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Faculty of Veterinary Medicine and Animal Science
Department of Animal Breeding and Genetics

Association of the *DMRT3* nonsense mutation with performance in Coldblooded trotters

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Association av *DMRT3* nonsens mutationen med prestation hos kallblodstravare

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1. Sammanfattning

I tidigare studier upptäcktes att en nonsens mutation i genen *DMRT3* har en stor påverkan på gångarter hos hästar, och möjliggör förmågan till alternativa gångarter, som pass och tölt. Mutationen i *DMRT3* har även påvisats ha en gynnsam effekt på hastighetskapaciteten och tävlingsprestation i travsport hos bland annat kallblods- och varmblodstravare. Syftet med den här studien var att undersöka associationen mellan mutationen i *DMRT3* genen och tävlingsprestation hos kallblodstravare. Totalt 769 kallblodstravare genotypades för *DMRT3* mutationen varav 485 hade startat minst en gång och 284 var icke-startade. Genotypen associerades med tävlingsprestation (antal starter, antal vinster, placering 1-3, prissumma, prissumma per start, oplacerade, och rekord tider). Prestationsegenskaperna definierades och erhölls vid olika åldrar: 3 års, 3 till 6 års, samt 7 till 10 års ålder. Vid tre års ålder tjänade hästarna som var homozygota för mutationen (AA) mer pengar än hästarna homozygota för vild typ allelen (C). Prestationsresultaten för den totala karriären, indikerade att genotyp CA hade bättre resultat jämfört med genotyp CC för majoriteten av egenskaperna ($P<0.05$). Hästar med genotyp CA tjänade även mest prispengar för den totala karriären ($P<0.05$). Den här studien tyder på att de hästarna heterozygota för *DMRT3* genen, har en fördelaktig association med tävlingsprestation. Resultaten från den här studien har en praktisk implikation inom avel och träning av kallblodstravare och kan hjälpa yrkesverksamma i att selektera de mest ideala hästarna för avel.

2. Abstract

In previous studies, a nonsense mutation in the gene *doublesex and mab-3 related transcription factor 3 (DMRT3)* was discovered to have a major influence on gait ability in horses. The mutation is permissive for the ability to perform alternate gaits, such as pace and tölt and has also been shown to have a favorable effect on speed capacity and harness racing performance in Coldblooded and Standardbred trotters. The main aim of this study was to investigate the association of the *DMRT3* genotype with performance in Coldblooded trotters. A total of 769 Coldblooded trotters were genotyped for the *DMRT3* mutation, where 485 had raced at least once and 284 were non-raced. The genotype was associated with harness racing performance (number of starts, victories, placings 1-3, earnings, earnings per start, unplaced, disqualifications, and race times). The performance traits were defined and obtained at different ages: 3 years, 3 to 6 years, and 7 to 10 years of age. At three years of age, the horses homozygous for the mutation (AA) earned more prize money compared to the horses homozygous for the wild-type allele (C). The performance results for the total career indicated that the genotype CA had better results for the majority of the traits, compared to horses homozygous for the wild-type allele ($P<0.05$). Horses with genotype CA also earned the most prize money for the total career ($P<0.05$). This study indicates that the heterozygotes at the *DMRT3* gene have a favorable association with harness racing performance. The results from this study have practical implications for breeding and training, in which the information from this study could assist industry professionals in selecting the most ideal horse for breeding Coldblooded trotters.

3. Introduction

3.1 Background

The domestic horse has for a long period of time been selected and bred in order to provide different breeds for racing. For instance, Arabian and Thoroughbreds are the two breeds mostly used for gallop racing. Another type of racing is harness racing, which is an important sporting event in countries such as Canada, Finland, Norway, Russia and Sweden (Thiruvenkadan et al. 2009). The natural gaits of a horse are, in order of increasing speed: walk, trot and gallop, or canter. Different breeds of horses show considerable variation in their pattern of locomotion and many breeds have been selected for the ability to perform alternate gaits such as tölt and pace (Andersson et al. 2012). In harness racing the horses run in a specified gait, usually trot or pace. Important horse breeds used in harness racing are Standardbreds, Finnhorses, and Norwegian and Swedish Coldblooded trotters (Thiruvenkadan et al. 2009). These breeds have been selected for the ability to trot or pace at high speed, where recent selection for speed in trot has resulted in increased racing speeds and a genetic improvement has been observed (Thiruvenkadan et al. 2009; Revold et al. 2010).

In Sweden, riding and horse racing are popular sports, where horse racing is dominated by harness racing (Thiruvenkadan et al. 2009). There are two distinct populations of trotters in Sweden: the Standardbred trotter and the Coldblooded trotter. The two breeds compete under similar conditions but in separate races (Thiruvenkadan et al. 2009; Revold et al. 2010). The Swedish Standardbred trotter is one of the most common horse breed used for harness racing. It is mainly developed from the American Standardbred, and solely bred for trotting performance (Revold et al. 2010; Andersson et al. 2012). The Coldblooded trotter is derived and evolved from the lighter of the two varieties of the North Swedish horse, which exists as both heavier and lighter types (Swedish Trotting Association, 2015a). Coldblooded trotters are tractable, robust horses and good trotters (Thiruvenkadan et al. 2009). The Coldblooded trotters constitute an important part of Swedish harness racing, and are highly appreciated for their energy, durability and reliability. Today, there are approximately 10 000 Coldblooded trotters in Sweden and the breed is mainly bred for use in harness racing (Swedish Trotting Association, 2015a). Swedish Coldblooded trotters are, together with the Norwegian and the Finnish trotters, the only Coldblooded trotters in the world. The overall breeding goal for the Coldblooded trotter is to preserve and further develop a sound, healthy, and competitive trotter through improvement of performance results, durability, conformation and temperament (Swedish Trotting Association, 2014a).

The horses should also function as sound breeding animals with good reproductive ability, but the inbreeding coefficient is relatively high in Coldblooded trotters (6% in 2007), mainly due to the use of an insufficient number of stallions in such a small population (Swedish Trotting Association, 2011 & 2014a). In 1998, a study by Klemetsdal demonstrated that inbreeding in Norwegian Coldblooded trotters impaired racing performance. Inbreeding does not only impacts performance negatively, but also eventually affects durability and fertility. When the number of individuals in a population is limited the risk of inbreeding depression increases (Revold et al. 2010). However, the number of stallions used for breeding has increased from 2014 to 2015, and the inbreeding coefficient is considered and closely monitored in the breeding program (Swedish Trotting Association, 2015c).

In 2012, an important discovery was the identification of a loss-of-function mutation in the gene *doublesex and mab-3 related transcription factor 3 (DMRT3*), which was shown to affect locomotion in horses (Andersson et al. 2012). The *DMRT3* mutation has shown to favorably affect performance in harness racing (Andersson et al. 2012; Jäderkvist et al. 2014; Jäderkvist et al 2015a). So far, studies on the influence of the *DMRT3* mutation on performance in the Coldblooded trotters are limited (Jäderkvist et al. 2014). Therefore, it is important to further investigate how racing performance is associated with the mutation in the *DMRT3* gene. Soundness and earned prize money are examples of important aspects of the industry of harness racing in Sweden, and the information from this study may be used to provide additional information when breeding for sound Coldblooded trotters and improved performance results.

3.2 Specific aims

The main aim of this study was to investigate the association of the *DMRT3* nonsense mutation with different performance traits in Coldblooded trotters.

4. *DMRT3* nonsense mutation

In 2012, Andersson et al. discovered a nonsense mutation in the *DMRT3* gene. A single base change from cytosine (C) to adenine (A) at nucleotide position 22,999,655 on chromosome 23 in horses causes a premature stop codon in the *DMRT3* gene. The *DMRT3* nonsense mutation in horses occurs in the last exon and the transcript is translated into a truncated protein. The premature stop codon in the *DMRT3* gene has been reported to have a major effect on the pattern of locomotion in horses, and is thus referred to as the 'Gait keeper' mutation (Andersson et al. 2012; Kristjansson et al. 2014). Andersson et al. (2012) also reported that Dmrt3 neurons have an important role in left/right coordination in mice and also in coordinating movement of the fore- and hindlegs. This was demonstrated using knock-out mice, in which mouse Dmrt3 is vital for normal development of a coordinated locomotor network in the spinal cord (Andersson et al. 2012).

In horses, the mutation in the *DMRT3* gene promotes the ability to perform alternate gaits, such as pace or four-beat ambling gaits (Andersson et al. 2012; Kristjansson et al. 2014; Jäderkvist et al. 2014). Horses without the nonsense mutation in *DMRT3* have the wild-type genotype (CC), and are usually only able to perform the natural gaits walk, trot and gallop/canter (Andersson et al. 2012). The Icelandic horse is an example of a multi-gaited breed, which is able to perform the additional gaits tölt and pace, in addition to walk, trot and gallop/canter. The mutation in the *DMRT3* gene was discovered in a genome-wide association study (GWAS), which showed that heterozygosity or homozygosity for the *DMRT3* mutation in Icelandic horses promotes tölt. For five-gaited Icelandic horses, homozygosity is required but not sufficient in order to have the additional ability of pacing (Andersson et al. 2012). The study by Andersson et al. (2012) showed that a complete loss of Dmrt3 on one allele in mice did not lead to any detectable phenotype, whereas heterozygosity for the *DMRT3* mutation in Icelandic horses promotes tölt, which indicates that the mutant protein acts in a dominant negative manner. However, it is still uncertain when and where the mutation arose (Promerová et al. 2014).

Furthermore, studies on the influence of *DMRT3* on classical riding traits in different breeds have been conducted. The studies indicated that homozygosity for AA was the least beneficial genotype due to difficulties with the gait trot and canter, and thus a likely selection against the mutated allele in *DMRT3* has occurred (Kristjansson et al. 2014; Jäderkvist et al. 2015a; Jäderkvist et al. 2015b). In a study by Promerová et al. (2014), 141 gaited and non-gaited breeds from all over the world was genotyped and it was found that the *DMRT3* mutation was present in various frequencies in all gaited breeds, whereas the non-gaited breeds were almost completely fixed for the wild-type gene. Exceptions were the breeds Standardbred and French trotters, used for harness racing, in which a high frequency of the *DMRT3* mutation was found. Most of the individuals from gaited breeds were homozygous AA, which is required for pacing. However, homozygosity for the mutation is not sufficient for pacing, since many Standardbred trotters (and some Icelandic horses) that are homozygous for the mutated allele do not pace (Andersson et al. 2012).

4.1 Harness racing performance associated with *DMRT3* in different breeds

In 2012, Andersson et al. reported a high frequency of the *DMRT3* mutation in Standardbred, and French trotters bred for harness racing. At high speeds, horse from such horse breeds have the ability to keep a symmetrical gait, such as trot or pace, without switching into gallop, which is the natural gait for horses at high speeds (Andersson et al. 2012). The American Standardbred seems to be fixed for the *DMRT3* mutation, whereas the Swedish Standardbred is nearly fixed (97%), possibly due to the import of French trotters, in which the frequency of the wild-type allele is fairly high (Andersson et al. 2012; Promerová et al. 2014). Andersson et al. (2012) showed that the mutated allele was associated with superior racing performance in Standardbred trotters, where horses homozygous for the mutation had significantly higher breeding values (BV) and increased earned prize money, compared to heterozygotes. In addition, similar results were shown in a study by Jäderkvist et al. (2014), in which Standardbred horses with genotype AA had superior BV for racing performance compared to CA horses. Standardbred trotters, which were homozygous AA, also had a higher percentage of victories, more earned money and the best time record, compared with the other genotypes for the age period of 3 to 5 years (Jäderkvist et al. 2014). Moreover, differences in trotting technique have been shown for the Standardbreds, in which horses with genotype CA had difficulties in sustaining a clean, even-beat trot at high speed (Andersson et al. 2012).

In a recent study of French trotters by Ricard (2015), the effect of the *DMRT3* gene was estimated by the effect at SNP *BIEC-620109*, which is in strong linkage disequilibrium (LD) with the mutation identified on *DMRT3* (r^2=0.91; Promerová et al. 2014). The results from the study by Ricard (2015) demonstrated that genotype CC at SNP *BIEC-620109* had negative effects on all traits associated with racing performance. It was also shown that during the whole career of the horses, the heterozygous horses achieved better results compared to horses homozygous for the mutated allele (AA) (Ricard, 2015).

In a study of the Finnish breed, Finnhorse, a significant difference in racing performance was shown, in which homozygotes for the mutated *DMRT3* allele (AA) had the highest proportion of victories and placings, they earned more prize money and had the best racing times. Additionally, the homozygous wild-type genotype CC was the least advantageous for trotting performance (Jäderkvist et al. 2015a).

4.2 Racing performance in Coldblooded trotters

Promerová et al. (2014) showed that the frequency of the mutated allele in *DMRT3* in Coldblooded trotters was 45%. However, only one study has examined the association of the *DMRT3* nonsense mutation with racing performance in these trotters. In one study by Jäderkvist et al. (2014) the Coldblooded trotters homozygous for AA had significantly higher BV, and at three years of age, the AA horses had the highest percentage of victories and placings, earned the most prize money and had the best racing times. Performance results remained significant for victories and placings for AA horses for the ages 3 to 6 years, but horses with the genotype CA were as good or even better regarding the other traits, such as earnings and racing times (Jäderkvist et al. 2014). The results suggested that homozygosity for the wild-type allele (CC) was associated with the poorest performance results, and the differences between CA and AA horses varied between the traits and were relatively small (Jäderkvist et al. 2014). However, the horses used in that study were not randomly selected and thus a larger study with randomly selected horses representing the population was desired. In addition, Jäderkvist et al. (2014) performed a blind study on trotting technique, in which a professional trainer was asked to group and judge horses on his race camp according to trotting capacity. This blind study was subsequently associated with the *DMRT3* gene. The results indicated that CC horses had difficulties in sustaining a clean, even-beat trot at high speed and instead tried to switch into gallop, while CA horses had a clean, even-beat trot and the majority of the AA horses went towards pace. In addition, the trainer in the study by Jäderkvist et al. (2014) graded the horses' trotting capacity, and the results showed a significantly better racing capacity for the AA and CA horses, compared to horses homozygous for the wild-type gene (Jäderkvist et al. 2014).

4.3 Factors affecting performance

Various factors affect the chances for a horse to become successful in harness racing. Environmental factors, such as diet, training, injuries, and random chance all contribute to the variation between individuals and influence performance. The conformation of a horse is also an important factor for long-lasting soundness in racing horses, (Dolvik & Klemetsdal, 1999; Kristjansson et al. 2014). Sound conformation and longevity are essential preconditions for a horse in harness racing to be able to compete several times in a year for many years (Thiruvenkadan et al. 2009). However, some, if not many, Coldblooded trotters used in harness racing have respiratory problems. A newly diagnosed upper respiratory tract disorder in Norwegian Coldblooded trotters, called dynamic laryngeal collapse, has been described. This disorder is caused by specific anatomical conditions in the throat region, and causes poor racing performance (Strand et al. 2009). In addition, Norwegian Coldblooded trotters seem to be predisposed to develop this disorder, compared to the Standardbred trotter (Strand et al. 2009).

The conformation of a horse is important for performance. Information about conformation can be used to pre-select sounder horses for racing and for breeding, and may also be used to aid in prediction of BV for diseases in the locomotory system (Dolvik & Klemetsdal, 1999). In Norwegian Coldblooded trotters, it was shown that conformation explained 10% of the variation observed in start status, and 12% of the variation in earnings at 3-5 years of age (Dolvik & Klemetsdal, 1999). In addition, genetic variants in several additional genes, or other gene(s) interacting with *DMRT3*, also influence racing performance (Andersson et al. 2012; Jädervist et al. 2014).

5. Material and methods

5.1 Horse material

Hair and blood samples were collected from a random sample of 1000 Swedish and Norwegian Coldblooded trotters, to accumulate approximately 500 raced horses (five 96-well plates), and approximately 300 horses that had never raced (three 96-well plates). The hair and blood samples were provided by the Animal Genetics Laboratory, Swedish University of Agricultural Sciences, Uppsala, Sweden, and by the Norwegian school of Veterinary Science in Norway. The Coldblooded trotters used in this study were born between 2000 and 2009. The distribution of year of birth for the horses that had raced at least once, and for horses that had never entered a race (non-raced horses), is presented in Table 1. The 500 raced and the 300 non-raced horses were used to ensure that the random sample accurately reflected the proportion of raced and non-raced horses in the population. The reason for the lower number of horses born in the year 2000, compared to the number of horses born 2001-2009, is because until the year 2000, the parentage control of Coldblooded trotters was done by DNA-typing from blood samples, and the DNA isolation from hair roots started in 2001 (Swedish University of Agricultural Sciences, 2016). Most of the blood samples from the year 2000 were not available.

The majority of the horses (n=485) had competed at least once and the samples were used for the analysis of racing performance traits. The other horses in the sample (n=284) were horses that never raced. The total sample consisted of 89 intact males, 347 mares, 332 geldings and one cryptorchid individual. The sample of raced Coldblooded trotters represented progeny from 111 sires with an average of 4.4 offspring per sire, where the number of offspring per sire ranged from 1-52. The non-raced horses consisted of progeny from 90 sires with an average of 3 offspring per sire, where offspring per sire ranged from 1-20. The whole sample of raced and non-raced horses (n=769) consisted of progeny from 128 sires with an average of 6 offspring per sire, where offspring per sire ranged from 1-72.

Table 1. Distribution of birth year and years of performance data for the sample of raced and non-raced Coldblooded trotters

Birth year	Total	Raced horses						Non-raced horses				
		Swedish	% [a]	Norwegian	% [a]	Total	Years of performance data [b]	Swedish	% [c]	Norwegian	% [c]	Total
2000	41	32	7	3	1	35	2-15	5	2	1	0	6
2001	102	31	6	25	5	56	2-14	40	14	6	2	46
2002	92	33	7	19	4	52	2-13	38	13	2	1	40
2003	89	37	8	20	4	57	2-12	32	11	0	0	32
2004	71	28	6	13	3	41	2-11	30	11	0	0	30
2005	77	28	6	20	4	48	2-10	28	10	1	0	29
2006	89	38	8	22	5	60	3-9	28	10	1	0	29
2007	81	32	7	14	3	46	3-8	34	12	1	0	35
2008	65	29	6	19	4	48	3-7	17	6	0	0	17
2009	62	25	5	17	4	42	3-6	20	7	0	0	20
Total	**769**	**313**	**65**	**172**	**35**	**485**		**272**	**96**	**12**	**4**	**284**

[a] % of total raced horses (n = 485)
[b] Years of performance data used in this study
[c] % of total non-raced horses (n=284)

5.1.2 Performance data

All analyzed performance data for the raced horses were obtained for the years 2003 to 2015 (Table 1). The sample used in this study included four Norwegian Coldblooded trotters (born in 2001, 2003 and 2005), which entered their first race when they were 2 years old. In Sweden, Coldblooded trotters are not allowed to enter a race before three years of age (Swedish Trotting Association, 2016). Since performance results were only available from four Norwegian trotters at the age of two, that information was included as their three year old performances in the analyses of all raced Coldblooded trotters.

Breeding index, or Best Linear Unbiased Prediction (BLUP), is based on the individual's own results (40% racing status and 60% earnings for Coldblooded trotters), as well as the forefather's results (Revold et al. 2010). BLUP is adjusted for the fixed effects of sex and age, and updated yearly (Swedish Trotting Association, 2014b). Inbreeding coefficient and BLUP for all horses used in this study were, together with phenotypic records, provided by the databases of the Swedish Trotting Association and the Norwegian Trotting Association.

The direct racing performance values included number of starts, number of victories, proportion of victories, number of placings (1-3), proportion of placings (1-3), earned prize money (SEK), earned prize money per start, number of unplaced, proportion of unplaced, number of disqualifications, proportion of disqualifications, and the time records for two different start methods: auto and volt start. Auto start is a start method in which the race starts by car, and volt start is a start method in which the horses circle in a specific pattern in pairs to hit the starting line as a group (Swedish Trotting Association, 2015b). It should be noted that, depending on which race the horses competed in; the race distances differed (1600-2000 m). Only the best racing times for each horse and the start method were used in this study. It should also be mentioned, that only horses where the number of disqualifications could be verified as accurate were included in the analysis (n=350). The provided information for the raced Norwegian trotters' earned prize money was in Norwegian currency (NOK). Therefore, an average exchange rate was calculated (μ=0.95) for the years 2003-2015 (Valuta, 2016), for the time period corresponding to the performance results for the sample of raced horses in this study. To set all earnings in Swedish currency, and achieve as accurate results as possible, this calculated average exchange rate of 0.95 was multiplied with the earned prize money in Norwegian currency.

The performance results for each trait were analyzed for all of the raced Coldblooded trotters in this sample (n=485) with performance data ranging from 2-15 years of age (Table 1). In addition, the different traits based on performance results were defined and obtained at different ages: 3 years, 3 to 6 years, and 7 to 10 years of age. In the sample of 485 raced horses; 268 horses raced at three years of age (155 born in Sweden and 113 born in Norway), 472 horses raced at 3 to 6 years of age (302 born in Sweden and 170 born in Norway), and 182 horses raced 7 to 10 years of age (113 born in Sweden and 69 born in Norway).

5.1.3 Summary statistics

Data were structured to facilitate the analysis using custom scripts written in the software program for statistical computing R (R Development Core Team, 2015). For the sample of raced horses (n=485), summary statistics for BLUP, inbreeding coefficient, number of starts, victories, placings (1-3), unplaced, disqualifications, earnings (SEK), earnings per start and racing times (auto and volt start) were calculated. For the sample of non-raced horses (n=284), summary statistics for BLUP and inbreeding coefficient were calculated. Earnings (SEK) were additionally calculated for the total sample of the raced including the non-raced horses (n=769). Summary statistics were also calculated for the reference population (born in 2000-2009; n = 7 782).

5.1.4 Isolation of DNA

DNA was isolated from hair roots using a standardized procedure. The hair roots were cut into separate wells in 96-well plates with five hair roots in each well. A solution containing 100 µl Chelex (5%) and 7 µl proteinase K was added to each well. The 96-well plates were centrifuged for one minute, then incubated at 56°C for 60 minutes and incubated at 95°C for ten minutes to inactivate proteinase K. DNA was also extracted from blood samples, in which 350 µl of blood was used and isolated by the Qiasymphony instrument (Qiagen, Hilden, Germany).

5.1.5 Genotyping

The samples were removed to a new 96-well plate where a total volume of 15 µl per well was added, consisting of 8.25 µl Master Mix (2.0X), 0.41 µl Genotyping Assay Mix (40X), 6.19 µl distilled water and 1.5 µl DNA. The samples were genotyped for the *DMRT3_Ser301STOP* SNP marker at nucleotide position 22,999,655 on chromosome 23. SNP genotyping was performed with the StepOnePlus Real-Time PCR System (Life Technologies) using custom-designed TaqMan SNP Genotyping Assays (Applied Biosystems) which included the following primers and probes: forward primer: 5´-CCTCTCCAGCCGCTCCT-3; reverse primer: TCAAAGATGTGCCCGTTGGA-3; wild-type probe: 5´-CTGCCGAAGTTCG; mutant probe: 5´-CTCTGCCTAAGTTCG-3´.

5.1.6 Statistical analysis

All statistical analyses were performed using the software program for statistical computing R (R Development Core Team, 2015). Each trait was tested for normality using the Shapiro-Francia test. In order to get normally distributed values, the traits earnings (SEK) and best racing times were transformed using ln(earnings + 1,000) and ln(racing time – 68.2), respectively (Árnason, 1994). The other non-normally distributed traits were log-transformed as log(trait + 10).

The analyses of BLUP and inbreeding coefficient between the three genotypes were performed using the Kruskal-Wallis test. Each trait for the sample of raced horses was analyzed using linear models in order to determine the association of the *DMRT3* genotype with performance. A single marker association analysis was performed using Analysis of variance (ANOVA). If significant results ($p<0.05$) were provided by ANOVA, a multiple comparison test was performed using Tukey's HSD-test ($p<0.05$). The difference in *DMRT3* frequency between the horses that have raced at least once, and the sample of non-raced horses, was analyzed using a linear model including the fixed effects of sex, age and country. The association analyses were then performed using ANOVA, followed by Tukey's HSD-test.

6. Results

6.1 Summary statistics

Performance traits for the sample of raced horses (n=485) are presented in Table 2. Descriptive data for the reference population (born between the years 2000-2009; n = 7 782) are presented in Appendix 1. Descriptive data for the sample of raced horses, stratified by sex, are presented in Supplementary Table 1 in Appendix 2. For additional descriptive data of earnings for the sample of raced horses (n=485) and for the total sample of raced and non-raced horses (n=769), see Supplementary Table 2 in Appendix 2.

Table 2. Summary statistics of performance traits for the sample of raced Coldblooded trotters (n=319-485)

Performance trait	Min	1st Quantile	Median	Mean	3rd Quantile	Max	SD
Number of starts	1.00	13.00	28.00	38.04	53.00	222.00	34.71
Victories	0.00	0.00	2.00	3.99	5.00	53.00	5.78
Victories (frequency)	0.00	0.00	0.065	0.094	0.139	1.000	0.11
Placings 1-3	0.00	2.00	7.00	11.29	15.00	101.00	12.99
Placings (frequency)	0.00	0.139	0.250	0.262	0.357	1.000	0.18
Unplaced	0.00	3.00	8.00	10.70	15.00	85.00	10.89
Unplaced (frequency)	0.00	0.194	0.278	0.298	0.375	1.000	0.19
Disqualifications	0.00	1.00	3.00	4.39	6.00	46.00	5.33
Disqualifications (frequency)	0.00	0.027	0.100	0.151	0.208	1.000	0.17
Earnings (SEK)[1]	0.00	31 250	97 620	229 800	250 500	3 189 000	397 097
Earnings per start	0.00	1 979	3 474	4 758	5 510	54 220	5 582
Racing time auto (seconds)[2]	80.10	86.50	88.60	88.99	91.00	177.40	6.04
Racing time volt (seconds)[2]	81.40	88.05	90.20	90.60	93.00	106.90	4.19

[1] Earnings (SEK) were transformed: ln(earnings + 1000)

[2] Racing time transformed: ln(racing time - 68.2)

6.2 Statistical analyses

The linear models used for each trait of the raced Coldblooded trotters are presented in Table 3. The age in the models included year, month and day of birth. Similar models were used for the analyses of three year-old, 3 to 6 year-old, and 7 to 10 year-old performances (Table 3). The difference in *DMRT3* frequency between the horses that have raced at least once, and the sample of non-raced horses, was analyzed using a linear model including the fixed effects of sex, age and country (Table 3).

Table 3. P-values for fixed effects, random effects and interactions used in the models for estimation of performance in the sample of raced Coldblooded trotters

	Fixed effect			Random effect				Interactions	
Performance trait	**Sex**	**Age**	**Country**	**Starts**	**Victories**	**Unplaced**	**Earnings (SEK)**	**Earnings (SEK)*Sex**	**Adjusted R^2**
No. of starts[a]	<0.01	<0.001	0.147	-	0.0006	-	<0.001	-	0.773
Victories[a]	<0.001	0.067	0.192	<0.001	-	-	<0.001	<0.001	0.6973
Placings (1-3)[a]	<0.001	0.051	0.030	<0.001	-	-	<0.001	<0.001	0.8633
Unplaced[a]	0.065	0.265	0.617	<0.001	-	-	<0.001	-	0.8684
Disqualifications[a]	0.916	<0.001	0.279	<0.001	-	<0.001	<0.001	-	0.3019
Earnings (SEK)[b]	0.121	<0.001	0.256	<0.001	<0.001	-	-	-	0.8072
Earnings per start[a]	0.090	0.002	0.023	-	<0.001	-	-	-	0.2877
Racing time auto (seconds)[c]	0.014	0.260	0.034	0.003	0.150	-	<0.001	-	0.6442
Racing time volt (seconds)[c]	0.044	0.052	0.516	<0.001	<0.001	-	<0.001	-	0.9017
Raced vs. Non-raced[a]	<0.001	0.197	<0.001	-	-	-	-	-	0.2162
BLUP	-	-	-	-	-	-	-	-	-

[a] Transformed values: log (trait + 10)
[b] Earnings transformed: ln (earnings + 1000)
[c] Racing time transformed: ln (racing time - 68.2)
- Not included in the analysis

6.3 Performance results for the raced Coldblooded trotters

The majority of the genotyped 485 raced horses were heterozygous CA (51.3%), 39.8% were homozygous wild-type CC, while only 8.9% were homozygous mutant AA (Table 7). The averages of BLUP and the inbreeding coefficient were not significantly different between the *DMRT3* genotypes (Supplementary Table 3 in Appendix 3). More than 50% of the genotyped horses started their first race at three years of age in each genotype (Table 7). When investigating the proportion of horses that entered their first race at the population level (n=7 782; Appendix 1), the results showed that 36.8% started their first race as three year olds.

The performance results for the sample of raced horses for the total career (2-15 year old performance) are presented in Table 4. The performance results for the sample of raced horses for three year old, 3 to 6 year old, and 7 to 10 year old performances are presented in Table 5. All significant results less than P<0.05 are presented. The distribution of earnings for the different genotypes is shown in Figure 1. The horses with genotype CA and CC have more outliers with horses that earned a lot, compared to the AA horses (Figure 1). The median is not as strongly influenced by the skewed values as the mean value, and is therefore a more suitable measure of the central tendency in this situation.

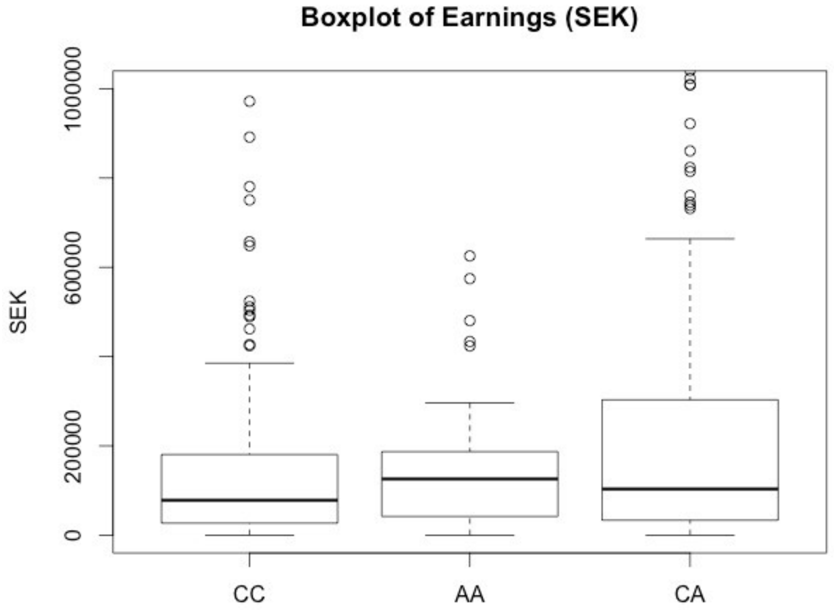

Figure 1. Boxplot of earnings (SEK) for the sample of raced Coldblooded trotters (n=485) according to *DMRT3* genotype.

The performance results for the sample of raced horses (n=485) for the entire career (2-15 years of age) showed that the majority of the results significantly differed between horses homozygous for CC and heterozygotes (Table 4). The CA horses had a higher percentage of victories, faster racing times (auto and volt start), and earned more money per start, compared to the homozygous CC horses. Horses with genotypes CA and AA had a higher percentage of placings (1-3). Earnings were significantly higher for the CA horses, compared to both AA and CC horses. In addition, for the total career, horses homozygous for CC had the highest percentage of disqualification, compared to the other genotypes (Table 4).

At the age of three, horses with genotype AA earned significantly more money and had the best racing time (volt start), compared to those with genotype CC. For the best racing time (auto start), the results showed a significant difference between heterozygous CA and homozygous CC. For the 3 to 6 year old performance, both CA and AA horses earned more money, had the highest percentage of victories and placings, compared to horses homozygous for CC. The horses with genotype CA had significantly higher earnings per start and faster racing times (auto start), compared to horses with genotype CC. Both AA and CA horses had significantly faster racing times (volt start) than CC. Performance results at the age of 7 to 10 years of age indicated higher earnings for both the CA and CC horses compared to AA horses. Horses with genotype CA had the best racing time for auto start, compared with the other genotypes, but were only faster than CC horses in racing time for volt start. Horses homozygous for CC had a significantly higher percentage of disqualifications than the other genotypes, both for 3 to 6 year-old, and 7 to 10 year-old performance (Table 5).

Table 4. Descriptive results of all performance traits for the sample of raced Coldblooded trotters (n=485) according to *DMRT3* genotype

	AA (n = 28-43)							CA (n = 171-249)							CC (n = 120-193)							P-value[1]		
Performance trait	**Min**	**1 st Quantile**	**Median**	**Mean**	**3rd Quantile**	**Maximum**	**SD**	**Min**	**1 st Quantile**	**Median**	**Mean**	**3rd Quantile**	**Maximum**	**SD**	**Min**	**1 st Quantile**	**Median**	**Mean**	**3rd Quantile**	**Maximum**	**SD**	**CA/CC**	**AA/CC**	**CA/AA**
Number of starts	1.00	10.00	25.00	31.09	43.00	114.00	25.58	1.00	14.00	31.00	40.74	58.00	210.00	36.36	1.00	14.00	26.00	36.10	49.00	222.00	34.08	0.005	-	0.002
Victories	0.00	1.00	2.00	3.35	5.00	20.00	3.88	0.00	0.00	2.00	4.52	6.00	53.00	6.43	0.00	0.00	1.00	3.47	5.00	29.00	5.18	0.01	-	-
Victories (frequency)	0.00	0.021	0.094	0.112	0.156	0.500	0.113	0.00	0.00	0.074	0.103	0.143	1.00	0.124	0.00	0.00	0.056	0.077	0.125	0.526	0.086	0.02	-	-
Placings 1-3	0.00	3.50	9.00	9.63	14.00	36.00	8.01	0.00	3.00	8.00	12.47	19.00	101.00	13.70	0.00	2.00	5.00	10.13	13.00	70.00	12.85	<0.001	-	-
Placings 1-3 (frequency)	0.00	0.185	0.319	0.304	0.394	0.750	0.179	0.00	0.154	0.271	0.283	0.382	1.00	0.185	0.00	0.115	0.222	0.226	0.316	0.790	0.162	0.004	0.02	-
Unplaced	0.00	2.00	6.00	9.19	13.50	35.00	9.12	0.00	3.00	9.00	11.91	17.00	67.00	11.69	0.00	2.00	7.00	9.47	13.00	85.00	10.02	<0.001	-	<0.001
Unplaced (frequency)	0.00	0.200	0.250	0.308	0.380	1.00	0.178	0.00	0.200	0.289	0.302	0.385	1.00	0.186	0.00	0.182	0.258	0.289	0.364	1.00	0.197	-	-	-
Disqualifications	0.00	0.00	1.00	2.49	4.50	9.00	2.84	0.00	1.00	3.00	3.86	5.00	46.00	5.08	0.00	1.00	4.00	5.66	8.00	37.00	6.10	<0.001	<0.001	0.01
Disqualifications (frequency)	0.00	0.00	0.058	0.092	0.159	0.400	0.109	0	0.023	0.105	0.144	0.183	1.00	0.159	0	0.087	0.200	0.238	0.321	1.00	0.223	<0.001	<0.001	-
Earnings (SEK)[2]	0.00	41 900	125 900	152 400	187 000	625 500	154 698	0.00	33 500	103 400	267 300	303 100	3 189 000	440 444	0.00	27 000	78 240	198 700	180 600	2 416 000	371 369	<0.001	-	0.04
Earnings per start	0.00	2 726	3 750	4 734	6 496	14 360	3 371	0.00	2 230	3 735	5 371	5 899	54 220	6 415	0.00	1 650	2 919	3 973	4 547	28 980	4 585	0.003	-	-
Racing auto (seconds)[3]	84.80	87.18	87.90	88.62	89.67	93.90	2.31	80.10	86.05	88.50	88.78	90.15	177.40	7.62	80.70	87.00	89.55	89.39	92.02	98.20	3.60	<0.001	-	-
Racing volt (seconds)[3]	84.70	88.25	89.70	90.65	91.65	102.80	4.01	82.40	87.25	90.00	90.27	92.80	106.90	4.24	81.40	88.50	90.60	91.04	93.60	104.40	4.14	0.003	-	-

[1] Multiple comparison test was performed using the Tukey's HSD-test

[2] Earnings transformed: ln (earnings + 1000)

[3] Racing time transformed: ln (racing time - 68.2)

\- Not significant (p>0.05)

Table 5. Descriptive performance results of 3 years, 3 to 6 years, and 7 to 10 years of age for the sample of raced Coldblooded trotters according to *DMRT3* genotype

Performance trait	AA							CA							CC							P-value[1]		
	Min	1 st Quantile	Median	Mean	3rd Quantile	Maximum	SD	Min	1 st Quantile	Median	Mean	3rd Quantile	Maximum	SD	Min	1 st Quantile	Median	Mean	3rd Quantile	Maximum	SD	CA/CC	AA/CC	CA/AA
3-year old	AA (n= 5-24)							CA (n= 35-143)							CC (n= 23-101)									
Number of starts	1.00	3.00	6.50	6.33	9.25	15.00	3.99	1.00	2.00	5.00	6.00	9.00	21.00	4.35	1.00	2.00	5.00	5.81	8.00	17.00	4.21	-	-	-
Victories	0.00	0.00	1.00	0.75	1.00	2.00	0.79	0.00	0.00	0.00	0.87	1.00	10.00	1.73	0.00	0.00	0.00	0.68	1.00	8.00	1.40	-	-	-
Victories (freq.)	0.00	0.00	0.079	0.088	0.171	0.286	0.094	0.00	0.00	0.00	0.101	0.167	1.00	0.18	0.00	0.00	0.00	0.083	0.125	0.667	0.153	-	-	-
Placings 1-3	0.00	0.00	2.00	2.08	4.00	6.00	1.95	0.00	0.00	1.00	2.33	3.00	17.00	3.13	0.00	0.00	1.00	1.92	3.00	10.00	2.38	0.05	-	-
Placings 1-3 (freq.)	0.00	0.00	0.261	0.245	0.341	0.833	0.229	0.00	0.00	0.250	0.291	0.500	1.00	0.296	0.00	0.00	0.200	0.236	0.429	0.900	0.249	-	-	-
Unplaced	0.00	0.75	1.50	1.54	2.00	5.00	1.32	0.00	0.00	1.00	1.45	2.00	11.00	1.58	0.00	0.00	1.00	1.18	2.00	6.00	1.24	-	-	-
Unplaced (freq.)	0.00	0.083	0.202	0.280	0.398	1.00	0.286	0.00	0.00	0.222	0.296	0.500	1.00	0.310	0.00	0.00	0.167	0.255	0.333	1.00	0.293	-	-	-
Disqualifications	0.00	0.00	0.00	0.47	1.00	3.00	0.80	0.00	0.00	0.00	0.61	1.00	5.00	0.97	0.00	0.00	1.00	1.05	1.00	7.00	1.41	0.003	0.03	-
Disqualifications (freq.)	0.00	0.00	0.00	0.126	0.100	1.00	0.262	0.00	0.00	0.00	0.159	0.250	1.00	0.279	0.00	0.00	0.092	0.223	0.321	1.00	0.317	-	-	-
Earnings (SEK)[2]	0.00	5 115	25 320	47 960	78 960	225 000	61 204	0.00	3 395	15 160	61 830	42 000	1 308 000	163 178	0.00	2 000	15 380	35 090	45 000	262 700	534 409	-	0.03	-
Earnings per start	0.00	2 015	3 278	6 128	7 677	32 140	8 087	0.00	944	3 480	6 136	7 014	93 440	10 760	0.00	950	2 579	4 358	5 663	32 830	5 656	-	-	-
Racing auto (seconds)[3]	88.90	91.20	92.20	92.02	92.40	95.40	2.35	85.90	89.70	91.40	91.47	92.65	97.30	2.57	89.20	90.85	92.40	92.87	94.10	102.50	2.99	0.03	-	-
Racing volt (seconds)[3]	89.30	92.10	94.00	94.56	96.20	104.90	4.17	86.80	92.60	95.10	95.83	98.70	111.30	4.42	86.40	93.15	95.40	96.04	98.80	106.50	4.35	-	0.03	-
3 to 6-year old	AA (n= 26-41)							CA (n= 150-243)							CC (n= 99-188)									
Number of starts	1.00	10.00	18.00	23.37	38.00	70.00	17.45	1.00	10.00	23.00	26.38	39.00	84.00	19.49	1.00	8.75	18.00	23.02	33.00	77.00	17.46	<0.001	-	-
Victories	0.00	1.00	2.00	2.88	4.00	19.00	3.30	0.00	0.00	2.00	3.28	4.00	24.00	4.30	0.00	0.00	1.00	2.61	3.00	27.00	4.07	0.005	-	-
Victories (freq.)	0.00	0.021	0.111	0.126	0.174	0.559	0.129	0.00	0.00	0.080	0.111	0.163	1.00	0.129	0.00	0.00	0.054	0.088	0.143	0.600	0.111	0.03	0.04	-
Placings 1-3	0.00	4.00	8.00	8.20	13.00	23.00	6.36	0.00	2.00	6.00	8.80	13.00	40.00	8.65	0.00	1.00	4.00	7.04	10.25	46.00	7.98	<0.001	<0.001	-
Placings 1-3 (freq.)	0.00	0.261	0.329	0.323	0.417	0.750	0.196	0.00	0.159	0.286	0.298	0.414	1.00	0.200	0.00	0.105	0.225	0.243	0.354	0.790	0.190	<0.001	0.001	-
Unplaced	0.00	2.00	4.00	6.10	11.00	20.00	5.83	0.00	3.00	6.00	6.97	10.00	26.00	5.70	0.00	2.00	5.00	5.60	8.00	19.00	4.77	<0.001	-	0.006
Unplaced (freq.)	0.00	0.177	0.243	0.293	0.357	1.00	0.198	0.00	0.167	0.258	0.288	0.364	1.00	0.201	0.00	0.149	0.250	0.287	0.354	1.00	0.224	-	-	-
Disqualifications	0.00	0.00	1.00	1.76	2.00	9.00	2.29	0.00	0.00	2.00	2.65	4.00	19.00	2.98	0.00	1.00	3.00	3.54	6.00	22.00	3.58	<0.001	<0.001	0.03
Disqualifications (freq.)	0.00	0.00	0.043	0.111	0.217	0.444	0.139	0.00	0.00	0.100	0.142	0.200	1.000	0.165	0.00	0.069	0.188	0.229	0.331	1.000	0.224	<0.001	<0.001	-
Earnings (SEK)[2]	0.00	38 950	98 500	128 100	149 700	523 200	132 062	0.00	24 590	72 500	179 300	210 300	2 228 000	289 055	0.00	19 590	54 370	133 200	131 000	1 954 000	235 199	<0.001	0.04	-
Earnings per start	0.00	2 768	3 895	4 963	6 762	16 350	3 801	0.00	2 204	3 781	5 417	6 613	55 030	6 671	0.00	1 517	2 873	4 178	4 985	28 980	4 871	0.001	-	-
Racing auto (seconds)[3]	84.80	87.32	88.90	89.12	90.07	95.00	2.52	81.60	87.00	88.75	89.49	90.78	177.40	7.88	81.30	87.20	89.80	89.88	92.45	98.20	3.41	<0.001	-	-
Racing volt (seconds)[3]	84.70	88.78	89.95	91.09	92.90	102.80	4.31	83.70	88.72	90.70	91.24	93.40	106.90	3.96	82.90	89.10	91.80	91.92	94.45	104.40	4.1	<0.001	0.01	-
7 to 10-year old	AA (n=14-18)							CA (n= 71-98)							CC (n= 44-66)									
Number of starts	1.00	3.75	13.00	16.22	19.75	87.00	19.53	1.00	5.25	18.00	25.34	39.75	96.00	22.96	1.00	9.00	15.00	23.45	35.75	97.00	21.94	-	0.004	<0.001
Victories	0.00	0.00	0.00	1.22	1.00	10.00	2.46	0.00	0.00	1.00	2.53	3.00	28.00	4.56	0.00	0.00	1.00	1.76	2.75	13.00	2.55	-	-	-
Victories (freq.)	0.00	0.00	0.00	0.048	0.092	0.222	0.067	0.00	0.00	0.043	0.074	0.118	0.433	0.096	0.00	0.00	0.046	0.067	0.108	0.500	0.087	-	-	-
Placings 1-3	0.00	0.00	1.50	3.78	6.75	23.00	5.81	0.00	1.00	3.00	6.91	9.00	54.00	8.98	0.00	1.00	3.00	5.50	6.75	34.00	6.81	<0.001	<0.001	<0.001
Placings 1-3 (freq.)	0.00	0.00	0.141	0.174	0.288	0.500	0.177	0.00	0.100	0.200	0.211	0.311	0.636	0.168	0.00	0.095	0.189	0.186	0.266	0.500	0.135	-	-	-
Unplaced	0.00	2.00	4.50	6.22	6.75	26.00	6.87	0.00	2.00	6.00	8.62	14.00	28.00	7.78	0.00	2.00	5.00	6.73	9.75	31.00	6.72	0.001	-	0.004
Unplaced (freq.)	0.00	0.250	0.421	0.415	0.613	1.000	0.265	0.00	0.250	0.349	0.380	0.500	1.000	0.252	0.00	0.189	0.286	0.346	0.494	1.000	0.234	-	-	-
Disqualifications	0.00	0.00	1.00	1.47	2.00	5.00	1.55	0.00	0.75	1.00	2.63	4.00	17.00	3.54	0.00	1.00	3.00	4.06	5.50	15.00	4.08	<0.001	<0.001	0.03
Disqualifications (freq.)	0.00	0.00	0.056	0.120	0.150	0.500	0.158	0.00	0.014	0.101	0.178	0.221	1.000	0.247	0.00	0.082	0.233	0.269	0.384	1.000	0.241	0.03	0.02	-
Earnings (SEK)[2]	0.00	4 956	23 020	62 120	66 840	475 300	111 433	0.00	15 500	53 900	168 200	173 600	2 140 000	330 993	0.00	15 440	40 140	114 000	125 600	1 587 000	233 547	-	0.03	<0.001
Earnings per start	0.00	952	2 402	2 903	4 910	7 626	2 358	0.00	1 258	3 283	4 371	5 474	32 660	5 078	0.00	1 281	2 482	3 328	4 133	22 670	3 781	-	-	-
Racing auto (seconds)[3]	85.20	87.82	89.60	89.72	91.50	93.90	2.66	80.10	85.70	87.70	87.74	89.95	94.50	3.12	80.70	87.15	88.80	89.11	92.00	95.00	3.49	<0.001	-	<0.001
Racing volt (seconds)[3]	84.70	89.20	90.50	90.26	91.50	97.30	3.22	82.80	86.75	89.00	89.16	91.05	99.50	3.51	81.40	87.88	89.45	90.12	92.38	100.50	3.73	0.03	-	-

[1] Multiple comparison test was performed using the Tukey's HSD-test. [2] Earnings (SEK) transformed: ln (earnings + 1000). [3] Racing time transformed: ln (racing time - 68.2). - Not significant (p>0.05).

6.4 Total sample of raced and non-raced Coldblooded trotters

6.4.1 Results for the sample of raced and non-raced combined

The frequencies of the *DMRT3* genotypes for the total 769 genotyped horses were 48% heterozygous CA, 40% homozygous CC and 12% homozygous AA, (Supplementary Table 1 in Appendix 3). Average BLUP was significantly higher for the raced compared to the non-raced Coldblooded trotters (P<0.01), but there was no significance for the average in inbreeding coefficient between the two groups (Table 6). When *DMRT3* genotype was included in the analyses of BLUP and inbreeding coefficient, the results were not significant between the genotypes for the raced, non-raced, or the total sample of the raced and non-raced horses (Supplementary Table 2 in Appendix 3). For additional information of earnings for the raced (n=485) Coldblooded trotters and total sample of raced and non-raced Coldblooded trotters (n=769), descriptive results are presented in Supplementary Table 2 in Appendix 2.

Table 6. Average BLUP and inbreeding coefficient between the sample of raced (n=485) and non-raced (n=284) Coldblooded trotters

	Raced (n = 485)	SD	Non-Raced (n=284)	SD	P-value[1]
BLUP	111.7	6.43	103.9	5.00	<0.01
Inbreeding coefficient	6.04	2.10	6.03	2.35	0.63

[1] P-value calculated using Kruskal-Wallis test

Genotype frequencies and proportions of horses when they started their first race at different ages according to *DMRT3* genotype are presented in Table 7. Of the sample of raced horses (n=485), a total of 29 (6%) horses started their first race at 6 years of age, and 13 (2.7%) horses started their first race at 7 years of age or older (Table 7).

The genotype frequency of AA horses was approximately twice as high in the group of non-raced horses compared to the group of raced horses, indicating that they less often make it to the racetrack (P<0.01 for AA vs. CA, and P<0.001 for AA vs. CC). The difference between CA and CC horses was not significant.

Table 7. Genotype frequencies, and proportions of *DMRT3* genotypes among raced and non-raced horses and different ages at first start (proportion of horses among all raced horses within this genotype in parentheses)

	Total (n)	AA	CA	CC
Raced	485	0.089	0.513	0.398
Non-raced	284	0.180	0.419	0.401
Among raced horses:				
Started first race as 3 year old	268	0.090 (0.558)	0.534 (0.574)	0.377 (0.523)
Started first race as 6 year old	29	0.034 (0.023)	0.448 (0.052)	0.517 (0.078)
Started first race as 7 year old or older	13	0.154 (0.047)	0.462 (0.024)	0.385 (0.026)

6.4.2 Results for the non-raced

For the 284 genotyped non-raced Coldblooded trotters, 18% were homozygous AA, 42% were heterozygous CA and 40% were homozygous CC (Table 7). Average BLUP, and the inbreeding coefficient, were not significantly different between the *DMRT3* genotypes of *DMRT3* for the non-raced horses (Supplementary Table 2 in Appendix 3).

7. Discussion

Although previous studies have presented a favorable association between performance and *DMRT3* genotype in other breeds (Kristjansson et al. 2014; Jäderkvist et al. 2014; Jäderkvist et al. 2015a), the association with racing performance in the breed Coldblooded trotter has not been fully explored. This study was performed to investigate the association between performance and *DMRT3* genotypes in Coldblooded trotters.

The results of the sample of raced horses' total career (2-15 year old performance) indicated a significant difference between horses heterozygous CA and horses homozygous for C for the majority of the traits. The CA horses had a higher percentage of victories and placings and faster racing times, compared to the CC horses (Table 4). It should be noted, that several horses have no earnings and some have earned a huge amount of prize money, thus making the distribution of earnings extremely skewed (see Figure 1). When the earnings are log-transformed, the values are more drawn towards the median. For example, horses heterozygous CA earned significantly more money in average for the total career, compared to the other genotypes. The higher mean value of earnings can be explained by that there are more outliers of the CA horses that earned a lot of money, compared to the AA horses, which appears to have the highest median value of earnings (Table 4). The same tendencies are shown for 3 years, 3 to 6 years, and 7 to 10 years of age (Table 5).

At three years of age, horses with genotype AA were significantly faster at racing time for volt start, compared to horses homozygous for CC (Table 5). For most of the other traits there were no significant differences at three years of age. However, in the study by Jäderkvist et al. (2014), the horses with genotype AA were significantly better than the other genotypes for most of the traits at three years of age, they had the highest earnings, and the highest percentage of victories and placings. In addition, the results from the study by Jäderkvist et al. (2014) indicated that horses with the genotype CA were as good or even better than the AA horses for some performance traits at older ages (3 to 6 years of age). Similar results were shown in this current study at 3 to 6 years of age, where both CA and AA horses not only earned more prize money, but they also had higher percentage of victories and placings, and faster racing time (volt start; Table 5). Although horses homozygous for the mutation appears to perform better early in life, the horses carrying the other *DMRT3* genotype seem to be faster at older ages and in general the racing times are improved. A possible reason for this is due to the fact that the horses with the AA genotype are less easy to train because of their tendency to fall in pace (Jäderkvist et al. 2014). Another reason could also be that the conformation of Coldblooded trotters homozygous for the mutation in the *DMRT3* is not fully capable of handling the high speed.

For the total career, and at 3 to 6 years, and 7 to 10 years of age, compared to the other genotypes CC homozygous horses had a significantly higher proportion of disqualification, (Table 4 and 5). Since the mutation in the *DMRT3* gene facilitates the ability to trot (or pace) at high speed without breaking into gallop (Andersson et al. 2012), one may hypothesize that horses with genotype CC are more frequently disqualified (because of breaking into gallop), which seems to agree with the results from this study. As mentioned, since only the horses where the number of disqualifications could be verified as accurate were included, the accuracy decreased due to loss of a random sample. However, it would be interesting to further investigate the cause of disqualification, since it is possible that horses with genotype AA are disqualified for switching into pace and CC horses for falling into gallop.

Jäderkvist et al. (2014) demonstrated that horses with genotype CA have a good trotting technique with a clean, and even-beat trot, which facilitates the training and driving. This might be the reason for horses with genotype CA in this study having the highest earnings (on average) and a higher number of starts. Interestingly, both Finnhorses and Standardbred trotters homozygous for AA had superior breeding values and performance results, compared to the other genotypes (Andersson et al. 2012; Jäderkvist et al. 2014; Jäderkvist et al. 2015a). In addition, Standardbred trotters with the genotype CA exhibited major difficulties in sustaining a clean trot at high speed (Andersson et al. 2012). It seems that there is a requirement for Standardbred trotters to have two mutated alleles in *DMRT3* (Jäderkvist et al. 2014), while in Coldblooded trotters only one mutated allele appears to be sufficient.

The purpose of including the non-raced Coldblooded trotters in this study was to investigate the potential influence of the *DMRT3* gene with the horses' probability of entering a race. The results indicate a significantly higher proportion of CA and CC horses entering a race, compared to the AA horses. This could be explained by the AA horses are more difficult to train for trotting due to their technique and tendency to fall into pace, which result in disqualification in racing. Although horses with genotype AA were significantly faster and earned more money than the CC horses at the age of three, they are not raced as often as horses with the other *DMRT3* genotypes. Perhaps not all Coldblooded trotters are fully adapted of being homozygous for the mutation and to trot at higher speeds. That might explain why homozygous AA performs well early in life, while not all horses are capable of handling the high speed and they possibly get injured, due to e.g. an unfavorable conformation. However, numerous other reasons could also explain why a horse never enters a race, for instance injuries, diseases, etc. It would be interesting to study this further, but for this study information concerning why the horses did not race was not available. In addition, since the frequency of the A-allele in Coldblooded trotters is 45%, compared to 97% in Standardbred, the number of horses homozygous for AA in Coldblooded trotters is low. In 1970, the frequency of the mutant allele in *DMRT3* reached over 30% and has been quite stable since then (Jäderkvist et al. 2014). It is difficult to predict if the frequency will increase in time, although perhaps it will not considerably increase any further, due to the good performance results of Coldblooded trotters heterozygous for the *DMRT3* mutation.

The average BLUP values (described in section 5.1.2) for the sample of raced Coldblooded trotters did not differ between the *DMRT3* genotypes. However, the results from the study by Jäderkvist et al. (2014) showed that horses homozygous for AA had significantly higher BLUP values than horses with the other genotypes. A probable explanation may be that the sample of horses used in the study of Jäderkvist et al. (2014) was not randomly selected, as the horses in the sample were in this study.

It can be argued that the two most important aspects in the horse racing industry are earnings and soundness. Since a lot of money and time are invested in horses, longevity and durability of racehorses is probably of considerable economic importance. A superior pedigree is important, although generating horses with good racing performance is a long and difficult path (Revold et al. 2010). As mentioned, the general breeding goal for the Coldblooded trotter is to preserve and further develop a sound, healthy, and competitive trotter through improvement of the horses' performance results (Swedish Trotting Association, 2014a). In order to achieve such a breeding goal, a first step would be to further address the issue of the relatively high inbreeding coefficient. In addition, it is important to consider other factors that may influence performance. Such factors could be either genetic factors, e.g. the presence of genetic variants associated with increased risk of developing disease, and an individuals response to diet, or environmental factors such as the trainer effect, racetracks, and injuries. Genetic variants in several additional genes, or other gene(s) interacting with *DMRT3*, also influence racing performance (Jädervist et al. 2014). Some of the environmental effects have changed over time, such as enhanced training methods, and improved racetracks, harnesses and sulkies (Thiruvenkadan et al. 2009). Interestingly, since some Coldblooded trotters used in harness racing have respiratory problems, and because Norwegian Coldblooded trotters seem to be specially predisposed to develop dynamic laryngeal collapse compared to the Standardbred trotter (Strand et al. 2009), this may also be the reason for the early end of a Coldblooded trotter's career. However, the relevance of genetic influences in that disorder could be a further consideration. In addition, Thiruvenkadan et al. (2009) suggested that a possible indicator of both health and of the soundness of a horse's conformation could be the of number of starts.

The identification of *DMRT3* and its influence on performance was a great achievement in the field of horse racing. However, more studies are needed to determine the relationship between *DMRT3* and other genes influencing performance. In the future, the results of this study could provide information for further studies when comparing *DMRT3* for example in other breeds. The studies by Andersson et al. (2012) and Promerová et al. (2014) not only showed that the frequency of the mutated allele in *DMRT3* differs between gaited and non-gaited breeds, but also among gaited breeds.

When it comes to selecting the most ideal horse for breeding or deciding which horse to buy for a higher chance of becoming a successful horse in harness racing, the implications are not straightforward. Even though results from different studies indicated that genotype CC was the least advantageous for trotting performance (Jäderkvist et al. 2014; Jäderkvist et al. 2015a; Ricard 2015), other affecting genes, which may influence performance of specific individuals, should be considered. Although CC horses seem to be disqualified more frequently, when looking at the outliers of CC horses in this study, there is a possibility with correct conditions and training that such horses could earn a lot of prize money (Figure 1). For example, some CC horses may be easier to train, compared to some AA horses.

Today, a genetic test is available, in which the breeder can test horses for *DMRT3* and use the information about a horse's genotype to select breeding animals. Genetic testing can be utilized to optimize the probability of achieving a faster genetic progress with improved racing performance in Coldblooded trotters (Capilet Genetics, 2016). In addition, the future implication of the results from this current study could provide valuable information for breeders, when performing a long-term breeding plan. For instance, a breeder could choose breeding animals according to a desired genotype. In addition, a breeder, trainer, or a new owner, could use the information provided from this study in order to see which horse has the probability of win often and earn a lot of prize money. Both the breeder, and a trainer/owner could also save money by selecting a horse, which has a high trotting capacity, and a high probability of becoming a successful horse in harness racing.

8. Conclusions

Based on the genotypes of the sample of 485 raced Coldblooded trotters, the results indicate, for most ages, that the CA horses earned the most money per start, had the highest percentage of victories and the best racing times (auto start), compared to horses with genotype CC. The genotype frequency differed between the sample of raced and non-raced horses (n=284), indicating that the horses homozygous for AA less often make it to the racetrack. Since the horses homozygous for AA had the best racing time (volt start) and earned the most money in average at 3 years of age, (compared to the CC homozygotes), they seem to have a high potential in becoming fast trotters early in life. Both CA and CC horses had significantly higher number of starts, compared to the AA horses. However, their tendencies to fall into pace could probably be the reason for the lower proportion of AA horses entering a race. Horses homozygous for the wild-type allele (C) had the highest percentage of disqualifications for all years of performance, except at three years of age, compared to the other genotypes. Consequently, the CA horses in this study had better overall performance results. This study shows a clear influence of the *DMRT3* nonsense mutation with performance and indicates a higher probability for Coldblooded trotters when they carry only one mutated allele to become successful in harness racing. The results from this study could have practical implications for training and for a long-term breeding plan, where the information from this study could help the industry professionals in selecting the most ideal horse for breeding Coldblooded trotters.

9. Participants contributions

I, the author of this thesis, wrote custom scripts in the software program R to structure the data, performed the statistical summaries, collected hair and blood samples, performed isolation of DNA and genotyping, and performed all statistical analyses in this study. I presented the results from this study for the research-group in the project "Genetic variation, performance and health in Coldblooded trotters". I, the author, also presented the results from this study at the Students World Championship at the breeding station Flyinge, Sweden. My supervisors Lisa Andersson, Kim Jäderkvist Fegraeus, Gabriella Lindgren, and Brandon Velie provided advice and guidance during this project, and assisted in finalizing this thesis.

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11. References

Andersson, L. S., Larhammar, M., Memic, F., Wootz, H., Schwochow, D., Rubin, C. J., Patra, K., Árnason, T., Wellbring, L., Hjälm, G., Imsland, F., Petersen, J. L., McCue, M. E., Mickelson, J. R., Cothran, G., Ahituv, N., Roepstorff, L., Mikko, S., Vallstedt, A., Lindgren, G., Andersson, L. & Kullander, K. (2012). Mutations in *DMRT3* affect locomotion in the horses and spinal circuit function in mice. *Nature*, vol. 488, pp. 642-646

Árnason, T. (1994). The importance of different traits in genetic improvement of trotters. *Proceedings of World Congress on Genetics Applied to Livestock Production*, August 7-12. University of Guelph, Guelph, Canada. Vol. 17, pp. 462-469

Capilet Genetics, (2016). Synchrogait® - ett användbart redskap för kallblodsentusiaster. Available at: https://www.capiletgenetics.com

Dolvik, N. I. & Klemetsdal, G. (1999). Conformational traits of Norwegian Cold-blooded trotters: heritability and the relationship with performance. *Acta Agriculturæ Scandinavica*, vol. 49, pp. 156-162

Jäderkvist, K., Andersson, L. S., Johansson, A. M., Árnason, T., Mikko, S., Eriksson, S., Andersson, L. & Lindgren, G. (2014). The *DMRT3* 'Gait keeper' mutation affects performance of Nordic and Standardbred trotters. *Journal of Animal Science*, vol. 92, pp. 4279-4286

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

Jäderkvist, K., Holm, N., Imsland, F., Árnason, T., Andersson, L., Andersson , L. S. & Lindgren, G. (2015b). The importance of the *DMRT3* 'Gait keeper' mutation on riding traits and gaits in Standardbred and Icelandic horses. *Livestock Science*, vol. 176, pp. 33-39

Klemetsdal, G. (1998). The effect of inbreeding on racing performance in Norwegian cold-blooded trotters. *Genet. Sel. Evol.*, vol. 30, pp. 351-366

Kristjansson, T., Bjornsdottir, S., Sigurdsson, A., Andersson, L. S., Lindgren, G., Helyar, S. J., Klonowski, A. M. & Árnason, T. (2014). The effect of the 'Gait Keeper' mutation in the *DMRT3* gene on gaiting ability in Icelandic horses. *Journal of Animal Breeding and Genetics*, vol. 131, pp. 415-425

Promerová, M., Andersson, L. S., Juras, R., Penedo, C. T., Reissmann, M., Tozaki, T., Bellone, R., Dunner, S., Horín, P., Imsland, F., Imsland, P., Mikko, S., Modrý, D., Roed, K. H., Scwochow, D., Vega-Pla, J. L., Mehrabani-Yeganeh, H., Yousefi-Mashouf, N., Cothran, E. G., Lindgren, G. & Andersson, L. (2014). Worldwide frequency distribution of the 'Gait keeper' mutation in the *DMRT3* gene. *Animal Genetics*, vol. 45, pp. 274-282

Revold, T., Larsen, S. & Ihler, C.F. (2010). Prediction of early race starts in Norwegian-Swedish Coldblooded Trotters. *Acta Veterinaria Scandinavica*, vol. 52:53

R Development Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria

Strand, E., Fjordbakk, C. T., Holcombe, S. J., Risberg, A. & Chalmers, H. J. (2009). Effect of poll flexion and dynamic laryngeal collapse on tracheal pressure in Norwegian Coldblooded trotter racehorses. *Equine Veterinary Journal*, vol. 41, pp. 59-64

Swedish University of Agricultural Sciences (2016-02-17). Varför härstamningskontrollera? Available at: http://www.slu.se/institutioner/husdjursgenetik/om-institutionen/hgenlabtest/hast1/varfor-harstamningskontrollera/ [2016-06-03]

Swedish Trotting Association, 2011. (2012-09-27). *Unikt avelsframsteg och negativ effekt av inavel*. Available at: https://www.travsport.se/article/2.292/1.6562

Swedish Trotting Association, 2014a. (2014-02-05). *Avelsvärdering av kallblodiga hingstar*. Available at: https://www.travsport.se/artikel/avelsvardering_av_kallblod

Swedish Trotting Association, 2014b. (2014-02-12). *Avelsindex.* Available at: https://www.travsport.se/artikel/avelsindex

Swedish Trotting Association, 2015a. (2015-02-03). *Hästarna*. Available at: https://www.travsport.se/artikel/hastarna;jsessionid=N6H9JQCfLbQ83DcS3WLTNClDTggW1nT8LVhv9QQFvvffZ2X20681!2127073853?dashboard=true [2015-05-17]

Swedish Trotting Association, 2015b. (2015-02-02). *Tävlingarna*. Available at: https://www.travsport.se/artikel/tavlingarna [2016-05-14]

Swedish Trotting Association, 2015c. *Årsstatistik 2015*. Available at: https://www.travsport.se/artikel/ovrig_statistik [2016-05-20]

Swedish Trotting Association, (2016-05-14). *Tävlingsregler*. Available at: https://www.travsport.se/artikel/tavlingsregler

Thiruvenkadan, A. K., Kandasamy, N. & Panneerselvam, S. (2009). Inheritance of racing performance of trotter horses: An overview. *Livestock Science*, vol. 124, pp. 163-181

Valuta (2016-06-04). Available at: https://www.valuta.se [2016-06-04]

12. Appendix 1

Descriptive data of the reference population of Coldblooded trotters born between 2000-2009, (n=7 782)

Number and gender proportion

	Total	Raced	Raced % (of total)	Non-raced	Non-raced % (of total)
Geldings*	3 021	2 259	29,0	762	9,8
Stallions	963	487	6,3	476	6,1
Mares	3 798	2 022	26,0	1 776	22,8
Total	**7 782**	**4 768**	**61,3**	**3 014**	**38,7**

* Three of them are cryptorchids

Proportion of Coldblooded trotters in different countries of origin, stratified by sex

	Swedish			Norweigan		
Gender	Total %	Raced % (of total SWE)	Non-raced % (of total SWE)	Total %	Raced % (of total NK)	Non-raced % (of total NK)
Geldings	38,0	24,5	13,5	40,4	37,5	2,9
Stallions	11,3	2,6	8,6	14,5	13,0	1,4
Mares	50,8	20,9	29,9	45,2	35,5	9,6
Total	**65,1**	**48,0**	**52,0**	**34,9**	**86,0**	**14,0**

Descriptive data, stratified by sex, for earnings (SEK) and earnings per start

	Min	1st Q	Median	Mean	3rd Q	Max	SD
Earnings (SEK)							
Population	0	0	21 650	141 100	150 000	10 020 000	348 172
Stallions	0	0	0	371 100	445 200	10 020 000	786 422
Mares	0	0	0	78 410	82 590	3 380 000	174 093
Geldings	0	0	58 050	146 600	190 500	3 189 000	235 319
Earnings per start							
Population	0	0	1 592	2 810	3 857	98 500	4 907
Stallions	0	0	0	5 545	7 325	98 500	10 016
Mares	0	0	0	2 023	2 963	68 280	3 693
Geldings	0	0	2 386	2 927	4 234	37 420	3 125

13. Appendix 2

Supplementary information

Table 1. Descriptive data, stratified by sex, for earnings (SEK), earnings per start, number of victories and racing time (auto and volt start), of performance data from 3 year old, 3 to 6 year old and total raced Coldblooded trotters

	N			Median			Mean			SD		
Performance trait	**3-year**	**3-6 year**	**Total raced**	**3-year**	**3-6 year**	**Total raced**	**3-year**	**3-6 year**	**All raced**	**3-year**	**3-6 year**	**All raced**
Earnings (SEK)												
Stallions	38	46	46	42 770	379 200	517 700	111 700	515 900	795 000	213 013	519 527	796 688
Mares	105	180	188	12 210	49 910	70 980	47 420	113 900	140 400	104 117	187 726	211 176
Geldings	125	246	251	12 230	62 040	93 950	30 930	110 300	191 900	89 113	141 580	304 330
Earnings per start												
Stallions	38	46	46	6 773	8 687	9 242	11 040	11 540	11 540	15 671	10 117	9 443
Mares	105	180	188	2 754	2 818	3 146	4 958	4 146	4 173	7 607	5 724	5 709
Geldings	125	246	251	2 243	3 024	3 302	3 788	3 832	3 939	5 456	3 156	3 171
Victories												
Stallions	38	46	46	1.50	8.00	8.50	1.76	8.57	11.20	1.95	7.01	10.20
Mares	105	180	188	0	1.00	1.00	0.68	1.91	2.28	1.46	2.56	2.92
Geldings	125	246	251	0	1.00	2.00	0.58	2.72	3.90	1.37	3.51	5.20
Race time auto												
Stallions	15	38	40	89.70	85.30	84.80	90.17	85.67	84.95	1.98	2.60	3.17
Mares	31	100	115	92.20	89.60	89.40	92.63	90.08	89.80	2.90	2.95	3.00
Geldings	17	137	164	92.20	89.30	88.80	92.55	90.32	89.42	2.42	8.03	7.60
Race time volt												
Stallions	38	46	46	92.25	86.85	85.60	92.60	87.60	86.72	3.67	3.36	3.74
Mares	91	166	174	95.50	91.40	90.50	96.25	92.17	91.56	4.05	4.20	4.22
Geldings	116	241	247	96.05	91.40	90.30	96.50	91.76	90.66	4.40	3.66	3.83

Table 2. Descriptive results, stratified by *DMRT3* genotype, for earnings (SEK) of the raced (n=485), and for the total sample of raced and non-raced Coldblooded trotters (n=769)

	Min	1st Q	Median	Mean	3rd Q	Max	Sd
Raced horses (n=485)							
AA	0	41 900	125 900	152 400	187 000	625 500	154 698
CA	0	33 500	103 400	267 300	303 100	3 189 000	440 444
CC	0	27 000	78 240	198 700	180 600	2 416 000	370 945
Raced and non-raced horses (n=769)							
AA	0	0	0	69 730	108 500	625 500	128 985
CA	0	0	35 250	180 900	185 300	3 189 000	383 104
CC	0	0	19 850	124 900	108 400	2 416 000	309 169

14. Appendix 3

Supplementary information of the total sample of raced and non-raced Coldblooded trotters

Table 1. Proportion of *DMRT3* genotype of raced, non-raced and the total sample of Coldblooded trotters in this study

	Number	AA	CA	CC
Raced	485	0.089	0.513	0.398
Non-raced	284	0.180	0.419	0.401
Total sample	769	0.122	0.479	0.399

Table 2. Average BLUP and inbreeding coefficient according to *DMRT3* genotype for the sample of raced (n=485), non-raced (n=284), and total sample of raced and non-raced Coldblooded trotters (n=769)

	AA	CA	CC	P-value[1]
Raced (n=485)				
BLUP	111 (6.55)	111.9 (6.49)	111.7 (6.35)	-
Inbreeding coefficient	5.77 (1.73)	6.09 (2.20)	6.03 (2.04)	-
Non-raced (n=284)				
BLUP	104.6 (4.96)	103.5 (4.69)	104.1 (5.33)	-
Inbreeding coefficient	6.56 (2.24)	5.96 (2.39)	5.85 (2.38)	-
Non-raced + raced (n=769)				
BLUP	107.5 (6.55)	109.2 (7.13)	108.9 (7.04)	-
Inbreeding coefficient	6.21 (2.05)	6.05 (2.26)	5.97 (2.17)	-

[1] P-value calculated using Kruskal-Wallis test

- Not significant (p>0.05)