

Estimation of genetic parameters for melanoma in the Old Kladruber horse

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ABSTRACT: The aim of this study was to assess the prevalence of melanoma to investigate a possible genetic variation of this trait in the Old Kladruber horse. A total of 564 grey varieties of the Old Kladruber horse, 238 males and 326 females, with five generations of ancestors ($n = 1245$ animals) were analysed. Melanoma status was recorded for different stages. Three different analyses were conducted: a linear animal model (LM) with melanoma classified into five categories, threshold animal model (TM) with melanoma classified into five categories and threshold animal model (TMb) with melanoma classified into two categories (0 = absence, 1 = presence). All models included the fixed effects of year of evaluation, age, line, sex, greying level, random direct genetic effect, and the effect of animal's permanent environment. Heritability for melanoma occurrence was estimated for LM – 0.09, for TM – 0.27, and for TMb – 0.11. The coefficient of repeatability was estimated for LM – 0.77, for TM – 0.90, and for TMb – 0.99. The values of the Pearson's correlation coefficient and Spearman's rank correlation coefficient among breeding values estimated by LM, TM, and TMb models were from 0.82 to 0.88 and from 0.83 to 0.90, respectively, for data with pedigree information and from 0.77 to 0.84 and 0.77 to 0.88, respectively, for a subset of animals with measurements. Results suggest that additive genetic variation of melanoma occurrence in the Old Kladruber horse seems large enough to be exploited in a specific breeding programme.

Keywords: melanoma; linear model; threshold model; horse; heritability; breeding value

The white colour in greying white horses, as one of the basic colour varieties in the Old Kladruber horse, is a result of progressive greying – loss of coat pigmentation with age. A white horse is born with pigmented hair (the most frequently dark bay horse or black horse) and the skin remains pigmented although the coat loses pigment in the process of greying. An exception may be the white head or leg markings, potentially partial vitiligo as a result of aging.

Increased susceptibility to dermal melanoma has been proved in greying white horses (Fleury et al., 2000a; Heizerling et al., 2001). Dermal melanoma usually occurs in a greying white horse at 5–6 years of age (Jeglum, 1999). However, scarce cases of the occurrence of early stages of melanoma were observed in three-years-old horses (Rodriguez et al., 1997). Jeglum (1999) reported that melanoma appears in some populations at adult age in up to 80% of individuals. In the French breed Fleury et al.

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Table 1. Occurrence and quantification of melanoma

Melanoma stage	Description
0	absence of melanoma
1	early stages of plaque type or nodules 0.5 cm in diameter
2	several nodules 0.5 cm in diameter or one nodule 2 cm in diameter
3	one nodule or more 5 cm in diameter, or subcutaneous melanoma
4	extensive melanoma covered with skin, skin destruction, metastases
5	extensive melanoma, moist surface, metastases, cachexia, health disorders

(2000a,b) reported 67% incidence of melanoma in horses older than 15 years. In a study conducted in Lipizzan horses Seltenhammer et al. (2003) found out melanoma in horses older than 15 years in 75% of cases. Rodriguez et al. (1997) reported 100% frequency of melanoma occurrence in horses older than 10 years. Fintl and Dixon (2001) documented melanoma occurrence in white horses of different breeds – Welsh Pony, English Thoroughbred, Highland Pony and Irish Draught Horse. Horses were older than eight years in all cases. Typical sites of melanoma occurrence in white horses are the region under the tail, perianal and anal regions, perineal region, udder, prepuce, lips and eyelids and surrounding tissues, and auricles (Fleury et al., 2000a).

The relationship between greying and the melanoma appearance and development has not been explained until now. But the influence of inheritance on this disease has been proved both in horses (Seltenhammer et al., 2003) as well as in other animals. Data in literature suggest an association between the process of greying and melanoma occurrence (Sölkner et al., 2004). However, Rieder et al. (2000) stated that greying cannot be considered as a direct cause of melanoma appearance.

The objective of the present paper was the analysis of melanoma prevalence in the Old Kladruber horse in relation to sire line, sex, year of birth, and age at a description of greying level and the selection of a suitable model for the estimation of genetic parameters and subsequent genetic analysis.

MATERIAL AND METHODS

Background data for this study were collected by a survey of 564 individuals of the grey variety of the Old Kladruber horse. The database included repeated measurements in 29% of animals (727 ob-

servations in total). The occurrence of dermal melanoma was evaluated by adspersion and palpation at typical sites of occurrence (perianal and anal regions, perineal region, lips and eyelids and surrounding tissues and/or auricles) in the interval of 4 years (2005–2008). Data were recorded at all grey varieties of the sire line: Generale (21%), Generale-Generalisimus (22%), Favory-Generalisimus (9%), Favory (21%), Sakramoso (20%), Rudolfo (7%). A point scale according to Sölkner et al. (2004) was used for the evaluation of melanoma occurrence and stage (Table 1). Repeated measurements were carried out to assess changes in occurrence of melanoma over time.

Statistical analysis

Normality test did not prove normality of the tested trait - melanoma occurrence ($P > 0.05$). Table 2 shows particular stages of melanoma development. Effects influencing the occurrence were analysed by a logit model. The occurrence of melanoma was converted to binomial distribution: 0 = melanoma absence (86%), 1 = melanoma presence (14%). A linear logistic model with binary variable was used for a subsequent statistical analysis. The dependent variable (y_i) may assume the values of 1 with probability of melanoma presence π_1 or the values of 0 with probability of melanoma absence $1 - \pi_1$, for the observation i . The conditioned mean value of the explained binary variable

Table 2. Frequency of occurrence of melanoma cases

Melanoma stage	0	1	2	3	4
Number of cases	602	58	28	7	7
%	86	8	4	1	1

is expressed as a nonlinear function of explanatory variables

$$g(\pi) = \ln \left[\frac{\pi_i}{1 - \pi_i} \right] = X \beta \quad (1)$$

where:

π = probability of melanoma presence

X = incidence matrix of fixed effects

β = vector of parameters of the independent variable

In relation to the conversion of a variable to a binary variable the preliminary analysis of fixed effects influencing the occurrence of melanoma was evaluated by the method of logistic regression. The used statistical model comprised the following effects:

$$\log \left[\frac{\pi_{ijk}}{1 - \pi_{ijk}} \right] = \text{Line}_i + \text{Sex}_j + \text{Year}_k + b_1 \text{Age} + b_2 \text{Age}^2 + b_3 L^* + e \quad (2)$$

where:

Line_i = fixed effect of the i^{th} line

b_1, b_2, b_3 = coefficients of fixed regression

Age = age at evaluation

Sex_j = fixed effect of the j^{th} sex

L^* = greying level

Year_k = fixed effect of the k^{th} year of evaluation

e = residual error

The evaluation of melanoma in all individuals was performed by one classifier only. This is the reason why the effect of the classifier was not included in the model equation. The age of the evaluated animals ranged from 2 to 21 years (mean $\pm$ SD, 7.2 ± 5.68). Most of the animals ($\sim 78\%$) were evaluated before 10 years of age.

The greying status was measured using Minolta CM-2500D Spectrophotometer (Konica Minolta Sensing, Inc., Osaka, Japan). The coat colour was measured on four parts (neck, shoulder, belly, croup) using L^* , a^* and b^* colour system. To quantify the level of grey colouring, our analysis was related to the parameter L^* only. The higher the L^* values, the more grey is the coat colour of a horse. Value L^* used is a mean of three consecutive measurements.

Estimation of genetic and environmental parameters

The fixed effect of line was included in the model (2) by breeders' proposal due to its routine use in practice. However, the effect of line is not a suitable

indicator for grouping the animals according to relationship. Because, the additive genetic value is theoretically the average of the parents plus a random Mendelian sampling (Falconer and Mackay, 1996). For this reason, the relationship matrix was used in the mixed model (3) for further analysis.

Considering the selected fixed effects according to the above-mentioned model (2), for the accurate estimation of variance components and genetic parameters linear model (LM), threshold model (TM) and threshold model with binomial distribution of melanoma occurrence (TMb) in the Old Kladruber horse were tested in the present study:

$$y_{ijk} = \text{Year}_i + b_1 \text{Age} + b_2 L^* + a_j + pe_k + e_{ijk} \quad (3)$$

where:

y_{ijk} = stage of melanoma development

b_1, b_2 = coefficients of fixed regression

Age = age at evaluation

L^* = greying level

Year_i = fixed effect of the i^{th} year of evaluation

a_j = effect of animal ($j = 1, \dots, 1245$) with $N(0, A\sigma_a^2)$

pe_k = permanent environment of animal ($k = 1, \dots, 564$) with $N(0, I\sigma_{pe}^2)$

e_{ijk} = residual error with $N(0, I\sigma_e^2)$

The GIBBSF90 computer programme (Misztal et al., 2002) was used for the estimation of variance and covariance components for linear model while THRGIBBS1F90 programme (Misztal et al., 2002), which applies the Bayesian approach by means of Gibbs sampling algorithm, was used for threshold model. According to the graphical representation the first 10 000 samples were excluded as burn-in. A total of 100 000 samples were used for the estimation of posterior mean and SD of variance components. The following population parameters were derived from the estimated variance-covariance components: σ_y^2 – phenotype variance [$\sigma_y^2 = \sigma_a^2 + \sigma_{pe}^2 + \sigma_e^2$], h_a^2 – coefficients of direct heritability [$h_a^2 = \sigma_a^2 / \sigma_y^2$], c^2 – the ratio of animal permanent environment variance to phenotype variance [$c^2 = \sigma_{pe}^2 / \sigma_y^2$], R – repeatability [$r_m = (\sigma_y^2 - \sigma_e^2) / \sigma_y^2$] (Falconer and Mackay, 1996). The pedigree set for estimation of genetic parameters comprised eight generations of ancestors ($n = 1245$ animals).

RESULTS AND DISCUSSION

Sölkner et al. (2005) and Hofmanová (2010) reported uncorrelated relationship between melano-

Table 3. Estimations of logistic model parameters and odds ratio for melanoma occurrence

Fixed effects		Estimated	S.E.	Significance	Odds ratio
Intercept		–12.515	2.364	***	–
	Generale-Generalisimus vs. Generale	0.844	0.487		2.324
	Favory-Generalisimus vs. Generale	0.310	0.595	ns	1.363
Line	Favory vs. Generale	–0.972	0.446	*	0.378
	Sakramoso vs. Generale	–0.208	0.524	ns	0.812
	Rudolfo vs. Generale	0.602	0.668	ns	1.827
Sex	female vs. male	–0.850	0.397	ns	0.918
Age		0.354	0.158	*	1.426
Age ²		–0.001	0.006	ns	0.998
<i>L</i> *		0.104	0.030	*	1.111
Year	2006 vs. 2005	–0.641	0.432		0.526
	2007 vs. 2005	–0.454	0.451	ns	0.635
	2008 vs. 2005	–1.075	0.468	*	0.341

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, $P < 0.1$, ns = not significant

ma occurrence and the level of grey colouring (0.10 and 0.03 respectively). The value of the correlations could be evaluated as low and therefore the above-mentioned effect may be included in the models.

Analysis of environmental effects

Table 3 illustrates the estimated statistical significance of particular fixed effects included in the logit model. Table 3 also shows the estimations of an odds ratio of melanoma occurrence in combination with the studied effects: line, age, sex, greying level and year of evaluation in the Old Kladruber horse.

As shown in Table 3, based on P -value there was a statistically significant difference between the other lines and line Favory ($P < 0.05$), in which the 0.38 times lower the odds of melanoma occurrence was estimated compared to sire line Generale. The observed statistically significant difference among the sire lines indicates a possible influence of inheritance factors. No statistically significant differences in the odds of melanoma occurrence were determined in the other lines that were included in the analysis. Based on the microsatellite analysis of DNA Vostrý et al. (2011) reported no pronounced genetic differences among the particular sire lines.

Statistical significance was also proved for the correlation between the effects of the value of parameter L with melanoma, which confirms an assumption that the loss of pigment may be the cause of dermal melanoma appearance. On the other hand, Rieder et al. (2000) and Hofmanová et al. (2010) found the influence of L value on melanoma occurrence to be statistically not significant at the evaluation on the same significance level ($P < 0.05$).

A higher odds of melanoma occurrence was estimated for stallions compared to mares. However, the effect of sex was found statistically not significant in this trait. Sundberg et al. (1977) reported higher frequencies of melanoma occurrence in males. Fleury et al. (2000b) recorded a higher melanoma occurrence in geldings compared to mares and stallions but the differences were on the boundary of statistical significance. The other authors are also convinced that sex does not influence the occurrence of melanoma (e.g. Smith et al., 2002).

Statistical differences in the year of evaluation were probably caused by influences of external environment. It is not assumed that in such a short period over which melanoma occurrence was analysed (2005–2008) a pronounced odds in the genetic composition of the population that would result in a higher melanoma occurrence took place.

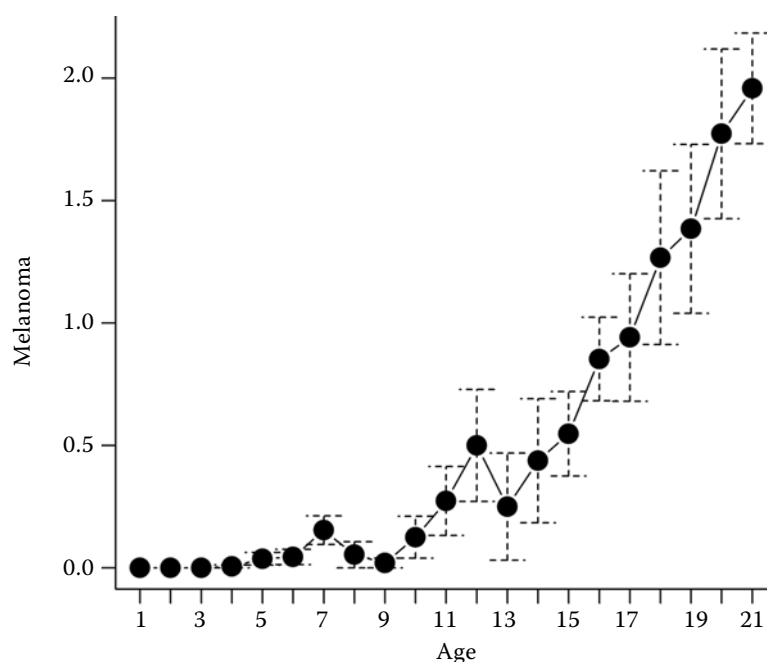


Figure 1. A relation of melanoma stage to age

The effect of animal age on melanoma occurrence was highly significant. Odds of melanoma occurrence is on average 1.43 times higher with each year of animal age. Figure 1 suggests a quadratic relationship of melanoma with age. However, this quadratic relationship was found statistically not significant in this trait. The course of phenotype values of melanoma development in Figure 1 corresponds with this finding. Figure 1 illustrates a progressive increase in melanoma stage with age. Early stages (values of 0.5–1) prevail in lower age categories. After the 20th year of age the average melanoma stage increases to 2. A high variability of melanoma stage was determined in all age categories. In the Kladruber population of white horses melanoma stage 5 – open stage – was not found out. These results support a hypothesis that the probability of melanoma occurrence increases with higher age. The high statistical significance of the effect of animal age corresponds to results of the above-cited authors. In some horse populations melanoma occurs in up to 80% of the individuals at adult age (Jeglum, 1999).

Like the animal age, greying level has a significant influence on melanoma occurrence. In the above-mentioned effect (greying level) a statistically significantly higher odds of melanoma occurrence was estimated for a higher greying level. In greying level (L^*) an odds of melanoma occurrence was estimated to be 1.11 times higher. The estimated higher odds of melanoma occurrence for a higher greying

level contradicts the assumption that the loss of pigment need not be a cause of the appearance of dermal melanoma (Rieder et al., 2000; Hofmanová et al., 2010).

Estimation of genetic parameters

Table 4 shows the estimations of variance components based on LM, TM, and TMb models. Higher values of all studied parameters were determined by threshold models (TM and TMb) compared to LM model. A large difference between LM and TM vs. TMb is probably caused by data transformation to binomial distribution.

Table 4 documents that the greatest influence on melanoma development is exerted by animal's permanent environment. In the particular models this effect explained 67% to 88% of total variance. The high values of animal's permanent environment variance and of repeatability coefficient (R) are caused by minimum odds in melanoma development with animal age. The high value of repeatability estimated by TMb model (0.99) is due to the expression of melanoma occurrence by a binary variable (yes, no) in which the stage of melanoma development is not considered.

Similarly like in variance components, higher values of heritability coefficient (h^2) were estimated by threshold models (TM and TMb). The highest h^2 value was estimated by TM (0.27). LM and TMb

Table 4. Estimations of variance components and genetic parameters with standard errors

	LM	TM	TMb
σ_a^2	0.02 (0.01)	0.28 (0.17)	6.78 (2.98)
σ_{pe}^2	0.16 (0.02)	0.67 (0.27)	54.10 (8.31)
σ_e^2	0.05 (0.00)	0.09 (0.03)	0.57(0.07)
σ_y^2	0.23	1.03	60.45
h^2	0.09 (0.04)	0.27 (0.16)	0.11 (0.05)
c^2	0.70 (0.05)	0.64 (0.17)	0.88(0.05)
R	0.79	0.91	0.99

σ_a^2 = additive genetic variance of direct effect, σ_{pe}^2 = variance of the effect of animal permanent environment, σ_e^2 = variance of the effect of residual error, σ_y^2 = phenotype variance, h^2 – coefficients of direct heritability, c^2 = the ratio of animal permanent environment variance to phenotype variance, R = direct repeatability

models showed similar values of h^2 (LM – 0.09 and TMb – 0.11). The higher value estimated by TM may be caused by a low frequency of melanoma of the higher stage in the analysed population. A comparison of the standard error of estimation with h^2 estimation shows that in TM the estimated value of h^2 did not reach the twofold value of standard error, therefore it is not possible to derive a statistically significant estimation of heritability coefficient by this model. Sölkner et al. (2005) reported similar values of heritability coefficient (0.12). Curik et al. (2002) estimated a higher value of heritability coefficient (0.24) in the population of 341 Lipizzan horses. Different values of h^2 estimated by Curik et al. (2002) compared to h^2 values obtained in the present paper may be due to different methods of estimation, different breed and different structure of data set. Most of the animals (~ 78%) were evaluated before 10 years of age. A difference between the Old Kladruber Horse and Lipizzan Horse is obvious from the stage of melanoma development. Stage 5 (exophytic growth of tumours which show wet surface and ulceration, metastases into different organs accompanied by paraneoplastic syndromes) has not been recorded

in the Old Kladruber Horse. The lower values of h^2 estimated in the present paper may also be caused by the age structure of evaluated horses. Sölkner et al. (2005) determined the value $h^2 = 0.19$ when only horses at 2 to 6 years of age were included in the evaluation. On the contrary, the analysis of only older horses showed an increase in h^2 to 0.36 (Sölkner et al., 2005). The increase in h^2 only in older horses is connected with the close correlation between melanoma stage and horse age.

Estimation of animal's genetic constitution for melanoma occurrence

Although it is not an intentionally bred population, the estimation of animal's genetic constitution for melanoma occurrence was expressed for illustration by means of the prediction of breeding value. Table 5 documents estimations of Pearson's (r_p) and Spearman's (r_s) rank correlation coefficients according to the prediction of breeding value for melanoma occurrence based on LM, TM, and TMb models for all animals included in the analysis. Correlation coefficients r_p and r_s showed

Table 5. Pearson's (above) and Spearman's (below) coefficients of correlation between LM, TM and TMb

	Entire			Partial		
	LM	TM	TMb	LM	TM	TMb
LM	–	0.86	0.88	–	0.78	0.84
TM	0.84	–	0.82	0.78	–	0.77
TMb	0.90	0.83	–	0.88	0.77	–

LM = linear animal model, TM = threshold animal model with melanoma classified into five categories, TMb = threshold animal model with melanoma classified into two categories

similar high positive values for both the subsets: Entire – comprising all horses including pedigree information, Partial – the evaluated horses only ($r_p = 0.86, 0.88, 0.82$ vs. $0.78, 0.84, 0.74$ and $r_s = 0.84, 0.90, 0.83$ vs. $0.78, 0.88, 0.77$). These high values of correlation coefficients in Table 5 indicate that there exist differences between both the sets. Table 5 documents that the entire data set, compared to the subset of partial results, showed a closer correlation among the breeding values estimated by LM, TM and TMb. This closer correlation corresponds to higher values of r_p and r_s in this data set. On the contrary, more pronounced changes in the rank of animals were observed in the subset of partial results. Smaller changes in breeding values and rank of animals in the entire set were caused by the fact that this set comprised also individuals without evaluation whose breeding value was estimated only on the basis of pedigree value. In these individuals fluctuations in the estimation of breeding value are not so large. Higher variability in horses with the evaluation of melanoma development may be influenced by a small set of animals and threshold evaluation of melanoma occurrence. In horses in the pedigree, whose breeding value is estimated on the basis of pedigree information, breeding value close to the population mean may be estimated in all tested models due to lower reliability. This may result in higher stability of breeding value estimations between the tested models.

Table 5 also shows that higher stability of breeding values was estimated between LM and TMb models compared to the remaining ones (LM and TM, TM and TMb). It may be influenced by data structure. Larger differences between breeding values predicted by TM model compared to the other models may be caused by a low percentage of higher stages of melanoma development. These conclusions also correspond to the lower statistical significance of heritability coefficient estimated by TM model. For these reasons TMb or LM model should be used for the estimation of genetic parameters and breeding value because higher stability of genetic constitution was estimated between them. TM model was found less suitable for the estimation of genetic variability and animal's genetic constitution for melanoma occurrence in the Old Kladruber horse. However, these conclusions may be influenced by a small population of the evaluated Old Kladruber horses. Therefore it is necessary to do further analyses.

Similar or higher values of correlation coefficients between threshold and linear models were

estimated in the other threshold traits, e.g. mastitis (0.99) (Heringstad et al., 2003) and calving ease (> 0.96) (Hansen et al., 2004). Some papers also compared suitability of threshold model with that of linear model both on simulated data (Gianola, 1982; Abdel-Azim and Berger, 1999) and on real data (Varona et al., 1999; Silvestre et al., 2007). The cited authors reported higher suitability of threshold models for categorical data compared to linear model. Abdel-Azim and Berger (1999) stated that comparable values between linear and threshold models are obtained only if categorical traits include more categories (ca. 10) that approximate categorical data to normal distribution. These authors also concluded that in categorical data with a low number of classes that were skewed from normal distribution threshold model should be preferred to data transformation or the formation of a new variable using a linear model was recommended. On the contrary, Matos et al. (1997) did not find out any marked differences between linear and threshold models used for the evaluation of categorical data.

CONCLUSION

The occurrence of dermal melanoma in the population of the Old Kladruber horses was confirmed and its prevalence and frequency of occurrence are related to some factors – age and genetic constitution, year of evaluation and greying level. The effect of sex on the frequency of melanoma occurrence was not proved.

The results document that TMb or LM should be used for the estimation of genetic parameters and subsequent genetic analysis of melanoma occurrence in the Old Kladruber horse. The use of TM is less suitable due to a small number of observations and low percentage of the higher stage of melanoma development in the evaluated population.

The results suggest that additive genetic variation of melanoma occurrence seems enough in the Old Kladruber horse to be exploited in a specific breeding programme.

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