

<https://doi.org/10.17221/136/2018-CJAS>

Estimation of genetic and non-genetic effects influencing coat colour in black horses

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Citation: Hofmanová B., Vostrý L., Vostrá-Vydrová H., Dokoupilová A., Majzlík I. (2019): Estimation of genetic and non-genetic effects influencing coat colour in black horses. Czech J. Anim. Sci., 64: 41–48.

Abstract: The aims of this study are the quantitative characterisation of the coat colour in the black variety of the Old Kladruher Horse breed, the estimation of the coat colour genetic parameters, and the determination of the factors that influence coat colour intensity. A total of 252 horses aged 1–24 years were included in the study. The colour parameters were taken using a Konica Minolta spectrophotometer CM-2500d at four different body parts during the summer and winter seasons, resulting in 1748 phenotypic records. The possible effects of sex, season, housing system, age and body part on the spectrophotometric parameters were analysed. The results confirmed that the intensity of the black coat colour can be influenced by a number of factors (sex, season, housing system, and age). The mares showed more red and yellow colour compared to the stallions. In the winter season, the horses had a lighter colour with a lower proportion of reddish-brown hairs. Horses kept outdoors, especially in the summer months, had a lighter colour with a higher proportion of red and yellow. Although the age was statistically significant, no specific trend was observed. The differences in colour found between body parts (neck, shoulder, belly, and croup) are in line with current knowledge – reddish fading in the blacks primarily occurs in the belly region. The estimated heritability values suggest possible multifactorial inheritance, especially with regards to the reddish tinge in hairs ($h^2 = 0.20$ to 0.29 according to body part).

Keywords: heritability; Old Kladruher; pigmentation; spectrophotometry; variability

The coat colour in horses is generally considered a qualitative trait which occurs on the basis of the Mendelian principles of inheritance. Currently, more than 10 major genes are known to participate in colour determination (reviewed by Thiruvendakadan et al. 2008; Rieder 2009). Three basic colours (chestnut, bay, black) are determined by the loci AGOUTI (*ASIP* gene) and EXTENSION (*MC1R*

gene) (Marklund et al. 1996; Rieder et al. 2001). Black coat colour is determined by the homozygous recessive genotype of the AGOUTI locus and at least one dominant allele at the EXTENSION locus (*a/a E/-*) (Sponenberg 1996).

Within the particular colour phenotypes, significant differences exist that cannot be explained by the Mendelian principles of inheritance and by

Supported by the Ministry of Education, Youth and Sports of the Czech Republic (S grant) and by the Ministry of Agriculture of the Czech Republic (Project No. QJ1510141).

the action of currently known major genes, for example differences in the level of greying (Curik et al. 2013; Hofmanova et al. 2015), the shades of chestnut or bay (Toth et al. 2006) or the variability of leopard complex spotting phenotypes (Druml et al. 2017). There are two different types of black coat colour – non-fading (jet or raven black) is charcoal black with a metallic or blueish shine and fading – black colour without shine, fading to a reddish-brown tinge, especially when exposed to the sunshine in the summer months. There have also been reported differences in the colour of new-born foals. The usual blacks are grey at birth, whereas jet blacks are exactly that from the beginning (Sponenberg 1996). The genetic determination of these two types of black colour is not known. The existence of dominant black in horses as a result of the presence of the E^D allele at the EXTENSION locus (Sponenberg and Weise 1997) has not been proven at the molecular level.

Although no direct relationship between coat colour and performance has been proven in horses in the past (Stachurska et al. 2007), it is considered to be aesthetically important in some breeds.

The Old Kladruber Horse, a baroque horse breed created for ceremonial use at the imperial court and by the high clergy, was used as a model population for the study into the basic quantitative parameters of black colour presented in this paper. The advantage of this breed for this study lies in the sufficient number of individuals bred in the same conditions and the reliable breeding documentation kept for a long time. The Old Kladruber Horse is the warm-blooded Czech autochthonous breed that originated at the end of the 16th century and which descends from Old Spanish and Old Italian horse breeds. In the beginning, there were horses of different colour varieties in the herd, which was later reduced to two, namely grey and black. The horses have maintained these two colours and their original baroque characteristics to this day. Besides their original use for ceremonies, the Old Kladruber Horse is used as a sports horse for driving events, dressage and pleasure riding.

At present, this unique breed is included in the Czech programme for the preservation of genetic resources and has been the subject of a number of studies focused on monitoring the genetic diversity of the population and the maintenance thereof in the future (Vostry et al. 2011; Janova et al. 2013; Kasarda et al. 2016; Vostra-Vydrova et al. 2016).

As of 31st December 2017, the population of the Old Kladruber Horse breed consists of some 1800 individuals, of which 52% are black. There are five sire lines within the black variety (Generalissimus, Sacramoso, Siglavi Pakra, Solo, Romke).

The coat colour is an important exterior trait in a breed that is primarily destined for ceremonial use. The grey colour has already been tackled in previous studies (Majzlik et al. 2010; Hofmanova et al. 2015). The black population of the breed exhibits both colour types mentioned above, with undesirable “fading black” occurring in a high proportion of individuals. Breeders’ efforts are currently focused on changing this proportion in favour of the more attractive jet blacks. The quantitative characterisation of the coat colour and the estimation of the coat colour genetic parameters as well as the determination of the factors responsible for the colour intensity being aims of the study, could be helpful to achieve this goal.

MATERIAL AND METHODS

In total, 252 horses (139 mares, 113 stallions) of 1 to 24 years of age were included in the study. These horses were the offspring of 28 sires (mean number of offspring per sire 7.9) and 106 dams (mean number of offspring per dam 2.1). All animals were kept in the same place, the National Stud Farm Slatiňany. In the summer season, the horses were divided into two groups – stabled (55%) and permanently kept outside (45%). There was no equipment covering the body of horses used during their stay at the pasture. Even though the breeding mares are usually kept separately from the stallions, there were many young horses of both sexes kept under the same conditions (permanently kept outside), therefore we evaluated sex and housing system as separate effects. The horses were divided into four different groups according to age (1–3 years, 3–7 years, 7–17 years, 18+ years).

Unfortunately, information on the fading or jet black colour of the horses is not included in the studbook record. On the basis of a subjective assessment, we determined only a small number of jet blacks (10%) in the population, which did not allow to evaluate them as a separate group in the statistical analysis.

Spectrophotometric measurements were taken during the summer (17th–18th August, 2015) and winter (20th–21st January, 2016) seasons. The groups

<https://doi.org/10.17221/136/2018-CJAS>

of horses measured were not quite identical, but both measurements were available for 80% of the animals. The measurements were taken on four body parts of each animal – neck (place with no mane covering), shoulder, belly and croup. A total of 1748 phenotypic records were obtained (4 or 8 per animal).

The measurements were taken by a Konica Minolta spectrophotometer CM-2500d (Konica Minolta, Japan) in accordance with Toth et al. (2006). The parameters measured under the CIE (Commission Internationale de l’Eclairage) system consisted of: L^* – lightness (0 = black, 100 = white), a^* – redness (–128 = green, +127 = red), b^* – yellowness (128 = blue, +127 = yellow). The L^* parameter is the most important for the black colour, as is the parameter of the green-red (a^*) axis for detecting possible fading of the hairs to a reddish-brown tinge. Each record of the parameter is the average of three consecutive measurements taken at the same spot (automatically done by the spectrophotometer).

PROC MIXED of SAS software (Version 9.3, 2012) using repeated statements for the purpose of repeated measurements was employed for the analysis of the effects responsible for the intensity of the black colour. The following model was used to analyse the possible factors affecting all three spectrophotometric parameters (L^* , a^* , b^*):

$$Y_{ijklmn} = \mu + AGE_i + SEX_j + SEASON_k + HOUSING_l + PART_m + pe_n + e_{ijklmn}$$

where:

- Y_{ijklmn} = parameter value (L^* , a^* , b^*)
- μ = overall mean
- AGE_i = fixed effect of age group ($i = 1$: 1–3 years, 2: 4–7 years, 3: 8–17 years, 4: 18+ years)
- SEX_j = fixed effect of sex ($j = 1$: male, 2: female)
- $SEASON_k$ = fixed effect of season ($k = 1$: summer, 2: winter)
- $HOUSING_l$ = fixed effect of housing ($l = 1$: stable, 2: free housing)
- $PART_m$ = fixed effect of body part ($m = 1$: neck, 2: shoulder, 3: belly, 4: croup)
- pe_n = permanent environment of the n^{th} horse
- e_{ijklmn} = residual error

The variances of genetic and non-genetic effects in the studied trait were estimated by the REML method using the DMU software package (Version 6, Release 4.7) (Madsen and Jensen 2008). The

following single-trait animal model was used for the estimation of the genetic parameters (heritability, repeatability):

$$Y_{ijklmno} = \mu + AGE_i + SEX_j + SEASON_k + HOUSING_l + PART_m + a_n + pe_o + e_{ijklmno}$$

where:

all the effects were the same as in the previous model

- a_n = random direct genetic effect of the n^{th} individual with $\sim N(0, \mathbf{A} \sigma_a^2)$
- pe_o = random permanent environment of the o^{th} horse with $\sim N(0, \mathbf{I}_{pe} \sigma_{pe}^2)$
- $e_{ijklmno}$ = random residual error $\sim N(0, \mathbf{I}_e \sigma_e^2)$
- $\mathbf{I}_{pe}, \mathbf{I}_e$ = identity matrices of the appropriate dimensions
- \mathbf{A} = matrix of additive genetic relationships among the individuals
- σ_a^2 = additive genetic variance of direct genetic effects
- σ_{pe}^2 = permanent environmental variance
- σ_e^2 = variance of residual error

The DMU package was also used for the calculation of the standard errors of the estimated genetic and non-genetic variances. The pedigree set for the estimation of the genetic parameters consisted of five generations of ancestors (629 animals).

Pearson correlation coefficients were calculated in order to characterise the differences/similarities between the body parts. Based on the correlation matrix, a Principal Component Analysis (PCA) plot was constructed.

RESULTS

The ranges of the observed L^* , a^* and b^* values were 15.54–31.18, 0.17–7.87, and 0.2513.68, with the mean values and standard deviations (in brackets) 21.91 (2.44), 2.27 (1.34), and 2.64 (1.78), respectively. The mean values and variability for the four measured spots and overall colour are shown in Figure 1.

Table 1 shows the differences between the stallions and the mares, between the summer and winter seasons, and between those horses housed in stalls and those horses kept permanently outdoors in the meadows. The difference in the L^* parameter between the mares and the stallions was at a level of statistical significance ($P = 0.02$), while statistically significant differences ($P < 0.001$) were determined for the a^* and b^* parameters. The mares had higher values (more red and yel-

Table 1. Values of Least Squares Means (LSMEAN) with standard errors (SE) for the gender, season and housing

	L*		a*		b*	
	LSMEAN	SE	LSMEAN	SE	LSMEAN	SE
Male	22.22 ^a	0.10	2.16 ^a	0.04	2.51 ^a	0.07
Female	22.02 ^a	0.13	2.99 ^b	0.05	3.70 ^b	0.09
Summer	20.83 ^a	0.10	3.09 ^a	0.04	3.32 ^a	0.07
Winter	23.41 ^b	0.13	2.06 ^b	0.05	2.89 ^b	0.09
Free housing	23.21 ^a	0.13	2.73 ^a	0.05	3.54 ^a	0.09
Stable	21.03 ^b	0.11	2.42 ^b	0.04	2.68 ^b	0.07

^{a,b}significant differences at $P < 0.001$

low colour). The season was another statistically significant factor influencing the intensity of the coat colour ($P < 0.001$ for all three parameters).

The differences in the parameters under study in relation to age are presented in Figure 2. Although age was a statistically significant factor in relation to all three of the parameters under study ($P < 0.001$), no trend was observed.

The differences between the body parts measured on the horses showed a statistical significance for all the parameters under study ($P < 0.001$). The values measured on the neck and the croup were lower compared to those measured on the shoulder and on the belly (Figure 3).

Estimations of variance components and genetic parameters (heritability) for overall coat colour and body parts are shown in Tables 2 and 3. The results reveal that the effect of the permanent en-

Table 2. Estimations of variance components and genetic parameters with standard errors (in brackets)

	L*	a*	b*
σ_a^2	0.61 (0.45)	0.18 (0.07)	0.43 (0.17)
σ_{pe}^2	1.70 (0.41)	0.18 (0.07)	0.36 (0.12)
σ_e^2	2.01 (0.09)	0.31 (0.13)	0.82 (0.03)
σ_y^2	4.33	0.67	1.61
h^2	0.14 (0.06)	0.27 (0.10)	0.22(0.10)
r^2	0.53	0.54	0.49
σ_{pe}^2/σ_y^2	0.39	0.27	0.27
σ_e^2/σ_y^2	0.46	0.46	0.51

σ_a^2 = additive genetic variance, 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 = coefficient of heritability, r^2 = coefficient of repeatability

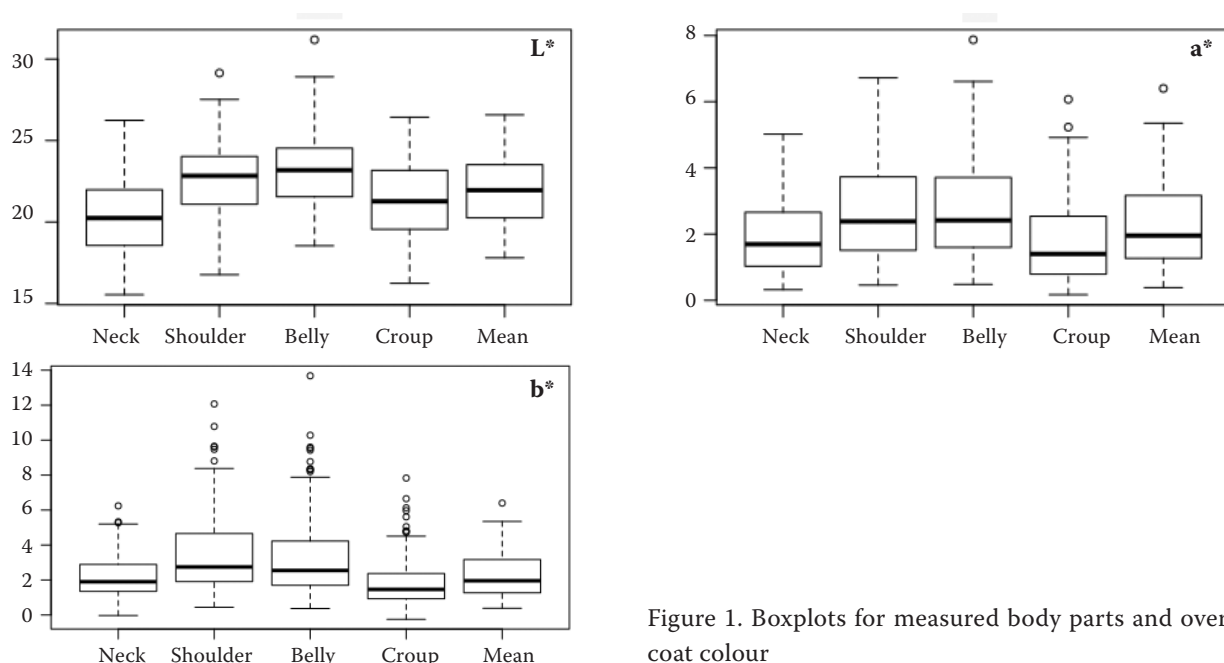


Figure 1. Boxplots for measured body parts and overall coat colour

<https://doi.org/10.17221/136/2018-CJAS>

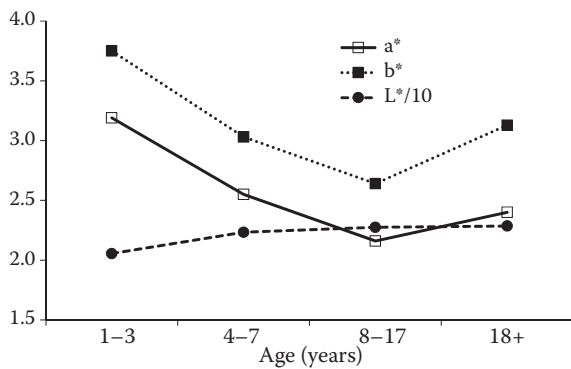


Figure 2. Least Squares Means values for age groups

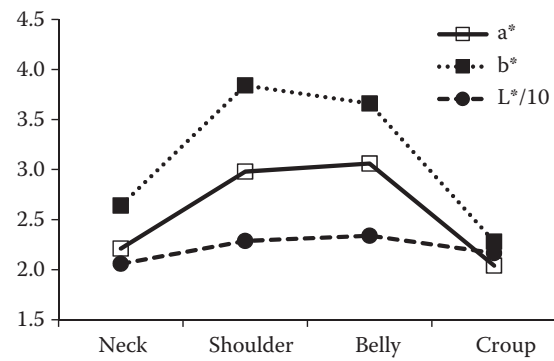


Figure 3. Least Squares Means values for body parts

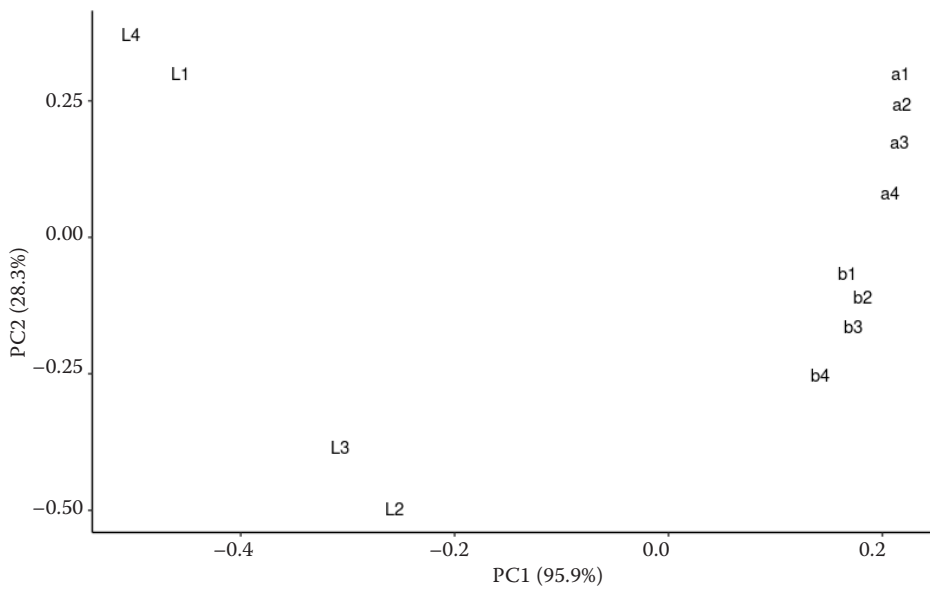


Figure 4. Principal component analysis of correlation matrix between four measured body parts (1 = neck, 2 = shoulder, 3 = belly, 4 = croup)

environment of an individual and the effect of residual error contribute the most to total variability, whereas additive genetic variance produces low values. For overall coat colour, the estimated heritability coefficients for the particular parameters ranged from 0.14 (L*) to 0.27 (a*) (Table 2). The estimated heritability coefficients for the body parts were in the

range of 0.01–0.33 (Table 3). Generally, the lowest heritability was indicated by the parameters for the colour at the shoulder, whereas the highest at the croup. The values for the a* and b* parameters were higher compared to those for the L* parameter.

The correlations between the measured points were high for all three parameters (Table 4). The

Table 3. Components of variance and heritability estimates for the particular body parts

Body part	L*				a*				b*			
	σ_a^2	σ_{pe}^2	σ_e^2	h^2	σ_a^2	σ_{pe}^2	σ_e^2	h^2	σ_a^2	σ_{pe}^2	σ_e^2	h^2
Neck	0.01	0.00	3.04	0.01	0.10	0.00	0.27	0.26	0.22	0.00	0.48	0.31
Shoulder	0.11	0.01	3.45	0.03	0.15	0.00	0.60	0.20	0.36	0.08	1.57	0.18
Belly	0.23	0.00	3.60	0.06	0.23	0.00	0.56	0.29	0.69	0.00	1.58	0.30
Croup	0.29	0.00	2.48	0.10	0.13	0.00	0.33	0.29	0.27	0.00	0.55	0.33

σ_a^2 = additive genetic variance, σ_{pe}^2 = variance of the effect of animal permanent environment, σ_e^2 = variance of the effect of residual error, h^2 = coefficient of heritability

Table 4. Pearson's correlation coefficients between body parts for L*, a*, b* parameters

	Neck	Shoulder	Belly	Croup
Neck	1	0.87, 0.91, 0.86	0.83, 0.90, 0.84	0.80, 0.87, 0.80
Shoulder		1	0.85, 0.89, 0.85	0.83, 0.89, 0.83
Belly			1	0.86, 0.92, 0.86
Croup				1

L* – the first value; a* – the second value; b* – the third value ($P < 0.0001$ for all values)

PCA plot based on the correlation matrix shows the separation of the L* parameter from a* and b* parameters according to the first principal component (PC1), which explains 96% of the variation in the data set. The second principal component (PC2) separates the measurements of the L* parameters at the neck and croup from those taken at the shoulder and belly (Figure 4).

DISCUSSION

The results reveal that the intensity of the black coat colour can be influenced by a number of external factors (sex, season, housing system, age). The values of the a* and b* parameters for the mares were higher (more red and yellow colour) than those for the stallions. The lighter tinge of hair in the coats of mares compared to stallions in blue duns (diluted colour derived from black) was reported by Stachurska et al. (2004) in primitive Polish breeds. Hofmanova et al. (2015) found differences in the level of greying between stallions and mares in Old Kladruber greys. The potential effect of sex hormones (mainly testosterone) on melanogenesis, and simultaneously on pigmentation, was described e.g. by Slominski et al. (2004). However, the hypothesis was not experimentally tested in horses.

In the winter season, the horses had a lighter colour with a lower proportion of reddish-brown hairs. The differences in colour intensity may be due to the seasonal change in coat; in winter the hair are stronger, fuller and longer. Taking into account its fast growth, the transport of melanin to the hair may not be sufficient. Kim et al. (2003) reported on the negative effect of low temperatures on the synthesis of melanin.

The analysis also confirmed the effect of housing. This fact is well-known among breeders, in particular with regards to the summer season when

changes occur in the colour intensity and tinge of the coats of those horses kept outdoors (they have a lighter colour with a higher proportion of red and yellow). These conclusions are in agreement with common knowledge on the influence of sunlight on changes in the black colour (Sponenberg 1996).

Although age was a statistically significant factor in relation to all three of the parameters under study ($P < 0.001$), no trend was observed. This is in contrast to the grey horses, where the values of the L* parameter continually increase until ca. 10 years of age when the greying process finishes and the horses attain their final coat colour (Curik et al. 2013; Hofmanova et al. 2015). For the black horses, the smallest difference in relation to age among the three measured parameters was found in the L* parameter. For the a* and b* parameters, the highest values were generally measured in young horses up to 3 years of age. Higher values of a* and b* parameters (more red and yellow colour) in foals compared with older horses was also presented by Stachurska et al. (2004) in blue duns. The colour changes with age are not so observable in non-greying horses, but the difference in colour especially between foals and adult horses was previously published (Sponenberg 1996).

The differences in colour found between specific body parts are in agreement with practical knowledge – reddish fading in blacks is primarily seen in the belly region (Sponenberg 1996). The question is why on this body part, because the croup is much more exposed to sunlight. The effect of the genealogical line was tested in a previous analysis. Despite the assumption made on the basis of breeders' observations that horses from the Romke line (the line founder stallion Romke of Friesian breed) had a darker colour, the effect of the line was not proved in any parameter (unpublished data). The explanation lies perhaps in the rotational mating plans used, with the result that every line has some genetic contribution

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from other black lines. In the population of Old Kladruber greys, for which the same mating system is used, no effect of the line on the speed and level of greying was proved. However, there were statistically significant differences between the particular lines with regards to the occurrence of melanoma and vitiligo (Hofmanova et al. 2015).

The estimated heritability values suggest possible multifactorial inheritance especially of the reddish tinge in hairs. Toth et al. (2006) published comparable heritabilities for the L^* parameter, whereas the within-colour class heritabilities for the a^* and b^* parameters were negligible. The L^* parameter is an important criterion with regards to greying in grey horses because its value corresponds to the total melanin content in the hairs (Toth et al. 2006). The heritability of greying in Old Kladruber greys was estimated as $h^2 = 0.52$ (Hofmanova et al. 2015) and comparable values for Lipizzaners were estimated by Curik et al. (2013).

The differences in the genetic determination of the fading and non-fading black colour have not been known until now. It is possible that the resultant phenotype can be influenced by minor genes with a cumulative effect. Theoretically, the black colour could also be influenced by the genotype at the EXTENSION locus. It can be assumed that the majority of Old Kladruber blacks are homozygous at this locus (E/E), nevertheless, due to the occasional segregation of chestnut foals, heterozygous individuals (E/e) also occur in the population. Unfortunately, the genotypes of the individuals were not available for this study. There is a possibility that the presence of recessive allele e , known as the “red factor”, could influence the occurrence of the fading to the reddish-brown tinge in the hairs. Rieder et al. (2001) reported the influence of the EXTENSION locus in bays – bays with a lighter shade of hair were heterozygous (E/e), whereas dark bays were homozygous (E/E). Druml et al. (2018) found a significant relationship between $A/a E/E$ genotype and darker shades in bay horses. Also Sakamoto et al. (2017), who studied segregation ratios by means of pedigree analysis, confirmed a relationship between AGOUTI (A/a) and EXTENSION (E/E) genotypes with darker shades of bays.

In the future, the presence of a polygenic component within the black coat colour could be solved by genomics. The availability of objective and exact information on animal phenotypes is a require-

ment for successful analyses. This and previous studies (Stachurska et al. 2004; Toth et al. 2006; Curik et al. 2013; Hofmanova et al. 2015; Druml et al. 2018) focused on determining the quantitative characterisation of coat colour in horses have proven that the Minolta Spectrophotometer is a suitable instrument for the acquisition of such data.

CONCLUSION

This paper is the first study into the black coat colour on a model population of 252 Old Kladruber horses, whereby the exact (spectrophotometric) measurements of the optical L^* , a^* and b^* parameters for coat colour were used. The evaluated results of the measurements indicate that the intensity and tinge of the black coat colour are mainly affected by environmental factors (season, housing system). Statistical analysis also suggests that the sex of a horse also has a potential effect, whereby the potential role of sex hormones should be verified experimentally. The effect of season and housing system was proved in all three spectrophotometric parameters. The L^* parameter showed stability in relation to age. The values of the a^* and b^* parameters, which describe the component colours of the spectrum, were higher in young horses up to the age of three years compared to adult individuals.

The estimations of heritability for the overall coat colour and measured body parts revealed the dominant effect of environmental factors on total variability. With regards to the effect of the genotype on the intensity and tinge of the black colour, the effect of the presence of the recessive allele at the EXTENSION locus should be assessed in future, as should the occurrence and action of yet unknown possible polymorphisms at the AGOUTI locus and/or polymorphisms in the other genes associated with coat colour.

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Received: 2018–07–12

Accepted: 2018–11–21