized Linear Models (also abbreviated: GLM) (Nelder and Wedderburn, 1972; McCullough and Nelder, 1989), which are models for parameter estimation for continuous or categorical response variables with distributions other than parametric. We continued our analysis for the Individual Test by comparing the sniffing responses the horses received from their group mates and the aggressive behaviour horses displayed among each other. For group 1, in which the respective group members showed no significant differences in sniffing responses, we tested the hypothesis that individual levels of aggressive behaviour displayed among particular pairs of horses may correlate with individual sniffing responses. For group 2, in which particular horses received significant sniffing responses, and which had one particularly aggressive horse, we compared the total amount of aggressive behaviour horses displayed towards specific group members with the received total amount of sniffing responses. We did this by applying multifactorial GLMs, with the donor's identity as factors.

Results

Sniffing frequencies

In general the mares and geldings in this study were highly motivated to pay attention to faecal samples, as they used the opportunities to sniff the faeces they were confronted with significantly above the chance level of 50% (Social Test, Binomial-Test: $n = 100$, $p < 0.001$; Individual Test, Binomial-Test: $n = 520$, $p < 0.001$). In the Social Test their sniffing response was equally significant when they sniffed own faeces, those of familiar horses, and those of unfamiliar horses from the opposite and the same sex (all: Binomial-Tests: $n = 25$, $p < 0.001$). During the 10 repetitions of the Individual Test their interest did not significantly decrease because the test day, i.e. the trial number, did not have any effect on the sniffing times (GLM, test-day: $n = 530$, $t = 0.13$, $p = 0.89$).

Social Test

General effects on sniffing time

For the Social Test we evaluated whether horses paid different amounts of attention to their conspecific's faeces depending on the latter's group membership and sex. We measured the amount of time horses sniffed their own faeces, their group members` faeces, and unfamiliar faeces from horses of the opposite and of the same sex. In all data the age and gender of the test horses did not have any significant effect (GLM, age: $N = 25$, $t = -0.76$, $p = 0.46$; sex: $N = 25$, $t = 0.41$, $p = 0.69$).

Comparison between own faeces, faeces from familiar horses, unfamiliar horses of opposite sex and unfamiliar horses of same sex

Horses sniffed their own faeces least (mean sniffing time 15 sec., SD = 9 sec., Fig. 2). The mean sniffing time at their own faeces significantly differed from the sniffing times at faeces from familiar group members (mean sniffing time 27 sec., SD = 15 sec., GLM: t = -642.06, p < 0.001), those of unfamiliar conspecifics of opposite sex (mean sniffing time 28 sec., SD = 14 sec., GLM: t = -684.42, p < 0.001) and those of unfamiliar conspecifics of same sex (mean sniffing time 30 sec., SD = 12 sec., GLM: t = -625.08, p < 0.001). However the sniffing time spent on faeces of familiar horses and those of unfamiliar horses of opposite sex and same sex did not significantly differ between the samples (GLM, familiar – unfamiliar opposite sex: t = -1.80, p = 0.09; familiar – unfamiliar same sex: t = -1.02, p = 0.32; unfamiliar opposite sex – unfamiliar same sex: t = -1.80, p = 0.08).

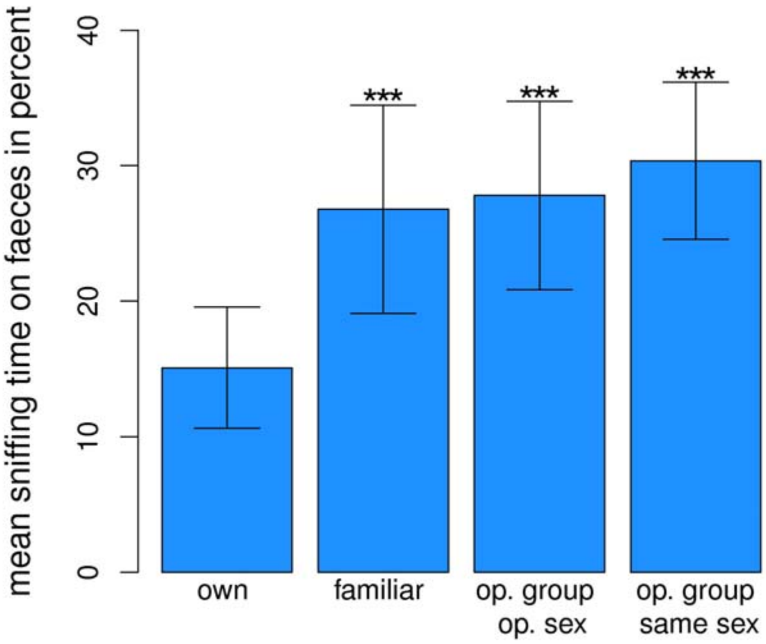

Fig. 2 Sniffing times Social Test. Fig. 2 depicts the variability around the mean of the percentages all horses sniffed at the faeces in the Social Test. Sniffing responses to familiar horses' faeces, and those of unfamiliar horses of the opposite and same sex significantly differ from those on own faeces. *** symbolise significant deviations from sniffing own faeces for p < 0.001. The error bars depict the standard deviation of the particular data set.

Individual Test

General effects on sniffing time

For the Individual Test, we investigated whether horses would discriminate between the faeces of their own group members. In a general comparison, we found that the identity of the faecal sample's donor caused a significant difference in sniffing time (GLM: N = 520, t = 425, p < 0.001), but none of the other possible effects on the sniffing time, such as the test day (GLM: n = 520, t = -0.13, p = 0.89), the position of the faeces (GLM: n = 520, t = 1.42, p = 0.16), or the order in which the faecal samples were collected (GLM: n = 520, t = 1.1, p = 0.27) were significant.

Group 1: Sniffing responses

When analysing the duration horses sniffed their group members’ faeces, in group 1, none of the particular donors’ faeces was sniffed significantly longer from all their group mates (GLM: n = 160, t = 1.208, p = 0.23, Fig. 3-1a).

Group 1: Sniffing responses versus individual aggressive behaviour

But differences occurred on an individual level, when comparing the aggressive behaviour horses displayed towards each other and the sniffing responses they received from their group mates. In group 1, we compared individual sniffing responses with individual levels of aggressive behaviour displayed among particular pairs of horses. The pairwise comparison between aggressive behaviour displayed and sniffing responses received was significant at the group level (GLM: n = 30, t = 2.673, p = 0.01, Fig. 3-1b). When analysing for pairwise linear relationships between the sniffing responses received and aggressive behaviour displayed for particular horses, the results for Farina (GLM: t = 2.337, p = 0.03), Alexia (GLM: t = 3.360, p = 0.003), and Sara (GLM: t = 2.505, p = 0.02) were highly significant, for Billy (GLM: t = 1.937, p = 0.06) and Peppermint (GLM: t = 1.908, p = 0.06) weakly significant, but not significant for Anouschka (GLM: t = 1.313, p = 0.2).

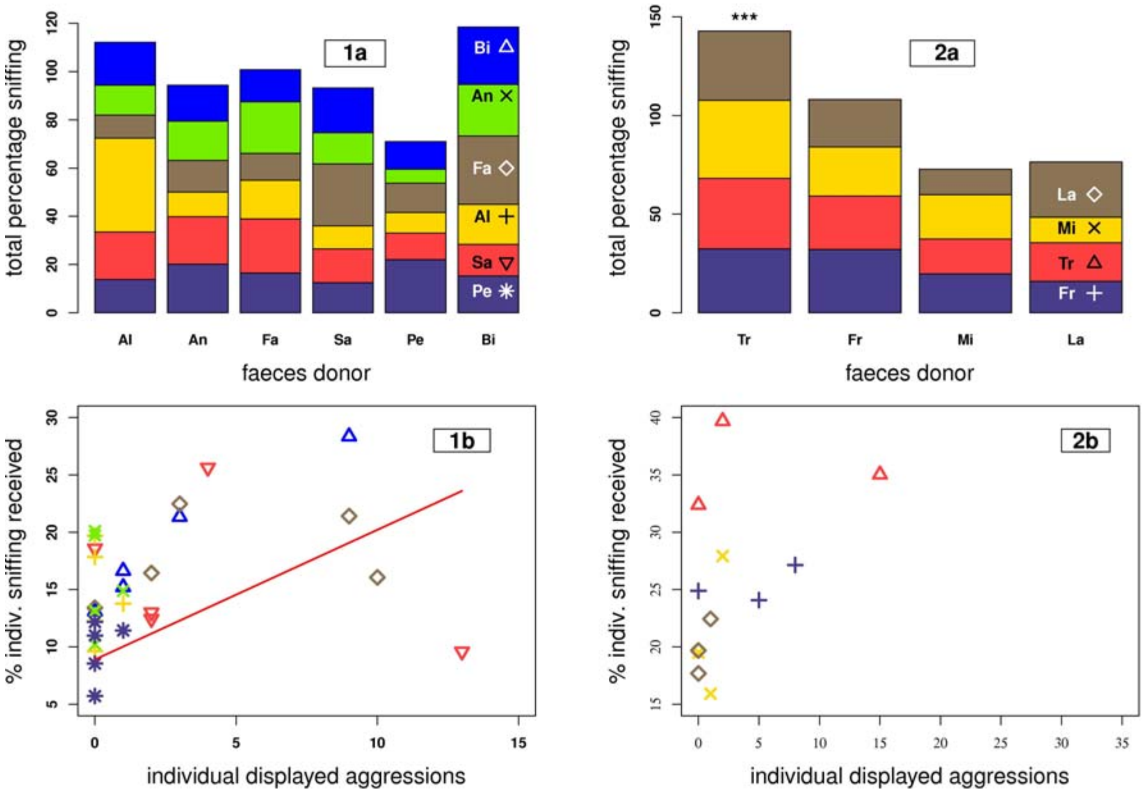

Fig. 3 Sniffing times Individual Test. Fig. 3-1a (group 1) and 3-2a (group 2) depict the respective donor’s faeces listed on the x-axis and the piled up total percentages of sniffing durations the test horses received from their group members on the y-axis. The horses’ identities are abbreviated (see table 1). *** symbolise significance for Traum ($p < 0.001$). Fig. 3-1b (group 1) and 3-2b (group 2) depicts the linear relationship of received, individual sniffing durations and displayed individual aggressions from particular horses, which is significant for group 1 on the group level ($p = 0.01$). In group 2 a positive linear relationship between received sniffing duration and displayed aggressions is again true for Traum ($p < 0.001$), but not for the whole group. Note that the sniffing on own faeces has been excluded from the comparison since horses cannot display aggressions against themselves

Group 2: Sniffing responses

In group 2 the horses significantly sniffed at particular group members' faeces (GLM: n = 160, t = 3.34, p < 0.001, Fig. 3-2a).

Group 2: Sniffing responses versus total aggressive behaviour

For group 2 the level of sniffing responses received showed a significant linear relationship to the total amount of aggressions displayed towards particular horses (GLM: n = 16, t = 5.758, p < 0.001). One particular horse (Traum) significantly displayed most of the aggressive behaviour and also received the significantly highest amounts of sniffing responses (GLM: t = 5.958, p < 0.001).

Control test soil

In the soil control test no significant sniffing response to any of the particular soil heaps was reported from the horses. The horses' sniffing duration did not significantly differ with the heap positions, i.e. not for the specific samples (GLM: n = 52, t = 1.39, p = 0.17).

Control test sheep

Also in the sheep control trial, the sniffing durations for the position, and thus the respective sheep donors` identity, did not vary (GLM: n = 52, t = -0.13, p = 0.9).

Discussion

As expected from the horse's social structure (Klingel, 1972; Tyler, 1972; Berger, 1977; Linklater, 2000; Moehlman, 2002; Feh, 2005) and its frequently observed marking behaviour (Linklater, 2000; Kimura, 2001; King and Gurnell, 2007) mares and geldings in this study are highly motivated to sniff faeces. They consistently pay attention to faeces they are confronted with, but invest more time in sniffing their conspecifics` faeces than their own. They tend to pay more attention to faeces from unfamiliar horses and most attention to unfamiliar faeces from horses of the same sex, which is not significant in this study, but corresponds with findings in stallions (Stahlbaum and Houpt, 1989; Rubenstein and Hack, 1992). Furthermore, in the Individual Test, the interest in the faeces of a group mate corresponds to the level of aggression they receive from that donor of the faecal sample. The more aggressions they receive from the respective horses the more they sniff the faeces of particular group mates. In group 1, the horses` individual sniffing responses are positively related to individual levels of aggressive behaviour displayed among particular pairs of horses. In group 2, one horse significantly received most sniffing responses and was particularly aggressive towards all the other group mates. Here the sniffing responses received show a significant linear relationship to the total number of aggressions displayed towards particular horses.

The first hypothesis, of horses being able to discriminate the group membership and the sex of faecal donors, can not be fully supported by this study even though the results hint at analogy to previous studies. Here horses clearly discriminated their own faeces from their conspecifics, but made no definite distinctions between the sexes or familiar and unfamiliar donors.

In contrast, hypothesis two, on the horse's ability to discriminate individual group mates by the smell of their faeces, can be supported. We suggest that the positive linear relationship between invested sniffing time and received aggressions from specific faecal donors indicate the recognition of potential competitors among group mates via the smell of their faeces in horses.

For both tests the horse's sniffing behaviour is not affected by the horse's sex, social rank, and age, the test day or, in general, the position of the faeces. The social rank has been calculated from the group members` aggressive interactions as well as from their approach behaviour (please compare to method section). This explains why a significant linear relationship for the sniffing durations exists with aggressive behaviour but not with the social rank of the horses. The lack of preference for specific positions indicates that the horses from this study were not strongly affected by position and side biases effects when sniffing faeces (see for review: Mandal et al., 2000).

To check for a possible visual effect, we conducted a control trial using soil samples instead of faeces because the limited visual acuity of horses (Timney and Keil, 1992) which could have resulted in confusion between faeces and soil heaps of similar size and colour. We also controlled for other olfactory cues generally present in faeces, such as fatty acids, alcohols, aldehydes, phenols, amines and alkanes, by exchanging the horse faeces with sheep faecal samples. To conclude, in the control tests we observed that horses are attracted by both sheep faeces and soil heaps, and thus by visual and olfactory cues. But, in contrast to their behaviour while sniffing horse faeces, there is no difference between the times spent on specific samples. This suggests that either cues for the identification of specific samples are missing in soil heaps and sheep faeces, or horses realise that the perceived cues are not of particular importance to them.

Variances from the overall correlation between sniffing times and aggressive behaviour may be affected by individual likes and dislikes as well as social hierarchies. These effects may be most apparent for the lowest ranking horses, which usually avoid displaying aggressive behaviour, as well as top ranking horses, which seldom receive aggressive actions and often do not have to compete for resources. Thus, for high ranking animals the cost of aggressive actions may outweigh the benefits (Pusey and Packer, 2003; Flack et al., 2005). On the other hand, dominant animals need to invest in affiliative behaviour for the maintenance of social bonds (Aureli and de Waal, 2000; de Waal and Tyack ,2003).

However, the physiological mechanisms of olfactory recognition through faeces are, as yet, unknown. The relevant substances could either be (1) non-volatile or (2) volatile. The first could act as primer and the latter as releaser pheromones (Kimura, 2001). Additionally, the memorisation of different scents may be affected by differing diets, which we counteracted by testing horses on similar diets for the present study. Furthermore, the horses from group 1 in this study showed an individual positive relationship between sniffing the faeces and received aggressions from particular horses. This suggests that their discriminative abilities cannot be explained by a possible steroid hormone level connected to the horses` aggressive behaviour, and thus the social position, alone (see for review: Mormède et al., 2007).

Whether individual recognition could be mediated by the presences of individual proteins similar to major histocompatibility complex proteins (MHCs) or major urinary proteins (MUPs) in faeces is to date unknown. It remains to be seen whether such proteins can be expressed, secreted and broken down by bacteria in the gastrointestinal tract.

In the future, it will be worthwhile investigating the horse's olfactory individual recognition by further behavioural tests and considering the crucial faecal components, their genetic expression, transportation to the gastrointestinal tract, excretion into and transportation through the gut, as well as the mechanism of olfactory recognition in horses. It may also be interesting to control for possible effects of kinship on the olfactory recognition. Even though mature horses disperse from their natal group in most cases, kin based recognition could still take place.

Acknowledgements

We wish to thank Katherine Albro Houpt for discussions on horses' dominance relationships, Christine Nicol for discussing the scientific background, Juergen Heinze and Bernd Kramer for helpful suggestions, Elisabeth D'Antoni and Kate Farmer for language corrections, Knut Krüger for the analysis in "R statistical environment" and all the owners of the horses who allowed us to test their horses and even helped with the tests. The study was supported by an HWP II grant of the University of Regensburg and an Excellence grant of the Bavarian Government. We declare that the experiments comply with the current laws of the country in which they were performed.

CHAPTER 7

The horse's (*Equus caballus*) propensity to use humans as local enhancement cues and adjust to the focus point of human attention

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Manuscript submitted to Animal Cognition

Abstract

We evaluated the propensity of horses (*Equus caballus*) to use human local enhancement cues and react to the focus of human attention when making feeding decisions.

We observed 60 horses in approaching a bucket with feed in a three-way object choice task when confronted with a) an unfamiliar or b) a familiar person in 6 different situations: a squatting position with alternating (1) or permanent gaze (2), in a standing position with permanent (3) or alternating gaze (4), or in a back-turned position gazing away, either a few meters from the bucket (5), or at the bucket (6).

When the person remained next to the correct bucket and faced the horse the correct bucket was chosen significantly above chance. However, when the test person turned around, and even more, when he/she was turned and distant from the buckets, their performance declined.

In the turned person situations the horses approached a familiar person and walked towards their focus of attention significantly more often than with an unfamiliar person. Additionally, in the squatting and standing person situations, some horses approached the person before approaching the correct bucket. This happened more often when the person was familiar.

We therefore conclude that horses can use humans as a local enhancement cue independently of their body posture or gaze consistency when the persons face them and horses seem to orientate on the attention of familiar more than of unfamiliar persons. We suggest that socialization and training improve the ability of horses to read human cues.

Keywords

Human-horse interaction, horse, attention-reading, position, familiarity

Introduction

This study focuses on the propensity of a domestic species, the horse (*Equus caballus*), to react to the focus of human attention in feeding decisions depending on the body posture and familiarity of the test person. Recent studies revealed large differences between species in recognising the focus of human attention, with dogs (*Canis lupus familiaris*) out-performing other species, including chimpanzees (Hare et al., 2002; Gácsi et al., 2004). The partially contradictory studies by Hostetter et al. (2007), Gácsi et al. (2004), Gácsi et al. (2005), Theall and Povinelli (1999), and Virányi et al. (2004) provoked lively discussion over whether to assess the studies on a behavioural or a cognitive representational level. While behavioural explanations (Povinelli and Vonk, 2003) emphasise the animal's ability to learn to use the focus of attention as a cue, cognitive explanations highlight the animal's understanding of the signaller's intentions (Tomasello et al., 2003). In humans and primates sensitivity to different states of attention has been proposed as the phylogenetic basis for theory of mind and language abilities (Povinelli and Eddy, 1996; Tomasello et al., 2003; Itakura, 2004). Generally, the ability to take advantage of other species' state and focus of attention is advantageous for the avoidance of predators and competitors, for resource allocation, and for communication. For example, hand raised ravens follow the direction of an experimenter's gaze towards distant locations and behind obstacles. As the bird's gaze-follow responses were affected by the type of the gazing, the age of the animals and also by learning, the authors suggested that cognitive mechanisms could direct the raven's visual co-orientation (Bugnyar et al., 2004). For prey animals, such as horses, a cross-species ability to use the focus of attention as a cue would provide particular survival benefits (Goodwin, 2002).

The superiority of dogs over other species in this area has been attributed to domestication (Call et al., 2003). Bräuer et al. (2004) and Schwab and Huber (2006) found that dogs adapted their behaviour according to the state of a human's attention, and Gácsi et al. (2004) found that dogs could distinguish between different states of human attention in fetching games and begging behaviour. Dogs recognised various cues associated with human attention, and the human's head orientation seemed to be an especially important factor. Dogs were more likely to choose those humans who oriented themselves towards the animals both in a food-begging situation and a toy-fetching game. Some dogs brought the object to the front of the person even when they were facing away, and they begged from the person facing them in preference to the person facing away (Gácsi et al., 2004; Virányi et al., 2004).

Such tasks have been determined to be cooperative in nature (Hare, 2001), and domestic species have especially been bred for cooperativeness with humans (Miklósi et al., 2003; Gácsi et al., 2005). The domestication theory has been supported by the fact that cooperatively working dog breeds outperformed independently working breeds in such tasks (Gácsi et al., 2009a). However, the strongest support for the domestication theory comes from the famous silver fox study. Foxes selected for their tameness and willingness to approach humans showed some characteristics of dog morphology and were better at giving their attention to humans, as well as in reading human cues, than a group of wilder foxes (Hare et al., 2005).

Nevertheless the significance of domestication for cognitive skills in reading human states of attention remains controversial, and recent studies in wolves and chimpanzees have provided mixed results. Theall and Povinelli (1999) reported that chimpanzees did not discriminate between an attentive (eyes open) and inattentive (eyes close) human, whilst Hostetter et al. (2001) showed that chimpanzees did discriminate between an attentive (facing toward) and inattentive (facing away) experimenter. In the case of dogs the head direction of humans seemed to be more important than the gaze, when comparing the performance of guide dogs of blind owners to pet dogs of sighted owners (Gaunet, 2008; Ittyerah and Gaunet, 2009). Furthermore, some studies showed wolves to be inferior (Hare et al., 2002; Miklósi et al., 2003; Virányi et al., 2008) and others superior (Udell et al., 2008) to dogs in their abilities to read human cues and recognise their state of attention.

Dogs may have inherited the ability to read human given cues from wolves through a process of selection and convergent evolution, or, as in chimpanzees, may have gained it through socialization to humans and training (Hare et al., 2002). Itakura et al. (2001) found that if chimpanzees were brought up by humans it made little difference in a food location task whether cues were given by another chimpanzee or a human. In a recent study Gácsi et al. (2009b) elaborate on a delayed emergence of socialisation in hand reared wolves compared with dogs. Dog pups outperformed hand reared wolf pups, but in adult animals hand reared wolves were as skilled in utilizing human pointing gestures as dogs.

As animals are tested by human experimenters in object choice tests, their relationship with the human should be considered. The familiarity of a human experimenter may be of central importance when animals are tested. Socialisation and training effects may be the key factors in the differing responses to familiar and unfamiliar persons in horses (Hausberger et al., 2008), as has been reported for approaches to unknown and known persons in dogs (Rappolt, 1979). Results for active approaches and interactions with known and unknown persons by cattle are partly contradictory. Rousing and Waiblinger (2004) found that although the cows' approaches to a test person were not affected by the familiarity of the human, the latency to touch an unknown person was shorter than with a known person, which they suggest could be caused by the cow's curiosity for novelties. On the other hand Breuer et al. (2003) found no difference in the interactions with familiar vs. unfamiliar humans in heifers. She claimed that positively handled heifers approached humans faster and interacted more with the person than their negatively handled counterparts (Breuer et al., 2003). Heart rate in cats differed according to whether they were petted by familiar or unfamiliar persons (Slingerland et al., 2008). Generally, horses show similar reactions towards familiar and unfamiliar humans (Henry et al., 2005; Lansade and Bouissou, 2008), and they discriminate familiar and unfamiliar persons at the same speed (Stone, 2010), which may be caused by the horse's generalization of positive and negative experiences from familiar to unfamiliar persons (Hausberger and Muller, 2002; Krueger, 2007; Hausberger et al., 2008). Some authors propose that early contact with foals can lead to positive and negative associations with humans (Landsade et al., 2004, 2005; Henry et al., 2005, 2006). Even the handler's relationship to the foal's dam shapes its behaviour towards humans in the future (Henry et al., 2005).

Horses are indeed a good model species on which to test the effects of domestication, socialisation, and training on the animal's ability to use the focus of human attention as a cue. During domestication, 2.500 – 5.000 years ago (Clutton-Brock, 1981), horses might have been selected for their ability to respond to human cues. Like goats, horses are not kept in close proximity to humans, but humans have relied on the performance of horses in battle, for farming and for transportation for centuries. The selection and training of horses for fast and subtle reactions to human cues has been of major importance from ancient times (Xenophon 426 – 355 b.C.) up until today. The skill of horses in responding to human facial and gestural cues is known from the case of Clever Hans in the early 20th century. Clever Hans was claimed to have the arithmetic skills of a 12 year old child and other extraordinary skills. Although subsequent observations revealed that he could not count, he was nevertheless extremely skilful in reading subtle human facial expressions and body movements, which he used to decide when to begin tapping with his hoof and when to stop. He even generalized the cues given by his trainer to unfamiliar persons (Pfungst, 1907).

Surprisingly few studies have addressed the horse's abilities to respond to human given cues, although horses have been shown to be able to read attention states when provided with body orientation, head orientation or gaze cues, and some horses walked around an "inattentive", turned-away person to attract attention (Proops and McComb, 2010). There have been two further studies published on the horse's performance in object choice tasks. Two out of four horses could use touch cues and one horse could use pointing cues in the McKinley and Sambrook (2000) study. Furthermore horses were able to use pointing gestures from both a standing and a squatting person when her hand was briefly held close (~ 10 cm) to the target, or her arm was permanently held (either close ~ 10 cm or distant ~ 80 cm) in direction of the target (Maros et al., 2008).

The present study is the first to address possible effects of human body posture and gaze, as well as the familiarity of the person, on the horse's propensity to use human cues for finding food. As the horses were allowed to observe the process of feed being placed in the bucket, valuable information will be gained on how horses prioritize their own knowledge compared to other information. In some previous studies, cueing by persons was necessary to encourage the horses to perform in object choice or in attention tasks (McKinley and Sambrook, 2000; Maros et al., 2008; Proops and McComb, 2010). However, there is only limited knowledge of how the horse's use of its own memory is influenced by the local enhancement of the presence of the person, the person's familiarity and the person's body posture, or potentially distracting cues, such as the person's gaze and person facing away from the focus of the test. We measured horses' choices in approaching a bucket with feed in a three-way object choice task when confronted with a) an unfamiliar or b) a familiar person in 6 different situations. The person was either in a squatting position and offering an alternating (1) or permanent (2) gaze, in a standing position offering a permanent (3) or alternating (4) gaze, or in a back-turned position gazing horizontally away, either distant, a few meters away from the bucket (5), or proximal, at the bucket (6).

For this study we hypothesize:

a) that the horse's choice of feed bucket may be affected by the person's body position (i.e. squatting, standing),
b) that the constancy of the person's gaze when facing the horses affects their choice (i.e. using permanent or alternating gaze),
c) that the horse's performance differs depending on the person's focus of attention (i.e. when he/she faces the horse or turns away),
d) that horses use humans as local enhancement cues for finding feed. When the person moves away from the feed buckets, and thus avoids providing local enhancement cues for a particular feed bucket, the horse's choice for a bucket should diminish, and their orientation on the person's focus of attention should be enhanced.
e) Finally, that the horse's performance may differ when tested with unfamiliar rather than familiar persons. This aspect in particular may provide data for the hypothesis that the horse's performance in object choice tasks is affected by their socialisation and training.

In addition to gaining new insights into the horse's use of human attention as a cue, an understanding of the influence of the human's body posture, gaze and familiarity on the horse's behaviour towards humans would facilitate the selection of suitable test persons and test situations in cognition tasks, or support the claim that persons should be removed from studies that should not be affected by human cueing.

Material and Method

Animals

We investigated the behaviour of 60 horses, including 27 Standard-breds, 1 Arab, 1 Arab-Trakehner-mix, 8 Trotters, 7 Haflingers, 4 Icelandic horses and 12 ponies. Among these were 2 stallions, 29 mares and 29 geldings, all aged between 3 and 28 years (mean age: 13.27, SD = 6.1). They were in 14 different locations, and all the horses were either constantly kept in open stabling with permanent access to pasture, or kept in social groups on pasture during the day and stabled in boxes overnight. In 9 locations we tested the horses with a familiar person and in the other 5 with an unfamiliar person. The horses' sleeping areas included bedding of straw or wood shavings. All horses were in excellent feeding condition; their feed was composed of hay twice a day and a compound feed once or twice a day, and in addition they had access to grass in their pastures. As far as was known, all horses had comparable histories with humans. They were all leisure horses trained in a conventional way.

Experimental area

We conducted the study in a part of the paddock or riding arena familiar to the horses. For the test, a 20m x 20m area was fenced off to prevent horses other than the particular test horse from seeing the test area from the outside. Nervous horses were tested in the proximity of a group mate that had already finished the test. Three feed buckets were placed on the ground 2 metres away from each other in a curved alignment (Fig.1) to ensure equal walking distances to all three buckets. A test person placed him/herself behind a randomly chosen bucket and was either

a) unfamiliar, for 32 of the test horses (19 horses were used for experiment 1 (N = 19) and 12 for experiment 2 (N = 12), plus 1 substitute for a side biased horse), or,
b) familiar, (through several years contact), for 28 of the test horses. Here 16 horses participated in experiment 1 (N = 16) and 12 horses in experiment 2 (N = 12). The familiar person was either the owner or the main caretaker of the particular horse.

An assistant led the horse by its halter to a central starting position. The distance from the starting position to the buckets had to be adjusted to the different conditions in the particular stable, but was always between 6 and 9 meters. Pieces of apple and carrot were used as incentives for the horse to approach the bucket.

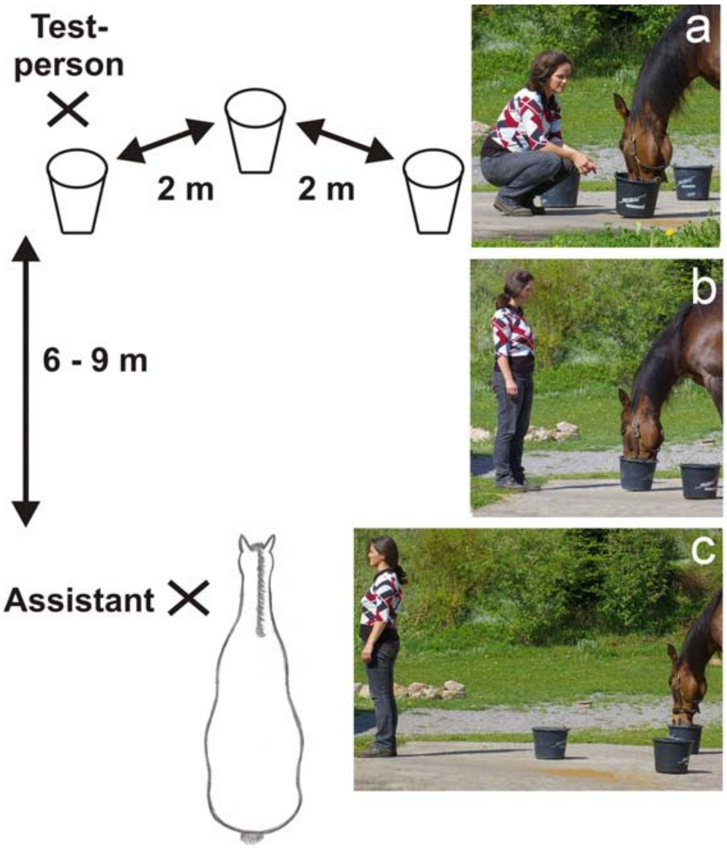

Fig. 1 Experimental set-up. Test situations: a) squatting person b) standing person c) turned-distant person

Habituation phase

Three persons took part in the test, a test person who gave the cues, an assistant who recorded the data, and another assistant who handled the horse. To ensure that all horses would approach the feed buckets reliably, and that the buckets would be equally marked with saliva, the handling assistant led each horse by its halter towards the buckets and allowed it to feed from all three. The horses fed until the buckets were empty and were allowed to check that there was no feed left in any of the three buckets. The assistant then led the horse to the starting position. He/she always approached and handled the horses by their halters from the left, as all the horses were used to being handled from this side. At the starting position the horse was offered feed from the test person's hand. Then the test person walked with further feed in his/her extended hand and placed it in one of the three buckets. Very cautious horses were allowed to follow the test person until he/she had reached the position behind the bucket at first, and later on they were held at the starting position by the assistant, as were most horses right from the beginning. The assistant faced a predetermined fixed point throughout this procedure, and released the horse, turning his/her back to the feed buckets and remaining in this turned position at the starting point. The horse was allowed to move freely in the experimental area. After it had made its choice of a certain bucket or approached the test person, the assistant turned around, approached the horse, took it by its halter, turned it either to the left or to the right (counterbalancing for side effects) and led it back to the starting position. The habituation phase was completed when the horses approached the feed buckets spontaneously and without hesitation when their name was called. The horses rapidly learned that the turned assistant would not intervene in their choice making procedure and ignored her/him during the tests. On average horses needed two to three trials but never longer than six trials to reach criteria.

Test phase

When the horse walked freely towards the feed buckets and the person after being released, we continued with the test phase. During the test phase the test person approached the horse, showed it some feed, walked towards one of the three buckets in a semi-random but predetermined order (making sure not to approach the same bucket more than twice in a row), and squatted down to place the feed in the bucket. The test person stayed in contact with the horse throughout the whole process by calling its name to keep its attention. The person then positioned him/herself behind one of the three buckets. In 6 test situations the persons could:

1) remain squatting behind the filled bucket and look back and forth between the bucket and the horse, (squatting/alternating gaze, Fig. 1a), or
2) squat, but hold visual contact to the horse without looking into the bucket (squatting/permanent gaze), or
3) stand behind the filled bucket and hold visual contact to the horse, without looking into the bucket (standing/permanent gaze, Fig. 1b), or

4) stand and look back and forth between the bucket and the horse (standing/alternating gaze), or
5) turn her back to the bucket and look horizontally away from the horse, then walk 3 meters towards the centre of the three buckets and remain in a standing, turned position (turned/distant, Fig.1c). This was to examine the horse's response to the focus of human attention without strong local enhancement effects for particular feed buckets.
6) Finally the person could stand in a turned position directly behind the bucket (turned/proximal).

The assistant released the horse, as in the habituation phase. The horse was then allowed to move freely in the test area and make its choice between the three buckets. It was allowed to eat the feed when it correctly approached the bucket in which the test person had previously placed the feed, but when it approached the wrong bucket it did not receive any food and was calmly led back to the starting position. A third person outside the test area documented the horse's behaviour on paper and continuously on video.

We conducted two experiments, experiment 1 with the test situations 1, 3 and 5, and experiment 2 with the test situations 2, 4 and 6. Experiment 2 was conducted to control for gaze and for local enhancement effects from persons close to the bucket (experiment 2.1) and additionally for order effects (experiment 2.2). Horses were given 6 consecutive choices in each test situation. Experiment 1 was conducted in this order: first the squatting/alternating gaze (1), then the standing/permanent gaze (3), and finally the turned/distant (5) situation. In experiment 2.1 we first controlled for gaze effects, as well as for local enhancement effects from persons close to the bucket, and therefore tested 6 horses in the following order: first the squatting/permanent gaze (2), then the standing/alternating gaze (4), and finally the turned/proximal (6) situation. To control for order effects in experiment 2.2, we reversed the trial order for another 6 horses, such that the turned/proximal (6) person situation was first, then the squatting/permanent gaze (2), and finally, the standing/alternating gaze (4) situation. All habituation and test trials were conducted in a single session, which did not last longer than 20 minutes for any horse. One horse from the second experiment had to be removed because of its strong side bias.

Test persons

Two persons participated in each test, which was recorded on video and an independent third person later wrote down the horses' choices and approaches to persons. Four different test persons participated in the unfamiliar person situation of experiment 1 and another four in experiment 2. They tested the horses in random order. In the familiar person situation, the test person was the horse's owner or caretaker. The assistant was the same, unfamiliar, person for all horses in experiment 1 and 2. Test persons wore the same clothes throughout all the trials.

Data collection

We recorded each horse's choices of feed bucket, distinguishing between correct, incorrect and no choice, and calculated the percentages of correct choices (Fig. 2). Additionally, we counted the cases in which the horses approached the test person without first feeding from the buckets. Instances where horses moved into the direction of the test person and then either stood motionless and faced the person, or passed the buckets and approached the person in the squatting or standing person situations were counted as approaches. Some of the horses fed from the bucket after approaching the person standing at the correct bucket. In the turned person situation, approaches were counted as in the squatting and standing person situation and, in addition, we recorded instances of horses passing the person and walking in the direction of the person's gaze (Fig. 3).

Data processing

Horses were used only for either an unfamiliar or familiar person test, as habituation effects would have confounded the results if they were tested both in unfamiliar and familiar test person situations. One horse from the familiar person group had to be deleted from the data as it refused to participate after 3 trials in the standing person situation, another horse in experiment 2 had to be replaced as it showed a strong bias for the left side. For each trial we recorded the choice of one of the three possible feed buckets (Fig. 2), and whether the horse approached the person before feeding from the bucket (Fig. 3). Even though we conducted six repetitions for each horse in each test situation we attached greater importance to the analysis of the first trial, as here the horse's behaviour is least distorted by learning and habituation effects (Mal et al. 1993). This procedure is justified by the results of many other studies which show an animal's success to be consistent over only a few repetitions (e.g. goats: Kaminski et al., 2005; dogs and cats: Miklósi et al., 2005; horses: Maros et al., 2008). However, as wolves improved their success rate with extensive training (Virányi et al., 2008) we will give the results for the total data as well.

Statistics

We applied the statistical software SPSS 15 and the R-project statistical environment (2009) to the data analysis. The likelihood of the horses' participation in the test, i.e. of making a choice when released, was tested with Chi square tests. Then, the complete binomial data for the first choices in experiment 1, as well as for whether horses approached the persons, were tested for effects of sex and breed, as were the data from experiment 2, which were additionally tested for order effects, with Generalized Estimating Equations (GEE). Further we applied a Binomial Test with a hypothesised probability of success at 33% for the bucket choice. Thereafter we checked the total choice and approach data for normal distribution with a Kolmogorov-Smirnov-Test. As not all variables were normally distributed, we continued with applying non parametric tests. Additionally, because data sample sizes were low and standard deviations relatively high, we followed the generally accepted procedure to enhance the robustness of the non parametric test by applying exact procedures. Friedman Exact tests were used for comparisons of the data from all the test situations within the unfamiliar and familiar person situations. Wilcoxon Exact Tests were applied to compare data from the different test situations within both the unfamiliar and familiar person situations. The comparison between data from unfamiliar and familiar person situations was done with Mann-Whitney U Exact Tests. Probabilities of choosing the middle bucket in the turned person situation were calculated with Chi square tests. All tests were two-tailed and the significance level was set at 0.05, which was corrected by a Sequential Bonferroni Procedure (Holm, 1979) after multiple testing.

Results

General effects on bucket choices and approaches to persons

The horses' participation

Before we analysed our test hypotheses we studied some variables that might have affected the horse's general performance. First was the horses' motivation to participate in the tests. Horses were very motivated to participate, which derives from their highly significant level of bucket choosing, no matter whether tested with unfamiliar (N = 92, df = 4, $p <0.001$) or familiar persons (N = 80, df = 6, $p < 0.001$). Only in few trials did horses make no choice of a feed bucket (Tables 1 and 2), which was most apparent for the familiar person situation when he/she turned away and remained distant from the buckets. These "no choice" trials are pretty much in line with instances of approaching the test person and may simply be explained by the fact that horses stayed with the person or walked in the direction of his/her gaze.

Sex and breed

We then analysed the total data sets for possible effects of the horses' sex and breed on the first bucket-choices and on whether they approached the test persons. For the squatting, standing, turned, unfamiliar and familiar person situations in experiment 1 and 2 we found neither of these were significant (first bucket choice: N = 180, sex: robust Z = 2.408, p = 0.92, breed: robust Z = 3.01, p = 0.99; approach to person: N = 157, sex: robust Z = 1.26, p = 0.9; breed: robust Z = -0.89, p = 0.81). Therefore the sex and breed of the horses are not taken into account for further analysis.

Order effects

Furthermore, the order did not influence either the outcome of the experiment 2 (first choice: N = 72, robust Z = -0.03, p = 0.51; total choice: N = 72, robust Z = -0.86, p = 0.8), or the likelihood of the horses approaching the persons (N = 72, Z = -0.55, p = 0.62), or walking in the direction of the person's gaze (N = 72, Z = -0.74, p = 0.5; Fig. 3). Therefore data for experiments 2, part 1 and 2, were analysed and presented in total for each test situation.

To clarify the complex results section, the main conclusions of each sub-point are given first, followed by the detailed results as needed.

Effect of the person’s body position (i.e. squatting, standing)

The test person’s body position did not have any consistent effect on the horses’ performance (i.e. there was no significant difference between the squatting and standing person situations; Fig. 2; Tables 1 and 2) in the first choices. Although the total data performance was significantly better with the squatting person than the standing person in the familiar person condition. Detailed results for experiment 1 and 2 are given as follows:

For the first bucket choices in experiment 1, horses chose the correct bucket significantly above chance level (squatting: unfamiliar: $N = 19$, $p \leq 0.001$; familiar: $N = 15$, $p \leq 0.001$; standing: unfamiliar: $N = 19$, $p \leq 0.001$; familiar: $N = 15$, $p \leq 0.001$, Table 1; Fig. 2a). Horses chose the correct bucket slightly, but not significantly, more often in the squatting than in the standing person situation (all $p > 0.05$).

Comparisons between the total bucket choices from experiment 1 revealed no difference between the squatting and standing person situation when horses were tested with an unfamiliar person ($N = 19$, $Z = -0.51$, $p = 0.8$), but when tested with a familiar person, there was a significant decrease from the squatting to the standing person ($N = 15$, $Z = -2.49$, $p = 0.016$; Fig. 2b). That is in the familiar person situation horses found the correct bucket better, when the person was squatting.

In experiment 2, both for unfamiliar and familiar test persons, horses performed similarly in the first bucket choice in the squatting and standing person situations ($N = 4$, $df = 2$, $\chi^2 = 5.29$, $p = 0.13$; Table 2; Fig. 2c). However in the total bucket choices, the performance significantly decreased from the squatting to the standing situation ($N = 24$, squatting – standing: $Z = -2.53$, $p = 0.01$, Fig. 2d), again for both, the unfamiliar and familiar persons.

Table 1 Individual counts of correct choices, experiment 1
** p = 0.001, * p < 0.05, Binomial test (hypothesised probability of success at 33%), [1]: 1 = correct, 0 = wrong, [2]: from 6 choices, [3]: within 6 trials, [4]: horse has been deleted from the data as it refused to participate in the test after 3 trials

	Horse name	Squatting / alternating gaze				Standing / permanent gaze				Turned / distant			
		First choice[1]	Total success[2]	Nr. no choice[2]	Approach to person[3]	First choice[1]	Total success[2]	Nr. no choice[2]	Approach to person[3]	First choice[1]	Total success[2]	Nr. no choice[2]	Approach to person[3]
Unfamiliar person	Diva	1	6**	0	0	1	4	0	0	1	5*	1	0
	Hamra	0	4	0	0	1	6**	0	0	0	2	2	2
	Malawit	1	6**	0	0	1	4	0	0	1	2	0	0
	Bibilotta	1	5*	0	0	0	5*	1	1	1	5*	0	0
	Angie	1	6**	0	1	1	6**	0	0	0	6**	0	0
	Leika	1	6**	0	0	0	4	0	0	1	3	0	0
	Fritzl	1	6**	0	0	1	6**	0	0	1	6**	0	0
	Luna	1	6**	0	0	1	6**	0	0	1	4	0	0
	Merlin	1	6**	0	0	1	5*	0	0	1	4	0	0
	Camillo	1	6**	0	0	1	6**	0	0	1	4	0	0
	Sissi	1	6**	0	0	1	6**	0	0	1	4	0	0
	Anja	0	2	0	1	1	5*	0	0	0	0	2	0
	Peppy	1	6**	0	0	1	6**	0	0	1	5*	0	0
	Billy	1	5*	0	NA	1	4	0	NA	0	4	0	NA
	Alexia	1	5*	0	NA	1	3	0	NA	0	3	0	NA
	Sara	0	4	0	NA	0	5*	0	NA	0	0	0	NA
	Farina	1	6**	0	NA	1	5*	0	NA	0	3	0	NA
	Peppermint	1	5*	0	NA	1	6**	0	NA	0	3	0	NA
	Anouschka	0	4	0	NA	0	6**	0	NA	0	3	0	NA
Mean total performance		79%	88%	0%	2.5%	79%	86%	1%	1%	53%	58%	4%	2.5%
Familiar person	Joschi	1	6**	0	0	1	6**	0	2	0	3	2	2
	Sunny	1	6**	0	3	0	3	0	1	0	4	0	0
	Bingo	1	6**	0	1	1	6**	0	0	0	1	3	2
	Pretty	1	6**	0	0	1	5*	0	1	0	3	0	0
	Sammy	1	6**	0	4	1	6**	0	1	1	4	1	1
	Bonita	1	6**	0	4	1	6**	0	2	0	0	2	2
	Bill Teiser	1	6**	0	1	1	5*	0	1	0	3	2	2
	Sheraz	1	6**	6	3	0	2	3	3	0	0	6	4
	Sambor	1	6**	0	2	1	5*	0	1	1	4	0	0
	Hjötra	1	6**	0	0	1	6**	0	0	0	4	1	1
	Mahranya	1	5*	0	0	1	6**	0	2	1	4	0	0
	Manon	1	5*	0	0	1	4	0	1	1	2	2	2
	Monty	1	6**	0	4	1	5*	0	1	1	3	1	1
	Romeo	0	4	5	3	1	4	2	3	0	0	4	3
	Graf Astor	1	6**	0	2	1	5*	0	2	0	2	2	2
	Hexi[4]	1	3	NA	0	NA	NA	NA	NA	NA	NA	NA	NA
Mean total performance		94%	93%	12%	28%	87%	82%	5.5%	23%	33%	41%	29%	24%

Table 2 Individual counts of correct choices, experiment 2
** p = 0.001, * p < 0.05, Binomial test (hypothesised probability of success at 33%), ^{1}: 1 = correct, 0 = wrong, ^{2}: from 6 choices, ^{3}: within 6 trials

	Horse name	Squatting / permanent gaze				Standing / alternating gaze				Turned / proximal			
		First choice^1	Total success^2	Nr. no choice^2	Approach to person^3	First choice^1	Total success^2	Nr. no choice^2	Approach to person^3	First choice^1	Total success^2	Nr. no choice^2	Approach to person^3
Unfamiliar person	Vittoria	1	5*	0	0	1	5*	0	0	0	1	0	0
	Savannah	1	4	0	0	1	2	0	0	1	1	0	1
	Fiona	1	4	0	0	1	4	0	0	1	2	1	1
	Gyula	1	6**	0	0	1	3	0	0	1	2	1	0
	Sarastro	1	4	0	0	1	4	0	0	1	5*	0	0
	Jeamy	1	6**	0	0	0	1	4	2	0	0	5	2
	Czaba	1	4	0	0	1	3	0	0	1	4	0	0
	Toffee	1	4	0	0	0	4	0	0	1	4	0	0
	Plainsman	1	6**	0	0	1	5*	0	0	0	2	0	1
	Nicolas	1	5*	0	0	1	3	0	0	1	2	0	0
	Eccos	1	4	0	0	1	4	0	0	1	4	0	0
	Giardino	1	6**	0	0	1	5*	0	1	1	2	0	0
Mean total performance		100%	80%	0%	0%	83%	60%	5.5%	4%	75%	40%	9%	7%
Familiar person	Lagsi	1	6**	0	1	1	5*	0	0	1	4	0	0
	Ronny	1	6**	0	0	0	4	0	1	1	4	2	3
	Naedingur	1	6**	0	0	1	5*	0	0	1	6**	0	0
	Lilly 1	0	3	0	0	0	4	1	3	0	2	2	2
	Wacker	1	6**	0	0	1	5*	0	2	1	4	0	1
	Prince	1	4	2	3	1	6**	0	5	1	5*	1	5
	Lilly 2	1	5*	0	0	1	6**	0	2	1	6**	0	1
	Rambo	1	6**	0	0	0	4	2	0	1	4	1	2
	Gustav	1	6**	0	1	1	6**	0	1	1	6**	0	3
	Zirkonia	1	6**	0	0	1	6**	0	0	1	6**	0	0
	Nevada	1	6**	0	0	1	6**	0	1	0	5*	0	1
	Lugana	1	6**	0	3	1	3	1	0	0	3	0	2
Mean total performance		92%	92%	3%	11%	75%	83%	5.5%	21%	75%	76%	8%	27%

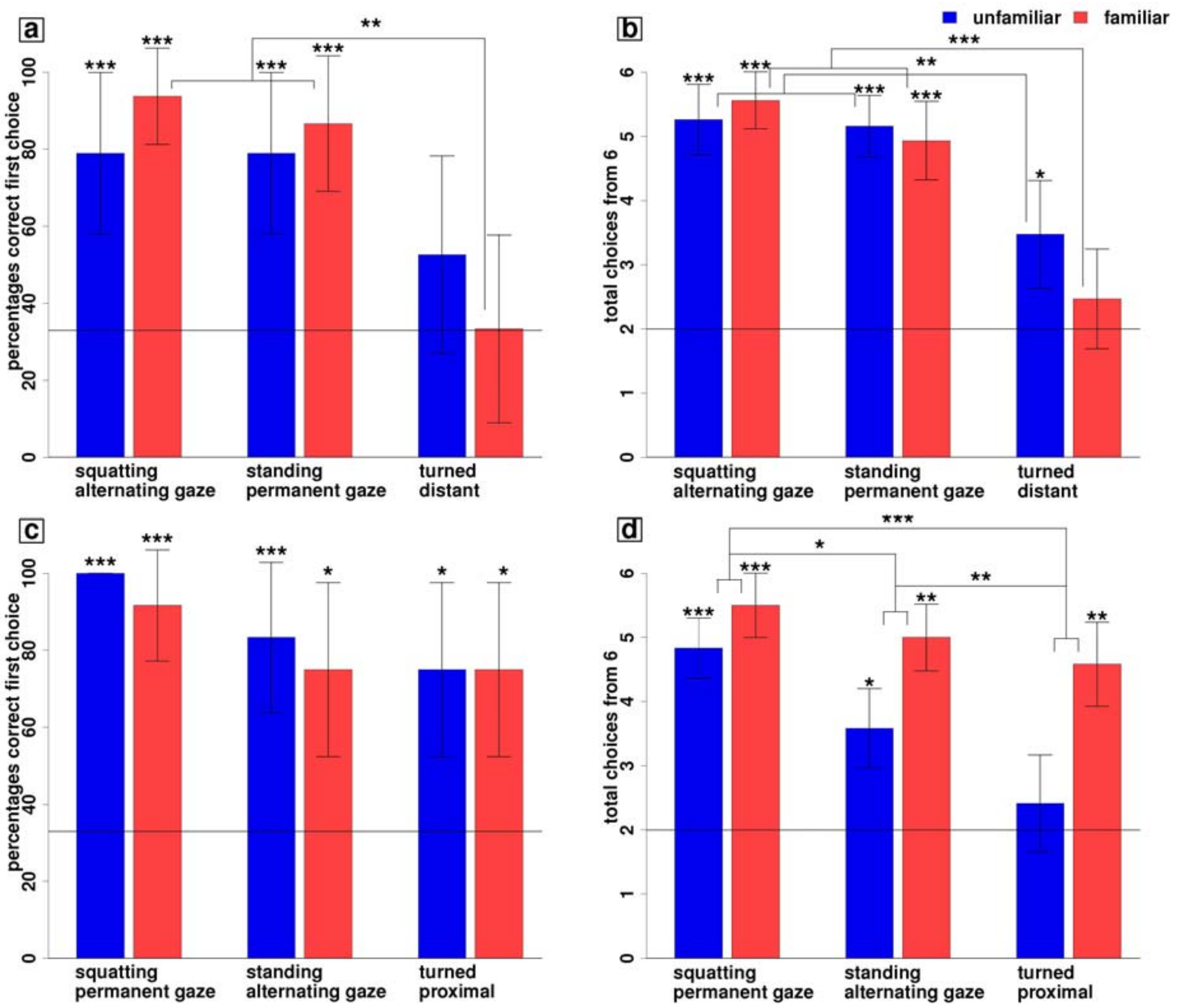

Fig. 2 The horse's choice for the feed bucket. a) depicts the horses' correct choices in the first trial of experiment 1, b) the correct total choices in experiment 1, c) the correct first choices in the experiment 2 and d) the correct total choices in experiment 2. First choices are given in percent, and total choices per six trials. The horizontal line indicates the baseline, obtained from the hypothesised probability of success at 33% for three options. Significant deviations between datasets or from the baseline are given with *** for $p < 0.001$, ** for $p \leq 0.01$ and * for $p \leq 0.05$

Permanent versus alternating gaze

There was generally no difference in performance between the permanent and alternating gaze situations (Fig. 2; Tables 1 and 2). The results for the first bucket choices in the squatting (N = 6, W = 377.5, p = 0.22) and standing person situation (N = 6, W = 423.5, p = 0.95; Fig. 2a and 2c) showed no significant differences, nor did the results for bucket choices in the squatting total data (N = 6, W = 478, p = 0.31; Fig. 2b and 2d). There were also no significant differences in approaches to persons with either permanent or alternating gazes in the first choice in the standing person situation (N = 6, W = 368, p = 0.52; Fig. 3a and 3b).

There was a significant difference between the permanent and alternating gaze for the total standing person data (N = 6, W = 549, p = 0.02; Fig. 2b and 2d), i.e. horses found the correct bucket more often when the person was gazing permanently at the horse. There were also differences when horses approached permanent and alternating gaze persons in the squatting situation (N = 6, W = 439, p = 0.05; Fig. 3a and 3b). Here, horses approached the person more often when he/she was alternating his/her gaze. However, the significant differences represent single cases in experiment 2 without general affirmation from overall comparisons and pairwise comparisons between all the test situations (all p > 0.05). They may as well be caused as side effect of irregularities in the individual horse's behaviour.

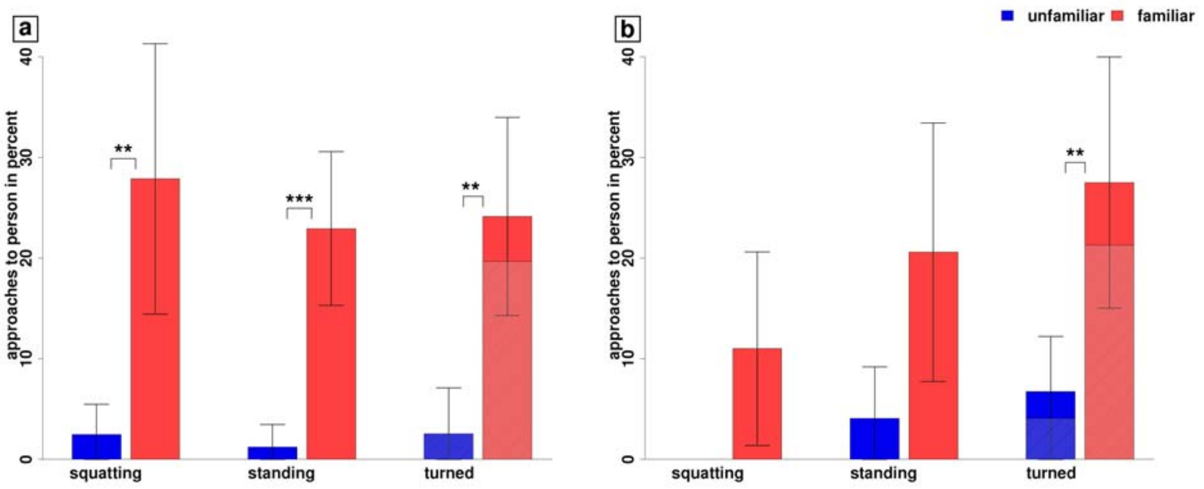

Fig. 3 Approaches to test persons. The two graphs show the percentages of approaches to the test person without prior feeding from the buckets, a) for experiment 1 and b) for experiment 2. The shaded parts of the two columns from the turned person situation stand for incidences in which the horses walked into the direction of the person's gaze after approaching him/her in this situation. Significant differences are given with *** for $p < 0.001$, ** for $p \leq 0.01$ and * for $p \leq 0.05$

Orientation on the person's focus of attention, comparison of test situations

Approaches to test persons

Horses may approach the person, possibly seeking their attention. In both experiments (exp. 1 and exp. 2) some horses approached the test person before feeding from the buckets (i.e. when the person stood close to the bucket), with no significant variation between test situations (N = 28, $\chi^2 = 0.47$, p = 0.8; Fig. 3). However, in experiment 2, horses approached the turned proximal person most frequently, less frequently the standing, and least the squatting person. Details for the approaches of individual horses are given in the tables 1 and 2. However, the differences between the test situations were not significant (N = 12, $\chi^2 = 5.28$, p = 0.07).

Performance when tested with a turned proximal person

Horses that adjust to the person's focus of attention are expected to decrease in their performance when the person turns away from the buckets (Fig. 2; Table 2), as they choose the focus of attention over the feed.

In fact, when the person turned and remained at the bucket (situation turned/proximal) in experiment 2, the horses' performance significantly decreased in comparison to the squatting and standing person situations for the total bucket choices (N = 24, standing - turned: Z = -2.69, p = 0.007, squatting- turned: Z = -3.35, $p \leq 0.001$; Table 2; Fig. 2d). First bucket choices were also lower in the turned/proximal person situation than in the squatting and standing person situations. However differences were not significant (N = 4, df = 2, χ^2 = 5.29, p = 0.13; Table 2; Fig. 2c).

Performance when tested with a turned distant person

We expected an additional decrease in the horse's performance when the person turned and moved away from the feed buckets. This expectancy was supported by the data. When the person turned and walked away from the buckets the horses failed to perform significantly above chance with the unfamiliar (N = 19, p = 0.06; Table 1; Fig. 2a) and the familiar person (N = 15, p = 0.597; Table 1; Fig. 2a) with their first choice. Their first choice performance was significantly lower in the familiar turned distant person situation than in the squatting and standing situations (N = 15, Z = -2.83, p = 0.008), but not when tested with unfamiliar persons. Additionally, the data for the total bucket choices show that the horse's performance significantly decreased from the standing to the turned distant person situation when tested both with an unfamiliar person (N = 19, Z = -2.98, p = 0.002; Fig. 2b; Table 1) and with a familiar person (N = 15, Z = -3.38, $p \leq 0.001$; Fig. 2b; Table 1).

Walking into the turned person's gaze direction

If horses orientate on the person's focus of attention they can be expected sometimes to walk into the direction of a turned person's gaze. The behaviour of the horses in this study met this expectation in the turned proximal and the turned distant person situations (Fig. 3). Large differences in this behaviour occurred when horses were tested either with familiar or unfamiliar test persons (see below).

Two horses that were tested with familiar persons even moved around the test persons in the squatting and standing situation and approached the feed buckets in direction of the person's gaze.

Unfamiliar versus familiar persons

Performance with unfamiliar versus familiar persons

In general, horses did not perform differently when tested with unfamiliar or familiar persons, both in experiment 1 (N = 35; first choice: squatting: Z = -1.23, p = 0.35, standing: Z = -0.17, p = 1, turned: Z = -1.11, p = 0.31; total choice: squatting: Z = -1, p = 0.34, standing: Z = -1.08, p = 0.6, turned: Z = -1.71, p = 0.09) and experiment 2 (N = 24; first choice: squatting: Z = -1, p = 0.75, standing: Z = -0.49, p = 0.75, turned: Z = 0, p = 1; total choice: squatting: Z = -0.61, p = 0.59, standing: Z = -2.07, p = 0.04, turned: Z = -2.04, p = 0.04; Table 2 and 3; all three not significant after Sequential Bonferroni Correction for multiple testing, Fig. 2d).

Attending to unfamiliar versus familiar persons` focus of attention

In contrast to the horses` performance we found a significant difference in their approaches to unfamiliar and familiar test persons. In both experiments (1 and 2), familiar persons were approached significantly more often than unfamiliar persons in the squatting ($Z = -2.81$, $p = 0.004$), the standing ($Z = -3.99$, $p \leq 0.001$), and the turned, distant person situation ($Z = -3.28$, $p = 0.003$; Fig. 3a).

Additionally, in the turned distant person situation, horses walked significantly more often in the direction of a familiar person's gaze than an unfamiliar person's gaze ($Z = -2.94$, $p = 0.004$; Fig.3a). The results were confirmed in experiment 2, only here the differences were not significant for the squatting ($Z = -2.14$, $p = 0.09$) and standing person ($Z = -2.1$, $p = 0.05$, not significant after Bonferroni Correction), but were significant for the turned, proximal person situation ($Z = -3.22$, $p = 0.001$), and when horses walked into the direction of the turned person's gaze ($Z = -3.58$, $p = 0.001$; Fig. 3b).

It is also interesting to note that 26 of the 28 horses tested with a familiar person (93%), approached the person at least once during all the trials, while only 11 of the 31 horses (35%) tested with unfamiliar people approached them ($N = 59$, $Z = -4.51$, $p < 0.001$). In the distant turned away person situation, 11 of the 15 horses approached the familiar person at least once (73%), significantly more than the 1 out of 13 horses tested that approached an unfamiliar person (8%) ($N = 28$, $Z = -3.44$, $p = 0.002$). There were no instances of a horse only approaching the person under any of the conditions, there was always a mixture of choosing the person and choosing a bucket.

Central bucket preferences in the turned, distant person situation?

For the turned/distant person situation in experiment 1 additional control for a possible central bucket preference is needed, as only in this situation did the test person move to and remain in a fixed central position. However, horses showed no preference for the middle bucket. As their first choice, horses chose the middle bucket at chance in the unfamiliar person situation (31%; $N = 13$, $\chi^2 = 1.08$, $p = 0.69$), and in the familiar person situation (33%; $N = 15$, $\chi^2 = 0.00$, $p = 1$). For the total choices in this situation the horses' performances were similar to the first choices, both with the unfamiliar person (39%, SD: 14.6%) and the familiar person (43.67%, SD: 27.1%) (unfamiliar: $N = 13$, $\chi^2 = 3.92$, $p = 0.32$; familiar: $N = 15$, $\chi^2 = 3$, $p = 0.75$). In comparison, the total choices for the middle bucket in the turned/distant person situation did not differ significantly between the unfamiliar and the familiar person group ($Z = -0.66$, $p = 0.75$).

Discussion

In this study, horses consistently used human local enhancement cues for finding food when persons remained facing the horse at the location of the feed, i.e. behind the filled bucket. In general, the person's body position (i.e. squatting or standing), and whether the person used a permanent or alternating gaze, did not have an effect on the horse's performance. There is no scientific evidence that there is any difference between a human's standing or squatting position in terms of influencing the horse's reactions and, according to our results, this is not a very important factor in an object choice task. However some horses preferentially approached the persons in a squatting position, which may indicate that horses find this posture more attractive.

When the persons turned around and faced away, the horses' performance declined, and declined further when the turned person moved to a distant position after depositing the feed. With the turned person situation our primary intention was not to investigate the horses' abilities for memorizing the placement of food, but rather whether the horses' motivation to approach the food would be affected by the person's changed body orientation and changed focus of attention, as reported for dogs (Gácsi et al., 2004; Virányi et al., 2004), and/or by the person's familiarity. The turned distant person removed the local enhancement cues provided by persons that face the horses and stay at the bucket and gave the horse a simple choice of either approaching the food or approaching the person.

The familiarity of the person did not affect the horses' performance in any of the three situations. In contrast, there were significant differences between approaches to unfamiliar and familiar persons themselves. Familiar persons were approached significantly more often than unfamiliar persons, when horses approached the squatting, standing and turned persons before feeding. Additionally, when the person turned and faced away from the feed buckets, horses walked into the direction of a familiar person's gaze significantly more often than with an unfamiliar person. This is in line with prior observations from social situations, where horses paid more attention to the behaviour of known than unknown conspecifics (Krueger and Heinze, 2008). Moreover, the animals' performance improved when tested with familiar conspecifics when they focused on a specific task (e.g. feeding) rather than dividing their attention across several tasks (e.g. aggression and feeding) (Griffiths et al., 2004).

Especially when horses were tested with familiar persons the difference between the horses' performance in the squatting and standing situations compared to the turned person situation can be explained by analysing the approaches horses showed towards the test persons without prior feeding from the buckets. Approaching the test person did not affect their performance in finding the food in the squatting or the standing person situations, nor in the turned person situation when the person stayed right at the bucket, as horses could then still feed from the buckets. However in the turned person situation when the person stayed a couple meters away from the bucket, the horses which approached the person did not return to the buckets afterwards, i.e. they did not make choices of feed buckets in these trials. An additional decrease in the horses' performance in both turned person situations has been caused by incidences in which horses walked into the direction of the person's gaze.

The approach to persons may indicate that some horses simply expected, or have learned, that food can be obtained from familiar persons. As the horse's long term memory has recently been shown to be excellent (Hanggi and Ingersoll, 2009) and a multitude of prior experiences with well known humans would affect the horse's behaviour towards specific persons (Hausberger et al., 2008), the horse's expectation of receiving food from familiar persons is not very astonishing. However only one horse nudged at the person after the approach, which may be considered attention demanding behaviour (Proops and McComb, 2010), so it is possible that the horses may rather have adjusted to the person's focus of attention. In this regard it may also be interesting that two horses even walked around familiar squatting and standing persons and approached the buckets from behind the person in direction of the person's gaze. These findings are in line with prior studies on horses (Proops and McComb, 2010) and dogs (Gàcsi et al., 2004), where some individuals placed themselves in line with the focus of a turned person's attention.

For some horses the need to adjust to another individual's focus of attention may even override their desire for food. Responding to the alertness, or simply changes in the attention, of other individuals of the same or other species may have survival benefits for a prey animal. Very simply put: when it comes to the necessity to flee from a predator, those that recognize a danger faster by adjusting to their own species or other species predator alarm behaviour, will have a survival benefit (Goodwin, 2002).

Both explanations confirm that at least some horses respond to the direction of human attention. Generally, our results indicate that the horse's willingness to orientate on a person's attention increases with the closeness of the relationship with that particular human, as horses showed orientation behaviour more often when the person was familiar to the horse.

As in other studies, we found large individual differences in approaching the test person, which could reflect the horses' different temperaments (Visser et al., 2001, 2002, 2003; Lansade et al., 2005; Lansade and Bouissou, 2008). However, we refrained from testing the horses with other persons as a control, as the results would have been influenced by habituation effects (Mal et al., 1993). Differences in response to the test person could also have been caused by kin or by breed specific temperament (Hayes, 1988; Houpt and Kusunose, 2000; Hausberger and Muller, 2002). Even though the horse's breed did not significantly affect our results, the sample size of this study does not allow for clear conclusions concerning kin and breed specific temperament effects. We can, however, support the evidence from other studies that gender is not decisive in the horse's behaviour towards humans (Visser et al., 2001, 2002, 2003; Hausberger et al., 2004; Henry et al., 2005).

We would like to conclude, that horses generally use humans as local enhancement cues when searching for food, and the horse's propensity to adjust to the person's focus of attention is affected by the human's familiarity, and body orientation. We confirm prior findings of horses being able to read gesticular cues (McKinley and Sambrook, 2000; Maros et al., 2008) and react to the human's focus of attention (Proops and McComb, 2010) analogous to other species (Hare et al., 2002). Furthermore, as horses respond to attention cues from familiar more strongly than from unfamiliar persons, and for horses tested with unfamiliar humans the food is of greater importance than the person's attention, we suggest that domestication is only partially responsible for the animal's choice to adjust to the human focus of attention. This is supported by the inconsistencies in human cue reading in other species (Theall and Povinelli, 1999; Hostetter et al., 2001; Virányi et al., 2004; Gácsi et al., 2005). On top of domestication, socialisation and training enhance the horse’s ability to read human cues as in other species such as apes (Povinelli and Eddy, 1996; Itakura et al., 2001), dolphins (Tschudin, 2001; Pack and Herman, 2004), seals (Shapiro et al., 2003; Scheumann and Call, 2004), ravens (Schloegl et al., 2008), parrots (Giret et al., 2009), and wolves (Gácsi et al., 2009b). Further studies on wild equids are urgently needed to clarify whether domestication set the stage for these abilities.

Behavioural explanations (Povinelli and Vonk, 2003), in terms of animals learning attention cues, and cognitive explanations (Tomasello et al., 2003) which emphasise the understanding of the signaller’s intentions, are both valid for this study. However, we may point out that the cognitive ability to read the focus of attention generally has to be present in order to learn how to apply it and use it as a cue. Additionally, the fact that the horse’s propensity to orientate on human attention differs when tested with unfamiliar and familiar persons and that some horses adjust themselves to the focus of human attention points to cognitive processes. This is further supported by the fact that no horse orientated on the attention every time, as one might expect if this was a behavioural response connected with previous experience of receiving food from the person. Furthermore, in the distant, turned person situation, where the horse had to make a clear choice between the person and the buckets, a significantly higher percentage of horses approached the familiar person than the unfamiliar person in the same position, but this decision was nevertheless taken on a trial by trial basis neither the bucket nor the human was consistently preferred by any horse. The horse’s choice is obviously connected to its relationship with the person and the representation of the person’s focus of attention. Further research is needed to establish which factors may influence the horse’s decision to approach the familiar human or the bucket in each instance. Horses may readily apply their cognitive abilities for generalizing and categorizing learning tasks (Hanggi, 1999) in social settings.

In closing, a word should be said about the possible distracting effect of the assistant on the test horse’s performance. Even though we were careful that the assistants controlled their behaviour and posture, we can not totally exclude the possibility that they may have influenced the horses’ behaviour subconsciously as they had seen the placement of the feed (Pfungst, 1907). We could have excluded this possibility by using a station and release system from which horses are released automatically with no further person needed. In several mammal species station and release systems are used on a regular basis, and were applied to equine studies in recent decades (e.g. Hanggi, 1999; Christensen et al., 2005). However, this was not practical for this field study. We chose to conduct our tests in a well habituated surrounding to avoid separation and novelty anxiety in our test horses. For most horses the test area was a part of their enclosure (all horses were kept in social housing systems with attached enclosures) or the riding arena. Solid wood fencing for a station and release system, with a pole at the entrance that could be removed by a hidden person outside, would not have been tolerated by the stable or horse owners in these premises. Additionally, changes in the well known areas would call for habituating the horses to the novelty. This can be very time consuming for some horses and the horse owners would not have been willing to invest so much of their time. For the test persons it is nearly impossible to habituate 60 horses in 14 different locations within a reasonable time frame. Finally, we could have used portable electric fencing. We actually tried to apply such systems prior to the tests. However some horses were frightened of electric fencing right from the beginning and would not remain calm and motivated for the test in an electrically fenced area. Other horses had prior experiences of going through electric fences that were not on charge. Applying electric station and release system without extensive training prior to the tests could have caused severe welfare problems for these horses.

Finally, we propose that when choosing test persons for future studies, a familiar person that faces the horse in a squatting or standing body posture, should be used when a strong interaction with the horse is needed, e.g. when responses to human cueing are studied. Unfamiliar persons and station and release systems should be used to achieve least affected test results.

Acknowledgements

We thank Jürgen Heinze, Katherine Albro Houpt and Susanne Waiblinger for theoretical support, Knut Krüger for helping with and Charlotte Hemelrijk for further advice for statistical analyses, as well as Julia Scharrer, Tobias Haertinger, Marion Füssel, Gudrun Schneider, Andreas Sailer, Anja Schlecht, Magdalena Schneider, and all the horse owners for the assistance at the tests. We also thank three anonymous referees for their help in improving earlier versions of the manuscript. The study was supported by an excellence grant from the Bavarian Government, and a HWP II grant from the University of Regensburg.

GENERAL DISCUSSION AND PERSPECTIVES

In this chapter I will first discuss the suitability of saliva and faecal samples for stress hormone analysis during the introduction of horses. Then, as my project is also intended to provide suggestions for horse owners, I will suggest a practical guideline as to how to reduce the aggression level during introductions. Finally, I will discuss my results in the light of the cognitive abilities of horses and will conclude with perspectives for further analysis and research.

Stress hormone analysis

The measurement of glucocorticoids appeared to be a good tool for investigating the horse's stress level during the introduction. Glucocorticoids, or their metabolites, can be measured in several body fluids or excreta, including plasma, saliva, milk, urine and faeces (e.g. Möstl and Palme, 2002). All methods have both advantages and disadvantages. In recent years the measurement of glucocorticoid metabolites (GCMs) in faeces has gained increasing attention for wild populations (Heistermann et al., 2006), wildlife management, and conservation as well as behavioural biology (Möstl and Palme, 2002; Touma and Palme, 2005), largely because it is a non-invasive and feedback-free sampling method. Even though horses excrete 41% of radioactive cortisol via faeces (Palme et al., 1996) which should therefore be well suited for detecting GCMs in faeces, the extraction procedure for horse faeces described so far (Merl et al., 2000; Gorgasser et al., 2007) is more complicated than in other species. In cooperation with the Vienna University of Veterinary Medicine (Institute of Biochemistry, Department of Biomedical Sciences/Biochemistry), the Ludwig-Maximilians-University Munich (Equine Clinic, Faculty of Veterinary Medicine) and the LVFZ Haupt- und Landgestüt Schwaiganger Pferdehaltung, I was able to conduct an ACTH Challenge Test in order to physiologically validate stress responses in blood, saliva and faecal samples of horses. By the combination of different extraction protocols and enzyme immunoassays I could greatly improve the method to detect GCMs in horse faeces (chapter 1). This method is superior to the assay used for the analysis of horse faeces so far, concerning the amplitude after ACTH induction and dexamethasone depression, as well as the stability of immunoreactive glucocorticoid metabolites. The simplified method poses big advantages, because it is faster and easier to apply and enhances accuracy and automatisation, while lowering laboratory expenses (e.g. less consumption of solvent, feasibility in simple equipped laboratories without pull-off devices), especially when adopted to huge quantities of faecal samples. The non-invasive sampling method is, on the one hand, particularly suitable when measuring stress in feral or wild horses for wildlife management, as well as in conservation and behavioural biology. On the other hand, it is urgently needed for the growing sector of equestrian sports and in view of the popular interest in the horse's welfare.

By analysing GCMs in faeces I could show that horses experience stress, when they are introduced into a new group together with an integration horse. However, it seems that not the introduction process itself is responsible for the elevated faecal GCMs, but the period when the horse stands together with an integration horse on a separate paddock. The analysis of the saliva samples gave less clear results and turned out not to be a good tool for analysing stress during the introduction of horses into new social groups. Saliva samples were useful, however, for detecting transport stress in horses in the studies of Schmidt et al. (2010a, 2010b). Our findings support the hypothesis that GCMs in faeces reveal only marked and prolonged increases in cortisol release, whereas cortisol immunoreactivity in saliva is sensitive to small and transient changes.

How to reduce the level of aggression during introductions: a practical guideline

My PhD project is also intended to provide practical suggestions for horse and stable owners. Even though animal welfare organisations propose group housing for horse welfare (BMELV, 2009), many owners stable their horses individually, for fear of aggressive interactions and because of injury risks. Above all, the introduction of horses into new groups arouses severe concerns in horse owners, because it is well known that, in social animals, encounters with unfamiliar conspecifics may elicit aggressive interactions while the social rank order is being established. I investigated three approved methods for the introduction of horses into new groups, namely the immediate introduction, the introduction after an observation period, and the introduction with an integration horse. In comparison to other introduction techniques, the introduction of horses together with an integration horse, i.e. one of the new group mates, caused significantly less interactions and resulted in lower aggressiveness (chapter 2). I therefore recommend introductions with group mates (Flauger and Krueger, submitted), which is in line with studies by Ullstein (1996), Kurtz et al. (2000), Zeitler-Feicht (2001) and the FN (2005). Moreover, my results from the observations of groups kept at best practice, at different stables, with varying group sizes, different age and sex composition support the findings that in artificial arrangements of group meetings nearly no injuries are observed except some superficial hairless spots, after groups with only 2-year-old mares, only 2-year-old stallions or mixed ages of only one sex had been composed (e.g. Hartmann et al., 2009; Jørgensen et al., 2009b). Indeed, we can remove the fear of horse and stable owners, because no severe injuries occurred during the introductions.

Whereas the introduction with integration horse leads to a decrease of aggression on group level, this technique seems to be the most stressful one for the introduced horse. In general, we can assure horse owners that the stress level during introductions into new groups is fairly low and that horses are perfectly able to deal with the conflict situation of being introduced to new group members. It is even possible that exactly this low level of stress leads to a positive effect, i.e. that the horses are less aggressive as under normal conditions. It would be desirable that more owners take the advice and keep their horses in group management for better welfare situations. Finally, I want to emphasize our formula derived from the interrelationship of aggression occurring among group members and the available space per horse (chapter 2). As the curve shows exponential characteristics, which means that from a defined available space per horse the aggressions among the horses decrease immensely and even approach zero when the enclosure size amounts to 300 m^2 per horse or more, horse owners should not be so anxious about the event of introduction, but rather take care that their horses have enough space so they can avoid contact to other horses if necessary.

Perspectives on cognitive abilities of horses

In my projects I studied the behavioural ecology of horses that were introduced into new groups. I reflected on how the behaviour of horses changes in different situations, different ecological contexts and with different possible factors of influence. However, one can even go a step further and describe not only how the behaviour changes, but search for answers to the questions of which information horses gain from the different situations, how they process, encode and store the information and change their behaviour according to this information. This leads to a perspective on the cognitive abilities which horses need when introduced into new groups.

In the study of chapter 5 I could demonstrate that domestic horses use social cognition and strategic decision making in order to decide where to feed in a social feeding situation. The social rank and the position of conspecifics affect the feeding strategy of horses. The results may be interpreted in terms of the horse's ability for concept formation on the ranks of their group mates, and lead to the conclusion that they are even able to assess their own relative rank position and to make cognitive decisions based on this knowledge. Concerning the horse's olfaction, which is suggested to be important in social discrimination between horses, I could show in chapter 6 that horses are capable of distinguishing both their own from their conspecifics' faeces and particular competitors among their group mates by the smell of their faeces. The ability of connecting the smell of faeces with individual animals and having a representation of these specific individuals hints at high cognitive capacities. Individual recognition is also very important during the introduction into new groups. The same may be true for urine. Hothersall et al. (2010) suggest that urine odour may contain some information enabling horses to discriminate between conspecifics. Moreover, horses show cognitive abilities because they are able to use humans as local enhancement cues when searching for food, and the horse's propensity to adjust to the person's focus of attention is affected by the human's familiarity and body orientation (chapter 7). Horses obviously make conscious decisions whether to adjust to humans depending on the person's importance and credibility. Only familiar persons are expected to display information, and it may be wise for a horse to stop feeding and rather adjust to a familiar person's changed focus of attention. Krueger and Heinze (2008) demonstrated that horses are able to differentiate between known and foreign, as well as dominant and subdominant conspecifics, and know their relative rank within the groups. Proops et al. (2009) showed that horses could match familiar individuals to their vocalisations.

All the studies mentioned discuss the cognitive abilities of horses, both in the interactions between horses and in the abilities of horses to use a stimulus, e.g. olfactory cues, to discriminate between horses as well as to use subtle cues in the interaction with humans. Against this background I discuss the introduction of horses into new groups (chapters 2 and 3). I propose that horses use their social cognitive abilities during the introductions because they are perfectly able to deal with this conflict situation. This can be seen in low injury rates, in the stress level which is much lower than expected, and in the fact that they are already able to establish their "permanent" rank position in the first-control observation. We can not fully support our hypotheses, however, that horses can use their cognitive abilities, when they are able to observe the new groups before joining them. I would have expected to see a big difference in the behaviour of the introduced horses when comparing the immediate introduction and the introduction after an observation period. My hypothesis was that I would see less aggression and stable social bonds in horses introduced after an observation period, because they are able to observe their new group mates before, observe the hierarchy and possibly use their cognitive abilities in order to find their final rank position more easily. But I did not find differences in social bonding for the different introduction techniques, and the reduced level of aggression cannot be attributed to the observation period, but rather to the calming effect of the integration horse. An alternative explanation would emphasise the fact that the cognitive abilities of horses are even better than was previously expected, that they do not need a prolonged observation period but can immediately infer the hierarchies of new groups when they are introduced. As horses have to change their groups under natural conditions, too, this seems to be a strategy for avoiding conflict situations. For animals living in groups it is beneficial to recognise individuals, track their social status and infer relationships among group members. Our results show that horses are very good at assessing their own rank position in comparison to their group members, even when confronted with unknown groups.

In the future I intend to have a closer look on the cognitive abilities of horses during the introduction into new groups through a combination of network analysis (Krause et al., 2009) and matrix tests (Hemelrijk, 1990a, 1990b). Network analysis has a long history in the mathematical and social sciences (Krause et al., 2009) and in recent years there has been a surge of interest in using network theory to analyse interaction patterns of various sorts (Newman, 2003; Proulx et al., 2005). The social network approach can yield new insights and questions for a broad range of issues in behavioural ecology including: cooperation, eavesdropping, dominance hierarchies, social learning, etc. (Sih et al., 2009; in Fig. 2 a network for the interactions between the animals of a group is shown.). In combination with network analysis I want to use matrix tests (Hemelrijk, 1990a, 1990b) which are the conventional statistical methods for analysing the quantity and quality of behaviours among animals. In cooperation with the Theoretical Biology Group at the University of Groningen I plan to have a closer look at social bonds in the groups and between specific group mates in the course of time, and analyse the reciprocity and interchange of behaviours with partial correlation tests of an actor- and a receiver matrix.

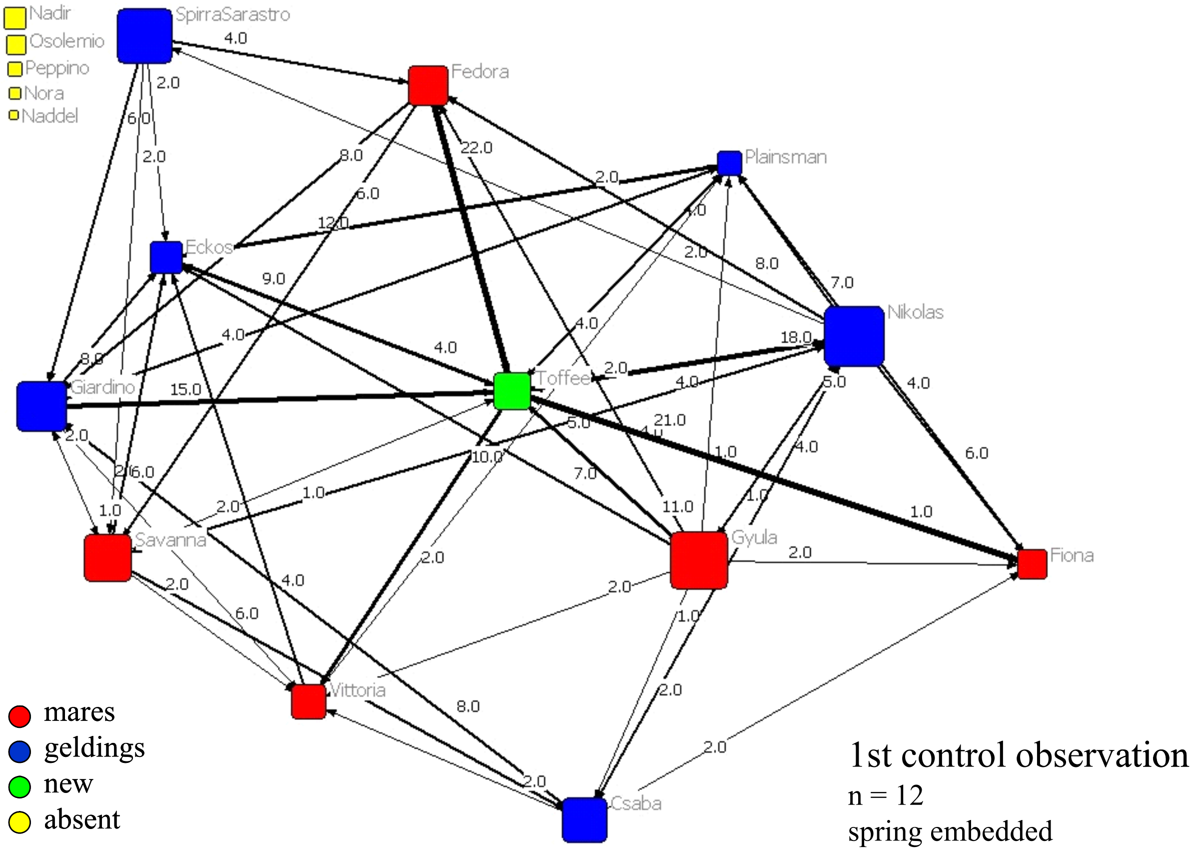

Fig. 2 Social network of a horse group where the horse Toffee (green) has been introduced six weeks before. Nodes represent the individuals, the size of the nodes correlates with their rank positions. Arrows between the nodes represent the interactions of the individuals, the bigger the arrow, the more interactions occur. The network is plotted with Ucinet 6/NetDraw

Moreover, during the experimental phase of my PhD thesis I collected data on the behaviour of Przewalski horses during the introduction into new groups. Since horses a are highly social species and still exist in different evolutionary stages, a comparative study between domestic horses, feral horses and Przewalski horses (the last remaining wild horses) concerning their behavioural ecology in the specific situation of the introduction into new social groups, can give valuable information about the evolution of individual social cognitive abilities in horses. The data shall be analysed for testing the hypothesis if convergent social evolution favours convergent cognitive evolution (Whiten and Byrne, 1988; Dunbar, 2000; De Waal and Tyack, 2003). It is likely that the social abilities of horses have changed with domestication and/or feralisation. Humans selected horses mainly for their performance, their beauty, their amenability and ability to cope with stressful situations. For example, Przewalski horses appear to act significantly more aggressively towards each other than domestic horses (Feh, 1988; Keiper and Receveur, 1992; etc.). On the other hand, during domestication the social abilities were considered to be less important. Therefore, it can be hypothesised that wild horses still show more effective social abilities than domestic horses, whereas domestic horses are more skilful in coping with stress.

The cognitive abilities of horses are not as obvious as in other species like primates, dogs, and social birds (Cheney and Seyfarth, 1990; De Waal and Tyack, 2003; Paz-y-Miño et al., 2004; Tomasello and Call, 2006). Although in recent years studies began addressing the horse's cognitive abilities (Krueger and Heinze, 2008; Hanggi and Ingersoll, 2009; Proops et al., 2009), these studies are less numerous compared to studies on other animal species. Therefore more studies are needed which further investigate the cognitive abilities of horses in different situations.

SUMMARY

Horses are a highly social species living in complex social systems which should require them to memorise and generalise social experiences and distinguish between familiar and unfamiliar conspecifics. In the main part of my thesis I concentrated on the specific conflict situation of a horse being introduced into a new social group, and investigated its behaviour and stress level. Horses were either introduced (1) immediately, (2) after an observation period, or (3) together with an integration horse after an observation period. Additionally, in the second part of my thesis I arranged several experiments to elaborate additional aspects which could affect the behaviour of horses during introductions.

In this study I could describe a simplified method for measuring stress through the analysis of faecal GCMs in horses. An enzyme immunoassay (EIA) for 11-oxoaetiocholanolone using 11-oxoaetiocholanolone-17-CMO: BSA (3α,11-oxo-A EIA) as antigen showed high amounts of immunoreactive substances. The new assay increases the accuracy of the test and lowers the expenses per sample; also storing of samples at room temperature after collection is less critical. This is a big advantage both in the field of wildlife management of equids and in the field of equestrian sports (**chapter 1**).

Comparing the different introduction techniques, the introduction with an integration horse led to significantly less total interactions and lower levels of aggression than the introduction of single horses, both immediately and after several days of observing the new group. Additionally, by observing the behaviour of the horses during everyday sociality I could develop a formula describing the interrelationship between expected aggression level and enclosure size per horse. The curve takes an exponential shape. Starting from a space allowance of 300 m^2 and more per horse, the amount of aggressions per hour approaches zero. For the reduction of aggression levels and injury risks in socially kept horses I recommend an enclosure size of at least 300 m^2 per horse (**chapter 2**).

I further investigated the stress level of the introduced animals. Horses which were immediately introduced did not show elevated faecal GCMs. In contrast, horses which were introduced after an observation period had slightly elevated values 2 and 3 days after the introduction. For horses introduced together with an integration horse faecal GCMs were significantly above the baseline value on the day of introduction and 1 day after it. These differences between introduction techniques indicate that the introduction event itself is not as stressful as previously assumed. Rather standing together with an integration horse and not being able to integrate immediately into the complete group elicits stress in horses (**chapter 3**).

In the commentary of chapter 4 several studies are discussed which failed to demonstrate social learning in horses. It is argued that they did not consider important aspects which could have an influence, such as the dominance status or the social background of the horses (**chapter 4**).

In chapter 5 a social feeding situation was investigated. The social rank as well as the position of conspecifics affected the feeding strategy of horses. Domestic horses used social cognition and strategic decision making in order to decide where to feed. When possible they tended to return to the same, continuously supplied feeding site and switched to an “avoidance tendency” in the presence of dominant horses or when another horse was already feeding there (**chapter 5**).

One possibility to recognize group members is through olfactory recognition. In chapter 6 it is shown that horses are able to distinguish their own from their conspecifics’ faeces. In addition, they paid most attention to the faeces of those group members from which they received the highest amount of aggressive behaviour (**chapter 6**).

Horses show cognitive abilities because they are able to use humans as local enhancement cues when searching for food, independently of their body posture or gaze consistency when the persons face them. Moreover, they seem to orientate on the attention of familiar persons more than of unfamiliar persons (**chapter 7**).

Altogether, the results of this thesis provide further support for the view that horses show good conflict resolution strategies. They are perfectly able to deal with the conflict situation of being introduced to new group members, and the introduction event itself is not as stressful as previously assumed. It is rather suggested that standing together with an integration horse and not being able to integrate immediately into the complete group elicits stress in horses. All additional experimental set-ups could demonstrate that horses are well capable of social cognition.

ZUSAMMENFASSUNG

Pferde sind eine hoch soziale Spezies, die in komplexen Sozialsystemen lebt. Dies lässt vermuten, dass sie sich an soziale Erfahrungen erinnern und diese generalisieren sowie zwischen bekannten und unbekannten Artgenossen unterscheiden können. Im Hauptteil meiner Doktorarbeit konzentrierte ich mich auf die spezielle Konfliktsituation der Eingliederung von Pferden in neue soziale Gruppen, wobei ich ihr Verhalten sowie ihren Stresszustand untersuchte. Die Pferde wurden entweder (1) sofort, (2) nach einer Beobachtungsphase oder (3) zusammen mit einem Integrationspferd nach einer Beobachtungsphase zu einer neuen Gruppe gesellt. Des Weiteren arrangierte ich im zweiten Teil meiner Arbeit verschiedene Experimente um zusätzliche Aspekte zu untersuchen, die ebenfalls einen Einfluss auf das Verhalten der Pferde während der Eingliederungen haben könnten.

In dieser Studie konnte ich eine vereinfachte Methode darstellen um den Stresszustand von Pferden anhand von Glucocorticoidmetaboliten im Kot zu messen. Ein Enzymimmunoassay für 11-oxoaetiocholanolone, bei dem 11-oxoaetiocholanolone-17-CMO: BSA (3α,11-oxo-A EIA) als Antigen verwendet wird, ergab eine hohe Anzahl an immunoreaktiven Substanzen. Dieser neue Assay erhöht die Genauigkeit des Tests, verringert die finanziellen Ausgaben und die Aufbewahrung der Proben bei Raumtemperatur ist weniger kritsch. Dies bringt einen erheblichen Vorteil sowohl beim Einsatz mit wild lebenden Pferden als auch bei Pferdesportveranstaltungen (**Kapitel 1**).

Bei einem Vergleich der verschiedenen Eingliederungstechniken zeigte sich, dass die Eingliederung eines Pferdes zusammen mit einem Integrationspferd zu signifikant weniger Interaktionen und Aggressionen führte als die sofortige Zusammenführung oder die Zusammenführung nach einer Beobachtungsphase. Außerdem konnte bei den Verhaltensbeobachtungen von Pferdegruppen im alltäglichen Sozialleben eine Formel entwickelt werden, die den Zusammenhang zwischen der Anzahl der erwarteten aggressiven Verhaltensweisen und dem Platzangebot pro Pferd darstellt. Die Kurve beschreibt eine Exponentialfunktion. Ab einem Platzangebot von 300 m^2 oder mehr pro Pferd nähern sich die erwarteten Aggressionen pro Pferd der Nulllinie an. Deshalb empfehle ich zur Reduzierung des Aggressionslevels und des Verletzungsrisikos von sozial gehaltenen Pferdegruppen ein Raumangebot von mindestens 300 m^2 pro Pferd (**Kapitel 2**).

Des Weiteren untersuchte ich den Stresslevel der neu eingegliederten Pferde. Pferde, die sofort zu der neuen Gruppe gesellt wurden, zeigten keine erhöhten Glucocorticoid-Werte im Kot. Hingegen hatten Pferde, die nach einer Beobachtungsphase auf die neue Gruppe trafen, leicht erhöhte Werte am zweiten und am dritten Tag nach der Zusammenführung. Bei Pferden, die zusammen mit einem Integrationspferd zu der Gruppe gesellt wurden, waren die Werte der Glucocorticoidmetaboliten im Kot am Tag der Eingliederung sowie einen Tag danach signifikant höher als der Basiswert. Diese Unterschiede, basierend auf den verschiedenen Eingliederungstechniken, deuten darauf hin, dass das Ereignis der Zusammenführung selbst für die Pferde nicht so stressig ist wie bisher angenommen. Vielmehr scheinen das Zuammenstehen mit dem Integrationspferd sowie nicht die Möglichkeit zu haben, sich sofort in die ganze Gruppe zu integrieren, den Stress auszulösen (**Kapitel 3**).

Im Kommentar von Kapitel 4 werden verschiedene Studien diskutiert, die soziales Lernen bei Pferden nicht nachweisen konnten. Es wird argumentiert, dass wichtige Aspekte nicht berücksichtigt wurden, die Einfluss gehabt haben könnten, wie zum Beispiel die Rangverhältnisse und die sozialen Erfahrungen der Pferde (**Kapitel 4**).

Im Kapitel 5 wurde eine soziale Futtersituation untersucht. Der soziale Rang sowie die Position des Artgenossen beeinflussten die Futterstrategie der Pferde. Es wurde gezeigt, dass domestizierte Pferde über soziale Kognition verfügen sowie bei der Wahl des Futtereimers strategische Entscheidungfindungen anwandten. Falls möglich, tendierten sie immer zum selben, stetig gefüllten Futterplatz zurückzukehren und wechselten zu einer „Ausweich-strategie“ bei der Anwesenheit eines ranghöheren Pferdes oder wenn ein anderes Pferd bereits dort fraß (**Kapitel 5**).

Eine Möglichkeit, um Gruppenmitglieder zu erkennen, ist die Erkennung mittels Geruch. In Kapitel 6 wird gezeigt, dass Pferde zwischen ihrem eigenen Kot und dem Kot von Artgenossen unterscheiden können. Außerdem zeigten sie die höchste Aufmerksamkeit für Kotproben von Gruppenmitgliedern, von denen sie am meisten Aggressionen erhielten (**Kapitel 6**).

Pferde zeigen kognitive Fähigkeiten, weil sie Menschen als „local enhancement cues“ verwenden, wenn sie nach Futter suchen, unabhängig von der Position und der Blickbeständigkeit des Menschen. Außerdem scheinen sie sich mehr an der Aufmerksamkeit von bekannten Personen als von unbekannten Personen zu orientieren (**Kapitel 7**).

Zusammenfassend liefern die Ergebnisse dieser Studie weitere Unterstützung für die Annahme, dass Pferde gute Konfliktlösungsstrategien zeigen. Sie können sehr gut mit der Situation umgehen, mit neuen Gruppenmitgliedern zusammengeführt zu werden. Im Gegensatz dazu scheinen vielmehr das Zusammenstehen mit dem Integrationspferd und nicht die Möglichkeit zu haben, sich sofort in die neue Gruppe zu integrieren, Stress auszulösen. Alle zusätzlich durchgeführten Testreihen konnten zeigen, dass Pferde gute soziale kognitive Fähigkeiten besitzen.

PUBLICATIONS AND PRESENTATIONS

Publications

Krueger K, Flauger B (2007) Social learning in horses from a novel perspective. *Behav. Processes* 76, 37-39

Krueger K, Flauger B (2008) Social feeding decisions in horses (*Equus caballus*). *Behav. Processes* 78, 76-83

Flauger B, Krueger K, Gerhards H, Möstl E (2010) Simplified method to measure glucocorticoid metabolites in faeces of horses. *Vet. Res. Commun.* 34, 185-195

Manuscripts submitted

Flauger B, Krueger K. Social interactions among horses (*Equus caballus*): Interrelations between aggression, enclosure sizes and introduction techniques. Submitted to *Appl. Anim. Behav. Sci.*

Krueger K, Flauger B. Olfactory recognition of individual competitors by means of faeces in horse *(Equus caballus)*. Submitted to *Anim. Cogn.*

Krueger K, Flauger B, Farmer K, Maros K. The horse's (*Equus caballus*) propensity to use humans as local enhancement cues and adjust to the focus point of human attention. Submitted to *Anim. Cogn.*

Manuscripts in preparation

Flauger B, Krueger K, Möstl E. Cortisol release, social bonds and social rank during the introduction of horses into new groups. To be submitted to *Horm. Behav.*

Krueger K, Flauger B. Socioecology of leadership in horses. In preparation

Flauger B, Cozzi A, Krueger K, Baragli P. Post-conflict behaviour in horses. In preparation

Presentations at meetings and workshops

Flauger B, Krueger K (2007) Ecology and evolution of equine cognitive abilities. (Poster) Meeting of the Ethologische Gesellschaft, Grünau, Austria

Flauger B, Krueger K (2007) Social feeding decisions in horses (*Equus caballus*). (Poster) 100th Meeting of the Deutsche Zoologische Gesellschaft, Cologne, Germany

Flauger B (2007) Cognitive abilities of horses concerning the integration into new social groups, with special regard to their stress level. (Talk) Group meeting, University of Pisa, Italy

Flauger B, Krueger K (2008) How do horses cope with the conflict situation of being integrated into new social groups? (Talk) Meeting „Conflict and Cooperation in Animal Societies", Debrecen, Hungary

Flauger B, Krueger K (2008) Social behaviour in domestic horses, wild horses (Przewalski horses) and „managed wild horses“. (Talk) Meeting of the Ethologische Gesellschaft, Regensburg, Germany

Krueger K, Flauger B (2008) Social feeding decisions. (Talk) Meeting of the Ethologische Gesellschaft, Regensburg, Germany

Flauger B, Krueger K (2008) Social feeding decisions in horses (*Equus caballus*). (Talk) 4th Meeting in Ecology and Behaviour of the Société Ecologique à Responsabilité Limitée, Toulouse, France

Krueger K, Flauger B (2009) Social and individual olfaction through horse faeces (*Equus caballus*). (Talk) Meeting of the Ethologische Gesellschaft, Göttingen, Germany

Flauger B, Krueger K, Gerhards H, Möstl E (2009) Simplified method to measure glucocorticoid metabolites in faeces of horses. (Talk) Annual Meeting of the Deutsche Zoologische Gesellschaft, Regensburg, Germany

Krueger K, Flauger B (2009) Horses (*Equus caballus*) show respect and trust in their owners. (Talk) Annual Meeting of the Deutsche Zoologische Gesellschaft, Regensburg, Germany

Krueger K, Flauger B (2009) Die Neigung der Pferde, sich am Fokus der menschlichen Aufmerksamkeit zu orientieren, hängt von der Körperhaltung und dem Bekanntheitsgrad der Person ab. (Talk) Workshop Training, Haltung und Belastung beim Pferd, Neustadt (Dosse), Germany

Flauger B, Krueger K (2009) The horse’s integration into new social groups, with special regard to the stress level. (Talk) Group Meeting, University of Groningen, Netherlands

INTERNATIONAL COOPERATION

- University of Veterinary Medicine, Vienna, Institute of Biochemistry, Department of Biomedical Sciences/Biochemistry
- Ludwig-Maximilians-University Munich, Equine Clinic, Faculty of Veterinary Medicine
- University of Muenster, Institute of Neuro- and Behavioural Biology
- Max Planck Institute for Evolutionary Anthropology, Department of Developmental and Comparative Psychology
- University of Pisa, Department of Veterinary Anatomy, Biochemistry and Physiology
- University of Groningen, Theoretical Biology Group
- Landschaftspflegeverband Mittelfranken
- Tierpark Nürnberg
- Tierpark Hellabrunn
- LVFZ Haupt- und Landgestüt Schwaiganger Pferdehaltung
- Stable and horse owners in Southern Germany

THANK YOU …

I think the “thank you pages” are the most widely read pages of PhD theses, therefore I will try to do my best!

Prof. Dr. Juergen Heinze
… for giving me the opportunity to work in his group
… for the supervision and support at all times

Dr. Konstanze Krüger
… for supervising my work and adopting me as her first PhD student
… for her encouragement whenever I needed it
… you are really too nice for this world! I hope our paths will cross in the future, both professional and private.

Prof. Dr. Erich Möstl
… for giving me the opportunity to collaborate with him and for allowing me to analyse the samples in his laboratory
… for agreeing to act as a reviewer of my thesis

Dr. Jürgen Stolz
… for all advice during my studies, my diploma and PhD thesis, and for not restricting his advice to the scientific realm

Knut and Tom Krüger
… for helping me with all sorts of statistical issues and computer problems
… for keeping me company during observations and for telling funny stories

Gudrun Schneider
… for talks and amusing times at the “mensa”, and during coffee, “kaba” and tea breaks
… for deciding to get your diploma thesis in our group. Continue with a PhD!
… it is worth waiting (insider…)

Angelika Oppelt
… for becoming my friend during our dissertation years

Marion Füßl
… for breaking up the daily routine with coffee sessions and for talking nonsense

Volker Schmid and Bartosz Walter
... for being good „office fellows“

TA’s, Fotolabor and office
Andi: … for the saliva-centrifugation-technique-invention
Birgit: … dear wichtl, do you remember the picture frame?
Doris: … for a lot of funny stories
Maria: … for advice during our coffee breaks
Renate: … for your helpfulness
Stefan: … when will you come visit me?
Tina: … for locking up the samples so that nobody will steal them ☺

all group members
… for their help with problems, both major and minor, in my daily routine

Verena, Wieky and Natalie
... for giving me call when new horses were introduced, for always taking time at our visits, and for good conversations

Dr. Henning Thies
… for correcting my English and for improving my knowledge of the English language
… it is great that friendship can cross generations

Dr. Doris Pfaffinger
… for proofreading and for convincing me to enjoy a lovely vacation in Newfoundland

Stable masters and horse owners
… for allowing me to observe and work with your horses
… for helping me collecting faecal and salivary samples, and sometimes even storing them in your freezers

Stefanie Kiener
… for the wonderful drawings for this thesis, even though I asked you only one week prior to the date of submission

Paula-Heike-Flotte Biene-Paperservice
… for everything, you know what I mean. Yeah, we did it!

Catja
… for always bringing me back into real life

Sonja, Andy and Sebastian including the whole animal crew
… the time with you in Brennberg was one of the best times in my life
… for everything and just stay in the way you are!

Klaus
… without words ♥

My parents and my dear brothers Thomas and Martin with family
… for all your support during my entire life
… for being there when I needed you

In memoriam: Mama and Billy

… to all the people I didn’t mention by name but who helped me with conducting test series and with whom I discussed my work and got helpful comments and ideas

Billy, Ziska, Lena, Nesmeli, Dareba, Frieda, Maya, Emma, …
… for distraction in my leisure time

MEDION laptop
… for holding on till the end of the thesis

Bayerische Eliteförderung
… for the financial support

Cooperation partners
… for their help

EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich, die vorliegende Dissertation selbständig und ausschließlich unter der Verwendung der angegebenen Quellen und Hilfsmittel angefertigt zu haben. Diese Arbeit wurde bisher weder einer Prüfungsbehörde vorgelegt noch veröffentlicht.

Regensburg, im Juni 2010

Birgit Flauger

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