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HOOF WALL SEPARATION DISEASE AND THE GENETICS OF IT IN THE CONNEMARA PONY

Diploma work
Completed in partial fulfilment of the Veterinary Medicine Diploma course of the Faculty of Veterinary Medecine, Budapest.

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Abstract

During my time as a student I come across many interesting cases but this one case about the pony X I couldn't let go. The reason it became so interesting was because when I first heard about this condition, Hoof Wall Separation Disease or HWSD it was not settled to be a recognised disease nor yet a genetic disorder. Thanks to some Connemara breeders I came in contact with the Connemara-pony blog spot. Where owners and breeders from all over the world could contact each other and share their stories. People started then to realise that they had a genetic problem in the Connemara breed where the hoof wall separates from the underlying structure and the affected pony bear its all weight on the sole instead of the hard hoof. At first there where many who questioned this theory and many of the breeders societys who didn't want to recognise this condition as a genetic problem. But now just a few years later the Connemara research group has developed and thanks to veterinarians and reasercher there are now a genetic test for this condition and more or less all the breeders' society encourage to test the breeding ponies.

Introduction

The Connemara pony

The Connemara ponies are known for their athleticism, versatility and good temperament. It is named after a wild rocky Connemara region in county Galway on Ireland's west coast. Where hardy ponies have existed for hundreds of years and where the breed first became recognized as a distinct type.
Connemara region has a very harsh landscape; wich is giving rise to a pony breed of hardy, strong individuals. Some believe that the Connemara pony developed from Scandinavian ponies that the Vikings first brought over to Ireland. For additional strength and stamina, Arabian, Hackneys and Thoroughbreds blood where added. Too much crossbreeding began to dilute the bloodlines so the Connemara pony breeder's society was founded in 1923 and works to preserve the breed type. The studbook was established in 1926 and today Connemara ponies are bred worldwide.
Today the Connemara pony is known as a good sports pony, ridden by both children and adults and competitive in show jumping, dressage and eventing but also endurance and shown in harness.

The Connemara Pony Breeder's Society of Ireland together with the British Connemara Pony society sets the original breed standards which follows:

The adult pony is usually 128-148cm in height, with a strong back, loins and hindquarters. Deep and broad through the ribs, and with a riding-type well laid back shoulder and well placed neck without undue crest, giving a good length of rein. The head should be of pony type, broad between the eyes, which should be large and appear kind. Deep but refined jaws, with clearly defined cheekbones. The ears should be of pony type (relatively short). The legs should be relatively short from the knees and hooks to the ground, with a strong, muscular upper leg, strong and well-defined knees and hocks, and well shaped hard feet that are of medium size. There action should be free, active and easy. Permitted colours are grey, black, brown, bay, dun, chestnut, palomino and dark eyed cream. The pony should be intelligent with a good temperament, suitable for adults and children; it should be hardy with good endurance sure footed around and able to jump.

Figure 1: Swedish Connemara stallion Poetic Kelly RC 108 year 2009

The structure of the hoof

The external surface is convex from side to side and slopes obliquely from edge to edge. In front the angel of inclination on the ground plane is 50° for forelimb and 55° for hind limb. It is gradually increasing by the sides and is about 100°at the heels.
The curve of the wall is wider on lateral than medial side and slope of the medial quarter is steeper than that of the lateral one.
The surface is smooth and is crossed by distinct ridges which are parallel with the coronary border and indicate variations in the actvitiy of the growth of the hoof. It is also marked by fine parallel striae which extend from border to border in an rectilinear manner and indicate the direction of the horn tubules.
The internal surface is concave from side to side and bears about 600 thiny primary laminae wich extends from the coronary groove to the basal border of the wall. Each laminae bears 100 or more secondary laminae on its surface so that the arrangement is pennate on a cross section.
The hoof is composed by epithelial cells which are more or less completeley keratinized except in its deepest parts, the stratum germinatum. The cells have not undergone corinification and by their proliferation, maintain the growth of the hoof. The cells are arranged to form horn tubes wich are united by intertubular epithelium, and enclose medullary cells and air spaces.
The dorsal hoof wall is composed of tubules of keratins, a protein which provide strength, hardness and insolubility due to disulfide bonds between and within the long chain fibrous molecules. There are dozens of different keratin molecules, with molecular weights in the range of 40–70 kDa and varying degrees of hardness and sulfur concentration. Terminally differentiated keratinocytes, originating from the coronary band, are arranged in specialized tubular and intertubular configurations in three distinct zones: the stratum externum, stratum medium and stratum internum.
Stratum externum comprises the periople and the stratum tectoric. Periople is composed by a soft nonpigmented tubular horn and becomes white when the hoof is soaked in water. It is continuous with the epidermis of the skin above and extends downwards for a variable distance. The stratum tectorium is a thin layer of horny scales, which gives the outer surface of the wall below the periople its smooth, glossy appearance.
Stratum medium forms the bulk of the wall and is the densest part of the hoof. Its horn tubules run in a parallel direction from the coronary to the basal border. In dark hooves it is pigemented except in its deep part.
Stratum internum is the laminar layer wich consists of the horny laminae and is nonpigmented. The primary laminae are narrow and thin and their origin at the lower margin of the coronary groove, but become wider and thicker distally.

At the junction of the wall and sole all the three layers are united by interlaminar horn to form the white line. Only the central part of the laminae becomes fully keratinized, they are composed of nontubular horn in the normal state.

Distribution of the density of the horn tubules reduces through the three main zones of the hoof wall. The greatest density of horn tubules is in stratum externum, where on the surface of the hoof wall they are slightly flattened to become more oval. Which is thought to help retain moisture of the hoof. The gradient in tubule density mirrors the gradient in water content across the hoof wall, with the innermost layers of the hoof having the highest relative water content, which confers high crack resistance.

Between the horn tubules is the intertubular horn, which binds the horn tubules together, this has intermediate filaments, which are laterally orientated fibers, which help to bind the horn tubules together and resist cracking.
The outer stratum externum is more rigid than the the more flexible stratum medium, while the stratum internum is the softest, this gradient of reducing stiffness helps the hoof capsule to transfer energy smoothly between the wall and the dermis and also allows the hoof capsule to expand in shape under loading, and return back to its original shape when the load is removed. Transfer of weight from the skeletal structures is primarily from distal phalanx via the hoof wall to the ground surface, the solar surface can only support a small proportion of the total weight of the horse before it causes pain.
In healthy horses, by the time the shock of the impact with the ground reaches the first phalanx, about 90% of the energy has been dissipated, mainly at the stratum internum.

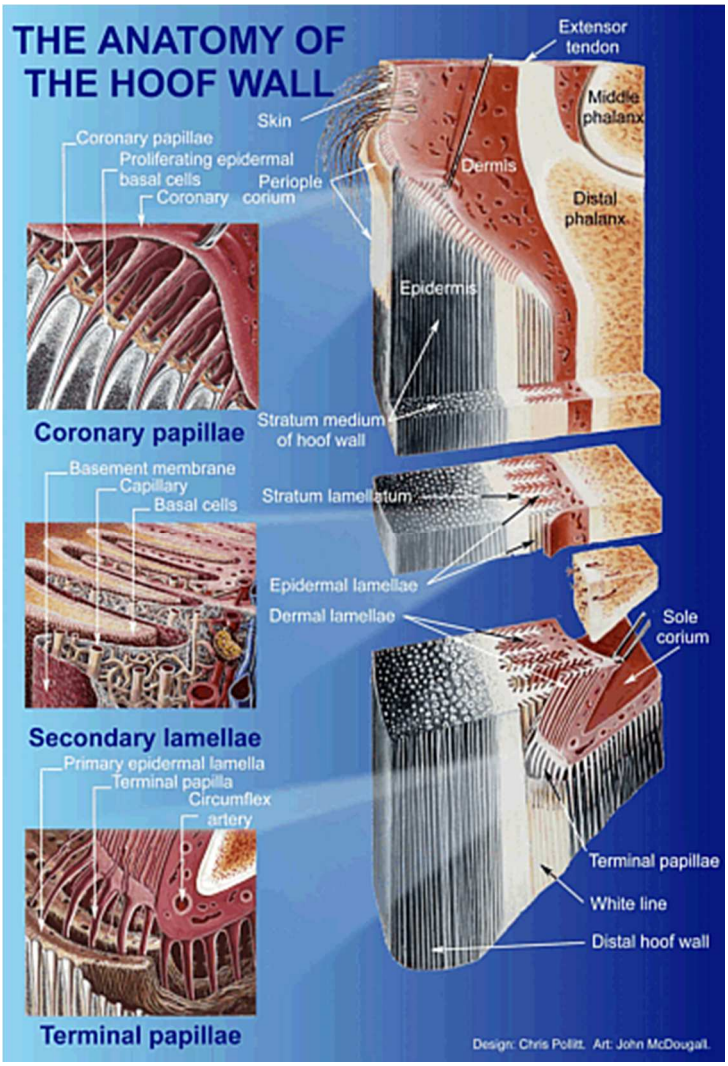

Figure 2: Hoof wall structure

The sole consists of tubular and intertubular horn. The tubules run parallel with those of the wall and very much in size.
The frog is composed of relatively soft horn, wich is much more elastic than that of the wall or sole, and is not fully keratinised. The horn tubes in it are slightly felxous.

The hoof is non vascular and receives its nutrients from the corium. It is also destitute of nerves.

Hoof Wall Separation Disease (HWSD)

Background

The Irish Connemara pony is known for being both athletic and surefooted. But a recent discovering and frustrating hoof condition can strike these ponies down before they even reach weanling age. This condition is called Hoof Wall Separation Disease or HWSD.

HWSD is troubling the Connemara pony because the parents of the affected pony are appears completely unaffected. Although affected individuals do share common bloodlines, it is problematic to predict if a particular breeding will produce a foal with unhealthy hooves. So the investigation of the underlying genetic cause of hoof wall separation disease have lead to inform these breeding decisions and has also provide insight into the disease pathophysiology. HWSD is a unique, verifiable and testable disease. It's a condition that has been identified in several different countries, in both local and imported stocks. This disease results in weight bearing borders of the hoof wall is breaking down and separates from the underlying structure, which leaves the pony to bear the weight on the sole of the hoof. This development can be intensely painful and prevents the ponies from moving easily or performing any work.

Diagnosing

Hoof wall separation Disease (HWSD) seen in Connemara ponies is an inherited condition. Clinical signs consistent with a receding dorsal hoof wall and secondary solar proliferation.
Wich means that the weight bearing borders of the hoof wall breaks away from the underlying structures leaving the pony to bear the weight on the sole instead of the dorsal hoof wall. This defects develops only in Connemara ponies aged 1 to 4 months of age and it only affects the dorsal hoof wall.
We see variable degrees of splitting within the dorsal hoof wall, mostly most prominent along the distal margin and variably spreading more proximally. The horn of the hoof wall is most likely to be brittle and easily broken while the horn of the sole appears stronger. The coronary band appears normal.
Distal extremity radiographs reveals no abnormalities. In some severe cases we can see coffin bone rotation, an indication of laminitis where the toe of the distal phalanx has dropped due to loss of lamellae support.
HWSD becomes severely painful despite careful management and it is a life long problem where their life quality reduces so euthanasia may be necessary. Even if the condition could be controllable, ponies can still develop chronic laminitis over time.

Robert Eustace of the Laminitis Trust in United Kingdom originally described this condition, as "Coconut-matting hooves" as the border of the hoof wall appear rough and frayed. Hoof samples of affected Connemara Ponies which had been referred to the Trust for Treatment, were analysed at the university of Edinburgh, where they found a malfunction of lipid metabolism in the extracellular matrix of the hoof wall between the tubular structures of the hoof wall. In simple terms there seems to be a lack of "waterproof glue" holding the hoof wall tubules together.

A confounding aspect of the problem is that it occurs in radically different environments without respect to wet or dry, hot or cold and appears early in life.

Figure 3:
This picture shows how HWSD causes the layers within the dorsal hoof wall to separate from each other. (The brown far right structure.)

Figure 3

Figure 4:
Here we see the calloused sole is typical of HWSD and the result of walking on the sole. If the callousing is left alone the pony will stay paddock-sound but will usually not be capable of any work.

Figure 4

Figure: 5

5A) Here we see the dorsal hoof wall separation at the sole and
5B) shows the proliferative horn on the solar aspect of the hoof.
5C) Shows us a sagittal section of a post mortem HWSD affected hoof that demonstrates that the dorsal hoof wall fissure occurs outside of the white line.

Figure 6

Figure 6:
7-month-old Connemara filly with HWSD with typical lesions: walking on the sole not on the wall as normal.

Distal hoof wall splitting does not, itself, result in lameness. Rather, repeated stress on the innermost layer of the lamellar interface results in separation of the interdigitating lamellae from the distal phalanx (laminitis).

Wall separations are commonly seen in the heel area of hooves, but this is different. In some cases the inner exposed hoof wall looked like steel wool fibres. Many cases of this type of dorsal hoof wall separation deteriorate to a diagnosis of laminitis.

At first it was easy to misdiagnose this condition with the white line disease or just poor hoof maintenance.

Differential diagnosis

Chronic selenium toxicosis

Clinical signs: Brittle dry hair, alopecia, abnormal hoof wall growth and lamness on all four legs. Theses signs reflects seleniums ability to bind to the sulphur containing amino acid methionine and cysteine, wich affects cell division and growth.
Compare to HWSD this abnormal hoof wall growth starts at the coronary band, while in HWSD we see proliferative horn on the solar aspect of the hoof.

Toe cracks, quarter cracks and heel cracks

Definition: Cracks of the hoof wall can either start at the bearing surface of the wall, or extend to a variable distance up the hoof wall, or cracks originating at the coronary band as the result of a defect in the band and extending downwards. They can vary from toe cracks, quarter cracks and heel craks depening on where on the foot they can be found.
Excessive growth of the hoof wall causing a splitting of the wall from lack of trimming of the feet is a common cause. Injury to the coronary band, producing a weak and deformed hoof wall wich can lead to cracks originating at the coronary band. There can also be weakening of the wall due to excessive drying or excessively thin walls.
Clinical signs: Obvious split in the hoof wall. Lamness may not be present, but it becomes evident if the crack extends in to the sensitive tissue or allowing infection to gain access to these structures.

Vertical tear of the hoof wall

It is most frequently seen at the medial quarter of the heel and is caused by tearing of the hoof wall from the underlying soft tissue of the hoof. The cause is unknown but seems to ouccur mostly in Thoroughbreds 2-3 years of age with small hoofs. Commonly these horses also have long toes, underrun heels, thin walls and flat soles. At necropsy a loss of the normal attachment occurs between the laminae that may extend to the solar surface, and hemorrages is usually present. It appears like a focal acute laminitis without rotation of the distal phalang.
Clinical signs: Hemorrhages can be seen through the wall on white hoofs. Degree of lamness can range from mild to severe. It is more difficult to appreciate lamness on soft ground but trotting on hard sufaces there is an obvious shortening of the stride. Hoof tester examination shows pain and an obvious lack of hoof strength. Unilateral perineural anesthesia of the palmar digital nerve usually relives the signs of pain and lamness.

Laminitis
Definition: inflammation of the sensitive laminae of the foot as a gross over simplification of a complicated interrelated sequence of events, that results in varying degrees of breakdown of the interdigitation of the primary and secondary epidermal and dermal laminae of the foot. If the loss is severe enough there will be a rotation and/or distal displacement of the distal phalang.
Clinical signs: Increased temperature of the hoof, pounding digital palamar pulse, increased vital signs and body temeperature, sweating, walking tenderly, constantly shifting of weight, positive response to hoof tester at the toe, "founder stance" in attempt to decrease the load on the affected hoof and tendency to lie down whenever possible.
In chronic cases there can be a change in the outward appearance of the hoof, with a concave hoof wall and also founders rings can be seen. Wich are growth rings that are wider at the heel than the toe. If the sole is either flat or concave just dorsal to the apex of the frog it indicates phalangeal displacement or penetration. Widening of the white line at the toe with or without brusing and clubbing of foot can also be seen.
In case of sinking, it may be possible to palpate a groove between the coronary band and the skin of the pastern.

White line disease
HWSD is clinically similar to white line disease witch causes a progressive separation of the inner structures from the outer hoof wall in horses of all breeds, age and sexes. And there is great debate about whether white line disease is caused by a bacterium or a fungus.

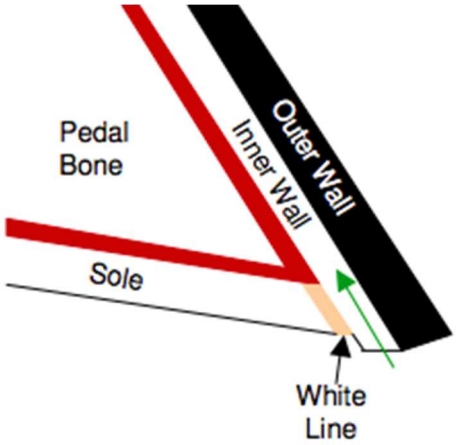

Figure:7

The green arrow (figure 7) shows where the white line disease infection would take place. As you can see, it is not the white line, but the inner wall that is most vulnerable. Other structures can become affected if the infection is severe, but this is uncommon. If the inner wall of the hoof is not so tightly bonded together as it should be (usually because of nutritional or metabolic imbalance), the organisms can potentially eat their way upward to the top of the inner wall, causing the outer wall to separate away.
It usually starts in the white inner hoof wall and eats its way upwards, weakening the attachment of inner and outer wall. In advanced cases, the disease has been known to eat through into outer wall, sole and even frog.

Treatment and management

In some cases, some combination or variation of supportive hoof orthotics, foot casts and glue-on shoes along the lines of laminitis treatment are used. Various topical treatment and nutritional supplements have been used. These efforts are under the heading of managing the condition but euthanasia is common.

In many instances, the solution consists of a temporary artificial repair rather then a long-term solution. Success is attained with the use of carefully considered foot care and shoeing principles. It is also apparent that hoof wall problems require farrier expertise, thus the development and subsequent maintenance of a solid working relationship with a farrier is essential.

Most avalible reports suggest removal of the separated wall, allowing exposure to the underlying damaged inner layer of horn, as well as removing the horny shelf that tends to retain moisture and foreign material. It is neccessery to remove the entire separated wall to the extent of reaching normal attached wall. Treatment management beyond this point varies with the amount of wall removed, the location of the removed wall, the shape of the foot, the environment and the overall management skills of the owner or caretaker. In most situations it is best to have the pony shod after resection of the wall to protect the remaining unaffected hoof wall, unsupported sole, and exposed lamellar tissues.
The remaining shape of the foot should dictate the design of the shoe. Clips or similar creative addittions to the shoe may help to secure such shoe. Foot bandages or glue-on shoes may be useful if sufficient wall has been removed, making it difficult to nail a shoe on. The damaged area can be filled in with various hoof wall repair materials, but it is important that the underlying lamellar tissues are fully keratinised.

It is belived that removal of the wall prevents the continued capture and retention of moisture and environmental debris as well as the potential for futher mechanical tearing, in combination with shoe designed to meet the needs of the affected foot or feets, it is the most important aspect of the therapy.

The best is to keep the affected pony in a reasonably dry area. Topical medication, such as 2% iodine or methiolate once a day, can be used to minimize further contamination as well as to provide the caretaker with a reason to examine and clean the involved area frequently.
It is not unusual to have to make repeated attempts at resection as the foot continues to grow if the growing horn is not attaching. In some cases, it is best to let the horse rest to prevent any undue mechanical forces during the repair process.

Ponies with acute partial or complete hoof wall avulsions should be treated as emergencies. In most cases the process of examination and initial treatment is facilitated with sedation and local anastesia.

This is a very painfull condition so NSAID is recommended. Unfortunately it can be too difficult to manage and the prognosis is often very poor, so the outcome is euthanasia in most of the cases.

Figure 8:
All the broken hoof is removed and the hoof is cleaned with iodine

Figure 9:
Kerckheart Aluminium shoe glued to repaired hoof. Play-dough is used on the inner border of the shoe to prevent the glue or shoe pressing onto the sole of the foot.

Materials and methods

Before now when we have a genetic test for this disease, it was only an affected foal with HWSD that would alert the breeder that both parents where carriers. As this may not be the first foal from this dam and sire, previous foals may already been produced and unknowingly sold on, as there were no indicatiors at the time there was a genetic problem.
When only a few ponies were carriers the chances of both parents being affected must have been small, the odd foal with poor quality hooves would have been seen as a random occurrence of bad luck. Other foals would have been born as carriers with good hooves, in this insidious way HWSD has secretly crept into the population and as the ponies have moved around the world, and so has HWSD.

Blood samples were collected at the University of California, Davis School of Veterinary Medicine William R. Pritchard Veterinary Medical Teaching Hospital. Additional samples were drawn by private veterinarians and mailed by individual Connemara owners. All animal samples were obtained following protocol number 17491 approved by the University of California Davis Institutional Animal care and Use Committee.

Samples of DNA in addittion to pedigrees and photographs were collected from 330 connemara ponies around the world, both affected and unaffected. A case-control allelic genome wide association analysis was performed with $n_{cases} = 15$ and $n_{controls} = 24$.

Phenotype

Two affected Connemara ponies were evaluated at the University of California, Davis (UCD) Veterinary Medical Teaching Hospital in 2011 by a board-certified equine internist. Following humane euthanasia, hoofes from three additional affected ponies were evaluated by a board-certified pathologist (VKA). Distal extremity radiographs were available from the five index cases. From these index cases and the histologic assessment of affected hooves, the phenotype of HWSD was established.

For additional cases, inclusion criteria as an HWSD case consisted of:

1. Connemara pony breed
2. Age of onset within the first six months of life
3. Clinical signs consistent with a receding dorsal hoof wall and secondary solar proliferation, supported by digital photographs of all four feet.

Inclusion criteria for unaffected animals in the genome wide association study consisted of:

1. Connemara pony breed
2. >2 years of age
3. No apparent hoof pathology, supported by digital photographs of all four feet when available.

Horse genetics

The last twenty years there have been many developments into the equine genome. The Horse Genome project that started in 1995 and later they completed the whole genome scanning of the horse in 2009 that is of major importance for all genetic research in the field of equine genomics. It is the work done by these groups that made it possible to discover the monogenic traits, and their diagnostic tests that can prevent further spreading of these diseases.
The current molecular genetic testing done on horses are either identification/parentage or disease/trait testing. It is based on polymerase chain reaction (PCR) for amplification of certain DNA targets. DNA test for monogenic traits are available at commercial laboratories and are frequently used today.
Further gene discovery projects and polygenetic traits are currently being investigated on the basis of the results and achievements of the whole genome scan and the Horse Genome project.
With the work of the Horse Genome Project there was developed several genetic maps that can map traits to chromosomes or sub chromosomal levels and later the gene sequence was developed.
The equine maps that have been developed includes;

- Radiation hybrid maps
- Comprehensive linkage maps
- Medium density horse gene map
- High density microsattelite maps
- Informative marker set for QTL (Quantitative Trait Loci) maps.

A whole genome shotgun library was sequenced at the Broad Institute in Cambridge Massachusetts and Bac ends sequenced at the university of veterinary medicine in Hannover and Helmholtz centre for infection research. Over one million single nucleotide polymorphisms (SNP) have been detected during the sequencing by the first horse with a whole genome scan and additional seven other breeds. The knowledge obtained in the scans is made available to the public, making it easier for future researches to discover new genes involved in equine diseases.

Quantitative trait loci (QTL) are genomic regions highly likely to contain genes influencing the traits of interest. To identify QTL responsible for certain diseases a high density linkage map of the whole genome with an equidistantly distribution are necessary. For the detection of QTLs responsible for equine diseases, different genetic markers are in use today, in equine genetics microsattelite markers and SNP has been most frequently used. The markers would be polymorphic, evenly spaced and give dense marker coverage and low genotyping error rate. SNPs are common and evenly distributed in the genome, but have less variability per marker and will need more SNPs to provide the same information as microsattelites. Microsattelites, which can have more alleles, are able to cover a larger area of the genes that will be beneficial when starting to look for QTLs. SNPs are able to provide a denser map compared with microsattelite and are often used when the region in interest are identified.
With this further insight and methods of investigating the equine genome, the search for polymorphic traits continuous all over the world. The draft of the gene sequence is now in place and details of the refinements are the next step in the research.

Genetics and tests for HWSD

This appears to be a congenital condition, if the pony has these problem it will be born with it and it can be seen in foals as young as 2-3 weeks of age. It is not an acquired condition but there are degrees of severity. Some can be managed and unfortunately some needs to be euthanized. Environment and nutrition may be contributing factors but do not appear to be the cause.

Year 2013 November 1-3 in West Palm beach, Fla. At the international equine conference on laminitis and diseases of the foot, Carrie Finno, DVM; PhD, Dipl. ACVIM presented recent study results suggesting that the Connemara HWSD is an autosomal recessive trait that can be avoided by careful breeding. She also explained that this disease is characterized by severe hoof wall separation during the first year of the Connemara ponies' life.

The team collected DNA samples, pedigree information and photographs of 330 Connemara ponies from around the world. They determined the genotypes of the 12 cases and 24 control horses and where able to identify a genome-wide significant candidate region of association or a specific region of the horses genome that appears to contain important genetic determinants for the trait being studied, in this case Connemara HWSD.

Based on their findings the team concluded that Connemara HWSD is an autosomal recessive trait. Autosomal chromosmes are not responsible for deciding the sex of the animal and therefore both males and females can carry the defective gene. Both parents must be carriers of the defective recessive gene in order to produce a chance of a foal with affected hooves. This means that each of the foals parents appear normal, but carry one normal allele and one defective allele (each gene has two alleles- one inherited from the mother and the other from the father). When both parents pass their defective alleles on to the foal (25% chance if both parents are carriers), the foal ends up with a pair of defective alleles, triggering disease expression. An average of one in every two offspring will be carrier, not displaying the syndrome but capable of passing the defect on. Because carrier ponies show no signs, there is no outward way to identify them and exclude them from the breeding program.
In theory, breeders could reduce or eliminate this disease prevalence by testing both parents to determine if they are carriers before breeding.

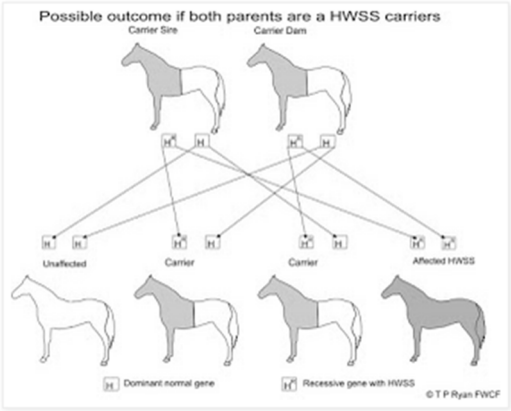

Figure 10:Possible outcome if both parents are HWSD carriers

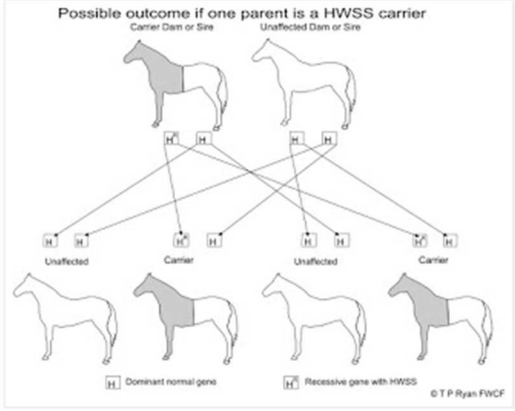

Figure 11:Possible outcome if one parent is a carrier

If one parent is a HWSD carrier and the other is not, then there will be a 2:4 chance of the foal being a carrier but will not have HWSD hoovs.

Genetic tests are needed for screening breeding stock. Such a test would allow over time, the level of HWSD within the population to be reduced or eliminated without compromising genetic diversity. Genetic diversity is a concern with an already small gene pool as the one for horses.

The genome for the horse was sequenced for the first time in 2007, opening the opportunity to isolate which genes may be involved in HWSD.

The researcher has focused on identifying the genes that are associated with the production of keratin, the protein that is used to produce horn and hair. DNA samples of both normal and affected ponies was collected and compared. The goal of this research was to identify carriers of HWSD with a simple blood test. So it can be easy to isolate and exclude HWSD from any future-breeding program.

12 cases and 24 controls where genotyped on the illumine Equine 74K single nucleotide polymorphism (SNP) array. Although small, theses groups provided sufficient power to identify a genome wide significant candidate region of association. Three additional affected foals and five obligate carriers were subsequently genotyped.

Result:

A region of homozygosity (1,8Mb) was identified in all 15 affected foals, consistent with an autosomal recessive mode of inheritance. There are tests now avalible to identify Connemara ponies that carry a mutaded gene responsible for this disease. It can determine if the pony are normal or carries one or two copies of the mutatation. Ponies that carry two copies of the mutation are highly likely to be affected by the disease. Some cases are milder and some more severe. The researchers led by Dr. Carrie Finno, identified at least one case where the pony did not appear to have clinical signs associated with having two copies of the mutation. This indicated that the mutation is not fully penetrant. However, it has very high penetrance of 96,8 percent.

The goal is to identify the molecular etiology of the disease in order to reduce its prevalence through genetic testing and to provide insight into this unique ectodermal structure. Genome-wide association analysis, coupled with whole genome next-generation sequencing, identified a frameshift variant in SERPINB11 associated with this novel, hoof specific phenotype in Connemara ponies.

Phenotypic description

Two clinically affected Connemara females were examined at 5-months and 1-year of age. The onset of hoof pathology in these two cases had become evident at 3 and 5-months of age, respectively. With the exception of the hooves, physical examinations revealed no other abnormalities; haircoat, underlying skin, mucous membranes and mucocutaneous junctions appearing normal. Abnormal sweating was not reported in either cases. In both cases, all four hooves displayed a dorsal hoof wall separation at the sole with a normal coronary band appearance. Proliferative horn was evident on the solar aspect of all four hooves. The 5-month old pony, which had markedly proliferative solar horn, were lame on both front feet at the walk while the yearling, which had undergone a recent hoof trimming, appeared sound at the walk. Distal extremity radiographs of both front feet and the dental arcade revealed no abnormalities.
Hooves from three additional HWSD-affected female ponies, aged 1, 4 and 5 years, underwent complete gross examination. Age of onset in these three cases was less than 6-months of age. In the three cases, all four hooves showed variable degrees of splitting within the dorsal hoof wall, most prominent along the distal margin and variably spreading more proximally. The horn of the hoof wall was brittle and easily broken while the horn of the sole appeared stronger. The coronary band appeared normal. All four feet were bisected sagittally. Coffin bone rotation, an indication of laminitis was evident in 2/3 ponies (aged 4 and 5 years). The white line was intact and there was no hyperaemia or scarring in the corium or lamina. In the one HWSD pony (1-year of age) with no evidence of coffin bone rotation, the distance of the white line to the horn capsule measured 1 cm and was consistent proximal to distal. This horn to white line distance is within the normal radiographic reference range reported for adult ponies. The dorsal hoof wall separation was outside of the white line. Histologic examination of coronary band, periople and proximal lamina, skeletal muscle and liver performed in one of three ponies (1-year of age) revealed no pathologic changes.

To investigate the underlying genetic cause of HWSD, a genome wide association study was performed and strong association between disease status and polymorphism in a two-megabase (Mb) region of the genome was observed. Sequencing of candidate genes within this region is currently under way and any functionally relevant genetic differences identified will be validated using a larger sample set.

Variant identification

The homozygous region identified in affected ponies (79,936,024–81,676,900 bp), contains a family of SERPINB genes. Based on the Equus caballus 2.0 genome assembly, horses have three additional copies of SERPINB3/B4 within this interval as compared to humans. The rest of the genes, orientations and order are conserved with respect to human. Due to limited information regarding comparative diseases related to the SERPIN gene family, whole-genome next generation sequencing was performed instead of selectively sequencing predicted candidate genes. Whole-genome sequencing was performed on two Connemara HWSD cases and two unaffected Connemara ponies that were homozygous reference for the associated haplotype. A published Quarter horse whole-genome sequence was used as an additional control.

Sequencing revealed a total of 9,758 single nucleotide variants (SNVs) within the interval, of which 363 segregated with HWSD in the two cases and three controls. Of these 363 SNVs, 16 were located within annotated genes or were fewer than 700 base pairs from the ATG. Although the promoters for the genes are not identified in horses, 700 base pairs where selected in order to cover key regulatory regions close to the start of translation. One additional SNV was chosen based on its location between SerpinB2 and SerpinB10. These gene-associated SNVs were selected for follow-up genotyping within a larger sample set. In addition, sequencing identified 1,230 small insertions and deletions (indels), of which 28 segregated with disease, 11 were within or close (<700 bp) to genes. Of these 11 indels, 6 were intronic, 1 was coding, and 4 were up/downstream. The coding and up/downstream indels were tagged for genotyping in a larger sample set. Of the 22 variants (17 SNVs and 5 indels) genotyped on the custom Sequenom panel, 21 passed quality control. Three variants were unique to the 23 affected Connemara ponies and heterozygous in 27 obligate carriers.

On chromosome 8, base pair 80,259,666 is located downstream of SERPINB2 and upstream of SERPINB10; 80,319,671 and the position of the four-base-pair deletion that failed quality control (80,319,673) are both within the rolling circle (RC) repeat element Helitron3Na_Mam located upstream of SERPINB8; 80,111,598 is in the fifth exon of SERPINB11. The insertion within SERPINB11 introduces a frameshift that first alters the two amino acids following residue 168, and then introduces a premature STOP codon. 55% of the protein is predicted to be truncated, including a large portion of the serpin scaffold and the entire reactive site loop.

Figure12: Connemara ponies have a genotype of “C/ CC”.

Table 1. Variants segregating with HWSD phenotype.

Genomic Location	Variant Type	Effect	gDNA	cDNA	Protein
chr8:80111598	1 bp-insertion	Frameshift *SERPINB11*	g.80111598_80111599insC or: g8883_8884insC	c.504_505insC	p. Thr169Hisfs*3
chr8:80259666	SNV	Downstream; 1431bp from *SERPINB2* and upstream; 2596 bp from *SerpinB10*	g.80259666T>C	N/A	N/A
chr8:80319671	12 bp-insertion	Upstream; 691bp from *SerpinB8*	g.80319671_80319683insTGAAAAATAAAT	N/A	N/A
chr8:80319673	4-bp deletion	Upstream; 677bp from *SerpinB8*	g. 80319673del	N/A	N/A

Four variants were unique to the 23 affected Connemara ponies and heterozygous in 27 obligate carriers.

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What are the SERPIN genes?

Genes in the SERPIN gene superfamily provide instructions for making proteins called serine peptidase inhibitors or serpins. Most serpins help control certain chemical reactions by inhibiting the function of proteins, specifically enzymes called serine proteases. A few serpins inhibit other types of proteins, and several do not have an inhibitory function.

Each serpin with an inhibitory role is responsible for blocking the activity of one or more proteins. Serpins binds to their target proteins to prevent them from completing any further reactions. Upon binding to a target, an irreversible change in the structure of a serpin protein occurs. Certain cells recognize when a serpin is bound to its target and clear these attached proteins from the bloodstream.

Researchers have identified at least 37 serpin genes in humans. These genes are divided into subgroups called clades based on various aspects of similarity. Each gene in this family is named with SERPIN, followed by a letter associated with its clade and a number assigned to the specific gene within the clade. For example, the first SERPIN gene in clade A is written as SERPINA1.

Serpin proteins are very sensitive to changes. Mutations that alter even a single amino acid in critical regions of these proteins can disrupt their usual functions. Abnormal serpin proteins often forms aggregates that can build up to toxic levels within cells. These protein aggregates may also cause a shortage of the inhibitor in areas where it is needed to control chemical reactions. Disorders caused by aggregates of abnormal serpins are called serpinopathies. Mutations in the SERPIN genes can cause various disorders, including a lung disease called emphysema, hereditary angioedema, a type of familial dementia, and thrombosis.

Gene expression

As potential regulatory variants were not identified in this analysis and in order to prioritize the four remaining candidate variants, expression analysis of SERPINB2, SERPINB8, SERPINB10 and SERPINB11 was performed. These were the four genes closest to segregating SNVs and indels. Evaluation of mRNA levels in coronary band samples from four HWSD-affected ponies and four unaffected controls revealed that coronary band SERPINB11 expression was significantly reduced in affected ponies. Relative Expression Software Tool (REST) analysis indicated down-regulation in the affected group by a mean factor of 16 and a probability that the difference between sample and control groups was due only to chance [P(H1)] of <0.001. By contrast, REST showed no difference in expression of SERPINB2, SERPINB8, or SERPINB10 between the affected and unaffected sample groups.

SERPINB11 was also found to be a very abundant transcript in the coronary band of an unaffected horse, relative to its levels in other tissues. RT-PCR showed gene expression in lung, stomach, skin, coronary band, and brain. Levels were subjectively highest in the stomach and coronary band. By contrast, SERPINB2 appeared most highly expressed in stomach and skin; SERPINB8 was widely expressed and most abundant in stomach and skin; SERPINB10 was minimally expressed in coronary band and most prominent in stomach and skin.

Penetrance, allele frequency, and carrier frequency

The SERPINB11 frameshift variant was found to be homozygous in a total of 31 affected ponies indicating complete penetrance. The severity of phenotype ranged from mild to severe. Mild being cleft between dorsal hoof wall and white line apparent on solar aspect of hoof but the pony was able to be maintained with frequent hoof trimming and shoeing. Within the entire 423-Connemara-pony data set, allele frequency was 18.7% and a total of 96 ponies were heterozygous for the SERPINB11 insertion. The heterozygous animals are all phenotypically unaffected by HWSD. Within a 324-pony subset of individuals unrelated to the affected animals, carrier frequency for the variant is 14.8%.

Genome-Wide Association

The original population of ponies used in this study was stratified, which was markedly improved by removing seven control ponies visualized as outliers on the multidimensional scaling plot. Alternatively, a mixed model approach could have been utilized to correct for the level of stratification; however, removal of the seven control ponies did not affect power to detect a significant association on ECA8. The associated region on ECA8 encompassed ~1.7 Mb, which contained 13 annotated SERPBINB genes. None of these genes have been previously associated with any of the human Eds nor was there any supporting evidence documenting expression of these genes in any ectodermal structures.

Whole genome sequencing reveals a frameshift variant in SERPINB11

Targeted resequencing of the ~1.7 Mb candidate region was considered; however, it has become more cost effective to perform whole-genome sequencing, when an autosomal recessive mode of inheritance is suspected. With Mendelian disorders, putative functional variants may more readily be uncovered on smaller sample sets using whole genome sequencing whereas if a more complex mode of inheritance was likely, capture re-sequencing could allow for many individuals to be sequenced for the particular region of interest at a relatively comparable cost.

Sequencing revealed 363 SNVs and 28 indels that segregated with HWSD, with 17 and 11, respectively, located within or adjacent to annotated genes. After genotyping a larger population of HWSD-affected and unaffected ponies, four segregating variants remained, including a 4-bp deletion that had failed Sequenom genotyping. Of these four variants, only one was coding. The insertion within SERPINB11 introduced a frameshift, leading to a premature stop codon. Based upon the severity of the HWSD phenotype, priority was placed on coding variants as the reseacher presumed the variant would alter the amino acid structure of the protein involved. In addition, q-RT-PCR data demonstrated decreased expression of SERPINB11 in coronary band tissue, where keratinocytes of dorsal hoof wall originate. The decision to focus on the one coding variant for HWSD was validated in this study; however, the reseacher acknowledge that non-coding variants have been increasingly associated with disease. The three other HWSD-segregating variants may be a part of the haplotype on which the frameshift variant originated.

Conclusions

The results of this study demonstrate a strong association of the SERPINB11 variant with the HWSD phenotype. Additionally, HWSD is the first disease to be described that results in a hoof-specific phenotype, with no other ectodermal structures affected. Further studies are necessary to determine the mechanism by which SERPINB11 maintains structural integrity of the hoof wall of healthy ungulates and if SERPINB11 plays a similar role in nail and claw health of non-ungulate species.

SNP and indel genotyping

A custom Sequenom SNP panel (GeneSeek, Lincoln, NE) was designed to genotype the 17 SNPs and 5 indels that segregated with the affected phenotype on an additional 369 Connemara ponies, 18 non-Connemara ponies, 50 Arabians, 51 Quarter Horses, and 50 Thoroughbreds. An additional 54 Connemara ponies were genotyped only for SERPINB11, using the forward primer CAAGGGGATGAGGGAGTTCT and reverse primer CCTCACTTAGCC- GAAAAGGA; the 296bp product was sequenced and assessed for the presence of a 1bp insertion.

Results

These figures cover six months from March 2015 to October 2015. As there are now three time periods covered – effectively a 12-month period – this information has been combined to provide an overview both internationally and by individual country. The information is now sufficient that trends and interpretation can be drawn from the data.
This raw data has been converted into graphs and charts to make the information more easily accessible. A short synopsis follows each of the graphs.

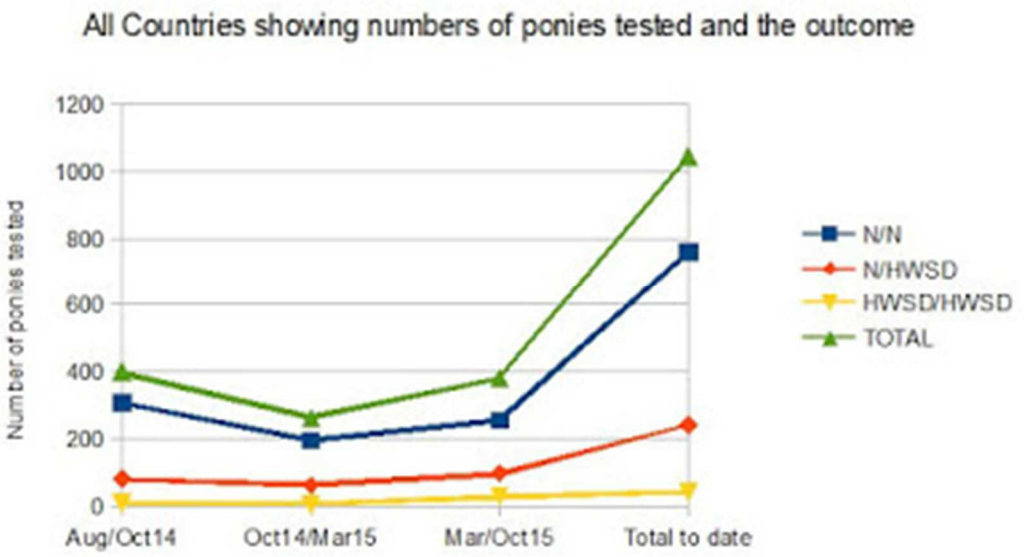

Figure 13: Shows the total number of ponies tested since August 2014

On the VGL website page it states *"**At the time that this test was released, approximately 15% of Connemara were carriers of HWSD (N/HWSD) however, the number of carriers can change with each generation. "***
Be aware that this figure of 15% was extrapolated from the population used for the HWSD research at Bannasch. The research population itself was biased, by virtue of the fact that as many affected ponies and their relatives made up the research cohort. Also important was the geographical origin/residence of the ponies from which samples were submitted. The carrier percentage across all countries, which is now becoming apparent, is in line with what the pedigree research initially suggested.

Some countries are showing a very much higher carrier rate than the average; this could be the result of owners realising which bloodlines appear to be a concern and then selectively testing ponies from those bloodlines. However the pedigree research indicated that with these particular countries, there was always going to be a higher carrier and affected rate because of the initial animals imported (and in some cases also subsequent imports) on which the breed was founded.

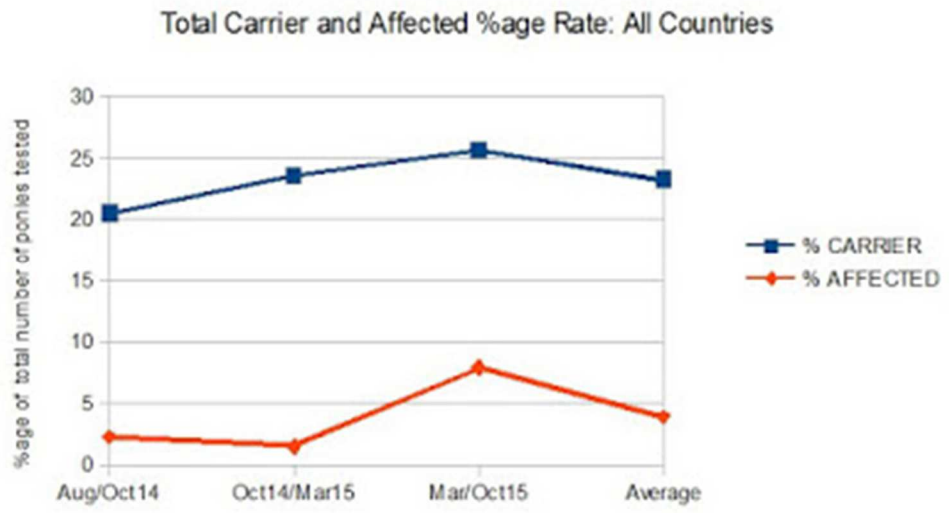

Figure 14

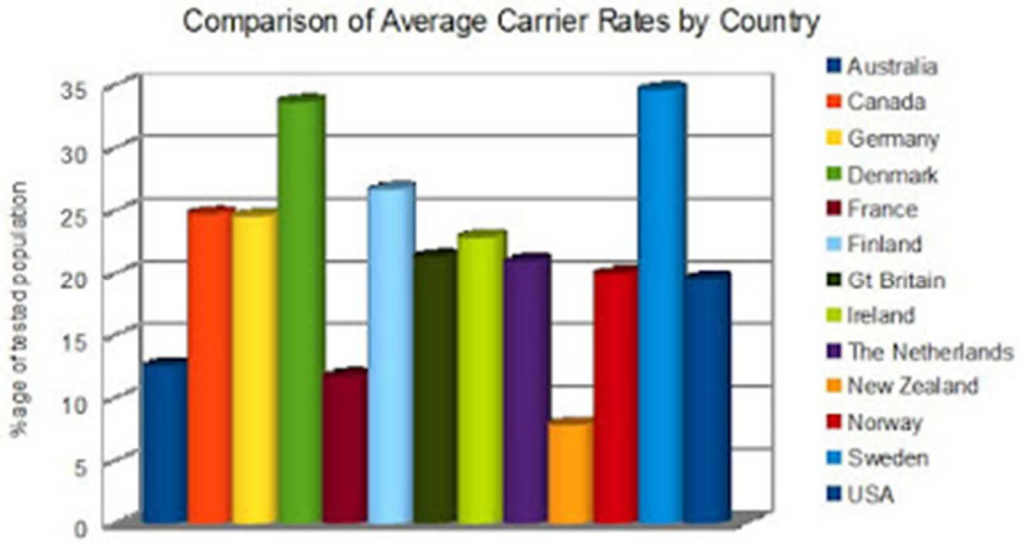

Figure 15: The difference of carrier rates between countries.

The differences with the affected rates between countries is not so obvious because in many cases owners are not choosing to test ponies with obvious/known hoof pathologies.

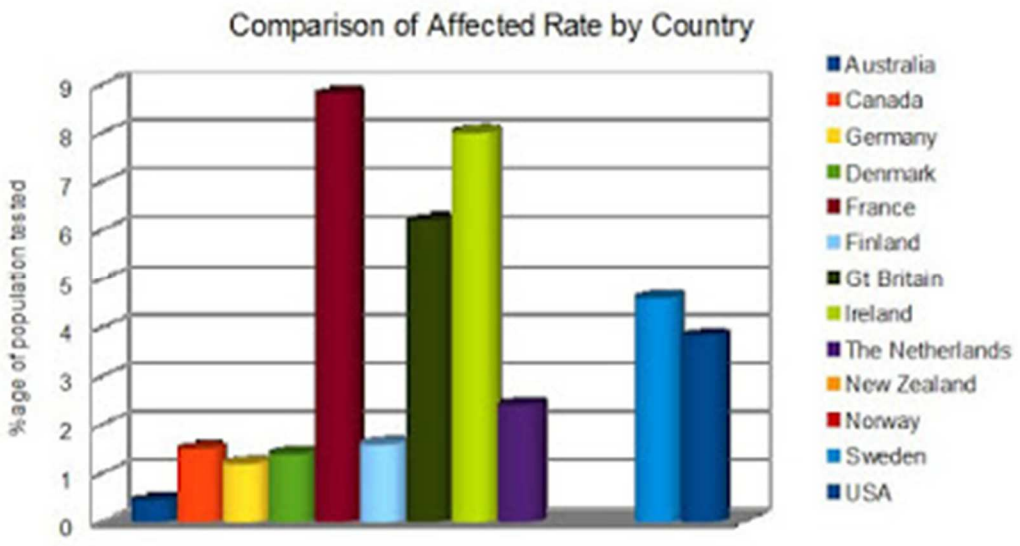

Figure 16: Comparison of affected rate by county

Conclusion

My personal opinion is that it is very important to put the spotlight on our genetic diseases. To encourage and teach our breeders and owners about these problems so they can breed for a healtier animal. We as humans, who are in control of the breeding and with that then are responsible for the outcome. The owners, breeders and the riders, we all want a healty animal and I hope that all the breeding society around the world do whats best for the breed to continue to improve the breed and not the opposite.
With a small gene pool as the horses I think it is imported to control the recessive traits and only use them with unaffected genes to rule out the outcome of the HWSD. So that this horses with recessive genes still can be used in the breeding program under controlled management. I recommend testing the ponies so that carriers can still be saftely bred to non-carriers in order to maintain diversity within the breed and to maintain other positive attributes. Carrier ponys must not be removed from the gene pool.

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Definitions

Acquired condition- A medical condition which developes post-fetally

Autosomal recessive trait- Two copies of an abnormal gene must be present in order for the disease to develop.

Codon- A triplet of adjacent nucleotides in the messenger RNA chain that codes for a specific amino acid in the synthesis of a protein molecule.

Congenital condition- A medical condition which developes pre-fetally

Frameshift- The addition or deletion of one or more nucleotides in a stand of DNA, which shifts the codon triplets of the genetic code of messenger RNA and causes a misreading during translation, resulting in an aberrant protein and therefore a mutation.

Genome- A full set of chromosomes; all the inheritable traits of an organism.

Genotype- The total sum of genes transmitted from parent to offspring.

Genome-wide association analysis- Examination of many common genetic variants in different individuals to see if any variant is associated between single-nucleotide polymorphism (SNP) and traits like major diseases. Theses studies normally comapre the DNA of two groups of particioants: those with the disease and those without the disease as controls.

Haplotype- A combination of closely linked DNA sequences on one chromosome that are often inherited together.

Homozygous- Having a identical pairs of genes for any given pair of hereditary characteristics.

Linkage maps- An abstract mathematical representation of genetic loci that conserves order of loci, which are spaced in such a way that the distances are algebraically additive; Conventually, a map is scaled so that as distances between loci become smaller the ratio of the map distnace to the value of the recombination fraction approaches and independently assorting loci are infinitely far apart.

Locus- The chromosomal position of a gene as determined but its linear order relative to the genes on that chromosome.

Megabase- Unit of length for DNA fragments that is equal to 1 million nucleotides. A megabase (Mb) is roughly equal to 1 centimorgan.

Microsattelites- A tract of repetitive DNA in which certain DNA ranging in lengt from 2-5 base pairs, wich are repeated typically 5-50 times. Microsatellites occur at thousands of locations in the genome and they are notable for their high mutation rate and high diveristy in the population.

Mutatation- A sudden departure from the parent type or more heritable characteristics, casued by a change in a gene or a chromosome.

PCR- Polymerase chain reaction: A technique for rapidly producing many copies of a fragment of DNA for diagnostic or research purposes

Phenotype- The appearence of an organism resulting from the interaction of the genotype and the environment.

Polymorphism- The presence of two or more distinct phenotypes in a population due to the expression of different alleles of a given gene, as human blood groups.

Quantitative Trait Loci- A section of DNA (the locus) that correlates with variation in a phenotype (the quantitative trait). The QTL typically is linked to, or contains the genes that controls that phenotype.

Single nucleotide polymorphisms- A DNA sequence variation occuring commonly within a population in wich a single nucleotide (A, T, C or G) in the genome differs between members of a biological species or paired chromosomes. Ex: two sequenced DNA fregments from different individuals, AAGCCT to AAGCTTA, contains a difference in a single nucleotide. In this case we say that there are two alleles. Almost all common SNPs have only two alleles. The genomic distribution of SNP is not homogenous, it occurs in non-coding regions more frequently than in coding regions or, in general, where natural selection is acting and fixing the allele of the SNP that constitutes the most favorable genetic adaptation.

STOP codon- A codon that stops the synthesis of a protein molecule.

Megabase- A length of double stranded DNA containing two million nucleotides, one million in each stand. Or a lengt of single stranded RNA containing one million nucleotides.

Whole genome next-generation sequencing- A laboratory process that determines the complete DNA sequence of an organisms genome at a single time. This entails sequencing all of an organisms chromosomal DNA as well as DNA contained in the mitochondria.

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- Figure 2: JON GOSCH

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- Figure 7: Own drawing of the white line in the hoof.

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