AN ABSTRACT OF THE THESIS OF

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Tobiano is a dominant pattern of white spotting that occurs in certain breeds of horses. This pattern is often desirable and some breeders will purchase a genetic test to help distinguish homozygous from heterozygous horses for breeding purposes. Some tobiano horses have smaller dark spots on the white patches of their coat, known as "ink spots" or "paw prints" which are commonly believed by breeders to be associated with homozygosity. Sixty-six tobiano Paint horses were surveyed to assess their genotype (homozygous or heterozygous for tobiano) and their phenotype (presence or absence of ink spots). Thirty-one of the horses with ink spots were then classified as having fewer than five, or five or more ink spots. The results indicate that ink spots do not occur at random ($p < .001$) and the presence of five or more ink spots is associated with homozygosity ($p < .001$).

Key Words: horse, tobiano, genetics, coat color, white spotting

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Ink Spots as an Indicator of Homozygosity in Tobiano Paint Horses

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes the release of my project to any reader upon request.

Annamaria Elizabeth Tadlock, Author

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INTRODUCTION

Pintos are horses with irregular white spotting over the body which come in several genetically and visually distinct patterns. Tobiano is a type of pinto that is characterized by large, smooth patches of white. The hooves and legs are often white, and white often crosses the spine at the hips or over the neck and shoulder. The eyes are dark, and the head and flank are often dark.

Like all pinto spotting patterns, tobiano shows variable expressivity. At the most extreme, a minimally marked tobiano will have a dark body with white on the legs and usually a white spot on the spine at the neck or tail; a maximally marked tobiano is often known as a “medicine hat” pinto, and the entire body may be white except for some pigment on the head or ears.

The tobiano pattern is found in many horse breeds, including the American Paint Horse, Tennessee Walking Horse, Missouri Fox Trotter, Icelandic Horse, Gypsy Vanner, Saddlebred, Miniature Horse, and American Mustang.

Tobiano is a desirable coat pattern in certain breeds of horses, and some breeders specialize in breeding tobianos. Paint horses are a breed of horse registered by the American Paint Horse Association, which is the largest registry for tobiano colored horses.

Tobiano is the result of a simple dominant allele, and according to Brooks et al., (2002) "Homozygous and heterozygous are usually phenotypically indistinguishable from one another". However, it is commonly believed that homozygous tobianos can be distinguished by the presence of ink spots-- small, dark spots that occur on the white patches on the coat, as illustrated in Fig 1.

Figure 1. Two examples of tobiano horses, one with and one without ink spots.
Sources, left: http://paintedfeatherfarms.com/hitter.asp
right: Kim Cox

A test is available to breeders to determine if a horse is homozygous or heterozygous. Tobiano horses carry a paracentric inversion on chromosome 3 which non-tobianos lack (Brooks et al, 2007), making it possible to distinguish heterozygous from homozygous tobianos by seeing if they have one or two chromosomal inversions.

For this project, owners of Tobiano paint horses were surveyed and data was collected about each horse's genotype (homozygous or heterozygous) and phenotype (presence of ink spots) to determine if there was a correlation between ink spots and homozygosity.

LITERATURE REVIEW

Coat Color in Mammals

Pigment cells in vertebrates develop from neural crest cells that migrate and differentiate into melanocytes, which produce melanin that affects the pigmentation of hair, skin, and eyes (Erickson, 1993). Mammals produce two main types of melanin that contribute to coat color, a black pigment called eumelanin and a red pigment called pheomelanin.

All variations in the coat color of horses is due to different combinations of these two pigments, or their absence which results in white hair and skin.

The three common base coat colors in horses are chestnut, bay, and black (Fig 2). These are the result of the interaction of two loci, the Extension and Agouti. The Extension locus controls the presence or absence of black pigment with two alleles, the dominant “E” allele which produces black pigment, or “e” which produces only red. The agouti allele “a” has no effect on the coat color, but the dominant allele “A” restricts black pigment to the mane, tail, and legs. A chestnut (or sorrel horse) has a red body without black and carries two copies of the “e” allele, while a black horse has one or two copies of the “E” allele. A bay horse, which has a red body with black mane, legs, and tail, carries at least one “E” and one “A” allele (Sponenberg, 2009).

Figure 2: Base Coat Colors in the Horse. Chestnut, black, and bay horse.
Sources, chestnut and bay: Annamaria Tadlock. Black: Holly Zech.

Animals with white spotting have been selectively bred for their aesthetic appearance. This is true for both companion animals and breeds of livestock. Many pathways influence the development, proliferation, and migration of melanocytes which are responsible for coat pigmentation. Mutations in the KIT gene, a gene that encodes the protein 'tyrosine-protein kinase Kit' or CD117, effect the migration of embryonic stem cells, and have been shown to affect hair and skin pigmentation, development of the gastrointestinal tract, sperm cells, and mast cells.

KIT mutations have been found to cause white spotting patterns in horses (Haase et al., 2009), cattle (Seitz et al., 1999), mice (Geissler, et al., 1988), pigs (Giuffra et al., 1999) and humans (Giebel and Spritz, 1991; Murakami et al., 2004).

Not all white spotting, however, is associated with the KIT gene. Several spotting variants in mice are due to mutations in other genes that influence melanocyte development (Baxter et al., 2004). Border collie dogs have a characteristic white spotting pattern that is not associated with the KIT gene (Metallinos and Rine, 2000) and leopard-spotting in horses is not associated with KIT either (Sponenberg, 2009).

White spotting patterns have been linked with various disorders and traits. In mice, some KIT mutations that result in white coat color are also linked with anemia and male sterility (Geissler et al., 1981) .

There is some evidence that spotting patterns can also be adaptive. Holstein cows with more white have been shown to have increased milk yield and fewer reproductive problems. Cows with more than 60% body white required fewer matings to conceive than cows with less white (King et al., 1988), and more horn flies were observed on darker colored cattle (Brown et al., 1994). Cows with more white also have more resistance to depressed milk yield caused by intense solar radiation when they were without shade (Hansen, 1990). Darker

pigmentation around the eye in cattle has been shown to decrease the incidence of eye lesions associated with the "cancer eye" condition in Hereford cattle (Anderson, 1991).

Variation in coat coloration may also be due to epigenetic or environmental causes. Dark spots in the wool of sheep are undesirable. A study conducted with Merino sheep found that dark spots could be induced in the wool by UV radiation (Forrest, 1986).

In mice, coat color can be influenced by the diet of the dam during pregnancy. Females fed a diet rich in genistein, a compound found in soy beans, during gestation gave birth to offspring with coat colors shifted from yellow agouti to a browner pseudoagouti coat. The offspring also had increased methylation at six sites upstream from the start site of the Agouti gene. The change in gene expression in offspring resulted in darker coat color and an increased resistance to obesity (Dolinoy et al., 2006).

Animals with the same genetics, such as identical twins or animals cloned from the same donor, often have different white markings. Five foals cloned from the stallion Smart Little Lena all had white markings on the face, but each foal's markings were different in the placement and extent of white, as illustrated in Fig 3. This is due to differences in cell migration during fetal development (Hinricks, 2006).

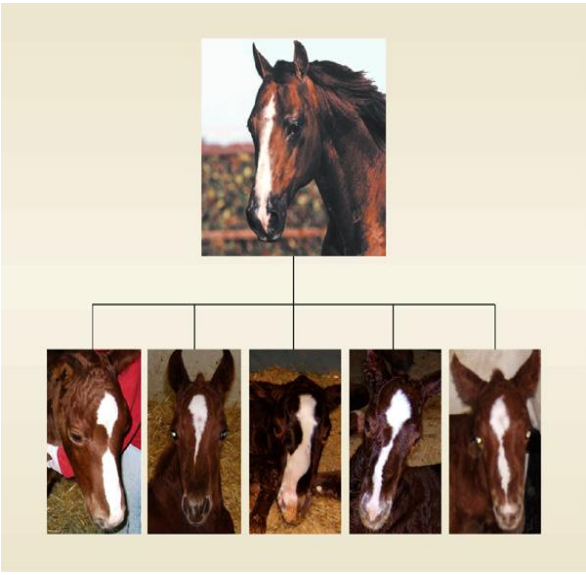

Figure 3. Facial markings of five identical cloned horses. Five foals cloned from the same donor horse, Smart Little Lena, showing different facial markings. Source: http://www.nature.com/nbt/journal/v24/n6/fig_tab/nbt0606-605_F1.htm

Many spotting patterns in mammals show variable expressivity in the amount of white spotting that occurs on the body. Selective breeding can produce animals with more or less white spotting. One example in the horse is the Clydesdale breed, in which the minimal sabino pattern of high white socks and a large white blaze are desired, but extensive body white is not (Fig 4). As a result of selective breeding, most horses of this breed are pintos that have little white over the body.

Figure 4: The Budweiser Clydesdales. An example of selective breeding for minimal sabino coloration.
Source: http://www.clarksvilleonline.com/2009/08/14/the-iconic-budweiser-clydesdales-pay-a-visit-to-historic-downtown-clarksville/

Classic breeding experiments in mice have shown that selection can increase the amount of white on the body. Dunn and Charles (1936) selectively bred piebald mice for increased body white, and were able to create a strain of mice that produced offspring with an average of 99% body white yet retained dark pigmented eyes. Animals with small black spots in the coat had the same proportion of all-white offspring as those that had no black at all, indicating some non-genetic influence on coat color.

White Spotting in Horses

In horses, white spotting patterns are dominant to solid, which is not always the case. In dogs, for example, white spotting patterns such as those found in the Border Collie are recessive, or codominant. (Schmutz et al., 2009)

White markings occur in non-pinto horses of all breeds, resulting in small amounts of white spotting on the face and legs. Horses can sometimes lack white markings completely, but even in most solid breeds, small white markings do occur. A horse that lacks any of the pinto body-spotting genes yet has white markings on the face and legs is considered a “solid”, along with horses with no white whatsoever.

Markings on the head and legs are classified by the placement and extent of the white and are often used to identify individual horses on registration papers. Markings on the face may be known as stars, strips, snips, chin spots, races, stripes, blazes, baldfaces, apronfaces, or bonnets depending on the extent of white. White markings on the legs may be known as white coronets, spots, white heels, half pasterns, white pasterns, white fetlocks, socks, or stockings depending on the extent.

White markings appear to be inherited quantitatively in some breeds and are thought to be related to a recessive allele that maps to the KIT gene. White markings tend to be more extensive on chestnuts than bays, and more extensive on bays than on black horses. Bay horses that are heterozygous for the extension allele (Ee) also have more extensive white markings that homozygous EE bays (Woolf, 1992).

The difference between white markings like those found on a solid horse, and white markings on pinto horses is that pinto patterns are characterized by extensive white spotting across the body.

There are currently four recognized non-symmetrical spotting patterns in horses: tobiano, splashed white, sabino, and frame overo. Each can be distinguished by the size and placement of white on the body, as illustrated in Fig 5. All show variable expressivity in the percent of body white present.

Figure 5: The four types of pinto coloration in horses. Left: sabino (top) and tobiano. Right: frame overo (top) and splash white.
Sources: sabino: http://www.starwood.com.au/5.html
tobiano: http://www.silverwoodfarm.com/sempatico.html
frame overo: http://equinegenetics.blogspot.com/2009/10/frame-overo-aka-lethal-white-and-why.html
splash white: http://www.myhorse.com/color-patterns-paint-horses.html

Tobiano is a dominant pattern that creates large white patches with smooth edges that often cross the spine at the neck and tail. The head and eyes are usually dark, and white facial markings are minimal, while white leg markings are usually present.

Homozygous and heterozygous tobiano horses are indistinguishable based on amount of white spotting. Tobiano horses may be anywhere from minimally marked with only white socks and a small white spot, to almost completely white, regardless of genotype. Some

tobiano horses have secondary dark spotting that occurs on the patches of white, known as "ink spots" or "paw prints". Some breeders have noticed horses with these spots are more likely to be homozygous than horses without them.

The tobiano pattern is thought to be caused by an inversion on chromosome 3 that disrupts the functioning of the KIT gene. Studies of tobiano and non-tobiano horses found that only tobianos carried this inversion, with homozygous horses carrying two copies (Brooks et al., 2008).

There is a genetic test available that distinguishes homozygous from heterozygous horses based on the number of chromosomal inversions a horse has. Many breeders that focus on producing tobiano colored foals test their breeding stock.

Sabino is characterized by white spotting on the legs with jagged edges, white on the face that usually includes the muzzle and chin but leaves the eyes dark, often spotting or roaning on the belly or flanks of the horse. White starts at the legs and chin of the horse and moves toward the body. There is sometimes ticking in the tail causing a ring-tail appearance known as “rabicano”.

New research has shown that the genetic control of sabino coloration is more complex than previously thought. There appear to be multiple mutations that can cause this pattern to occur.

One mutation is the sabino-1 mutation, which is due to a single changed base pair in intron 16 of the KIT locus on chromosome 3 which affects the functioning of exon 17. This allele acts as an incomplete dominant, with homozygous horses having much more white on the body, often making the horse completely white (Brooks and Bailey, 2005). The sabino-1 allele has been found in Tennessee Walking Horses, Miniature Horses, American Paint

Horses, Shetland Ponies, Missouri Fox Trotters and other breeds. However, it does not occur in Arabians or Clydesdales, two breeds that are noted for having sabino markings.

Other mutations that cause sabino coloration in breeds include a single base pair nonsense mutation in exon 15 of the KIT locus in the Franches-Montagne breed, a missense single base-pair change in exon 4 in the Arabian, a single base-pair change in exon 12 in the Camarillo White breed, and a single base-pair change in exon 13 in the Thoroughbred.

Sabino Clydesdales, Arabians, and American Paint Horses have sabino patterns that appear to be polygenic rather than due to a single allele, and offspring often resemble their parents in the amount of white present.

Another potential type of sabino has been observed in cases when two solid parents have had loudly marked sabino offspring, which then go on to produce loudly marked offspring themselves. This has led to speculation about a possible genetic masking of sabino in some horses (Sponenberg, 2009).

In breeds where high white socks and blaze are desired but body spotting is not, selective breeding has produced horses that produce fairly consistently marked offspring; an example is the Clydesdale breed.

Because sabino horses in different breeds have subtly different phenotypes and are now being shown to have a different genotypes as well, they may actually be distinct patterns that are all being lumped under the term “sabino”.

Splashed white is a pattern where white occurs on the face and legs, and the body is white ventrally. The white areas have crisp, often straight edges, giving the appearance that the horse was dipped in white paint. The head often has a large white blaze or bald face or may be completely white, and the eyes are often blue. There is also an association between deafness and the Splashed White color; many, although not all, horses with this pattern are

deaf. Some breeders believe that if there is white around the eyes of a horse it is more likely to be deaf (Sponenberg, 2009).

Splash is thought to be due to a dominant gene although some breeders believe it is actually an incomplete dominant, with homozygous horses showing greater amounts of white spotting (Behning, 2008).

Frame overo is a dominant pattern of white that occurs on the sides of the abdomen and neck and spreads horizontally. White usually does not cross the spine and the legs are solid. There is usually a lot of white on the face and the eyes are often blue. The edges of the white are often jagged.

The frame mutation substitutes isoleucine for a lysine in the Edothelin B receptor gene, affecting the development of melanocytes and enteric nerve cells (Metallinos, et al., 1998).

Homozygosity in frame horses is also known as “Overo Lethal White Syndrome” because homozygous foals do not survive more than a few days after birth. They are born almost or completely white and have an underdeveloped colon which results in intestinal obstructions and death within days. A similar disorder is also found in humans, known as Hirschsprung's disease (Puri and Shinkai, 2004)

Lethal white foals can be prevented by mating frame overos only to horses that do not carry the allele. A genetic test is available to detect the presence of the frame overo allele. This is useful in horses that carry multiple pattern genes, or display minimal white, making identification of frame by phenotype alone difficult.

A fifth type of white pinto spotting, called manchando, may exist. It is incredibly rare and has only been found in Argentina, in a few horses of different breeds. Its appearance in Argentina may be because the culture there is more fascinated with variations of horse color

and more likely to bring odd colors to attention (Sponenberg, 2009). It has been also speculated manchando could be due to an environmental, rather than genetic, cause.

There is a symmetrical white spotting pattern that occurs in horses known as the leopard complex, or LP gene, and is a characteristic of some breeds, such as the Appaloosa. The leopard complex is a single incomplete dominant that creates patches and spots of white across the topline of the horse, often starting on the top of the hips and spreading from there (Sponenberg, 1982).

The colors produced by the leopard complex are varied (Fig 6), with some common expressions being small white spots over the hips, a white blanket over the rump with or without dark spots, a mostly white body with or without dark spots, and a type of roan that creates white hairs over the body leaving bony prominences dark, known as “varnish roan” or “Appaloosa roan” that increases with age. A horse may exhibit one or several of these

Figure 6: Examples of patterns created by the leopard-complex. Left: Snowcap (top) and leopard. Right: varnish roan (top) and spotted blanket. Sources:
snowcap:
http://www.jgappaloosas.com/rollsoffspring.htm
spotted blanket:
http://www.onlyequus.com/breeds/appaloosa/
varnish roan:
http://www.crosscreeksporthorses.com/forsale.html
leopard appaloosa:
http://www.rainbowridgefarmappaloosas.com/dream_photos.html

patterns at one time-- for example, a horse may be varnish roan with a white blanket over the hips and have leopard spots.

Horses that are homozygous tend to have fewer dark spots on the white areas and are sometimes referred to as “fewspots”. This mutation also creates skin that has flecks of unpigmented areas, referred to as “mottled skin”, a white sclera around the eye, and often striped hooves (Appaloosa Horse Club) .

The LP allele is directly associated with congenital stationary night blindness, which affects the animal's ability to see in low-light conditions. Horses that are homozygous for LP have severely limited gene expression of TRPM1 (transient receptor potential cation channel, Subfamily M, Member 1), just .05% that of non-LP horses (Bellone, et al., 2008).

The same low levels of TRPM1 expression are found in both pigmented and unpigmented skin in homozygous LP horses. Heterozygous horses have expression that is intermediate between non-LP and homozygous LP horses. The TRP proteins are thought to help regulate intracellular Ca2+ concentration. Low levels of calcium interfere with the ability of bipolar cells in the eye to transmit nerve impulses, creating night blindness in homozygous horses. TRPM1 is also thought to play a role in melanocyte function.

A horse that does not carry any of the spotting genes is considered a true solid. These horses may have no white at all, or more commonly may have white markings-- small amounts of white on the face and legs. In some breeds, such as the American Quarter Horse, pinto patterns are undesirable and only solids are allowed for registration.

The definition of solid varies according to the standards of different breeds. The American Quarter Horse Association, for example, will consider a horse solid if white markings are limited to the face or the knees and hocks of a horse. Because this has resulted

in selection for minimal pintos, the patterns frame overo, sabino, and leopard-complex all occur rarely with minimal expression in the Quarter Horse breed.

Horses that carry a spotting gene may be registered as solid if their white spotting falls within the breed standard's acceptable white rules. This can occur with any white pattern but seems to be more common with frame overo, splashed white, and sabino since the white does not cross the spine. A pinto foal resulting from “solid” parents is known as a crop-out. Such horses are rare but do occur from matings of minimally marked horses.

MATERIALS & METHODS

Tobiano horse owners were located through Dreamhorse.com, a horse sales website. Sale listings with photos were used to identify tobiano horses. Horses that appeared to have pinto coloration due to multiple genes-- for example, toveros, or horses carrying both the tobiano and frame overo genes, which often have blue eyes-- were excluded. Since foal coat color can change as an animal matures, foals were also excluded. Photos too small or not clear enough to identify the horse as a tobiano were also excluded. A survey was sent to every owner of a tobiano horse that fit the above criteria.

Owners were asked three questions: Has this horse been tested as being homozygous or heterozygous? Does this horse have any “ink spots”-- smaller dark spots on the white areas of the body? Are the parents both tobianos or is either solid? Photos of every horse were viewed, confirming the presence or absence of ink spots. Ermine spots-- small, dark spots that appear around the coronet band above the hoof in horses of all colors with white socks- were not counted as ink spots.

Horses were identified as being homozygous only if they were tested as homozygous and had two tobiano parents, or in one case, if both parents were confirmed as tested homozygous tobianos. Horses were identified as being heterozygous either if they tested as heterozygous, or if they had a solid, non-tobiano parent.

In any case where an owner was unsure or provided conflicting information (for example, if they said the horse had ink spots but the photos showed none, or if owner was unsure of parentage), the data were not used.

Horses were then placed into one of four categories: Homozygous with ink spots, homozygous without ink spots, heterozygous with ink spots, heterozygous without ink spots. Numbers are presented in table 1. A Fisher's exact test was performed to see if there was an association between homozygosity and ink spots.

During the data collection process, it appeared that some of the homozygous horses had more ink spots than most of the spotted heterozygous horses. The number of ink spots on the body of the horses were then counted, and horses were classified as either having five or fewer ink spots, or more than five ink spots. Horse with fewer than five ink spots were omitted from the counting if photos were not available for both sides of the horse.

A Fisher's exact test was performed to test the association between homozygosity and number of ink spots.

RESULTS AND DISCUSSION:

Of the 66 horses included in the data sets (table 1), there were twenty-four homozygous horses with ink spots; no homozygous horses without ink spots; twelve heterozygous horses with ink spots; and thirty heterozygous horses without ink spots. These results indicate that ink spots are not random, but rather are associated with homozygosity ($p < .001$).

The horses with ink spots were then classified as having more than five ink spots, or five or fewer ink spots, using photos of both sides of each horse. We were able to obtain photos of both sides of 31 of the horses in our data set, and counted the number of ink spots on those horses. There were eleven heterozygous horses, four of which had more than five ink spots, and seven with five or fewer, and twenty homozygous horses, all of which had greater than 5 ink spots (table 2).

Table 1. Numbers of homozygous/heterozygous horses observed with and without ink spots

	Homozygous	Heterozygous
Ink Spots Present	24	12
No Ink Spots	0	30

Table 2. Numbers of homozygous/heterozygous horses observed relative to numbers of inkspots

	Homozygous	Heterozygous
>5 Ink Spots	20	4
≤ 5 Ink Spots	0	7

Ink spots do not occur at random but are associated with homozygosity ($p < .001$), and among all horses with ink spots, those with greater than five ink spots are more likely to be homozygous ($p < .001$).

CONCLUSION:

The presence and number of ink spots appears to be due to both variable expression and variable penetrance. Heterozygous horses may have ink spots; however homozygous horses always have ink spots and have more inks spots than heterozygotes.

While no homozygous horses without ink spots were found, it should be noted that all horses in the survey had a body white percentage of approximately 20-80% white. No minimal tobianos (those with white restricted to the legs and face) were contained in our survey. Inclusion of such horses may give different results as less body white may leave less room for ink spots to be expressed.

Ink spots appear to be a fairly good indicator of homozygosity in tobiano horses. Both the presence and the number of ink spots can help predict the genotype. Horses with ink spots are not necessarily homozygous, however homozygous horses always have ink spots, and tend to have more ink spots, than do heterozygous tobiano horses.

This information may be valuable to tobiano breeders when deciding which horses they choose to breed or test for homozygosity.

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