Gene expression and mechanical properties regulating two phenotypes of horse's skin appendages: hoof strength and hair structure.

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Independent project in Biology, 30 credits

Swedish University of Agricultural Sciences, SLU

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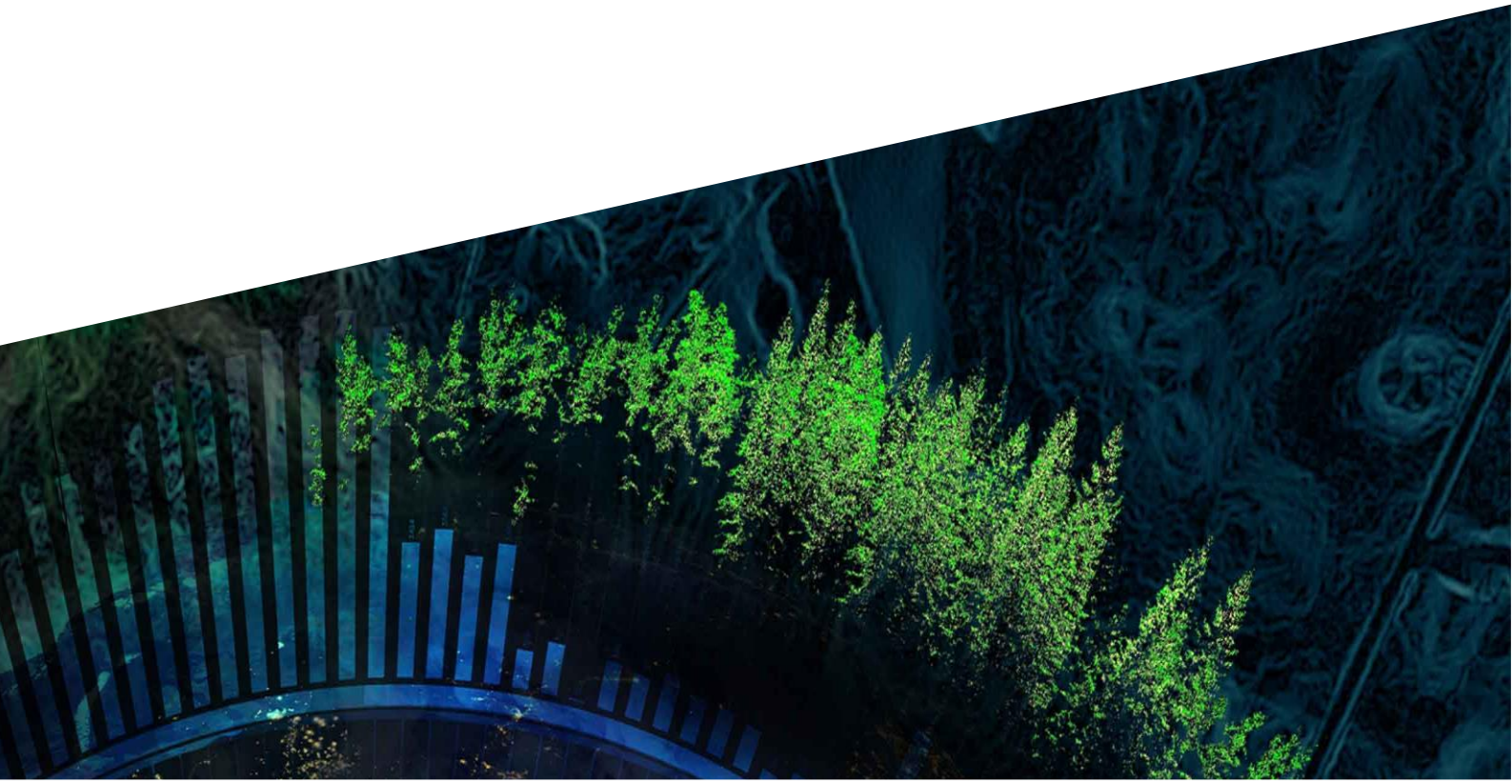

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Keywords: horse, hair, hoof quality, RNA-seq, gene expression, mechanical properties, barefoot racing.

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ABSTRACT

Background:

The hair follicle and hoof are two parts of the horse's skin appendages that emerge from the dermis and epidermis. Different phenotypes characterize these two integument structures and determine their quality. In this project, we were interested in studying two phenotypes, the strength of the hoof and two types of horse hairs. In the first study, an RNA-seq experiment was performed to investigate the gene expression characterizing the quality of the hoof of harness racing Standardbred horses. The aim of this study was to explain the ability of horses to race barefoot for successive races. The second study aimed to understand the mechanical properties of horses' phenotype#1 and phenotype#2 hair by measuring the hair diameter and tensile parameters.

Results:

The hoof study implicated the involvement of four differentially expressed genes encoding proteins with functions in keratinization, vascularization, and development of the horses' hooves. These results may explain the underlying mechanisms for the ability of horses to race barefoot frequently. In the hair study, the diameter and tensile measurements done on the phenotype#1 and phenotype#2 hairs showed thicker diameter measurements in the phenotype#2 hair and higher strain and toughness modulus in the phenotype#1 hair.

Conclusions:

In the hoof study, we detected four differentially expressed genes involved in different biological processes controlling the hoof quality, which could explain the ability of horses to race barefoot for successive races. The immune system-related gene that was significantly differentially expressed may be involved in the quality and integrity of the hoof through an unrevealed new mechanism. In the hair study, the analysis of hair diameter and tensile measurements showed significant differences in the diameter, the strain, and the toughness modulus between phenotype#1 and phenotype#2 hair, which could reveal differences affecting the quality of the two types of hairs.

Keywords: horse, hair, hoof quality, RNA-seq, gene expression, mechanical properties, barefoot.

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Abbreviations

SLU:	Swedish University of Agricultural Sciences
RNA:	Ribonucleic acid
DNA:	Deoxyribonucleic acid
cDNA:	Complementary DNA
RNA-seq:	RNA sequencing
PCR:	Polymerase chain reaction
qPCR:	Quantitative PCR
mRNA:	Messenger RNA
dNTPs:	Deoxynucleotide triphosphates
RNAse:	Ribonuclease
dUTP:	Deoxyuridine Triphosphate
cAMP:	Cyclic adenosine monophosphate
UVB:	Ultraviolet B
TCHH:	Trichohyalin
EDAR:	Ectodysplasin A receptor
FGFR2:	Fibroblast growth factor receptor 2

1 INTRODUCTION

Horse skin appendages are defined by different structures that derive from the dermal and epidermal layers [1]. The hoof and the hair are both highly keratinized tissues that grow from the basal epidermal cells [2] and the hair follicle, respectively [3]. The hoof is a limb structure made up of different parts, the coronet, the wall, the frog, the sole, and the heels [2]. The hair consists of four distinct compartments: the medulla, cortex, intercellular material, and the cuticle, structured from inside outwards [3]. Many studies were implicated in understanding the equine integumentary system [4-8]. During my master's thesis, I was involved in two projects aimed at understanding the biology behind hoof and hair structures in horses.

The hoof plays a crucial role in the ability of the horse to survive and function. It is considered a barrier that protects the internal structures and the tissues beneath from external conditions [9]. It also supports the horse's weight and sustains its balance [10]. Hence, ensuring the soundness of the hoof is essential to maintain the horse's health. For example, for athletic horses, it is crucial to maintain a strong and healthy hoof to race with better performance [11, 12]. The soundness of the hoof is affected by many factors like the age of the horse, hoof trimming, physical activity [13], arteriovenous activity and the ability of the hoof to eliminate waste materials [14]. Also, the horse's nutrition [15] and the availability of some necessary minerals like calcium, copper and zinc is strongly involved in the growth of the hooves and the quality of the keratinized structures [14]. Moreover, the adaptation of the horses to environmental conditions is involved in determining the strength of the hoof. For example, the Baicha Iron hoof horses that belong to the Chinese Mongolian horses are characterized by their strong hooves because of the morpho-anatomical adaptations to the rugged mountain terrain [16]. In harness racing, some horses possess strong hooves, so they can race barefoot frequently, while others cannot cope with the barefoot condition for successive races. The understanding of the genetic factors behind this diversity is important as the horse runs faster, on average, running 0.7 seconds/km faster when barefoot compared with shod [11].

The color, length, and thickness of horse hair have attracted humans since prehistory, and the artificial selection based on human preference enhanced the variation of these phenotypes [17]. Moreover, the study of coat coloration has significant relevance in understanding the history of current breeds and comprehending breeding preferences [17]. The biological properties of horse hair were studied for medical and cosmetic applications in humans, like the development of keratin [18, 19] and the possibility of using it as a suture material in many surgeries [20]. The selective breeding over generations of domestication created different hair types [21].

The hair cycle consists of different transitions of stages: the anagen stage is the active phase of hair growth, followed by the catagen, which is the regression phase, and finally, the telogen phase, which is the quiescence stage [22]. Hair's units affect the constitution and the shape of the hair and therefore influence the appearance of the shaft [23-25]. Different signaling pathways are involved in the differentiation of hair follicles and the growth cycle of the hair by controlling the transition from one phase to another [26]. The mutations in the genes controlling these pathways could affect hair cycling and hair follicle morphogenesis [27]. Also, the nutrition of the horse was demonstrated to affect the hair content in many essential elements like zinc, copper, potassium, sulfur, and iron that influence the mechanical properties of the hair [28].

The first aim of this study was to investigate the pattern of gene expression implicated in the quality of the hoof of Standardbred horses that have competed in harness racing. As a second aim, we were interested in studying the mechanical properties of another skin appendage, the horse hair, and, more specifically, the phenotype#1 and phenotype#2 hair type. Several experiments were performed to achieve our aims (Figure 1):

- An RNA-seq experiment was performed to explore how gene expression influences hoof quality and thereby identify the critical genes implicated in that process.
- A qPCR experiment was performed to validate the RNA-seq results.
- Diameter and tensile measurements were done on hair samples to identify the mechanical properties of phenotype#1 and phenotype#2 hair types.

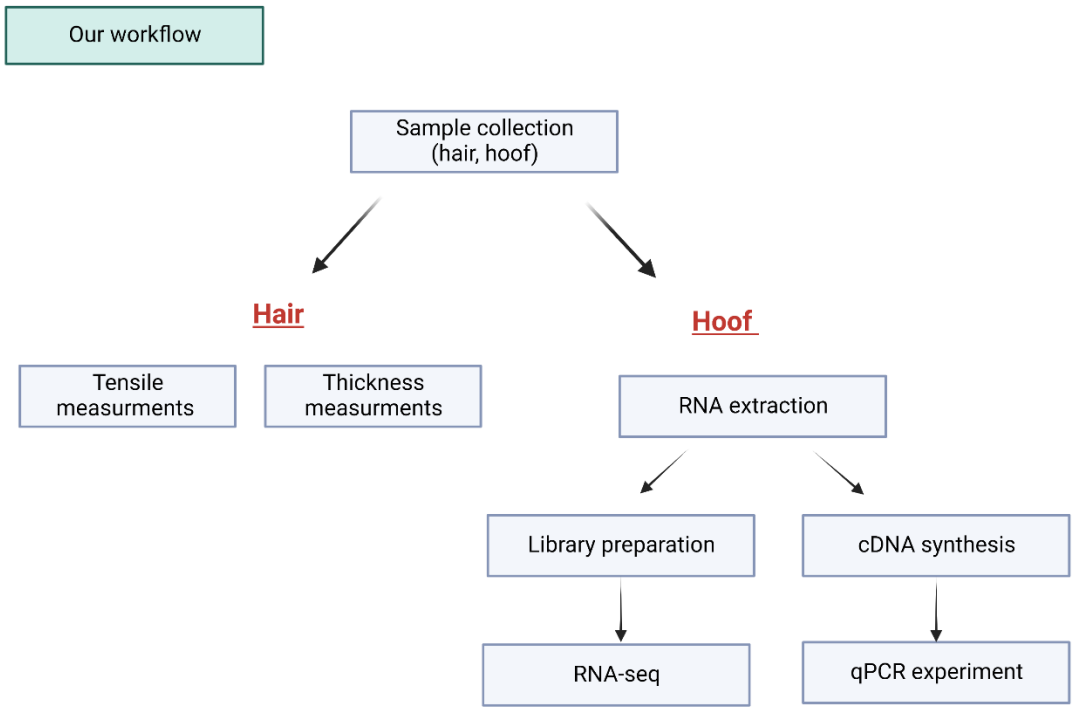

Figure 1: The workflow used to accomplish hoof and hair studies.

2 MATERIALS AND METHODS

2.1 ANIMALS AND SAMPLE COLLECTION

Hoof study:

After being euthanized at the slaughterhouse, we collected the left and right hind hooves from Standardbred trotters. The hooves were then directly transported on ice to the laboratory. The horses selected for this study were aged from 5-20 years and must have raced a minimum of eight times. The horses were divided into two groups. The barefoot racing group (n=10) included the horses that have

raced 3 times barefoot during 31 days, at least once during their career. The second group comprised seven horses (n=7) that have raced barefoot, but a minimum period of 45 days must separate between two races because of limitations in wear and tear of the hind hoof. All the needed data are provided by the Swedish Trotting Association and confirmed by horse trainers.

Hair study:
Hairs were plucked from Icelandic horses. Ten horses with phenotype#2 hair and five horses with phenotype#1 hair were used for diameter and length measurements, while nine horses with phenotype#1 hair and seven horses with phenotype#2 hair were used for tensile measurements. These were the available samples at the present time.

2.2 SAMPLE EXTRACTION

Hoof study:
The left hind hooves were sectioned with a band saw at the growth zone at the coronary band circumference and the solar surface from one corner to the other. Four pieces were obtained. The tissue was taken from the lateral slice at the coronary band, then stored at -70°C for RNA extraction.

Hair study:
20 to 25 hairs were plucked from the horse's forelock and placed in plastic bags identified with the id of the horse.

2.3 RNA EXTRACTION

10-20 mg of tissue taken out from hoof samples were used to perform the RNA extraction. After the homogenization of hoof samples using the Precellys Evolution instrument (Bertin Instruments, Montigny-le-Bretonneux, France), the RNA was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer´s protocols.

2.4 LIBRARY PREPARATION

Sequencing libraries were prepared from 500 ng total RNA using the TruSeq stranded mRNA library preparation kit (Illumina, San Diego, CA, USA), including polyA selection. Unique dual indexes were used. The library preparation was performed according to the manufacturer's protocol (#1000000040498). The quality of the libraries was evaluated using the Fragment Analyzer (Advanced Analytical Technologies, Inc., Santa Clara, CA, USA) using the DNF-910 dsDNA kit. The adapter-ligated fragments were quantified by qPCR using the Library quantification kit for Illumina (KAPA Biosystems, Wilmington, MA, USA) on a CFX384 Touch instrument (Bio-Rad, Hercules, CA, USA) before cluster generation and sequencing.

2.5 RNA-SEQUENCING

Library preparation and sequencing were performed by the SNP&SEQ Technology Platform, a national unit within the National Genomics Infrastructure (NGI), hosted by Science for Life Laboratory in Uppsala, Sweden (https://www.scilifelab.se/units/ngiuppsala/). Sequencing was carried out on an Illumina NovaSeq 6000 instrument (NSCS v 1.7.0/ RTA v 3.4.4) according to the manufacturer's instructions

(Illumina). Demultiplexing and conversion to FASTQ format were performed using the bcl2fastq2 (2.20.0.422) software provided by Illumina (http://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html). Additional statistics on sequencing quality were compiled with an in-house script from the FASTQ files, RTA, and BCL2FASTQ2 output files. The RNA-seq data were analyzed using the best practice pipeline nf-core/rnaseq (https://nf-co.re/rnaseq)[29].

2.6 cDNA SYNTHESIS

The reverse transcription of RNA into cDNA is used in many applications, including library preparation in RNA-seq experiments. In transcriptomics, the cDNA is used to measure the level of mRNA reverse transcribed into cDNA to quantify or evaluate the gene expression level by qPCR. In this study, the synthesized cDNA was used in a qPCR experiment. The complementary DNA (cDNA) was generated from messenger RNA (mRNA) using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, San Francisco, CA, USA). The cDNA synthesis was performed as described below:

The master mix was prepared, and then 10 µL were pipetted into each well. This 10 µl of the master mix should contain 2 µl of 10xRT buffer, 0.8 µl of 25x dNTP mix, 2 µl of 10xRT random primers, 1 µl of reverse transcriptase, 1 µl of RNAse inhibitor, and 3.2 µl H_2O. After that, a volume of 10 µl of mRNA with a concentration of 20 ng/µl was prepared for each sample and added to the ten µl of the master mix present in each well of the 96-wells reaction plate. The plate was then briefly centrifuged to spin down the contents and remove the bubbles. Then it was placed in a ProFlex PCR thermal cycler (Applied Biosystems™) to perform the reverse transcription. After that, the temperature and the time were set as described in Table 1, and the reaction volume was adjusted to 20 µl on the software. Reverse transcriptase was the enzyme used to perform the reverse transcription of mRNA into cDNA using the added dNTPs. Also, the RNase inhibitor was used to prevent the degradation of RNA molecules by the RNase enzyme and maintain their integrity.

Table 1: Temperature and time set on the ProFlex PCR thermal cycler to perform the cDNA synthesis.

Temperature (°C)	Time (minutes)
25	10
37	120
85	5
4	Hold

The final cDNA solution was diluted (1:8) by adding 140 µl of water to get 160 µl.

2.7 QUANTITATIVE POLYMERASE CHAIN REACTION

Quantitative polymerase chain reaction (qPCR) was used to quantify the expression of certain genes of interest. Specific primers were designed for each target gene. This reaction is performed in the thermal cycler StepOnePlus™ Real-Time PCR System (Thermo Scientific, Applied Biosystems™) that quantifies the fluorescent emissions that are proportional to the quantity of cDNA present in each well after each PCR cycle. At the end of the experiment, the melting curve was performed to confirm that the PCR mix was not contaminated and that the primers were specific to the target genes. The forward and reverse primers were designed for the target and the B-actin housekeeping genes. The B-actin forward primer sequence is 5'-CGAGCACGATGAAGATCAAG-3', and its reverse primer sequence is 5'GTGGACAATGAGGCCAGAAT 3'.

In each well of a 96-well plate, a volume of 10 µl of a master mix was added. This volume contained 5 µl of the SYBR™ Green PCR Master Mix (2X), 0.09 µl of forward and reverse primers, 2.82 µl of water and 2 µl of DNA template. The SYBR™ Green Master Mix (Applied Biosystems™) contained SYBR™ Green I dye, AmpliTaq Gold™ DNA Polymerase, dNTPs with dUTP, Passive Reference 1 (ROX), and optimized buffer components. The prepared PCR plates were placed in the thermal cycler to perform the quantification process. The temperature and the time were set, and the reaction volume was adjusted to 10 µl in the software StepOne v2.3 as in (Figure 2).

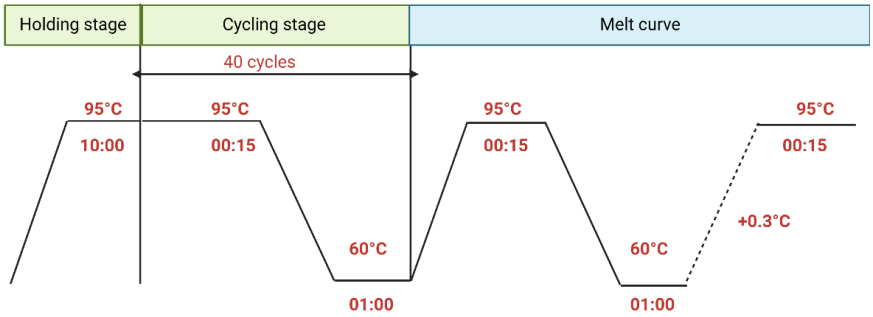

Figure 2: Temperature and time were set to perform reverse transcription of cDNA in the thermal cycler (StepOnePlus™ Real-Time PCR System). The settings of the SYBR method, the quantitation experiment's type, the number of PCR wells, and the reaction volume per well, were set on StepOne v2.3 software. The holding and cycling stages and the melting curves were visualized as quantitative curves, during the process of the experiment.

2.8 MICROSCOPY

The digital microscope Nikon ECLIPSE 80i (Nikon, Tokyo, Japan) was used to measure each horse's hair diameter. Phenotype#1 and phenotype#2 hairs are the two distinct phenotypes of horse hair included in this study. These phenotypes were defined by looking at the horse's photos.

The length of each hair was measured then the midpoint of the hair was maintained between the coverslip and the microscope slide. The hair with the slide was placed on the digital microscope connected to the NIS-elements D software (Nikon imaging software), used to visualize the hair with 10X magnification and to measure the diameter in µm. Ten hairs were taken from each horse, and the diameter was checked for each hair by measuring it in three sections S1, S2, and S3. For each section,

the diameter was noted in three points P1, P2, and P3 (Figure 3). In total, the diameter was measured in nine points for each hair, which gave nine points*ten hairs = 90 points. The minimum and the maximum diameter values were extracted, and the mean of the measurements was calculated in order to compare them in both phenotypes. Besides, the length of the ten hairs that belong to each horse was measured, and then the mean of these measurements was calculated and compared to the mean measurements of the diameter.

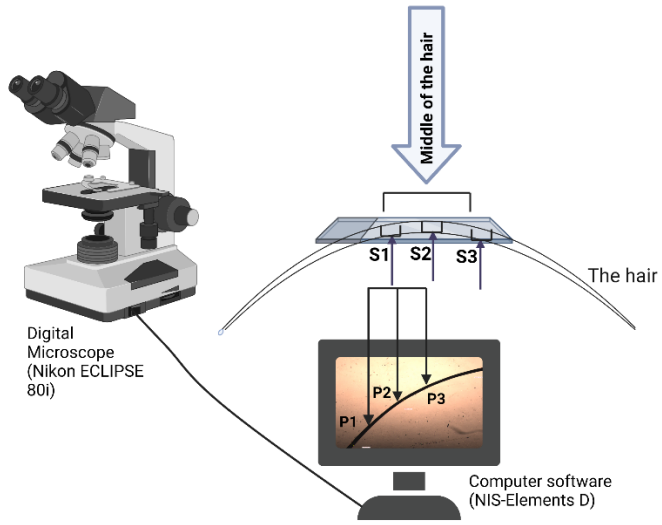

Figure 3: The hair is set in its midpoint between the slide and the coverslip and then placed on the microscope Nikon ECLIPSE 80i. NIS-Elements software is connected to the microscope to visualize the hair and measure the diameter in three sections in its middle (S1, S2, S3). In each section, the diameter was measured in three points (P1, P2, P3). In total, the diameter of each hair was measured in 9 points 10 hairs = 90 points for each horse.*

2.9 TENSILE MEASUREMENTS

In this study, we were interested in analyzing the strain, strength, Young's modulus, toughness modulus, and diameter of the phenotype#1 hairs taken from seven horses *vs*. the phenotype#2 hairs taken from nine horses. The measurements were done on five to ten hairs taken from each horse. The diameter was measured prior to the tensile test using the digital microscope (Nikon ECLIPSE 80i). The significance of the difference in the parameters between phenotype#1 and phenotype#2 hairs was tested using a student's t-test. Pearson correlation coefficient was used to quantify the correlation between the parameters, and the significance was detected by calculating the T-scores and then the P-values.

The machine "5940 Series Single Column" (Instron, 825 University Avenue Norwood, MA, USA) was used to test the hairs to obtain their mechanical properties by applying a displacement on the material and measuring the force (N) and the distance (m). From these measurements, the strain (mm/mm) (relative deformation, it is the ratio of the change in length/original length), the stress (pressure inside the hair), Young's modulus (related to the stiffness of the material), and the toughness modulus (energy per unit of volume to break the hair) were calculated as described in Greco & Pugno [30].

The holder of the hair was prepared by cutting an A4 paper in the form of a square. Then a little window was carved in the middle of the holder; the same dimensions were fixed for all the prepared holders.

The middle section of the hair was cut and glued on the upper and lower sides of the holder. After mounting the holder with the hair, the paper is cut from the left and right sides, as shown in Figure 4. The software launches the tensile measurements (Blue Hill Universal). The strain rate was 6 mm/min, and the gauge length of the hair was approximately 10 mm (Figure 4).

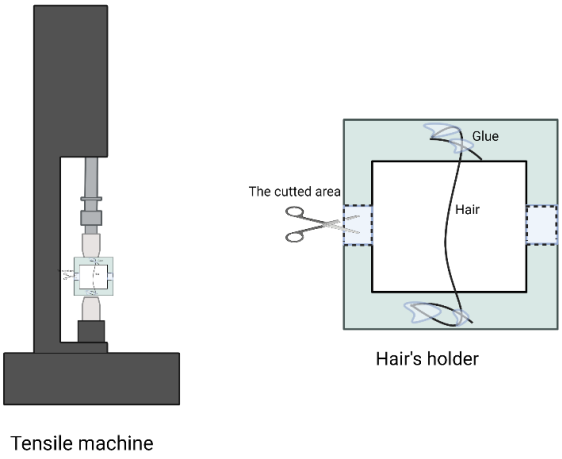

Figure 4: Hair holder preparation and its mounting on the tensile machine 5940 Series Single Column.

2.9.1 Illustrations to clarify the tensile parameters

The strain quantifies the deformation (change in the length (**ΔL**)/original length (**L0**)) (Figure 5).

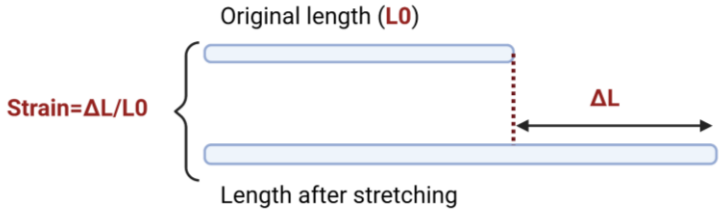

Figure 5: A schematic representation of the strain. It is the result of the change in the length(ΔL) ratio to the original length (L0).

Young's modulus (GPa) measures the material's ability to avoid its deformation along an axis when tension or compression is applied to it (Figure 6).

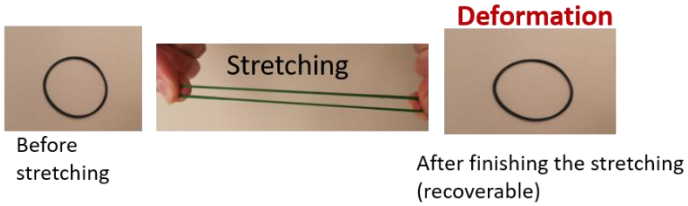

Figure 6: A schematic representation of young's modulus using an elastic material. The original form of the materiel is recoverable after the stretching applied on it.

The strength (MPa) is the ability of the material to resist its impairment when a force is applied to it (Figure 7).

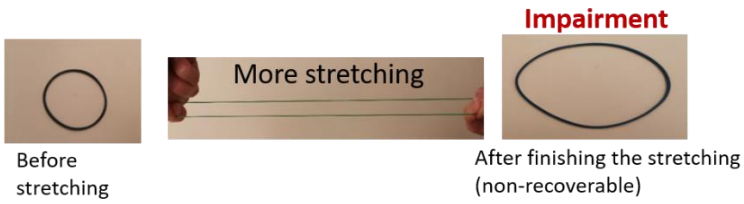

Figure 7: A schematic representation of the strength using an elastic material. The original form of the material is non-recoverable after the stretching applied on it.

The toughness modulus (Mj/m^3) is the capacity of materiel to absorb the force and deform or impair without breaking (Figure 8).

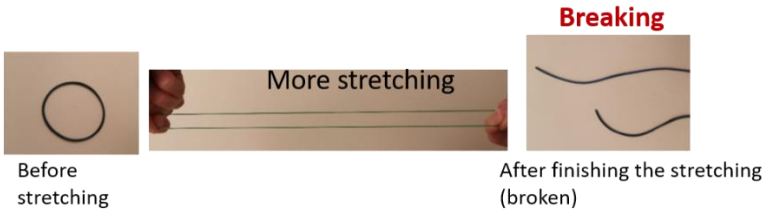

Figure 8: A schematic representation of the toughness modulus using an elastic material. The original form of the materiel is broken after the stretching applied on it.

3 RESULTS

3.1 RNA SEQUENCING SHOWED SIGNIFICANTLY DIFFERENTIALLY EXPRESSED FOUR GENES IN THE HOOF OF THE RACING BAREFOOT AND RACING SHOD HORSES

The results of RNA-seq revealed 886 genes with a P-value < 0.05. However, the analysis showed one gene (A) with an adjusted P-value < 0.05 while it revealed 3 genes (B, C, and D) with 0.05 < adjusted p-value < 0.1 considered as close to significance (Table 2).

Table 2: The significant genes expressed in the horses racing barefoot frequently or not often barefoot.

	NUMBER OF GENES
P-VALUE < 0.05	886
0.05 < ADJUSTED P-VALUE <0.1	3 (Genes B, C, D)
ADJUSTED P-VALUE < 0.05	1 (Gene A)

The significant genes were differentially expressed in both groups of horses. Genes A and B were overexpressed in the hooves of horses that were able to race barefoot for successive races, while genes

C and D were overexpressed in the hooves of horses that were not able to race barefoot frequently (Figure 9).

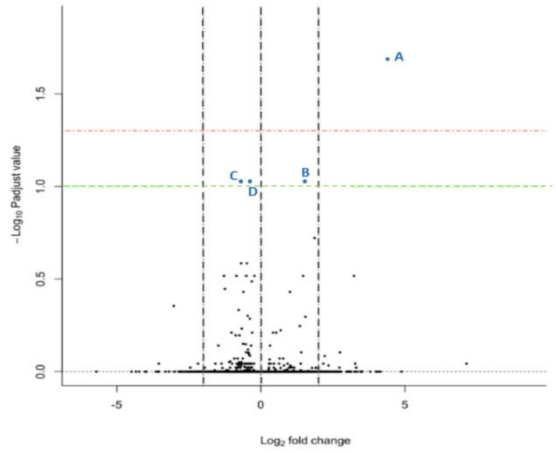

Figure 9: Volcano plot with the log2 fold change in gene expression in the shod and barefoot horses on the x-axis and the statistical significance (−log10 adjusted P-value) on the y-axis.

3.2 THE QPCR EXPERIMENT CONFIRMED THE RNA-SEQ RESULTS

The qPCR results confirmed that the genes C and D were overexpressed, while Gene A expression was absent in the hooves of horses that were unable to race barefoot for successive races. Also, it validated the overexpression of gene B and the significant expression of gene A in the hooves of the horses that were able to race barefoot for successive races (Figure 10).

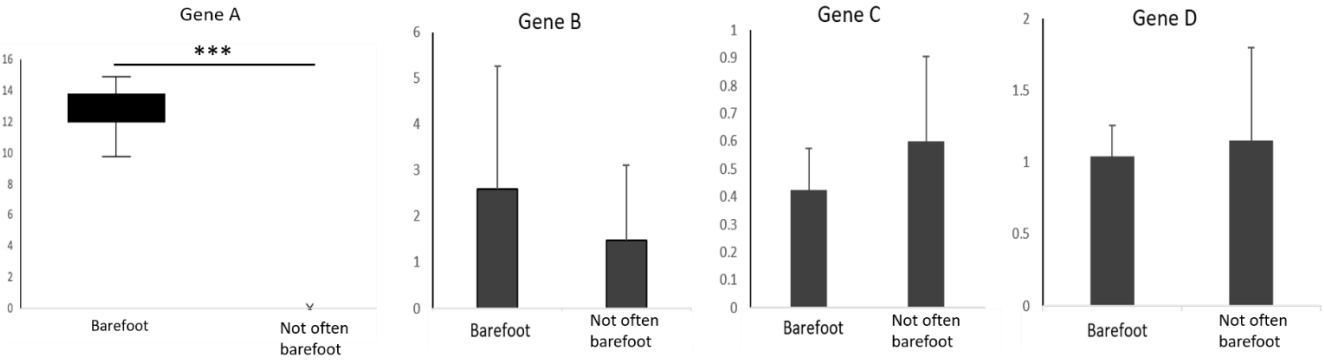

*Figure 10: Bar chart showing the ΔCt values of the gene A and the mean fold change of the genes B, C, and D in horses racing barefoot frequently and horses not often barefoot. ΔCt of the gene A = Ct (gene A) – Ct (housekeeping gene (b-actin)). Student's T-test *** P<0.001.*

3.3 THE MICROSCOPY HAIR MEASUREMENTS SHOWED A SIGNIFICANT DIFFERENCE BETWEEN PHENOTYPE#1 AND PHENOTYPE#2 DIAMETERS.

The minimum, maximum and mean measurements of the diameter were taken for phenotype#2 hair from ten horses and for phenotype#1 hair from five horses. These measurements were compared for the two types of hair. We found these measurements to be significantly higher in the phenotype#2 hairs compared to the phenotype#1 hairs (P<0.0001). Student's t-test was used to test the significance of this difference (Figure 11 a-c).

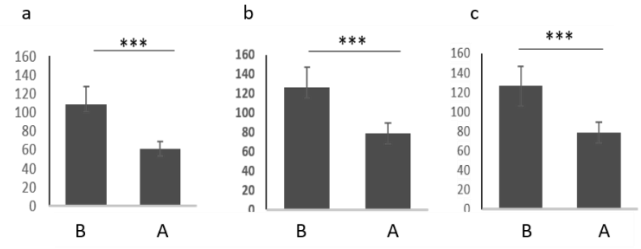

*Figure 11: The minimum (a), maximum (b) and mean (c) measurements of the diameter (µm) taken on horse's phenotype#1 (A) and phenotype#2 (B) hairs. Student's T-test *** P<0.001.*

3.3.1 The length and the diameter were positively correlated

The mean of the diameter measurements and the mean of the length of the hairs were significantly positively correlated (0.762) (P-value <0.05) (Figure 12). The correlation between the parameters was quantified using Pearson's correlation coefficient, and the significance was detected by calculating the T-scores and then the P-values.

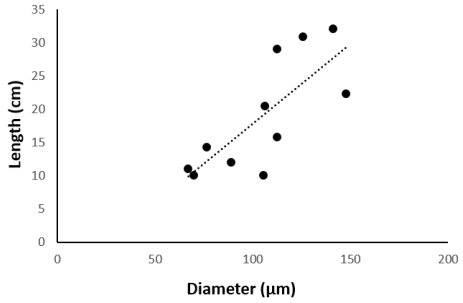

Figure 12: Correlation plot between the mean of the length (cm) and the mean measurements of the diameter (µm) of the hairs. Correlation = 0.762, P-value <0.05. Pearson correlation coefficient was used to quantify the correlation and detect the significance by calculating the T-scores and then the P-values.

3.4 TENSILE MEASUREMENTS SHOWED A SIGNIFICANT INCREASE IN THE TOUGHNESS MODULUS AND THE STRAIN OF THE PHENOTYPE#1 HAIR AND DIFFERENT CORRELATIONS BETWEEN THE MEASURED PARAMETERS.

The strain and the toughness modulus were found to be significantly higher in phenotype#1 compared to phenotype#2 hairs (P-value<0.05). While the difference in the strength and Young's modulus between phenotype#1 and phenotype#2 hairs was not significant (Figure 13). Student's T-test was used to test the significance of different parameters between phenotype#2 and phenotype#1 hairs (P-value<0.05).

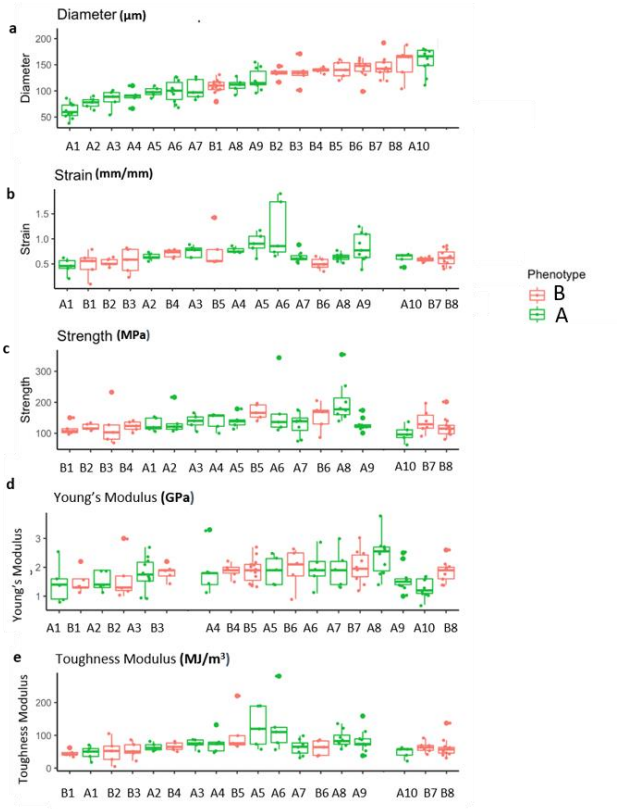

Figure 13: Boxplots showing the diameter (µm)(a), strain (mm/mm) (b), strength (MPa) (c), and Young's modulus (GPa) (d) and the toughness modulus (MJ/m3) (e) measurements done on the horse's phenotype#1 (A) and phenotype#2 (B) hair. The parameters are shown on the y- axis, and the horses with phenotype#1 (A) hairs (n=9) and phenotype#2 (B) hairs (n=7) are represented on the x-axis. (Pa: Pascale unit, J/m3: Joule/cubic meter).

The diameter was found to be significantly negatively correlated with the strain (-0.5), the strength (-0.63), Young's modulus (-0.54), and the toughness modulus (-0.61) in phenotype#2 and phenotype#1 hairs (P-value<0.05). (Figure 14). Young's modulus was shown to be significantly positively correlated to the strength (0.69) ($p<0.05$). But the positive correlation between the young's modulus and the toughness modulus was not significant (Figure 14). Pearson's correlation coefficient was used to quantify the correlation between the parameters, and the significance was detected by calculating the T-scores and then the P-values.

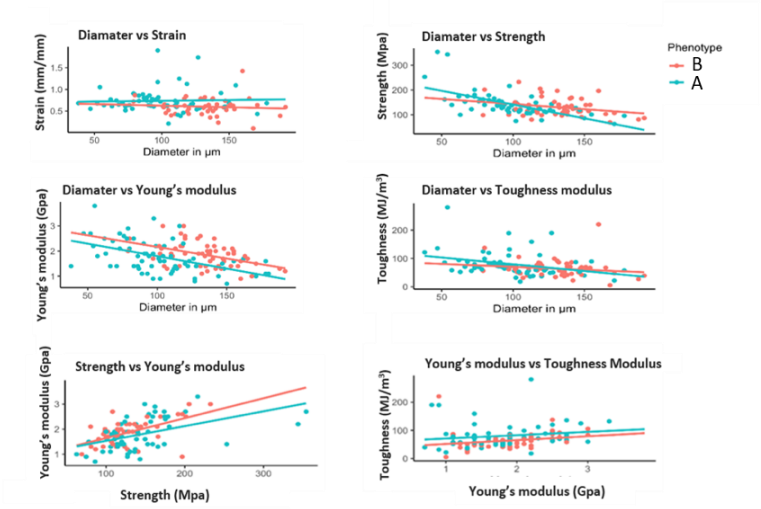

Figure 14: Correlation plots between the diameter (µm), young's modulus (GPa), strain (mm/mm), strength (MPa), and toughness modulus (MJ/m3) measured on the phenotype#2 (B) and phenotype#1 (A) hairs taken from seven and nine horses respectively. For each horse, five to ten hairs were tensile measured.

4 DISCUSSION

In this project, we were interested in studying the hoof and the hair of Standardbreds and Icelandic horses, respectively. The RNA-seq experiment was performed to investigate the genes involved in the quality of the hoof of horses that could race barefoot frequently and of those who could not race unshod for successive races. We could reveal four differentially expressed genes coding for proteins that may influence the hoof quality. These genes are involved in the immune system, the keratinization process, and the vascularization of the hoof. In the other part of this study, we investigated and compared the mechanical properties of phenotype#1 and phenotype#2 hair by measuring the diameter and by tensile measuring the hairs. In the text below, we will discuss the results of hoof and hair studies, respectively.

As a result of our first aim, the RNA-seq displayed overexpression of genes A and B in the horses' hooves that could race barefoot frequently. Though, in the hooves of horses unable to race barefoot for successive races, genes C and D were shown to be significantly overexpressed. These results were validated by the qPCR experiment. The identified differentially expressed genes encode proteins that are involved in different biological processes influencing hoof quality. The gene B encodes a universal sulfate donor, implicated in the assimilation of sulfur and the metabolism of sulfate. Therefore, it can be involved in the keratinization process as keratin is a highly sulfated intermediate filament protein. Moreover, keratin is the main component of the horse's hoof [31], and the strength and the rigidity of the hoof are proportionally related to the number of disulfide bonds that connect the intermediate filaments of keratin and prevent water loss and make a strong barrier that protects the internal tissues of the hoof [32]. Thus, the overexpression of gene B in the horses that frequently race barefoot could contribute to the hoof strength, which could be partly related to the sulfur content and, therefore, to the keratinization process. Gene A is the other overexpressed gene in horses' hooves that could race

barefoot for successive races. This gene encodes a protein involved in the immune system by neutralizing bacterial and viral infections and activating the complement system to lyse pathogens. The implication of this gene in the hoof quality still needs more analysis but may be related to wound healing. On the other hand, the hooves of horses that were unable to race barefoot frequently showed overexpression of the genes C and D. Gene C encodes a neuronal calcium sensor protein involved in regulating the cAMP level, in the signaling, and the differentiation of keratinocytes. The upregulation of the gene C was revealed to be involved in reducing the migration and the proliferation of keratinocytes [33] which can explain the sensitive structure of the hooves of horses that were unable to race barefoot frequently. Gene D encodes a protein that mediates thiamine (vitamin B1) transport across the mitochondrial and cellular membrane and promotes energy metabolism and production [34]. It was shown that overexpression of the gene D could result in the dilation of the peripheral arteries and increased blood flow [35]. Besides, the inappropriate dilation of arteriovenous anastomose (AVAs) could lead to a shunt in the blood through those dilated capillaries instead of through the capillaries that transfer the nutrients to the epidermal structures of the hoof [36, 37]. Thus, the upregulation of the gene D can impact the vascular reactivity of the arteries and capillaries that supply the hoof and therefore affect its quality.

In a second aim, to analyze the mechanical properties of phenotype#1 and phenotype#2 hairs, we compared the diameters of the hairs. We found that phenotype#2 hairs were characterized by a higher diameter than phenotype#1. The diameter was positively correlated to the length of the hair. Therefore, we were interested in linking the diameter to hair's mechanical properties by tensile measuring the strain, strength, young's modulus, and toughness modulus of the hairs. The diameter was negatively correlated to these parameters, but young's modulus was positively correlated to the toughness modulus and strength. In addition, phenotype#1 hair was characterized by a higher strain and toughness modulus than phenotype#2 hair.

The structure and the composition of the hair compartments affect the constitution and the shape of the hair and therefore influence the appearance of the shaft resulting in different types of hair. They are also involved in determining hair' mechanical properties. The cortex, the middle layer of the hair and the thicker one, consists of keratin filament proteins and keratin-associated proteins. It gives strength and elasticity to the hair [28]. Also, disulfide crosslinking is involved in the toughness of the cuticle [39]. Further, the negative correlation between the diameter and the strength of the hair can be explained by the fact that the longer the hair shaft is, the more it is exposed to environmental stresses, like UVB radiation, pollution, and chemical products [40]. These factors affect protein content, cuticle quality, and structural elements of the hair [40]. Also, it leads to the disturbance of disulfide bonds [41] between cysteine amino acid residues. This disruption increases hair shaft porosity and irregularities and thus affects the mechanical properties of the hair by decreasing the tensile strength [41].

Likewise, it was shown that in human hair, when the thiol groups are closer together, the formation of disulfide bonds becomes easier and thus gives more strength to the hair [25]. Hence, the deformation of the hair, represented by the strain, is higher in such type of hair, as it can stretch and elongate more.-Furthermore, as the strength is positively correlated to the toughness modulus, young's modulus, and strain, we can conclude the decrease in these parameters when the diameter and the length of the hair increase. Unlike the toughness modulus and strain parameters, we couldn't find any significant difference in the strength and Young's modulus (related to the stiffness of the material) when comparing phenotype#2 and phenotype#1 hairs. The significance may change by increasing the number of hair samples or considering the length of the hair and avoiding variations in hair length in each horse.

Finally, it will be interesting to investigate the genetic factors acting on the difference in the mechanical properties of the different hair types. Several studies in humans found a high implication of some genes in determining the hair's diameter and texture. These factors differed between the populations. For example, the EDAR and FGFR2 genes were associated with the thickness of the hair of Asian human populations [42, 43], while the TCHH gene was found to act on the texture of the hair of people originating from the north of Europe [44]. Others suggested that the *VEGF* gene affects the size and the growth of the follicle and the hair of mice by promoting the vascularization of the hair follicle during its cycling [45].

5 CONCLUSION AND PERSPECTIVES

Different factors contribute to the quality of a horse's skin appendages. On the hoof, genes A and B were found to be significantly differentially expressed in the hoof of horses that were able to race barefoot for successive races. In contrast, genes C and D were overexpressed in horses that don't cope with the unshod condition for successive races. The observed difference in the expression level of gene A was significant, while the differential expression of genes B, C, and D was close to significance. These genes were affecting the quality of the hooves by acting on the keratinization, vascularization, and growth of this integumentary structure. Further investigations are needed to understand the immune system's involvement in the enhancement of hoof quality; likely, gene A encodes a protein that is implicated in tissue repair and wound healing. On the other hand, the study of the mechanical properties of horses' phenotype#1 and phenotype#2 hairs showed a higher toughness modulus and strain than the phenotype#2 hair.

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