Linköping University | Department of Physics, Chemistry and Biology
Master Thesis, 60 hp | Educational Program: Applied Ethology and Animal Biology
Spring 2017 to spring 2018 | LITH-IFM-A-EX-18/ 3463-SE

Relation between different lifestyles and hair cortisol in horses (*Equus caballus*)

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Avdelning, institution
Division, Department

Department of Physics, Chemistry and Biology
Linköping University

Datum
Date
11/05/2018

Språk
Language

- [ ] Svenska/Swedish
- [x] Engelska/English
- [ ] ______________

Rapporttyp
Report category

- [ ] Licentiatavhandling
- [x] Examensarbete
- [ ] C-uppsats
- [ ] D-uppsats
- [ ] Övrig rapport
- [ ] ___________

ISBN

ISRN: LITH-IFM-A-EX--18/3463--SE

Serietitel och serienummer Title of series, numbering

ISSN

URL för elektronisk version

Titel
Title

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Sammanfattning
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Nyckelord
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1 Abstract

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2 Introduction

Traces of domestication of horses go as far back as around 4000 BC, in the late Neolithic period (Mills and McDonnell 2005, Minero and Canali 2009). Ever since that time, humans have used horses for several purposes such as food, farming, transport and eventually sports and leisure (Clutton-Brock 1981). Although horses have undergone domestication for more than 6000 years, it has been shown that this has not affected their behavioural patterns (Christensen et al. 2002).

Horses are social animals that spend most of their time in groups, walking and grazing, thus it is important to provide horses with an environment where they can express these behaviours (Minero and Canali 2009, McGreevy 2004). An animal’s welfare can be described as its state when coping with its environment, this includes behavioural and physiological coping mechanisms (Broom 2008). Equine welfare is a growing research topic and horse behaviour has been thoroughly studied to understand and better fulfil their needs (Cooper et al. 1998, Visser and Van Wijk-Jansen 2012; Dalla Costa et al. 2014; Viksten 2016). Management conditions such as housing style and feeding regimes can have an impact on horses' well-being. Indeed, if an animal is confronted with an inadequate environment it will be likely to experience stress which might be reflected in its behaviour (Cooper and Mason 1998; Visser and Van Wijk-Jansen 2012).

Stress can be defined as an unpredictable, uncontrollable environmental challenge that can result in adverse consequences for an animal, eventually reducing fitness (Broom 2008; Koolhaas et al. 2010). According to this definition, the presence of a stressor can affect an animal's health, and if prolonged could impair welfare. Indeed, it is thought that it is the animal's perception of a particular situation that can result in stress, and the state resulting from this perception is the welfare (Veissier and Boissy 2007). Welfare includes both physical and mental aspects thus it's assessment needs to consider both physiological and behavioural parameters (Viksten 2016).

The hypothalamic-pituitary-adrenocortical axis (HPA axis) is a central endocrine pathway involved in the stress response in mammals. However it is important to keep in mind that it also has implications in other biological processes such as reproduction, immune responses and energy balance (Habib et al. 2001, Keay et al. 2006, Dunn 2007). When the HPA axis is activated, the hypothalamus will release CRH (corticotropin-releasing hormone) this will, in turn, activate the anterior pituitary gland which will release ACTH (adrenocorticotropic hormone). The ACTH will induce the secretion of corticosteroids, including cortisol, by the adrenal cortex (Jansen et al. 2015) (Figure 1).

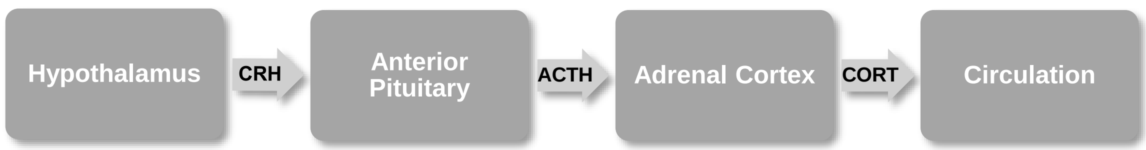


Figure 1. Activation pathway of the hypothalamic-pituitary-adrenocortical axis to produce cortisol. CRH: corticotropin-releasing hormone; ACTH: adrenocorticotropic hormone; CORT: Cortisol.

The activity of the HPA axis has often been evaluated in human and animal stress research and is commonly assessed by measuring glucocorticoids (Meyer and Novak 2012). In most mammals, cortisol is the main glucocorticoid secreted following a stress response thus, within seconds of stressor exposure, cortisol levels will rise in the blood circulation (Keay et al. 2006, Macbeth 2013). This increase in cortisol allows the organism to deal with the stressor by modulating the immune system and energy stocks (Russel et al. 2012). However, repetitive activation of the HPA axis and prolonged elevated cortisol can have a negative impact on physiological functions such as digestion, reproduction or growth (Habib et al. 2001). Although cortisol is often measured to assess stress levels, its link to chronic stress is still unclear. Indeed, studies on wild animals have shown that there is no predictable endocrine response to chronic stress. In fact, chronic stress can induce increases or decreases in glucocorticoids and this variation might be influenced by the species and the nature of the stressors (Dickens and Romero 2013). Furthermore, results are not consistent when it comes to cortisol variation in long-term stress (Pawluski et al. 2017). For instance, previous research on chronic stress has described blood cortisol concentrations to increase in pigs exposed to noise stress (Otten et al. 2004). Others have shown a decrease in HPA activity in socially isolated calves (Van Reenen et al. 2000) and no variation in cortisol levels in outdoor- or indoor-housed ewes (Casamassima et al. 2001, Pawluski et al. 2017).

Cortisol measurements are usually carried out on blood plasma, urine, milk, saliva and faeces samples (Meyer and Novak 2012; Peric 2014). The use of these matrices can be problematic as sampling can be invasive and time sensitive (Meyer and Novak 2012; Peric 2014). Using hair as a matrix for measuring cortisol levels offers a non-invasive alternative for assessing HPA activity over weeks or even months (Meyer and Novak 2012; Peric 2014). After cortisol is released into the circulation by the HPA axis, it can be incorporated in the hair through the blood supply of follicular cells responsible for the growth of the hair shaft. Hair cortisol reflects the general adrenocortical activity over a period of time and offers the opportunity to study cortisol levels retrospectively (Meyer and Novak 2012). It is a promising marker for chronic stress and has been used as an indicator of HPA activity in several species such as humans (Karlén et al. 2011, Russel et al. 2012), dogs (Roth et al. 2016), bears, caribous (Macbeth 2013) and cows among others (Comin et al. 2013).

Although hair cortisol has been assessed in several species, only few recent studies evaluated hair cortisol concentrations in horses (Comin et al. 2012, Montillo et al. 2014, Duran et al. 2017). Hair cortisol measured in shaven withers hair in young foals showed that cortisol concentrations can indeed be measured in horse hair (Comin et al. 2012). Montillo et al. (2014) also analysed withers hair in foals and found that environmental factors (temperature, rainfall and day length) did not influence hair cortisol levels. Recently, Duran et al. (2017) carried out measures on mane and tail hair in adult horses to assess long-term stress. Their results suggest that horse hair cortisol concentrations can vary in response to exposure to a stressor (Duran et al. 2017). Hence, hair seems to be a promising matrix to measure cortisol variation in horses although a standardisation of body location and hair type needs to be done.

Animal personality can be described as individual behaviour that is different from that of other conspecifics and consistent over time and across context (Stamps and Groothuis 2010). Although research on animal personality has become popular in the last two decades (Jensen 2017), horse personality is yet to be more thoroughly studied. For instance, it has been shown that personalities in horses can be breed typical (Lloyd et al. 2008). Moreover, Hausberger et al. (2008) suggest that horse personality can influence the human-horse relationship. Animal personality can be an important tool to ensure optimal individual welfare (Müller and Schrader 2005). Indeed, since individuals have different ways of coping with a situation, their experienced well-being might vary. Taking that into account, personality assessments may provide important individual information to influence management practices and thus improve welfare (Tetley and O'Hara 2012).

2.1 Aims

This study was conducted to gain further knowledge of hair cortisol levels in adult horses and ponies of several breeds. The first aim of this project was to determine if two types of hair and body locations differed for measuring hair cortisol concentrations in horses. The second objective of the study was to investigate whether horses having different lifestyles and management regimes varied in behaviour and/or hair cortisol levels. The third aim was to analyse if personality can influence a horse's hair cortisol levels.

3 Materials and Methods

3.1 Animals

A total of 153 horses and ponies from seven different stables in Östergotland, Sweden, were recruited to participate in this study. Horses' ages ranged from 3 to 28 years, however the age of 18 horses was unknown. Stable A provided 16 riding school Icelandic horses (mean age: 15.9±1.4 years, one unknown age); Stable B had 14 riding school Icelandic horses (mean age: 13.1±2.1 years, 7 unknown ages); Stable C contained 13 riding school Icelandic horses and 12 riding school Shetland ponies (mean age: 13.8±1.3 years); Stables D and E were both riding schools with respectively 46 and 23 horses and ponies of various breeds (mean age D: 14.1±0.8 years, 8 unknown ages and mean age E: 11.2±0.8, 2 unknown ages); Stable F had 16 Swedish Trotters (mean age: 6.3±1.4 years) and Stable G had 13 Swedish Trotters (mean age: 5.2±0.5 years). More information about the horses' age, sex and breed can be found in Appendix 1. In all further sections of this manuscript the stables will be referred to as Icelandic A, B or C, Riding school D and E and Trotter F and G. No animals were harmed for this experiment. No special ethical permit was required since hair cutting in domestic horses is part of grooming routines

3.2 Hair cortisol analysis

3.2.1 Sample collection

Two hair samples were collected from each horse in September 2017. A strand of mane was cut behind the head collar position using scissors and a second sample was cut using clippers on the left side of the horse, in front of the withers and above the scapula (Figure 2). Both samples were cut or shaven as close to the skin as possible, in cases where the hair was too thick, or the horse reacted defensively to clippers, only scissors were used. The collected hair samples were stored in labelled plastic zip bags in dry conditions at room temperature until further analysis.

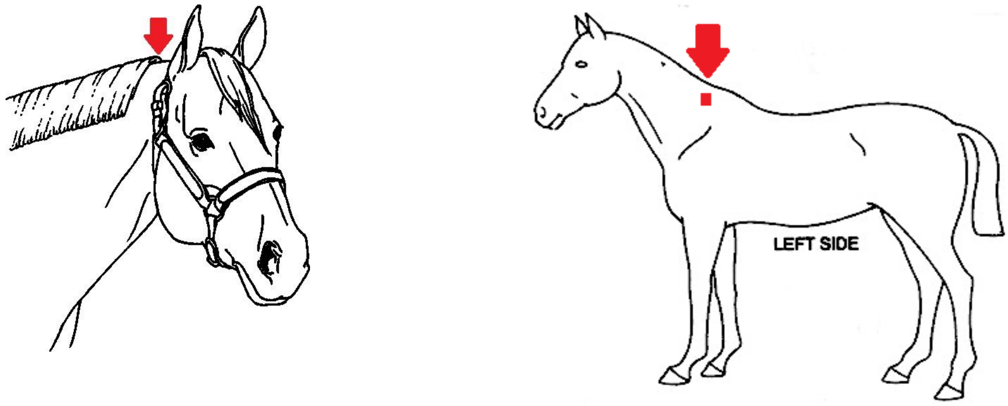


Figure 2. Illustration of the hair cutting positions. Left: Position of first hair sample, strand of mane behind head collar position. Right: Position of second hair sample, clipped hair from the left side, in front of the withers and above the scapula.

3.2.2 Cortisol extraction and radioimmunoassay

Cortisol extraction and analysis were done according to methods previously described by Karlén et al. (2011) and Roth et al. (a2016). Due to differences in length of the different hair samples, it was decided to calibrate the segment lengths according to the shortest samples collected. Thus, one centimetre (cm) of hair closest to the skin was cut into smaller (1-2 mm) pieces and weighed to approximately 7 mg, the exact weight was recorded for further calculations. The hair samples were then frozen in liquid nitrogen and pulverized with steel beads using a Ratch Tissue lyser II (2 minutes at 23 Hz). 1 ml of methanol was added to each sample after which they were shaken overnight in a tube shaker. On the following day the samples were centrifuged (23G, 1 minute, 4°C). 800 µl of the supernatant was taken and put in a Savant Speed-Vac Plus SC210A to evaporate (1.5 hours, -OH mode). The remaining pellets were stored in the refrigerator until further analysis.

The cortisol pellets were quantified using a Radioimmunoassay technique. Standards as well as high and low controls were made with different concentrations of exogenous cortisol and RIA buffer (Bovine serum albumin, Phosphate buffer, Triton-X-100), this allowed for a standard curve for sample measurements. The previously extracted horse hair cortisol pellets were dissolved in 150 µL RIA buffer. 50 µL of this dissolved solution was taken and 100 µL of primary antibody (Anti-cortisol rabbit antibody) was added to it. After a 48-hour incubation period, 100 µL of radioactive conjugated cortisol was added to each tube, these were then incubated for 24 hours. After this delay 75 µL of SAC-CEL (Solid phase second anti-rabbit antibody coated cellulose suspension) was added to all samples. After 30 minutes, the reaction was stopped by adding 2 mL of water. The samples were then centrifuged for 15 minutes (3000 rpm; 4°C). The water was removed using a decanting tool and the tubes were placed in a gamma counter. Each sample was counted for 300 seconds and the output was calculated by a software (Multicalc) which gave measures in CPM and nmol/L. A manual conversion (see equation below) was then performed in order to obtain measures in pg of cortisol per mg of hair:

$$\frac{([\text{Cortisol nmol/L}] \times \text{Molar mass}_{\text{Cortisol}}) \times \text{Volume}_{\text{Dissolved RIA buffer}}}{\text{mass}_{\text{Hair sample}} \times \text{Volume proportion}_{\text{taken after extraction}}}$$

3.3 Behavioural observations

Observation sessions were held in the autumn (from early September to late October). A total of four hours of observation were held at each stable. Observations were carried out for two days per stable (two hours/day), with at least two weeks between each observation day. The time was divided in four 30 minutes sessions spread out from 8.30 a.m to 12.15 p.m., with approximately 20 minutes waiting time between each session. Horses were observed when two or more individuals were together on pasture. Rotational focal sampling was used, switching between individuals in a randomised manner every two minutes. A random horse was selected at the start of the observation session, when rotating to another individual, the horse to its right was selected. 1/0 interval sampling was carried out, with 30 second intervals. Occurrence was noted for states and frequency for events (in italics in table 1). Inter-observer reliability was accounted for by comparing data collected with a second observer in the first four hours of observation (Mann Whitney U tests $p > .05$ for all observed behaviours) (Appendix 2). For all remaining observation sessions, the same observer collected all data. Social, stereotypical, locomotion and feeding-related behaviours were recorded by completing ethogram worksheets at the time of the observations. A full description of the analysed behaviours can be found in the table below (Table 1).

Table 1: Ethogram of behaviours observed on horses out on pasture. The behaviours in italic represent events, while the non-italic behaviours are states.

Behaviour	Definition
Social interactions[3]	
Biting	Reaching for conspecific with an open mouth while flattening ears
Kicking	One or two legs extended in the air towards conspecific while flattening ears
Pushing	Moving a conspecific by applying physical pressure to its body
Head threat	Extending neck towards conspecific while flattening ears
Stomping	Stamping one or two feet against the ground near a conspecific
Mutual sniffing	Nostrils of two conspecifics are in immediate proximity
Flehmen response	Extending neck and rolling back upper lip, exposing teeth
Play behaviour[1]	Biting, kicking as previously described but with ears mobile, in forward or axial position. Also includes pursuit of a conspecific
Grooming:	
● *Mutual*	Two horses simultaneously nipping or rubbing each other's neck, withers and/or back
● *One-way*	One horse nipping or rubbing a conspecific's neck, withers and/or back. Includes horse nipping or rubbing its own body.
Vocalization[3]	
Neighing	Long and loud sound emitted by a horse with mouth slightly open
Snorting	Expulsion of air through the nostrils creating a repetitive sound
Stereotypic behaviours[3]	
Crib-biting	Putting pressure on stationery structure with teeth then inhaling deeply
Weaving	Shifting body weight from side to side repeatedly
Pacing	Walk/trot or canter back and forth repeatedly in a given location
Locomotion[3]	
Walk	Four beat movement where two or three hooves are on the ground
Trot	Two beat movement where two or no hooves are on the ground
Canter/Gallop	Three beat movement where two or three hooves are on the ground/Four beat movement where all hooves are off the ground in each stride

Lying down	Horse is on the ground with four legs in contact with the ground
Standing still: [2]	
● Resting	Body usually supported by 3 legs, ears rotating laterally, eyelids drooping. Neck may drop below horizontal
● Alert	Head held higher than neck, eyes wide open
● Withdrawn	Stretched neck, similar neck and back height. Eyes wide open and no ear or head movements
Feeding-related behaviours[3]	
Grazing/Browsing	Teeth and lips are in immediate proximity with grass/leaves. Steps may be taken.
Eating roughage	Teeth and lips are in immediate proximity with roughage
Drinking	Lips are in immediate proximity with water

References: Hausberger et al. 2012[1], Fureix et al. 2012[2], McGreevy 2004[3]

3.4 Personality and lifestyle assessment

An online questionnaire in Swedish was sent to the stable staff. The first twelve questions investigated general information about the individual horses such as height, breed, age, and sex of the horse. Some questions were management-based such as the average hours of training per week, the intensity of that training, the average hours per week the horse is out on pasture, the average hours per day the horse has access to roughage, etc. A full list of the sent questions can be found in the appendix (Appendix 3).

The horse personality questionnaire (HPQ), developed by Lloyd et al. (2007) was adapted and translated to Swedish for this study. The survey was made of 25 behaviourally defined adjectives (BDA) which were each followed by a definition; the owner had to score each BDA accordingly to the degree by which the horse expresses this behaviour. The scoring was done using a seven-point Likert-type scale where 1 means no expression, 4 means average expression and 7 means total expression. The full list of BDAs and their description can be found in Table 2. Additionally, a final question was added to the questionnaire concerning the horse's hierarchy rank which was asked to be scored from 1 (low rank) to 7 (high rank).

Table 2: List of adjectives used to determine the personality type of horses and their description (English version adapted from Lloyd et al. 2007; in the present study the adjectives and their descriptions were provided in Swedish)

Adjective	**Description**
Active	Moves around a lot, does not like staying still for a long time
Aggressive	Causes harm or potential harm to other individuals, both horse and human
Apprehensive	Seems to be anxious about everything, fears or avoids any kind of risk
Confident	gets their will through, can control other, rather dominant, individuals
Curious	Willingly explores new situations
Eccentric	Shows stereotypes, unusual mannerisms and exaggerated behaviour
Equable	Reacts to others in an even, calm way; not easily disturbed
Excitable	Overreacts to any change, easily excited, highly strung
Fearful	Retreats readily from others or from outside disturbances
Insecure	Hesitates to act alone; seeks reassurance from others
Irritable	Reacts negatively with little provocation
Motherly	Provides warm receptive secure base for others, is tender and caring
Opportunistic	Seizes a chance as soon as it arises
Playful	Initiates play and joins in when play is solicited
Popular	Sought out as a companion by others
Protective	Prevents harm or possible harm to others
Slow	Moves and rests in a relaxed manner, moves slowly and deliberately, not easily hurried
Sociable	Seeks companionship of others
Subordinate	Gives in readily to others, submits easily and does not put up a fight to defend self
Tense	Shows restraint in posture and movement; carries the body stiffly which suggests a shrinking tendency, as if to pull back and be less conspicuous
Understanding	Responds in a discriminating and appropriate manner to the behaviour of others
Suspicious	Does not trust others readily (human and horse), trusts few individuals
Reliable	Can be trusted to do things or behaves well might also be considered a safe horse to be with
Stubborn	Does not give in easily, not very cooperative
Intelligent	Learns new things easily/fast benefits from mental stimulation

References : Lloyd et al. 2007, Stevenson-Hinde et al. 1980, Morris et al. 2002

When creating the HPQ, Lloyd et al. extracted six components (using principal component analysis) each with different loadings for all individual BDAs. In order to calculate personality scores for the studied horses, the rating for each BDA in the survey was multiplied by the previously determined loadings (See Appendix 4). This allowed for a final score of the following personality traits: Dominance, Anxiousness, Excitability, Protection, Sociability and Inquisitiveness (Lloyd et al. 2007).

An alternative, less time-consuming, questionnaire was sent out to obtain information about horses for which we had not received completed personality surveys. This questionnaire inquired the following:

- Number of hours for which the horse is out on pasture on a regular day in autumn
- Number of hours for which the horse has access to roughage on a regular day in autumn
- Number of hours per week for which the horse is training on a regular week in autumn
- Horse name, date of birth, sex
- Horse's hierarchy rank in the group (1-7 with 1 being the lowest and 7 the highest)
- Whether or not the horse has been competing in the last 6 months

Three lifestyle groups were determined from these questionnaires. The first group was named Free Roaming and included stables in which the horses lived outdoors for more than 18 hours a day all year. The second group, Riding School, included the stables in which horses spent the night indoors in the winter and had regular riding classes. The last group was called Trotter Racing and consisted of stables in which horses were trained to compete in trotter harness racing.

3.5 Statistical analysis

Due to the non-normal distribution of hair cortisol data (Shapiro-Wilk test for non-normality, $p < .05$), non-parametric statistics were used. All correlations were evaluated by Spearman correlation tests. Differences between groups were assessed using Kruskal-Wallis H tests and when differences were found, follow-up pairwise comparisons with adjusted p-values were conducted. Differences between two variables were evaluated with Mann-Whitney U tests. All statistical tests were performed with IBM SPSS statistics 25.

4 Results

4.1 Hair cortisol analysis

Mane and withers hair cortisol measures were correlated to each other ($r_s(151)= .477$, $p < .001$) (Figure 3) and did not differ significantly ($U =12,183$; $n1 = n2 = 151$; $p=.354$). The mean mane hair cortisol concentration was 7.619 ± 0.518 pg/mg and the mean withers hair cortisol concentration was 8.993 ± 0.702 pg/mg. Due to these results, only mane hair cortisol was considered for further analyses and is henceforth referred to as hair cortisol. Sex had no effect on hair cortisol levels in the mane or withers ($p > .05$ with both comparisons) and age was not correlated to either mane or wither hair cortisol concentrations ($p > .05$ with both comparisons).

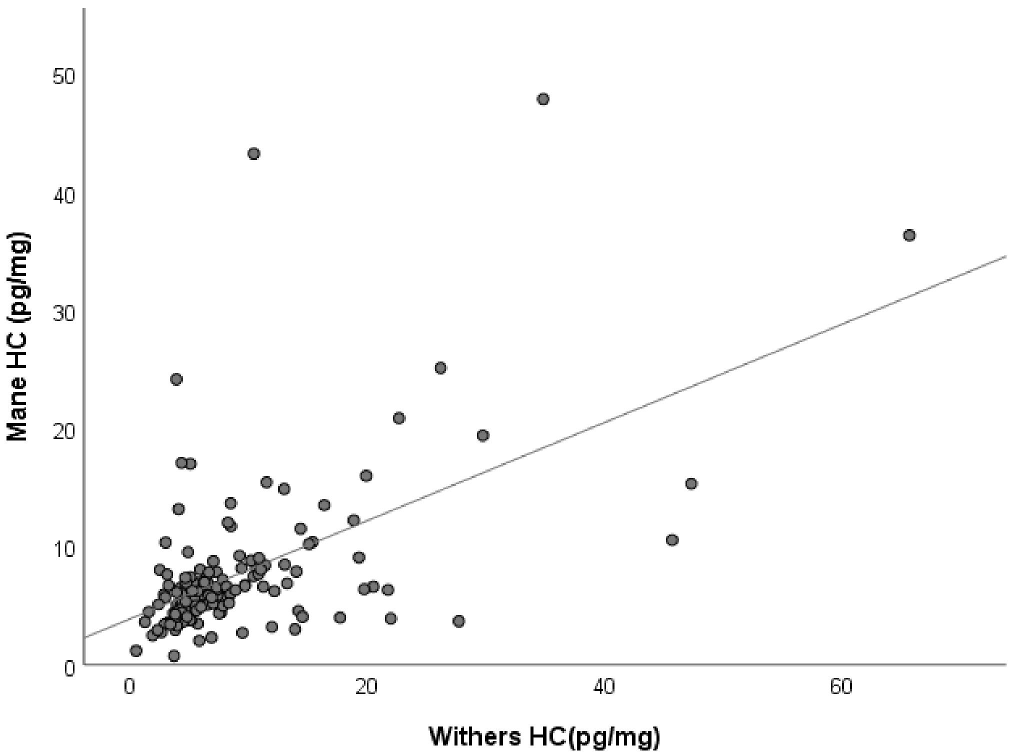


Figure 3. Significant positive correlation between mane and withers hair cortisol concentrations

Hair cortisol measures differed significantly across the stables ($H(6) = 18.633$, $p= .005$). Follow up pairwise comparisons showed that Icelandic A had significantly lower hair cortisol concentrations than Icelandic B ($p= .003$; $z= -3.779$) and than Icelandic C ($p= .019$; $z= -3.320$) (Figure 4).

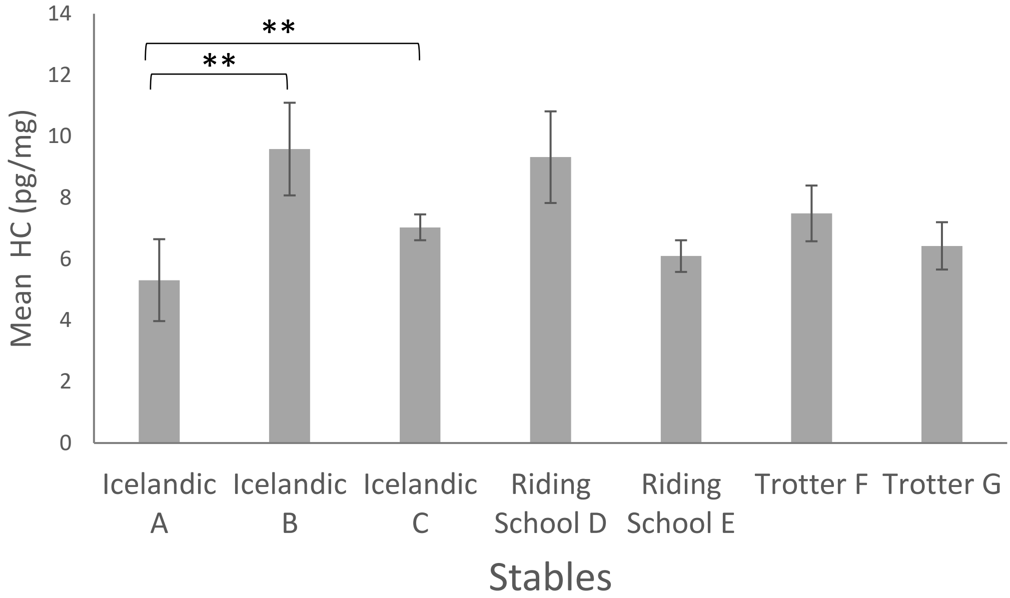


*Figure 4. Mean hair cortisol concentrations across the different stables (Mean ± SE). **: p< .01*

Hair cortisol levels were significantly affected by lifestyle (H(3) = 16.356 , p = .001). Follow-up pairwise comparisons with adjusted p-values showed significant differences in hair cortisol between the Riding School and the Free Roaming lifestyles, with slightly higher hair cortisol levels in the Free Roaming group (p=.007, z= 3.065). No other significant differences were found among the lifestyles (p> .05) (Figure 5).

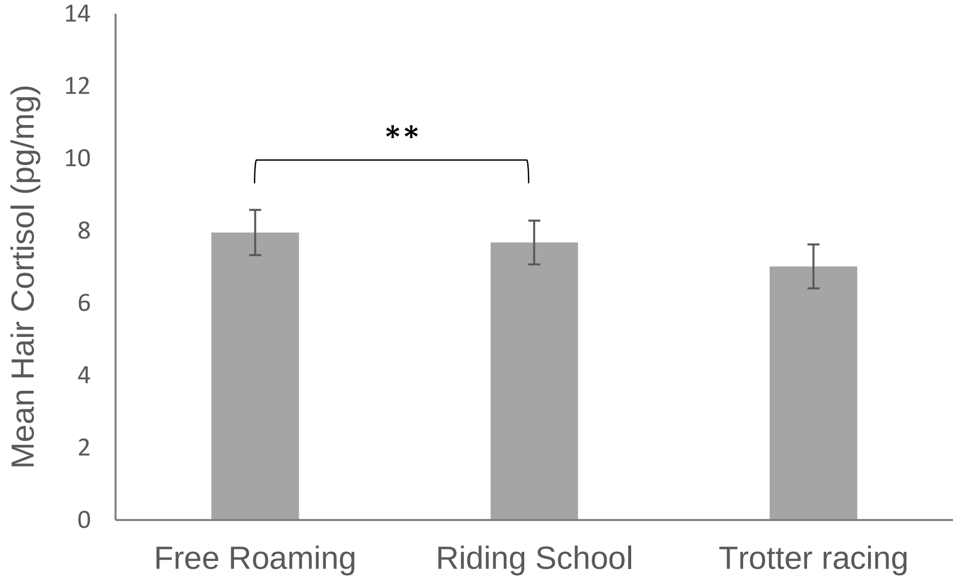


*Figure 5. Mean hair cortisol across the different lifestyles (Mean ± SE). **: p< .01*

4.2 Behaviour observations

No stereotypic and only very few vocalizing behaviours were recorded in all observation sessions, it was therefore decided to remove these behavioural categories from further analyses. The remaining behaviours were pooled into categories for analyses, the list of behaviours included in the different categories can be found in table 3. The categories were analysed as the sum of occurrences of the included behaviours per observation session.

Table 3. Behaviours included in different categories.

Total Social	Social + (positive)	Social - (Negative)	Feeding	Locomotion	Resting
Biting	Mutual sniffing	Biting	Grazing/ browsing	Walk	Lying down
Kicking	Flehmen	Kicking	Eating roughage	Trot	Standing still
Head threat	Play	Head threat		Canter/ gallop	Resting
Pushing	Grooming (mutual & one-way)	Pushing			
Stomping		Stomping			
Mutual sniffing					
Flehmen					
Play					
Grooming (mutual & one-way)					

4.2.1. Behaviour categories across stables

There was a significant difference in total social behaviours between the different stables ($H(6)=20.736$; $p= .002$). Follow-up pairwise comparisons showed that Icelandic B had significantly fewer total social behaviours than Trotter F ($p=.028$; $z=-3.208$), Riding School D ($p=.011$; $z=-3.477$) and Icelandic A ($p=.002$; $z=3.885$) (Figure 6). Positive social behaviour differed significantly between the different stables ($H(6)=21.177$; $p= .002$). Follow-up pairwise comparisons with adjusted p-values showed that Icelandic A had more positive social behaviours than both Icelandic B (p= .011; z= 3.462) and Riding School E (p= .032; z= 3.167) (Figure 6). Feeding behaviour differed significantly between the different stables (H(6)=22.982; p= .001). Pairwise comparisons showed significant more feeding behaviours in Riding School E than both Riding School D (p=.001; z=-4.118) and Icelandic A (p= .008; z= -3.543) (Figure 6). There was a statistical difference in locomotion behaviour between the stables (H(6)=15.904; p= .014). Follow-up pairwise comparisons showed significantly higher locomotion behaviour in Trotter F when compared to Icelandic C (p= .020; z=-3.304) and to Icelandic B (p= .022; z= -3.281) (Figure 6). Resting behaviour differed significantly between the stables (H(6)=26.782; p< .001). Follow-up pairwise comparisons showed that Riding School E had significantly fewer resting behaviour occurrences than Icelandic C (p= .040; z= 3.102), Riding School D (p= .001; z= 4.071) and Icelandic A (p= .001; z= 4.202) (Figure 6). No significant difference was found between the different stables regarding negative social behaviours (H(6)=10.822; p= .094) (Figure 6).

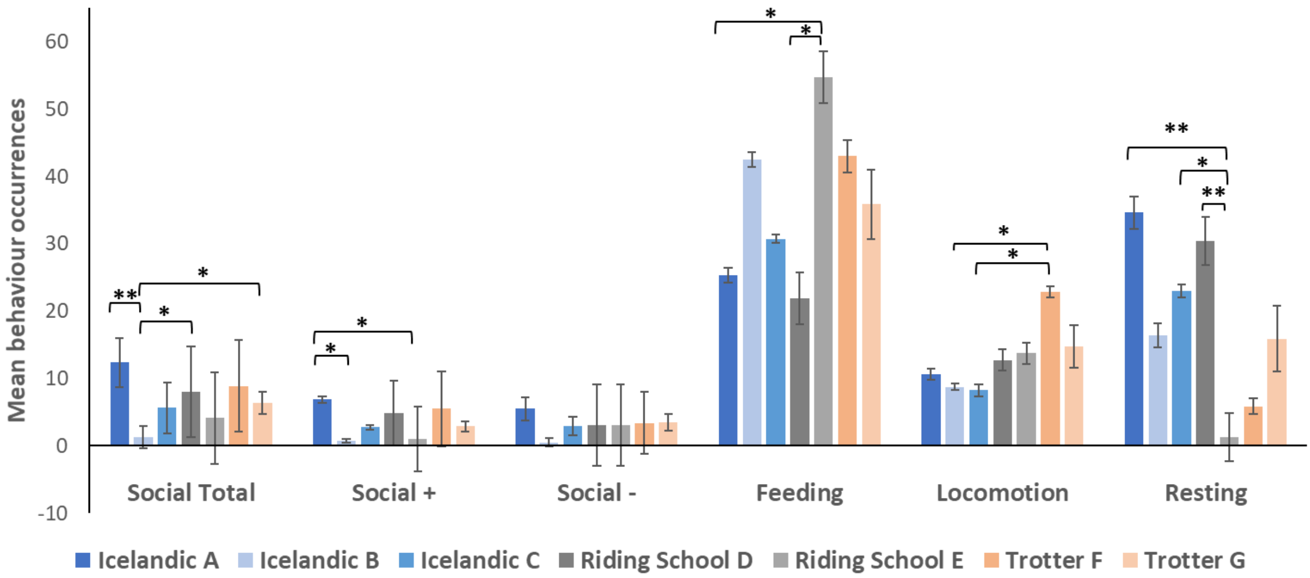


*Figure 6. Behaviour categories per stable (Mean ± Standard Error). Behaviour occurrences are expressed as a mean per observation session. Significant results are reported in the text. *: p< .05; **: p< .01*

4.2.2. Behaviour categories across lifestyles

Three types of lifestyles were established according to routine and management techniques: Free roaming (Icelandic B and Icelandic C); Riding School (Riding School D, Riding School E, Icelandic A); Trotter racing (Trotter F and Trotter G). Behaviours across lifestyles were measured as the mean of behaviour occurrences per observation session in all stables included in the lifestyle type.

Total social behaviours were significantly different across lifestyles ($H(2)=8.053$; $p= .018$). Follow-up pairwise comparisons showed that the Riding School lifestyle had significantly more total social behaviours than the Free Roaming lifestyle ($p= .018$; $z= -2.748$) (Figure 7). Similarly, positive social behaviour differed significantly between the different lifestyles ($H(2)=7.825$; $p= .020$). Follow-up pairwise comparisons showed that the Riding School lifestyle had more positive social behaviours than the Free Roaming lifestyle ($p= .021$; $z= -2.698$) (Figure 7). No significant differences were found between the different lifestyles when looking at negative social behaviours ($H(2)=2.309$; $p= .315$), feeding behaviours ($H(2)=2.066$; $p= .356$) or locomotion behaviours ($H(2)=5.712$; $p= .057$). Resting behaviour differed significantly between the different lifestyles ($H(2)=7.825$; $p= .020$). However, follow-up pairwise comparisons with adjusted p-values showed no significant differences between specific lifestyles ($p > .05$) (Figure 7).

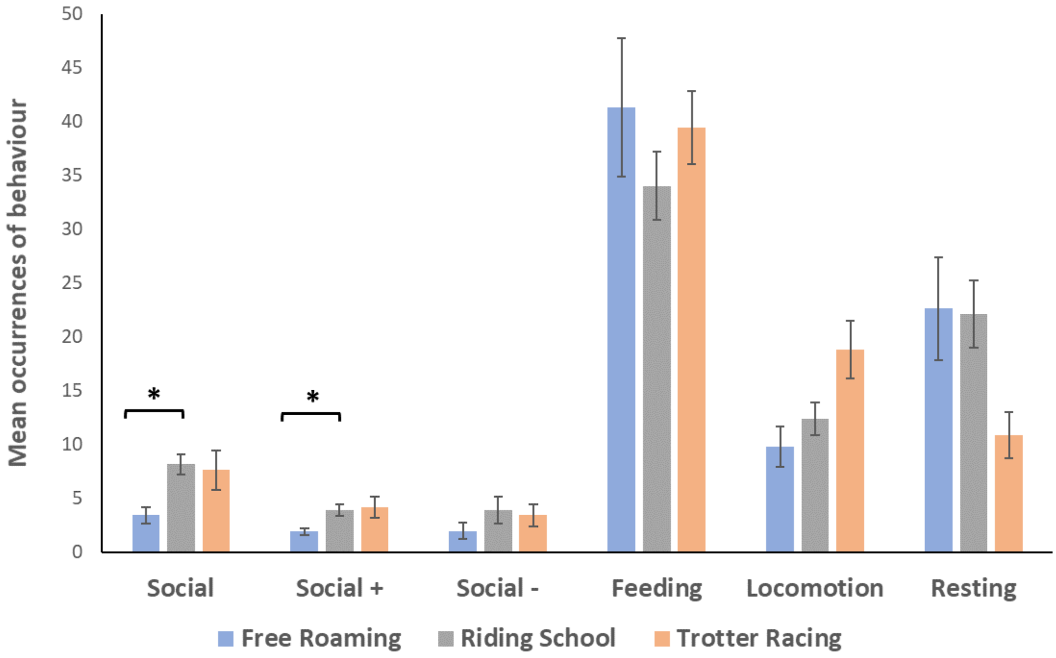
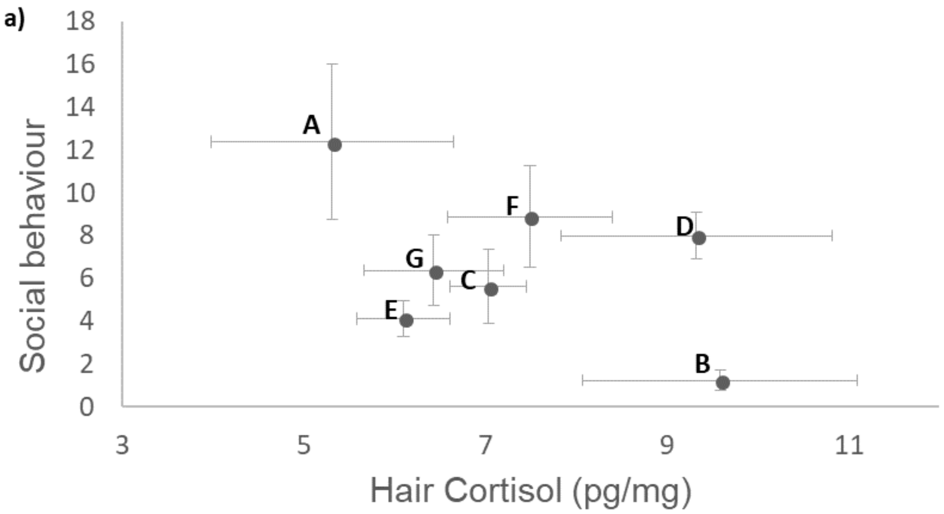
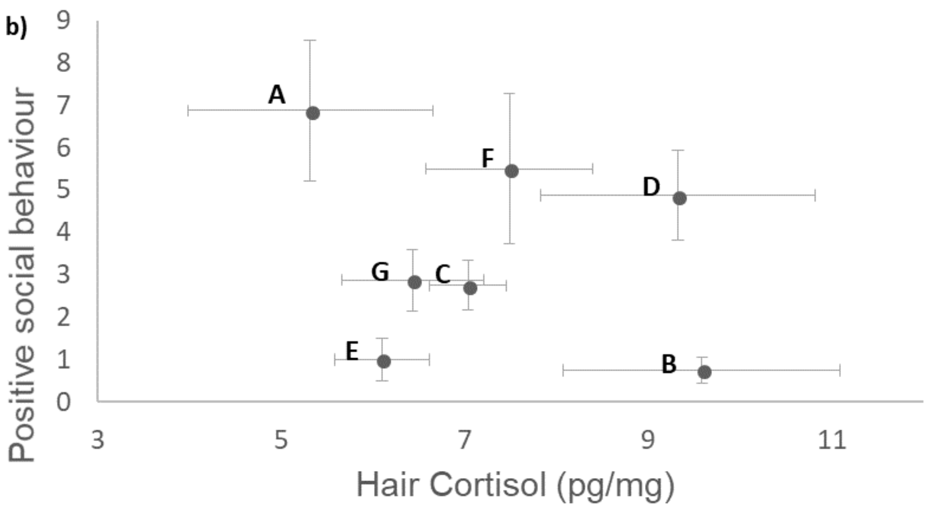


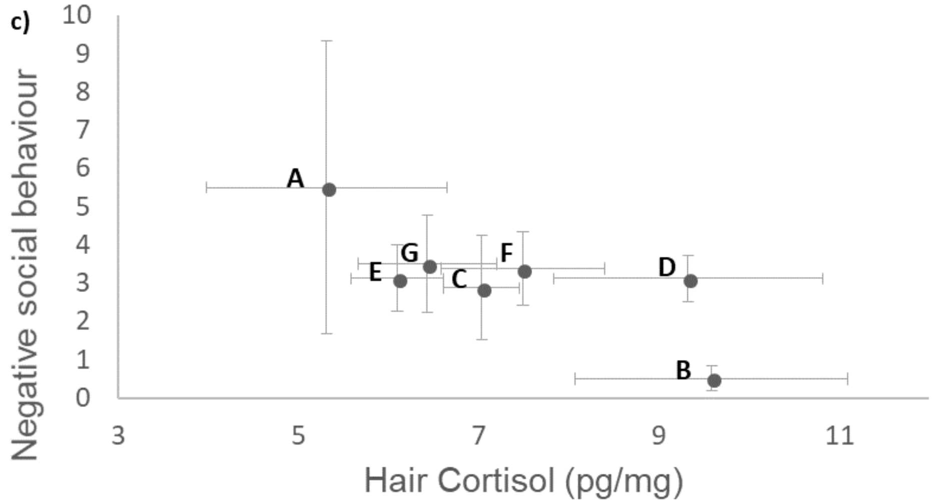
*Figure 7. Behaviour categories across lifestyle groups (Mean ± SE). Behaviour occurrences are expressed as a mean per observation session. *: p< .05*

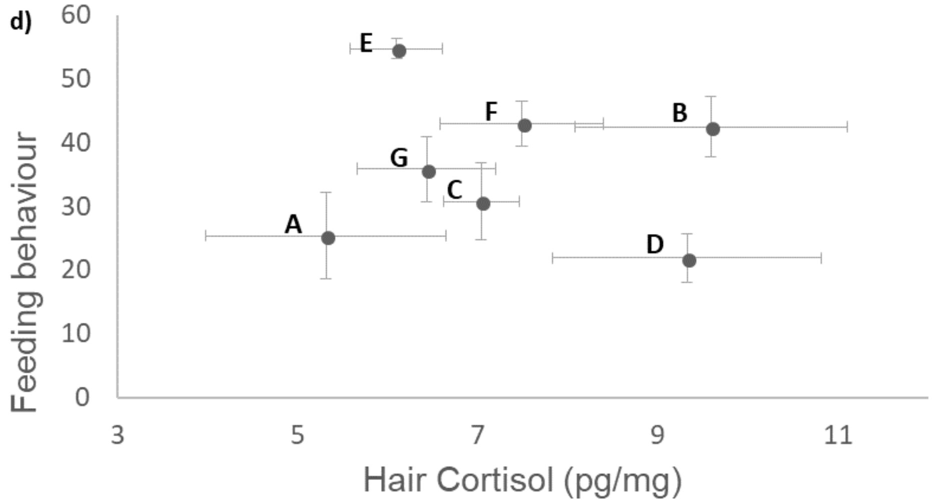
4.3 Behaviour and hair cortisol

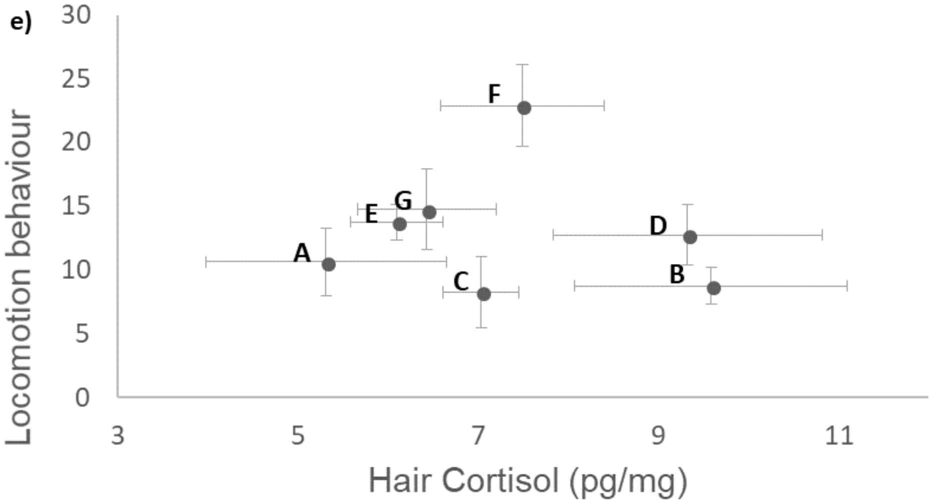
Behaviour category means were established for all stables and compared to mean hair cortisol per stable. There was no significant correlation of hair cortisol and total social behaviour across the stables (r(7)=-.357, p> .05) (Figure 8. a). Similarly, positive social behaviour was not correlated to hair cortisol per stable (r(7)=-.357; p> .05) (Figure 8. b). However, there was a negative correlation trend between negative social behaviour and hair cortisol per stable (r(7)=-.685; p=.090) (Figure 8. c). No significant correlations were found between hair cortisol concentration and feeding behaviour(r(7)=-.036; p>.05) (Figure 8. d), locomotion behaviour (r(7)=-.143; p> .05) (Figure 8. e) or resting behaviour across stables(r(7)=-.357; p>. 05) (Figure 8. f).






c)
Negative social behaviour
A
B
C
D
E
F
G
Hair Cortisol (pg/mg)


d)
Feeding behaviour
A
B
C
D
E
F
G
Hair Cortisol (pg/mg)


e)
Locomotion behaviour
A
B
C
D
E
F
G
Hair Cortisol (pg/mg)

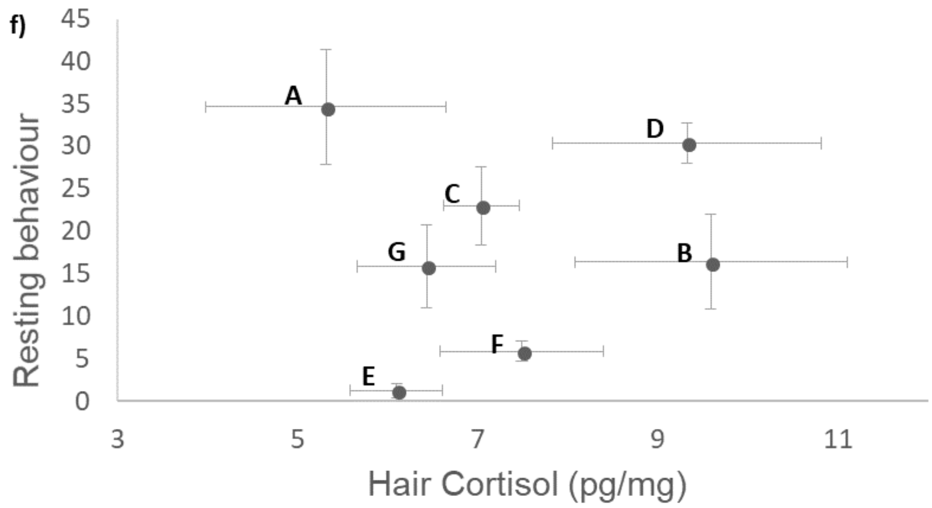


Figure 8. Behaviour categories, expressed as mean of mean occurrences per observation session, and Hair Cortisol per stable (Means ± SE). Data labels on each data point indicate stable name (A-G). a) Social behaviour and hair cortisol concentration per stable b) Positive social behaviour and hair cortisol concentration per stable c) Negative social behaviour and hair cortisol concentration per stable d) Feeding behaviour and hair cortisol concentration per stable e) Locomotion behaviour and hair cortisol concentration per stable f) Resting behaviour and hair cortisol concentration per stable.

4.4 Personality and hair cortisol

Personality scores were established with the Horse Personality Questionnaire (Lloyd et al. 2007) for 42 horses from three different stables (Icelandic B, Icelandic C and Riding School D). The personality score “Dominance” was significantly negatively correlated to hair cortisol (r(42)= -.366; p= .017) (Figure 9, a). There was also a significant negative correlation between “Anxiousness” and hair cortisol (r(42)= -.478; p= .001) (Figure 9, b). Furthermore, “Excitability” and hair cortisol were significantly negatively correlated (r(42)= -.318; p= .040) (Figure 9, c). A negative correlation trend was found between “Protection” and hair cortisol (r(42)= -.273; p= .080) (Figure 9, d) and there was a negative correlation trend between mane HC and “inquisitiveness” (r(42)= -.264; p= .091) (Figure 9, f). No correlation was found between “Sociability” and mane HC (r(42)=.158; p>.05)(Figure 9, e).

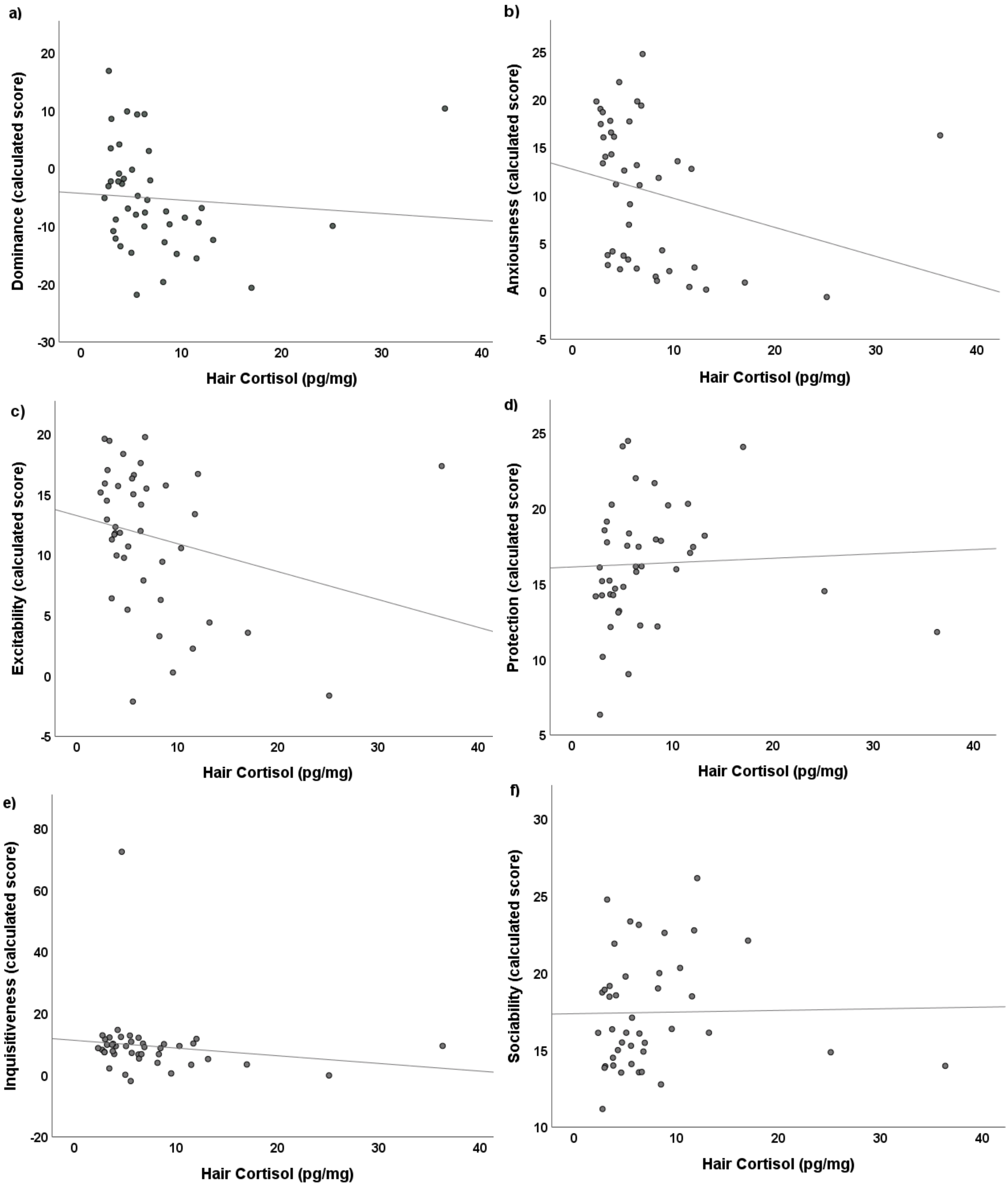


Figure 9. Personality scores and hair cortisol. a) Dominance and hair cortisol ($p < .05$) b) Anxiousness and hair cortisol ($p < .01$) c) Excitability and hair cortisol ($p < .05$) d) Protection and hair cortisol e) Sociability and hair cortisol f) Inquisitiveness and hair cortisol

Since Dominance, Anxiousness and Excitability were all negatively correlated with mane hair cortisol, the relationships between these traits were analysed. It was found that these three traits were strongly positively correlated with each other (Table 4).

Table 4. Correlation coefficient and significance for Dominance, Anxiousness and Excitability

	Dominance	Anxiousness	Excitability
Dominance	-		
Anxiousness	r(42)=.735; p< .001	-	
Excitability	r(42)= .690; p< .001	r(42)=.671; p< .001	-

Hierarchy rank was analysed but no significant difference in hair cortisol was found between low-ranked horses (ranked 1 and 2 in the survey) and high-ranked horses (ranked 6 and 7 in the survey) ($U = 286$; $n1 = 24$; $n2 = 32$; $p = .105$).

4.5 Management and hair cortisol

There were no significant differences in cortisol levels between horses that spent less than eight hours outdoors per day and those that spent 18 hours or more outdoors per day ($U=1000$; $N1=64$; $N2=39$; $p=.181$). Horses that spent less than seven hours per week training did not have significantly different cortisol levels than horses that spent more than 7 hours per week training ($U= 1489$; $N1= 41$; $N2=82$; $p=.303$).

5 Discussion

To our knowledge, this is the first time that different hair types (thin body hair and thick mane hair) were assessed and directly compared for hair cortisol in horses. The results of this study show that both mane and withers are suitable locations for sampling hair to analyse cortisol concentrations. Additionally, there is a link between certain personality types and hair cortisol levels. However, management did not have an influence on the cortisol levels measured in horses.

According to Duran et al. (2017) 2 cm of horse mane or tail hair should contain the cortisol accumulated during one month. Since in the current study one centimetre was cut, the hair cortisol levels that are reported reflect approximately two weeks prior the hair cutting. Hair cortisol concentrations from both body locations were positively correlated and did not significantly differ from each other. This suggests that similar amounts of cortisol were incorporated in both hair types. However, for convenience purposes, it was decided to focus on the values obtained from the mane samples for further analysis since mane hair collection was easier thus preferable for further research. Indeed, mane sampling did not require the use of clippers and mane hair thickness seemed to vary less between individuals than wither hair. However, plucking the mane hair would be a better approach than cutting as it would allow for more precise measurements of segments proximal to the follicle. Although the current study did not find differences in hair cortisol concentrations among body regions, Duran et al. (2017) reported a difference between mane and tail hair cortisol levels. Since results are not consistent and insufficient on this matter, it would be preferable to collect hair from a same body region when analysing horse hair cortisol. Breed difference in hair cortisol was not assessed in the current study, however it was previously reported that hair cortisol levels did not differ between two tested breeds (Duran et al. 2017).

In the current study, mean hair cortisol concentrations were similar in the three lifestyle groups but were significantly higher in the Free Roaming than the Riding School. If compared to existing literature, this might indicate that the riding school horses are more affected by chronic stress than the free roaming horses (Alexander et al. 1988, Mills et al. 1997). Indeed, research on horses shows a decrease in glucocorticoids in response to chronic stress. In fact, several studies have shown that chronic pain and chronic social stress lead to a decrease in plasma cortisol (Alexander et al. 1988, Mills et al. 1997). Moreover, horses with compromised welfare showed a decrease in plasma and faecal cortisol (Pawluski et al. 2017).

The presence of stereotypic behaviours can be linked to environmental deficiencies (McGreevy 2004). In the current study, no stereotypic behaviours were recorded in any stable at any time during data collection which suggests an absence of adverse management conditions. The observed differences in behaviours among stables could be influenced by a number of factors such as the size of the enclosure, the number of horses in the group, whether there was food available at the time of observations etc. Due to all the stated confounding factors, no conclusions can be made for the reasons underlying the observed behavioural differences between stables. However, pooling the stables into lifestyle groups gives a better insight into general behavioural patterns according to routine. For instance, the fact that the Free Roaming group expressed significantly fewer social behaviours than the

Riding School group could be explained by overcompensation of riding school horses and ponies that were housed indoors during the night, usually individually without possibility of social contact. Recent research on enclosure size has found that the bigger the pasture, the fewer social interactions between horses (Majecka and Klawe 2018), thus it would be interesting to investigate pasture size in relation to behaviour and hair cortisol in future studies. The Trotter Racing group tended to have higher locomotion behaviour occurrences when compared to the Free Ranging group. Although this difference was not statistically significant it could be reflecting the fact that since the Free Ranging horses were continuously outdoors, their activity bouts were spread out over the day while the horses that were outdoors for limited amount of time exercised at the moment of the observations. There might also be a breed influence since Swedish trotters tend to be selected for their stamina, however further research would be needed to confirm this suggestion.

The fact that no behaviour category was significantly correlated to hair cortisol levels could be due to the low number of data points (7 stables). However, it is interesting to note that the stable (Icelandic A) which had the highest social and resting behaviour occurrences also had the lowest cortisol mean. It has been previously found that horses having social interactions had lower faecal glucocorticoid levels than socially isolated horses, this might also be the case with hair cortisol and requires further investigation (Yarnell et al. 2015). Furthermore, horses undergoing exercise have been shown to have an increase in plasma cortisol (Gordon et al. 2007). Thus, the elevated resting behaviour occurrences could result in the absence of such an increase in cortisol and explain the case of stable A mentioned above. As all the current behavioural results reflect group behaviour, it would be interesting for future studies to investigate the link between individual behaviour and hair cortisol levels.

Individual variations in behaviour were, however, investigated by the assessment of personality. In fact, results showed that dominance, anxiousness and excitability were traits that were negatively correlated to hair cortisol levels. This means the higher horses were scored on either of these components the lower their hair cortisol concentration. Previous research in humans has shown a decrease in hair cortisol concentrations in humans with general anxiety disorder (Steudte et al. 2011). This is in accordance to horses in the present study which, when scored as highly anxious had lower hair cortisol levels. Moreover, dominance, anxiousness and excitability scores were all correlated with each other. This implies that when horses were given high scores in dominance-related questions in the personality survey, they were also scored high in anxiousness and excitability. When interpreting these personality results it is important to be aware that the scores were attributed by humans and that there might be bias or influence according to the scorer’s personal relationship with an individual horse or the situations in which the horse has been observed by this scorer. Although previous research has shown that personality scores in dogs were not related to salivary cortisol concentrations, further research is needed to increase our understanding of the relationship between personality and cortisol levels in horses (Carrier et al. 2013).

In the present study, the intensity of training and the time spent outdoors did not seem to have an effect on cortisol levels. However, since previous research has shown an increase in cortisol with physical activity, it would be interesting to further investigate management factors that may influence hair cortisol concentrations (Gordon et al. 2007).

Future research needs to investigate hair cortisol in horses and the factors that may affect its variation. For instance, better knowledge of seasonal variation of cortisol in hair, and hair growth in horses would be critical in expanding the use of hair as a matrix to measure cortisol variation in horses. Hair cortisol is a promising tool to provide information on chronic stress and thus could be used in welfare assessments.

In conclusion, the present results showed that hair cortisol levels from both mane and withers did not significantly differ from each other and were highly correlated. Additionally, they varied with lifestyle. Interestingly, personality traits were strongly correlated with hair cortisol levels. These results validate the analysis of cortisol in horse hair and provide more knowledge about this method that is promising in investigating factors underlying chronic stress in horses.

5.1 Societal and ethical considerations

The societal aspects of this research include validating a non-invasive method of sampling cortisol concentrations in horses. This technique is an alternative to restraining and sometimes painful invasive methods such as blood sampling. Knowledge on horse personality can also have an impact on management practices, for example knowing if a horse is anxious can help owners to anticipate potential stressful situations. This research may therefore have an impact on people working with horses on a regular basis.

Better knowledge on chronic stress can lead to better handling and reduction of this stress, which in turn can have a positive impact on domestic horses' welfare. Horse behaviour research is expanding and thus increasing knowledge which will positively impact everyday lives of domestic horses. Efforts need to be made to communicate scientific results to horse professionals in order for them to have an impact and make a difference in improving horses' daily lives.

6 Acknowledgements

I am grateful to all the stables who welcomed us for this study, as well as all the stable staff involved. I thank Lina Roth for her supervision throughout the study and Ann-Charlotte Svensson for her patience and guidance in laboratory analyses. I would like to acknowledge Per Jensen and Ann-Sofie Sundman for their advice, especially concerning the statistical aspects of this research. I thank Josefine Henriksson for her help and company in the field and Enya Van Poucke for her help in the lab and her review of this thesis. Additionally, I am grateful to Matthias Laska and Andrea Cabrera for their review and feedback on this manuscript. Finally, I thank Henri Sauve for his continuous support and encouragement.

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Appendix

Appendix 1. General information about all horses included in the study.

Stable	Horse number	Age	Sex	Breed
A	1	17	M	Icelandic
A	2	16	M	Icelandic
A	3	19	M	Icelandic
A	4	20	M	Icelandic
A	5	12	F	Icelandic
A	6	13	F	Icelandic
A	7	17	M	Icelandic
A	8	18	M	Icelandic
A	9	24	M	Icelandic
A	10	N/A	M	Icelandic
A	11	8	M	Icelandic
A	12	10	M	Icelandic
A	13	26	M	Icelandic
A	14	13	M	Icelandic
A	15	18	M	Icelandic
A	16	7	M	Icelandic
B	1	9	M	Icelandic
B	2	13	F	Icelandic
B	3	N/A	N/A	Icelandic
B	4	N/A	N/A	Icelandic
B	5	18	F	Icelandic
B	6	9	F	Icelandic
B	7	12	M	Icelandic
B	8	N/A	M	Icelandic
B	9	N/A	N/A	Icelandic
B	10	N/A	N/A	Icelandic
B	11	N/A	N/A	Icelandic
B	12	N/A	N/A	Icelandic
B	13	8	F	Icelandic
B	14	23	M	Icelandic
C	1	16	M	Icelandic
C	2	7	M	Icelandic
C	3	9	M	Icelandic
C	4	5	F	Icelandic
C	5	21	M	Icelandic
C	6	18	F	Icelandic
C	7	8	M	Icelandic
C	8	18	M	Icelandic
C	9	9	F	Icelandic
C	10	19	F	Icelandic

C	11	9	F	Icelandic
C	12	15	F	Icelandic
C	13	16	M	Icelandic
C	14	11	M	Shetland
C	15	21	F	Shetland
C	16	19	M	Shetland
C	17	18	M	Shetland
C	18	11	F	Shetland
C	19	28	F	Shetland
C	20	4	M	Shetland
C	21	18	M	Shetland
C	22	8	F	Shetland
C	23	6	M	Shetland
C	24	22	F	Shetland
C	25	10	F	Shetland
D	1	12	F	Grade pony
D	2	21	F	Grade pony
D	3	10	M	Swedish half-blood
D	4	9	F	Grade horse
D	5	8	M	Grade pony
D	6	14	F	Grade horse
D	7	7	M	Swedish half-blood
D	8	25	M	Swedish half-blood
D	9	15	F	Swedish half-blood
D	10	11	M	Swedish half-blood
D	11	14	F	Connemara
D	12	10	M	Swedish riding pony
D	13	18	M	N/A
D	14	19	F	Danish Riding pony
D	15	16	F	Grade pony
D	16	18	F	Danish Riding pony
D	17	10	F	Swedish half-blood
D	18	18	F	Grade pony
D	19	8	M	Welsh part bred
D	20	13	M	Swedish half-blood
D	21	12	M	Welsh pony
D	22	15	M	Grade pony
D	23	15	M	Grade pony
D	24	14	F	N/A
D	25	10	M	Grade horse
D	26	14	F	Grade horse
D	27	N/A	M	N/A
D	28	N/A	F	N/A
D	29	24	F	N/A
D	30	N/A	M	N/A
D	31	20	M	N/A

D	32	8	F	N/A
D	33	13	F	N/A
D	34	9	M	N/A
D	35	N/A	M	N/A
D	36	7	F	N/A
D	37	N/A	M	N/A
D	38	20	M	N/A
D	39	N/A	M	Swedish half-blood
D	40	14	M	N/A
D	41	22	F	N/A
D	42	N/A	M	N/A
D	43	13	M	N/A
D	44	18	F	N/A
D	45	N/A	F	N/A
D	46	13	M	N/A
E	1	13	F	Halfinger
E	2	14	F	Danish Riding pony
E	3	6	F	Swedish riding pony
E	4	5	F	N/A
E	5	9	F	N/A
E	6	11	M	N/A
E	7	N/A	M	N/A
E	8	11	M	Swedish half-blood
E	9	10	M	Latvian half-blood
E	10	17	F	Swedish half-blood
E	11	17	M	N/A
E	12	13	F	N/A
E	13	15	F	Polish half-blood
E	14	9	F	N/A
E	15	9	F	Irish riding pony
E	16	10	F	Connemara
E	17	17	M	New Forest
E	18	6	F	Swedish riding pony
E	19	10	M	N/A
E	20	6	F	Swedish riding pony
E	21	11	F	Shetland x Gotland pony
E	22	17	M	Shetland x appaloosa
E	23	N/A	N/A	N/A
F	1	10	M	Swedish trotter
F	2	7	M	Swedish trotter
F	3	6	M	Swedish trotter
F	4	5	F	Swedish trotter
F	5	5	F	Swedish trotter
F	6	7	F	Swedish trotter

F	7	6	F	Swedish trotter
F	8	3	M	Swedish trotter
F	9	5	M	Swedish trotter
F	10	5	M	Swedish trotter
F	11	16	M	Swedish trotter
F	12	5	M	Swedish trotter
F	13	3	F	Swedish trotter
F	14	3	F	Swedish trotter
F	15	3	F	Swedish trotter
F	16	11	M	Swedish trotter
G	1	8	F	Swedish trotter
G	2	7	M	Swedish trotter
G	3	3	F	Swedish trotter
G	4	3	F	Swedish trotter
G	5	4	F	Swedish trotter
G	6	4	F	Swedish trotter
G	7	3	F	Swedish trotter
G	8	3	F	Swedish trotter
G	9	7	M	Swedish trotter
G	10	6	M	Swedish trotter
G	11	8	M	Swedish trotter
G	12	6	M	Swedish trotter
G	13	5	M	Swedish trotter

Grade horse/pony: horse or pony whose lineage is unknown
N/A: Information not available

Appendix 2. Results of inter-observer reliability assessment. Data recorded for each behaviour in 8 observation sessions were compared between observer 1 and 2.

Behaviour	**Test results**
Biting	U= 35.5, n1=n2=8, p= .684
Kicking	U= 32, n1=n2=8, p= 1.000
Head threat	U= 37.5, n1=n2=8, p= .553
Pushing	U= 32, n1=n2=8, p= 1.000
Stomping	-
Sniffing	U= 36, n1=n2=8, p= .535
Grooming one-way	U= 25.5, n1=n2=8, p= .461
Grooming Mutual	-
Playing	-
Flehmen	-
Grazing	U= 31, n1=n2=8, p= .916
Drinking	U= 32, n1=n2=8, p= 1.000
Eating roughage	U= 32.5, n1=n2=8, p= .958
Walking	U= 32.5, n1=n2=8, p= .958
Trotting	U= 29, n1=n2=8, p= .727
Cantering/galloping	U= 32, n1=n2=8, p= 1.000

Lying down	-
Standing still – Resting	U= 35.5, n1=n2=8, p= .685
Standing still – Alert	U= 33, n1=n2=8, p= .916
Standing still – Withdrawn	-
Neighing	U= 36, n1=n2=8, p= .317
Snorting	U= 32, n1=n2=8, p= 1.000
Crib biting	-
Weaving	-
Pacing	-

- *: Behaviour not observed*

Appendix 3. List of general information questions in the first questionnaire (translated from Swedish)

- Horse's name
- How long have you known the horse for?
- Horse's height (cm)
- Horse's sex
- Approximately how many hours does the horse go out on pasture in a regular autumn day?
    - 0-2h
    - 3-5h
    - 6-8h
    - 9-11h
    - 12-14h
    - 15-17h
    - 18h or more
- Approximately how many hours does the horse have access to roughage on a regular autumn day?
    - 0-2h
    - 3-5h
    - 6-8h
    - 9-11h
    - 12-14h
    - 15-17h
    - 18h or more
- Approximately how many hours is the horse trained/ridden in a regular autumn week?
    - 0h
    - 1-3h
    - 4-6h
    - 7-9h
    - 10-12h
    - 13-15h
    - 16-18h
    - 19h or more
- How intense is the horse's work?

  - 1 – Mostly low intensity, with slight raise in heartrate
  - 2 – Mostly low-medium intensity
  - 3 – Mostly medium intensity
  - 4 – Mostly medium-high intensity
  - 5 – Mostly High intensity, with a big raise in heartrate
- Does this horse compete and if so, how often?
  - No, this horse does not compete
  - Approximately 1-2 times a month
  - Approximately 3-4 times a month
  - More than 4 times a month

Appendix 4. PCA results from Lloyd et al. 2007 identifying 6 personality components and corresponding loadings for all BDAs

BDA	**C1 (Dominance)**	**C2 (Anxiousness)**	**C3 (Excitability)**	**C4 (Protection)**	**C5 (Sociability)**	**C6 (Inquisitiveness)**
Reliable	-0.785	-0.181	-0.262	0.139	0.066	-0.228
Subordinate	-0.772	0.18	0.134	0.07	0.062	-0.032
Equable	-0.656	-0.522	-0.255	0.05	0.089	0.201
Eccentric	0.419	0.37	0.291	0.251	0.121	0.414
Effective	0.612	-0.096	0.203	0.009	0.298	0.079
Stubborn	0.729	0.23	0.17	-0.179	0.01	0.45
Aggressive	0.794	0.077	0.124	0.314	-0.086	-0.024
Irritable	0.837	0.292	0.27	-0.069	-0.015	0.004
Suspicious	0.06	0.75	0.086	-0.265	-0.243	0.013
Insecure	0.077	0.805	0.081	0.084	0	0.044
Tense	0.352	0.822	0.206	-0.054	0.11	-0.092
Apprehensive	0.015	0.907	0.22	-0.048	0.104	-0.022
Fearful	-0.008	0.915	0.067	-0.028	0.085	0.122
Active	0.18	0.236	0.777	0.032	0.411	0.207
Slow	-0.306	-0.228	-0.768	0.123	-0.274	-0.042
Excitable	0.18	0.549	0.709	-0.038	0.036	0.247
Intelligent	-0.031	0.106	0.731	0.367	-0.064	0.279
Understanding	-0.627	-0.038	-0.007	0.679	0.006	-0.166
Motherly	-0.372	-0.006	0.006	0.832	0.078	0.039
Protective	-0.05	-0.132	-0.033	0.842	0.343	-0.072
Sociable	-0.174	0.265	0.036	0.277	0.76	0.329
Playful	0.008	0.086	0.403	-0.049	0.785	0.105
Popular	-0.086	-0.117	0.067	0.428	0.803	-0.83
Curious	0.02	-0.194	0.268	-0.06	0.332	0.697
Opportunistic	0.195	0.064	0.214	-0.066	0.028	0.807