Genetic background of temperament traits in Standardbred trotters

Genetisk bakgrund till temperamentsegenskaper hos varmblodstravare

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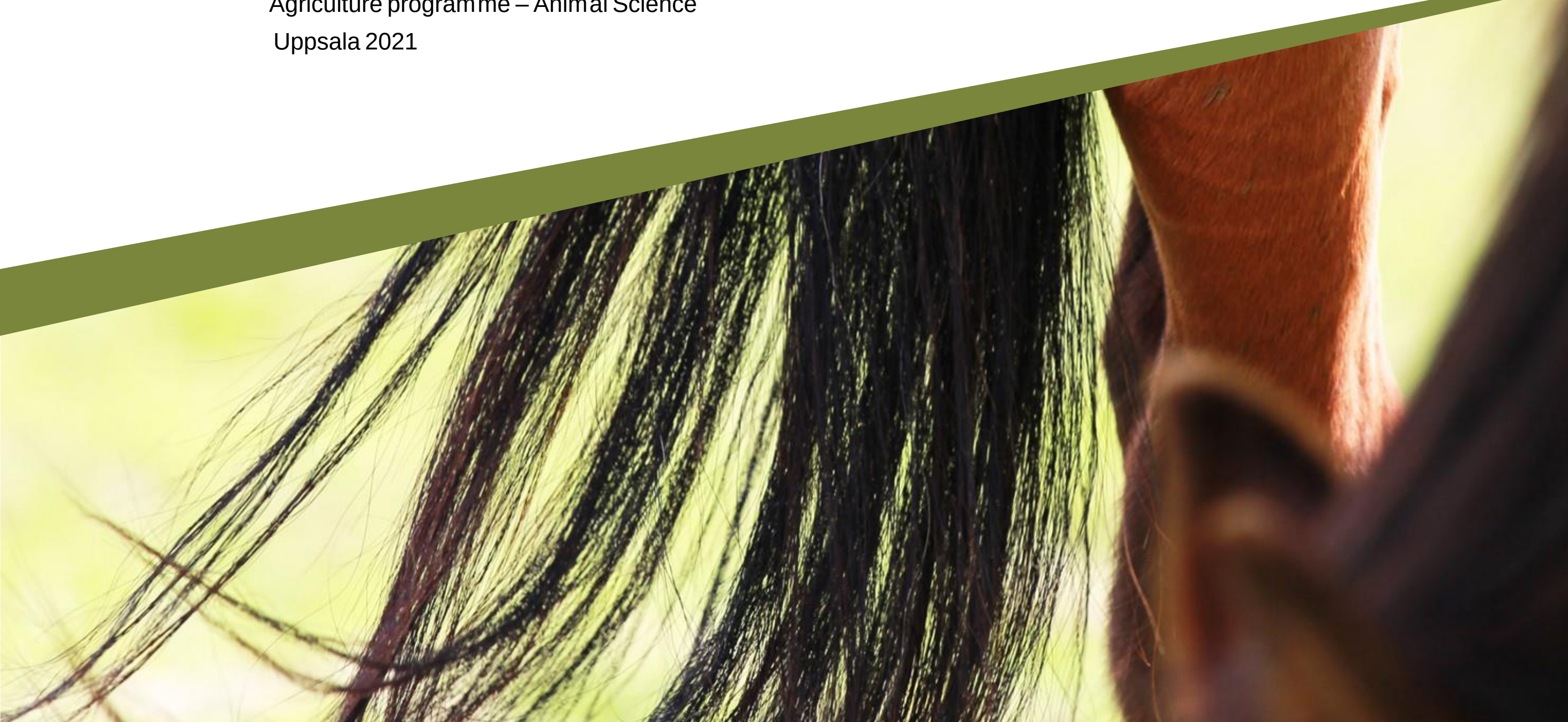

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Abstract

The genetic background of temperament traits in horses has been subject for research for many years to obtain understanding of domestication, breed differences and welfare. Previous studies have found heritabilities ranging from low to moderate for temperament. There are also a few studies who have found genetic regions or genes correlated to temperament in horses, but also genes found important for performance that possibly are linked to temperament. There are various methods used in research to assess temperament. Previous studies have for example used objective methods to assess temperament such as novel object tests. Subjective temperament assessment is also a widespread method used at e.g., young horse-riding tests, but also in the form of surveys aimed at trainers or caretakers of horses. Positive attributes of using a survey are that information about temperament traits that are not expressed at test stations/field tests can be obtained, for example traits related to learning and stress.

In this thesis, temperament assessments of Standardbred trotters were performed by collection of survey responses and DNA, to investigate the genetic background of 13 traits related to temperament at competition and to stress. Factor analysis was applied to find underlying variables explaining the temperament of Standardbreds. By using different statistical models, trait data obtained from a survey including 376 horses were analysed. Variance components for the temperament traits were estimated using linear animal models. In addition, the aim was to, as far as possible, prepare and sequence DNA obtained from the horses in the study. And if possible, perform an association analysis for one of the temperament traits.

Three factors were extracted from the factor analysis: anxiousness, tractability, and excitability. The factor anxiousness included mainly low self-control, memory of unpleasant events and fearfulness. Having a high score for this factor was significantly correlated with performing a stereotypic behaviour. The second factor named tractability, described cooperative horses that easily learned the task of competing and had a high will to win. The third factor, excitability, was dominated by nervousness and excitability. A similar factor has previously been found in Coldblooded trotters; a breed also bred for harness racing. This gave indications of that this temperament characteristic might be favourable when competing in trotting races.

Heritability estimates ranged from 0 to 0.42, where learning and cooperation were the traits with the highest heritability (h^2=0.42 for both). In total, 288 samples were prepared for sequencing whereof 96 samples were sequenced. Based on the heritability estimations and trait score distributions, the trait excitability was chosen to be included in an association analysis. Significant differences between the case and control groups were found at a few loci.

In conclusion, despite the small data material, genetic variation in several of the temperament traits was found and significant heritabilities could be estimated. The next step would be to correlate the traits in this thesis to performance data and to look at the genetic correlation between these traits. The heritability estimations also gave a preliminary indication on which traits could be worth studying further on a molecular genetic level. The preliminary analysis of the whole genome sequencing data showed interesting results worth further investigation with data from more horses included.

Keywords: Temperament, Standardbred trotter, heritability, low-pass whole genome sequencing

Sammanfattning

Den genetiska bakgrunden för temperamentsegenskaper hos hästar är ett ämne som studerats under många år för att få en djupare förståelse för hästars domesticering, rasskillnader och välfärd. De tidigare studier som finns inom området har skattat låga till medelhöga arvbarheter. Ett fåtal studier har hittat potentiella regioner eller gener korrelerade till temperamentsegenskaper, inklusive gener som visats vara viktiga för prestation och som eventuellt är kopplade till temperament. Det finns ett flertal metoder för att studera temperament inom forskning. Tidigare studier inom området har exempelvis använt objektiv bedömning så som reaktivitetstest (Novel object test). Subjektiv bedömning av temperamentsegenskaper hos hästar är en vanligt förekommande metod som används vid ridhästtester men också i form av enkätundersökningar riktade mot tränare och skötare. Fördelen med en enkät är att den fångar temperament som inte alltid kan mätas i en testsituation, så som egenskaper relaterade till lärande och stress.

I denna studie har temperamentsegenskaper hos den varmblodiga travhästen studerats genom insamling av enkätsvar samt DNA. Vidare så analyserades den genetiska bakgrunden till 13 egenskaper relaterade till temperament under tävlingsmomentet och stress. Faktoranalys användes för att undersöka underliggande faktorer som förklarar varmblodstravarens temperament. Med hjälp av olika statistiska modeller analyserades temperamentsegenskaper från totalt 376 individer. Varianskomponenter estimerades med linjära djurmodeller för temperamentsegenskaperna. Utöver detta så var målet att i möjligaste mån förbereda och sekvensera DNA från hästar med enkätsvar samt om möjligt, utföra en associationsanalys för en av temperamentsegenskaperna.

Tre faktorer extraherades genom faktoranalys, dessa var ängslighet, medgörlighet och upphetsning. Den första faktorn ängslighet, inkluderade främst egenskaperna låg självkontroll, minne av otrevliga händelser samt rädsla. Höga poäng i denna faktor var signifikant korrelerat till att uppvisa en stereotypi. Faktor 2, medgörlighet, inkluderade främst egenskaperna vilja att vinna, läraktighet och samarbetsvilja. Den tredje faktorn, upphetsning, inkluderade främst nervositet och upphetsning. En liknande faktor har i en tidigare studie hittats hos kallblodstravare och skulle möjligtvis kunna vara en temperamentsegenskap som är fördelaktig i travtävlingar.

Egenskapernas arvbarhet skattades vara mellan 0 till 0.42, där läraktighet och samarbetsvilja hade de högst skattade arvbarheterna (h^2=0.42 för båda). Totalt förbereddes 288 DNA-prover varav 96 inkluderades i sekvenseringen. Baserat på egenskapernas arvbarhet och poängfördelning så valdes egenskapen upphetsning ut till kommande associationsanalys. Signifikanta skillnader mellan fall- och kontrollgrupper återfanns vid ett flertal loci.

Slutligen, trots ett litet datamaterial, sågs en genetisk variation hos ett flertal av de analyserade temperamentsegenskaperna och signifikanta arvbarheter kunde skattas. Nästa steg är att korrelera temperamentsegenskaperna till prestationsegenskaper och att skatta genetiska korrelationer mellan dessa. Arvbarhetsskattningarna i detta arbete gav en indikation på vilka egenskaper som kan vara intressanta att studera vidare på en molekylärgenetisk nivå. Den preliminära analysen av data från helgenomsekvenseringen visade intressanta resultat som bör analyseras vidare med fler hästar inkluderade.

Nyckelord: Temperament, varmblodstravare, arvbarhetsskattning, helgenomsekvensering

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Abbreviations

AM	Animal Model
BLUP	Best Linear Unbiased Prediction
CBT	Coldblooded trotter
FA	Factor Analysis
GWAS	Genome Wide Association Study
HPQ	Horse Personality Questionnaire
PCA	Principal Component Analysis
QC	Quality Control
SB	Standardbred
SBR	Spontaneous Blink Rate
SNP	Single-Nucleotide Polymorphism
TB	Thoroughbred
WGS	Whole Genome Sequencing

1. Introduction

Each year, about 4400 Standardbred (SB) trotter mares are bred in Sweden. The studbook is open, and the majority of horses have American- or French blood lines (Swedish Trotting Association 2021a). It is described in the breeding objectives for the Swedish SB that breeders should put emphasises on breeding competitive and cooperative individuals suitable for harness racing, the horses should be easy to handle and have a strong desire to win (Swedish Trotting Association 2021a). The temperament of modern sport horses is highly valued and so is the physical ability to perform (Graf et al. 2013; Bartolomé & Cockram 2016). Even though SB horses are bred for competing, studies show that temperament within horse breeds still has a significant variation (Visser et al. 2001). Assessment of advantageous temperament characteristics for elite sport horses as well as measuring individuals' ability to cope with stress during equine competitions have been subject to research for many years. Assessment of stress level is often highlighted from an animal welfare perspective. However, stress is not necessarily exclusively an unfavourable reaction when it comes to competition events, the "fight or flight" reaction is the biological response when the horse must run to escape from predators (Bartolomé & Cockram 2016). The cascade of biological reactions during the "fight or flight" response is what makes the horse a superior competitor and sport animal. Bartolomé & Cockram (2016) also highlights the threshold between stress and distress, when the horse is unable to recover from the temporary stress, it leads to distress followed by behaviour changes such as stereotypes, muscle loss, fertility problems and immuno-suppression. Little is known about the impact of genetics on behaviour and susceptibility to stress among trotting horses. Different temperament traits in horses have been estimated to have heritability values ranging from low (h^2=0.15) to moderate (h^2=0.40) according to a review by König von Borstel (2013).

Up to this date there are no functional genomic studies of temperament traits in horses, but knowledge based on previous research in other species have resulted in some candidate gene approach studies of temperament genetics in horses. A single nucleotide polymorphism (SNP) in the serotonin receptor 1A (*HTR1A*) have been correlated to tractability in Thoroughbred horses (Hori et al. 2016). In the same breed polymorphism in the dopamine D4 receptor gene (*DRD4*) have been linked to vigilance and curiosity (Momozawa et al. 2005b). At the same SNP, Ninomiya et al. (2013) found a significant correlation to frustration level during feeding time.

Another candidate gene study by Song et al. (2017) found a SNP in monoamine oxidase A (*MAOA*) causing more aggressive horses and a SNP in the androgen receptor (*AR*) causing more docile horses.

In this thesis the aim was to assess temperament characteristics linked to stress, cooperation, learning and will to win in the population of Swedish and Norwegian SBs by using a survey aimed at trainers of SB trotters in Sweden and Norway. In addition, the aim was to prepare and sequence DNA obtained from selected horses in the study and perform an association analysis for one of the temperament traits. Low density whole genome sequencing was performed with riptide DNA library preparation protocol, including 96 horses with survey data.

The outcome of this thesis may help to explain the biological background of temperament traits linked to stress, cooperation, learning and will to win. The results will give preliminary estimations of heritability of these temperament traits and possibly associate one trait with the genotypes for some of the horses. The results may give some guidelines for further research on how to select SBs with suitable temperament for harness racing for breeding.

2. Literature review

2.1. The Standardbred trotter

Standardbred (SB) trotters originates from Thoroughbred horses selected for their trotting ability, and the studbook was created 1871 in the US (Petersen et al. 2013). In the US, two subpopulations was later constructed where SB's performing pace belonged to one group and SB's performing trot the other group (Cothran et al. 1987).

Standardbreds are bred for harness racing, a sport where a driver in a sulky is pulled by the horse in trot at high-speed competing against other horses at a racetrack. In Sweden there are in total 3700 trainers, 3300 of these are amateur trainers and 400 are professional trainers (Swedish Trotting Association, 2021b). The racetracks in Sweden are normally 1000 meters long and distances allowed at competition are 1140, 1640, 2140, 2640, and 3140 meters (Swedish Trotting Association 2021a). Standardbreds start to compete from the month of July the year they turn two up to the age of ten (mares) and 14 (geldings and stallions) according to the regulations and rules for Swedish trotting (Swedish Trotting Association 2021a). Before entering a real competition, the horse must have at least one approved qualification race to assure that the horse is trained enough to make it through a real race without a negative impact on animal welfare (Swedish Trotting Association 2021c).

In Sweden, the SB trotter is not allowed to pace in races. The Swedish Trotting Association is responsible for the breeding evaluation of SB trotters in Sweden. All horses have indices estimated with Best Linear Unbiased Prediction (BLUP) animal model (AM). The indices are predicting the genetic value for harness racing performance where 100 represents the mean indices in the population. BLUP-values are based on the horses' performance: number of starts, proportion of races where the horse got placed (1-3), earnings, earnings per start, best time, and racing status. The heritability of each trait, correlations (phenotypic and genotypic), additive genetic relationship, genetic base group, and adjustments for fixed effects are considered in the current statistical model for genetic evaluation (Árnason 1999).

2.2. Methods of temperament assessment in animals

2.2.1. Definition of temperament

In a review article by König von Borstel (2013) the author come to the conclusion that there is confusion and uncertainty of which words should be used in research to describe the personality of horses. However, temperament seem to be the term breeding associations use in their breeding objectives to describe the personality of the breed. The Swedish SB trotters do not have temperament included in the breeding objective, but according to the breeding regulations the horses should have good temperament traits which means that the horses should be cooperative, easy to handle and have a strong desire to win (Swedish Trotting Association 2021a). Therefore, the word temperament will be used in this thesis. In human psychology, temperament can be formed into four different categories according to Goldsmith et al. (1987). The author describes that reactivity to stimuli is one of the cornerstones of understanding temperament, but also the activity level which is defined as how fast or slow the individual moves and think. The two final categories are emotionality which represent the span of happy and sad emotions and sociability, how individuals strive to socialize. Later a five factor model was presented which is now a well-known practise used to describe personality traits in humans (Digman 1990). This method aims to distinguish between individual's personality traits, traits that are independent of situation and culture. The five factors, meant to cover most of the variance are; Extraversion, agreeableness, conscientiousness, neuroticism and openness (Digman 1990).

2.2.2. Objective and subjective temperament tests in horses

There are various methods used in science to assess temperament of horses. Novel object tests, tests where trained judges rate behaviours, physical measurements and surveys are just a few examples (König von Borstel 2013). Possible applications of behaviour assessments in horses are to match the horse to an owner or to a specific task the horse is supposed to perform, or to provide a basis for selection when breeding. In the following text, both subjective and objective methods used in research are discussed including pros, cons, estimations of their reliability, if they are valid or not, and if they are possible to repeat. These are all important criterions of temperament assessment in horses according to König von Borstel (2013).

Objective temperament assessments

Research studies applying objectivity in temperament tests tries to minimize potential bias due to personal perception. Novel object tests are constructed to measure and quantify horse's reaction to a new and unfamiliar stimulus. The following novel object test method is described by Visser et al. (2001) and used as a standard method in multiple studies.

During a novel object test, the horse is first walked and then let into a box of normal size. The door of the box is opened, and the horse thereby gets access to enter an enclosed arena to habituate for a couple of minutes. Thereafter a novel object is introduced in the arena. By looking at the footage from the video cameras placed in the box and arena, behaviours and time budget can be registered with the help of an ethogram. Repeatability of novel object tests (in arenas, led, under rider etcetera) range from r=0.1-0.81 according to an review by König von Borstel (2013) where a higher r number equals better repeatability. Numerous studies have used novel object tests to rate fearfulness in horses (Visser et al. 2001, 2002; Christensen et al. 2008).

Novel object tests have also been combined with heart rate monitor measurements (Visser et al. 2002). By observing shifts in different heart rate measurements, one can differentiate between the parasympathetic and sympathetic activation of the nervous system to help explain the biological background of the novel object test. Visser et al. (2002) could explain the reaction of young Dutch-warmbloods by looking at heart rate variability during a novel object test where less trained horses showed more intense reactions in terms of heart rate and heart rate variability. They concluded that this test could help to assess temperament defined as the emotional state of the horses and their reaction to a stimulus by measuring fearfulness, and thereby predict how individuals react to changes in the everyday environment.

Objective assessment methods are also commonly used to assess stress level in horses. Heart rate variability measurements have in several studies been used to measure stress response and is now a verified tool for assessing stress level in horses (von Borell et al. 2007). Becker-Birck et al. (2013) demonstrated how heart rate increased during competition (in addition to the increase due to physical exercise), but an increased heart rate was also found when the horses were prepared for the competition in the stable. An increased cortisol level and decreased heart rate variability were also found, indicating that competitions cause stress in horses. Cayado et al. (2006) found hormone responses in terms of elevated cortisol levels during competition, where horses with less experience had significantly higher cortisol levels, illustrating a greater stress response.

Subjective temperament assessments

Another method to evaluate temperament in horses is to let trained judges, trainers, owners, or caretakers fill in standardised surveys about the horse's temperament. At breeding assessments, trained judges often rate temperament traits of importance for the breed. Assessment of potential breeding horses performed by judges at test stations are common in warmblood sport horses. In a survey aimed at breeding associations of warmblood horses almost 60 % reported that they included behaviour as one of the traits in their breeding objectives (Koenen et al. 2004). On a scale ranging from 0-10 where 0 indicated no importance of the trait and 10 high importance of the trait, the mean value of behaviour reported by Koenen et al. (2004) was 8.0± 1.1. To date, there are no similar studies about importance of temperament traits (relative weights) when it comes to breeding associations of SB trotters.

König von Borstel et al. (2013) highlighted potential drawbacks of subjective temperament assessments in horses based on survey responses from judges and test riders in Germany. Temperament evaluated by judges at test stations gives an idea of the temperament at the test but not over time. Also, judges expressed lack of consistency and standardised definition of scores, but also bias due to expectations of the horse based on the pedigree. König von Borstel et al. (2013) argued that traits of importance often are missed out, for example behaviours only performed at home such as stereotypes and traits related to cooperation. Some traits examined were also believed to measure the same temperament, therefore actions need to be taken to eliminate traits that measure the same thing. König von Borstel et al. (2013) discussed the risk of inconsistency between judges at different test stations, which would make horse owners select test station with higher mean scores for temperament related traits to improve the test results.

Judges and trainers perception of the same horses temperament have been shown to differ (Diverio et al. 2010). A possible advantage of letting someone familiar with the horse rate temperament traits is that the results of the evaluation are based on the horse's temperament in different environments. This helps to avoid the previous mentioned problems at test stations. Principal component analysis (PCA) or factor analysis (FA) are commonly used methods to reduce the number of traits and thereby find the most important temperament traits characterising the breed (Momozawa et al. 2005a; Staiger et al. 2016; Sigurðardóttir et al. 2017). The outcome of PCA and FA have in previous studies associated breed specific temperament traits with genetic variance in the breed (Hori et al. 2016; Staiger et al. 2016) but also given heritability estimations of temperament traits (Sigurðardóttir et al. 2017).

Surveys carried out among a group of caretakers of Thoroughbreds have also proved to be consistent, indicating high inter-observer reliability for temperament traits like trainability, anxiety and affability (Momozawa et al. 2005a). With the intention to evaluate temperament in a more objective manner, Roberts et al. (2016) compared survey results with dopamine levels of 100 horses in the UK. The non-invasive method of measuring blink rate as an estimation of dopamine concentration have successfully been carried out in primates, where an elevated dopamine level was found to be correlated with a faster blink rate (Taylor et al. 1999). The owners of the horses in the study by Roberts et al. (2016) were asked to reply to a survey consisting of temperament traits thought to be related to dopamine levels. The results showed a significant correlation between two temperament traits; docility and anxiety with the spontaneous blink rate (SBR). Roberts et al. (2016) concludes that this non-invasive method of estimating dopamine level can help to differentiate between anxious horses having a higher SBR and docile horses having a lower SBR.

The reliability of surveys have also been investigated in order to evaluate horses' temperament in comparison with novel object tests by Momozawa et al. (2003). Results showed that caretakers' survey responses for the same horse were significantly associated with the horses' behaviour and heart rate during a novel object test. More specifically, anxious horses had a higher heart rate during the novel object test ($r=0.318$, $P<0.01$). Horses tending to get excited at competition events also showed a higher heart rate at the novel object test ($r=0.346$, $P<0.01$). In conclusion Momozawa et al. (2003) explained that surveys aiming at persons that know the horse very well is an reliable method to assess horses temperament.

2.2.3. Temperament tests in other species

In dogs, temperament evaluation is a commonly accepted practice. In Sweden, owners with a dog registered in the Swedish kennel club can choose to let their dog perform a mentality test. This test is open to all breeds and consists of ten elements such as willingness to make contact, play, cooperation, fearfulness, reaction to new situations, loud noises etcetera (Swedish working dog association 2017). The results from the mentality test are thereafter put together with the pedigree to help select the appropriate dogs for breeding. Svartberg & Forkman (2002) evaluated the temperament of more than 150 000 dogs of different breeds with the help of the standardised mentality test used in Sweden at the time. Trained judges rate the dog's mentality in 33 traits, five factors (underlying temperament traits) could be formed to explain the mentality of the dogs. Results were shown to differ between breeds indicating different selection strategies in different breeds. Another well-known practice used to evaluate temperament in dogs is the C-barq survey (Hsu & Serpell 2003). The C-barq survey consists of 68 questions about the dog's temperament where the owner should rate each question in a scale ranging from 0 never to 4 always. This survey can help to evaluate temperament of dogs and find indications of behaviour problems (Hsu & Serpell 2003).

2.3. Temperament characteristics in different horse breeds

The horse personality questionnaire (HPQ)

Characterisation of breed-specific temperament are often carried out by letting an owner or trainer reply to a survey about the horse's temperament as previously mentioned. One standardised survey called Horse Personality Questionary (HPQ) including 25 temperament traits has been used in multiple studies to differentiate between breed specific temperament. Lloyd et al. (2007) developed this model based on successful surveys used to assess temperament in primates. In the study by Lloyd et al. (2007), 44 horses of different breeds and crossbreeds common in the UK were included in the final principal component analysis (PCA). This analysis method aims at reducing the number of traits explaining a temperament characteristic. The analyses revealed six factors, presented in descending order with the first factor explaining most of the variance: dominance, anxiousness, excitability, protection, sociability, inquisitiveness.

A work by the same authors, aimed at exploring how different breeds loaded on factors also extracted by PCA with the HPQ as a basis (Lloyd et al. 2008). Warm blooded horses as Arabians, Thoroughbreds, Quarter horses and Appaloosas were included. Also, Irish draught horses were included as well as ponies (Highland ponies, Shetland ponies, Welsh-ponies, and Cobs). The number of horses included varied from 61 to 281 per breed and the factors extracted from the PCA were the same as in the previous work by the authors. In the factor named dominance, aggressive, stubborn, reactive, and dominant breeds could be distinguished. The factor scores for the eight breeds did not show much variation in this factor, all breeds were loading on the negative side, indicating calmness and cooperativeness. Thoroughbreds were the most dominant and the draught horses the least dominant breed. The second factor Lloyd et al. (2008) named anxiousness, included traits like fearful, being tense and insecure. This factor showed large differences between breeds where Thoroughbreds were the most anxious breed and Highland pony the least anxious breed. The third factor excitability described excited horses, that tended to move fast and did not like to stand still. Thoroughbreds and Arabian horses were the two breeds that were most excited. The level of excitement were the most obvious breed difference among the factors constructed. Lloyd et al. (2008) concludes that temperament characteristics such as anxiousness and excitability are of high importance depending on the task the horse is supposed to perform, and therefore these significant breed differences have occurred during selection.

Olsen & Klemetsdal (2017) assessed temperament in horse breeds common in Norway using the same HPQ developed by Lloyd et al. (2008) but with 13 additional traits included (in total 43 traits). The breeds included were Norwegian

Coldblooded-trotter, Dole horse, Fjord horse and Lyngen horse. The Norwegian coldblooded-trotter, also called the Swedish-Norwegian coldblooded trotter (CBT) due to the common studbook, is used for harness racing and originates from the Swedish draft horse, the Norwegian Dole horse but also from the Standardbred trotter (Bjørnstad et al. 2000). Therefore, the results from Olsen & Klemetsdal (2017) can be of interest for the current thesis. From each breed, between 214 and 281 horses were included. With factor analyses, five factors were constructed for all breeds except one. The exception was the factors constructed for the CBT where six factors fulfilled the criteria of eigenvalues >1. The unique factor for this breed included excitability, with speedy and tense loading on the positive side, and slow loading on the negative side. Olsen & Klemetsdal (2017) interpret this factor as an important temperament characteristic for performance in harness racing. The rest of the factors were shared by all breeds, these factors were related to "fight or flight reactions" and different aspects related to cooperation with owner/trainer.

Other surveys used to assess temperament in horses

Other studies of temperament characteristics have used surveys based on Momozawa et al. (2005a)'s work. In Momozawa et al. (2005a), temperament characteristics of Thoroughbred horses were investigated. By factor analysis a factor named anxiousness was extracted that explained most of the variance of Thoroughbred horses' temperament. Traits included in this factor were vigilance, panic, nervousness, skittishness, timidity, and excitability. A factor named trainability described horses that easily remembered things they learned, were easy to train, they were concentrated, not affected by the surroundings and patient. Affability, the third factor represented cooperative, friendly, and docile horses.

Staiger et al. (2016) also used this survey to assess temperament characteristics of Tennessee Walking horses. Results showed that similar factors could be extracted, the factor which explained most of the variance was again anxiousness. This factor included traits like excitability, skittishness, nervousness, and panic on one side and concentration and docility on the opposite side. The factor with the second highest variance was named tractable and represented horses which were easy to train, remembered what they learnt, and that were concentrated and docile. Horses loading on the opposite side in this factor were stubborn and emotionally inconsistent. The Tennessee Walking horse's temperament could also be explained by two extra factors concerning social traits (Staiger et al. 2016).

2.4. Heritability of temperament traits in horses

Estimation of variance components are of great importance when evaluating which traits should be used for selecting appropriate horses for breeding. With the help of heritability estimations, it is possible to sort out to what degree the difference between individuals for a phenotype is impacted by additive genetic variance. Data material used in estimations of heritability of temperament traits have in previous studies been collected using various methods. A summary of results of heritability estimations for different temperament traits in horses are presented in Table 1.

In a study by Sigurðardóttir et al. (2017) the heritability of temperament traits in Islandic horses was estimated based on judges' and riders' observations in a field test. The results were also compared with those from a survey aiming at owners or trainers of the assessed horses, traits in the survey were thereafter analysed with factor analysis and heritabilities were estimated. The trait cooperation was estimated to have a heritability (h^2) of 0.31 when analysing riders' scores (also shown in Table 1). For the factor "general cooperation" formed by owners and trainers scores in the survey, the heritability was 0.05. The heritability of the trait nerve strength was estimated to 0.39, 0.04 and 0.24 based on scores given by riders, judges, and owners/trainers, respectively. The temperament trait spirit, which is a general score for the horse's attitude to work and its appearance was estimated to have heritability of 0.08 and 0.28 based on scores from riders and judges, respectively. One must also notice the high standard errors for some of the traits including cooperation, nerve strength assessed by judges and spirit assessed by riders.

In Swedish warmblood horses, heritability for temperament during loose jumping and under rider have been estimated from data obtained at field tests by Viklund et al. (2008). During two annual field tests of young horses (young horse test and riding horse quality test), temperament scores from more than 4000 horses at young horse tests and more than 12 000 horses at the riding horse quality test were obtained. Heritability of temperament during loose jumping at the young horse test was estimated to 0.23. At the riding horse quality test, the heritability for temperament at loose jumping and under rider was 0.17 and the heritability for temperament for gaits under rider was 0.41. A more recent study estimated the heritability for the trait temperament during loose jumping in the same breed at the young horse test in the years 2013-2016 to 0.42 (Viklund & Eriksson 2018). At the young horse test, the trait temperament is incorporated in an assessment criterion also including general impression and loose jumping. At the riding horse quality test, temperament is included in an assessment criterion including general impression but also rideability. In Danish Warmbloods, the horses' reactivity during a conformation test (field test) was assessed in a 3- point scale by Rothmann et al. (2014). The horses got different scores depending on how much they moved around when they were supposed to stand still. The heritability was reported to be low for this trait, h^2=0.17 (Rothmann et al. 2014).

In Haflinger horses, two temperament traits were assessed for almost 4000 horses by nine judges in a study by Samoré et al. (1997). The horses got a score on a scale that ranged from 0 to 10. In the first temperament trait, 0 corresponded to a docile horse and 10 to a rebellious horse. In the other temperament trait, 0 corresponded to irritable and 10 to passive. Results showed that both traits had a low heritability, h^2= 0.06 and 0.02, respectively.

Table 1. Heritability estimations (h^2) of temperament traits in different horse breeds and reference to article (Ref.)

Breed	**No. of horses/ recordings**	**Trait**	h^2	**Ref.**
Islandic horses	231	Cooperation	0.31	(Sigurðardóttir et al. 2017)
	451	Nerve strength	0.39	
	443	Spirit	0.08	
Jeju and Thorough-bred cross	659	Patience	0.40	(Kim et al. 2018)
	659	Sensitivity	0.53	
Haflinger	3902	Temperament	0.06	(Samoré et al. 1997)
		Docility	0.02	
Andalusian	1273	Temperament	0.08	(Molina et al. 1999)
Danish Warmblood	323	Reactivity	0.17	(Rothmann et al. 2014)
Several breeds (incl. French trotter)	702	Alone in the area	0.42	(Hausberger et al. 2007)
		Novel Object	0.29	
		Handling travel	0.24	
		Opening box	0.36	
		Memorisation	0.17	
Swedish Warmblood	434	Temperament gaits	0.31	(Gerber Olsson et al. 2000)
	1145	Temperament free jumping	0.23	
	1179	Temperament jumping under rider	0.33	
Swedish Warmblood	16 504[a]	Temperament gaits	0.41	(Viklund et al. 2008)
	4110[b]	Temperament free jumping	0.23	
	16 504[a]	Temperament jumping under rider/free jumping	0.17	
Swedish Warmblood	3410	Temperament free jumping	0.42	(Viklund & Eriksson 2018)

[a] Riding horse quality test

[b] Young horse test

2.5. Genes or genomic regions for temperament in horses

2.5.1. Previous research of genes associated with temperament in horses

In horses there are 2 470 quantitative trait loci (QTL) reported to be associated with phenotypic traits (Hu et al. 2013). The only publication in the Horse QTL database examining temperament characteristics of horses is published by Staiger et al. (2016). By doing a factor analysis of temperament traits in a survey aimed at Tennessee Walking horses, three factors were used as phenotypes to associate with genomic regions by applying a single nucleotide polymorphism (SNP) array. The array used for this study contained 65 000 SNPs and a genome wide association study (GWAS) was done. For the phenotype anxiousness, Staiger et al. (2016) found two genes located in the associated region; 5-hydroxytryptamine receptor 1A *(ALDH18A)* and hydroxysteroid 17-beta dehydrogenase 3 *(HSD17B3)*. Also, in the second phenotype analysed, tractable horses, two genes were located in the associated region; protein kinase C beta *(PRKCB)* and DLG associated protein 1 *(DLGAP1)*. For the third phenotype, agonistic, the adenylate cyclase 2 *(ADCY2)* gene were found to be located in the associated region.

The *ALDH18A1* gene found in the region associated with anxious horses have in humans been associated with mutations causing degradation of neurons and spasticity (Koh et al. 2018), but also with Alzheimer's disease and Downs syndrome (Patel et al. 2011). The *HSD17B3* gene is involved in the metabolism of testosterone, it is mainly expressed in the testis of men (Yazawa et al. 2020).

Mutations in the *PRKCB* gene, located in the region associated with tractable horses have previously been associated with autism in humans (Lintas et al. 2009). By studying the same gene in mice, Wu et al. (2007) found that stress before birth, resulted in the protein encoded by *PRKCB* was suppressed causing impaired memory ability and learning ability. The second gene that was located in the area associated with tractable horses, *DLGAP1,* has been linked to cognitive flexibility i.e., the ability to adapt to a changed situation, new stimulus and "learn by doing mistakes" (Fan et al. 2018). This gene has recently been associated with attention deficit hyperactivity (ADHD) (Fan et al. 2018).

In Table 2, a list of studies on molecular genetics of temperament traits in horses are presented. The study with the largest number of horses is by Hori et al. (2016), where 167 Thoroughbreds were genotyped for the *HTR1A* gene. Results showed a significant association between tractability and a SNP, where the G allele was associated with higher tractability and the A allele with lower tractability (Hori et al. 2016). There was also a sex effect where mares with the A allele were shown to be less trainable in four out of five tractability traits/phenotypes examined, compared to only one for stallions/geldings. In Thoroughbred horses and Thoroughbred crosses, Momozawa et al. (2005b) and Ninomiya et al. (2013) associated temperament phenotypes to a genomic region covering the Dopamine D4 receptor gene *(DRD4)*. Momozawa et al. (2005b) reported a missense mutation, a G to A substitution (causing an amino acid change) in *DRD4* associated with more curious and less vigilant horses. Looking at the same SNP as Momozawa et al. (2005b), Ninomiya et al. (2013) instead found that horses reported as frustrated during feeding time were correlated with having the A allele. The Dopamine D4 receptor gene have also been found to interact with glutamate ionotropic receptor NMDA type subunit 2B (*GRIN2B*) causing ADHD, a gene known to be correlated to memory and attention difficulties (Kim et al. 2018a). Interestingly, Velie et al. (2018) found an association between earnings of CBT and the *GRIN2B* gene.

Table 2. Genes significantly associated with temperament traits in horses, type of DNA change, consequence, and reference article (Ref.)

Breed	**No. of horses**	**Gene**	**Trait**	**DNA change**	**Consequence**	**Ref.**
TB	167	*HTR1A*	Tractability	Non-synonymous mutation	Lower tractability	(Hori et al. 2016)
TB	136	*DRD4*	Vigilance and curiosity	Non-synonymous mutation	A allele: High vigilance and low curiosity	(Momozawa et al. 2005b)
TB TB cross OLD	19 1 1	*DRD4*	Frustration	Non-synonymous mutation	A allele: Less frustrated G allele: More frustrated	(Ninomiya et al. 2013)
TB	16	*MAOA*	Aggressiveness Docility	Silent	T>C	(Song et al. 2017)
		AR		Silent	G>T	
TW	113	*ALDH18AI*	Anxiousness	-	Minor: C Major: A	(Staiger et al. 2016)
		HSD17B3		-	Minor: C Major: T	
		PRKCB	Tractability	-	Minor: G Major: T Minor: G	

DLGAP I			Major: A
ADCY2	Agonistic	-	Minor: T Major: C

TB: Thoroughbred horses

TB cross: Thoroughbred cross

OLD: Oldenburg

TW: Tennessee Walking horses

3. Material and Methods

3.1. Data collection and data material

The 376 horses included in this project were Standardbred trotters who had started at least one race. The horses were born between 1995-2018, and the data included 152 mares, 186 geldings and 38 stallions. Collection of behavioural data and hair from the tail to obtain DNA took place in Sweden and Norway, data was collected during a period from end of April 2019 to end of March 2021. Data were obtained from 124 horses in 2019, from 114 horses in 2020, and from 138 horses in 2021. Some of the horses were selected based on that they had participated in previous studies so that DNA samples already were in place. All horses were randomly selected, and they had started at least one race with the current trainer. The horses were a mix of active and retired trotters. Both professional and amateur trainers participated and there were in total 121 trainers. In this study 265 horses were trained by a professional trainer, 78 horses had an amateur trainer and 33 horses had been trained by both professional and amateur trainers.

The 376 horses were sired by 148 stallions and were from 347 unique dams. The number of offspring per sire is presented in Figure 1 and ranged from 1-20 where 78 sires had one offspring and 70 sires >1 offspring. The mean number of offspring per sire was 2.54. Out of 347 dams, 319 had one offspring, 27 had two offspring and one have three offspring. Pedigree information had previously been provided by the Swedish Trotting Association that gave permission to use it for the study.

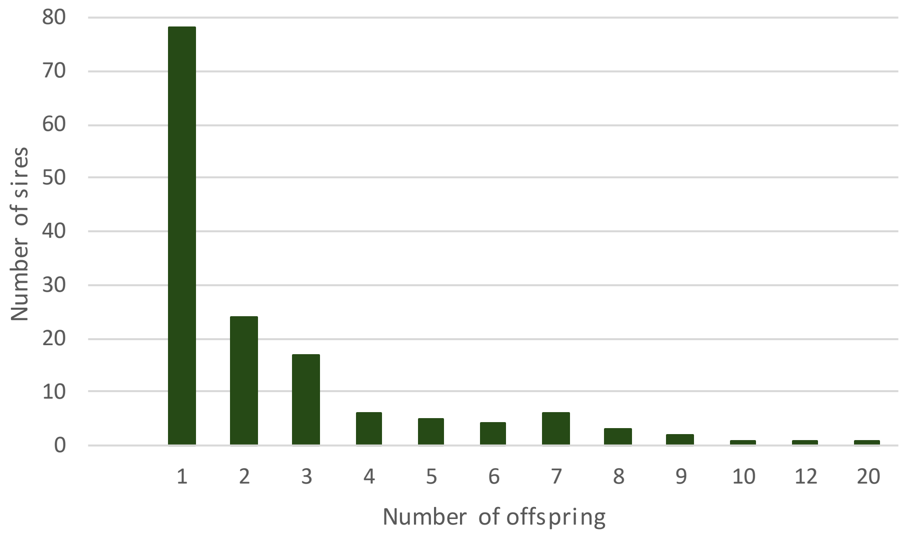


Figure 1. Distribution of offspring per sire. Number of offspring ranged from 1-20 where 78 stallions had one offspring and three stallions had 10, 12 and 20 offspring, respectively.

3.1.1. Survey

In this thesis, a pre-existing survey adapted from Momozawa et al. (2005a) and Staiger et al. (2016) prior to this MSc- project was used. The survey, including 13 behaviour traits with response options on a scale from 1 to 7, was used to describe the temperament of the horses at trotting races. The trainer was asked to rate how often they observed the temperament trait in competition where 1 corresponded to never, 2 rarely, 3 occasionally, 4 sometimes, 5 often, 6 usually and 7 always. The temperament traits and their descriptions are presented in Table 3. In addition, questions were asked about regular health and behaviour problems such as stereotypes. The answer options for the question about frequent health problems were yes or no, if yes the trainer was asked to give a brief description. The trainer was also asked to rate how often he or she observed stereotypic behaviours such as pawing, crib biting, windsucking and weaving on the scale 1-7 described previously. Besides the translation from Swedish to English, some of the traits in the survey were renamed when analysed in this study in order to more logically follow the definition given for the scale in the survey. These were appetite described on a scale from never poor appetite to always poor appetite, that was renamed as poor appetite in this study. The trait self-control described on a scale from never tends to lose control to always tends to lose control was renamed as low self-control. The original survey can be seen in Appendix 1.

Table 3. Traits included in the survey and their description, points were given on a scale that ranged from 1-7 for each trait

Trait	Description	1. Never ⟷	7. Always
Nervousness	Tends/tended to become nervous	Never nervous	Always nervous
Excitability	Tends/tended to get excited or agitated easily	Never excited	Always excited
Fearfulness	Tends/tended to be afraid easily (e.g. novel environments)	Never afraid	Always afraid
Concentration	Tends/tended to be focused and unaffected by the environment	Never focused, never unaffected by the environment	Always focused Always unaffected by the environment
Learning	Tends/tended to learn the task of competing quickly	Never learns the task of competing	Always learns the task of competing
Memory	Tends/tended to memorize/remember unpleasant events	Never memorizes or remembers unpleasant events	Always memorizes or remembers unpleasant events
Cooperation	Tends/tended to be cooperative, have good attitude (e.g., willing to work/no resistance)	Never cooperative, bad attitude, no willingness to work	Always cooperative, always good attitude, strong willingness to work
Will to win	Tends/tended to desire to win	Never desires to win	Always desires to win
Stubbornness	Tends/tended to be obstinate once it resists a command	Never stubborn	Always stubborn
Low self-control	Tends/tended to panic, escape and lose control (e.g. impossible to handle or stop / damage itself)	Never panics, escapes, or lose control, easy to handle	Always panics, escapes and lose control, hard to handle
Recovery	Tends/tended to relax quickly	Never relaxes after races	Always relaxes after races
Poor appetite	Tends/tended to have poor appetite	Never poor appetite between competition events	Always poor appetite between competition events

The survey was available as an online- version and distributed by the Swedish Trotting Association (Svensk Travsport) to an email list of all registered trainers in Sweden. Also, the project was advertised in the Swedish Standardbred breeders' association magazine Travhästen. Moreover, the survey was distributed in relevant Facebook groups. Professional trainers in the area nearby campus were contacted via phone calls to plan visits to their farm to fill in the survey and collect hairs. In total, pulled hairs or blood was obtained from 332 horses. The total number of surveys collected was 377.

3.2. Statistical analyses

Data editing was performed with the Statistical Analysis Software (SAS) package (SAS Institute Inc., 2012). Horses with less than two (out of 13) trait observations were excluded. In total, 376 of the 377 horses had two or more trait observations. Except the questions asked for in the survey, the data was complemented with registration number of each horse. Twenty-two horses did not have official Swedish registration numbers and for these new unique numbers were constructed to be able to add the horses to the pedigree file. The pedigree of these 22 horses could be found on the Norwegian trotting associations website. There were no horses with unknown pedigree.

New variables such as age groups were created in SAS. In total, five age groups were created and represented the age of the horse when the survey was filled in. The definition of age groups and distribution of horses in each age group is shown in Table 4.

Table 4. Division of horses in age groups, age, number of horses in each group (frequency) and corresponding percentage

Age group	Age in years	Frequency	Percent
1	2-3	85	23
2	4-5	129	34
3	6-7	70	19
4	8-10	52	14
5	11-24	40	11

Descriptive statistics were performed in SAS. All 13 traits were tested for normality according to Anderson-Darling's test. All traits had a significance level of p=<0.005 indicating that no traits were normally distributed. The skewness and kurtosis of each trait was also examined. Spearman's rank correlation coefficients were estimated between traits using proc corr (Appendix 2). For results in this thesis, the null hypothesis was rejected, and results considered significant if p-values were <0.05 (*), <0.01 (**) or <0.001 (***).

3.3. Factor analysis

Factor analysis (FA) is a well-known method used in animal temperament research to explain underlying temperament attributes based on correlations of measured traits (Budaev 2010). Factor analysis calculations estimates an error term for each trait which helps to correct the loadings on each factor from being overestimated. This property of FA makes it superior to use over principal component analysis, a method which instead aims at reducing traits without estimating an error term (Budaev 2010). The FA was performed in SAS using Proc factor statement (method=print). The Kaiser Meyer Oklin (KMO) test was applied to test if the correlation matrix was appropriate for FA, KMO should be over 0.7. The KMO for the data material in the current thesis was 0.81 which means the data was appropriate for FA.

The decision of how many factors should be used is commonly based on each factor's eigenvalue, a value which represent the proportion of variance the factor explains in the dataset. The most widespread rule is that factors with eigenvalues >1 are accepted. However, there exist other criteria based on if the factors are realistic or not, one can also plot the factors and draw a line where the factors form a horizontal line called a scree plot test (Williams et al. 2010). In this thesis, three factors were included in the analysis but only one would have been used based on the eigenvalue >1 criterion. The eigenvalue of the reduced correlation matrix for factor 1 was 3.6 and the proportion of common variance explained by the factor was 0.38. The second factor had an eigenvalue close to 1 (0.97) and the proportion of common variance explained by the factor was 0.35. The third factor had an eigenvalue of only 0.62 but was kept for further analysis due to its logical factor loadings. The proportion of common variance explained by factor 3 was 0.27. Orthogonal varimax rotation was implemented to adjust the x- and y-axis to better fit the data. On each factor, the traits included receive different loadings. These loadings are considered as significant for the factor if the loading is > |0.4| (Budaev 2010). Horses with missing data received loadings representing the mean value of that trait, therefore all 376 horses got scores for each factor and could be included in further analysis.

3.4. Genetic parameters

Using Proc mixed statement in SAS, different linear models were tested to find significant effects for the 13 traits in the survey but also for the factors created. For example, including the fixed effect of the horse having regular health problem was tested, but found to not be significant for any of the traits or factor scores. The fixed effects of sex (mare, gelding, or stallion), level of trainer licence (amateur, professional or both), age group of the horse at the time of the survey (2-3, 4-5, 6-7, 8-10, or 11-24 years old) were found to be significant for several of the traits and chosen for the statistical model. For the trait stereotype, the effect of the 13 traits was tested to see if horses with specific temperament characteristics were more prone to show or not to show stereotypic behaviour. The effect of the factors constructed were also tested for and included as fixed regressions.

The random effect of individual trainers was found to be significant in some cases, and therefore additional comparisons were made between variance components from models including both random effect of sire and random effect of trainer. The sire variance component did not seem affected by inclusion or exclusion of the random trainer effect, except for the trait stubbornness. The effect of the trainer was removed from the final model because only 20 out of 121 trainers replied to the survey for more than three horses.

Heritabilities were estimated with the DMU software which analyses multivariate mixed models and estimate variance components (Madsen & Jensen 2013). The pedigree file that contained information about the horse's ancestry for up to 7 generations included 5835 horses. The linear model used for variance estimations is presented below.

$$Trait\ Score\ or\ Factor\ Score = \mu + Sex + Trainer\ License + Age\ group + Animal + e$$

Where μ is the mean value for the trait score or factor score. Sex is a fixed effect (mare, gelding, and stallion), trainer license is a fixed effect of level of trainer of the horse (professional, amateur or both) and age group is fixed effect with five age classes: 2-3, 4-5, 6-7, 8-10 and 11-24 years old. Animal is the random genetic effect of the individual $\sim ND(0, A\sigma_A^2)$, and e is the random residual $\sim ND(0, I\sigma_e^2)$.

Heritability estimates were calculated as:

$$h^2 = \frac{\sigma_A^2}{\sigma_P^2}$$

Where σ_A^2 is the additive genetic variance and σ_P^2 is the phenotypic variance.

Also in DMU, a random trainer effect was included in preliminary analyses, but this had very little impact on the heritability estimates, except for the trait stubbornness.

Traits and factors that showed $h^2 > 0.2$ in the single trait analyses were also included in bivariate analyses to estimate genetic and residual correlations. Genetic correlations were estimated as:

$$r_g = \frac{\sigma_{A1}\sigma_{A2}}{\sqrt{\sigma_{A1}^2 \times \sigma_{A2}^2}}$$

Where r_g is the genetic correlation between trait 1 and 2, σ_A is the additive genetic standard deviation and σ_A^2 is the additive genetic variance.

Environmental or residual correlations were estimated as:

$$r_E = \frac{\sigma_{E1}\sigma_{E2}}{\sqrt{\sigma_{E1}^2 \times \sigma_{E2}^2}}$$

Where r_E is the environmental or residual correlation between trait 1 and 2, σ_E is the standard deviation and σ_E^2 is the variance.

3.5. DNA sequencing

3.5.1. Data material

Horses selected for sequencing were individuals with complete surveys plus hair samples. Horses with blood samples and horses with missing surveys were excluded. Due to the small sample material, there was no possibility to select certain individuals for genotyping based on other criteria. In total, 288 samples were prepared and sent for the low coverage whole genome sequencing. Due to the fact that this thesis was made as a pilot study for a larger project and that a new protocol was used (Riptide™ High Throughput Rapid DNA Library prep), a decision was made to only sequence one plate (96 horses) before the completion of this MSc thesis.

The trait distributions were compared for the 96 sequenced horses and the full data, and the included 96 horses were found to be a representable sample for the whole dataset. The 96 horses were shown to have nearly identical means, kurtosis and skewness as the ones presented for the 376 horses in Table 5. The number of observations for each temperament trait reached from 96 (for nervousness, excitability, and the factors) down to 83 for the trait stereotype. The 96 horses included were born between 1995 and 2017, and the surveys were collected in 2019.

There were 39 mares, 41 geldings and 16 stallions included in the dataset. Most of the horses (70) were trained by professional trainers, 20 were trained by amateurs and six horses had been trained by both. The 96 horses were sired by 68 stallions, where 50 stallions had one offspring, ten stallions had two offspring, six stallions had three offspring and two had four offspring. The 96 horses were offspring to 88 mares, 80 mares had one offspring and eight mares had two offspring.

3.5.2. Sequencing analysis

One trait was analysed due to the limited time available. The decision was based on the distribution of scores for the trait, if the horses could be divided into case and controls for the trait, and the estimated heritability. Therefore, the trait excitability was chosen. The control group of the trait excitability consisted of horses receiving score 1 (never excited), this group included 19 horses. A second control group with 38 horses was also constructed to find significant regions associated with the trait, this group included horses with score 1 and 2 (never to rarely excited). Horses that were included in the case group where those receiving score 5 (often excited), 6 (usually excited) and 7 (always excited). This group consisted of 24 horses.

3.5.3. DNA extraction

Forty hairs including hair roots from each horse were cut into tubes and centrifuged. After centrifugation, 186 µl 5 % cortex 100 Resin and 14 µl Proteinase K with a concentration of 20 mg per ml were added to each tube. The plates were incubated at 56 degrees Celsius for two hours at 600 rpm followed by ten minutes incubation at 95 degrees Celsius to inactivate Proteinase K. The plates were thereafter incubated at room temperature for half an hour to cool down.

The plates were once again centrifuged and the content in each tube except for the chelex in the bottom was transferred to new plates. DNA concentration was measured with Qubit and Nanodrop spectrophotometer (Thermo Fisher scientific Nanodrop 8000 spectrophotometer).

Qubit protocol

A working solution containing Qubit Buffer (199 × n µl) and Qubit reagent (1 × n µl), where n is the number of samples, + 2 standards for calibration of the Qubit instrument were mixed. The working solution was dispensed into two tubes (190 µl in each) and then 10 µl of the standards were added. The remaining working solution was dispensed into tubes (198 µl working solution + 2 µl sample in each tube). All tubes were vortexed and incubated at room temperature for two minutes before they were read in the Qubit fluorometer.

Evaporation and dilution of DNA

Minimum input of DNA for the riptide protocol was 12.5 ng/µl and samples with lower concentration were left to evaporate on a heat block at 37 °C until half of the volume was left, then concentrations were measured again with Qubit. The evaporation procedure was repeated until samples contained at least 12.5 ng/µl. In the next step, all samples were diluted in new plates to the correct input concentration according to the riptide protocol. The DNA concentration in the protocol was set to 50 ng with a maximum volume of 4 µl. The volume of sample DNA needed was calculated by $V_{sample} = 50/C_{sample}$. Then nuclease free water was added to reach 4 µl with 50 ng DNA in each well, $V_{Nuclease\ free\ water\ to\ add} = 4 - V_{sample}$.

3.5.4. Library preparation

Three plates consisting of 288 horses were prepared for sequencing. The kit used for this study was the Riptide^{TM} High Throughput Rapid DNA Library prep. The protocol consists of four primary steps.

1: Extension and termination of primer A
2: Bead capture and extension of primer B
3: PCR and amplification

4: Size selection

The first step of the reaction was to add primers to new plates. Since the GC content of EquCab3 is 41,8 % according to Kalbfleisch et al. (2018), the protocol recommended to use a 1:1 combination of low GC primer and high GC primer. A master mix containing dNtP, enzyme buffer and enzyme was added to the primer plates. The DNA samples were thereafter added to the primer plate after incubation at 98 °C for 1 minute. The plates were placed in the PCR for extension, following this program:

1. 92 °C for 3 minutes
2. 16 °C for 5 minutes
3. Ramp: 0,1 °C per second up to 68 °C
4. Hold at 68 °C for 15 minutes
5. Hold at 4 °C

After this step, each horse had an individual barcode with 8 nucleotides followed by a random sequence with 12 nucleotides. The products from the 96 wells were thereafter transferred to one single tube containing EDTA. After this step, three tubes each containing products from one 96 well- plate were obtained. SPRII beads was added to the tubes to collect the "Polymerase chain reaction (PCR) products", the tubes were placed on a magnetic stand and the supernatant containing everything that was not attached to the DNA could be discarded. After washing the beads with ethanol and thereafter dissolve the DNA from the SPRI beads with Tris-HCI, the eluted DNA could be transferred to a new tube.

Step two started with adding Capture beads to new tubes, the tubes were placed on the magnetic stand and the supernatant discarded. HS buffer was used to resuspend the capture beads and the tubes where once again placed on magnetic stand and the supernatant was discarded. HS buffer was added again together with the eluted DNA which have been heated up to 95 °C for 3 minutes. Thereafter the beads with DNA attached were washed with sodium hydroxide and bead wash buffer before reaction 2 started. Enzyme buffer 2, dNTP 2, primer B and nuclease free water was added, and the tubes were incubated at 24 °C for 20 minutes. After this step, the beads were washed once again, and a complementary strand had been created.

Step three was a PCR reaction. Universal primers, barcodes, and PCR amplification mix was added to each tube. The three tubes were placed in the PCR machine with the following settings:

1 cycle	98 °C for 2 minutes
8 cycles	98 °C for 20 seconds
	60 °C for 30 seconds
	72 °C for 30 seconds
1 cycle	72 °C for 5 minutes
	4 °C hold

After this step, each plate (tube) had a unique barcode and the DNA strands had been amplified.

The fourth step was a size selection, depending on the Illumina sequencing read length, the amount of SPRI beads 2 volumes differed. Since the read length for the current project was 2x150, 70 µl followed by 30 µl of SPRI beads 2 was added to remove small fragments ending up in the supernatant. The beads were washed with ethanol and Tris HCI was added to resuspend the DNA from the beads. The tubes were placed on a magnetic stand and the supernatant containing the library could be transferred to new tubes and sent for sequencing.

3.5.5. Lowpass whole genome sequencing

Illumina sequencing

The DNA was sequenced with Illumina Novaseq6000 with S4 Flow cells. The aim was to have 2x coverage, and an insert size of 350 base pairs (bp) paired end reads was targeted, yielding 300 bp sequenced (150+150). Haploid genome length of the horse is 2.41 Gb (Kalbfleisch et al. 2018). The Illumina S4 flow cell can read 2.0-2.5 x 10^9 base pairs per flow cell. After quality control of the three tubes, the decision was made to only proceed to sequencing with one tube (96 horses). The tube that was chosen to proceed with passed the quality control and had satisfactory fragment lengths. Of the 96 samples sequenced, the mean depth was 3.45X. The minimum depth was 0.01X and the maximum depth was 14.1X.

Illumina sequencing is a sequencing by synthesis technology where the flow cells, which contains oligonucleotides binds to the DNA-fragments. DNA-polymerases are then attached to replicate the fragments binding to the oligonucleotides. The template strand is washed away and thereafter the complementary strand is replicated with the help of DNA polymerase. The two strands are amplified multiple times, the reverse strands are washed off and the sequencing begins. When the correct nucleotide attaches to the strand it emits a light which can be detected by a laser. After two index reads, sequencing of the reverse strand begins. Forward and reverse strands are paired, and continuous rows are formed, these can later be aligned with the horse genome.

After Illumina sequencing, each individual was separated based on their unique barcodes attached during the library preparation.

Quality control, variant detection, statistical analyses, and association analysis

The quality control (QC) was performed on the output from the sequencing (fastq files, one for each horse). The QC was done with FastaQC software package (Andrews 2019). The alignment of the data was done following Li & Durbin (2009)'s work, Burrows-Wheeler Alignment tool and then Sequence Alignment/Map tool software was applied for the analysis. The data was aligned to the horse genome EquCab3.0 from NCBI. Due to the limited time, an already existing pipeline was used to analyse the data and best practice according to Auwera et al. (2013) was applied to sort out variants of good quality. Traits of interest were divided into case and control groups. The amount of fixed alleles was examined with a F_{ST} (fixation index- test) in Poopolation2 with a window size of 50 kb (Kofler et al. 2011). The F_{ST} scores were converted to Z scores and to corrected for type I errors that could occur if the null hypothesis is rejected when it should not be rejected (Bonferroni adjustment). Significance level was set to $p < 0.05$. The F_{ST} score is a measure of population differentiation and range from 0 (allele frequencies within the case or control group are the same) to 1 (one allele is fixed in the case group and another allele is fixed in the control group) (Wright 1951).

4. Results

4.1. Descriptive statistics

For the 13 traits analysed, means, standard deviation (SD), skewness and kurtosis are shown in Table 5. Number of observations ranged from 376 for nervousness down to 358 for the trait will to win. Some traits like will to win had less observations as a result of 1) a young horse with only one or a few starts, resulting in that the trainer could not yet tell if the horse showed a high will to win or not, or 2) the horse had only been in training at the current trainer for a short while, therefore the trainer could not yet tell if the horse showed a high will to win or not. All traits had a minimum value of one and maximum value of seven, where 1 corresponds to "never" and 7 corresponds to "always". Definitions of each trait can be seen in Table 3. Skewness ranged from -1.51- 2.36. Negative skewness could be seen in concentration, learning, cooperation, will to win, and recovery. This indicates that the "tail" of the normal distribution curve is longer on the left side. Traits with positive values; stereotype, nervousness, excitability, fearfulness, memory, stubbornness, low self-control, and poor appetite are skewed to the left and have a longer "tail" on the right side. The kurtosis ranged from -0.83-5.37. High values are sometimes a sign of outliers but also equals a heavy "tail". Low values are instead representing a lighter "tail". Traits with logarithmic properties were in preliminary analyses transformed with log 10 transformation in SAS. However, these actions did not much improve the normality of the residuals which was the main goal of the data transformations. Normally distributed residuals would make the estimations of variance components in DMU more correct but in this study, the raw data was kept instead. It should be noted that the distributions of residuals from the linear models used generally were closer to normal distribution than those of the raw trait scores were.

Table 5. Number of horses (N), means, standard deviations (S.D), skewness and kurtosis for each variable (trait) included in the survey. All traits had a minimum value of 1 and maximum value of 7

Variable	N	Mean	S.D	Skewness	Kurtosis
Stereotype	363	1.60	1.36	2.34	4.49
Nervousness	376	3.14	1.73	0.60	-0.59
Excitability	375	3.14	1.70	0.42	-0.83
Fearfulness	374	2.46	1.47	0.98	0.18
Concentration	372	5.01	1.61	-0.82	-0.01
Learning	368	5.75	1.29	-1.51	2.51
Memory	372	2.98	1.81	0.69	-0.66
Cooperation	372	5.77	1.34	-1.34	1.69
Will to win	358	5.17	1.73	-0.68	-0.64
Stubbornness	369	1.63	1.19	2.32	5.37
Low self-control	371	1.93	1.42	1.62	1.91
Recovery	367	5.59	1.43	-1.44	2.02
Poor appetite	367	2.36	1.66	1.38	1.20

4.2. Correlations

Spearman's rank correlation coefficients based on the raw trait scores are presented in Appendix 2. Some of the strongest and significant ($p<0.0001$) correlation coefficients (r_s) for each trait that were estimated are presented here. Horses with stereotypes were found to be more stubborn (r_s=0.23). Nervous horses were more excited (r_s=0.61), and fearful (r_s=0.31). Nervous horses also tended to remember unpleasant events (r_s=0.33) and they had poor appetite after competitions (r_s=0.21). These horses were also less concentrated at competitions (r_s=-0.26) and did not recover as well after competitions (r_s=-0.26). Excitability was positively correlated to: Fearfulness (r_s=0.40), stubbornness (r_s=0.28), memory of unpleasant events (r_s=0.24). Excitability was negatively correlate to recovery (r_s=-0.35) which means that excited horses did not recover well after competitions.

The trait fearfulness was positively correlated to low self-control (r_s=0.50) and memory of unpleasant events (r_s=0.40). Fearfulness was negatively correlated to concentration (r_s=-0,50). Concentration was positively correlated to learning (r_s=0.45) and cooperation (r_s=0.38) and negatively correlated to low self-control (r_s=-0.32) meaning that concentrated horses did not tend to lose control of themselves. Learning was significantly correlated to cooperation (r_s=0.54), will to win (r_s=0.44) and recovery (r_s=0.43). Learning was negatively correlated to low self-control (r_s=-0.42), indicating that horses learning the task of competing did not tend to lose control of themselves.

Memory, a trait with the definition "tend to remember unpleasant events" was significantly correlated to low self-control (r_s=0.53) but also stubbornness (r_s=0.29). Cooperative horses had a significant higher will to win (r_s=0.42) and did also recover better after competitions (r_s=0.37), they were less stubborn (r_s=-0.34) and did not tend to lose control of themselves (r_s=-0.36). Stubborn horses did also have a lower self- control (r_s=0.37).

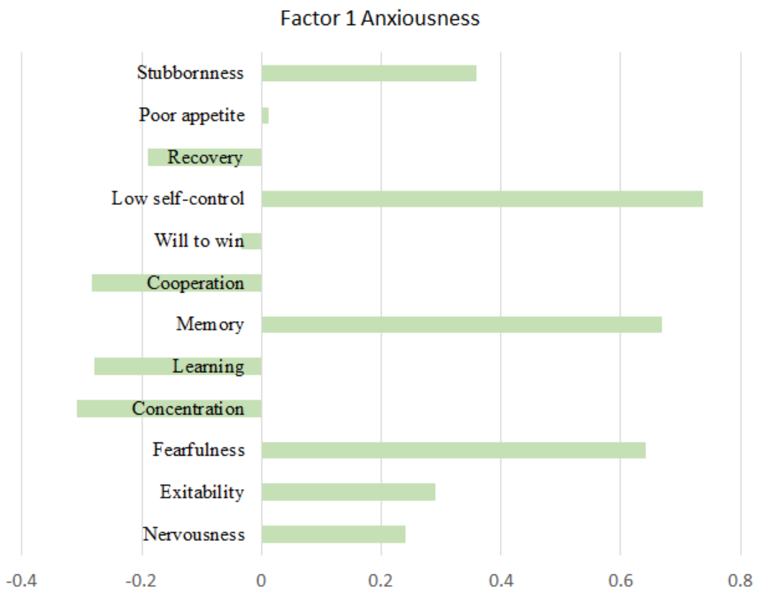
4.3. Factor analysis

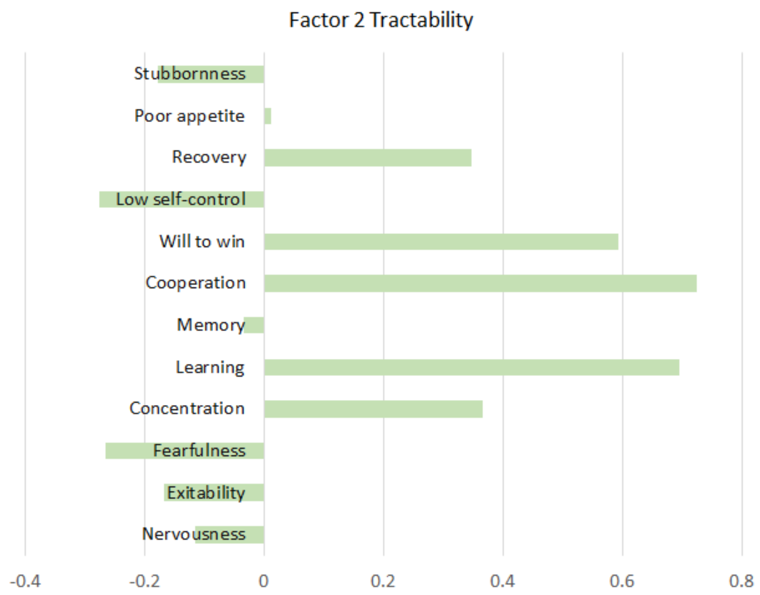
Three factors were constructed to illustrate underlying latent variables factor loadings are presented in Table 6. Factor 1 explained 38 % of the common variance. In factor 1, three traits fulfilled the criterion with loadings >|0.4|, Low self-control (0.74), memory (0.67) and fearfulness (0.64) loaded strongly on the positive side. Also, stubbornness loaded on the positive side (0.36). Factor 1 represent horses that tend to lose control and panic, they tend to get afraid easily and remember unpleasant events. They are stubborn and do not concentrate on their task. They also find it difficult to learn the task of competing and to recover after competitions. Factor 1 was therefore named anxiousness.

Table 6. Factor loadings after orthogonal rotation, bold numbers are loadings > |4|

Variable	Factor1 Anxiousness	Factor 2 Tractability	Factor 3 Excitability
Nervousness	0.24	-0.12	**0.73**
Excitability	0.29	-0.17	**0.69**
Fearfulness	**0.64**	-0.27	0.19
Concentration	-0.31	0.37	-0.17
Learning	-0.28	**0.70**	-0.12
Memory	**0.67**	-0.03	0.16
Cooperation	-0.28	**0.72**	-0.11
Will to win	-0.03	**0.59**	0.03
Low self-control	**0.74**	-0.28	0.20
Recovery	-0.19	0.35	-0.32
Poor appetite	0.01	0.01	0.35
Stubbornness	0.36	-0.18	0.06

Factor 2 explained 35 % of the common variance. In factor 2, there were also three traits with loadings >|0.4|. Cooperation (0.72), learning (0.70) and will to win (0.59). Factor 2 was therefore named tractability, representing cooperative horses that quickly learns the task of competing and have a strong will to win. Factor two also represent horses that recover well after competitions, they are also concentrated and unaffected by the environment, these traits had a loading of 0.35 and 0.37, respectively. The third factor extracted explained 27 % of the common variance. In factor 3, two traits loaded strongly on the positive side, nervousness (0.73) and excitability (0.69). In factor 3 poor appetite also loaded on the positive side (0.35) and recovery loaded on the negative side (-0.31). Factor 3 represent horses that tend to get nervous and excited at competitions, they experience a suppressed appetite after competitions and do not recover well afterwards. Factor 3 was therefore named excitability. A figure illustrating factor loadings as bar charts is presented below (Figure 2).


Factor 1 Anxiousness
Stubbornness
Poor appetite
Recovery
Low self-control
Will to win
Cooperation
Memory
Learning
Concentration
Fearfulness
Exitability
Nervousness
-0.4
-0.2
0
0.2
0.4
0.6
0.8


Factor 2 Tractability
Stubbornness
Poor appetite
Recovery
Low self-control
Will to win
Cooperation
Memory
Learning
Concentration
Fearfulness
Exitability
Nervousness
-0.4
-0.2
0
0.2
0.4
0.6
0.8

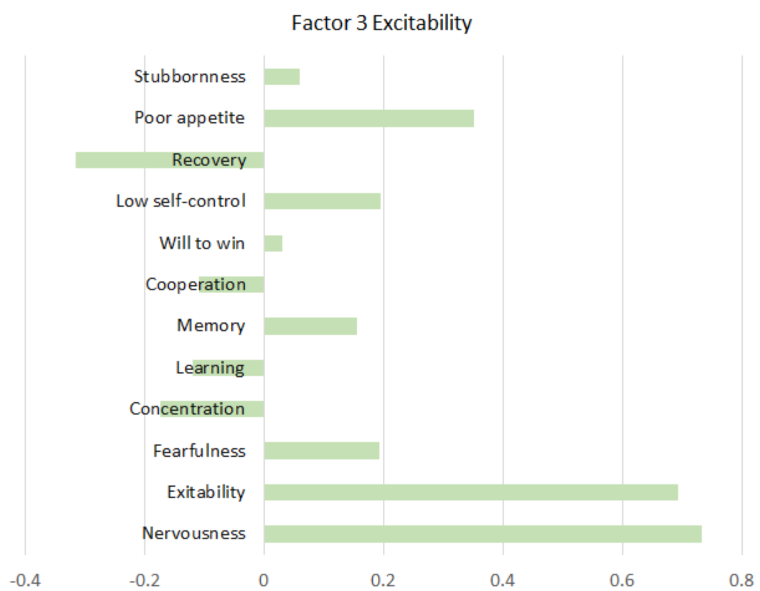

Factor 3 Excitability
Stubbornness
Poor appetite
Recovery
Low self-control
Will to win
Cooperation
Memory
Learning
Concentration
Fearfulness
Exitability
Nervousness
-0.4
-0.2
0
0.2
0.4
0.6
0.8

Figure 2. Factors extracted and their loadings, factor 1 anxiousness, factor 2 tractability and factor 3 excitability. Traits with loadings >|0.4| are considered as important for the factor.

4.3.1. Linear model mixed procedure

From the mixed linear models examined in SAS, the final estimations of effects on selected traits are shown in Table 7 together with their significance level. Stereotype was the only trait for which the effect of the three factor scores was included as fixed regressions. The effect of the factors on the trait stereotype is not presented in Table 7 but factor 1 (anxiousness) had a significant effect on the trait (p=0.0138). Horses with higher factor scores for anxiousness were also more prone to perform stereotypical behaviours. Factor 2, tractability was also significantly correlated to stereotype (p=0.0286). Horses with positive factor 2 scores (tractable horses) had less stereotypical behaviours.

The effect of sex

The sex of the horse had a significant effect on if the horse tended to get nervous at competitions or not (p=0.0042). Stallions were significantly less nervous than mares and geldings. Mares tended to get more nervous at competitions in comparison to both stallions and geldings (not significant). The effect of sex was also significantly correlated to learning ability (p=0.0035), geldings did not learn the task of competing as good as mares or stallions did. The sex of the horse had a significant effect on the appetite (p=0.0403), where mares had significantly lower appetite after competitions compared to stallions but also lower appetite in comparison to geldings. For the second factor constructed named tractability since it described cooperative horses that easily learned the task of competing and had a high will to win. The sex of the horse had a significant effect on how tractable the horse was (p=0.0497). Geldings were significantly less tractable than stallions. The third factor named excitability included traits like nervousness and excitability, which loaded strongly on the positive side. The sex of the horse had a significant effect on how nervous and excited the horse was at competitions (p=0.0076). Mares and geldings got significantly more nervous and excited than stallions, but mares also tended to get more nervous and excited than geldings.

The effect of age

The age of the horse (age group) had a significant effect on if the horse tended to get excited at competitions (p=0.0021). Younger horses tended to get more excited at competitions than older horses. For the trait fearfulness, the age of the horse had a significant effect on if the horse tended to get afraid easily at competitions or not (p=0.0074). The overall trend was that horses in younger age groups tended to be more fearful. Younger horses were also less concentrated in comparison to the older, more experienced horses (p=0.0042). Not surprisingly, the age of the horse did also play a role in how easily the horse learned the task of competing. Older horses with more experience seemed to have learned the task of competing compared to younger horses (p<0.001). For the trait cooperation, there was no significant difference between age group 2-3 and 4-5 but the horses got significantly more cooperative between the rest of the age groups where the oldest horses were the most cooperative ones (p=0.0006). Also, the older horses in age group 8-10 and 11-24 had a significantly higher will to win than horses in the younger age groups (p=0.0001). The age of the horse also had a significant effect on the trait tractability (p<0.0001). Younger horses, belonging to group 2-3 years old and 4-5 years old were significantly less tractable than the older horses.

The effect of trainer licence

The trainers' licence (professional or amateur) had a significant effect on three traits and one of the factors. For the trait concentration, the trainers licence had a significant effect (p=0.0094). Horses which have been trained by professional trainers were significantly more concentrated. Horses trained by amateur trainers were significantly more cooperative than horses trained by professional trainers (p=0.0097). Trainer licence had a significant effect on if the horse performed a stereotypic behaviour (p=0.0003). Horses trained by amateur trainers had significantly higher indices to show stereotypic behaviours than horses trained by professional trainers. Horses trained by amateur trainer were also significantly more tractable than horses trained by professional trainers (p=0.0444).

Table 7. The effect of sex, age group and trainer on the traits and factors in the mixed linear model. For the trait stereotype three additional effects were tested for their significance on the trait. p-values are given as; <0.05 (*), <0.01 (**) and <0.001 (***) and non-significant p-values as ns

Trait	Sex	Age group	Trainer Licence
Nervousness	**	ns	ns
Excitability	ns	**	ns
Fearfulness	ns	**	ns
Concentration	ns	**	**
Learning	**	***	ns
Memory	ns	ns	ns
Cooperation	ns	***	**
Will to win	ns	***	ns
Stubbornness	ns	ns	ns
Low self-control	ns	ns	ns
Recovery	ns	ns	ns
Poor appetite	*	ns	ns
Factor 1	ns	ns	ns
Factor 2	*	***	*

Factor 3	**	ns	ns
Stereotype[a]	ns	ns	***

[a] Out of the three factors included in the model (not shown in table), factor 1 anxiousness and factor 2 tractability had a significant effect on the trait

4.4. Heritability of temperament traits

The result from the estimations of variance components in DMU are shown in Table 8. After exploring different effects in the model and their impact on the variance components and the heritability of the traits, the same fixed effects were included for all traits for simplicity, and since the cost in degrees of freedom was low. The results seemed relatively robust despite the small data material in that the heritability estimations did not change much when including different effects in the model. Stubbornness was the only exception and differed greatly between the current model and a model including the trainer as a random effect. Therefore, the heritability estimation for stubbornness (0.61) is likely an overestimation in the current model and may in fact be considerately lower, as was indicated when including the random individual trainer effect (h^2 =0.14, data not shown). The highest heritability (0.42) was estimated for learning and cooperation. The heritability of excitability, nervousness, factor 3 excitability, and factor 2 tractability were shown to be moderate (0.20-0.33). Memory, low self-control, and factor 1 anxiousness had low heritabilities of 0.13-0.16.

The estimated heritability was zero or close to zero for six of the traits, including fearfulness, concentration, will to win, recovery, poor appetite and stereotype.

Table 8. Variance components where $\sigma^2 P$ is the phenotypic variance, $\sigma^2 A$ is the additive genetic variance and $\sigma^2 e$ is the residual variance. The estimated heritabilities (h^2) for the traits and factors (F) are also presented. Standard errors are shown as subscripts and bold estimates are considered as significant

Trait	**N**	h^2	σ^2 **P**	σ^2 **A**	σ^2 **e**
Nervousness	376	$0.20_{0.16}$	2.93	$0.58_{0.49}$	$\mathbf{2.35_{0.48}}$
Excitability	375	$0.33_{0.17}$	2.81	$0.92_{0.51}$	$\mathbf{1.89_{0.47}}$
Fearfulness	374	$0.09_{0.13}$	2.10	$0.20_{0.28}$	$\mathbf{1.91_{0.30}}$
Concentration	372	$0.00_{0.12}$	2.48	$0.00_{0.29}$	$\mathbf{2.48_{0.33}}$
Learning	368	$\mathbf{0.42_{0.21}}$	1.57	$0.65_{0.35}$	$\mathbf{0.92_{0.31}}$
Memory	372	$0.16_{0.14}$	3.29	$0.54_{0.46}$	$\mathbf{2.75_{0.47}}$
Cooperation	372	$\mathbf{0.42_{0.19}}$	1.67	$\mathbf{0.70_{0.34}}$	$\mathbf{0.98_{0.30}}$
Will to win	358	$0.00_{0.12}$	2.85	$0.00_{0.34}$	$\mathbf{2.85_{0.39}}$
Stubbornness	369	$\mathbf{0.61_{0.20}}$	1.41	$\mathbf{0.86_{0.32}}$	$0.56_{0.27}$
Low self-control	371	$0.13_{0.13}$	1.98	$0.26_{0.26}$	$\mathbf{1.72_{0.27}}$
Recovery	367	$0.07_{0.13}$	2.02	$0.14_{0.25}$	$\mathbf{1.87_{0.28}}$
Poor Appetite	367	$0.06_{0.13}$	2.70	$0.15_{0.34}$	$\mathbf{2.55_{0.38}}$

F1 Anxiousness	376	$0.13_{0.13}$	0.69	$0.09_{0.09}$	$\mathbf{0.59_{0.09}}$
F2 Tractability	376	$0.22_{0.18}$	0.63	$0.14_{0.12}$	$\mathbf{0.49_{0.11}}$
F3 Excitability	376	$0.31_{0.18}$	0.66	$0.21_{0.12}$	$\mathbf{0.46_{0.11}}$
Stereotype	362	$0.01_{0.14}$	1.78	$0.02_{0.25}$	$\mathbf{1.75_{0.27}}$

Genetic (r_g) and residual correlations from the bivariate analysis for traits with $h^2 >$ 0.2 and for factor 1 anxiousness, factor 2 tractability and 3 excitability are shown in Table 9.

*Table 9. Genetic correlations from the bivariate analyses above diagonal and residual correlations below diagonal. Standard errors are listed as subscripts, bold estimates are significant (SE*2< estimate). Correlations where the convergence criteria were lowered are not considered as significant*

	Learning	**Cooperation**	**Excitability**	**Nervousness**	**Factor 1**	**Factor 2**	**Factor 3**
Learning	X	**$0.65_{0.207}$**	$-0.60_{0.343}$	$-0.62_{0.412}$	**$0.92_{0.378}$**	**$0.90_{0.113}$**	$0.38_{0.400}$
Cooperation	**$0.49_{0.17}$**	x	$-0.39_{0.338}$	$0.39_{0.434}$	$-1.00_{0.367}$*	$1.00_{0.179}$*	$0.28_{0.358}$
Excitability	$-0.05_{0.20}$	$-0.24_{0.18}$	x	**$0.82_{0.247}$**	$1.00_{0.362}$*	$0.27_{0.471}$	**$0.85_{0.115}$**
Nervousness	$-0.14_{0.18}$	**$-0.51_{0.19}$**	**$0.59_{0.09}$**	x	$0.71_{0.450}$	$0.50_{0.552}$	$1.00_{0.095}$*
Factor 1	$-0.15_{0.14}$	$-0.13_{0.14}$*	$0.18_{0.13}$*	$0.23_{0.12}$	x	$-1.00_{0.647}$*	$0.68_{0.453}$
Factor 2	**$0.77_{0.08}$**	$0.81_{0.05}$*	$-0.12_{0.16}$	$-0.30_{0.16}$	$0.05_{0.13}$*	X	$0.42_{0.439}$
Factor 3	$-0.03_{0.20}$	$-0.38_{0.22}$	**$0.84_{0.05}$**	$0.05_{0.13}$*	$0.06_{0.14}$	$-0.21_{0.18}$	x

*Convergence criteria was set to 10^{-4} instead of 10^{-7} due to convergence problems

4.5. Whole genome sequencing analysis

Excitability was the only trait included in the analysis of the whole genome sequencing data. All 96 horses included in the analysis had a score for excitability. The mean value for the age of the horses was 5.2 with an SD of 3.1, the youngest horse was 2 years old, and the oldest horse was 24 years old. The mean value for the trait excitability was 3.23 with an SD of 1.73, and the median value was 3. The kurtosis was -0.9 and the skewness was 0.32. The trait distribution was compared for the 96 sequenced horses and the full data (Table 5), and the included 96 horses were found to be a representable sample for the whole dataset.

The results from the whole genome sequencing analysis for the trait excitability are shown in Figure 3 and Figure 4. The case group consisted of horses receiving score 5, 6 and 7 (often, usually, and always excited). In Figure 3, the control group consisted of horses with score 1 and 2 (never and rarely excited). Each dot in the Manhattan plots corresponds to a 50 Kb window that includes up to 40 SNPs per window. In the Manhattan plot, the order of the chromosomes on the x-axis is random. As seen in Figure 3, the F_{ST} values range from low for most regions up to approximately 0.13 for one region. An F_{ST} value of 0.13 indicates a low genetic differentiation between the case and control group. According to Frankham et al. (2002), an F_{ST} of 0.15 is indicating significant differences in allele frequencies in the region. However, looking at the results from Figure 3 the case and control groups are significantly different at all points above the green line which corresponds to the Bonferroni significance threshold.

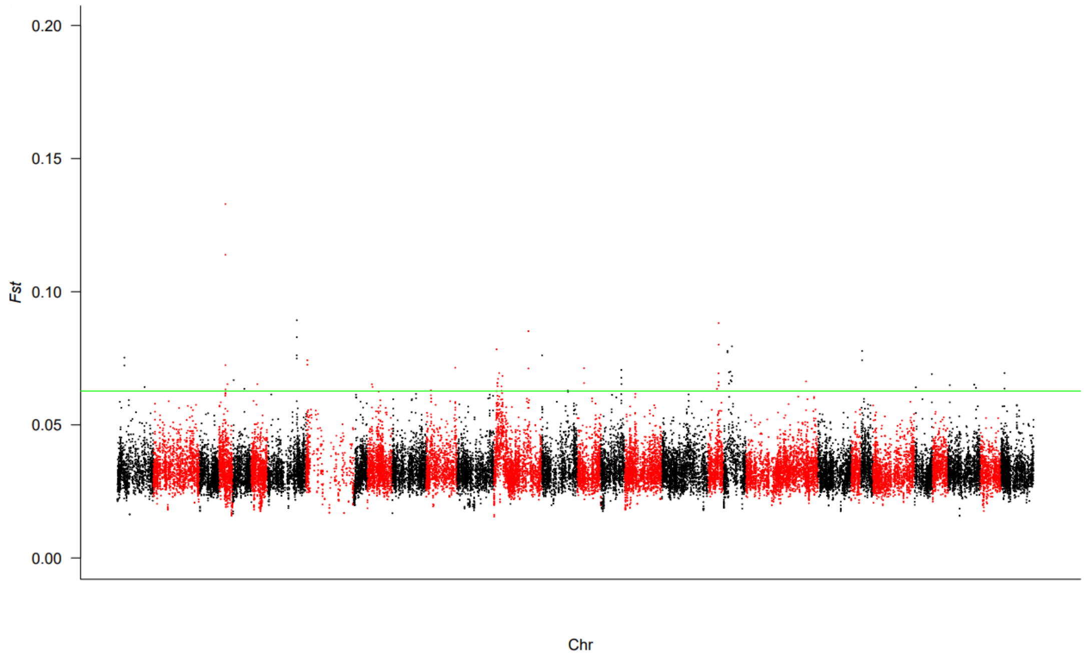


Figure 3. Manhattan plot displaying population differentiation between horses receiving score 1 or 2 and horses receiving score 5, 6 and 7 for the trait excitability. The x-axis represents the chromosomes (scrambled) and the y-axis the F_{ST} score (fixation index). The green line is the significance threshold after Bonferroni adjustments.

In Figure 4, the control group consisted of horses with score 1 (never excited). Results show that by including horses in the control group that are more extreme compared to the case group, some of the false positive regions could be removed. In Figure 4, two regions marked as a and b contained a lot of windows in the same region. This indicates a more reliable result than single points. The most interesting result of the analysis of the whole genome sequencing data is therefore considered to be the loci marked as a and b in Figure 4.

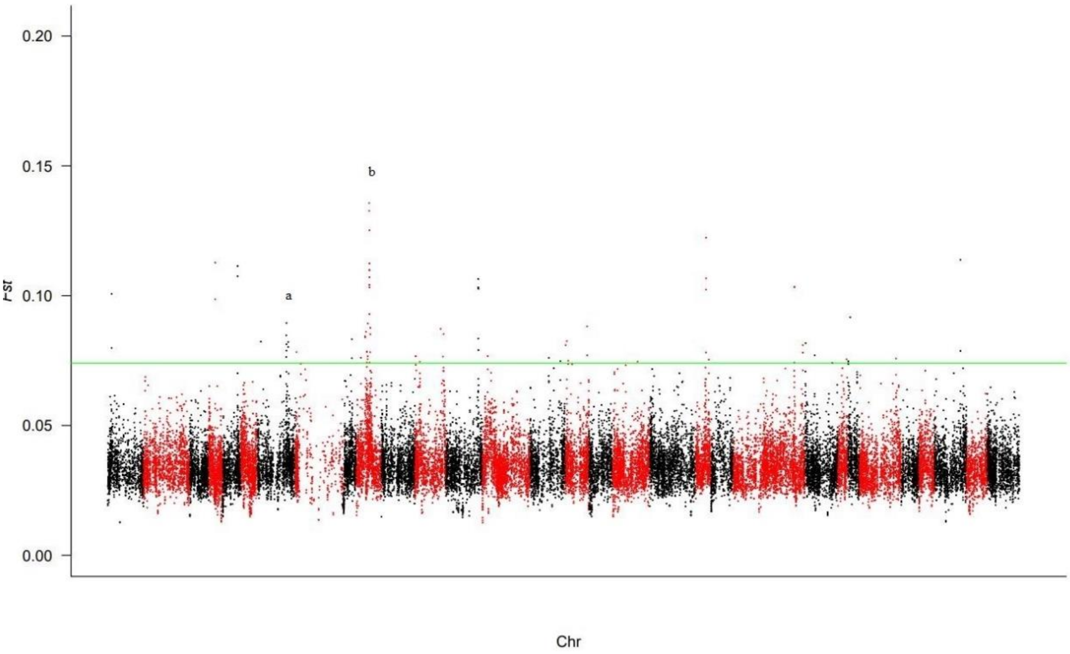


Figure 4. Manhattan plot displaying population differentiation between horses receiving score 1 and horses receiving score 5, 6 and 7 for the trait excitability. The x-axis represents the chromosomes (scrambled) and the y-axis the F_{ST} score (fixation index). The green line is the significance threshold after Bonferroni adjustments. Interesting regions are marked as a and b.

5. Discussion

5.1. Materials and methods

5.1.1. Data material

The data material in this study consisted of 376 horses with survey responses. As mentioned earlier, several different strategies were applied to reach out to trainers of SB trotters. It is therefore not possible to find out how many trainers received the information about the project and thereof, response rate could not be calculated. The majority of surveys were collected by visiting professional trainers located nearby campus, amateur trainers were mainly reached by posts in Facebook groups and with the help of the email list of active trainers the Trotting Association offered us to reach out to. To visit professional trainers was an efficient way of collecting both survey replies and hairs to extract DNA from. The percentage of professional trainers in the study (17 %) was higher than the percentage of professional trainers in Sweden (10 %) (Swedish Trotting Association 2020b). Possible implications of this might be that horses trained by professional trainers are trained on a different level with potential to reach the top level. Horses trained by amateur trainers might instead be held for fun with less demands to bring in money.

5.1.2. Interpretation of the survey

By looking at the distribution of answers on the 1-7 scale for each question, it became clear that results were skewed. For learning and cooperation, most of the horses received higher scores. Also, for the trait stubbornness and low self-control, nearly 70, and 60 % respectively of the horses received score 1 (never). By collecting more data, as a suggestion around 1000 individuals from both amateur and professional trainers, the chance of a more representative data material increases. Despite that, the definition of stubborn and memory (of unpleasant events) should preferably be clarified. The trait memory was referring to if the horse remembered unpleasant events it has experienced. No effects were significant for this trait. Possible reasons why no effects were significant for this trait might be different interpretations of the trait, many trainers also expressed that the horse never had experienced any unpleasant events.

Although, surveys very similar to the one used in the current study are proven to have high inter-observer reliability (Momozawa et al. 2005a) and to be correlated to biological reactions to stimuli (Momozawa et al. 2003; Roberts et al. 2016). One must still remember the subjective character of a survey and the fact that different trainers' perception of the scores may vary.

5.1.3. Material and Methods applied

Statistical analysis of the temperament survey

As mentioned earlier, the data was not normally distributed. Not having normally distributed data and performing analyses assuming data is normally distributed might give unreliable results. This also applies to the analyses in DMU which assumes normally distributed residuals. A way to solve this problem is by transformation of the data. Skewed data with logarithmic properties are subject for log transformation, and by that making the data more appropriate for analyses (FENG et al. 2014). Although, after log transformations and preliminary analysis in SAS, these changes did not have a large effect on the distribution of residuals or preliminary heritability estimations for the traits. However, other types of transformations could be investigated in further studies.

The results from the raw correlations appeared as reasonable. However, some of the estimations of genetic correlations between traits from the bivariate analysis (Table 9) were less logical. Due to difficulties to reach the convergence criteria, the criteria were lowered from 10^{-7} to 10^{-4} for five of the bivariate analyses. The genetic correlation between excitability and factor 1 anxiousness was 1.00 compared to factor 3 excitability (0.85). Since excitability had a strong positive loading (0.69) on factor 3 excitability, but a weak positive loading on factor 1 anxiousness (0.29) it is doubtful that the genetic correlation to factor 1 would be higher than to factor 3. Also, the genetic correlation between learning and factor 1 was surprisingly high (0.92), compared to the genetic correlation between cooperation and factor 1 (-1.00). A more logical result would have been a negative genetic correlation between learning and factor 1 anxiousness since it loaded on the negative side on that factor (-0.28).

As the results showed, the survey seemed to be able to distinguish a genetic variation in temperament characteristics for some of the traits like learning, cooperation, and excitability. For other traits like will to win, poor appetite, stereotype, anxiousness, recovery, low self-control, fearfulness, stubbornness, concentration, memory, and factor 1, the variation explained by additive genetic variance were low and the residual variance was high.

As König von Borstel et al. (2013) highlights, subjective temperament scoring at assessment of potential breeding horses might be biased due to a number of factors such as the horse's pedigree, inconsistency between judges etcetera. Applying the survey in the current thesis as a basis for selection might also cause false results if trainers give more favourable scores to reach higher breeding values. The method of evaluating horse's temperament through a survey is probably limited to assess temperament characteristics of different breeds and the genetic background of these traits in research.

Selection criteria for sequencing and statistical analysis

The original plan was to sequence 288 horses (three full plates) but due to the uncertainty of how the new riptide protocol had worked, only one third of the horses were sequenced. If this would have been the plan from the beginning, horses could have been selected for genotyping based on certain criteria instead of being a random sample. Assuming that the case/control analysis still would have been performed, only horses belonging to the extreme groups (score 1 vs score 7) could have been included. Then no horses with prepared samples would have been discarded (horses with scores in the middle of the scale were in the current thesis discarded from the sequencing analysis) and the results would have had more power to reach significance.

5.2. Temperament of Standardbred trotters

5.2.1. Factor analysis and trait heritabilities

The aim of this thesis was to assess temperament characteristics in SB trotters located in Sweden and Norway. With the help of FA, three factors were extracted from the analysis but only one had an eigenvalue >1. In spite of this, three factors were included in further analysis based on their logical content. Budaev (2010) lists some of the knowledge gained from research on animal temperament when it comes to FA and highlights the fact that other methods than the eigenvalues>1 criterion are accepted in research when deciding on how many factors should be extracted. Williams et al. (2010) proposed the use of "scree plot test" or to look at the relevance of the factor loadings when deciding what factors should be extracted.

Factor 1 Anxiousness

The factor explaining most of the variance in the temperament of SB's was named anxiousness. This factor described horses that tended to lose their self-control, remember unpleasant events and were fearful. Other traits in this factor with slightly lower loadings were stubbornness but also bad concentration and bad cooperation. Fear and anxiety are two closely connected emotional states but also an important attribute of the “fight and flight reaction” to unfamiliar stimulus (Davis 1998).

Momozawa et al. (2003) found quite similar results in Thoroughbreds which also is a breed used for horse racing, where the factor explaining most of the variance also was named anxiety and included nervousness, excitability, stubbornness, friendliness, and attachment to specific person. A later study on Thoroughbreds by the same research group extracted a factor named anxiousness explaining the largest proportion of the variance (Momozawa et al. 2005a). Except the traits loading on this factor found in their previous study, traits like panic, sketchiness, timidity, emotionally inconsistent and vigilance were also belonging to this factor. In native Norwegian horse breeds, the factor explaining most of the variance was shared among the four breeds and named anxiousness (Olsen & Klemetsdal 2017).

Also in Tennessee Walking horses, anxiousness accounted for most of the variance in the breed (Staiger et al. 2016). This temperament characteristic seems to be shared among horse breeds and is not unique for the trotter or other breeds selected for racing. One must consider that there still exists differences between the breeds when it comes to the level of anxiousness. Roberts et al. (2016) found a correlation between horses reported as anxious and elevated dopamine levels measured though spontaneous blink rates. Horses maintained for pleasure and ponies were reported less anxious than horses used for equine sports in this study. One of the conclusions drawn by Roberts et al. (2016) is that environmental factors such as how the horses are stabled/held and daily routines may differ depending on the usage of the horse and result in different levels of anxiety. In the current study, there was no registration of information about how the horses were stabled (loose housing system or box stalls etcetera). Therefore, there might be information missing to draw conclusions of environmental impact on the horse’s anxiety level in the current thesis. Even though traits included in the factor named anxiousness in the studies mentioned previously slightly differ from the traits included in this study, it seems reasonable that factor 1 anxiousness can be influenced by environmental factors not covered in this thesis. No effects were found to be significant for this factor based on the single trait model tested for in SAS, and the heritability for this factor was low (0.13) and the standard error was high.

Furthermore, the factor extracted in this thesis named anxiousness had a significant effect on the trait stereotype. Horses with higher factor 1 score (fearful horses that tend to lose their self-control and remember unpleasant events) had a significantly elevated risk of performing stereotypic behaviours. The elevated dopamine levels (Roberts et al. 2016) found correlated to anxiety in horses, have in studies on rodents been linked to a higher activity level and increase in stereotypic behaviours (Garner & Mason 2002). The main focus in this thesis was not to assess risk-factors for stereotypic behaviour in SB. However, the very primary results based on the small data material in this study could serve as a starting point for further analysis on possible stressors causing stereotypic behaviour and further analyse to what extent this might affect welfare and performance in SB's.

The two traits, with strongest loadings on factor 1 was low self-control and fearfulness. Two traits that could be of great importance for the handling of the horse at the competition as well as during the race. The age of the horse had a significant effect on how fearful the horses were at competitions. Not very surprisingly, it seems like horses get less fearful when they grow older and gain more experience. Heritability estimations strengthens this conclusion since the inherited component in this trait was low (h^2=0.09) and environmental factors impacts this trait to a great extent. Contradictory, results from von Borstel et al. (2010) showed significant differences in fear reaction to a novel object in warmblood horses bred for dressage compared to those bred for show-jumping suggesting a possible genetic influence. There were no estimations of variance components for fearfulness in their study to compare the results from the current thesis to, but the age of the horse had a significant effect supporting the idea of more experience will led to less fear and anxiety. König von Borstel (2013) has in a review article listed heritability estimations for reaction to novel objects, h^2 ranged from 0.24-0.40 and with standard deviations ranging from 0.08-0.24. This might be an indication of that it is more trustworthy to assess fear with novel objects tests than surveys.

To connect this factor to human psychology research, this factor would be closely linked to neuroticism, also supported by Kristiansen & Kuczaj II (2013)'s findings when assessing temperament in horses. Neuroticism separates anxious individuals from calm individuals and have in other species also been liked to fearfulness, reactiveness and excitability (Gosling & John 1999).

Factor 2 tractability

The second factor extracted represented highly cooperative horses that easily leans the task of competing and have a strong will to win. The eigenvalue of this factor almost fulfilled the criterion >1 to be extracted (0.97). A similar factor were extracted by Roberts et al. (2016) and (Momozawa et al. 2005a) named trainability. All effects: sex, age and trainer licence had a significant effect on this factor. Younger horses were significantly less tractable than older horses. These results are reasonable since older horses must have understood their task and what the trainer demands from them. But there is also a possibility that horses that do not understand or do not learn the task of harness racing will be sorted out during their first years of competing. This might cause bias if horses in the older age groups only consist of horses with a long career and not those with shorter racing careers. There might be improvements for the division of horses in age groups, for example the addition of if the horse is active or not when replying to the survey, but also to weigh in the number of years the horse was/have been in training. In our survey, only time in training was asked for. Many horses with injuries are convalescent for a longer period, not asked for or considered in this type of survey. This “problem” might be difficult to correct for because many horses move around to different trainers during their career which means it will be difficult to track down the history of the horse.

Interestingly, the sex of the horse had a significant effect on how tractable the horse was. Stallions were significantly more tractable than geldings, but also more tractable than mares (not significant). Stallions did also learn the task of competing better than geldings. Staiger et al. (2016) found no significant differences between sexes for the trait tractability in Tennessee Walking horses, but Momozawa et al. (2005b) did. The effect of sex on learning have also been examined in studies with objective measurements. A study focusing on learning ability of young horses found that mares were better at learning the task of opening a box than males (stallions and geldings) (Wolff & Hausberger 1996). In young SB trotters, Cape & Vleck (1981) found no effect of sex on learning. The different results of the effect of sex seem to be consistent and there are to this date not clear if or why the gender of the horse would influence cooperation, tractability, or learning abilities. Also, why the interpretation of what a tractable/cooperative horse is differ between trainers shown in the current thesis, might be due to the subjective character of the question. Learning ability in horses are of great importance for the horse and human interaction, and is a multidimensional trait. König von Borstel (2013) highlights other research in the area concerning the debate about using positive or negative reinforcement during equine training and how it affects animal welfare.

For factor 2, the heritability was estimated to 0.22 in the current thesis. For Standardbreds, the corresponding heritability for trainability has been estimated to 0.10 (Cape & Vleck 1981). One must consider that the standard error in their study (0.32) as well as in the current study (0.18) were high. In the study by Cape & Vleck (1981) there were few horses participating (159) and lastly, these horses were trained for auction and not for trotting races. Traits with high loadings on this factor in the present study was cooperation (h^2=0.42), learning (h^2=0.42) and will to win (h^2=0). The estimation for cooperation is higher than those reported previously for willingness to work (0.10-0.29) (König von Borstel 2013). Interestingly, tractability had a significant effect on the trait stereotype. Horses with higher values for tractability (cooperative, high will to win and easy taught) had less stereotypes. This result are in line with Hausberger et al. (2007a) findings that horses with stereotypes have more difficulties to learn a new task than horses without stereotypes.

Applying this result to the five factors of personality in humans, this factor seems to correspond to agreeableness (Digman 1990). In animal research, Gosling & John (1999) have described animals in this factor as cooperative, not aggressive, tender-mindedness (sympatric) and they trust humans easily. A temperament attribute that logically would be good to strive for in horse breeding. In Norwegian horse breeds, (including the CBT) Olsen & Klemetsdal (2017) did instead draw the connection between cooperation, hard-working, not stubborn and willing to learn to conscientiousness. The factor conscientiousness describes persons who do not act impulsively, they follow rules and are self- disciplined, a factor thought to be unique for humans and primates (Gosling & John 1999).

Factor 3 excitability

The third factor extracted in this thesis was named excitability since the loading for excitability was 0.69 and for nervousness 0.73, which are indications of a very strong connection between these two traits and factor 3. Due to the low eigenvalue of this trait (0.62) the relevance of this factor can be questioned and after all, the inclusion of this factor was based on other criteria as discussed before. Roberts et al. (2016) found a very similar factor explaining almost 7% of the variance in their study. This strengthens the evidence of another underlying temperament attribute not to be confused with factor 1 anxiety where excitability and nervousness had low loadings in the current thesis. In Roberts et al. (2016) the factor named excitability also included low self-reliance, active, impulsive and eccentric. Lloyd et al. (2008) also reported anxiousness and excitability as two different factors where Arabian horses and Thoroughbreds were reported as the breeds with the highest scores for excitability. In CBT's, a corresponding factor to excitability seemed to be unique for the breed and included speedy (hot temperament), not slow (quick, hurried) and excited (Olsen & Klemetsdal 2017). Standardbreds and Coldblooded trotters are like Thoroughbreds and Arabians bred for racing and therefore temperament characteristics such as excitability (but also anxiety) seem to be selected for based on these findings. Sex had a significant effect on factor 3, mares and geldings got significantly more excited at competitions than stallions. However, Roberts et al. (2016) found no effect of sex on excitability and Lloyd et al. (2008) did not examine the effect of sex.

One trait with a loading very close to 0.4 on factor 3 was poor appetite. Mares were shown to have significantly lower appetite after competitions than stallions. Previous studies on hormone concentrations of leptin after exercise in SB trotters have shown significant differences between sexes (Kędzierski & Kapica 2008). Elevated leptin levels supress appetite, and mares were shown to have significant higher levels of leptin in plasma compared to stallions/geldings after exercise. This supports the results found in this study.

Genetic correlations

Significant genetic correlations from the bivariate analysis (Table 9), were found between learning and cooperation r_g= 0.65 but also between excitability and nervousness r_g=0.82. The next step would be to include performance data of traits used in the current statistical model for breeding evaluation of SB trotters in

Sweden, e.g., number of starts, proportion of races where the horse got placed (1-3), earnings, earnings per start, best time, and racing status. In Swedish Warmbloods bred for dressage and show jumping, genetic correlations between temperament at the riding horse quality test and competition points/placings during the horses' lifetime was estimated to 0.00-0.93 (Wallin et al. 2003). The temperament at the loose jumping test as a 4-year-old seemed to be important for competition success later in life for horses bred for show- jumping (r_g= 0.91-0.91). Wallin et al. (2003) also found temperament under rider for 4-year-old horses bred for dressage important for performance in dressage competitions later in life (r_g= 0.75). Estimations of genetic correlations between temperament traits assessed with a survey and performance traits might give indications of whether temperament is a trait with potential to select for in SBs.

5.3. Whole genome sequencing analysis

Based on the results shown in Figure 4, significant differences between the groups were found at a few loci. The vertical lines with dots marked with a and b are the most interesting results since further analyses might remove significant "lonely dots". Still, more data is needed to remove remaining false positives. If the remaining 192 horses are sequenced, it is possible to only include horses with score 6 and 7 and remove horses with score 5 from the case group. Even better results might be achieved if more data is collected to reach at least 20 horses in total with score 7. Comparing the two extreme groups (horses with score 1 and score 7) will improve the power of the analysis and might increase the F_{ST} score at regions of differentiation. The next step of the analysis would be to extract SNPs and with the help of the software SNPeff, annotate SNPs to find their location and potential effect on genes.

A recommendation for further analyses would be to correct the scores for the effect of age, before dividing the horses into case and control groups, since the age of the horse had a significant effect on how excited the horses were at competition when analysed in the bigger data material. Average relationship within and between groups would also be interesting to investigate.

6. Conclusion

In this thesis, three factors explaining temperament characteristics of SB trotters were found: anxiousness, tractability, and excitability. Anxiousness and tractability were considered to be associated with neuroticism and agreeableness, respectively. Those are temperament characteristics frequently used in academic psychology in humans (Digman 1990). The factor anxiousness was found to be significant for performing stereotypic behaviours, further analysis of this factor and its correlation to stress, alternatively, distress is recommended. The factor tractability, characterised by having a high will to win, be cooperative and learn the task of competing easily would be a subject for further genetic analysis and how it interacts with performance in harness racing. Heritability estimates ranged from 0-0.42, where learning and cooperation had the highest estimates of 0.42 for both. The small data material was a limiting factor in this study. Despite that, the preliminary analysis of the WGS data showed interesting results worth further investigation with data from more horses included.

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Appendix 1

Table 10. The survey used in the thesis with trait names, trait descriptions and response options on a scale from 1-7. The trainer was asked to rate how often he or she observe/d the specific behaviour in competition

Trait	Description	1 Never	2 Rarely	3 Occasionally	4 Sometimes	5 Often	6 Usually	7 Always
Nervousness	Tends/tended to become nervous							
Excitability	Tends/tended to get excited or agitated easily							
Fearfulness	Tends/tended to be afraid easily (e.g. novel environments)							
Concentration	Tends/tended to be focused and unaffected by the environment							
Learning	Tends/tended to learn the task of competing quickly							
Memory	Tends/tended to memorize/remember unpleasant events							
Cooperation	Tends/tended to be cooperative, have good attitude (e.g. willing to work/no resistance)							
Will to win	Tends/tended to desire to win							
Stubbornness	Tends/tended to be obstinate once it resists a command							

Self-control	Tends/tended to panic, escape and lose control (e.g. impossible to handle or stop / damage itself)
Recovery	Tends/tended to relax quickly
Appetite	Tends/tended to have poor appetite between competitions events

Appendix 2

Table 11. Trait correlations, significance level and number of observations for the 13 traits included in the study. p-values are given as; <0.05 (*), <0.01 (**) and <0.001 (***) and non-significant p-values as ns

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Stereotype (1)	1	0.10	0.09	0.11	-0.12	0.00	0.13	-0.09	-0.07	0.23	0.10	-0.04	0.08
Significance level		*	ns	*	*	ns	*	ns	ns	***	*	ns	ns
N	362	362	361	360	358	354	358	358	344	355	357	353	353
Nervousness (2)	0.10	1	0.61	0.31	-0.26	-0.30	0.33	-0.19	-0.12	0.17	0.29	-0.26	0.21
Significance level	*		***	***	***	***	***	**	*	**	***	***	***
N	362	376	375	374	372	368	372	372	358	369	371	367	367

Excitability (3)	0.09	0.61	1	0.40	-0.30	-0.31	0.24	-0.32	-0.08	0.28	0.40	-0.35	0.17
Significance level	ns	***		***	***	***	***	***	ns	***	***	***	***
N	361	375	375	374	372	368	372	372	358	369	371	367	367
Fearfulness (4)	0.11	0.31	0.40	1	-0.50	-0.38	0.40	-0.32	-0.24	0.26	0.50	-0.28	0.06
Significance level	*	***	***		***	***	***	***	***	***	***	***	ns
N	360	374	374	374	372	368	372	372	358	369	371	367	367
Concentration (5)	-0.12	-0.26	-0.30	-0.50	1	0.45	-0.23	0.38	0.30	-0.14	-0.32	0.36	-0.12
Significance level	*	***	***	***		***	***	***	***	**	***	***	*
N	358	372	372	372	372	367	371	371	357	368	370	366	366
Learning (6)	0.00	-0.30	-0.31	-0.38	0.45	1	-0.21	0.55	0.44	-0.27	-0.42	0.43	-0.07
Significance level	ns	***	***	***	***		***	***	***	***	***	***	ns
N	354	368	368	368	367	368	368	368	358	365	367	367	367
Memory (7)	0.13	0.33	0.24	0.40	-0.23	-0.21	1	-0.15	-0.07	0.29	0.53	-0.12	0.09

Significance level	*	***	***	***	***	***		**	ns	***	***	*	ns
N	358	372	372	372	371	368	372	372	358	369	371	367	367
Cooperation (8)	-0.09	-0.19	-0.32	-0.32	0.38	0.55	-0.15	1	0.42	-0.34	-0.36	0.37	-0.13
Significance level	ns	**	***	***	***	***	**		***	***	***	***	**
N	358	372	372	372	371	368	372	372	358	369	371	367	367
Will to win (9)	-0.07	-0.12	-0.08	-0.24	0.30	0.44	-0.07	0.42	1	-0.10	-0.16	0.24	-0.02
Significance level	ns	*	ns	***	***	***	ns	***		ns	**	***	ns
N	344	358	358	358	357	358	358	358	358	355	357	357	357
Stubbornness (10)	0.23	0.17	0.28	0.26	-0.14	-0.27	0.29	-0.34	-0.10	1	0.37	-0.21	0.05
Significance level	***	***	***	***	**	***	***	***	ns		***	***	ns
N	355	369	369	369	368	365	369	369	355	369	369	365	365
Low self-control (11)	0.10	0.29	0.40	0.50	-0.32	-0.42	0.53	-0.36	-0.16	0.37	1	-0.29	0.10
Significance level	*	***	***	***	***	***	***	***	**	***		***	0.0654

N	357	371	371	371	370	367	371	371	357	369	371	367	367
Recovery (12)	-0.04	-0.26	-0.35	-0.28	0.36	0.43	-0.12	0.37	0.24	-0.21	-0.29	1	-0.22
Significance level	ns	***	***	***	***	***	*	***	***	***	***		***
N	353	367	367	367	366	367	367	367	357	365	367	367	367
Poor appetite (13)	0.08	0.21	0.17	0.06	-0.12	-0.07	0.09	-0.13	-0.02	0.05	0.10	-0.22	1
Significance level	ns	***	**	ns	*	ns	ns	**	ns	ns	ns	***	
N	353	367	367	367	366	367	367	367	357	365	367	367	367