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Polymorphism in the BIEC2-808543 locus and its association with growth curve in Brasileiro de Hipismo horse breed

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ABSTRACT - Our objective was to evaluate whether the single nucleotide polymorphism (SNP) BIEC2-808543, identified in some horse breeds, also occurs in the Brasileiro de Hipismo (BH) breed. In addition, we verified if this SNP is related to the growth curve profile of these animals for the variables body mass, height at withers, and height at croup, using nonlinear mixed models. For the DNA isolation, we collected blood samples from 167 young BH horses. We obtained the genotypes of these animals using the polymerase chain reaction-restriction fragment length polymorphism technique. For the association studies of this polymorphism with the growth curve in foals, we selected three traits: body mass, height at withers, and height at croup. Polymorphism C/T exists in BH horses and is significantly associated with the evaluated traits. Animals that presented the TT genotype were smaller and lighter when compared with animals of the CT and CC genotypes. By the Akaike information criterion, the model that best described the growth curve for the body mass variable is the Brody model associated with the power of the mean variance function. For the height at withers variable, the best-fit model was von Bertalanffy, adjusted without polymorphism effect in parameter b, associated with the asymptotic variance. For the height at croup trait, the model that best described the growth curve was Brody model, associated with asymptotic variance. This polymorphism represents a good molecular marker. Nonlinear models are promising for describing growth curves in horses, particularly by the possibility of associating SNP effects to model parameters.

Keywords: *LCORL* gene, nonlinear models, SNP

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1. Introduction

Since the domestication of horses, more than 5,000 years ago, equine species have served humans in various ways, such as in agriculture, in wars and as a means of transportation. They are currently highlighted in the leisure (Raudsepp et al., 2019) and competition industry. The animals used for sport and leisure activities in Brazil total 1.1 million heads, with an estimated economic movement of approximately 1 billion dollars per year (MAPA, 2016). Horses with better performance will be of greater value in the sports industry. In this sense, it is necessary to carry out studies to accelerate the genetic gain of these animals. Horses of the Brasileiro de Hipismo (BH) breed are suitable for dressage,

jumping, full riding contest, and endurance sports. Animals of this breed already participated in several national (in Brazil) and international competitions, such as the 2016 Olympics that took place in the city of Rio de Janeiro, Brazil. Therefore, studies about animal growth and the selection of athlete horses demand records of morphometric evaluations, such as height at withers, height at croup, and body weight (Padilha et al., 2017), as they predict specific breed growth rates. There are literature reports based on some of these measures to evaluate growth traits for BH, such as Santos et al. (1992), who evaluated the growth of foals up to one year old, and McManus et al. (2010), who evaluated young animals until adulthood. However, those studies did not account for genetic polymorphisms.

Height at withers is one of the main linear measurements used to select horses, mainly for sports activities, and to define the breed standard determined by breeders' associations (ABCCH, 2013). Nonetheless, there is a large variation between breeds for this trait. The genetic control of this trait in horses is similar to that observed in other domestic mammals, such as cattle (Pryce et al., 2011) and dogs (Chase et al., 2002), in which there is a small number of loci with alleles of greater effect on height (Makvandi-Nejad et al., 2012).

Signer-Hasler et al. (2012) carried out studies of genomic-wide association in Franches-Montagnes horses and identified two quantitative trait loci for height at withers located on chromosomes 3 and 9, which together explain 18.2% of the variance of the estimated breeding values for this trait. According to these authors, the marker on the autosomal chromosome 3 of *Equus caballus* L. (ECA 3) is located near the genes that encode protein. This single nucleotide polymorphism (SNP), identified as BIEC2-808543, corresponds to a T/C transition, in which allele C is associated with increased height at withers. In the same year, Makvandi-Nejad et al. (2012) reported four *loci* on chromosomes 3, 6, 9, and 11 in several horse breeds, capable of explaining 83.5% of the height variations, and pointed out the SNP BIEC2-808543 as the most significant.

Given the importance of the polymorphism identified in the papers cited above, we assumed that animals with different genotypes might have different growth profiles. Thus, we aimed to check for the SNP BIEC2-808543 existence in the BH breed and if this genetic variation is related to growth curve profiles of body mass, height at withers, and height at croup traits by using the methodology of nonlinear models.

2. Material and Methods

2.1. Animals and measurements

The institutional ethics committee on livestock use approved all procedures on animals (case no. 23083.001452/2017-98). The evaluated horses belong to Coudelaria e Campo de Instrução de Rincão, which is the only horse husbandry unit of the Brazilian Army. The farm, located in São Borja, state of Rio Grande do Sul, Brazil (55°35'00" S, 28°45'40" W, and 130 m above sea level), is used to produce horses for various purposes, such as military ceremonies, sports, and employment in operations to guarantee law and order. A large part of the crossbreeding is performed based on the pedigree and performance traits for the choice of animals, and the trait selected depends on the desired purpose. The period of evaluation of the horses lasted from January 2013 to March 2015, covering animals from four different generations, from 2010 to 2013. Animals are raised in an extensive system on the farm, in which mares graze on pastures with their offspring until foals reach six months old. In sequence, the managers wean, mark, and separate foals in groups according to sex, which are thus maintained until three years old. After this phase, the army selects and distributes horses to the military units spread across the Brazilian territory.

We recorded the two linear measurements with a measuring stick and to obtain body mass, we used scales. For linear measurements, we kept the foals in a forced station and took the measurements on the left side of the body according to a methodology adapted from Pinto et al. (2005; 2008) and Godoi (2012). We measured the height at withers by taking a vertical distance from the highest point

of the interscapular region, located in the space defined by the fifth and sixth spinous process of the thoracic vertebra, to the ground. Height at croup was taken as the vertical distance from the highest point of the croup, over the sacral tuberosity, to the ground. We obtained measurements from animals of both sexes from birth to 867 days of age. For the morphometric traits, we used 161 animals (161 measures of height at withers and 161 measures of height at croup) and for the body mass, we used 167 animals (167 measures). We did not measure the same animal at different ages, which is equivalent to say that we measured each trait only once on each animal, even though the animals were of different ages (Table 1).

Table 1 - Genotypes, number of observations (n_o), mean, standard deviation (SD), and range limits for the dataset

Genotype	n_o	Average	SD	Minimum	Maximum
		Height at withers			
TT	14	1.38	0.19	1.00	1.53
TC	40	1.48	0.11	1.14	1.62
CC	107	1.45	0.15	0.98	1.67
		Height at croup			
TT	14	1.41	0.17	1.06	1.54
TC	40	1.50	0.10	1.18	1.62
CC	107	1.47	0.15	1.00	1.69
		Body mass			
TT	21	195.38	154.28	43.50	433.00
TC	31	299.93	170.81	55.00	499.00
CC	115	242.83	178.84	48.00	546.00

TT, TC, and CC represent the three different genotypes for the BIEC2-808543 polymorphism in Brasileiro de Hipismo horse breed.

2.2. Genotyping

We sampled approximately 8 mL of blood from the jugular vein of each animal in sterile vacutainer tubes containing 8% EDTA and froze the collected samples for transportation. Thence, we thawed, homogenized, and washed the samples with phosphate buffer saline (PBS; 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, and pH 7.4) three times with centrifugations at 735 × *g* for 10 min. We transferred the pellet to 2-mL tubes for storage at –20 °C and took small aliquots for DNA extraction.

We extracted the genomic DNA by using 100 µL of the cell lysate and 500 µL of the buffer described by Doyle and Doyle (1987). We kept the samples at 65 ° C for 1 h, stirring them occasionally. After this time, we let the samples cool to room temperature and added 500 µL of chloroform and isoamyl alcohol in a 24:1 ratio and stirred them. We centrifuged the samples at 10,000 × *g* for 10 min and transferred the supernatant to 2.0 mL tubes. We added 250 µL of isopropanol at 4 °C and kept the samples for 30 min at this same temperature. After this time, we centrifuged the samples at 12,000 × *g* for 20 min. We discarded the supernatant and washed the pellet (DNA) twice in 75% ethanol. After the evaporation of alcohol, we diluted the DNA in ultrapure water and quantified it in a spectrophotometer at a wavelength of 260 nm.

The polymerase chain reaction (PCR) technique amplified the fragments of interest in a final volume of 20 µL. Each reaction contained approximately 100 ng of genomic DNA, 1 unit of *Taq* DNA polymerase, 0.2 mM of each deoxyribonucleotide (dNTP), 2.0 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl, plus 4.0 pmoles of each primer. Metzger et al. (2013) described the primer sequences used. The thermal cycler (Applied Biosystems® ProFlex™ PCR System) program consisted of an initial denaturation step at 94 °C for 4 min, 35 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 40 s and a final extension step at 72 °C for 10 min.

The restriction endonuclease *Bsr*I cleaved the PCR adequately amplified samples by a technique called PCR-Restriction Fragment Length Polymorphism (PCR-RFLP). These fragment-digesting reactions were carried out in a final volume of 10 µL, following the recommendations of the enzyme manufacturer (BioLabs Inc., New England). We used 5% polyacrylamide gel electrophoresis (PAGE) to evaluate all fragments subjected to the PCR-RFLP technique and stained the fragments with $AgNO_3$.

Macrogen (Macrogen INC, Seul Korea) sequenced samples that differed in the cut pattern according to the chain termination technique (Sanger et al., 1977). We edited the generated electropherograms and compared the sequences to each other using *ChromasPro* software (version 2.4). We aligned the edited sequences with the NC_009146 sequence deposited in GenBank, using the ClustalW2 program (available at http://www.ebi.ac.uk/Tools/msa/clustalw2/). We evaluated these sequences for cutting sites using Restriction Mapper (version 3) and Webcutter 2.0 (available at http://www.restrictionmapper.org/ and http://www.firstmarket.com/cutter/cut2.html) softwares, respectively.

2.3. Statistical analysis

For data analysis, we used the methodology of nonlinear mixed models. The general structure of the growth models was $Y_t = \mu_{Y_t} + e_t$, for $Y_t \sim \text{Normal}(\mu_{Y_t}, \sigma^2_{Y_t})$, that is, variable Y_t follows a normal distribution with mean and variance functions μ_{Y_t} and $\sigma^2_{Y_t}$ (Tables 2 and 3).

Table 2 - Nonlinear growth models used in the study

Model	Equation
Brody (1945)	$\mu_{Y_t} = Y_f - b * \exp(-kt)$
Gompertz (Winsor, 1932)	$\mu_{Y_t} = Y_f * \exp\{-b[\exp(-kt)]\}$
Logistic (Pearl and Reed, 1923)	$\mu_{Y_t} = Y_f/\{1 + b[\exp(-kt)]\}$
von Bertalanffy (1957)	$\mu_{Y_t} = Y_f\{1 - b[\exp(-kt)]\}^3$
Michaelis-Menten (López et al., 2000)	$\mu_{Y_t} = (bK^n + Y_f t^n)/(K^n + t^n)$
Richards (1959)	$\mu_{Y_t} = bY_f/[b^m + (Y_f^m - b^m)\exp(-kt)]^{1/m}$

μ_{Y_t} is the mean of the weight of the animal, height at withers, or height at croup at specific time t; Y_f represents the weight of the animal, height at withers, or height at croup at maturity; k is the specific maturity rate or specific growth rate; b is a scale parameter; K represents the time (days) in which half the adult size is reached; n is a scale, dimensionless parameter; and m is a dimensionless parameter that scales growth to metabolic processes.

Table 3 - Variance functions used in the study to correct records for heterogeneity of variance over time in nonlinear parameter estimation

Model of variance	Equation
Homogeneous	$\sigma^2_{Y_t} = \sigma^2$
Exponential[1]	$\sigma^2_{Y_t} = \sigma^2_0 * \exp(ct)$
Asymptotic[2]	$\sigma^2_{Y_t} = \sigma^2_0 + \sigma^2_b\,[1 - \exp(-st)]$
Power of the mean[3]	$\sigma^2_{Y_t} = \sigma^2\,(\mu_{Y_t})^{2\psi}$

$\sigma^2_{Y_t}$ is the variance of the weight of the animal, height at withers or height at croup; σ^2 is the residual homogeneous variance.
[1] Exponential variance contains the initial residual variance ($\sigma^2_{Y_t} = \sigma^2_0$) associated with the variable (live weight, height at withers, or height at croup) at birth ($t = 0$), which increases exponentially during growth at a rate (1/t), for time expressed in days.
[2] The asymptotic variance describes an increasing behavior in which $\sigma^2_{Y_t} = \sigma^2_0$ is the birth-related variance. Parameter σ^2_b is the increase in asymptotic variance over time, and s (1/d) represents the rate of first-order increase in $\sigma^2_{Y_t}$.
[3] Residual variance scaled by an exponent (ψ), as a function of the expected mean, μ_{Y_t}.

For all models presented, parameter Y_f represents weight, height at withers, or height at croup of the animal at maturity; parameter k is the specific maturity rate or specific growth rate; b is a scale parameter; and the error term was assumed normally and independently distributed, $e_t \sim \text{Normal}(0, \sigma^2_{Y_t})$. For the Generalized Michaelis-Menten equation, parameter K represents the time (days) in which half the adult size is reached, and n is a scale, dimensionless parameter. In the Richards equation, m is a dimensionless parameter that scales growth to metabolic processes.

Attempts have been made to correct records for heterogeneity of variance over time in nonlinear parameter estimation (Matis and Hartley, 1971; Bard, 1974; Rocha et al., 2015). Thus, the variance was modeled (Table 3). In the first equation ($\sigma^2_{\dot{Y}_t} = \sigma^2$), σ^2 is the residual homogeneous variance. The exponential variance contains the initial residual variance ($\sigma^2_{\dot{Y}_t} = \sigma^2_0$) associated with the variable (live weight, height at withers, or height at croup) at birth (t = 0), which increases exponentially during growth at a rate (1/t), for time expressed in days (d). The asymptotic variance describes an increasing behavior in which $\sigma^2_{\dot{Y}_t} = \sigma^2_0$ is the birth-related variance. Parameter σ^2_b is the increase in asymptotic variance over time, and s (1/d) represents the rate of first-order increase in $\sigma^2_{\dot{Y}_t}$. The last equation represents the residual variance scaled by an exponent (ψ), as a function of the expected mean, μ_{Y_t}.

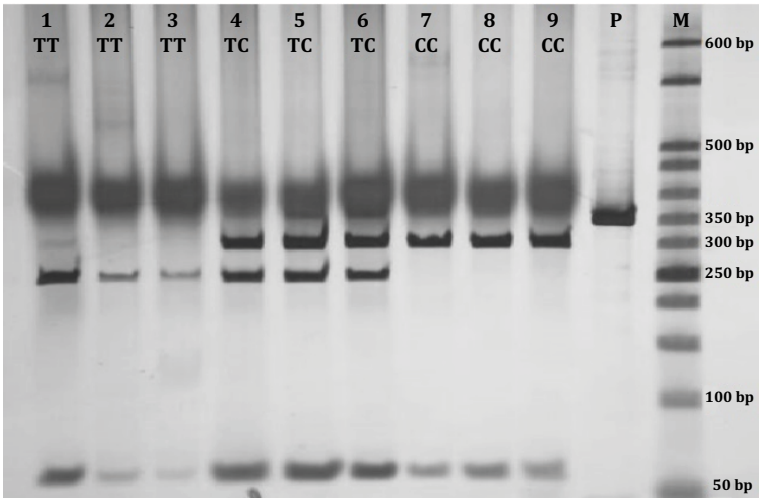
The models were adjusted for growth data using the NLMIXED procedure of the SAS software (Statistical Analysis System, version 9.0; Vonesh (2012)). The corrected Akaike criterion information (AICc; Akaike, 1974; Sugiura, 1978) is an output of the NLMIXED procedure; therefore, the smallest AICc yielded the largest model probability and the smallest evidence ratio, and the model with the smallest AICc was selected as the best one (Littell et al., 2006). The fixed effects in the models were genotypes TT, TC, and CC, and the polymorphism effect on each parameter of the model was evaluated to reduce parameterization and avoid overfitting.

Although our sample consisted of 167 animals for body weight and 161 for the linear measures, the number of degrees of freedom was sufficient for detecting significant SNP effects, which was one of the major findings of the study. Besides, we used the Information-Theoretic approach, which is the state-of-the-art theory for multimodel selection. The most suitable model presented a high probability of model and allowed to identify significant effects of the SNP concerning the evaluated traits.

3. Results

3.1. Animal genotyping

Using the PCR-RFLP technique, we identified three genotypes for the BIEC2-808543 polymorphism in BH horses: TT and CC, which are homozygotes, and TC, which is a heterozygote. The TT genotype is recognized by the occurrence of two cutting sites in the DNA fragment, with 347 base pairs (bp), resulting in smaller fragments of 55, 57, and 235 bp. The CC genotype has a single cutting site in the original fragment, resulting in two fragments of 55 and 292 bp, respectively. The TC genotype is identified by the presence of all four fragments (55, 57, 235, and 292 bp) (Figure 1). For the population



Lines 1 to 9 - 347 base pair (bp) fragments after digestion by the *Bsr*I enzyme.
Lane P - fragment without digestion; M - 50 bp marker (Ludwig Biotecnologia Ltda, Brazil); TT and CC - homozygous genotypes of Brasileiro de Hipismo breed horses; TC - heterozygous genotype.

Figure 1 - Representative image of 5% polyacrylamide gel showing the DNA fragments of the BIEC2-808543 (C/T) polymorphism region.

used in this study, we estimated the allele frequency of 0.79 and 0.21 for alleles C and T, respectively, and the genotypic frequencies of 0.66, 0.25, and 0.09, for CC (n = 107), CT (n = 40), and TT (n = 14) genotypes, respectively. We found that the genotypic proportions are not in the Hardy-Weinberg equilibrium (P<0.05).

Through automatic sequencing, it was possible to confirm the identity of the amplified samples and the confirmation of the SNP BIEC2-808543 (C/T) in BH horses. The sequences generated in this study, aligned with the reference sequence available on GenBank (access number NC009146), show the differences between these fragments (Figure 2). The C/T polymorphism is highlighted in bold and identified by the letter Y (from pYrimidine), and the cutting region by the enzyme is underlined. The generated electropherograms also showed new polymorphism in our samples. The new polymorphism corresponds to a G/A transition, identified with the letter R (from puRine), which results in the appearance of another cutting site for the restriction enzyme *Bsr*I when base A is present.

```
209         ----------TGCATGTTCTTGAGAGAAACCAAATTTGCCTGGCTAGAGAAGCATTC↓CAG 50
861         GCCATCTATTTGCATGTTCTTGAGAGAAACCAAATTTGCCTGGCTAGAGAAGCATTC↓CAG 60
NC009146    GCCATCTATTTGCATGTTCTTGAGAGAAACCAAATTTGCCTGGCTAGAGAAGCATTC↓CAG 60
                      ********************************************** ***
209         YTTATTTCTGTACCCCAAAGGCAGAATCACAGGGAACACAATAGTGCAAACTTAAATGAC 110
861         YTTATTTCTGTACCCCAAAGGCAGAATCACAGGGAACACAATAGTGCAAACTTAAATGAC 120
NC009146    TTTATTTCTGTACCCCAAAGGCAGAATCACAGGGAACACAATAGTGCAAACTTAAATGAC 120
             ************************************************************
209         AACCTCTACAAAGAATATTCCTCTCTCAACTCTCCTCTCTCTATGCTATCCGGTCAAAGC 170
861         AACCTCTACAAAGAATATTCCTCTCTCAACTCTCCTCTCTCTATGCTATCCRGTCAAAGC 180
NC009146    AACCTCTACAAAGAATATTCCTCTCTCAACTCTCCTCTCTCTATGCTATCCGGTCAAAGC 180
            ************************************************** ********
209          CAGAGGTGGAGAGTACCTATATGTTAATGCGTTGAATATATTAATAACAAACATTTGTTT 230
861          CAGAGGTGGAGAGTACCTATATGTTAATGCGTTGAATATATTAATAACAAACATTTGTTT 240
NC009146     CAGAGGTGGAGAGTACCTATATGTTAATGCGTTGAATATATTAATAACAAACATTTGTTT 240
             ************************************************************
209         TGAACTCCATTTTAAATTAGCAATGAGACTTTCAGATGGAGAGCTCACTGGA↓AGCCTGTG 290
861         TGAACTCCATTTTAAATTAGCAATGAGACTTTCAGATGGAGAGCTCACTGGA↓AGCCTGTG 300
NC009146    TGAACTCCATTTTAAATTAGCAATGAGACTTTCAGATGGAGAGCTCACTGGA↓AGCCTGTG 300
            ************************************************** ********
209          TTTGTGAACAATGTTGGTTAATCATTGAACCAGCCT---------- 326
861          TTTGTGAACAATGTTGGTTAATCATTGAACCAGCCT---------- 336
NC009146     TTTGTGAACAATGTTGGTTAATCATTGAACCAGCCTATGAACTTGCC 347
             ***********************************
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NC009146 - reference sequence (GenBank access number); 209 and 861 - identification number of the Brasileiro de Hipismo breed horses; A - adenine; T - thymine; C - cytosine; G - guanine.
The SNP C/T is identified by letter Y (from "pYrimidine"), and letter R identifies the new G/A polymorphism (from "puRina"), both highlighted in bold. The restriction sites of the *Bsr*I enzyme are underlined.

Figure 2 - Alignment between DNA sequences for the BIEC2-808543 polymorphism region in horses.

3.2. Body mass

The nonlinear model that best described the growth curve for the body mass variable came from the association between the Brody model (1945) and the Power of the Mean (PoM) variance function (Table 4), which is weighed according to the current live weight of the animal over time. That is, over time, the variance increases in a nonlinear behavior as well, until a plateau is reached. However, we can observe that the AICc differences (Δ) between the Brody and the Richard and von Bertalanffy models

(adjusted without polymorphism effect in parameter b), both associated with the PoM variance, were similar to the selected model. Therefore, they can also be used to describe this variable. With the comparison between the evidence ratios (ER) of these same models, we can see that von Bertalanffy–b, when compared with the Brody model, is 1.7 times lower than the model probability, calculated as the ratio $W_{Brody}/W_{von\ Bertalanffy–b}$.

Table 4 - Akaike values and likelihood criteria for the best-fitted models for each variable analyzed in Brasileiro de Hipismo horses

Model	Variance	AICc	Δ	W	ER	Variable
Brody	PoM[2]	1432.8	0	0.392	1.0	
Richard	PoM	1433.8	1.0	0.238	1.6	Body mass
von Bertalanffy - b[1]	PoM	1433.9	1.1	0.226	1.7	
von Bertalanffy - b[1]	Asymptotic[3]	−541.3	0	0.118	1.0	
Gompertz	Asymptotic	−541.2	0.1	0.112	1.1	
Brody - b[1]	Asymptotic	−541.0	0.3	0.102	1.2	
Logistic - b[1]	Asymptotic	−541.0	0.3	0.102	1.2	Height at withers
Brody	Asymptotic	−540.5	0.8	0.079	1.5	
Gompertz	Asymptotic	−540.4	0.9	0.075	1.6	
von Bertalanffy	Asymptotic	−540.4	0.9	0.075	1.6	
Brody	Asymptotic	−577.0	0	0.115	1.0	
von Bertalanffy	Asymptotic	−576.6	0.4	0.094	1.2	
Gompertz	Asymptotic	−576.4	0.6	0.085	1.3	
Logistic	Asymptotic	−575.7	1.3	0.060	1.9	Height at croup
von Bertalanffy - b[1]	Asymptotic	−574.5	2.5	0.033	3.5	
Brody - b[1]	Asymptotic	−574.4	2.6	0.031	3.7	
Gompertz - b[1]	Asymptotic	−574.2	2.8	0.028	4.0	

PoM = power of the mean; AICc - Akaike information criterion corrected for small samples; W - model probability; ER - evidence ratio.
[1] Adjusted model without polymorphism effect in parameter b.
[2] Residual variance scaled by an exponent (ψ), as a function of the expected mean, μ_{Y_t}.
[3] Asymptotic variance describes an increasing behavior in which $\sigma^2_{Y_t} = \sigma^2_0$ is the birth-related variance. Parameter σ^2_b is the increase in asymptotic variance over time, and s (1/d) represents the rate of first-order increase in $\sigma^2_{Y_t}$.

We evaluated the influence of the polymorphism for each parameter of the selected nonlinear model. We observed a difference in the profiles of the growth curves for the body mass trait (Figure 3 A, B, and C), which reinforces the relation of the SNP BIEC2-808543 (C/T) with the curve profile for this trait.

Parameter Yf of the Brody model predicts the asymptotic body mass at maturity. We verified that animals with the TT genotype are lighter compared with those of TC and CC genotypes, as revealed by their disjoint 0.95 confidence intervals (0.95CI), whereas body mass at maturity of the TC and CC genotypes presented joint 0.95CI (Figure 4).

The Brody model (1945) admits that the specific growth rate is inversely proportional to the assessed trait and age of the individual. However, we were not able to detect differences regarding specific growth rates of smaller animals (TT) from heterozygous animals (TC) and larger animals (CC).

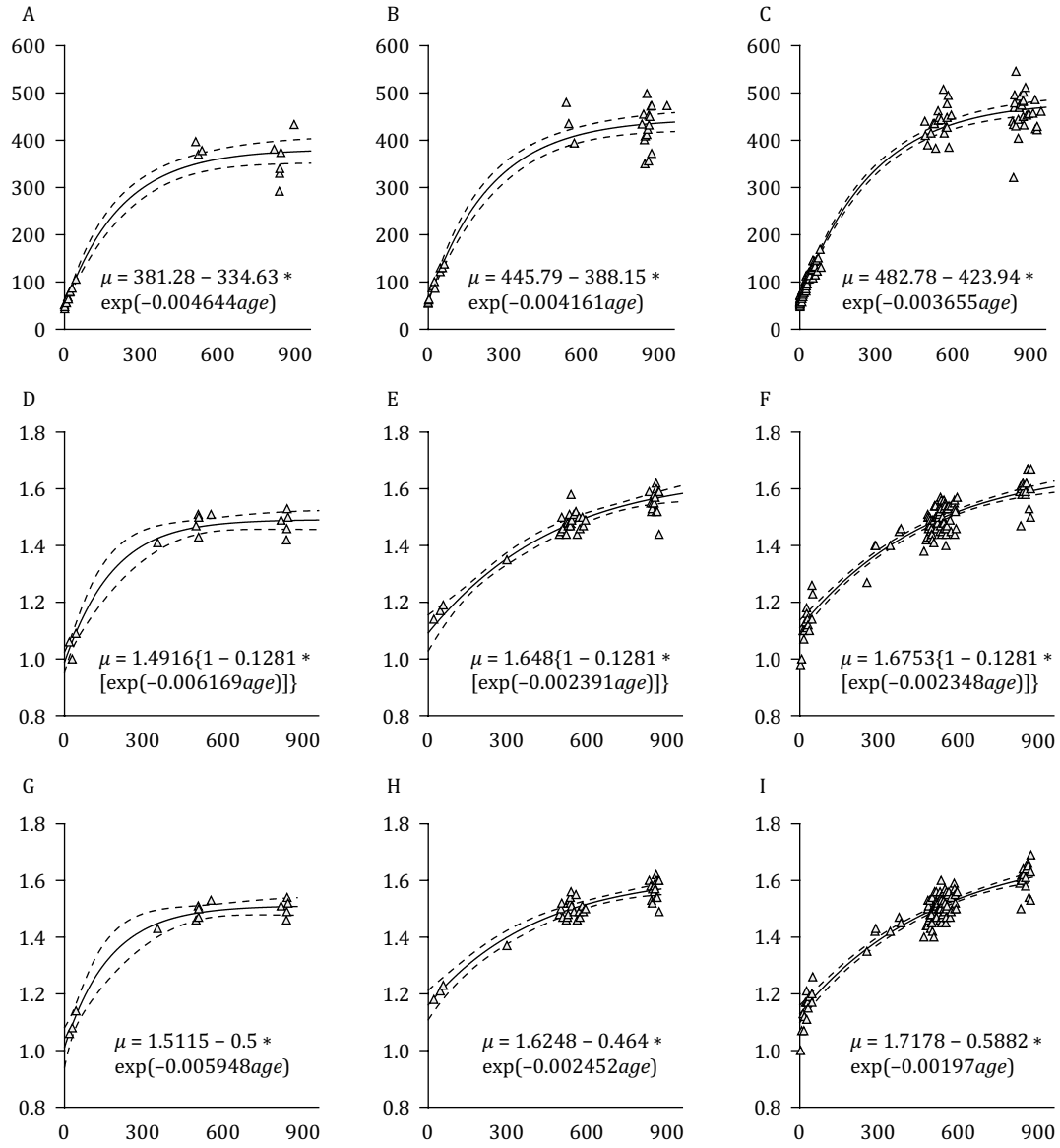
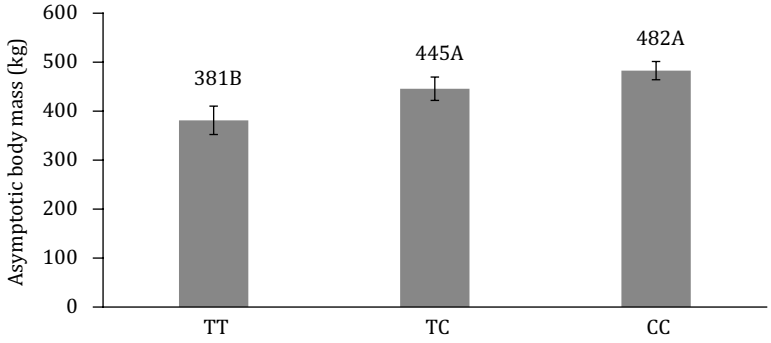


Figure 3 - Observed data (Δ), predicted values (solid line), and 0.95 confidence intervals (dashed lines) for the TT, TC, and CC genotypes for body mass (graphs A, B, and C, respectively), height at withers (graphs D, E, and F, respectively), and height at croup (graphs G, H and I, respectively) of Brasileiro de Hipismo breed horses. The genotypes correspond to the SNP BIEC2-808543.



TT, TC, and CC correspond to the genotypes of Brasileiro de Hipismo breed horses.
Values above the bars indicate the mean body weights for each genotype, and the different letters indicate differences revealed by disjoint 0.95 confidence intervals.

Figure 4 - Effect of TT, TC, and CC genotypes for SNP BIEC2-808543 on body mass at maturity (Yf), according to the Brody model with power-of-the-mean variance.

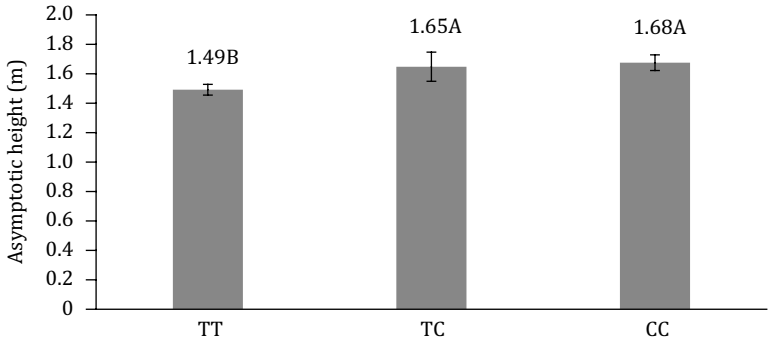
3.3. Height at withers

The nonlinear model that best described the growth curve for height at withers was the von Bertalanffy model (1957), adjusted without polymorphism effect in parameter b, and associated with the asymptotic variance (Table 4), i.e., the variance increases over time until it plateaus, along with the asymptotic height at withers.

We evaluated the influence of the polymorphism in each parameter of the selected model and observed a difference in the growth curve profiles for the trait (Figures 3 D, E, and F), which indicates the influence of the BIEC2-808543 (C/T) polymorphism on the growth curve profile of this variable.

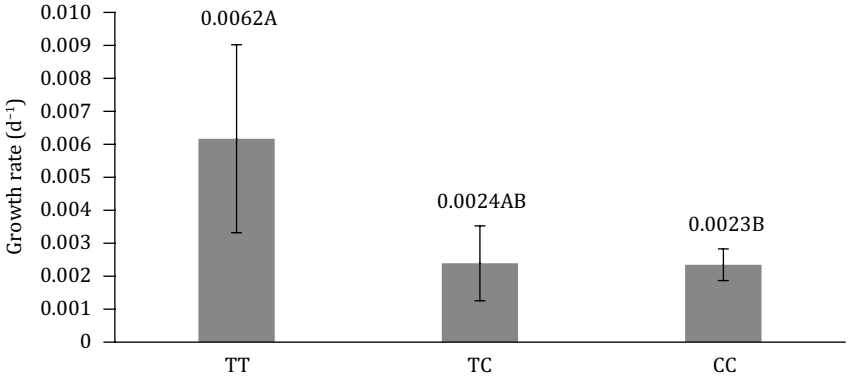
According to the von Bertalanffy model (1957), parameter Yf predicted the asymptotic height at withers at maturity. Thereby, animals with the TT genotype were smaller compared with those of the TC and CC genotypes, as revealed by disjoint 0.95CI (Figure 5).

Using the parameter k of the von Bertalanffy model (1957), which represents the specific growth rate related to height at withers, we verified that the specific growth rate of the shorter animals (TT genotype) was faster than of the CC genotype carriers. Nonetheless, we were not able to detect differences between TC and TT genotypes, nor between TC and CC genotypes (Figure 6).



TT, TC, and CC correspond to the genotypes of the Brasileiro de Hipismo breed horses.
Values above the bars indicate the mean height at withers for each genotype, and the different letters indicate differences revealed by disjoint 0.95 confidence intervals.

Figure 5 - Effect of TT, TC, and CC genotypes for SNP BIEC2-808543 on height at withers at maturity (Yf), according to the von Bertalanffy model with asymptotic variance.



TT, TC, and CC correspond to the genotypes of the Brasileiro de Hipismo breed horses.
Values above the bars indicate the rates for each genotype, and the different letters indicate differences revealed by disjoint 0.95 confidence intervals.

Figure 6 - Effect of TT, TC, and CC genotypes for SNP BIEC2-808543 on the growth rate (k) referring to height at withers, according to the von Bertalanffy model with asymptotic variance.

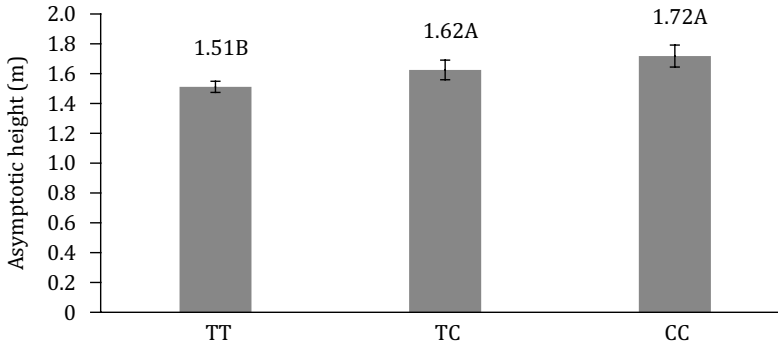
3.4. Height at croup

In the evaluation of growth for the variable height at croup, the nonlinear model from the association of the Brody model (1945) with an asymptotic variance, which was a function of time, was the one that best described the growth of BH horses (Table 4).

We observed a difference in the growth curve profiles for height at croup (Figure 3 G, H, and I) when we evaluated the influence of polymorphism in each parameter of the selected nonlinear model.

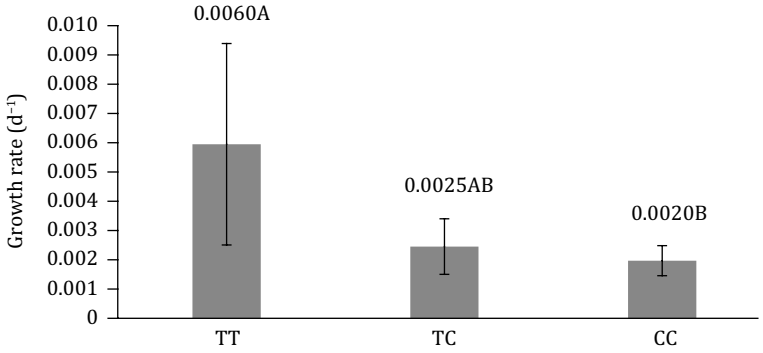
According to the Brody model (1945), parameter Yf predicts the asymptotic height at croup at maturity, and animals with the TT genotype were shorter than those carriers of TC and CC genotypes, as revealed by disjoints 0.95CI (Figure 7).

Shorter animals (TT genotype) showed a faster specific growth rate (parameter k) if compared with those animals taller at the croup (CC genotype carriers). However, we could not detect differences in the specific growth rate of height at croup between the TC and CC genotypes because of joint 0.95CI (Figure 8).



TT, TC, and CC correspond to the genotypes of Brasileiro de Hipismo breed horses.
Values above the bars indicate the mean height at croup for each genotype, and the different letters indicate differences revealed by disjoint 0.95 confidence intervals.

Figure 7 - Effect of TT, TC, and CC genotypes for SNP BIEC2-808543 on height at croup at maturity (Yf), according to the Brody model with asymptotic variance.



TT, TC, and CC correspond to the genotypes of the Brasileiro de Hipismo breed horses.
Values above the bars indicate the growth rates for each genotype, and the different letters indicate differences revealed by disjoint 0.95 confidence intervals.

Figure 8 - Effect of the TT, TC, and CC genotypes for SNP BIEC2-808543 on growth rate (k) referring to height at croup, according to the Brody model with asymptotic variance.

4. Discussion

4.1. Genotype frequencies and gene function

Regarding genotypic frequencies, the absence of Hardy-Weinberg equilibrium ($P<0.05$) can be explained by the lack of random mating and, consequently, by the differential selection pressure on the genotypes, since there is a minimum height accepted for breeding selection, probably favoring the CC genotype.

The association of this polymorphism with conformation in horses leads to an important question: what is the function of this polymorphic region? According to Metzger et al. (2013), SNP BIEC2-808543 modifies a probable binding site for the transcription factor D of the RNA polymerase II (TFIID). According to the authors, the allele C is associated with reduced expression of the *LCORL* gene, located downstream to this polymorphism. The *LCORL* gene is expressed about 56% less in animals of CC genotype and about 40% less in animals of CT genotype, compared with the expression of this gene in smaller horses, those with TT genotype.

It is widely known in molecular genetics that the initiation of transcription by RNA polymerase II requires the participation of several basal transcription factors, with the TFIID factor being one of the first to interact with the core of the promoter. The BIEC2-808543 C/T polymorphism predates the *LCORL* gene and is located in the TATA box of the promoter. The current understanding is that allele T would make it possible to anchor the TFIID, unlike allele C, which possibly modifies this binding site. For Metzger et al. (2013), the TFIID transcription factor and *LCORL* gene, which is also a transcription factor, are central components of the gene transcription apparatus involved in bone development. Although some information is available on this gene, its function is still unclear. In a review study (Naccache et al., 2018), the authors found a strong association between osteochondrosis in horses and the polymorphism in question, which alerts us to the problem of animal selection by a single criterion or without knowing more comprehensively the effects of the gene marker.

In addition to the C/T polymorphism discussed above, the polymorphism identified in the current study (A/G) was not evaluated for its association with conformation traits in horses, given the small number of animals showing this variation. The region verification of this new polymorphism through *Nsite* (Solovyev et al., 2010) and SIGNAL SCAN (Prestridge, 1991) shows that the polymorphism is in a region with rich gene signaling.

4.2. Statistical analyses

Traditional methodologies (nonlinear least squares) are restricted to the assumption that the variance is homogeneous, i.e., constant (Araújo et al., 2012; Marinho et al., 2013; Teleken et al., 2017; Hojjati et al., 2018; Ribeiro et al., 2021). Thus, the intrinsic assumption is that the same variance occurs throughout the development of the animal and even between animals. Another limitation of these methods is the impossibility of associating fixed effects on the parameters in a single adjustment; in this case, the effect of the polymorphism is associated with each parameter of the model. Therefore, the nonlinear mixed models fit heterogeneous variances and even nonlinear variance functions, and this tool is flexible to combine fixed effects, its interactions, and random effects (Pinheiro and Bates, 2000; Littell et al., 2006; Vonesh, 2012). Therefore, they can describe growth profiles more accurate and precisely.

4.3. Variables

In the literature, we found several studies associating the BIEC2-808543 polymorphism with height and other horse traits in different breeds (Signer-Hasler et al., 2012; Metzger et al., 2013; Tetens et al., 2013). Unlike other studies, we found that there is an influence of SNP BIEC2-808543 C/T on body mass, height at withers and height at croup at maturity (Figures 4, 5, and 7) and on

growth rate (Figures 6 and 8). Nevertheless, the asymptotic point of body mass, height at withers, and height at croup for each genotype is not necessarily the weight or the height of these animals in adulthood, but the average to be reached regardless of seasonal conditions. Likewise, there are no studies on the association of the referred SNP with growth curve parameters as an attempt to estimate the effect of polymorphism on the growth profile of horses, such as those carried out by our team (Figure 3). Early knowledge of animal genotypes can optimize management efficiency on the farm since animals with a homozygous TT genotype tend to be lower and have a faster growth rate than animals with TC and CC genotypes and a pattern different from the growth curve.

4.3.1. Body mass

McManus et al. (2010) evaluated five nonlinear models (Brody, Richards, Gompertz, Logistic, and Weibull) to describe the growth profile of Hanoverian, BH, and Thoroughbred and Thoroughbred × BH crossbred raised by the Brazilian Army. The authors recommended the Brody model for describing the growth curve, as in our study. However, those authors observed that the Brody and Weibull models similarly fitted this variable and recommend the Brody model as the best because of the parsimony criterion. Santos et al. (2007) did not achieve the same conclusion. Among the same five models described previously, they selected those that presented the lowest combined mean squared errors, which was the Weibull model as the best for describing body mass growth of grazing Pantaneiro horses. Nevertheless, those studies did not correct the heterogeneity of variances over time and longitudinal correlation, as we used in this study. In addition, our results indicate that a model averaging process is necessary for predictive purposes (Burnham and Anderson, 2004).

4.3.2. Height at withers

Santos et al. (1999) recommended the Weibull model for describing the growth curve of the height at withers in Pantaneiro horses, whereas Santos et al. (2007) recommended the Richards model (1959) for this same variable and breed. McManus et al. (2010) stated that the Brody model best described the growth of the Brazilian Army horses measured at the height at withers. These differences in the choice of models probably occurred due to the number of animals and observations in each study, since all the authors tested the same five nonlinear models: Brody, Richards, Gompertz, Logistic, and Weibull. Those authors also used ordinary nonlinear least squares. The framework we used here roots on nonlinear models; this structure groups information from all animals and growth records in a single matrix of variance, which also accounts for heterogeneity and longitudinal correlations over time (Pinheiro and Bates, 2000). The models differed little from each other (Table 4); what defined the model that best described the variance about the growing height at withers was the asymptotic variance, which was the differential approach we used in this study.

We found an association of allele C of SNP BIEC2-808543 with taller horses, the same way as found by Signer-Hasler et al. (2012), with Franches-Montagnes horses. In another study (Metzger et al., 2013), the same polymorphism seems to be associated with height at withers within and between horse breeds. The TT genotype is more frequent in animals below 148 cm height (e.g., ponies); the common CT genotype occurs in animals between 130 and 160 cm height; and the highest frequency of the CC homozygote occurs in taller animals, i.e., more than 160 cm.

4.3.3. Height at croup

The models differed little from each other, likewise for the other traits studied. Therefore, we chose the Brody model with an asymptotic variance to describe height at croup (Table 4).

We did not find studies on growth profiles of height at croup variable, possibly because it is more common to measure the height of horses at the withers. However, Pinto et al. (2005), with principal component analysis, evaluated morphometric measurements in Mangalarga Marchador animals.

The authors reported that in newborn animals, the height at croup trait is one of the components necessary to explain a minimum percentage of 80% of the total existing variation and discard the need to measure height at withers for growth assessment. According to their analysis, this variable does not show any significant variation or is strongly related to one of the selected variables, and it is possible to reduce the number of morphometric variables from 25 to nine in male foals and seven in female foals of the same age. In that same study, when analyzing one-year-old foals, height at croup, together with hip width, were able to explain about 62.8% of the total existing variation, which is evidence of the importance of this variable in the growth analysis of horses, regardless of the height at withers.

5. Conclusions

The BIEC2-808543 polymorphism occurs in Brasileiro de Hipismo horses and suffers selection pressure due to the preferential crossings performed based on the animal's phenotype. Although all horses were bred in a standard condition, we attributed the differences in growth to the genotypes. The nonlinear mixed models are promising for the description of growth curves in horses, particularly by the possibility of associating SNP effects to model parameters. This technique is also useful to model variances over time more properly, which accounts for heterogeneous variances observed in the growth profiles of animals.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: R.A.M. Vieira and M.A.M. Soares. Data curation: E.C.X. Costa and M.A.M. Soares. Formal analysis: R.A.M. Vieira and L.S. Glória. Funding acquisition: M.A.M. Soares. Investigation: E.C.X. Costa and R.A.M. Vieira. Methodology: E.C.X. Costa, R.A.M. Vieira, L.S. Glória and M.A.M. Soares. Project administration: M.A.M. Soares. Resources: M.A.M. Soares. Software: R.A.M. Vieira. Supervision: R.A.M. Vieira and M.A.M. Soares. Validation: E.C.X. Costa and L.S. Glória. Visualization: E.C.X. Costa and L.S. Glória. Writing-original draft: E.C.X. Costa. Writing-review & editing: R.A.M. Vieira, L.S. Glória and M.A.M. Soares.

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