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Discriminant Analysis of Colour Measurements Reveals Allele Dosage Effect of *ASIP/MC1R* in Bay Horses

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ABSTRACT

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Considering the variability of bay coat colour, we aimed to investigate the association of different shades of bay with *ASIP* and *MC1R* genotype combinations and we studied the discrimination between the bay and black coat colour. We phenotypically characterized coat colour using a spectrophotometer. The measurements were based upon international standards as defined by the CIE L*a*b* colour system and we phenotyped five different body parts (neck, armpit area, shoulder, belly, croup) of 43 bay and 14 black horses kept under standardized conditions. From the five measuring points a stepwise discriminant analysis revealed that chromacity and luminescence of armpit area and luminescence of the neck were the most important traits to differentiate between black and bay horses, whereas it was shown that the red colour spectrum of neck, luminescence of the neck, and luminescence of the armpit area grouped bay horses according to their *ASIP* and *MC1R* genotype combinations. Within the group of bay horses the analyses identified a single *ASIP/MC1R* genotype combination (*A/a E/E*) where colour variables differed significantly from the three remaining genotype groups. *A/a E/E* horses were characterized in all body parts except in the armpit region by significant darker shades (lower luminescence, less chromacity). Regarding classifications of coat colour, we found in the significant cluster of *A/a E/E* horses the coat colour categories seal brown and dark (mahogany) bay. Overall, we were able to show that the characterization of equine coat colour based upon international standards as defined by the CIE L*a*b* colour system represents a valuable tool for a precise description of colour variation and association analyses.

Keywords: horse; coat colour; *ASIP*; *MC1R*; CIE L*a*b

Bay coat colour occurs at high frequencies within numerous breeds at a global level (Reissmann et al. 2016). The mode of inheritance of bay, black, and chestnut coat colour was clearly understood throughout the 20th century. The recessive mode of chestnut inheritance was firstly published in 1906 by Hurst (Hurst 1906) and the interaction between two loci resulting in bay, black, and chestnut coat colour was clearly described by Munckel (1929). This still valid basic concept could be confirmed on a molecular level in the 1990s by the detection of two causal mutations: a single base substitution (C901T; AF288357) in *MC1R* (melanocortin-1-receptor) on chromosome 3, and an 11-bp deletion in exon 2 (AF288358) in *ASIP* (agouti signaling protein) on chromosome 22 (Marklund et al. 1996; Rieder et al. 2001). Although the gene interaction for *ASIP* and *MC1R* in bay horses is well described, the huge phenotypic variability in colour shades ranging from light bay to seal brown remains unclear. Within the framework of cross-species sequencing and mutation analyses, which resulted in the detection of the 11-bp deletion in exon 2 (AF288358) in *ASIP* (Agouti locus), Rieder

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et al. (2001) also investigated allele effects of *MC1R*, *ASIP*, and *TYRP1* (tyrosinase-related protein 1) on colour phenotypes of horses, which were derived from classification. For *MC1R* and *ASIP* associations Rieder et al. (2001) chose a twofold classification, differentiating between bay and dark bay. Whereas *A/a* and *A/A* horses did not differ in shades, a significant difference between bay *E/e* heterozygous and dark bay *E/E* homozygous horses was found. In a recent pedigree analysis, which was based upon the breeding records and coat colour classifications of 153 778 Thoroughbred horses, Sakamoto et al. (2017) analysed the segregation ratios of offspring in relation to different mating combinations. From the segregation ratios Sakamoto et al. (2017) concluded that the allele status of *ASIP* and *MC1R* affects the shading of bay colour.

Regarding the phenotypic variability of bay/brown horses, researchers had to deal with the problem of colour definition, which conventionally is based upon visual inspection and classification. For example Rieder et al. (2001) distinguished between the categories bay and dark bay, Sakamoto et al. (2017) differentiated bay, dark bay, and brown. In this study we aimed to objectively describe and quantify coat colour variation in bay horses using a spectrophotometer in order to characterize equine coat colour shades according to standardized international procedures as defined by the Commission Internationale de l'Eclairage (CIE) and the L*a*b* colour system. In order to discriminate different shades of bay, especially the dark shades of bay, e.g. brown, mahogany bay and seal brown, we also included black horses as a reference. Based upon CIE L*a*b* colour measurements we wanted to investigate if the genotype combinations of *MC1R* and *ASIP* correspond with the observed coat colour variation.

MATERIAL AND METHODS

The animals (32 Shagya Arabians and 25 Noriker horses) included in this study were selected to represent the complete phenotypic variability of bay ranging from wild bay to seal brown. We specifically selected these two breeds, because we can assume due to the rotating mating strategies between bay, black, and chestnut horses that all *ASIP*/*MC1R* genotype combinations exist. 32 Shagya Arabian horses (30 bay, 2 black) from the Slovak National stud farm of Topoľčianky and 25 Noriker horses (13 bays, 12 black) from the Austrian stud farm Ossiacher Tauern were phenotyped using a Chroma Meter CL-100 (Konica Minolta, Japan). In order to minimize environmental effects which may influence coat colour variation, we measured horses originating from two herds, which were kept under standardized conditions (free running herds on pastures of about 20 ha with free access to shelters throughout the whole year; the same feeding and training program). Each herd was measured on one day (Shagay-Arabians on June 13, 2017 and Noriker horses on June 17, 2017, daylength approximately 16 h). As especially in bay horses black and red/yellow pigment is not equally distributed over the whole body, measurements were taken from five different body regions including neck, shoulder, armpit area, belly, and croup (Figure 1). As classifications of coat colour differ across breeds and countries, we adapted the colour nomenclature derived from the studbooks to the systematics of Sponenberg (2009). Colour as defined by the CIE L*a*b* colour space consists of three axes defining variation from black to white (L*) at a scale from 0 to +100, blue to yellow (b*) at a scale from –100 to +150, and green to red (a*) at a scale from –170 to +100. The

Black *a/a E/E* | Seal brown *A/a E/E* | Brown *A/A E/E* | Bay *A/A E/e* | Light bay *A/A E/e*

Figure 1. Black and bay coat colour phenotypes by classification category and *ASIP* and *MC1R* genotype
circles on the left horse indicate five measuring points (neck, shoulder, armpit area, belly, and croup)

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colour saturation (chroma; C) can be calculated according to the following formula:

$$C = \sqrt{(a^*)^2 + (b^*)^2}$$

Genomic DNA was extracted from hair root samples using the nexttec™ Tissue & Cells Kit (nexttec™, Germany) following the manufacturer's protocol. The *ASIP* and *MC1R* genotypes were obtained by applying a pyrosequencing approach on a PyroMark Q96 MD pyrosequencer according to the manufacturer's protocol (QIAGEN, Germany).

In total 1140 measurements (57 horses, 5 measuring points, 3 axes per point, chroma per point) were used for correlation analysis in order to characterize differences/similarities between body parts and variability in colour of body regions. To visualize this variability the measured L*a*b* values were transformed into computer colours using the RGB (red, green, blue) colour space in Adobe Photoshop©. We further applied different statistical differentiation techniques in order to identify the optimal measuring points for (a) the discrimination between black and bay colour and (b) the discrimination between genotype combinations of *ASIP* and *MC1R* in horses of bay coat colour. The canonical discriminant function results in variables which maximize the differences between given groups (black or bay; *ASIP*/*MC1R* genotypes), whereas the stepwise discriminant function ranks the phenotypic traits necessary to gain this discrimination. In a generalized linear model we additionally tested the level of significance between means of colour measurements by genotype groups applying a correction for multiple levels according to Tukey and Kramer. All statistical analyses and graphical representations were performed using the SAS software package (Statistical Analysis System, Version 9.1, 2009).

RESULTS

Out of the 57 genotyped horses 14 animals were black (11 *a/a E/E*; 3 *a/a E/e*). The 43 sampled bay horses covered the whole range from light (wild) bay, over bay, brown, dark bay to seal brown (Figures 1 and 2). 14 of the bay horses had the genotype *A/a E/E*, 6 horses were *A/a E/e*, 11 horses were *A/A E/e,* and 12 horses were *A/A E/E*. From the horses with genotype *A/a E/e*, 100% were classified as bay, among the *A/a E/E* horses 71% were classified dark bay, 21% seal brown, and 8% dark brown. Within the genotype group *A/A E/e* 55% of the horses were light bay, 36% were bay, and 9% brown, and in the group *A/A E/E* 50% were classified as bay, 33% light bay, and 17% brown.

Within the black sample L* values ranged from 18.9 (croup) to 26.9 (armpit area) at an overall mean of 22.4 (Table 1 and Figure 3). Bay horses were characterized by L* values from 19.9 (croup) to 50.9 (armpit area) at an overall mean of 32.4. Chroma varied from 1.5 to 9.1 (mean 4.4) in black and from 1.7 to 26.3 (mean 16.6) in bay horses (Figure 3). The red colour spectrum described by the a* value reached a mean of 2.7 (min. 0.5; max. 5.3) in black horses and was therefore partially overlapping with bay horses who had a mean of 8.7 (min. 0.6; max. 11.9). In the yellow spectrum (b* value), the differences between black horses (mean 3.5; min. 1.0; max. 7.8) and bay horses (mean 14.1; min. 1.4; max. 23.6) were more pronounced across all measuring points. For all measured colour traits (L*a*b*, chroma) within the measuring points (neck, shoulder, armpit area, belly, and croup) of 57 horses, highly significant differences ($P < 0.001$) between black ($n = 14$) and bay ($n = 43$) horses were found.

The correlations between the measuring points varied from 0.53 (L* value croup and a* value armpit area) to 0.97 (for chroma shoulder and a* value neck) and were all highly significant ($P < 0.0001$) (Supplementary Table S1 in Supplementary Online Material (SOM)). The correlation structure as illustrated in Figure 4 by means of Principal component analysis (PCA) shows, that the colour measurements from the armpit stand far away, especially the a* value, which represents the red spectrum within the colour space. Colour variables of the croup (a* value, b* value, and chroma) form together another cluster, whereas the L* values (brightness) for measuring points neck, shoulder, and croup are away from the main cluster. The main cluster contains measurements from the belly, shoulder, and neck, which are characterized by high inter-correlations ranging from 0.81 to 0.99.

When applying a stepwise discriminant function on the colour data in order to structure the animals according to their base colour (black or bay) we could observe that the chroma measured at armpit was ranked first (partial R-square (R-sq.) of 0.86) within the equation, followed by L* values for armpit (partial R-sq. of 0.11), and neck (partial R-sq. of 0.16).

As a conclusion these three variables (chroma armpit area, L* armpit area; L* neck) described the

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Figure 2. Reproduced RGB colours (RGB colour model; red, green, blue) according to individual L*a*b* measurements categories: 1 = black horses of genotypes *a/a E/e* and *a/a E/E*; 2 = bay genotype *A/a E/E*; 3 = bay genotype *A/A E/e*; 4 = bay genotype *A/A E/E*; 5 = bay genotype *A/a E/e*. The first column represents the measurement of neck, the second column the measurement of armpit area, and the third column the measurement of belly

main phenotypic differences between black and bay horses, where bay horses showed a three times higher chromacity and a twice higher brightness in the armpit than black horses.

When considering the *ASIP* and *MC1R* genotype for the discriminant function of black and bay horses, we got a similar ranking of colour variables and luminescence which separated the animals according to their genotype: (1) Rank chroma armpit area (R-sq. = 0.85), (2) Rank a* value neck (R-sq. = 0.45), (3) Rank L* value armpit area (R-sq. = 0.28), (4) Rank L* value neck (R-sq. = 0.22).

Analysing only bay horses of different shades the measuring point armpit area loosed its power of discrimination, as the first rank got occupied by the a* value neck (R-sq. = 0.58), followed by L* value neck (rank 2; R-sq. = 0.16), and L* value armpit area (rank 3; R-sq. = 0.20). Therefore the main criterion for discriminating between the genotype combinations of bay horses was the proportion of red pigment (a* value) on the neck.

The canonical discriminant analysis clearly separated the black horses (*a/a E/-*) from the bay horses. Within the bay horses the genotype

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Table 1. Mean values, standard deviation (SD), minimum and maximum values for L*, a*, b*, and chroma (C) variables measured at five different body regions for black and bay horses

Measuring point	Variable	Black				Bay			
		mean	SD	min	max	mean	SD	min	max
Neck	L*	20.61	0.82	19.30	21.90	29.00	4.44	19.90	40.90
Shoulder		22.64	1.38	20.00	25.40	30.52	3.86	23.00	39.70
Armpit		24.49	1.38	21.70	26.90	41.15	4.80	27.90	50.90
Belly		23.42	1.65	21.00	25.80	34.01	4.45	21.70	42.60
Croup		20.85	1.06	18.90	22.40	27.34	4.10	19.90	36.20
Neck	a*	1.81	0.54	1.00	2.70	8.38	2.79	1.50	11.30
Shoulder		2.70	1.09	1.10	4.30	8.38	2.45	1.20	10.90
Armpit		4.00	0.89	2.20	5.30	9.44	1.49	4.90	11.70
Belly		3.42	1.10	1.50	4.80	10.02	2.08	2.20	11.90
Croup		1.78	0.85	0.50	3.30	7.21	2.88	0.60	11.10
Neck	b*	1.96	0.61	1.00	2.90	12.30	4.41	2.20	19.70
Shoulder		3.33	1.15	1.50	5.10	12.61	3.90	2.20	19.60
Armpit		5.74	1.29	3.40	7.80	19.47	2.73	11.40	23.60
Belly		3.95	1.51	1.80	6.30	16.21	4.25	2.70	21.90
Croup		2.31	0.88	1.40	3.70	10.01	4.24	1.40	17.60
Neck	C	2.70	0.68	1.49	3.64	14.92	5.10	2.66	22.28
Shoulder		4.31	1.49	2.06	6.67	15.17	4.52	2.56	22.24
Armpit		7.00	1.53	4.05	9.11	21.67	2.88	14.22	26.25
Belly		5.24	1.82	2.34	7.74	19.09	4.59	3.48	24.78
Croup		2.94	1.18	1.49	4.88	12.36	5.06	1.72	20.19

group *A/a E/E* formed a distinct cluster (Figure 5). The ANOVA and the pairwise comparisons of means between the four genotype groups of bay horses confirmed these results and identified that *A/a E/E* horses had a distinct coat colour shade, which differed significantly from all other bay horses in the sample. For the genotype *A/a E/E*, all measuring points except the armpit area showed

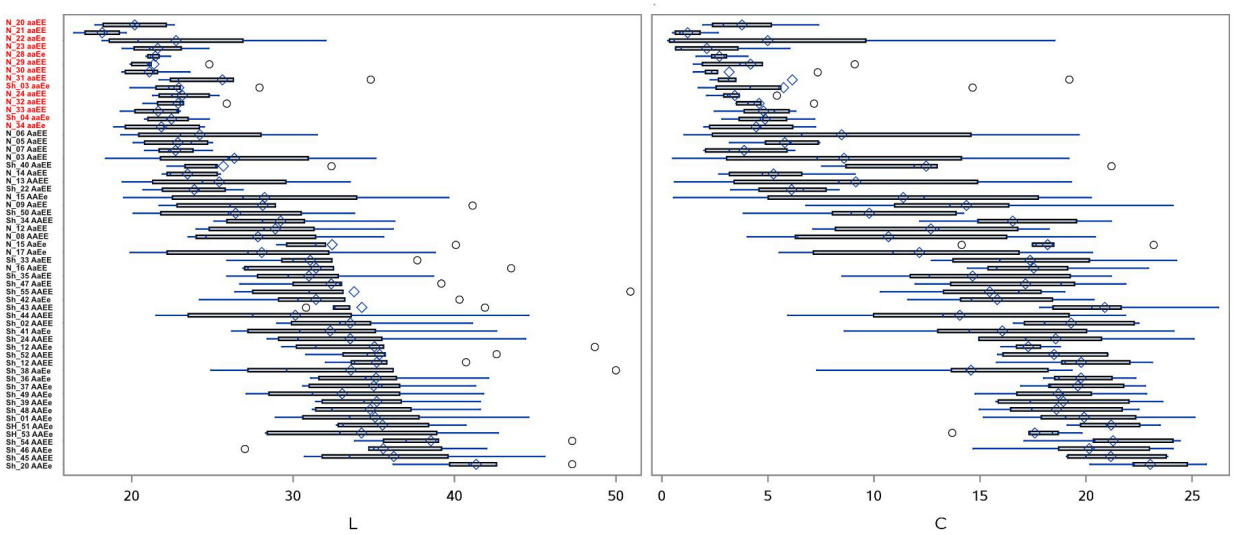

Figure 3. Box plots of L* values (left) and chroma (C) (right) across five measurements per animal animals are sorted by L* values, black horses are marked by red IDs, and bay horses by black IDs

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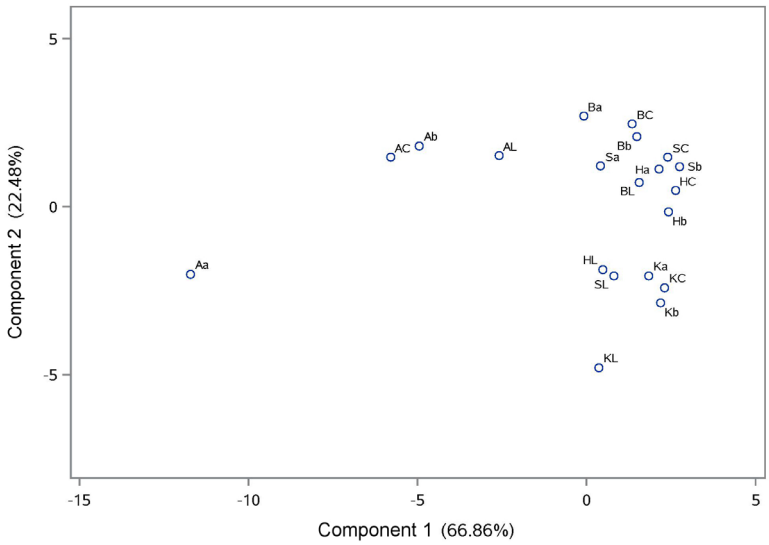

Figure 4. Principal component analysis of correlation matrix between 5 measuring points of 57 horses
the first letter indicates: A = armpit area, B = belly, S = shoulder, H = neck, K = croup; the second letter indicates colour variable: a = a* value, b = b* value, C = chroma, L = L* value

significant differences. The measurement points with the highest R-sq. values (ranging from 0.52 to 0.58) explained by the genotype effect were the neck (L*, b*, chroma), croup (a*, b*, chroma), and shoulder (a*, b*, chroma). All these body parts were characterized by darker pigmentation in *A/a E/E* horses. On average *A/a E/E* horses had by about 25–40% lower L* values. The a* and b* values and the chroma were by about 30–80% lower indicating a loss in chromacity of colour.

In the scatter plot of Figure 5 we can observe, that within the genotype cluster *A/a E/E* the animals were phenotypically classified as dark (mahogany) bay or seal brown. The coat colour category seal brown describes a horse with predominantly occurring black hair all over the body except around the muzzles, eyes, flanks, and armpit area, where lighter shades from yellow to red occur. Our data showed that the L*a*b* values of seal brown horses measured at neck, belly, and croup did not differ from those in black horses. The crucial factor to discriminate black from seal brown horses were the colour values of the armpit area, where the chromacity is three times higher and the luminescence is 40% higher (Table 2).

Dark bay (mahogany bay) horses are characterized by black pigmented legs up to knees and hocks and a darker shade occurring at the upper part of the body (back and croup). The *A/a E/E* horses classified as dark bay did not differ in the croup measurements from black horses. Colour variables from the neck were slightly higher than in black horses, whereas the belly region showed a higher variation of L* values. Especially the lower part

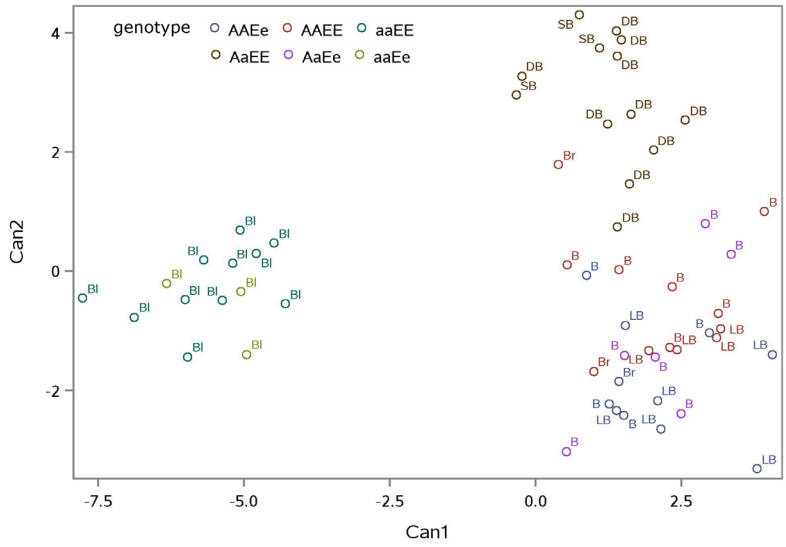

Figure 5. Plot of the first two Canonical variables separating the different genotypes by colour measurements in the sample
legend next to the scatters identifies the coat colour classifications from the stud book (Bl = Black, SB = Seal brown, DB = Dark bay, Br = Brown, B = Bay, LB = Light bay)

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Table 2. Values of Least Squares Means for the four genotype groups of *ASIP* and *MC1R* for all five measuring points

Measuring point	Variable	R-square	*A/a E/e*	*A/a E/E*	*A/A E/e*	*A/A E/E*
Neck	L*	0.52	29[a]	23.84[b]	32.36[a]	29.11[a]
	a*	0.58	9.85[a]	4.23[b]	10[a]	8.37[a]
	b*	0.52	13.08[a]	5.9[b]	15.44[a]	11.85[a]
	chroma	0.55	16.39[a]	7.33[b]	18.44[a]	14.56[a]
Shoulder	L*	0.49	31.63[a]	16.61[b]	33.77[a]	30.51[a]
	a*	0.55	9.78[a]	5.45[b]	9.57[a]	9.19[a]
	b*	0.57	14[a]	8.07[b]	15.63[a]	13.24[a]
	chroma	0.58	17.1[a]	9.77[b]	18.34[a]	16.14[a]
Armpit area	L*	0.17	41.97	36.82	42.21	40.63
	a*	0.18	9.95	8.37	9.68	9.93
	b*	0.32	20.05[a]	15.97[b]	20.12[a]	20.28[a]
	chroma	0.32	22.38[a]	18.12[b]	22.34[a]	22.59[a]
Belly	L*	0.37	35.4[a]	27.92[b]	35.94[a]	33.51[a]
	a*	0.38	10.72[a]	6.73[b]	10.56[a]	10.39[a]
	b*	0.36	16.88[a]	10.13[b]	17.91[a]	16.27[a]
	chroma	0.37	20.02[a]	12.18[b]	20.83[a]	19.34[a]
Croup	L*	0.48	28.8[a]	21.87[b]	29.86[a]	26.59[a]
	a*	0.55	8.18[a]	2.61[b]	8.68[a]	7.42[a]
	b*	0.53	11.1[a]	3.5[b]	12.76[a]	9.61[a]
	chroma	0.54	13.8[a]	4.43[b]	15.46[a]	12.1[a]

[a,b]significant differences at $P < 0.01$

of the body (armpit area, belly) was characterized by more chromacity.

DISCUSSION

The precise quantification of equine coat colour using a spectrophotometer according to standardized international procedures has been applied from Stachurska et al. (2004), Lackner (2006), Toth et al. (2006), Pielberg et al. (2008), and Curik et al. (2013). With this approach several aspects concerning equine coat colour as environmental and age effects (Stachurska et al. 2004; Curik et al. 2013) and quantitative genetic parameters (Curik et al. 2006; Lackner 2006; Toth et al. 2006) were analysed. Allele dosage and substitution effects of single loci on grey coat colour variation in Lipizzan horses have been studied extensively by Pielberg et al. (2008) and Curik et al. (2013). In this study we aimed to characterize and examine single loci effects (*ASIP* and *MC1R*) on coat colour variability in bay and black horses. Although *ASIP* and *MC1R* and their interaction have been studied (Rieder et al. 2001; Sponenberg 2009; Sakamoto et al. 2017), the reasons for bay coat colour variability ranging from light bay to seal brown are not fully resolved. This led Sponenberg (2009) postulate a dosage effect and the existence of multifactorial genetic control. From the four different genotype combinations of *ASIP* and *MC1R* (*A/A E/E, A/a E/e, A/A E/e,* and *A/a E/E)*, which result in bay coat colour, we could confirm a significant association between the genotype *A/a E/E* with darker shades of bay. This genotype group was also identified by discriminant analysis, whereas all other three genotype combinations could not be differentiated from each other. Rieder et al. (2001) published that bay *E/E* horses have darker shades, whereas the *ASIP* status (*A/A* or *A/a*) of the animals was not simultaneously considered in the analyses. For this locus the authors could not find a difference in colour shade between *A/a* and *A/A*. Also Sakamoto et al. (2017), who studied segregation ratios by means of pedigree analysis, treated the Extension and Agouti loci separately. For the Agouti locus the authors compared the expected number with the observed number of

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brown, bay, and dark bay horses of offspring from bay × bay and black × bay matings. The expected relation between the hypothesized *A/a* genotype and a dark bay phenotype could be significantly confirmed by the segregation ratio. Applying the same concept and a hypothesized association of *E/E* genotype with darker shades of bay on a second data set (chestnut × bay; bay × bay/black matings), a significant association of *E/E* genotype with darker shades of bay was found.

Our data generally confirm these findings, whereas only the combination of the genotypes *A/a* with *E/E* resulted in a statistically confirmed distinct cluster. This genotype group was represented by bay horses with the lowest mean L* value and lowest chromacity indicating less production of phaeomelanin (red, yellow pigment), therefore showing a dosage effect of heterozygous *ASIP* (*A/a*) status in combination with homozygous *MC1R* (*E/E*) status.

According to melanogenesis of bay coat colour our results let us conclude that two copies of the *E* allele (coding melanocyte-stimulating hormone (MSH)) result in an altered production of eumelanin. In the case, an *E/E* horse has just one copy of the *A* allele (coding MSH receptor antagonist), the downregulating effect on the action of MSH on melanocytes is minimized compared to an individual with the *A/A* genotype. As a result the *A/a E/E* phenotype is located between black (genotype a/a E/E; no action of MSH receptor antagonist) and bay (genotype *A/A E/E*; higher action of MSH receptor antagonist), indicating an incomplete dominant mode of inheritance for the *ASIP* locus, which is visible in the presence of two copies of the Extension allele.

Also in chestnut/sorrel horses, where the occurrence of high colour variation is object of speculations, the application of this method in a population where the whole range of colour shades is present, should reveal new insights.

CONCLUSION

In the present study it has been shown that the measurement of equine coat colour based upon international standards as defined by the CIE L*a*b* colour system represents a valuable tool for the precise description of colour variation. We could demonstrate that the combination of the genotypes *A/a* with *E/E* resulted in a statistically confirmed distinct phenotype cluster, represented by bay horses with the lowest mean L* value and the lowest chromacity indicating less production of phaeomelanin (red, yellow pigment). In order to discriminate between genotype combinations of bay horses, we found that spectrophotometrically determined proportions of red pigment and brightness measured on the neck area revealed the most meaningful results. Such phenotypic data offer a high analytical power regarding statistical and genomic analyses on a finer scale.

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