Exploring the Horse Genome to Elucidate the Genetics of Gaits and Athletic Performance

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Abstract

The athletic nature of the horse and the large number of diverse horse breeds provides an opportunity to study the genetics of locomotion pattern and performance in mammals. The overall aim of this thesis was to get a better understanding of the genetics behind gaits and performance in the horse. This thesis compiles four papers on four different horse breeds. In studies I and II we describe the effect of a known mutation in the *doublesex and mab-3 related transcription factor3 (DMRT3)* gene on harness racing performance in Swedish-Norwegian Coldblooded trotters and Finnhorses. Previous studies have demonstrated a major impact of the gene on harness racing performance results in Standardbreds. While the gene clearly is important for harness racing performance in both Coldblooded trotters and Finnhorses, the most successful genotype differed between the two breeds. The homozygous mutant (AA) Finnhorses were most successful on the racetrack but had difficulties in performing a good canter in riding. For Coldblooded trotters the CA horses were the better race horses overall, even though the AA horses performed well at young ages.

While previous studies have reported that homozygozity for the *DMRT3* mutation (AA) is required for a horse to be able to pace, not all AA horses can pace. To understand more about the genetic regulation of pace, in study III we compared the genomes of AA Icelandic horses with and without the ability to pace. We performed a genome-wide association study and identified a potential candidate region that contained a gene known to influence memory and learning ability.

In study IV we utilized the close relationship between the Coldblooded trotter and the North-Swedish draught horse to identify novel genes influencing harness racing performance. The two breeds are genetically similar but have been selected for different traits. By comparing the genomes of the two breeds with the genome of Standardbreds, we identified five top regions where the Coldblooded trotters and Standardbreds were similar but together differed from the North-Swedish draught horse. One of the regions identified contained five single nucleotide polymorphisms (SNPs) that were significantly associated with racing performance in Coldblooded trotters.

In conclusion, this research shows that carefully selected horse materials can serve as models to gain deeper knowledge on the genetics of performance and locomotion pattern. It is also vital to contextualize the importance of these genes within each horse breed.

Keywords: Equine, Fst, GWAS, harness racing, locomotion pattern, pace

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Dedication

To my family and friends

Plans are nothing; planning is everything
Dwight D. Eisenhower

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Jäderkvist Fegraeus K**, Lawrence C, Petäjistö K, Johansson MK, Wiklund M, Olsson C, Andersson L, Andersson LS, Røed KH, Ihler C-F, Strand E, Lindgren G & Velie BD. 2017. Lack of significant associations with early career performance suggest no link between the *DMRT3* "Gait Keeper" mutation and precocity in Coldblooded trotters. *PloS One* 12(5): e0177351. https://doi.org/10.1371/journal.pone.0177351.

II **Jäderkvist Fegraeus K**, Johansson L, Mäenpää M, Mykkänen A, Andersson LS, Velie B.D, Andersson L, Árnason T & Lindgren G. 2015. Different *DMRT3* genotypes are best adapted for harness racing and riding in Finnhorses. *Journal of Heredity* 106(6), 734-740.

III **Jäderkvist Fegraeus K**, Hirschberg I, Árnason T, Andersson L, Velie BD, Andersson LS & Lindgren G. To pace or not to pace: a pilot study of four- and five-gaited Icelandic horses homozygous for the *DMRT3* "Gait Keeper" mutation. In Press in *Animal Genetics.*

IV **Jäderkvist Fegraeus K**, Velie BD, Axelsson J, Ang R, Hamilton NA, Meadows JRS & Lindgren G. Selective sweep mapping using a unique Nordic horse model revealed *EDN3* as a candidate gene for harness racing performance. *Manuscript.*

Papers I-III are reproduced with the permission of the publishers.

Related work by the author

(Not included in the thesis)

1. **Jäderkvist K***, Andersson LS*, Johansson AM, Árnason T, Mikko S, Eriksson S, Andersson L & Lindgren G. 2014. The *DMRT3* 'Gait keeper' mutation affects performance of Nordic Standardbred trotters. *Journal of Animal Science* 92, 4279-4286.

2. **Jäderkvist K**, Kangas N, Andersson LS & Lindgren G. 2014. Gaitedness is associated with the *DMRT3* 'Gait keeper' mutation in Morgan and American Curly horses. *Animal Genetics* 45(6), 908-909.

3. **Jäderkvist K**, Holm N, Imsland F, Árnason T, Andersson L, Andersson LS, Lindgren G. 2015. The effect of the *DMRT3* 'Gait keeper' mutation on riding ability traits and gaits in Standardbred and Icelandic horses". *Livestock Science* 176, 33-39.

4. Velie BD, **Jäderkvist K**, Imsland F, Viluma A, Andersson LS, Mikko S, Eriksson S & Lindgren G. 2015. Frequencies of polymorphisms in *myostatin* vary in Icelandic horses according to the use of the horse. *Animal Genetics* 46(4), 467-468.

5. Eriksson S, **Jäderkvist K**, Dalin A-M, Axelsson J & Lindgren G. 2015. Prevalence and genetic parameters for cryptorchidism in Swedish-born Icelandic horses. *Livestock Science* 180, 1-5.

6. François L, **Jäderkvist Fegraeus K**, Eriksson S, Andersson LS, Tesfayonas YG, Viluma A, Imsland F, Buys N, Mikko S, Lindgren G & Velie BD. 2016. Conformation traits and gaits in the Icelandic horse are associated with genetic variants in *Myostatin* (*MSTN*). *Journal of Heredity*. doi: 10.1093/jhered/esw031.

7. Johansson MK, **Jäderkvist Fegraeus K**, Ekesten B* & Lindgren G*. 2017. The refractive state of the eye in Icelandic horses with the Silver mutation. *BMC Veterinary Research* 13:153. doi: 10.1186/s12917-017-1059-7.

8. Staiger EA, Almén MS, Promerová M, Brooks SA, Cothran EG, Imsland F, **Jäderkvist Fegraeus K**, Lindgren G, Mehrabani Yeganeh H, Mikko S, Vega-Pla JL, Tozaki T, Rubin CJ & Andersson L. 2017. The evolutionary history of the *DMRT3* 'Gait keeper' haplotype. *Animal Genetics*, doi: 10.1111/age.12580.

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Abbreviations

ACTN3	Sarcomeric α-actinin 3
aDNA	Ancient deoxyribonucleic acid
BP	Before Present
CKM	Creatine kinase, muscle
CPG	Central Pattern Generator
DMRT3	Doublesex and mab-3 related transcription factor 3
DNA	Deoxyribonucleic acid
EBV	Estimated Breeding Value
EDN3	Endothelin 3
ERE1	Equine Repetitive Element 1
FAANG	Functional Annotation of Animal Genomes
Fst	Fixation Index
Gb	Giga Bases
GEBV	Genomic Estimated Breeding Value
GRF	Ground Reaction Force
Grin2B	Glutamate ionotropic receptor NMDA type subunit 2B
GWAS	Genome-wide association study
IBD	Identity-by-descent
LD	Linkage Disequilibrium
MAS	Marker-assisted selection
MCOA	Multiple Congenital Ocular Anomalies
mRNA	Messenger ribonucleic acid
MSTN	Myostatin
mtDNA	Mitochondrial deoxyribonucleic acid
NGS	Next-generation sequencing
OLWS	Overo Lethal White Syndrome
PCA	Principal Component Approach
PCR	Polymerase Chain Reaction

PDK4	Pyruvate dehydrogenase kinase, isozyme 4
QQplot	Quantile-Quantile plot
QTL	Quantitative Trait Locus
QTN	Quantitative Trait Nucleotide
RNA	Ribonucleic acid
SNP	Single Nucleotide Polymorphism

1 Introduction

1.1 Horse domestication and breed creation

Horses (*Equus ferus caballus*) have, since their domestication, played important roles in society, and today horses are kept for both business and pleasure. The exact time period for horse domestication has been debated, and many different time periods and locations have been proposed, with time suggestions ranging from the early Bronze Age (5000-3900 years Before Present [BP]) to the Neolithic Age (8000-6000 years BP) (Levine, 2005, Outram *et al*., 2009, Schubert *et al*., 2014). Since then and until about 100 years ago horses were mainly used for transportation and warfare, but today most horses are kept for sport or recreation purposes (Levine, 2005). In some countries there is also a horsemeat industry (Martuzzi *et al*., 2001, Belaunzaran *et al*., 2015). While almost all existing horse breeds today are bred and controlled by humans, a few wild horse populations still exist. However, only one, the Przewalski´s horse (*Equus ferus ssp. przewalskii*), is a true wild horse that has never been domesticated (Goto *et al*., 2011). The Przewalski´s horse became extinct in the 1960´s but was bred in captivity and re-introduced to the wild in the beginning of the 1990´s (Ryder & Wedemeyer, 1982, Ryder, 1993, King, 2005). Today the Przewalski´s horse is listed as endangered with about 2,000 individual horses present in the population (King *et al*., 2015). It is considered a subspecies of the wild horse (*Equus ferus*) and possesses 66 chromosomes, compared to 64 in the modern horse (Benirschke *et al*., 1965, King *et al*., 2015). Due to this difference in chromosome number, the Przewalski´s horse is considered a sister taxon to the wild ancestors of domestic horses (Kavar & Dovč, 2008). A recent study suggested that the Przewalski´s horse and the domestic horse population split about 45,000 years ago (Der Sarkissian *et al*., 2015). While it is possible that domestic horses originate from one founder population, most research on horse origin using mitochondrial DNA (mtDNA) demonstrates that it is more likely that several distinct horse populations were involved in the domestication process (Lister *et al*., 1998, Vilá *et al*., 2001, Jansen *et al*., 2002, Lippold *et al*., 2011). The patrilineal diversity in domestic horses is significantly lower, likely due to the

use of a limited number of stallions in the breeding of the domestic horse (Lindgren *et al*., 2004, Kavar & Dovč, 2008, Wallner *et al*., 2017). Following domestication of the horse, selection and breeding of horses created a large number of diverse breeds. Today about 1,400 different horse breeds of various sizes, shapes and colors exist in the world, according to the Domestic Animal Diversity Information System database of the Food and Agriculture Organization of the United Nations (FAO) (FAOSTAT, 2017). However, there are likely duplicate breeds reported, as some breeds may have slightly different names in different countries. The different breeds are used for many diverse purposes, including agricultural work, jumping, racing, driving and exhibitions. According to FAO, in 2014, there were about 58 million horses in the world (FAOSTAT, 2017).

1.2 Horse breeding

As for the domestication time, it is difficult to determine exactly when humans started to breed horses, i.e. deliberately planning the breeding and keeping track of pedigrees. Today, a large proportion of the breeds are meticulously monitored in studbooks and the newborn foals are registered and marked, most often with a chip. Many breeding organizations also require hairsamples from newborn foals to be sent for parentage control. The studbooks for the breeds can be either open or closed. An open studbook means that the book is open for all horses that meet the breed specific requirements. A closed studbook only accepts horses where the parents are registered in the same studbook. While natural matings are still the most common reproduction method in many breeds, the use of artificial reproduction techniques such as artificial insemination (AI) and embryo transfer (ET), provide the opportunity to breed stallions and mares from different countries (Brinsko & Warner, 1992, Allen, 2005, Hinrichs, 2013). Another reproduction technique that can be applied is cloning. By cloning it is possible to create an individual that is an exact genetic copy of the animal donor. The first mammal clone from an adult somatic cell was the sheep Dolly, which was cloned in 1996 and the first horse was cloned in 2003 (Campbell *et al*., 1996, Galli *et al*., 2003).

1.2.1 Introduction of genetics and genomics into horse breeding

Historically, the breeding and selection of horses was solely based on pedigree and appearance of the horses. Today, the use of genetic information is becoming more and more important in the breeding work. By sequencing an individual's genome, every base of the DNA (deoxyribonucleic acid) is charted. The first human was sequenced in 2001 (Lander *et al*., 2001) and the first horse genome sequenced was published in 2009 (Wade *et al*., 2009). Since then there has been a rapid development of new technologies and techniques within the field of genomics. This increase in the number of analysis tools available has resulted in a sharp reduction of the costs to analyze the genome. A classic example is the cost associated with sequencing the human genome.

The costs for the project in which the first human genome was sequenced and analyzed were approximately $300 million (Lander *et al.*, 2001). Today, the cost for sequencing the mammalian genome ($3x10^9$ base pairs [bp]) is getting close to a $1000 per sample (Wetterstrand, 2015). As a result, the possibilities for using genomic tools in the breeding of animals are growing each year. A lot of genomic horse research is being conducted all over the world, thus increasing the availability of commercial genetic tests for horse breeders to use in their work. Already today there are a number of genetic tests available, where breeders can test their horses for genetic variants associated with different genetic diseases, coat color and/or performance. Examples of tests available include the test for the eye defect multiple congenital ocular anomalies (MCOA) that is linked to the Silver coat color, and skeletal atavism in Shetland ponies (Andersson *et al.*, 2011, Rafati *et al.*, 2016). By testing the breeding animals, it is possible to avoid breeding two horses that are carriers of the same disease alleles, thus minimizing the risk of breeding affected offspring.

1.3 Marker-assisted selection, genomic breeding values and genomic selection

The use of traditional breeding methods, i.e. selection of the superior animals to produce offspring, has been successful for the genetic improvement in many ways. However, the efficiency of these methods is lower if traits are difficult to measure, if the heritability is low or if it is difficult or costly to measure many animals in a short period of time (Eggen, 2014). These traits include for example fertility and resistance to diseases (Eggen, 2014). To improve the breeding for these types of traits, the use of genomic information has become important (Eggen, 2014). In the beginning of the 1990´s the marker-assisted selection (MAS) technology was introduced. The concept is that instead of measuring the actual trait of interest, a marker (biological or DNA/RNA) that is known to be associated with the trait is measured and used to predict the phenotype (Eggen, 2014). However, the limitation of the MAS technology is that it requires prior knowledge about the markers that are used (Eggen, 2014).

The first study that described the concept of genomic selection was published in 2001 (Meuwissen *et al.*, 2001). The idea of genomic selection is that a large number of genetic markers (single nucleotide polymorphisms, SNPs) are used to select which animals should be used in breeding (Eggen, 2014). A reference population of animals with accurate phenotype records is genotyped for a large number of SNPs. Using the information from the reference group, genomic breeding values (GEBVs) can be estimated without having complete knowledge about the genes in the genome (Eggen, 2014). As a large number of SNPs are used in the estimation, it is assumed that there is at least one SNP in close linkage disequilibrium (LD) with the causative mutation (Goddard & Hayes, 2009, Eggen, 2014). Based on the prediction equation retrieved from

the reference population, GEBVs can then be calculated for another group of selection candidate animals that have been genotyped for the same SNPs but lack phenotype records (Goddard & Hayes, 2009, Eggen, 2014). Using genomic selection will improve the selection response and reduce the generation interval, especially when selecting for traits that are limited to one sex or traits that appear later in life (i.e. fertility) (Eggen, 2014). While the use of genomic selection is increasing one of the challenges is the need for a large reference population to accurately be able to estimate GEBVs, as well as the costs that comes with the genotyping (Goddard & Hayes, 2009).

In horses the use of genomic selection is not very common, although it has been demonstrated to potentially reduce the generation interval in horse breeding programs (Haberland *et al*., 2012). As the accuracies of breeding values do not increase significantly until the offspring of a horse starts to compete, introducing genomic breeding values would improve the accuracies of the breeding values for young horses, especially for horses that do not have any competition data or offspring (Haberland *et al*., 2012). This will enable selection of horses at an earlier age, reducing the generation interval and potentially increasing the genetic progress of the breed (Haberland *et al*., 2012).

1.4 Monitoring inbreeding level

An intensive selection for a specific trait, especially in combination with a small population size, may increase the level of inbreeding. Inbreeding (i.e. breeding of related individuals) can increase the risk of inheriting rare defects or diseases due to an increased homozygosity for rare recessive alleles. It may also lead to a loss of important genetic variants. Partly due to the heavy selection for performance, a significant increase in inbreeding level has been observed in several breeds, for example Thoroughbreds and Standardbreds (Árnason, 2011, Binns *et al*., 2011).

Traditionally the individual inbreeding level has been estimated using pedigree information (Boyce, 1983). However, as the genetic relationship level may differ from the pedigree relationship level, to accurately estimate the inbreeding level in an individual, the estimation needs to be done on a genetic level. There are several different molecular tools available that can be used to analyze and monitor the genetic inbreeding level (Bruford *et al*., 2017).

1.5 Trait definition, pleiotropy and inheritance patterns

Within genetics, the traits or phenotypes can be classified into two different groups: monogenic or polygenic, depending on whether they are affected by a single gene or a combination of many genes. Monogenic traits, also referred to

as Mendelian traits, are explained by one gene with very little, if any, environmental impact. Polygenic traits on the other hand, also called multifactorial or complex traits, are influenced by a combination of several genes and environmental factors. As a result, polygenic traits are generally more difficult to study genetically compared to monogenic traits (Glazier *et al*., 2002). Different traits can also be classified as qualitative and quantitative. A qualitative trait is categorized into discrete groups, for example blood groups (A, B, AB, 0). Quantitative traits show a continuous variation, for example length measurements. A large number of QTLs (quantitative trait locus) have been identified, but revealing the causative mutation is more challenging (Andersson, 2009). Despite this, there are examples of studies where the causative mutation, or QTN (quantitative trait nucleotide), for a complex trait has been identified (Grisart *et al*., 2002, Van Laere *et al*., 2003, Clop *et al*., 2006).

Some of the loci that have been identified in genetic studies show pleiotropic effects. This means that the same gene influences more than one phenotype. Some of the well-known pleiotropic associations are those between coat color and diseases (Reissmann & Ludwig, 2013). A few examples are the Silver coat color and the eye defect MCOA, the Grey coat color and melanoma, and the Overo coat color and Overo lethal white syndrome (OLWS) (Santschi *et al*., 1998, Pielberg *et al*., 2008, Andersson *et al*., 2011).

The inheritance patterns for different traits can be categorized into dominant or recessive and autosomal or X-linked. All organisms have two copies of each chromosome, one that it is inherited from the mother and one that is inherited from the father, and the chromosomes contain the individuals DNA (deoxyribonucleic acid). For a dominant inheritance pattern only one allele is required for the phenotype to be displayed. For a recessive inheritance pattern, two copies of the same allele are required for the phenotype to be displayed. If the inheritance pattern is autosomal or X-linked depends on whether the SNP is located on one of the autosomal chromosomes or on the X-chromosome. Many of the rare genetic diseases show a recessive inheritance pattern, which means that both parents need to be carriers of the genetic variant for the offspring to show the phenotype.

1.6 Locomotion pattern of the horse

The horse is an explosive and very athletic animal. For centuries humans have used horses for transportation and competitions. As a consequence, horses have long been selected for their locomotion pattern and gaits, and for many breeds the gaits are considered the most important features. All horses possess four different gaits: walk, trot, canter and gallop. These gaits are classified based on stride length, stride duration and speed (Barrey, 2013). In addition, a large number of breeds are also able to perform a variety of additional gaits, and

these breeds are referred to as “gaited” or sometimes “ambling” horse breeds. There are about 80 gaited breeds in the world and these breeds can perform many different, often breed-specific, gaits. The majority of the alternative gaits are classified as ambling gaits, and some breeds also have the ability to perform the lateral gait pace (Barrey, 2013).

1.6.1 Definition of the gaits

The gaits of a horse can be defined as a coordinated rhythmic movement of both the limbs and the body, which together produces forces for movement (Barrey, 1999). The definition of a stride is a full cycle of limb movement (Barrey, 1999). There is no clear beginning or end of a stride as it is a continuously repeated pattern. A complete limb cycle includes a stance phase and a swing phase (Barrey, 1999). The stance phase is when the limb is in contact with the ground and the swing phase is when the hoof is lifted up from the ground (Barrey, 1999).

All gaits can be divided into either symmetrical or asymmetrical depending on whether the limbs are considered to be used equivalently or if they are employed differently (i.e. if there is symmetry between the left and right sides or not) (Barrey, 1999, Robilliard *et al*., 2007, Barrey, 2013). The symmetrical gaits include walk, trot, tölt and pace, while canter and gallop are considered asymmetrical gaits (Barrey, 1999, Robilliard *et al*., 2007, Barrey, 2013). Another way of classifying gaits is to divide them into stepping/walking gaits and running gaits (Barrey, 2013). The difference between the stepping/walking gaits and the running gaits is that for walking gaits there is always at least one foot in contact with the ground. For the running gaits there is at least one suspension phase in each stride, when no foot is in contact with the ground (Barrey, 2013).

Walk is the slowest gait, it is a four-beat gait with no suspension and with a speed of approximately 1.2-1.8 meters per second (m/s), corresponding to 4.5-6.5 kilometers per hour (km/h) (Barrey, 2013). Other examples of walking gaits are tölt, paso, running walk and stepping pace, which are performed at speed varying between 3.4 and 5.3 m/s, equivalent to 12-19 km/h (Barrey, 2013). The running gaits can be divided into trot, pace, canter and gallop. The trot is a two-beat diagonal gait, with a speed of 2.8-14.2 m/s, or 10-50 km/h (Barrey, 2013). Pace is a lateral symmetric gait, where the horse moves the legs at the same side of the body at the same time. The speed of pace can vary between 9 and 16m/s, or 32-58 km/h (Barrey, 2013). The higher speed obtained in pace compared to trot is likely due to fewer coordination problems in the pace, as there is less problem of limb interference compared to the trot (Barrey, 2013). Canter and gallop are two descriptions of the same asymmetric gait, performed at different speeds. The canter is a slower three-beat gait, while the increase in speed makes the gait four-beat (Barrey, 2013). Gallop is the

fastest gait a horse can perform with a speed of up to 20 m/s, equivalent to 72 km/h (Barrey, 2013).

1.6.2 Assessment of locomotion pattern and gaits in horses

Gaits and locomotion pattern are considered very important traits for many breeds and the gaits are often evaluated at competitions and breeding shows. Assessment of locomotion pattern in horses is traditionally done by having one or more persons visually evaluating the horse and scoring the different gaits. Although some people are very good in evaluating horses, it is still a subjective evaluation. As such, the evaluation is influenced by a number of external factors, for example the experience of the person evaluating the horse and the environment where the evaluation takes place (Parkes *et al.*, 2009, Clayton & Schamhardt, 2013). Also, by judging a horse visually it can be difficult to observe small differences or changes in gait pattern, especially at higher speeds. Therefore the use of different objective measuring-techniques is becoming more common to overcome these obstacles and increase the accuracy of the locomotion pattern evaluation.

Traditionally, studies of locomotion pattern in horses are performed using two different approaches: kinetics and kinematics (Barrey, 1999). Kinetics is a concept that involves the study of forces (i.e. causes of motion). Kinematics, on the other hand, focuses on velocity and acceleration (i.e. motion changes). It includes measures of timing, distance and angles (Barrey, 1999, Clayton & Schamhardt, 2013). The first kinetic study that used sensors to measure the forces of the hooves in different gaits was performed in 1873. Even though the techniques have developed since then, we still today, almost 150 years later, use the same measurement principles of the gaits (Marey, 1873, Barrey, 1999). For kinematic studies a common approach is to film the horses in movement. Already in 1887 the first kinematic study was performed, by using chronophotography (Muybridge, 1887).

For locomotion analysis in horses there are a number of different techniques available. For kinetic studies one important concept is the ground reaction force (GRF) (Clayton & Schamhardt, 2013). The GRF is the force that is exerted back from the ground during the stance phase of a stride, when the hoof is put down on the ground. The magnitude of the GRF is the same as the force from the hoof, and the GRF is described by its magnitude, direction and point of application (Clayton & Schamhardt, 2013). The force from the hoof can be divided into three different force components that act in a vertical, longitudinal or transverse direction (Clayton & Schamhardt, 2013). The GRF can be measured using either a force plate or force shoes (Clayton & Schamhardt, 2013). Other instruments used in kinetic studies to measure the transmission of forces and accelerations through the body are strain transducers, ultrasonic transducers, accelerometers, gyroscopes and magnetometers attached to the segment (Clayton & Schamhardt, 2013).

For kinematic studies one common method used involves optical motion capture (Clayton & Schamhardt, 2013). It is based on markers that are attached to the skin or the bones of the horses, mainly on the legs and back, and then the horses are filmed (Barrey, 1999, Clayton & Schamhardt, 2013). There are three different types of marker-methods used: passive markers, active markers or marker-less methods (Clayton & Schamhardt, 2013). The passive markers are made of a reflective material that will reflect light, while the active markers are usually LED lights that are flashed in different patterns. The marker-less method uses a pattern of recognition software to track the area of interest (Clayton & Schamhardt, 2013). By using the optical motion capture it is possible to make animations and perform gait analysis. To perform this kind of studies multiple cameras are needed to record the activity, especially to get three-dimensional models (Clayton & Schamhardt, 2013). Therefore, these kinds of studies are mainly performed in laboratory environments (Clayton & Schamhardt, 2013).

For studies outside the laboratory the wireless sensor technique can be used. Small sensors are attached to different parts of the horse's body and with the help of accelerometers and transducers signals are transferred to a computer where the data can be analyzed. The first study that used a wire-less sensor system for gait analysis was performed in 1994 (Barrey *et al*., 1994). With the wireless sensor system it is possible to evaluate the gaits not only on a treadmill but also in the field on different ground surfaces (Clayton & Schamhardt, 2013). The treadmill is a very good and useful tool for gait analysis as it is possible to control both speed and the surrounding environment. However, studies have demonstrated differences in the stride kinematics on a treadmill compared to ground locomotion, and with the wire-less systems these differences can be accounted for (Barrey *et al*., 1993, Buchner *et al*., 1994). The results from sensor-based systems are comparable with the results from the video-based motion analysis systems and the systems can accurately capture most of the variation in head nod hip hike and back movement. Despite that it is important to be aware of the limitations that exists when it comes to accuracy, precision and repeatability (Keegan *et al*., 2004, 2011, Warner *et al*., 2009, Pfau *et al*., 2016a, 2016b).

Most gait analysis systems today are used to evaluate lameness in horses. Several studies have shown that the agreement between veterinarians visually examining mildly lame horses is low, and the agreement level is affected by the experience levels of the veterinarians (Keegan *et al*., 1998, 2010, Hammarberg *et al*., 2016). Also, the lameness score differed depending on which type of scoring system that was used (Hewetson *et al*., 2006). In addition, one study reported movement asymmetries also in horses perceived as free from lameness by their owners (Rhodin *et al*., 2017). As such, even though there are some limitations of the sensor systems used, the use of the

objective evaluation systems make it easier to detect the mechanical changes that occur in lame horses (Pfau *et al*., 2016a). In addition to lameness evaluation, the evaluation of locomotion pattern is useful also for prediction of performance in horses (Barrey, 1999). Studies have demonstrated that the pattern of locomotion influences performance in different disciplines and this information could for example be used to predict the potential performance ability in young horses (Deuel & Park, 1991, 1993, Barrey *et al*., 1995, Barrey & Galloux, 1997, Barrey, 1999).

1.7 The *DMRT3* mutation – a single base change with major impact on a complex trait

1.7.1 Identification of the "Gait Keeper" mutation in horses

Locomotion pattern and performance are complex traits influenced by both genes and environment. Therefore the findings from a genome-wide association study (GWAS) on pacing ability in Icelandic horses were quite amazing (Andersson *et al*., 2012). The study compared Icelandic horses that were four-gaited (walk, trot, canter, tölt) and five-gaited (walk, trot, canter, tölt, pace) using the 50K Equine SNP chip array. Tölt is a four-beat ambling gait while the pace is a two-beat lateral gait. The results revealed one significant SNP on equine chromosome 23 (Chr23:22,967,656). By sequencing additional horses with different gait pattern, the causative mutation was identified (Chr23:22,999,655). The mutation, a change from cytosine (C) to adenine (A), was located in the second exon of the *doublesex and mab-3 related transcription factor3* (*DMRT3*) gene (Andersson *et al*., 2012). It introduces a stop codon and results in a truncated protein lacking 174 amino acids (about 40%) of the normal protein (Andersson *et al*., 2012). While all gaited horse breeds analyzed were either homozygous mutant (AA) or heterozygous (CA), most of the horses considered to be non-gaited (i.e. with only walk, trot, canter and gallop) were homozygous for the wild-type allele (CC) (Andersson *et al*., 2012). Interestingly, the frequency of the homozygous mutant genotype (AA) was high in Standardbreds, a breed used for harness racing. This suggested that there was an effect of the gene also on the ability to trot in high speed. Further investigation of the association between the mutation and harness racing performance demonstrated a significant impact of the gene on both racing performance traits and trotting technique in Standardbreds (Andersson *et al*., 2012)

1.7.2 Characteristics of the *DMRT3* gene and its encoded protein

In addition to the effects on locomotion pattern in horses, the DMRT3 protein was found to be critical for development of a normal locomotor network in mice (Andersson *et al*., 2012). The *DMRT3* gene is a member of the doublesex and mab-3 related transcription factor gene family, which includes eight different *DMRT* genes (1-8) mainly involved in sexual development (Hong *et*

al., 2007). As a transcription factor, the DMRT3 protein is involved in regulating the transcription by binding to specific DMRT3 binding sites at regulatory DNA sequences (Latchman, 1997, Murphy *et al*., 2007). The *DMRT* genes all share a conserved cysteine-rich DNA binding motif called the DM domain, but except for that they show little sequence conservation (Raymond *et al*., 1998, Murphy *et al*., 2007, Kopp, 2012). The DM domain is a zinc-finger like DNA binding motif that interact mainly with the minor groove of the DNA (Zhu *et al.*, 2000, Murphy *et al*., 2007). The DMRT proteins can bind DNA as heterodimers, and sometimes the heterodimer binding is even more efficient than the binding of the homodimer (Murphy *et al*., 2007). In mice and chicken embryos the *DMRT3* gene is mainly expressed in forebrain, neural tube and nasal placode but it is also expressed in mice testes and ovaries (Smith *et al*., 2002, Kim *et al*., 2003). Like the other *DMRT* genes the *DMRT3* gene has been suggested to be involved in sexual development, and some male mice from a *DMRT3* knockout model demonstrated sexual development abnormalities (Kim *et al*., 2003). Interestingly, the null mice also displayed shorter lifespan due to dental problems, compared to the wild-type mice (Ahituv *et al*., 2007). Although there have been several studies that demonstrated where the *DMRT3* gene is expressed and how it influences locomotion pattern, which genes that are targeted by the DMRT3 protein is still unknown.

In mammals the *DMRT3* gene is expressed in the dI6 subdivision of spinal cord neurons. These are inhibitory neurons that cross the midline of the spinal cord, so called commissural neurons, which can synapse onto motor neurons (Andersson *et al*., 2012). The neurons are involved in neuronal circuits or networks referred to as central pattern generators (CPGs) (Kiehn, 2006, Goulding, 2009, Nishimaru & Kakizaki, 2009, Dyck *et al*., 2012, Vallstedt & Kullander, 2013). These networks are responsible for the output patterns required for movement, by controlling the timing and pattern of muscle contractions (Kiehn, 2006, Nishimaru & Kakizaki, 2009, Dyck *et al.*, 2012). There are five different classes of neurons present in the ventral spinal cord, including the dI6 neurons (Dyck *et al*., 2012), and those can further be divided into different subpopulations (Goulding, 2009). The activity of the CPGs in the spinal cord is regulated by locomotor commands that originate from neurons in the brainstem and midbrain, and the CPGs have the ability to function without any sensory inputs (Kiehn, 2006, Goulding *et al*., 2009). The CPGs are important for a large part of the motor neuron activity that is crucial for a number of different body functions (Nishimaru & Kakizaki, 2009).

Recent studies have demonstrated the importance of the *DMRT3* gene in locomotion pattern control (Andersson *et al*., 2012, Larhammar, 2014, Perry, 2016). Silencing of the gene created mice with impaired coordination of movements, alterations in gaits and difficulties coordinating left-right alternations (Andersson *et al*., 2012, Larhammar, 2014, Perry, 2016). In null

mice the output signal from the CPG was uncoordinated and had an irregular rhythm (Andersson *et al*., 2012). Also, the *DMRT3* knockout mice displayed major difficulties to run at high speed and they demonstrated significantly longer stride lengths compared to control mice (Andersson *et al*., 2012). Later studies further characterized the importance of the *DMRT3* gene for locomotion pattern and showed that the gene is critical for the development of dI6 neurons (Larhammar, 2014, Perry, 2016).

Although the size of the dI6 subset of neurons did not differ between wild-type and null mice, the null mice had fewer commissural neurons and they displayed an increase of *Wilm´s Tumor 1* (*WT1*) positive neurons that also belong to the dI6 interneurons. In addition, when *DMRT3* was deleted in the mice there was an increase in the expression of *DMRT1* (Andersson *et al*., 2012). By analyzing spinal cord tissue from horses, Andersson *et al*. (2012) demonstrated that *DMRT3* mRNA (messenger RNA) was present in both wild-type and mutant horses, in a similar pattern as in mice. The DNA binding capacity of the protein was not affected by the mutation but the interaction with other protein/s may be defective (Andersson *et al*., 2012).

1.7.3 The origin of the *DMRT3* mutation in horses

Recent research has focused on identifying when and where the *DMRT3* mutation first arose in horses (Wutke *et al*., 2016, Staiger *et al*., 2017). One study suggested, based on the *DMRT3* genotype findings in ancient DNA, that the origin of gaitedness goes back to approximately 2900-2850 years BP in the medieval England (Wutke *et al*., 2016). From the Brittish Isles the ambling horses would then have spread to Iceland, likely around the 900´s (Wutke *et al*., 2016). A later study on 69 different horse breeds suggested, based on the low diversity in sequences from mutant chromosomes, that it is likely that the mutant *DMRT3* chromosomes arise from a common ancestral sequence, no later than 10,000 years ago (Staiger *et al*., 2017). As such, the mutation probably occurred sometime around the time of the domestication of the horse, and then spread around the world (Staiger *et al*., 2017). The study also suggested that the *DMRT3* nonsense mutation is causal, as no other sequence polymorphisms encompassing the *DMRT3* gene showed stronger associations with the locomotion traits (Staiger *et al*., 2017). While the mutation is present in ancient horse DNA from as early as 2900 years BP, none of the wild-horse populations genotyped have been carriers of the mutation (Promerová *et al*., 2014, Staiger *et al*., 2017).

1.7.4 The frequency and the effect of the *DMRT3* mutation in different horse breeds

As demonstrated in several studies the frequency of the mutated A-allele is high in all of the gaited horse breeds (Andersson *et al*., 2012, Promerová *et al*., 2014). On the other hand, in breeds classified as non-gaited almost all horses were homozygous wild-type (CC) (Andersson *et al*., 2012, Promerová *et al*.,

2014). This suggests not only a favorable effect of the mutation on ambling and lateral gaits, but it also indicates a possible negative effect of the mutation on the basic gaits walk, trot and canter. Indications of the negative association between the *DMRT3* mutation and the basic gaits have been demonstrated in several different breeds (Andersson *et al*., 2012, Kristjansson *et al*., 2014, Jäderkvist *et al*., 2015, Jäderkvist Fegraeus *et al*., 2015).

1.7.4.1 Standardbreds

The Standardbred is the major horse breed used for harness racing in the world (Figure 1). It has been bred for racing, trotting or pacing, for many generations and they are found in many countries in the world, including The United States, Canada, France, Sweden, Norway, Finland, Italy and Spain (Thiruvenkadan *et al*., 2009). As demonstrated in the study by Andersson *et al*. (2012) the mutation in *DMRT3* affects the horses' ability to perform a clean, well-synchronized trot at high speed (Andersson *et al*., 2012). As a consequence, the large majority of Standardbreds are homozygous AA, and the frequency of the mutated allele appears to have been stable for at least 60 years (Andersson *et al*., 2012, Jäderkvist *et al*., 2014a, Promerová *et al*., 2014). While the American Standardbreds are fixed for the mutation, the frequency of the C-allele is significantly higher in the French trotter. It has even been suggested that the CA genotype is favorable for performance at older ages in this breed (Promerová *et al*., 2014, Ricard, 2015). The difference in genotype frequency observed between the French trotters and Standardbreds from other countries may be due to selection for slightly different attributes as there are differences in race types and regulations between the countries.

Figure 1. A Standardbred trotter. Photo: Robert Fegraeus

The *DMRT3* mutation allows the horses to race at high speed either in trot or pace, without transitioning in to gallop, which would be the natural gait in higher speeds in wild-type horses (Andersson *et al*., 2012). As a result, Standardbreds homozygous for the mutation performs significantly better at the racetrack compared to CA and CC horses, with higher earnings and faster times (Andersson *et al*., 2012, Jäderkvist *et al*., 2014a). In the United States Standardbreds are used for either pace or trotting races and the mutation is fixed in the American population (Promerová *et al*., 2014). Recent research has identified 7 SNPs that can be used to genetically differentiate a Standardbred trotter from a Standardbred pacer (McCoy *et al*., 2017). Many Standardbreds can also be difficult to use for riding, like show jumping or dressage, due to their difficulties at the canter. A study on Swedish Standardbreds demonstrated significant differences in riding ability traits between homozygous AA and heterozygous CA horses (Jäderkvist *et al*., 2015).

1.7.4.2 Coldblooded trotters and Finnhorses

The Swedish-Norwegian Coldblooded trotter is a breed originating from the North-Swedish horse and the Norwegian Döle horse (Figure 2) (Bohlin & Rönningen, 1975, Thiruvenkadan *et al*., 2009). While North-Swedish horses were mainly used for agricultural and forestry work, the Coldblooded trotters have been strictly selected for harness racing throughout the last century. This has created a breed with the appearance of a light draught horse but with a mentality more similar to Standardbreds. As the *DMRT3* mutation was shown to strongly influence performance in Standardbreds, the hypothesis has been that the same genotype would also be favorable in the Coldblooded trotters (Andersson *et al*., 2012). A study in 2014 demonstrated a significant favorable effect of the AA genotype on performance at 3 years of age, with significantly higher earnings, more wins and placings as well as faster race times, compared to CA and CC horses (Jäderkvist *et al*., 2014a). However, at older ages, there were few significant differences between the genotypes, and it was even suggested that the CA horses would earn more money (Jäderkvist *et al*., 2014a). Also, unlike in Standardbreds, no effects of the *DMRT3* mutation on riding ability traits were observed in the Coldblooded trotters (Jäderkvist *et al*., 2015). As the previous study on performance in Coldblooded trotters only included about 170 horses and the results suggested that the CA horses might earn more money at older ages, we have investigated the effect of the *DMRT3* mutation in a larger sample of raced and unraced Coldblooded trotters (Study I).

Figure 2. A Coldblooded trotter. Photo: Robert Fegraeus

Another, phenotypically similar but genetically different breed to the Swedish-Norwegian Coldblooded trotters is the Finnish Coldblooded trotter, the Finnhorse. There are four different types of Finnhorses: the trotting type, the riding type, a miniature type and a draught horse type (Figure 3). It is allowed to cross the different types, and the majority of the horses are used for harness racing (Hippos, 2017). To further understand the effects and the function of the *DMRT3* gene we investigated the frequency of the *DMRT3* mutation in a cohort of Finnhorses either used for harness racing or traditional riding (Study II).

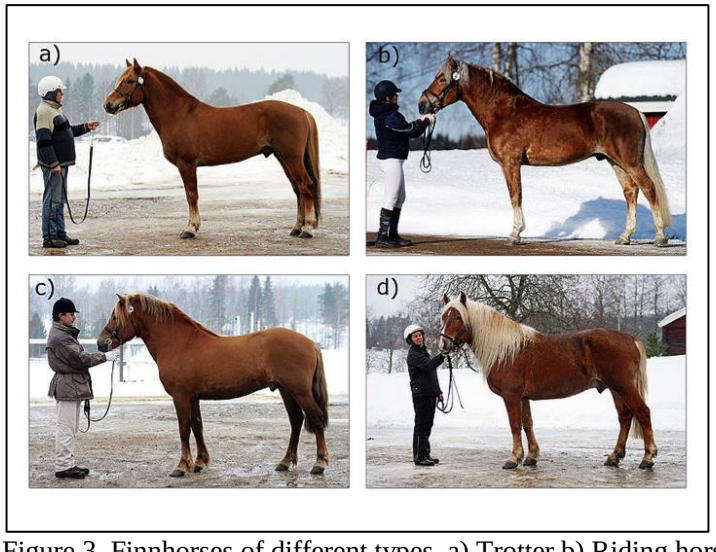

Figure 3. Finnhorses of different types. a) Trotter b) Riding horse c) Miniature d) Draught horse. Photo: Johanna Rautio/Wikimedia Commons

1.7.4.3 Icelandic horses

The Icelandic horse is the only horse breed present in Iceland (Figure 4). Most Icelandic horses are either four-gaited (walk, trot, canter and tölt) or five-gaited (walk, trot, canter, tölt and pace). While the five-gaited horses exclusively are homozygous (AA) for the nonsense mutation in *DMRT3*, most four-gaited horses are heterozygous CA or homozygous CC (Andersson *et al*., 2012, Kristjansson *et al*., 2014, Jäderkvist *et al*., 2015). In concordance with studies on other breeds the *DMRT3* mutation appears to not only influence gaiting ability, but also the quality of the gaits in Icelandic horses. Recent studies have demonstrated favorable effects of the AA genotype on breeding evaluation scores for tölt. However, the CA genotype was favorable for the evaluation scores for the basic gaits walk, trot and canter (Andersson *et al*., 2012, Kristjansson *et al*., 2014).

Figure 4. An Icelandic horse in tölt. Photo: Wikimedia Commons

Despite the strong association between *DMRT3* genotype and number of gaits in Icelandic horses, the *DMRT3* gene does not provide a complete explanation for the phenotypic variation observed. While the mutation undoubtedly is important for the ability to pace, not all AA horses pace (Andersson *et al*., 2012, Kristjansson *et al*., 2014, Jäderkvist *et al*., 2015). As for all other complex traits the environment plays an important role, for example training of the horses, but it is also highly likely that other genes have an influence. Therefore, in this thesis we have investigated the genetic differences between four- and five-gaited Icelandic horses homozygous for the *DMRT3* mutation (AA) (Study III).

The gait tölt is the most important trait in Icelandic horse breeding evaluation (FEIF, 2017) and it is highly valued by both breeders and owners. Five-gaited

horses have been favored in breeding, which is reflected by the increase of the A-allele in the population during the last 30 years (Kristjansson *et al*., 2014). As previously reported, the *DMRT3* gene influences the quality of Icelandic horse gaits and one study also showed an effect of the mutation on the ability to learn how to tölt. In addition, the *DMRT3* gene appears to influence which gaits the horses chose on pasture and under saddle (Jäderkvist *et al*., 2015). The CC Icelandic horses were significantly more difficult to teach to tölt, and there are several examples of CC Icelandic horses that are classified as three-gaited (i.e. they lack the ability to perform any kind of ambling gaits). Although three-gaited Icelandic horses exist in the population they are not very common, and the fact that some CC Icelandic horses are able to tölt is noteworthy. Especially since very few CC horses of other breeds are able to perform any alternative gaits (Andersson *et al*., 2012, Promerová *et al*., 2014). On the other hand, the lack of alternative gaits in CC horses of other breeds may also be due to lack of training for the particular gaits. The ability of some CC Icelandic horses to perform tölt is likely due to other genes under selection that favors lateral gaits, as the Icelandic horses have been bred for tölt for thousands of years. Also, intensive training for gait performance increases the likelihood that the horse will be able to perform the gait.

1.7.4.4 Other gaited horse breeds

Since the discovery of the mutation in 2012 a number of studies have investigated the frequency and the effect of the *DMRT3* mutation in different breeds (Andersson *et al*., 2012). Many of the gaited breeds are fixed for the mutation, but there are a number of breeds where the mutation segregates and thus it is possible to investigate the effect of the mutation on the variation in gait pattern. The association of the *DMRT3* mutation with gaiting ability has been demonstrated in breeds such as the Mangalarga Marchador, American Saddlebreds, Tennessee Walkers, American Curly and Morgan horses as well as a number of Chinese horse breeds (Jäderkvist *et al*., 2014b, Han *et al*., 2015, Patterson *et al*., 2015, Regatieri *et al*., 2016, Staiger *et al*., 2016). The studies demonstrated that while there is no doubt that the *DMRT3* mutation is important for gaiting ability, there are clearly other genetic factors influencing the gaits (Jäderkvist *et al*., 2014b, Han *et al*., 2015, Patterson *et al*., 2015, Regatieri *et al*., 2016, Staiger *et al*., 2016, Fonseca *et al*., 2017).

1.8 Other known genes influencing performance in horses

1.8.1 Performance genes

In addition to *DMRT3* there are other genes known to influence performance in horses. Many studies on performance have focused on racing breeds, and several candidate genes have been identified. These include the *creatine kinase, muscle* (*CKM*), the *cytochrome c oxidase, subunit 4, isoform 2* (*COX4I2*), the *sarcomeric α-actinin 3* (*ACTN3*), *Myostatin* (*MSTN*) and the

Pyruvate dehydrogenase kinase, isozyme 4 (*PDK4*) genes (Gu *et al*., 2010, Hill *et al*., 2010a, 2010b, Thomas *et al*., 2014). A large number of candidate genes important for performance also exist in humans (Schröder *et al*., 2011). Although potential performance genes have already been identified in horses, they do not explain all of the phenotypic variation observed. Performance is a complex trait influenced by a combination of genes and environmental factors. Therefore, in this thesis we have performed a genome scan of Standardbreds, Coldblooded trotters and North-Swedish draught horses with the aim to identify novel genes important for harness racing performance (Study IV).

1.8.2 The influence of *MSTN* on performance

One of the performance genes that has been thoroughly studied in horses during the last few years, particularly in Thoroughbreds, is the *MSTN* gene (Binns *et al*., 2010, Dall´Olio *et al*., 2010, 2014a, 2014b, Hill *et al*., 2010a, 2010c, Tozaki *et al*., 2010, 2011a, 2011b, McGivney *et al*., 2012, Petersen *et al*., 2014, Velie *et al*., 2015, François *et al*., 2016). *MSTN* is located on the equine chromosome 18 (ECA18) and the protein is a member of the transforming growth factor β family. The gene is expressed in skeletal muscle, and it is involved in the regulation of skeletal muscle growth (McPherron *et al*., 1997). In horses the gene has been associated with variations in body composition and conformation as well as competition performance and best racing distance in Thoroughbreds (Binns *et al*., 2010, Dall´Olio *et al*., 2010, 2014b, Hill *et al*., 2010a, Tozaki *et al*., 2010, 2011a, 2011b, Santagostino *et al*., 2015, François *et al*., 2016). A number of genetic variants have been reported in the horse *MSTN*, although none with such dramatic phenotypic effects as observed for knockout mutations in other species (Grobet *et al*., 1997, McPherron & Lee, 1997, Mosher *et al*., 2007).

Previous studies on best racing distance and performance in Thoroughbreds have reported a strong association with a SNP (C>T) in one of the introns of *MSTN* (Hill *et al*., 2010a, 2010c). However, more recent studies suggests that an insertion of an Equine Repetitive Element (ERE1) retrotransposon in the promotor region of *MSTN* is the genetic variant that is targeted by the selection for short-distance racing (Petersen *et al*., 2014, Santagostino *et al*., 2015). This insertion is in LD with the previously reported SNP, and it influences the gene expression of *MSTN* (Hill *et al*., 2010a, 2010c, Santagostino *et al*., 2015). The insertion is associated with an increased proportion of type 2B muscle fibers and the frequency is high in horses used for short-distance races (Petersen *et al*., 2013, 2014, Santagostino *et al*., 2015).

1.8.3 Epistatic regulation and breed specific gene effects

As demonstrated in several studies, the gaiting phenotypes differ between breeds and not all horses with the *DMRT3* mutation perform alternative gaits. This is partly due to environmental factors but it also strongly indicates an epistatic gene regulation. Epistatic gene regulation, or epistasis, is a

phenomenon where one gene influences the expression of other genes (i.e. gene interactions) (Phillips, 2008). This is for example the case with coat color in animals, where one gene variant may inhibit the activation/phenotypic effect of other genes, creating different coat colors or pattern (Phillips, 2008). Also, the effect of the genes may vary between different breeds, either due to genetic background or epistasis.

1.9 Genetic methods to study gaits and performance in horses

To better understand which genetic factors influence locomotion pattern and athletic performance in horses we have a wide variety of genetic tools to use. Depending on whether a candidate region is known or not there are different approaches used. If there is a gene with known functions, one can focus the study on that specific gene in order to investigate potential associations with other similar traits. However, for many genes there is limited knowledge about the function, and another approach is therefore necessary. Consequently, for many studies, especially studies on complex traits, the initial step is to perform association studies using a large number of genetic markers spread over the whole genome. If an association is detected between the trait of interest and any of the markers, this is usually followed up and verified in additional samples with the aim to identify the causative mutation. Once the mutation is known, functional studies are performed to determine how the mutation influences the phenotype of interest.

1.9.1 Genome-wide association studies (GWAS)

Genome-wide association studies were implemented in the beginning of the 21st century as a way to identify genetic risk factors for different traits (Bush & Moore, 2012). To perform a GWAS two things are required: a sample set with genetic variation and a variable phenotype. The genetic variation is due to mutations in the form of SNPs, insertions, deletions or inversions of one or several nucleotides. For GWA studies SNPs are traditionally used. A SNP is the specific nucleotide position in the genome, where individuals or chromosomes differ from each other (i.e. where there are different nucleotides present in the DNA sequence). To be classified as a SNP the frequency of the minor allele should be higher than 1% (Brookes, 1999). SNPs are abundant in the genome and there are more than 23 million SNPs in the horse genome (Schaefer *et al.*, 2017).

The basic concept of GWAS relies on the assumption that SNPs are in LD with SNPs affecting disease or other phenotypes (Bush & Moore, 2012). LD simply means that there is a non-random association of alleles at two or more different loci (Slatkin, 2008). Recombination events that occur during meiosis break down LD and the LD between SNPs can be affected by selection, gene flow, genetic drift as well as mutations (Slatkin, 2008). When performing a GWAS all samples are genotyped for a large number of SNPs. The exact number of

SNPs used for GWAS studies varies between species but in horse there are SNP arrays available for 50,000 up to 2,000,000 SNPs (McCue *et al*., 2012, Schaefer *et al*., 2017). All SNPs are analyzed simultaneously for association with the phenotype of interest, either case-control differences (categorical phenotypes) or a quantitative measure (Bush & Moore, 2012). It is usually preferred to analyze quantitative measures as this gives more power to the analysis and it increases the possibilities to find a significant association (Bush & Moore, 2012). As not all SNPs in the genome are analyzed, most GWAS do not reveal the actual causative mutation, but more often SNPs that are in LD with a causative mutation. The results of a GWAS will be influenced by a number of factors such as sample size, SNP density and effect size of the mutation. Another important factor that may influence the results and that can cause false positive associations, is population stratification. When a population is divided into different groups it is possible to find associations between the phenotype of interest and SNPs that do not have any linkage to a causative mutation. In a GWA comparing horses with and without a disease there is a chance that the horses that have the disease are related and therefore share a larger proportion of the genotypes. A quantile-quantile (QQ) plot can be used to visualize population stratification, by plotting the observed versus the expected *P*-values for the association analysis. If the distributions compared are identical the plot will follow a 45° line indicating that there are no significant associations present.

Also, when performing a large number of association tests it is important to correct for multiple-testing. For each association test performed the likelihood of obtaining a false positive association is 5 % (provided a *P*-value of <0.05). This can be corrected for using the bonferroni-correction where the *P*-value is divided by the number of association tests. An example is shown in study III where 670,000 SNPs were used and the *P*-value threshold was <0.05, yet the bonferroni corrected *P*-value was $0.05/670{,}000=7.4\text{x}10^{-8}$. Although often used in GWAS, one limitation of the bonferroni-correction is that it assumes that all tests are independent. In GWAS this is not usually the case as many markers will be in LD. As such, bonferroni is often considered too stringent for GWA analysis. Another way to correct for multiple testing is to use permutations. This method was used in the GWAS performed in study III in the current thesis. The permutation test is performed by randomly assigning the samples as cases or and controls and observing how often you get a *P*-value lower than the *P*-value observed is the association analysis. Most often a *P*-value less than 0.05 is considered significant, and the number of permutations required varies between from 10,000 up to 1,000,000 depending on the size of the expected *P*-value.

1.9.2 Identity-by-descent (IBD) mapping, single SNP genotyping and association analysis

A GWAS is usually the first step towards identifying novel genes with

influence on a specific phenotype. When a region has been identified as associated with a phenotype the next step is to follow up and narrow down the region in other horses. As all individuals in a population derive from common ancestors they will share some segments of their genome, so called identity-by-state (IBD) regions. During meiosis the IBD segments will be broken up during the recombination process, and for each generation the IBD segments will become shorter. IBD-mapping can be used to identify the causative mutation. In a case-control approach, if there is an IBD-region that is only present in the case group, it is likely that the region contains a causative mutation (Albrechtsen *et al*., 2008). IBD mapping can have a very high power even with few individuals, under the assumption that there is a shared causative mutation present in the cases (Albrechtsen *et al*., 2008).

When the mutation is known, it needs to be verified in other, independent individuals to make sure that the association holds up. This is usually done by genotyping the individuals for the SNP and performing a single-SNP association analysis. Single SNP testing can also be used for commercial testing. For example, if Mendelian SNPs for specific diseases have been identified it is possible to test animals before they are used for breeding, to verify whether or not they are carriers of a disease allele. There are several different techniques available for SNP genotyping, and they all, with a few exceptions, require the Polymerase Chain Reaction (PCR) step (Kim & Misra, 2007). PCR is a technique that was developed for amplification of an organisms DNA or RNA. In the current thesis single SNP genotyping was performed in study I, II and IV using the TaqMan Real Time PCR assays from Thermo Fisher (Livak, 1999). Other methods that have been developed to genotype single SNPs are dynamic allele-specific hybridization (DASH) (Howell *et al*., 1999), molecular beacons (Tyagi & Russell Kramer, 1996), tetra-primer ARMS PCR (Ye *et al*., 2001), restriction fragment length polymorphism (RFLP) (Botstein *et al*., 1980) and pyrosequencing (Nordström *et al*., 2000).

The association between a single SNP and a phenotype can be analyzed using different statistical methods. One method commonly used is linear models, which was used to analyze the data in study I. In study I the associations between *DMRT3* genotype and different harness racing performance traits were analyzed. All traits were analyzed for associations with the mutation as well as a number of so-called covariates. For example for the trait earnings the covariates used in the model included number of starts, sex and birthdate. The covariates were included in the model to account for the variation in performance that may be due to factors other than the SNP of interest. For instance, if all horses with a certain genotype are stallions, chances are that those horses will have better performance records than the other genotypes, although the differences are mainly due to the difference in sex and not genotype.

1.9.3 Selective sweep mapping and Fst-analysis

Selection in animals can be identified on a genetic level by so called selective sweeps in the genome. When there is selection for a specific allele in a population the variation for that SNP as well as the SNPs surrounding it will decrease, and eventually the alleles will become fixed. This will create regions in the genome with a high proportion of homozygosity (Maynard Smith & Haigh, 1974). These regions in the genome can be used to identify which parts of the genome are under selection in different breeds by performing a fixation index (Fst) analysis (Akey *et al.*, 2009, Petersen *et al.*, 2013, Ramey *et al.*, 2013). This was done in study IV in the current thesis where we compared the genomes of Coldblooded trotters, Standardbreds and the North-Swedish draught horses. Coldblooded trotters and North-Swedish draught horses share the same ancestor but the breeds have been selected for different purposes. While the North-Swedish draught horse is mainly used for heavy work, such as forestry and agricultural work, the Coldblooded trotters have developed into a pure racehorse breed.

The aim of study IV was to identify regions under selection for performance, by comparing the Coldblooded trotters and North-Swedish draught horses with the racehorse breed Standardbreds. We calculated a sliding window Fst across the three breeds. The fixation index is a measure of how different two populations are based on the genetic structure. Fst was calculated for each SNP according to Wrights definition: Fst= var(p)/(p(1-p)), where p is the average minor allele frequency for the two breeds compared (Brown, 1970). The Fst value for each SNP varies between 0 and 1, 0 meaning that the allele frequencies of the two breeds compared are exactly the same and 1 meaning that the two breeds are fixed for opposite alleles. The average Fst value between two breeds gives an indication of the genetic relationship between the two breeds. An average Fst value of 0 between two breeds indicates that they are the same breed while 1 means that the two breeds are completely unrelated.

In study IV in the current thesis the three breeds were divided into two sets, set A which included the Coldblooded trotters and the Standardbreds and set B which included all the Coldblooded trotters and the North-Swedish draught horses. The average Delta Fst was calculated from windows of five SNPs, by using $\Delta Fst = Fst[Set\ B] - Fst[Set\ A]$. The five top windows with the highest Delta Fst value, where the Fst for set A was low and the Fst for set B was high, were selected for further investigation. From each of the five regions the SNP with the highest single Fst value was selected for genotyping in additional horses.

1.9.4 Whole-genome sequencing

Whole-genome sequencing means that every single nucleotide in the genome is analyzed. This method produces large amounts of data for every individual. The first DNA sequencing was performed in the beginning of 1970 where a

small stretch of DNA was analyzed (Wu, 1970). Since then there has been a rapid development of new technologies and today it is possible to sequence the whole genome of any individuals, if the DNA analyzed is of acceptable quality. Sequencing analyses of ancient DNA (aDNA) is usually more complicated, due to degradation of the DNA (Nair, 2014). The first full genome to be sequenced was the genome of the bacteriophage φX174 in 1977 (Sanger *et al*., 1977a).

Before the introduction of whole-genome sequencing the most common sequencing method was the Sanger sequencing. The method was developed by Frederick Sanger and his colleagues in the end of the 1970s (Sanger & Coulson, 1975, Sanger *et al*., 1977b). The method is still used today, mainly for sequencing shorter pieces of DNA. However, for large scale DNA sequencing and whole genomes, the faster and more effective sequencing technology referred to as "Next-generation sequencing" (NGS) or "massively parallel sequencing" is used. The first commercially available platform for NGS analysis, GS 20, was developed in 2005 by 454 Life Sciences (Margulies *et al.*, 2005). Today there are a number of different sequencing technologies available on the market (Goodwin *et al*., 2016, Mardis, 2017). The NGS technology can be used for analyzing different types of both DNA and RNA. With the introduction of a third generation of sequencing techniques, which is currently under development, it becomes possible to sequence individual molecules. These new methods also provide significantly longer reads than the previous techniques used (Bleidorn, 2016).

Even though there are a number of different sequencing options and the cost for sequencing a genome has drastically decreased (Wetterstrand, 2015), it is still common to genotype individuals on SNP arrays instead, especially for studies that involve a large number of individuals. The main reasons for that are that even though sequencing provides more detailed information than SNP array it is still relatively expensive to whole-genome sequence many individuals. In addition, sequence data requires a large amount of storage and more computational analyses of the data.

1.9.4.1 Sequencing of the horse genome

A first draft of the horse genome sequence was completed in 2007 and shortly after a second version (EquCab2) was released (Wade *et al*., 2009). The horse sequenced was a Thoroughbred mare called Twilight. The size of the horse genome is about 2.7 Giga bases (Gb) and the predicted number of protein-coding genes from EquCab 2 was 20,322 (Wade *et al*., 2009). About 1.2 million SNPs were identified and a SNP map of more than one million markers was created using sequence data from seven horses of different breeds (Wade *et al*., 2009). Following the development of new technologies, especially in the field of NGS, a new updated version of the Equine genome (EquCab3) is now near completion (Kalbfleisch *et al*., 2017).

1.9.5 Functional genomics

When a genetic variant has been verified to be associated with a specific phenotype the next step is often to study the function of the variant. The focuses of these studies are usually gene transcription, translation, gene expression and protein-protein interactions. Functional experiments can be performed on DNA or RNA level by modifying the gene or genetic region of interest and observing the effects on the phenotype. These kinds of studies are commonly referred to as functional genomic studies and encompass all research that aims to define the functions of genes in the genome (Gibson & Muse, 2009). Although the function of a protein in one species may differ from another species, the characterization of a protein in one species often gives a good idea about the function in other species (Gibson & Muse, 2009). There are three main approaches to study functional genetics on gene level: forward genetics, reverse genetics and fine-structure genetics (Gibson & Muse, 2009).
The aim of the forward genetics approach is to identify which genes that affect a specific phenotype. This is done by inducing random mutations or looking at natural mutations in the genome of an organism, and then studying which phenotypes that are displayed. Based on the phenotype observed the work is focused on identifying the causative mutation (Gibson & Muse, 2009). The second approach, reverse genetics, has the DNA sequence as a starting point and aims to identify phenotypes that occur after disruption of one or several genes, so called knock-in or knock-out experiments (Gibson & Muse, 2009). The fine-structure genetics approach includes manipulation of the structure and regulation of specific genes, to characterize novel functions and interactions of the genes (Gibson & Muse, 2009).

1.9.6 Transgenic animals and genome editing

Using transgenic animals to study gene function is a common approach in today's genetic research. The phrase transgenic is used to describe the introduction of foreign DNA into the genome, including both the nuclear and the mitochondrial genome (Hickman-Davis & Davis, 2006, Pinkert, 2014). The first method to genetically modify animals was reported in the 1980´s (Wells, 2010). Since then a number of different species, including mouse, rat, rabbit, pigs, sheep, goats and cattle have been genetically modified (Wells, 2010).

There are different techniques used to create transgenic animals, including pronuclear injection, embryonic stem cell transfer, nuclear transfer, homologous recombination and RNA interference (RNAi) (Hickman-Davis & Davis, 2006, Pinkert, 2014). The basic procedure to create a transgenic mouse via embryonic stem cells includes the following steps: introduction of a transgene into stem cells derived from embryonic stem cell lines, injection of the modified stem cells into a host blastocyst and injection of the host blastocyst into a surrogate mother. The chimeric offspring can then reproduce and produce homozygous transgenic offspring (Gibson & Muse, 2009).

Genome editing is very similar to transgenic animals, with one major difference. While transgenic animals carry a foreign piece of DNA in their genome (Gibson & Muse, 2009), genome edited animals can be created by modifying, adding or deleting pieces of the individuals DNA. Genetic manipulation of animals is a useful tool in research. It can be used not only for studying gene function, but also to create animal models of human diseases, to improve resistance to disease and to treat inherited or spontaneous diseases (Wells, 2010). While the use of genome editing in larger animals such as pigs and cattle has so far been limited, it would be useful for human medical research and medicine, as for some traits these animals are more similar to humans compared to mice and rats (West & Gills, 2016). With the introduction of site-specific nucleases such as clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9, it is now possible to edit the genome with more precision, to avoid problems with poor regulation of gene expression and variability associated with random integration (West & Gill, 2016). By changing the expression of different genes and studying the phenotype, it is possible to obtain an increased knowledge about which functions certain genes have. The use of genomically modified animals is also a powerful method to study gene function in living animals.

1.10 Solving the puzzle

Working with genetics is like solving a puzzle. Many small pieces are put together to create a bigger picture. To solve the genetic puzzle we need to use a combination of different approaches and methods. In the ideal scenario we start by testing for associations between a phenotype of interest and a large number of genotypes. We then genotype additional animals to verify the associations and we perform sequence analysis to narrow down the number of associated genetic variants. As a last step we perform functional studies to understand how the identified mutation/s influences the phenotype. Although it is important to remember that most studies do not fall into this category of ideal scenarios, all results obtained will add small but important pieces to the bigger puzzle. This will get us closer to solving the large and very complex picture of genetic regulation of different phenotypes.

2 Aims of the thesis

This PhD project was divided into two parts. The overall aim of the first part was to present an investigation of the effect of the *DMRT3* mutation in two different harness racing breeds. The overall aim of the second part was to identify novel genes influencing gaits and racing performance in horses.

Specific aims of the thesis:

- Investigate the importance of the *DMRT3* mutation for early career performance in Coldblooded trotters
- Determine the effect of the *DMRT3* mutation on harness racing performance at different ages in a large sample of raced and unraced Coldblooded trotters
- Investigate the effect of the *DMRT3* mutation on racing performance and riding ability traits in Finnhorses
- Compare four- and five-gaited Icelandic horses with the same *DMRT3* genotype to identify novel genes influencing pacing ability
- Compare the genomes of Standardbreds, Coldblooded trotters and North-Swedish draught horses to identify novel genes important for harness racing performance

3 Summary of studies (I-IV)

This thesis comprises four studies (I-IV) with the aim to investigate the genetic regulation of horse performance and locomotion pattern. Studies I-II were performed to evaluate the effect of the *DMRT3* gene on harness racing performance in Coldblooded trotters and Finnhorses. In the third study (III) we compared four- and five-gaited Icelandic horses with the same *DMRT3* genotype in order to identify novel genetic factors influencing pacing ability. The last study (IV) compared the genomes of Coldblooded trotters, North-Swedish draught horses and Standardbreds, with the aim of identifying novel genes important for harness racing performance.

3.1 Study I – Lack of significant associations with early career performance suggest no link between the *DMRT3* "Gait Keeper" mutation and precocity in Coldblooded trotters

To investigate the effect of the *DMRT3* mutation on harness racing performance in Swedish-Norwegian Coldblooded trotters, 770 raced (n=485) and unraced (n=285) horses were genotyped for the *DMRT3* SNP. The horses were born between the years 2000 and 2009. We analyzed racing performance data for the years 2003 to 2015. Three age intervals were defined, 3, 3-6, and 7-10 years of age, and included in the performance analyses. The performance traits analyzed in the study included number and frequency of wins and placings (1-3), earnings and race times, as well as how many times the horse had been disqualified for galloping. In the study we also compared the *DMRT3* genotype frequencies between raced and unraced horses.

3.1.1 Results and discussion

Most of the performance results at 3 years of age did not differ significantly between genotypes and there were no differences in how many of the horses that started to compete at that age. The average age for the first start did not differ between the genotypes. However, there was a significant difference in the frequency of the AA genotype between raced and unraced horses (Figure

5). Only 45 % of the AA horses raced compared to 68 % of the CA horses and 63 % of the CC horses.

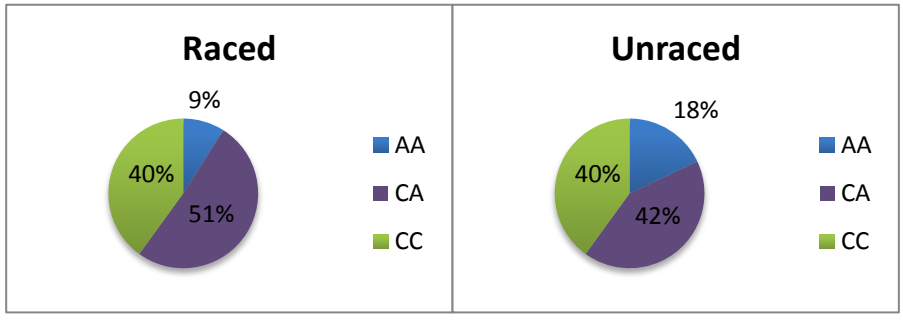

Figure 5. *DMRT3* genotype frequency in raced and unraced Coldblooded trotters

At older ages, the CA horses performed significantly better than the CC horses for almost all traits but there were no significant differences between CA and AA horses for the ages 3 to 6 years (Table 1). Despite the higher number of placings for the AA horses compared to the CC horses, they earned less money (Table 1). Interestingly, for the age interval 7 to 10 years there was a significant difference in number of starts between the genotypes, with AA horses starting less often. This suggests that the AA horses end their career earlier than horses with the other genotypes. Also, the AA horses that did compete at those ages had the lowest number of placings and earnings. The findings suggest that the AA genotype is not as advantageous for performance in Coldblooded trotters as it is for Standardbreds and Finnhorses, especially since the ability to start racing is one of the most important traits for a successful racehorse.

For all age intervals studied, the CC horses had the highest number of disqualifications. This finding is in concordance with a previous study on trotting technique in Coldblooded trotters where the CC horses had more problems to coordinate their trot, compared to CA and AA horses (Jäderkvist *et al.*, 2014). The low starting frequency for the AA horses may also be influenced by trotting technique, as a previous study reported an increased preference for the unwanted gait pace in AA Coldblooded trotters (Jäderkvist *et al.*, 2014). Overall, the most desirable *DMRT3* genotype in Coldblooded trotters appears to be the heterozygous CA, which is different from the situation in both Standardbreds and Finnhorses (Andersson *et al.*, 2012, Jäderkvist *et al.*, 2014, Jäderkvist Fegraeus *et al.*, 2015). If the AA Coldblooded trotters are more precocious than the other genotypes, as was suggested in the previous study (Jäderkvist *et al.*, 2014), we would have expected a higher proportion of AA horses racing at 3 years of age as the desire for precocious Coldblooded trotters is strong. Although the starting frequency

for the AA horses was low, the AA horses that made it to the racetrack performed well up to six years of age, as indicated by high median values for earnings and victories. While successful at young ages, the AA horses did not perform as well for the older ages, where the median values for earnings and victories were the lowest of the three genotypes.

Overall the results demonstrate the importance of studying different breeds to fully understand the effects of specific genes. The study also provides valuable knowledge for the Coldblooded trotter industry on what effect the *DMRT3* gene has in the breed.

Table 1. Mean and median racing performance results for Coldblooded trotters at 3 to 6 years of age according to *DMRT3* genotype

	AA (n=26-41)			CA (n=149-243)			CC (n=99-188)			P-value[2]		
Trait[1]	**Mean**	**Median**	**SE**	**Mean**	**Median**	**SE**	**Mean**	**Median**	**SE**	**AA/CA**	**CA/CC**	**AA/CC**
No.of starts	23.4	18.0	2.7	26.4	23.0	1.3	23.0	18.0	1.3	0.27	**0.002**	0.89
No of wins	2.9	2.0	0.5	3.3	2.0	0.3	2.6	1.0	10.3	0.95	**0.007**	0.11
Wins (freq.)	0.126	0.111	0.020	0.111	0.080	0.008	0.088	0.054	0.008	0.68	0.11	0.13
No of placings (1-3)	8.2	8.0	1.0	8.8	6.0	0.6	7.0	4.0	0.6	0.93	**<0.001**	**0.007**
Placings (freq.)	0.323	0.329	0.031	0.298	0.286	0.013	0.243	0.225	0.014	0.70	**0.005**	**0.03**
No of unplaced	11.0	7.0	1.5	12.0	11.0	0.6	11.0	9.0	0.6	0.31	**0.004**	0.93
Unplaced (freq.)	0.499	0.471	0.034	0.480	0.462	0.014	0.490	0.500	0.016	0.84	0.88	0.96
No of disqualifications	1.8	1.0	0.4	2.7	2.0	0.2	3.5	3.0	0.3	0.24	**0.04**	**0.007**
Disqualifications (freq.)	0.111	0.043	0.024	0.142	0.100	0.013	0.229	0.188	0.019	0.65	**<0.001**	**0.003**
Earnings (SEK)	128,100	98,500	20,625	179,300	72,500	18,543	133,200	54,370	17,154	0.72	**<0.001**	**0.03**
Earnings/start (SEK)	4,963	3,895	594	5,417	3,781	428	4,178	2,873	355	1.00	**0.007**	0.19
Race time auto (sec/km)	89.1	88.9	0.4	89.5	88.8	0.5	89.9	89.8	0.3	0.76	**0.002**	0.39
Race time volt (sec/km)	91.1	90.0	0.7	91.2	90.7	0.3	91.9	91.8	0.3	0.86	**0.005**	0.06

[1] Transformed values were used for the analysis: $\log_{10}$, ln(earnings + 1 000) and ln(race time - 68.2)

[2] A multiple comparison test was performed using Tukey´s HSD test. Significant results ($P \leq 0.05$) in bold

3.2 Study II – Different *DMRT3* genotypes are best adapted for harness racing and riding in Finnhorses

In Study II we investigated the effect of *DMRT3* genotype on harness racing performance and riding traits in Finnhorses. One-hundred and eighty Finnhorses used for harness racing and 59 Finnhorses used for riding were genotyped for the *DMRT3* mutation. The trotters were born between 1999 and 2010 and the riding horses were born between 1991 and 2011. All harness racing horses had competed at least once. The racing performance traits analyzed included number of starts, wins and placings, as well as earnings and race times. Associations between *DMRT3* genotype and the performance traits were analyzed for three age intervals: 3, 3-6 and 3-10 years of age. Data for the riding horses were collected via an owner-questionnaire, where the rhythm, balance and transitions for each gait were scored on a scale from 1 (poor) to 6 (perfect). The questionnaire also included additional questions about the gaits of the horse as well as competition experience.

3.2.1 Results and discussion

The frequency of the A-allele was significantly higher in Finnhorses used for harness racing compared to the horses used for riding (Table 2).

Table 2. Allele frequencies in trotting and riding Finnhorses

Finnhorse type	**n**	**A**	**C**	***P*-value**[1]
Harness racing	180	0.63	0.37	
Riding	59	0.43	0.57	
Total	239	0.58	0.42	<0.001

[1]Fisher´s exact test was performed in R

3.2.1.1 Harness racing performance

At 3 years of age there were only two genotypes present, CA and AA, and there were no significant differences in performance between the two groups. Only 25 out of 180 horses (14%) started their racing career at 3 years of age and most horses started to compete at 4 years of age. There were no differences observed between the genotypes for age at first start. For the older ages, 3 to 6 and 3 to 10 years of age, the AA horses performed significantly better than the C-horses, with more wins and placings, higher earnings and faster race times (Table 3).

Table 3. Average racing performance results for Finnhorses for the age period 3 to 6 years (standard errors in brackets)

Performance trait	**AA (n=41-52)**	**CA (n=60-83)**	**CC (n=13-19)**	**P-value[1]**
No. of starts	21.7 (1.8)	26.9 (1.9)	24.2 (3.3)	0.23
Wins (freq.)	0.234 (0.030)	0.116 (0.013)	0.061 (0.013)	**<0.001**
Placings (freq.)	0.464 (0.032)	0.324 (0.020)	0.217 (0.025)	**<0.001**
Earnings (euro)[2]	20,659 (4216)	11,411 (1695)	4,026 (1155)	**0.009**
Time rec. volt[2,3]	89.9 (0.6)	91.5 (0.6)	92.9 (0.8)	**0.005**
Time rec. auto[2,3]	88.4 (0.7)	89.2 (0.5)	91.9 (0.9)	**0.006**

[1]A Wald test was performed in PLINK, significant values in bold
[2]For these variables transformed values were used for the calculations: ln(earnings+100), ln(time record-68.2)
[3]Best average race time for 1 kilometer, in seconds

3.2.1.2 Riding performance traits

For the riding performance traits the CA and CC horses got significantly better scores than the AA horses for most of the traits analyzed. While the gait scores for the AA horses differed significantly from the C-horses, there was only one significant difference between CA and CC horses (rhythm in extended canter, *P*=0.05) (Table 4).

Table 4. Average gait scores for Finnhorses with different *DMRT3* genotypes

Trait[1]	**AA**	**CA**	**CC**	**P-value[2]**		
	n=13-14	**n=22-23**	**n=22**	**AA/CA**	**AA/CC**	**CA/CC**
Coll. canter						
Rhythm	2.50 (0.31)	4.57 (0.22)	4.23 (0.26)	**<0.001**	**<0.001**	0.32
Balance	2.71 (0.38)	4.30 (0.25)	4.14 (0.25)	**<0.001**	**0.003**	0.63
Transitions	2.57 (0.36)	4.04 (0.26)	3.82 (0.25)	**0.002**	**0.006**	0.53
Ext. canter						
Rhythm	2.71 (0.30)	4.52 (0.23)	3.82 (0.26)	**<0.001**	**0.01**	**0.05**
Balance	2.79 (0.37)	4.09 (0.27)	3.91 (0.26)	**0.007**	**0.02**	0.64
Transitions	2.64 (0.36)	3.87 (0.25)	3.64 (0.22)	**0.007**	**0.02**	0.50
Coll. trot						
Rhythm	2.38 (0.29)	4.34 (0.24)	4.41 (0.21)	**<0.001**	**<0.001**	0.83
Balance	2.38 (0.29)	4.43 (0.20)	4.55 (0.19)	**<0.001**	**<0.001**	0.68
Transitions	2.38 (0.27)	4.30 (0.20)	4.45 (0.18)	**<0.001**	**<0.001**	0.57
Ext. trot						
Rhythm	2.85 (0.32)	4.09 (0.24)	3.68 (0.26)	**0.003**	**0.05**	0.25
Balance	2.85 (0.41)	3.95 (0.25)	3.73 (0.24)	**0.02**	**0.05**	0.52
Transitions	2.77 (0.39)	3.95 (0.26)	3.64 (0.24)	**0.01**	0.06	0.38
Walk						
Rhythm	4.00 (0.30)	4.91 (0.21)	4.50(0.18)	**0.02**	0.14	0.15
Balance	4.08 (0.33)	4.95 (0.17)	4.68 (0.22)	**0.01**	0.12	0.33
Transitions	3.85 (0.34)	4.77 (0.19)	4.55 (0.23)	**0.01**	0.08	0.44
Jumping ability	3.54 (0.37)	4.13 (0.28)	4.32 (0.25)	0.22	0.08	0.62

[1] Scale from 1 (poor) to 6 (perfect)
[2] Pairwise comparisons were made using Student´s t-test, significant values are in bold

This study is a good example of how *DMRT3* influences different traits in the breed. As previously demonstrated the frequency of the CC genotype is high in breeds used for traditional riding, suggesting that the genotype is favorable for the ability to canter (Jäderkvist et al., 2015, Promerová *et al*., 2014, Jäderkvist *et al*., 2015). Even though the Finnhorses used for riding and harness racing are all considered to be the same breed, this study demonstrated a clear difference in genotype frequency between the two groups. While AA was the most favorable genotype for harness racing, riding horses with the AA genotype received poor evaluation scores for the riding ability traits. In the future these results may be used in the breeding process and the selection of horses for the different disciplines. Given the strong association between *DMRT3* and gaits and performance in Finnhorses the difference in genotype frequency between riding and trotting horses will likely be even bigger in the future than it is today.

It is interesting to note that even though Finnhorses and Coldblooded trotters are very similar phenotypically, the effect of the *DMRT3* mutation is clearly different. While the exact reasons for this are still unknown, one could speculate that it may be due to different training strategies or conformation differences. In studies I and II we observed a difference between the breeds for when the horses started their first race. While most Finnhorses start to race when they are four years old, most Coldblooded trotters start competing already at three years of age. This means that the Finnhorses have more time for training before they start to race, and this may have an influence on the risk for injuries. One of the theories for the low number of starts for AA Coldblooded trotters is that they are very talented and can run very fast early in life. This early speed training may increase the risk for injuries. If the Coldblooded horses started to compete one year later maybe the performance results would look different.

3.3 Study III - To pace or not to pace: a pilot study of four- and five-gaited Icelandic horses homozygous for the *DMRT3* "Gait Keeper" mutation

As previously mentioned, the *DMRT3* mutation appears to be required for pacing ability in horses. However, even though homozygozity for the mutation is required, only about 70% of the AA Icelandic horses are reported to pace (Andersson *et al*., 2012, Jäderkvist *et al*., 2015). Therefore in Study III we performed a genome-wide scan and compared four- and five-gaited Icelandic horses with the *DMRT3* genotype AA, to investigate whether there are other genetic factors involved in the regulation of pace. An owner questionnaire was used to determine the gait preferences of the horses. To obtain as many four-gaited AA horses as possible and avoid getting four-gaited CA horses, we advertised online for four-gaited horses with two five-gaited parents. The horses were selected based on the results from the questionnaire. All horses

were genotyped for the *DMRT3* mutation and any CA horses were excluded from the study. After genotyping of *DMRT3* 55 horses (20 four-gaited, 35 five-gaited) born between 1986 and 2010 remained and were included in the study. The horses were genotyped for 670,000 SNPs using the 670K Axiom Equine Genotyping Array. A genome-wide association (GWA) analysis of the gaits (four- or five-gaited, 0/1) was performed using a principal component approach ("egscore" function) (PCA).

3.3.1 Results and discussion

The genotyping failed in one individual and after quality control (QC) 54 individuals (19 four-gaited, 35 five-gaited) were analyzed for 356,741 SNPs. No SNPs demonstrated significant associations with the ability to pace (Figure 6). However, one region on chromosome 6 showed a suggestive association. There were two closely located SNPs that resulted in the lowest *P*-values (Table 5). These SNPs were located close to the glutamate ionotropic receptor NMDA type subunit 2B (Grin2B) gene (chr6. 41,227,875-41,626,797) (Wade *et al.*, 2009). As demonstrated in Figure 6 one SNP on chromosome 34 also showed a suggestive association with the trait. As horses only have 32 chromosomes pairs, in the GWA analysis chromosome 34 was classified as unknown chromosome.

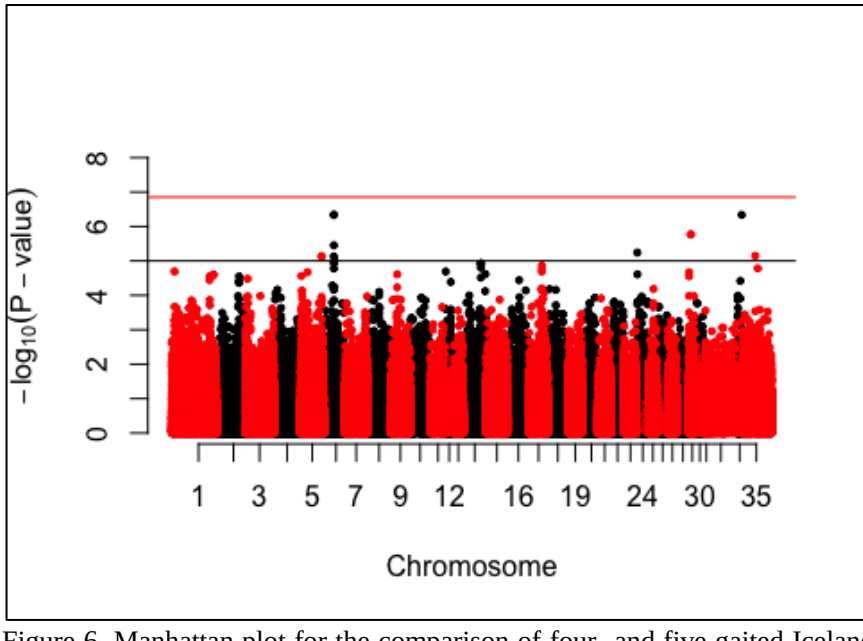

Figure 6. Manhattan plot for the comparison of four- and five gaited Icelandic horses homozygous for the *DMRT3* mutation (AA). The red line indicates the Bonferroni-corrected significance threshold ($P<1.4x10^{-7}$); the black line indicates the threshold for suggested genome-wide significance ($P<1x10^{-5}$)

Table 5. Top 10 SNPs from the GWAS analysis

SNP Name	Chromosome	Position	*P*-value unadjusted	*P*-value adjusted (genome-wide)*
AX-104875752	6	41,206,762	$4.6x10^{-7}$	0.29
AX-104542743	6	41,218,272	$4.6x10^{-7}$	0.29
AX-103922132	und	17,547,779	$4.6x10^{-7}$	0.29
AX-103516804	29	1,112,0750	$1.7x10^{-6}$	0.68
AX-103497403	29	11,151,705	$1.7x10^{-6}$	0.68
AX-104844167	6	41,292,483	$3.5x10^{-6}$	0.86
AX-104841069	24	2,138,716	$5.7x10^{-6}$	0.93
AX-104349116	X	55,026,040	$7.2x10^{-6}$	0.96
AX-103477519	5	89,752,224	$7.4x10^{-6}$	0.96
AX-104225161	5	89,794,839	$7.4x10^{-6}$	0.96

*After 10,000 permutations

Although limited by the small sample material, the findings from Study III may warrant further investigation, as the Grin2b gene is involved in neural regulations in mice and humans and the gene is considered to be an important factor for learning and memory (Tang *et al*.,1999, Zamanillo *et al*., 1999). In addition to the GWA analysis we also calculated the chip heritability for pace (i.e. how much of the variation observed is explained by all the genotyped SNPs combined). The heritability for pace previously reported is 0.6-0.7 (Albertsdóttir *et al*., 2011). The chip heritability estimate in the current study was considerably lower (0.18) and this supports the theory that most of the variation in pace is explained by *DMRT3*. However, it also provides evidence that there are other genetic factors, in addition to *DMRT3*, that determines whether a horse can pace or not. Future studies will benefit from including not only horses that have never paced, but also horses that are more difficult to get to pace and that prefer other gaits. Likely, the genetic regulation of pace in addition to *DMRT3* is complex and involves factors such as conformation and willingness of the horse.

3.4 Study IV – Selective sweep mapping using a unique Nordic horse model revealed *EDN3* as a candidate gene for harness racing performance

With the aim to identify novel genes important for harness racing in study IV we performed an introgression study. Here we utilized the close relationship between the Coldblooded trotter and the North-Swedish draught horse and the fact that Coldblooded trotters have long been selected for harness racing performance (Bohlin & Rönningen, 1975). In addition, it is well known that before parentage testing was introduced in the breed in 1969, Coldblooded trotters were crossbred with Standardbreds to improve the racing performance. Study IV includes a Delta-Fst analysis, combined with a performance

association analysis in 400 Coldblooded trotters to identify regions that have been selected for performance.

For the first part of the study we obtained blood samples from 11 Coldblooded trotters, 19 North-Swedish draught horses and 12 Standardbreds, in total 42 horses. The draught horses were all approved breeding stallions and the trotters were elite-performing horses selected based on EBV and pedigree. DNA was extracted and the samples were genotyped on one of two different Illumina SNP50 Genotyping BeadChips. After merging the data it was divided into two sets to be used for the calculation of Fst between the breeds: Set A included all the Coldblooded trotters and Standardbreds and Set B included all the Coldblooded trotters and the North-Swedish draught horses.

The association between each SNP and harness racing performance was investigated in 400 Coldblooded trotters using linear models. If there were any significant associations between a SNP and performance we also performed haplotype analysis for that region. In addition to the performance association analyses we genotyped 1,634 horses of different breeds for the top SNP identified in the Delta-Fst analysis.

3.4.1 Results and discussion

The Delta Fst-analysis provided five regions on different chromosomes where the Standardbreds and Coldblooded trotters were genetically similar but together differed from the North-Swedish draught horse (Table 6). From the five SNPs tested for association with racing performance, only one appeared to have a significant impact on the performance traits analyzed (g.22: 45748491C>T), and the CC genotype seemed to be negatively associated with the majority of traits (Table 7). Also, four SNPs in high LD with the associated SNP (r^2 = 0.92-0.94) were significantly associated with racing performance.

Table 6. Five top-regions from the Delta Fst analysis

Chr.	SNP window (bp)	Delta Fst value (window of 5 SNPs)	Z-transformed Delta Fst	SNP for association analysis (bp)
22	45,748,491- 45,752,522	0.34	5.67	45,748,491
7	67,498,458-67,594,705	0.33	5.41	67,498,458
10	49,931,991-50,002,425	0.30	5.09	49,931,991
15	88,492,813-88,577,552	0.27	4.61	88,565,665
11	2,153,152-2,521,729	0.25	4.37	2,517,091

Table 7. Coldblooded trotter performance results for SNP g.22:45748491C>T

Genotype	**TT (n=106-173)**		**TC (n=112-167)**		**CC (n=15-38)**		***P*-values[2]**		
Performance trait[1]	**Mean**	**Median**	**Mean**	**Median**	**Mean**	**Median**	**TT vs TC**	**TT vs CC**	**TC vs CC**
No. of starts	37.6	29.0	37.7	26.0	23.1	18.0	0.94	**0.01**	**0.02**
No. of wins	4.1	2.0	4.1	2.0	1.9	1.0	0.72	**0.006**	**0.01**
No. of placings (1-3)	11.1	6.0	11.5	7.0	6.0	3.0	0.08	0.10	**0.007**
Wins (freq.)	0.10	0.07	0.09	0.07	0.06	0.02	0.28	0.84	0.39
Placings (freq.)	0.24	0.23	0.28	0.27	0.20	0.16	0.46	0.66	0.38
Earnings (SEK)[3]	235,900	81,000	249,300	102,200	97,280	42,290	0.12	0.27	**0.04**
Earnings/start (SEK) [3]	4,734	3,152	4,965	3,917	3,108	2,332	0.21	**0.02**	**0.003**
Time record voltstart (sec/km)	90.8	90.4	90.5	90.0	92.6	91.8	0.09	0.15	**0.01**
Time record autostart (sec/km)	88.8	88.6	88.8	88.6	90.2	90.4	0.95	0.31	0.33

[1)] Transformed values were used for the analysis: $\log_{10}$, ln(earnings + 1 000) and ln(race time - 68.2)

[2)] Linear model analyses were performed in R. Significant results ($P \leq 0.05$) in bold

[3)] SEK=Swedish Kronor

For the top-region on ECA 22 we also performed haplotype analysis using 7 SNPs, including the 5 significant SNPs observed in the single SNP association analysis. There were four haplotypes present in the population: TGTAAAG, GGTAAAA, TTCGGGA and GTCGGGG, with the SNP identified in the Delta-Fst analysis on position 3. The TGTAAAG haplotype was the most common (0.34) and it was nominated as the base haplotype. We observed a significant effect of the haplotype TTCGGGA on the number of starts and number of victories ($P \leq 0.05$). Apart from that, none of the haplotypes showed any significant associations with racing performance.

When genotyping additional breeds for the top SNP we observed a high frequency of the TT genotype in breeds considered to be high-performing, i.e. Standardbreds, Coldblooded trotters, Finnhorses, Thoroughbreds and Warmbloods. On the other hand, the frequency of the CC genotype was high in North-Swedish draught horses, Exmoor, Shetland ponies, Gotlandsruss and Icelandic horses (Table 8).

Table 8. Genotype frequencies for SNP g.22:45748491C>T in 14 different breeds

Breed	**TT**	**TC**	**CC**	**n**
American Curly	0.84	0.15	0.01	87
Ardennes	0.00	0.00	1.00	7
Thoroughbred	0.99	0.00	0.01	91
Finnhorse	0.37	0.47	0.16	157
Fjordhorse	0.00	0.00	1.00	20
Gotlandsruss	0.07	0.25	0.69	153
Icelandic horse	0.05	0.42	0.53	167
Swedish Warmblood	0.94	0.05	0.01	77
Coldblooded trotter	0.46	0.47	0.07	183
North-Swedish draught horse	0.02	0.15	0.83	53
Standardbred	0.73	0.25	0.02	250
Shetland- and minishetland	0.18	0.47	0.35	104
Exmoor	0.00	0.03	0.97	271
American Miniature	0.29	0.64	0.07	14

The top region identified on ECA 22 was located close to the gene *Endothelin3* (*EDN3*). The *EDN3* gene encodes for a ligand that binds to an endothelin receptor (Baynash *et al*., 1994, Hosoda *et al*., 1994). The gene is mostly known for its impact on the development of melanocytes and enteric neurons and no previous studies have reported any associations between mutations in the gene and performance (Baynash *et al*., 1994, Hosoda *et al*., 1994, Lee *et al*., 2003, Stanchina *et al*., 2006). However, there have been reports of associations between the *EDN3* gene and high blood pressure and cardiovascular disease risk in humans (Levy *et al*., 2009, International Consortium for Blood Pressure Genome-Wide Association Studies, 2011). This, and the fact that the gene is

involved in the regulation of vasopressin release from the hypothalamus, could be a possible explanation for the association observed in the current study (McKeever *et al*., 2002). Also, another type of endothelin, *EDN1* may be of importance for performance. One study observed an increase in the concentration of the EDN1 protein after exercise in horses, and another study suggested a possible association of *EDN1* and asthma in horses (Benamou *et al*., 1998, McKeever *et al*., 2002). Except for the region close to *EDN3* no other regions associated with performance were identified in the Delta Fst analysis and none of the already known performance genes were detected in the top regions (Binns *et al*., 2010, Gu *et al*., 2010, Hill *et al*., 2010a, Andersson *et al*., 2012, Thomas *et al*., 2014). The reasons for lack of significant associations may be that Delta Fst analysis detected genes important for other traits than performance, the small sample size used for the Fst estimation, low genetic variation for the SNPs analyzed or the low number of SNPs used in the analysis. Also, the Fst analysis was performed for windows of five SNPs and it is possible that single SNPs with a high Fst value were not discovered because the other SNPs in the same window had a low Fst value. In addition, it is also possible that some SNPs were not discovered because the allele frequencies differ between all three breeds. One example is the *DMRT3* nonsense mutation, where North-Swedish draught horses and Standardbreds were more or less fixed for the opposite alleles. However, the majority of the Coldblooded trotters were heterozygous. For that reason the gene was not discovered in the top regions in the Delta Fst analysis, although we know that the gene is important for harness racing performance. This may be the situation also for other SNPs.

4 Concluding remarks, future prospects and practical applications

In this thesis we have investigated the genetics behind locomotion pattern and performance in horses. We demonstrated a significant effect of the *DMRT3* mutation on harness racing performance in two different breeds and we also discovered novel potential candidate genes for locomotion pattern and harness racing performance. While the ability to pace appears to be mainly controlled by the *DMRT3* mutation, clearly other genes influence the trait. As demonstrated in this thesis the genetic background of pacing ability and harness racing performance is complex, with several genes involved in the regulation of these traits. Identification of a major gene for a complex trait, like *DMRT3*, is probably a rare event, as most traits are affected by a large number of genes with small effects. Mapping the genetic regulation of complex traits is far more challenging than for a monogenic trait, as there may be hundreds or even thousands of genes with small effects that control a complex trait. In addition, the environment will have an effect on the trait. Nevertheless, knowledge about the genetics of complex traits is still crucial for genetic applications in agriculture and human medicine.

The results from study I and II provided information about the *DMRT3* gene and the effect in different breeds. As there is a genetic test available for the *DMRT3* mutation the information and knowledge gained from these studies will be an aid in the selection process and breeding of these horses. Also, it is a useful tool for planning the training of the horses, as some horses may require more extensive training before they are ready to compete while others need to be more carefully trained to avoid injuries. In study III we identified a potential candidate gene for pacing ability in horses. Interestingly, the SNP was located close to a gene that is known to be an important factor for memory and learning ability. Possibly the ability to pace is influenced by a horse´s ability to learn new tasks. This finding would be interesting to follow up in additional

horses to confirm whether or not some horses have a genetic variant that makes it easier for them to learn new things. The study included four-gaited Icelandic horses homozygous mutant for *DMRT3* that had never showed any signs of pace and these horses were compared with five-gaited AA Icelandic horses that were natural pacers. Any four-gaited horse that had showed signs of pace, even if it was just one time, were excluded. As no SNPs reached genome-wide significance, in the future it would be interesting to also include horses that have shown pace, but that were more difficult to teach to pace, or horses that just showed pace a few times. One way could be to compare Icelandic horses that are homozygous AA and evaluated for breeding as four- respectively five-gaited. Horses with limited pacing ability are often not trained in pace as the training may impair the quality of the tölt. As such, some of the horses evaluated as four-gaited may have the ability to pace, but they are not trained for it. Including Icelandic horses with limited pacing ability in the analysis would also make it possible to increase the sample size and thereby increase the statistical power. It would also be interesting to study pacing ability in other breeds, for example the Coldblooded trotters. There are some AA Coldblooded trotters that have a lot of problems with pace, while other AA Coldblooded trotters only trot. Comparing these two groups would provide additional information about which genes control pacing ability.

In study IV we utilized SNP array data to identify selective sweeps for performance in Coldblooded trotters. To continue the search for new performance genes we have obtained pooled whole-genome sequence data from Coldblooded trotters, Standardbreds and North-Swedish draught horses. We will perform the same type of Delta Fst analysis in this data set to hopefully confirm the findings in study IV and perhaps also identify additional regions that may be of importance for performance. To follow up the associations found between the *EDN3* gene and racing performance we will perform functional experiments to investigate the role of this gene in horses. Our group is a member of the Functional Annotation of Animal Genomes (FAANG) consortium (www.faang.org). The aim of the FAANG project is to create an infrastructure that can be used to improve the fundamental understanding of biology and better understand the link between genotype and phenotype. Another aim of FAANG is to provide high quality functional annotation of the animal genomes. Through the FAANG project we will have access to various types of gene expression and epigenetic data that we can use in our studies, for example through the "adopt a tissue" initiative. With the help of equine researchers all over the world, a large number of horse tissues have been sequenced (RNA) to provide an atlas for the horse research community. By funding the sequencing of several tissues the research group will get access to the raw sequence files as soon as they are available. In addition, the funding will also assist in creating a better atlas of RNA sequences from various tissues to be used by the whole horse genome community.

The optimal racehorse should have a correct conformation, willingness to run fast, be resistant to diseases and injuries, it should mature early in life and it should be durable. For some of these traits a large fraction of the phenotype is controlled by genetics. As such, even though we know about a handful of genes that influence horse performance, there is much more left to discover. Therefore, our future studies will not focus as much on the already known performance genes, but more on the discovery of novel genes influencing performance in horses. This will be done using both selective sweep mapping and performing GWA analyses for performance traits in different breeds. Currently, GWA analyses are being conducted for performance traits in Coldblooded trotters and this project also includes an investigation of the genetic diversity of the breed. In addition, we aim to continue to work with the genetics of gaits and locomotion pattern in different breeds by for instance studying horses with the same *DMRT3* genotype but with different gait pattern. Further studies of the quality of the gaits would be interesting to perform, to understand more about the genetic regulation of locomotion pattern in mammals.

While the *DMRT3* gene clearly is important for the development of a normal locomotor network in mammals, the exact functions of the gene are still unknown. To better understand the role of the gene in the regulation of neuronal development it is necessary to study which genes that DMRT3 binds to and which genes that bind to and regulate the expression of *DMRT3*. Also, although the gene has major impacts on locomotion pattern and performance in horses, it is very important to remember that the gene is only one part of the big puzzle. It is easy to create misleading selection in a breed if the results from the research are not correctly interpreted. Therefore, it is important that the industry is provided with correct information from the researchers on what is found and how the results should be interpreted and used.

To summarize, this thesis has confirmed the importance of the *DMRT3* gene for locomotion pattern and harness racing performance and it also showed that many other factors are important for which gaits a horse can perform or how fast they can run on the racetrack. The search for novel genes will continue with the aim to add more knowledge to the big and complex genetic puzzle underlying racing performance and locomotion pattern.

5 Sammanfattning

På grund av sin atletiska natur och det stora antalet olika hästraser är hästen är en utmärkt genetisk modell för att studera rörelsemönster och gångarter hos däggdjur. Hos många hästraser är rörelser och förmågan att trava eller galoppera i högt tempo högt värderade egenskaper. Målet med denna avhandling var att få en bättre förståelse för genetiken som påverkar gångarter och prestation hos hästar. Avhandlingen består av fyra artiklar som behandlar fyra olika hästraser. I artikel I och II beskrivs effekten av en känd mutation i genen *doublesex and mab-3 related transcription factor3 (DMRT3)* på prestationsegenskaper hos svensk-norska kallblodstravare och Finnhästar. Tidigare studier har visat en stor effekt av genen på prestation hos varmblodstravare. Resultaten från studie I och II visade att även om genen är viktig för prestation hos både kallblodstravare och Finnhästar så skiljer sig den mest framgångsrika genotypen mellan raserna. Finnhästar som är homozygota för mutationen (AA) var mer framgångsrika på travbanan men hade en sämre galoppförmåga som ridhästar. För kallbloden så presterade CA hästarna bättre överlag, även om AA hästarna presterade bra som unghästar.

Tidigare studier har rapporterat att en häst måste vara homozygot för *DMRT3* mutationen för att kunna gå i passgång. Trots detta kan inte alla AA hästar gå i pass. För att försöka förstå mer om den genetiska regleringen av passgång hos hästar har vi i studie III jämfört genomet hos islandshästar som är homozygota (AA) för *DMRT3* mutationen men med eller utan förmåga att gå i passgång. Vi genomförde en genomisk associationsstudie och identifierade en potentiell kandidatregion som innehöll en gen som är känd för att påverka minne och inlärning hos andra arter.

I studie IV utnyttjade vi det nära släktskap som finns mellan den svensk-norska kallblodstravaren och den nordsvenska brukshästen för att identifiera nya gener som påverkar prestationsförmåga. De två raserna är genetiskt lika men har selekterats för olika egenskaper. Genom att jämföra genomet hos de två raserna med genomet hos varmblodstravare kunde vi identifiera fem topp-regioner där kallblodstravarna och varmblodstravarna var lika genetiskt men skiljde sig från den nordsvenska brukshästen. En av regionerna innehöll fem

enbaspolymorfier (SNPs) som alla var signifikant associerade med prestation hos kallblodstravare.

Sammanfattningsvis visar vår forskning att noga utvalda hästmaterial kan fungera som modeller för att få en djupare kunskap om genetiken bakom prestation och rörelsemönster och att det är nödvändigt att studera betydelsen av dessa gener hos olika hästraser.

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