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Running Head:

White spotting in the American Paint Horse

Title:

Impact of white spotting alleles, including *W20*, on phenotype in the American Paint Horse

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Abstract:

The American Paint Horse Association (APHA) officially records pedigree and performance information for their breed; these registered stock-type horses are valued for utility in work on the farm and ranch and as pleasure horses. As the name of the breed implies, the breed is also valued for attractive white spotting patterns on the coat. Current APHA procedures utilize visual inspections of photographs to determine if white spotting phenotypes exceed threshold anatomical landmarks considered by the association as characteristic of desirable patterns. Horses with sufficient white patterning enter the "Regular" registry, rather than the "Solid Paint-Bred" division. Genetic studies have now defined causative sequence variants for 35 alleles likely contributing to white spotting phenotypes in the horse. Here, the allele frequency was calculated for nine white spotting alleles commonly found in the American Paint horse breed using a sample of 1,065 APHA registered animals. Epistatic interactions were documented among five spotting loci, and with the *MC1R* and *ASIP* genes controlling black and red pigment production. The *W20* allele within the *KIT* gene, in the absence of other spotting loci, was strongly associated with the APHA-defined white spotting phenotype ($p = 1.86 \times 10^{-18}$), refuting advertisements reporting that *W20* acts only as a modifier of other white spotting patterns. An empirical definition of the action of these loci on the desired APHA-defined white spotting phenotype will allow for a more accurate application of genome-assisted selection in this breed, providing breeders with a powerful tool to increase the marketability of their horses.

Key words:

KIT

coat color

ASIP

MC1R

W20

American Paint Horse

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Introduction:

Paint Horses themselves were not new to the equine industry—colorful pinto horses have for decades been staple stablemates across the United States, as favorites among some horsemen for their flashy coats and versatility but shunned by traditionalist Quarter Horse breeders for their white spotting patterns. “Cropout” horses—those of Quarter Horse or Thoroughbred descent born with excessive white markings on their coats, and therefore refused registration with the American Quarter Horse Association—along with other colorful stock that sported white spotting patterns became the foundation of a new breed. The American Paint Stock Horse Association was founded in 1962, the brainchild of Rebecca Tyler Lockhart, a well-respected and well-connected North Texas horsewoman with a penchant for colorful stock horses (Hood, 1987). The fledgling association’s directives were outlined: collect, preserve and record the pedigrees of Paint stock horses; publish a stud book; maintain a registry; and regulate the breed’s exhibition, publicity, sales and racing. Known as the American Paint Horse Association (APHA) since 1965, the association originally counted 3,800 registered Paint Horses and 1,300 members at that time (Hein, 2012). Since its inception, registration of the Paint Horse has been based primarily on a combination of pedigree and phenotype in order to select for the white-patterned, stock-type horse valued by APHA’s founders. Initially, APHA’s bloodline requirements remained “open,” meaning horses could be registered with APHA so long as they met the phenotypic requirements of a spotted stock-type horse. As of January 1, 1980, the association requires all fully-registered APHA horses to have a sire and dam registered with either the American Paint Horse Association, the American Quarter Horse Association or The Jockey Club, a standard that remains today (APHA, 2018).

During the APHA registration process, horses are designated into one of two sub-registries based on the presence or absence of qualifying natural white markings on their coats: the Regular Registry or the Solid Paint-Bred Registry. Regular Registry status is granted for horses that meet white spotting requirements as outlined in the *APHA Rule Book* (APHA, 2018). The patterns desired by the APHA extend beyond what is typically observed as an average white marking on the face or legs (Haase et al., 2013) and exclude markings created by the *Leopard* locus (Bellone et al., 2013).

With more than 1.09 million horses registered with its organization as of May 2019, the APHA ranks as the second-largest equine breed association in the world. According to the American Horse Council Foundation’s 2017 National Economic Impact Study, the equine industry is directly responsible for $50 billion in U.S. economic impact, nearly 988,400 jobs, and $38 billion in wages,

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salaries and benefits; when expanded to include all associated impacts, total contributions include $122 billion to the U.S. economy and 1.7 million jobs (American Horse Council Foundation, 2019). While the American Paint Horse is valued for conformation and performance traits, the white spotting pattern (reflected by the registry designation of either Regular or Solid Paint-Bred) exerts an overwhelming influence on the value of individual horses (Brooks et al., 2007).

In the horse, at least 35 known white spotting polymorphisms underly many of the coat patterns valued by members of the APHA (OMIA, 2019). Yet, the population frequencies of these alleles lack investigation, and despite the easily applied Mendelian inheritance patterns for most of these alleles, genetic testing remains underutilized in the industry. Recent work highlighting "exceptions" to proposed qualitative phenotypes for these loci emphasizes the need for application of impartial and quantitative assessments capturing the full breadth of phenotypes resulting from these variants (Druml et al., 2018). The goals of this study aim to: establish allele frequencies for known white spotting alleles in the American Paint Horse, investigate association of these loci with a phenotype uniformly defined by the APHA thresholds for Regular Registry status, identify any interactions between loci, and to assess the accuracy of estimating the genetic value for production of spotting patterns using photo-based phenotypes.

Materials and Methods:

Definition of APHA Registration Type

When registering a Paint Horse today, the applicant provides identifying information pertaining to the foal, including its date of birth, color, pattern, sex, parentage and ownership. This information, in combination with breeding records required to be on file with the association, confirm the foal was produced within the typical gestational range anticipated for the proposed breeding date. The APHA requires parentage verification via genetic testing when the horse is the product breeding with transported or frozen semen, embryo transfer or vitrified embryo transfer, or other special situations, such as when registering a horse over age 10, and for all Quarter Horses and Thoroughbreds applying for registration as Paint Horses (APHA, 2018).

The applicant provides a minimum of four full-color photographs of the horse, showing the entire animal from the left side, right side, front and rear. Additional photographs of spotted areas of the coat might be required by APHA staff to verify that the pattern meets registration guidelines in

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terms of the location, size and requirement for underlying skin to also be unpigmented (APHA, 2018). The photographs become part of the horse's permanent record at APHA and are used to identify the animal throughout its life. Trained APHA staff members determine the registration category for each horse by visual inspection to confirm that a horse's natural white spotting patterns meets APHA's standard. All horses in this study were examined using photographs submitted by the owner. The quality of the submitted photographs was variable and added additional challenges to the interpretation of photographic evidence of the pattern relative to the APHA standards.

In brief, unpigmented areas characterizing APHA-accepted white spotting patterns must include both white hair and skin and are found on the face extending beyond a reference line from the base of the horse's ear to the outside corner of the eye to the corner of the mouth and under the chin to the opposite corner of the mouth (Figure 1). On the body, qualifying depigmented areas must occur above a line on the leg above the center of the knee or hock. These spotting patterns must include a minimum of two inches of solid white hair with some underlying unpigmented skin in the qualifying area. The Solid Paint-Bred Registry is used for horses who meet the parentage requirement but lack the minimum requirement for white spotted skin and coats as defined in the rulebook (APHA, 2018).

Retrospective Registration Records

The American Paint Horse Association provided data for 1,071 horses born and registered between 1992 and 2018 including: registered name and number, registration type (solid, overo, tobiano or tovero), age, and sex for each horse, as well as the registry and registration type of the sire and dam for that horse. The original photos submitted by the applicant for evaluation of the white spotting pattern on the horse at the time of registration were also provided by the APHA and used to confirm predicted phenotypes by visual inspection from a single experienced observer (SAB). Twelve horses were excluded from the study due to missing demographic details in the record (n = 10) or when the registration was later rejected by APHA (n =2).

Genotypes

Each horse was genotyped for the following nine spotting pattern loci using a commercial service (Veterinary Genetics Laboratory, University of California, Davis): *KIT- TO, SB1, W5, W10, W20* or *N* (Brooks and Bailey, 2005; Brooks et al., 2007; Haase et al., 2009; Hauswirth et al., 2013), *EDNRB- O* or *N* (Metallinos et al., 1998; Santschi et al., 1998; Yang et al., 1998)*, MITF- SW1, SW3* or *N* and *PAX3- SW2* or *N* (Hauswirth et al., 2012). Additionally, two known pigmentation loci were

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genotyped for analyses of epistasis: *MC1R- E* or *e* (Marklund et al., 1996)*, ASIP- A* or *a* (Rieder et al., 2001)*.*

Statistical analyses

Analyses were conducted in the JMP Pro v14.1.0 package (SAS Institute Inc.). To measure the impact of the *MC1R-ASIP* signaling system on white spotting, a subset of 368 horses that possessed only one spotting allele (controlling for any effect due to epistasis between multiple spotting loci) was utilized and all horses possessing a *Tobiano* allele (avoiding the impact of linkage between *MC1R* alleles and *TO)* were excluded*.* Given the well documented interaction between alleles at *ASIP* and its antagonistic target, the *MC1R* receptor, a logistical model was constructed comparing the APHA registry phenotype (Regular or Solid Paint-Bred) with the linear ranking of the genotypes by the predicted signaling activity and base color phenotype. Thus, the *E/- a/a* genotype, likely resulting in a constitutively active *MC1R* receptor and the black base color, was scored "0"; the wild-type genotype *E/- A/-*, predicted to have normal signaling activity and a bay base color, was given a "1"; and the *e/e* genotype, resulting in a loss of *MC1R* signaling regardless of the *ASIP* genotype and a chestnut base coat, received a score of "2".

Results and Discussion:

Phenotype distribution varies for each white spotting genotype

The 1,065 horses registered with the APHA in the examined time-period included 412 stallions, 91 geldings and 562 mares submitted for registration at a mean age of 1.07 years. Among this group, 777 were designated to the Regular Registry, while 282 entered the Solid Paint-Bred category. Despite the large population examined, this sampling did not represent an unbiased observation of all foals produced from APHA-registered breeding since it is likely that not all foals are submitted for registration (especially if the foal is unlikely to achieve Regular Registry status) and, furthermore, records are not retained by the APHA on horses ultimately rejected for registration.

Genotypic and allele frequencies for each of the nine variants examined are presented in Table 1. Presence of just a single alternate allele resulted in APHA designation to the Regular Registry for all spotting alleles except *O* (*EDNRB), SW1 (MITF), SW2 (PAX3),* and *W20 (KIT).* As

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expected, no horse homozygous for the *O* allele was observed, as this genotype causes the well-documented Lethal White Overo Syndrome (Metallinos et al., 1998; Santschi et al., 1998; Yang et al., 1998). However, a single horse homozygous for the *SW2* allele was identified, which is a state previously hypothesized to be lethal based on comparisons to similar variants in the *PAX3* gene of other species (Hauswirth et al., 2012).

Photographic estimation of spotting patterns miscategorizes 17% of horses

APHA registered 110 horses as Solid Paint-Bred based on visual examination of photographs, but for which we found at least one white spotting allele. Based on our inspection of the photographs, the extent and distribution of white markings in these horses fell below the guidelines of the *APHA Rule Book* (2018) but were more extensive than the common white markings described as “socks” on the limbs or a “blaze” in other studies (Haase et al., 2013). One horse categorized as “Solid” possessed a total of five white spotting alleles and an entirely white coat. Thus, although this animal has a high breeding value for white spotting, it was registered in the less-valued Solid Paint-Bred category because the horse did not exhibit at least two inches of contrasting colored hair in his coat, per APHA registration guidelines. In these cases, phenotyping by photograph and use of the *APHA Rule Book* description for Regular Registry white spotting patterns missed horses with genetic value for production of white spotting patterns.

For 98 horses recorded by APHA in the Regular Registry based on their photographic phenotype, genetic testing results did not identify possession of any of the nine white spotting alleles evaluated as part of this study. In some cases, white spotting patterns in these horses may have been due to genetic markers not tested in this study, or which have not yet been discovered. Visual inspection of the registration photographs for these 98 horses revealed that 31 possessed patterns consistent with white spotting loci. These 31 included many phenotypes resembling those generated by alleles known but not genotyped in this cohort, including *SW4* (Hauswirth et al., 2013), *SW5* (Henkel et al., 2019), and more than 27 other alleles at the *KIT* locus. We also observed among these 31 horses some well-recognized patterns with yet unknown genetic etiology (*i.e.* the “Rabicano” pattern) and two horses with previously undocumented and distinct white spotting phenotypes. Phenotypes for the remaining 67 (68%) horses fell within the described range for common white markings of the face and legs (Haase et al., 2013), or typified the roan and grey coat colors. Roan and grey do introduce white hair into the coat, but in a more interspersed manner than desired by the

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APHA (Marklund et al., 1999; Sundstrom et al., 2012). The predicted phenotype from genotypes of the nine spotting variants investigated here agreed with the Regular Registry (spotted) classification in 95.4% of these horses. In total, the photo-based designation to the Regular or Solid Paint Bred categories disagreed with the genotyped presence or absence of a white spotting allele in 17% of horses submitted for registration.

The W20 allele results in white spotting, independent of other alleles

The first published observation of the *W20* allele came from a study searching for white spotting patterns with phenotypes that left the majority of the skin surface totally unpigmented, and therefore did not attempt to document less extensive spotting phenotypes (Haase et al., 2007). Subsequently, the authors examined this variant in a larger set of horses (n= 52) and noted a compound heterozygote effect creating extensive white patterning in horses with the *W5/W20* genotype, and that presence of a single *W20* allele among horses with a white spotting phenotype more expansive than the typical white markings (Hauswirth et al., 2013). The lack of data specifically addressing the phenotype produced by the *W20* allele led to claims that there is no, or minimal, impact from the *W20* allele on white spotting. However, in this study photographic assessment of 539 APHA horses possessing only the +/+ (n= 270), *W20/*+ (n= 225) and *W20/W20* (n= 34) genotypes (no other spotting alleles at any of the 8 remaining loci tested) revealed that *W20* is indeed significantly associated with white spotting patterns on the coat and is a common variant among Regular Registry APHA horses (Chi-square test under a dominant model, $p= 1.86 \times 10^{-18}$, Table 2). Clearly, *W20* generates a white spotting phenotype as defined by the *APHA Rule Book,* and this genotype will be valuable in future genomic selection schemes for improving production of white spotted foals.

Epistasis between loci additively increases white spotting phenotypes

Excluding the one horse possessing five alleles and an entirely white coat but considered a Solid Paint-Bred under current rules, horses with an increasing number of spotting alleles are more likely to exhibit a phenotype that exceeds the APHA threshold for the Regular Registry (Chi-square = 238.41, $p < 0.0001$, Table 3). Thus, the nine spotting alleles examined here act epistatically,

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increasing the proportion of white on the horse above the APHA Regular Registry phenotype threshold.

Although the *MC1R* signaling pathway primarily contributes to pigment switching between eumelanin and pheomelanin, loss-of-function alleles at the *MC1R* locus can reduce migration of melanocyte stem cells (Chou et al., 2013). Linkage between *MC1R* alleles and the *KIT* locus, also on ECA3, could produce an association with white spotting alleles irrespective of any biological action. Indeed, in this dataset, significant linkage exists between the *Tobiano* allele, a 36 Mb paracentric inversion lying within the 41 Mb span between *KIT* and *MC1R* (n = 1065, chi-square = 117.326). Linkage was not observed between the *W20* allele (within the *KIT* coding sequence) and the *MC1R* locus. Therefore, linkage observed between *MC1R* alleles and the *Tobiano* allele likely arises solely from recombination suppression generated by the *Tobiano* paracentric inversion, and not from the physical proximity of these two genes on the q-arm of ECA3.

Before assessing association of spotting phenotype with *MC1R* and *ASIP*, we selected a subset of horses that possessed just one spotting allele (avoiding any coincidental effect of epistasis across multiple spotting alleles) and excluding any horse with a *TO* allele (likely to produce association due to recombination suppression on ECA3). Among these 368, horses we observed a significant effect of the *MC1R-ASIP* signaling system on the APHA-defined Regular Registry white spotting phenotype (Table 3, p = 0.0136), with the *e/e* genotype producing the most extensively marked (Regular Registry) horses.

The sex of the individual horse did not significantly impact the registry designation when examined within 465 horses possessing just one white spotting allele (Chi-square test, p = 0.209). Across the entire sample population of 1,065 horses, stallions were more likely to enter the Regular Registry than mares or geldings (p< 0.0001), but this may reflect a tendency for applicants to submit registrations on colts with some prospect as future stallions, rather than an influence of sex or castration on white spotting phenotype.

No evidence for unknown white spotting phenotype "modifying" alleles

Given the historical selective pressure to eliminate white spotting phenotypes in the American Quarter Horse breed (AQHA, 2019) and the rarity of white spotting phenotypes within the Thoroughbred breed (often used in the production of American Quarter Horses), APHA breeders are

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concerned that undiscovered “modifying” alleles capable of reducing the white spotting phenotypes may be present in the American Quarter Horse population. As APHA rules permit cross-breeding with American Quarter Horses or Thoroughbred horses, as well as registration of horses from these breeds that exhibit approved white spotting phenotypes, in-flow of “modifying” alleles could negatively impact the expression of white spotting phenotypes on APHA horses in future generations. 335 registrations were examined where the sire of the submitted horse belonged to the APHA Regular Registry and the dam was registered as either Solid Paint-Bred (n= 189) versus those with dams from the American Quarter Horse Association or The Jockey Club (Thoroughbred) registries (n= 146). No significant difference was observed in the Regular vs. Solid Paint-Bred registry status of foals from dams belonging to the Solid Paint-Bred Registry compared to those with dams registered with the AQHA or The Jockey Club (Chi-square test p= 0.704). Finally, at the genome-wide scale, the genetic background of horses from the APHA and AQHA registries is very similar (Petersen et al., 2013), and written history documents many AQHA-registered foundation animals at the inception of the APHA. Thus, insufficient time has passed for selective pressure to cause these two populations of horse to diverge, nor to establish any such white spotting “modifying” alleles in the American Quarter Horse, should they exist.

Conclusions

This study documents that we now know most of the genetic markers responsible for the iconic color patterns valued by APHA breeders. These loci are valuable tools for prediction of the genetic value of breeding stock, thereby optimizing future production of spotted foals. Identification of phenotype-altering epistatic interactions between the nine spotting loci tested, as well as with the *MC1R-ASIP* signaling system, will improve genotype-based phenotype predictions. Genetic tools could rapidly improve the accuracy of selection for white spotting in the horse and may provide significant economic savings compared to the time-consuming photo-analysis approach currently used to register APHA horses.

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Tables and Figures:

Table 1. Genotype counts and allele frequencies for some previously published white spotting variants among APHA registered horses within each of the two registration categories. Data are presented as genotype counts for the homozygous reference (Hom Ref), heterozygous (Het) and homozygous alternate (Hom Alt) states, as well as the frequency of the reference (*f*Ref) and alternate (*f*Alt) alleles.

	Regular Registry (n= 783)					**Solid Paint-Bred Registry (n= 282)**					**Total (n= 1065)**				
	Hom Ref	Het	Hom Alt	*f*Ref	*f*Alt	Hom Ref	Het	Hom Alt	*f*Ref	*f*Alt	Hom Ref	Het	Hom Alt	*f*Ref	*f*Alt
EDNRB- O (Frame Overo)	580	203	0	0.87	0.13	264	18	0	0.97	0.06	844	221	0	0.90	0.11
PAX3- SW2 (Splashed White)	767	15	1	0.99	0.01	273	9	0	0.98	0.03	1040	24	1	0.99	0.01
MITF- SW1 (Splashed White)	690	90	3	0.94	0.06	264	17	1	0.96	0.07	954	107	4	0.95	0.05
MITF- SW3 (Splashed White)	779	4	0	1.00	0.00	282	0	0	1.00	0.00	1061	4	0	1.00	0.00
KIT- TO (Tobiano)	582	142	59	0.83	0.17	282	0	0	1.00	0.00	864	142	59	0.88	0.12
KIT- SB1 (Sabino1)	755	27	1	0.98	0.02	282	0	0	1.00	0.00	1037	27	1	0.99	0.01
KIT- W05 (Variable White)	783	0	0	1.00	0.00	282	0	0	1.00	0.00	1065	0	0	1.00	0.00
KIT- W10 (Variable White)	781	2	0	1.00	0.00	282	0	0	1.00	0.00	1063	2	0	1.00	0.00
KIT- W20 (Variable White)	407	326	50	0.73	0.27	197	74	11	0.83	0.17	604	400	61	0.75	0.25

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Table 2: Horse designation to the Regular or Solid Paint-bred Registry divisions by genotype and genotype combinations (for simplicity, the table reports only genotypes/combinations observed in at least three horses.)

		Registry Category		
	Genotype	Regular	Solid Paint-bred	% Regular
	N/N	100	173	36%
Single Locus Genotypes	*N/SW2*	7	3	70%
	W20/W20	24	10	71%
	N/W20	166	58	74%
	N/SW1	33	9	79%
	N/O	64	10	86%
	N/SW3	3	0	100%
	N/TO	97	0	100%
	TO/TO	57	0	100%
	N/SB1	8	0	100%
Multi-allele Genotypes	*N/SW1, N/W20*	16	6	73%
	N/O, N/SW1	7	1	88%
	N/O, N/W20	82	7	92%
	N/TO, N/SW1	5	0	100%
	N/TO, N/O	5	0	100%
	N/TO, N/W20	25	0	100%
	N/O, N/SB1	4	0	100%
	N/SB1, N/W20	6	0	100%
	N/O, W20/W20	19	0	100%
	N/SW1, W20/W20	4	0	100%
	N/TO, N/O, N/W20	7	0	100%
	N/O, N/SW1, N/W20	9	0	100%
	N/O, N/SW1, W20/W20	3	0	100%

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Table 3: Horse counts for the Regular and Solid Paint-bred phenotypes compared to the total number of white spotting pattern alleles at the nine loci tested, and genotypes at the *MC1R* and *ASIP* coat color loci.

		Registry Category		
		Regular	Solid Paint-bred	% Regular
Total # of White Spotting Alleles (n= 1064)	*0*	100	173	37%
	1	386	79	83%
	2	243	29	89%
	3	51	0	100%
	4	3	0	100%
MC1R -ASIP Geno. (n=368)	*E/-, a/a*	13	7	65%
	E/-, A/-	57	23	71%
	e/e	219	49	82%

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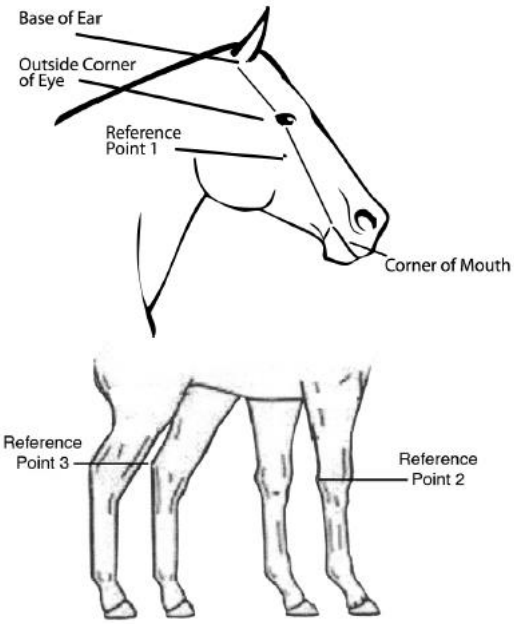

Figure 1: Anatomical landmarks on the face and limbs of the APHA horse are used to determine the extent of white spotting patterns, and to designate each registered horse to either the Regular or Solid Paint-bred sub-registries. To enter the Regular registry, a proposed horse must possess at least 2” of white hair, as well as underlying unpigmented skin, on the body surface beyond these reference lines.