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Effect of the glycogen synthase 1 (*GYS1)* mutation on performance traits in 169 Noriker draft horse stallions – a retrospective study

Thomas Druml[1], **Gertrud Grilz-Seger**[2], **and Gottfried Brem**[1]

[1]Institute of animal breeding and genetics, Veterinary University Vienna, Veterinärplatz 1, 1220 Vienna, Austria
[2]independent researcher: Pöckau 41, 9601 Arnoldstein, Austria

Correspondence to: Thomas Druml (thomas.druml@vetmeduni.ac.at)

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Abstract. The aim of this study was to investigate the effect of glycogen synthase 1 (*GYS1)* mutation on performance traits in Noriker draft horse stallions. Individual scores of 32 performance traits and the final performance score were obtained from 169 stallions that took part in the standardized stationary 30-day performance test throughout the years 2002 to 2014. In 2014 the stallions have been genotyped for the *GYS1* mutation resulting in 105 non-mutation-carriers, 57 heterozygous, and 7 homozygous animals. The mean frequency of animals carrying the mutation was 38 % (64 of 169). The final performance score and 32 single performance traits were analyzed using a linear model including the *GYS1* mutation genotype, the testing year and age as fixed effects. For the final performance score no effect of the *GYS1* mutation was found. In three single traits – driving ability, drafting manner, and kindness in the discipline heavy-load lodging – significantly lower scores for heterozygous horses than for non-carrier animals were obtained. Homozygous animals did not differ significantly from both groups. Our results from this retrospective study suggest no effect of the *GYS1* mutation on performance traits and on the final performance score.

1 Introduction

Polysaccharide storage myopathy (PSSM) was first described in nine horses from Quarter Horse-related breeds by Valberg et al. (1992). Valentine et al. (1997) were the first authors who documented PSSM in draft horses. Based on retrospective histopathological and medical records in eight Belgian and Percheron horses, Valentine et al. (1997) documented clinical symptoms which varied from overt weakness and muscle atrophy in two horses at the age of 2 and 3 years to recumbency with inability to rise in horses at the age of 4 to 21 years. In the year 2005, Valentine expanded the PSSM-associated symptoms in draft-horse-related breeds to abnormal hind limb gate, poor muscling, poor performance, exercise intolerance, severe rhabdomyolysis, spontaneous recumbency with inability to rise and "episodic colic" (Valentine, 2005).

In the early stage of the debate about the PSSM complex a heritable background, where environmental effects are modifying the disease pattern, was assumed. Hence, the influence of the mode of dietary modification on the management of clinical signs of PSSM was investigated (Ribeiro et al., 2004; Valentine, 2005). Furthermore, the effect of standardized exercise on metabolic muscle parameters have been analyzed in clinical case/control studies of Quarter Horse-related breeds and Thoroughbreds by De la Corte et al. (1999) and Ribeiro et al. (2004). These studies underline the positive effect of daily exercise and a low-starch and high-fiber diet on clinical symptoms in PSSM-affected horses.

In 2008 McCue et al. (2008a) identified an incompletely dominant inherited mutation (Valberg et al., 2011; Naylor et al., 2012; McCoy et al., 2014) in the skeletal muscle glycogen synthase 1 (*GYS1*) gene. The guanine (G) to adenine (A) base transition in exon 6 of the *GYS1* gene leads to an arginine (R) to histidine (H) amino acid substitution at codon 309 causing a gain of function, which results in an increase in glycogen synthase activity (generally referred to as G926 to A926 DNA mutation or R309 to H309

amino acid mutation; McCue et al., 2008a). With the detection of the point mutation (referred as R309H mutation and here in the text generally named *GYS1* mutation, chr. 10:18944308; dbSNP rs68898610; NCBI, https://www.ncbi.nlm.nih.gov/snp/?term=rs68898610, last access: 30 October 2016), the PSSM diagnosis became more differentiated and elaborated following a grading system of muscle biopsies. PSSM type 1 diagnosed histological samples are characterized by amylase-resistant periodic-acid Schiff (PAS) positive inclusions and PSSM type 2 diagnosed samples possess increased amylase-sensitive glycogen. The association of the *GYS1* mutation (genotypes for the R309H mutation are noted R / R – non-carrier; R / H – heterozygous carrier; H / H – homozygous carrier) with histopathologically confirmed PSSM type 1 horses varied from 65 to 87 %, where the association was found to be highest in draft horse breeds (McCue et al., 2008b). These results initiated a special interest in the *GYS1* mutation frequencies in draft horse breeds. McCue et al. (2010), Baird et al. (2010), and Druml et al. (2016) studied the *GYS1* mutation frequencies in 23 European and American samples of healthy draft horses.

The results of these studies indicate moderate to high frequencies of animals carrying the *GYS1* mutation (McCue et al., 2010; Baird et al., 2010), whereas the R / H and H / H horses did not show clinical symptoms at the time of sampling. So far, the incidence of clinical signs in horses carrying the *GYS1* mutation has not been studied at a population level; furthermore, it is not known if this gene mutation affects the general performance ability in draft horses. Submaximal exercise tests in draft horses and draft-horse-related breeds were conducted by Schwarz et al. (2011), Naylor et al. (2012), and Schröder et al. (2015). Amongst other things, the authors studied parameters the glucose metabolism in blood, resting, and post-exercise muscle enzyme activity in relation to the *GYS1* mutation genotypes of the probands.

The aim of this work was to determine the effect of the *GYS1* mutation on the performance traits in 169 Noriker stallions, which were trained to a high work level during the obligatory stationary standardized performance test in four different equestrian disciplines.

2 Material and methods

2.1 Data collection and performance traits

For this study we analyzed the performance data of 169 healthy Noriker stallions that took part in the obligatory stationary 30-day stallion's performance test in the years 2002 to 2014. DNA for genotyping the *GYS1* mutation was available from 164 stallions actually in use in the year 2014. For five additional stallions from earlier periods with R / R and H / H parents, it was possible to derive the genotype from the data published in Druml et al. (2016). The 169 stallions included in this study were born between 1999 and 2011, having a mean age of 3.82 years ±0.57 (ranging from 3.41 to 9.65 years) at the testing event. During the performance test all stallions were stabled on straw in the same boxes, having unlimited access to water, and were fed the same diet throughout the training process. The diet consisted of 20 kg hay on average, 4–6 kg concentrates according to the individual need and mineral supplementation. Four to six weeks before the performance test the stallions were individually trained by their owners.

The stationary performance test for Noriker stallions includes a training phase lasting 4 weeks and 2 days and a final test. The positive completion of the performance test is the precondition for the stallions to be registered as breeding stallions. Before the performance test a veterinarian check is conducted, and the training and fitness status of the stallions is examined by a supervision commission consisting of a veterinarian, the training leader, and one member of the breeders association. Throughout the whole training period the stallions and their health status are supervised by the same commission. During the performance test the stallions were trained at one facility by a team of professional riding and driving trainers in the disciplines riding, driving, working, and heavy-load lodging according to a training plan. The horses were worked 1 h per day (Monday–Friday), starting with riding, driving, and later proceeding to heavier work (working and heavy-load lodging). On Saturday the stallions were exercised in a training mill, and on Sunday they were rested in the boxes for 24 h without exercise.

The athletic development of the stallions was evaluated weekly by the training instructor and two judges from the breeding commission. In the four disciplines riding, driving, working, and heavy-load lodging, 11 physical and eight psychological traits were classified using an evaluation scale from 1 to 10 with a 0.5-point increase (Table S1 in the Supplement). In the final test 13 traits (9 physical traits and 4 psychological traits) within the four disciplines were scored by a team of external professional classifiers from the breeding association and the Austrian Fédération Equestre Internationale (FEI) driving and riding association. Finally, the performance ability of stallions is evaluated by the final performance score, which represents the weighted sum of mean evaluations including all 32 traits from the training and the final test (Table S1). The lower limits for a positive testing result are 60 points in the final performance score, e.g., six points in each single testing trait. Throughout the years 2002 to 2013 the *GYS1* status of the stallions was not known to breeders and the breeding organization, as the stallions were genotyped in 2014. Therefore, the test results and evaluations can be considered to be unbiased with regard to the horses' *GYS1* mutation genotype.

2.2 Genotyping and statistical analysis

In total, 164 Noriker stallions actually used in the breeding program of the year 2014 were genotyped for the *GYS1* mutation. Additionally, the genotype of 5 out of 73 stal-

lions not available anymore (already dead or not in the breeding program) could be derived from pedigree data using genotypic information from Druml et al. (2016). Due to this situation and the fact that the Noriker horse represents an endangered breed with a limited census, phenotypic and genotypic information from 169 Noriker stallions were available in this study. Genomic DNA, when not available from paternity testing, was extracted from hair root samples using the nexttec™ Tissue & Cells-kit (nexttec™, Hilgertshausen, Germany) following the manufacturer's protocol. The genotype of the *GYS1* mutation of each horse was obtained by applying a pyrosequencing approach on a PyroMark Q96 MD pyrosequencer (Qiagen, Hilden, Germany), where the following forward and reverse PCR primers for *GYS1* G926 to A926 DNA mutation at position 10:18944308: 5'-TGAAACCATGGGACCTTCTCC-3' and 5'-AGCTGTCCCCTCCCTTAGAC-3' were used, and the sequencing primer was 5'-CGAATCCAGGAGTTTGTG-3'. The PCR product was 230 bp long including the *GYS1* mutation in the center. The analyzed sequence was G/ATGGCCAT (McCue et al., 2008a).

Descriptive statistics and graphs concerning the scoring data were calculated using the software package SAS v.9.1 (SAS Inc., 2003). After data processing and quality control of the scoring data (checking for outliers and missing data), the residuals of scores were tested for normality. For testing fixed effects and to evaluate the effect of the *GYS1* mutation on the performance traits, the following linear model was applied: $Y_{jklm} = \mu + \text{year}_j + \text{age class}_k + GYS1_l + \varepsilon_{jklm}$, where year_j accounts for the effect of the testing years 2002 to 2014, age class_k takes differences due to age in the testing year (age classes 1 to 9) into account, and GYS1_l stands for the genotype effect of the *GYS1* mutation (R / R, R / H, H / H). In this study we aimed to analyze phenotypic raw data instead of using breeding values, which may distort the genotype effect of the *GYS1* mutation that cannot be separated from other effects due to limited population size (cf. Curik et al., 2013). In order to test for significant differences between trait means, the pairwise comparisons of LS (least square) means from the linear model were adjusted for multiple levels according to Tukey and Kramer (Tukey, 1949), and significant differences ($p < 0.05$) are indicated by superscripts.

3 Results

Of the 169 Noriker stallions 105 animals did not carry the mutation (R / R), 57 were heterozygous (R / H), and 7 stallions were homozygous (H / H). The frequency of animals carrying the mutation in this sample was 38 % (64 of 169), and the estimated allele frequency of the *GYS1* mutation was 21 %. In total 5408 individual evaluation scores from the performance tests of the years 2002 to 2014 were available for the statistical analyses. Mean values, standard deviations, significance levels for the fixed effects in the linear model, and the proportion of variance explained by the model (R^2) are presented in Table 1. For the training scores we could observe a shift from lower mean values in the years 2002 to 2007 to higher values in the latter years. This situation can also be noticed in the highly significant effect of training year in Table 1, where the R^2 of these models is 2 times higher than in the testing block. This might also be explained by the increasing professionalism of horse owners and the growing interest of draft horse breeders in equestrian disciplines throughout the recent years. Therefore, owners put more effort into the preparation of stallions before taking part in the performance test.

The effect of the *GYS1* mutation genotype (R / R, R / H, H / H) on the final performance score, which is the selection criteria for passing the performance test, was not significant and no differences in LS means between non-carriers, heterozygous, and homozygous animals were observed. Within all 19 traits in the four disciplines of the training block, scores did not show significantly differing LS means with regard to *GYS1* mutation genotypes. Only in the testing block did the effect of *GYS1* mutation genotype reach a significant level ($p < 0.05$) within the linear model (Table 1) in four traits: driving ability, drafting manner, willingness to work, and kindness in the discipline heavy-load lodging. Pairwise comparisons of LS means corrected for multiple levels show (Table 2) that the scores of the heterozygous stallions (R / H) were significantly lower than in non-carriers (R / R) in the three behavioral traits driving ability, drafting manner, and kindness in the discipline heavy-load lodging. In contrast homozygous stallions (H / H) did not differ significantly from R / R or R / H horses. A difference between *GYS1* mutation genotypes was not proven for all 32 phenotypic performance traits of Noriker stallions. The distribution of individual scores over the testing years 2002 to 2014 for the final performance score and the trait willingness in driving are shown in Fig. 1. The scores of homozygous animals (H / H) marked in red are distributed on the left and right side of the sample mean, reaching maximal values in the testing years 2014 and 2003 and submaximal values in the testing years 2014, 2013, and 2011 (Fig. 1).

4 Discussion and conclusions

In this study we analyzed the influence of the *GYS1* mutation on the final performance score and on 32 performance traits in 169 Noriker stallions. The frequency of animals carrying the *GYS1* mutation in this sample was 38 % (64 of 169). Based on the 30-day stationary and standardized performance test, all ways of utilization, which are commonly requested from a multipurpose medium-sized draft horse, are tested at a high-performing level. We could demonstrate that the *GYS1* mutation has no significant effect on the testing results of Noriker stallions represented by the final performance score. During the 4-week training period, no significant differences between R / R, R / H, and H / H horses

Table 1. Mean values, standard deviations (SDs), significance levels for the effects of the linear model, and level of determination (R^2) for performance traits within the training block and testing block of the performance test for 169 Noriker stallions.

Disciplines	Traits	Mean	SD	*GYS1*	Year	Age	R^2
Training block							
Riding	Kindness	8.08	0.64	n.s.	***	n.s.	0.39
	Willingness to learn	7.66	0.54	n.s.	***	n.s.	0.28
	Performing capability	7.93	0.53	n.s.	***	n.s.	0.36
	Walk	7.26	0.65	n.s.	***	n.s.	0.25
	Trot	7.37	0.61	n.s.	***	n.s.	0.30
	Gallop	7.37	0.60	n.s.	***	n.s.	0.30
	Riding ability	7.75	0.65	n.s.	***	n.s.	0.25
Driving	Kindness	7.64	0.48	n.s.	***	**	0.39
	Willingness to learn	7.45	0.40	n.s.	***	*	0.29
	Performing capability	7.48	0.48	n.s.	***	**	0.37
	Walk	7.33	0.56	n.s.	***	n.s.	0.30
	Trot	7.44	0.49	n.s.	***	*	0.41
	Driving ability	7.46	0.49	n.s.	***	**	0.33
Working	Drafting manner	7.61	0.51	n.s.	***	n.s.	0.37
	Concentration	7.58	0.55	n.s.	***	n.s.	0.33
	Kindness	7.55	0.56	n.s.	***	n.s.	0.33
Heavy-load lodging	Drafting manner	7.64	0.53	n.s.	***	n.s.	0.43
	Willingness to work	7.58	0.56	n.s.	***	n.s.	0.35
	Kindness	7.61	0.55	n.s.	***	n.s.	0.37
Testing block							
Riding	Walk	6.69	0.70	n.s.	n.s.	n.s.	0.17
	Trot	6.71	0.78	n.s.	n.s.	n.s.	0.13
	Gallop	6.76	0.70	n.s.	n.s.	n.s.	0.20
	Riding ability	6.73	0.87	n.s.	n.s.	n.s.	0.11
Driving	Walk	6.72	0.85	n.s.	n.s.	n.s.	0.19
	Trot	6.81	0.65	n.s.	n.s.	n.s.	0.11
	Driving ability	6.74	0.88	**	n.s.	n.s.	0.18
Working	Drafting manner	7.35	0.72	n.s.	n.s.	n.s.	0.16
	Concentration	7.40	0.71	n.s.	*	n.s.	0.17
	Kindness	7.45	0.76	n.s.	**	n.s.	0.21
Heavy-load lodging	Drafting manner	7.35	1.03	*	n.s.	n.s.	0.22
	Willingness to work	7.35	1.04	*	*	n.s.	0.21
	Kindness	7.45	1.05	*	*	n.s.	0.24
Final performance score	Weighted total score	102.8	18.54	n.s.	n.s.	n.s.	0.12

GYS1: effect of glycogen synthase 1 mutation in the linear model; year: effect of testing year in the linear model; age: effect of age classes in the linear model; R^2: level of determination of the linear model.
Levels of significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
n.s.: not significant.

could be detected in any of the 19 scored performing traits. A significant effect of the *GYS1* mutation could be found in four traits of the testing block: driving ability, drafting manner, willingness to work, and kindness in the discipline heavy-load lodging. In three of these traits (driving ability, drafting manner, and kindness in the discipline heavy-load lodging), heterozygous animals (R / H) showed significantly lower scores ($p < 0.05$) compared to non-carrier animals (R / R) after a correction for multiple levels (Table 2). The homozygous animals (H / H) did not differ significantly from the population. A significant deviation of R / H horses from R / R horses only could be observed in the three behavioral or psychological traits, where heterozygous horses showed on average a half score lower testing result. The reasons for these findings are unclear and need further investigation.

In this long-term retrospective study, the genotype of the stallions was not known in the performance evaluation from the testing years 2002 to 2013. None of the stallions participating in the performance tests showed PSSM-associated

Table 2. Multiple comparisons of mean scores from the performance test in 169 Noriker stallions between homozygous glycogen synthase 1 gene mutation carriers H / H, heterozygous carriers R / H, and non-carrier stallions R / R in the testing block traits driving ability, drafting manner in heavy-load lodging, willingness to work in heavy-load lodging, and kindness in heavy-load lodging.

Trait	R / R	R / H	H / H
Testing score driving ability	6.91[a]	6.47[b]	6.62
Testing score drafting manner in heavy-load lodging	7.49[a]	7.11[b]	7.79
Testing score willingness to work in heavy-load lodging	7.49	7.10	7.67
Testing score kindness in heavy-load lodging	7.60[a]	7.14[b]	7.92

[a, b] Values within a row with different superscripts differ significantly at $P < 0.05$.

clinical symptoms such as training intolerance and/or exertional rhabdomyolysis throughout the 15-year testing period (supervision commission ARGE Noriker, personal communication, 2015). The absence of clinical signs (weakness, training intolerance, muscle atrophy, rhabdomyolysis) was also mentioned by studies performing and evaluating submaximal exercise tests in draft horses (Naylor et al., 2012) and draft-horse-related breeds (Schwarz et al., 2011; Schröder et al., 2015). Naylor et al. (2012), who studied 125 Belgian and Percheron horses, argued that the low level of work demanded in their study was the reason for the absence of clinical signs in 54 *GSY1*-mutation-carrying animals. In addition, the same authors underline that the muscle histopathological changes in homozygous *GYS1* mutation carrier animals (H / H) are more severe. In our study we demonstrated that the performing status of homozygous H / H stallions does not deviate significantly from the sample mean in all traits. In the long term of 15 years H / H horses often had high-performing results (see year 2014, 2013, 2003; in Fig. 1). Schwarz et al. (2011), who conducted a submaximal exercise test in 50 Haflinger horses consisting of 5 min of walk, followed by 20 min trotting on the lunge, also did not detect clinical signs in nine R / H animals, which the authors attributed to the horses' management. Schröder et al. (2015) conducted a submaximal treadmill exercise test (2 min of walk, 13 min of trot) in seven R / R and seven R / H Haflinger horses. In this study no horse showed PSSM-related clinical signs. The authors mention that the mean age of 8 years of the horses tested could be the reason for the absence of symptoms, an argument for which they refer to the publication of Firshman et al. (2005). In this study 103 Belgian draft horses were examined for PSSM, shivering and for signs of weakness. The authors conclude that clinical signs of weakness as a pre-indicator for PSSM may be relevant at

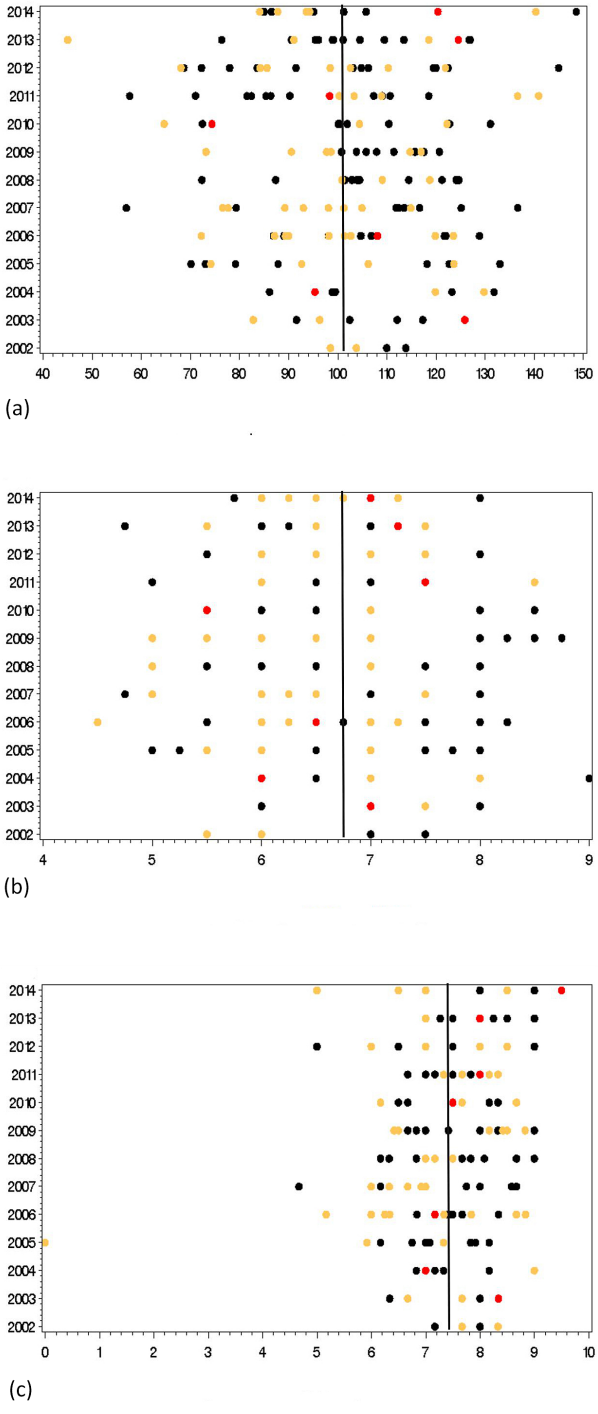

Figure 1. Individual scores from the performance test of 169 Noriker stallions in the testing years 2002 to 2014 for **(a)** the final performance score, **(b)** driving ability, and **(c)** willingness to work in heavy-load lodging. Non-carrier stallions of the glycogen synthase 1 mutation (R / R) are marked by black dots, heterozygous animals (R / H) are marked by orange dots, and homozygous animals (H / H) are marked by red dots. The overall mean score is indicated by a vertical line in each of the plots.

a later stage of age than observed (the age of 30 horses with signs of weakness reported by the owners was 8.0 ± 4.6 years within a range from 2 to 18 years). However, in one of the first descriptions of clinical signs of PSSM in draft horses, Valentine et al. (1997) reported severe clinical signs of PSSM in Belgian and Percheron horses of the age of 2 to 3 years.

Although Naylor et al. (2012) reported significant differences between the PSSM type 1 histopathology and subclinical symptoms of H / H, R / H, and R / R horses, they did not find clinical symptoms in 125 horses studied given the severity of their histopathology observed. This result is in accordance with our findings. Whereas Naylor et al. (2012) conclude that horses that are being worked hard may be more prone to muscle damage and rhabdomyolysis, we can counter this argument with our data. Performance tests in horse breeding are designed to evaluate horses under a high work load. Under these conditions we did not find an effect of the incompletely dominantly inherited *GYS1* mutation on performance traits. Nevertheless it is unclear to what extent environmental effects play a role in the expression of clinical signs in *GYS1*-mutation-carrying horses. Several authors mention the protective effect of regular and consistent exercise on PSSM diagnosed foals and adult horses (Byrne et al., 2000; De la Corte et al., 1999; Firshman et al., 2005). In the Noriker horse breed this positive environmental factor may be a central point, as nearly all colts that proceed to the performance test at the age of 3 years have been reared under semi-feral conditions in free-running stallion herds on pastures 1000 m above sea level. In order to study the impact of such environmental effects (rearing, feeding, and management) on the incidence of clinical signs of PSSM, we suggest collecting and analyzing long-term biographies of horses with both positive (H / H; R / H) and negative (R / R) *GYS1* status.

5 Data availability

Data are available on demand. As the genetic data and the phenotypic data are the property of the breeder associations, agreement of the ARGE Noriker (association of Austrian Noriker horse breeders) is necessary.

The Supplement related to this article is available online at doi:10.5194/aab-2-453-2016-supplement.

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