

# Reproductive traits in Pura Raza Española mares manifest inbreeding depression from low levels of homozygosity

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## Abstract

Inbreeding depression is a genetic phenomenon associated with the loss of fitness and mean phenotypic performance due to mating between relatives. Historically, inbreeding coefficients have been estimated from pedigree information. However, the onset of genomic selection programs provides large datasets of individuals genotyped using SNP arrays, enabling more precise assessment of an individual's genomic-level inbreeding using genomic data. One of the traits most sensitive to issues stemming from increased inbreeding is reproduction. This is particularly important in equine, in which fertility is only moderate compared to other livestock species. To explore this further, we evaluated the effect of inbreeding on five reproductive traits (age at first foaling (AFF), average interval between foalings (AIF), total number of foalings (NF), productive life (PL) and reproductive efficiency (RE)) in Pura Raza Español mares using genomic data. Residual predicted phenotypes were obtained by purging these traits through the REML ( $wg_{Residual}$ ) and ssGREML ( $g_{Residual}$ ) approaches in reproductive data of 29,847 PRE mares using the BLUPF90+ program. Next, we used pedigree-based ( $F_{ped}$ ) and ROH-based genomic ( $F_{ROH}$ ) inbreeding coefficients derived from 1018 animals genotyped with 61,271 SNPs to estimate the inbreeding depression (linear regression). Our results indicated significant levels of inbreeding depression for all reproductive traits, with the exception of the AIF trait when  $F_{ped}$  was used. However, all traits were negatively affected by the increase in genomic inbreeding, and  $F_{ROH}$  was found to capture more inbreeding depression than  $F_{ped}$ . Likewise, REML models (ssGREML) using genomic data for estimated predicted residual phenotypes resulted in higher variance explained by the model compared with the models not using genomics (REML). Finally, a segmented regression analysis was conducted to evaluate the effect of inbreeding depression, revealing that the levels of genealogical and genomic homozygosity do not manifest uniformly in reproductive traits. In contrast, the levels of inbreeding depression ranged from low to high as homozygosity increased. This analysis also showed that reproductive

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traits are very sensitive to inbreeding depression, even with relatively low levels of homozygosity.

**KEYWORDS**

fertility, genomic inbreeding, horse, runs of homozygosity, segmented regression

## 1 | INTRODUCTION

Inbreeding is caused by mating between relatives and is associated with a loss of fitness and average phenotypic performance, a phenomenon known as inbreeding depression (Howard et al., 2017; Leroy, 2014). Recently, increased inbreeding has become a concern in livestock populations, as it can be an unwanted consequence of both artificial selection and the widespread use of reproductive biotechnology. It can negatively affect economically important traits, such as fitness, health, reproduction, adaptability, performance and yield (Antonios et al., 2021; Azcona et al., 2020; Doekes et al., 2020, 2021; Zhang et al., 2022). There are three genetic mechanisms which may cause inbreeding depression: partial dominance, overdominance and epistasis. However, it has been shown that the major proportion of inbreeding depression is caused by partial dominance which is caused by the accumulation of deleterious recessive alleles previously hidden in the heterozygosity state (reviewed by Charlesworth and Willis (2009)). These alleles are commonly present at low frequencies in livestock populations, but the occurrence in deleterious recessive homozygotes is increased by the inbreeding, thus exposing deleterious effects. In addition, it is also demonstrated that phenotypic superiority of heterozygous over homozygous genotypes exists in some particular loci, known as the overdominance effect (Crow, 1970). In this case, the increase in inbreeding tends to reduce the frequency of heterozygous genotypes. Finally, epistasis hypothesis refers to a reduction in the combination of favourable heterozygous genotypes across multiple loci (Jain & Allard, 1966), but it is commonly mentioned as the least important genetic mechanism involved in the occurrence of inbreeding depression.

Inbreeding depression has been assessed using different approaches. The standard approach is to *regress* the phenotype of the trait of interest on the inbreeding coefficient, by including inbreeding values as a covariate in a REML mixed model, which provides a refined inbreeding depression estimate when correcting for the effects in the model (Gómez et al., 2009). However, the increasing availability of genomic information currently offers an alternative way of estimating the genome-wide homozygosity in a more accurate way. Among the different homozygosity estimation methods developed using genomic information,

the assessment of autozygosity across chromosomal segments using runs of homozygosity (ROH) has emerged as a valuable tool for estimating inbreeding, due to its overall flexibility and its ability to quantify the contribution of genomic homozygosity ( $F_{ROH}$ ) to inbreeding depression (Hill et al., 2023).

The rise in inbreeding is a significant concern in various equine breeds, notably the Pura Raza Española (PRE) horses. Despite a substantial global population of nearly 250,000 individuals across 60 countries, the PRE breed exhibits high levels of inbreeding (Perdomo-González et al., 2022; Poyato-Bonilla et al., 2022). This trend is common in many European equine breeds due to closed population structures, resulting in inbred matings from limited founders and breeding animals available for trait selection within studs.

While inbreeding depression is extensively studied in livestock species like cattle (Baes et al., 2019; Gutiérrez-Reinoso et al., 2022), sheep (Antonios et al., 2021) and pigs (Saura et al., 2015; Zhang et al., 2022), research in horses remains minor, being particularly focused in the effect of inbreeding in morphology (Giontella et al., 2020), or athletic aptitude (Hill et al., 2022; Todd et al., 2018). Previous studies have reported inbreeding depression in body measurements in the PRE breed (Gómez et al., 2009), as well as for morphological traits and defects (Poyato-Bonilla et al., 2020). A recent study indicated an adverse impact on reproductive traits in the PRE (Laseca et al., 2022), but did not quantify the magnitude of effects on phenotypic level.

The aim of this study was to estimate and quantify the effect of inbreeding depression on reproduction traits in Pura Raza Español horses using a genomic approach.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals and phenotypic dataset

The initial reproductive dataset included 344,707 foaling records of 78,986 breeding mares and 8133 studs included in the PRE studbook. Records were obtained from the breed's studbook up to 2022, including only foaling records from mares born after 1970, when the breed's official parentage control was established. Small breeders (producing

less than 10 foals per year) and mares mainly used for leisure or sports activities (first foaling after 7 years old, with an interval between first and second foaling, and last and penultimate foaling over 5 years) were also excluded to avoid a negative bias in reproductive traits (high probability that reproductive variables do not reflect the reproductive aptitude of the animal but rather decisions made by the breeder), according to the methodology validated by our group (Gómez et al., 2020). The final dataset included 29,847 breeding mares belonging to 6939 studs.

The phenotypic characterization of the mares' fertility was based on five reproductive traits: age at first foaling (AFF), average interval between foalings (AIF), total number of foalings (NF), productive life (PL, calculated individually for each mare from the age at first foaling and the age at which the mare was culled from the breeding herd or the age at her last foaling in the case she has not finished her productive life) and reproductive efficiency (RE, defined as the number of total foalings relative to the optimal (maximal) number of foalings the mare could have had during her entire lifetime; Perdomo-Gonzalez et al., 2020).

## 2.2 | Genomic data

A total of 1018 mares belonging to the PRE breed were genotyped. Briefly, the DNA was isolated from blood or hair samples using DNeasy Blood & Tissue Kit (Qiagen), following the manufacturer's protocol. The horses were genotyped with medium density GGP Equine Array (NEOGEN), and over 70,000 evenly distributed SNPs were profiled. The raw genotypic data were filtered using PLINK software v1.9. Only SNP markers showing a high-quality genotyping rate (call-rate > 0.95), with a known genomic position located on the autosomes and X chromosome, were kept (61,271 SNPs). No minor allele frequency or linkage disequilibrium filtering was used, following the latest estimation guidelines for runs of homozygosity (ROH) (Meyermans et al., 2020).

## 2.3 | Predicted residual phenotypes

The predicted residual phenotypes (equivalent to the purged phenotype for the effects included in the model) were obtained for each animal and reproductive trait, using a multivariate animal model, as follows:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Za} + \mathbf{e}$$

where  $\mathbf{y}$  is the vector of observations of reproductive traits;  $\mathbf{b}$  is the vector of fixed effects (coat colour, geographical area and birth stud size and age at last foaling as covariates);  $\mathbf{a}$

is the random additive genetic effect, and  $\mathbf{e}$  is the random residual effect;  $\mathbf{X}$  and  $\mathbf{Z}$  are incidence matrices relating observations to fixed and random additive genetic effects respectively.

The additive genetic effect was modelled using two different genetic covariance structures: the  $\mathbf{A}$  matrix, denoting the pedigree-based additive genetic relationship, for the classical evaluation, and the  $\mathbf{H}$  hybrid matrix, which integrates the pedigree and genomic data to establish relationships among individuals, for ssGBLUP. The latter ( $\mathbf{H}$ ) was derived by blending  $\mathbf{A}$  with the genomic relationship matrix  $\mathbf{G}$ , calculated following VanRaden (2008) as follows:

$$\mathbf{G} = 0.95 \frac{\mathbf{SS}'}{2 \sum_{i=1}^n p_i(1-p_i)} + 0.05 \mathbf{A}$$

where,  $n$  is the number of SNP markers,  $p_i$  is the allele frequency of marker  $i$ ,  $\mathbf{A}$  is the pedigree relationship matrix and  $\mathbf{S}$  is a centred incidence matrix of SNP markers.

This combined approach allowed us to obtain two types of predicted residual phenotypes: one from the model with genomics ( $g_{\text{Residual}}$ ) and the other from the model without genomics ( $wg_{\text{Residual}}$ ). Finally, variance components, estimated breeding values (EBVs), genomic estimated breeding values (GEBVs) and predicted residual phenotypes ( $g_{\text{Residual}}$  and  $wg_{\text{Residual}}$ ) were estimated, applying restricted maximum likelihood (REML) and ssGREML approaches for conventional and genomic evaluations respectively. The analyses were performed with the BLUPF90+ package (Misztal et al., 2022).

## 2.4 | Estimation of inbreeding and homozygosity

Two inbreeding estimation methods were employed in this study: the pedigree-based inbreeding coefficient ( $F_{\text{ped}}$ ), and the genomic inbreeding coefficient based on ROH ( $F_{\text{ROH}}$ ).

The  $F_{\text{ped}}$ , defined as the probability that the two alleles at any locus in an individual are identical by descent (IBD), according to Wright (1922), was computed using Endog program (Gutiérrez & Goyache, 2005). This is an estimate of an animal's homozygosity level by the presence of common ancestors in both paternal and maternal pathways. This parameter is a probabilistic statistic since it measures the expected (theoretical) homozygosity level of any animal that presents these common ancestors at the same pedigree site (expected average homozygosity level). In our case, the pedigree used for the estimation of the inbreeding coefficient ( $F_{\text{ped}}$ ) is deep and reliable. The animals considered in the study had at least 10 complete

generations and up to 19 known ancestral generations. Moreover, the inclusion of animals in the studbook requires confirmation through molecular markers since the early 1980s. As a result, the PRE population has over 40 years of proven parental information.

The  $F_{ROH}$  is defined as the percentage of the genome covered by ROH, according to McQuillan et al. (2008). This parameter aims to estimate the level of homozygosity along the genome produced by the presence of common ancestors (identity-by-descent, IBD) by detecting homozygous regions which are unlikely produced by identity-by-state. We first estimated all the ROH for each animal using the *slidingRuns* procedure in the DetectRUNS Package (Biscarini et al., 2018) in R (R-Core-Team, 2022), following the parameters used in the study by Laseca et al. (2022): size of window = 50, minimum number of SNPs = 100, threshold = 0.05, maximum gap between consecutive SNPs = 100,000 bps, minimum length of ROH = 1,000,000 bps, maximum number of SNP with opposite genotype = 1 and maximum number of missing genotypes = 1. Thereafter,  $F_{ROH}$  values were determined for each individual using the *summary.runs* procedure from the same package.

## 2.5 | Estimation of the degree of inbreeding depression

The estimation of pedigree-based and genomic inbreeding depression was performed by regressing the predicted residual phenotypes  $wg_{Residual}$  (predicted residual phenotypes applying REML) and  $g_{Residual}$  (predicted residual phenotypes applying ssREML) of the trait of interest on the

pedigree-based ( $F_{ped}$ ) or genomic-based ( $F_{ROH}$ ) inbreeding coefficients, using two methodological approaches: classical and segmented linear (piecewise) regressions. In the latter, the expression of each reproductive variable is interpreted as a linear piecewise function over  $F_{ped}$  or  $F_{ROH}$ , with each segment separated by a breakpoint (i.e. a point in time when the effect on the dependent variable changes). We conducted a segmented regression analysis to ascertain whether linear relationships exist within specific segments across the  $F_{ped}$  and  $F_{ROH}$  analysis interval. This was prompted by the observation of a predominantly cubic relationship between the two variables. The classical linear regression was fitted with the *predicted residual phenotypes* ( $wg_{Residual}$  and  $g_{Residual}$ ) with the *Reg* procedure of SAS, while the segmented regression was performed using only the  $g_{Residual}$  ones, with the *Nlin* procedure of SAS. In addition, the Chow breakpoint test (Chow, 1960), based on autoregressive analysis with the *autoreg* procedure of SAS v.9.4 (Cary, US) was used to assess statistically whether there are any structural changes in the relationship between the dependent and independent variables (breakpoint).

## 3 | RESULTS

### 3.1 | Predicted residual phenotypes and inbreeding coefficients

The descriptive statistics of the five reproductive traits (predicted residual phenotypes) analysed in PRE mares are shown in Table 1. The mean predicted residual phenotypes were similar in  $wg_{Residual}$  and  $g_{Residual}$  in all the

**TABLE 1** Descriptive statistics of the reproductive traits evaluated (predicted residual phenotypes) in 1018 Pura Raza Español mares, estimated heritability and estimated inbreeding coefficients.

	Traits	Mean	SD	Minimum	Maximum	CV (%)	$h^2$
$wg_{Residual}$	AFF (years)	4.93	1.68	1.99	9.01	34.20	0.23
	AIF (months)	19.90	2.13	12.52	25.98	10.70	0.03
	NF	5.00	3.71	-0.51	16.14	74.30	0.12
	PL (years)	11.76	8.14	-5.47	39.00	69.20	0.04
	RE (%)	52.80	11.79	24.25	84.72	22.30	0.24
$g_{Residual}$	AFF (years)	4.97	1.68	1.99	9.06	33.70	0.23
	AIF (months)	19.89	2.18	12.38	26.03	10.90	0.02
	NF	5.00	3.77	-1.19	16.73	75.40	0.11
	PL (years)	11.41	8.16	-5.62	38.27	71.50	0.04
	RE (%)	52.80	12.25	23.79	90.72	23.20	0.22
Inbreeding	$F_{ped}$	0.09	0.06	0.00	0.37	65.90	-
	$F_{ROH}$	0.14	0.07	0.00	0.46	49.10	-

Abbreviations: AFF, age at first foaling; AIF, average interval between foalings; CV, Coefficient Variation (%);  $F_{ped}$ , pedigree-based inbreeding coefficient;  $F_{ROH}$ , genomic inbreeding coefficient;  $g_{Residual}$ , predicted residual phenotype with a model with genomic data;  $h^2$ , heritability; NF, total number of foalings; PL, productive life; RE, reproductive efficiency; SD, standard deviation;  $wg_{Residual}$ , predicted residual phenotype with a model without genomic data.

five traits, but the maximum values of the  $g_{\text{Residual}}$  were slightly higher than those of  $wg_{\text{Residual}}$ , except for PL, while the minimum values of the predicted residual phenotypes were lower for  $g_{\text{Residual}}$  than for  $wg_{\text{Residual}}$ . The coefficient of variation ranged from 10.70% to 74.30% for  $wg_{\text{Residual}}$  and from 10.90% to 75.40% for  $g_{\text{Residual}}$  (Table 1).

The mean pedigree-based and genomic inbreeding coefficients were 0.09 and 0.14, respectively, showing a moderately high positive correlation of 0.78. In addition, both inbreeding coefficients showed a remarkably narrow confidence interval and a relatively wide prediction interval (Figure 1).

### 3.2 | Effect of inbreeding on reproductive traits

A significant inbreeding depression ( $p < 0.05$ ) was observed in all reproductive traits ( $wg_{\text{Residual}}$  and  $g_{\text{Residual}}$  predicted residual phenotypes) based on  $F_{\text{ped}}$  and  $F_{\text{ROH}}$ , except for the AIF with  $F_{\text{ped}}$  (Table 2). The classical regression coefficient was positive for AFF and AIF and negative for NF, PL and RE. Similar effects were observed between  $wg_{\text{Residual}}$  and  $g_{\text{Residual}}$  predicted residual phenotypes with both coefficients ( $F_{\text{ped}}$  and  $F_{\text{ROH}}$ ), but somewhat higher with the  $g_{\text{Residual}}$ , particularly for RE. For example, a 10% of the increase in  $F_{\text{ped}}$  and  $F_{\text{ROH}}$  for  $wg_{\text{Residual}}$  was associated with an increase in AFF of 0.37 and 0.31 years, and 0.15 and 0.28 months in AIF respectively. In addition, NF decreased to 0.76 and 0.54, PL to 2.73 and 2.98 years and RE was reduced to 3.26% and 2.58%, for  $F_{\text{ped}}$  and  $F_{\text{ROH}}$  respectively. On the other

hand, for each 10% of the increase in  $F_{\text{ped}}$  and  $F_{\text{ROH}}$ -based inbreeding coefficients in the  $g_{\text{Residual}}$ , an increase in AFF (0.37 and 0.31 years respectively), and AIF (0.24 and 0.43 months respectively) were estimated. While a 10% increase in  $F_{\text{ped}}$  and  $F_{\text{ROH}}$  caused a decrease by 0.80 and 0.65 in FN, 2.79 and 3.05 years in PL and 3.98% and 3.89% in RE respectively. In addition, the percentage of variance explained (%Var) was slightly higher by  $F_{\text{ROH}}$  than by  $F_{\text{ped}}$  in most of the cases, except for NF ( $wg_{\text{Residual}}$  and  $g_{\text{Residual}}$ ) and RE ( $wg_{\text{Residual}}$ ). In the same sense, the %Var was higher using  $g_{\text{Residual}}$  than  $wg_{\text{Residual}}$  (on average 46.97% higher, with a minimum of 1.96% for AFF and 131.25% for AIF with  $F_{\text{ped}}$ ) (Table 2).

### 3.3 | Segmented regression effect of inbreeding on reproductive traits

The segmented regression analysis determined the existence of breakpoints (changes in trends or changes between the segments) for the reproductive traits. Only the  $g_{\text{Residual}}$  predicted residual phenotypes were used, because the estimates in the classical regression were a better fit than with  $wg_{\text{Residual}}$  predicted residual phenotypes. Table 3 shows these breakpoints (BP), the inbreeding coefficients and the slopes, defined as the different estimates of the inbreeding depression detected in the regression curves. In all cases, the analysed traits showed two breakpoints, with the sole exception of RE using  $F_{\text{ROH}}$  as regressor, which showed only one ( $p < 0.05$ ). The amplitude between the breakpoints was greater when  $F_{\text{ROH}}$  was used to estimate inbreeding depression than when using  $F_{\text{ped}}$ , except for

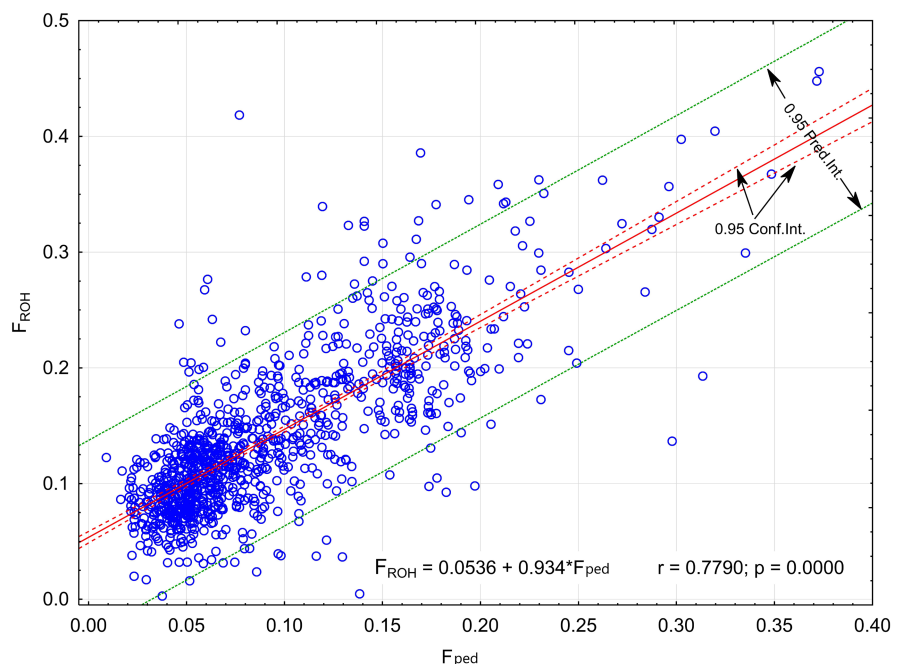


FIGURE 1 Scatterplot of the relationship between pedigree-based inbreeding ( $F_{\text{ped}}$ ) and genomic inbreeding ( $F_{\text{ROH}}$ ) in 1018 mares genotyped, with a 95% confidence interval of the mean and individual prediction.

**TABLE 2** Estimates of the effect of inbreeding depression on reproductive traits in pedigree-based inbreeding and genomic inbreeding by classical linear regression in 1018 Pura Raza Español mares.

Predicted residual phenotypes	Traits	$F_{ped}$			$F_{ROH}$		
		Estimate	SE	%var	Estimate	SE	%var
$wg_{Residual}$	AFF	3.68***	1.02	1.53	3.06***	0.85	1.53
	AIF	1.51	1.35	0.16	2.79*	1.14	0.75
	NF	-7.57***	2.04	1.34	-5.40**	1.70	0.98
	PL	-27.33**	10.40	3.39	-29.78***	9.17	5.08
	RE	-32.58**	7.39	2.39	-25.84***	6.26	2.10
$g_{Residual}$	AFF	3.69***	1.02	1.56	3.11***	0.85	1.60
	AIF	2.37	1.38	0.37	4.29***	1.16	1.71
	NF	-7.96***	2.07	1.43	-6.49***	1.73	1.37
	PL	-27.95**	10.41	3.53	-30.48***	9.18	5.30
	RE	-39.83***	7.64	3.31	-38.92***	6.43	4.42

Abbreviations: AFF, age at first foaling (years); AIF, average interval between foalings (months);  $F_{ped}$ , pedigree-based inbreeding coefficient;  $F_{ROH}$ , genomic inbreeding coefficient;  $g_{Residual}$ , predicted residual phenotype with a model with genomic data; NF, total number of foalings; PL, productive life (years); RE, reproductive efficiency (%); SE, standard error; %Var, the proportion of the variance in the response of variable that can be explained by the predictor variable;  $wg_{Residual}$ , predicted residual phenotype with a model without genomic data.

\*\*\* $p \leq 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ .

Regressor	Traits	Intercept	Slope 1	Slope 2	Slope 3	BP 1	BP 2
$F_{ped}$	AFF	4.66	3.94	2.99	3.61	0.15***	0.22***
	AIF	19.69	3.89	0.53	1.52	0.10**	0.31*
	NF	5.68	-10.40	-4.19	-6.39	0.12***	0.31***
	PL	13.72	-17.92	-50.16	-31.53	0.15***	0.25***
	RE	56.19	-30.87	-56.21	-41.66	0.20***	0.34***
$F_{ROH}$	AFF	4.55	1.95	5.09	3.85	0.17***	0.38***
	AIF	19.30	7.11	0.16	2.99	0.14*	0.32*
	NF	5.90	-6.86	-5.75	-6.30	0.17***	0.32***
	PL	15.63	-44.86	2.17	-24.75	0.15***	0.25***
	RE	58.14	-39.44	-27.91	-	0.25***	-

**TABLE 3** Estimates of inbreeding depression on reproductive traits in pedigree-based and genomic inbreeding by segmented regression in 1018 Pura Raza Español mares.

Abbreviations: AFF, age at first foaling (years); AIF, average interval between foalings (months); BP 1, Breakpoint 1 for inbreeding coefficient; BP 2, Breakpoint 2 for inbreeding coefficient;  $F_{ped}$ , pedigree-based inbreeding coefficient;  $F_{ROH}$ , genomic inbreeding coefficient; NF, total number of foalings; PL, productive life (years); RE, reproductive efficiency (%).

\*\*\* $p \leq 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ .

PL, which showed the same breakpoints, and for the combination RE, which only presented one breakpoint with  $F_{ROH}$ . The breakpoints in the inbreeding depression estimation with  $F_{ped}$  ranged from 0.10 (for AIF) to 0.20 (for RE) and from 0.22 (for AFF) to 0.34 (for RE) (BP1 and BP2 respectively). Likewise, in the inbreeding depression estimation with  $F_{ROH}$ , the breakpoint 1 ranged from 0.14 (for AIF) to 0.25 (for RE) and breakpoint 2 ranging from 0.25 (for PL) to 0.38 (for AFF). Therefore, it was observed that the breakpoint for inbreeding depression estimated with  $F_{ROH}$  shifted slightly towards higher values in comparison with the estimation obtained using  $F_{ped}$ .

For AFF, a 10% increase in inbreeding affected estimates of inbreeding depression more in the first segment ( $0 < F_{ped} < 0.15$ ) (0.39) when using  $F_{ped}$  as a regressor, while the 10% increase in inbreeding in the second phase ( $0.17 < F_{ROH} < 0.38$ ) showed a more intense effect (0.51) using  $F_{ROH}$ , that is, the trait was more affected by inbreeding after the first breakpoint. The AIF trait had a similar trend of increasing inbreeding when using  $F_{ped}$  and  $F_{ROH}$ . This trait was most affected by a 10% increase in inbreeding in the first phase (0.39 for  $F_{ped}$  and 0.71 for  $F_{ROH}$ ), while after that, the effect was maintained and then rose again in the final phase. The trend of the NF trait to heightened

inbreeding was consistent for both inbreeding coefficients. Although, the value of the first slope for a 10% increase in inbreeding ( $-1.04$ ) using  $F_{\text{ped}}$  was almost double that obtained when using  $F_{\text{ROH}}$  ( $-0.69$ ). The other two estimates were similar for  $F_{\text{ped}}$  and for  $F_{\text{ROH}}$  (slope 2 =  $-0.42$  and  $-0.58$  and slope 3 =  $-0.64$  and  $-0.63$ , respectively, for a 10% increase in inbreeding). As for PL, when using  $F_{\text{ped}}$ , the trait was most affected by a 10% increased inbreeding after breakpoint 1 (with a slope of  $-5.2$ ). Conversely, in the case of  $F_{\text{ROH}}$ , PL was more affected ( $-4.49$ ) before breakpoint 1 ( $0 < F_{\text{ROH}} < 0.15$ ), and remained stable until breakpoint 2, after which it exhibited a renewed increase. Finally, RE presented only one breakpoint when using  $F_{\text{ROH}}$ , showing a greater negative effect in the first segment ( $0 < F_{\text{ROH}} < 0.25$ ) of the 10% increase in inbreeding. In contrast, with  $F_{\text{ped}}$ , the three slopes were strong ( $-3.09$ ,  $-5.62$ ,  $-4.17$  slope 1, 2 and 3 respectively) with a 10% increase in inbreeding, although the greatest negative effect arose in the slope 2. All these trends are shown in [Figures S1–S10](#).

## 4 | DISCUSSION

The objective of this study was to demonstrate how inbreeding affects the reproductive traits of Pura Raza Española mares. The PRE breed has a closed breeding system in which only animals belonging to the breed can be used for breeding. As a result, mating of related individuals is inevitable, leading to increased inbreeding in their offspring (Perdomo-González et al., 2022). Additionally, many breeders have conducted intense line breeding, seeking the maximum genetic resemblance to outstanding animals in their studs. One case in point is the Carthusian lineage, which due to closed breeding with only Carthusian breeders, and the relatively small effective size, has experienced dramatic increases in inbreeding throughout its history (Poyato-Bonilla et al., 2022; Valera et al., 2005).

Several studies have shown that reproductive traits are among the traits most affected by inbreeding depression in several species (Charlesworth & Willis, 2009; Martikainen et al., 2018; Saura et al., 2015). Although horses frequently show high levels of inbreeding (e.g. in breeds such as PRE (Poyato-Bonilla et al., 2022) or Thoroughbred horse (Hill et al., 2023)) and a moderate reproductive ability, very few studies have addressed the influence of inbreeding on reproductive traits (Cothran et al., 1986; Klemetsdal & Johnson, 1989; Kownacki, 1965; Müller-Unterberg et al., 2017). This may be because it is hard to find an easily and reliably obtained estimator related to reproductive performance. The main difficulty lies in differentiating whether the poor reproductive performance of a mare (e.g. a long interval between foals, a very late age

at first foaling) is due to poor genetic potential, or to the breeder's reproductive management (e.g. not covering the mare until she has participated in sporting competitions or leisure activities for several years, or simply showing an unwillingness to mate their animals to produce foals in a particular season). But also, fertility traits are not taken into account in selecting individuals since choosing the pairings for mating is mostly on the basis of a combination of sport performance (Todd et al., 2018), morphological traits (Poyato-Bonilla et al., 2021) and pedigree lineages. In a previous study wherein fertility traits based on pedigree were developed and validated in PRE, we demonstrated that including mares from small-size studs can bias the results, since reproductive management and breeding decisions can mask the fertility potential of their mares (Gómez et al., 2020). Based on these results, we applied filters within broodmare studs (e.g. long interval between foals, a very late age at first foaling unsustainable for a breeding stud), and discarded a large number of mares from studs with less than 10 active broodmares. This resulted in retaining only 38% of the original available population and allowed us a more accurate estimation of the genetic and non-genetic effects in the whole population. The resulting more accurate predicted residual phenotypes were used to estimate the level of inbreeding depression. Likewise, this estimation of the predicted residual phenotypes was performed following the classical BLUP REML methodology ( $w_{\text{Residual}}$ ), with additional genomic information used from a single-step BLUP ( $g_{\text{Residual}}$ ) with the **H** relationship matrix.

Traditionally, inbreeding estimates have been based on pedigree information, but the current availability of genomic data enables us to make more accurate estimates of homozygosity levels and employ more refined approaches in detecting inbreeding depression. One of the advantages of using genomic data in inbreeding depression studies is that it can be estimated in populations where the pedigree records are scarce and/or incomplete (Cassell et al., 2003; Kardos et al., 2015), together with the added benefit that it measures the homozygosity of the genome (Keller et al., 2011) instead of the expected homozygosity per offspring, as with the pedigree-based inbreeding coefficient. The use of SNP genomic data may be constrained by the number of genotyped individuals, which is typically lower than the dataset from a pedigree. In the case of the PRE horse, the advantage of genomic information over genealogical information is to a minor extent related to the depth and/or reliability of the pedigree: All studbook entries must be confirmed with molecular markers since the 1980s. Furthermore, the animals considered in this study had at least 10 complete generations and up to 19 known ancestral generations. Thereby, our results showed moderately high correlations between

pedigree-based ( $F_{\text{ped}}$ ) and genomic estimations  $F_{\text{ROH}}$  (0.78) (see Table 1) in comparison with recent studies in other breeds such as Polish Cold-Blooded horses (0.56) (Velie et al., 2019), the Norwegian–Swedish Coldblooded trotter (0.44) (Polak et al., 2021) or the extreme case of the Mangalarga Marchador horse (0.02) (Bizarria dos Santos et al., 2021). This supports that our breed has more accurate and complete pedigree records in comparison to other horse breeds. Similar results have been reported in other species such as pigs, cattle or sheep, with a correlation between  $F_{\text{ped}}$  and  $F_{\text{ROH}}$  of 0.63 (Saura et al., 2015), 0.66 and 0.61 (Doekes et al., 2019; Makanjuola et al., 2020) and 0.60 (Antonios et al., 2021) respectively. Furthermore, the linear correlation observed between  $F_{\text{ped}}$  and  $F_{\text{ROH}}$  showed tight confidence limits and relatively large prediction limits (Figure 1), which is due to the very nature of both variables, in that  $F_{\text{ROH}}$  measures the degree of genomic homozygosity ‘in a real state’, whereas  $F_{\text{ped}}$  is an estimator of the probability of the degree of homozygosity obtained by descent (Keller et al., 2011).

To evaluate the effects of inbreeding, we calculated the classical linear regression coefficients between the different inbreeding values of the mares ( $F_{\text{ped}}$  and  $F_{\text{ROH}}$ ) and their predicted residual reproductive phenotypes. The challenge of obtaining reliable reproductive data at the population level is significant, but the filters employed guarantee the high reliability of these parameters in the sample. Accordingly, we consider that the use of this reduced database will not result in less representativeness of the broodmare population. On the other hand, the sample of genotyped mares is derived from a larger set of approximately 4000 genotyped mares by the ANCCE, collected as part of a comprehensive genome association research project in the PRE horse breed and for genomic selection. Among these mares, only 1018 met the specified criteria, being breeding livestock or mares exclusively utilized for reproduction. Thus, the selection of genotyped animals provides good representation of the broodmare population within the breed. All this involves a statistical challenge in terms of having a non-uniform distribution of  $F_{\text{ped}}$  and  $F_{\text{ROH}}$  data across the analysed interval, resulting in heterogeneous precision in the estimates. However, the advantage is that the results guaranteed a robust representation of the breed’s variability and provided an accurate representation of the extent of inbreeding depression experienced by the breed for these traits.

Our results showed the existence of statistically significant inbreeding depression for all the reproductive traits, except for the average foaling interval trait when  $F_{\text{ped}}$  was used ( $w_{\text{gResidual}}$  and  $g_{\text{Residual}}$ ). In general, the estimates with genomics ( $g_{\text{Residual}}$ ) and without ( $w_{\text{gResidual}}$ ) in the REML estimation model were very similar in sign and magnitude. However, when comparing  $F_{\text{ped}}$  and  $F_{\text{ROH}}$

results,  $g_{\text{Residual}}$  estimates were 22% higher (on average). Similarly, percentage of the variance explained (%Var) was always higher when using  $g_{\text{Residual}}$  (on average 47% higher), but the total percentage of variability explained by inbreeding was low, which could be explained by the high variability of reproductive traits throughout the animal’s life in relation to the influence of inbreeding levels. This great variability in the reproductive traits of mares is probably due to external effects of the animal, such as management, breeder’s decisions and even those of a genetic nature, such as imprinting (which occurs when the expression of a gene varies with the sex of the parent from which it was inherited and is caused by an epigenetic effect) (Perdomo-González et al., 2023) or transmission ratio distortion (TRD, defined as the deviation from the expected Mendelian inheritance of alleles from heterozygous parents to offspring) (Laseca et al., 2023). Similarly, when comparing  $F_{\text{ROH}}$  and  $F_{\text{ped}}$ , it has verified that  $F_{\text{ROH}}$  was more suitable (standard errors are minor) for estimating inbreeding depression in all reproductive traits except NF  $g_{\text{Residual}}$ , and NF and RE  $g_{\text{wResidual}}$ . The increase of %Var was on average 88% in the  $g_{\text{Residual}}$  and 75% in the  $g_{\text{wResidual}}$  predicted residual phenotypes. Likewise, the increase in estimation in  $w_{\text{gResidual}}$  when using  $F_{\text{ROH}}$  was 5.53%, and in  $g_{\text{Residual}}$  10.74%, compared to  $F_{\text{ped}}$ .

Previous studies on reproductive traits in other species such as swine (Saura et al., 2015; Zhang et al., 2022) or dairy cattle (Bjelland et al., 2013; Pryce et al., 2014) have also supported the use of genomic inbreeding instead of the pedigree-based inbreeding coefficient to estimate inbreeding depression. For example, the effect of inbreeding depression was two to three times greater when based on genomic inbreeding compared to pedigree-based inbreeding for Holstein and Jersey breeds (Pryce et al., 2014). Doekes et al. (2019) also reported that  $F_{\text{ped}}$  captures less inbreeding depression than  $F_{\text{ROH}}$  and suggested that this could be explained by the random nature of recombination and segregation, which is captured by genomic measures but not by pedigree. Furthermore, since there will be more measurement error in pedigree than in genomic inbreeding estimates, it follows that the slope is more likely to be attenuated or ‘flattened’ to zero for  $F_{\text{ped}}$ , a statistical phenomenon known as *regression dilution* (Hutcheon et al., 2010). Knowing the genomic relationship of parents in a mating does not assure us of knowing the actual genomic inbreeding of the offspring. Nevertheless, the probability of more accurately determining the inbreeding of potential progeny will be higher than when using pedigree-based inbreeding coefficients. Hence, genomic inbreeding ( $F_{\text{ROH}}$ ) becomes a crucial tool for livestock breeders.

The classical regression coefficient was positive for AFF and AIF. According to our results, there is a clear



trend that shows that the higher the level of inbreeding, the lower the mare's reproductive aptitude: increased inbreeding relates to an increase in the age at first foaling and longer mean inter-foaling interval of the mare. For NF, PL and RE, the negative regression coefficient would be compatible with a worse breeding performance: increased inbreeding relates to lower number of foalings, shorter productive life and reduced reproductive efficiency (Table 2). These results agree with the study by Perdomo-González et al. (2021), who reported similar effects of inbreeding (sign and magnitude) on reproductive traits in the same breed, using  $F_{ped}$  as a regressor and a classical approach with a linear regression model. They informed that a 10% increase in inbreeding was associated with an increase in AFF and AIF of 0.06 years and 0.43 months, respectively, and with a decrease in NF, PL and RE of 0.17 foalings, 0.76 years and 2.34% respectively. Because they used the uncorrected phenotypic data, these results are not directly comparable with our predicted residual phenotypes ( $wg_{Residual}$  and  $g_{Residual}$ ). A study similar to ours, but not directly focused on reproductive aspects was conducted by Hill et al. (2023). They investigated the impact of inbreeding on the durability (number of race starts) of the North American Thoroughbred horse. Their findings revealed a significant decrease in the number of race starts as  $F_{ROH}$  increased. Specifically, a 10% increase in  $F_{ROH}$  was linked to a reduction of 3.5 race starts compared to horses with an average inbreeding coefficient. This result could be extrapolated to our findings for the 'productive life' trait (a 10% increase of  $F_{ROH}$  was associated with a decrease of about 3 years of PL), since both traits measure the horse's productivity, either in sport performance or reproduction. Besides that, the increase in phenotypic values for the 'calving interval' trait (CI; similar to the average interval between foalings in the horse) caused by increased inbreeding has been demonstrated in other species such as cattle using the inbreeding coefficient based on pedigree or genomic data (Doekes et al., 2019, 2020; Pryce et al., 2014).

On the other hand, our methodology presents an additional advantage over other studies carried out to date on this topic: it models the relationship between inbreeding and the phenotypic parameter of interest without assuming a linear relationship. In other words, the effects of inbreeding on the phenotype do not exhibit the same intensity consistently as inbreeding increases. This observation is recognized by breeders in livestock management. In our case, we performed an estimation of the second- and third-degree polynomial regression (data not shown). Our results showed that the cubic curve had a better fit ( $R^2$  adjusted) than the linear and quadratic curves for all the reproductive traits except for RE and AIF with  $F_{ped}$  and

NF with  $F_{ROH}$  as regressor (data not shown). This implies the presence of different intensities in the relationship between reproductive traits and inbreeding when inbreeding increases, that is, different levels of inbreeding depression can be expected with increasing levels of homozygosity. Therefore, a segmented regression analysis was proposed to determine the existence of different slopes in certain levels of inbreeding depression of the reproductive trait. To our knowledge, this study is the first to use the segmented regression technique to estimate the effect of inbreeding depression in any animal species. In this regard, determining where a significant change in the relationship between  $F_{ped}/F_{ROH}$  and each trait may occur indicates greater flexibility for selecting matings.

Our results showed that reproductive traits are highly sensitive to inbreeding depression even with relatively low levels of homozygosity (even  $<0.10$  with  $F_{ped}$  or  $0.14$  with  $F_{ROH}$  in the case of AIF). This has also been described in the case of dairy cattle (Doekes et al., 2019). Table 3 (and the graphs in Figures S1-S10) shows how the inbreeding depression varied in the different stretches of homozygosity. In many of these reproductive traits, a more severe inbreeding depression was obtained as the homozygosity section considered increased, although in some traits, a greater intensity was observed in the first section (e.g. AFF and AIF with  $F_{ped}$ ). Furthermore, in some traits (e.g. RE or PL with  $F_{ped}$ ), the intermediate homozygosity stretch inbreeding depression intensity was significantly lower than in the earlier and later interval. Explaining the variability in behaviour based on the type of regressor ( $F_{ROH}$  or  $F_{ped}$ ) and the considered trait proves highly challenging. This difficulty arises from the distinct nature of the homozygosity parameter, which may be attributed to the common ancestors ( $F_{ped}$  and  $F_{ROH}$ ) and the founders ( $F_{ROH}$ ). These common ancestors might be distant or recent in time, leading to potential scenarios where the detrimental effects of consanguinity may or may not have been purged. Another explanation is the state, where there may be other evolutionary effects at play, such as the existence of census bottlenecks throughout the breed's history, the very nature of their character (which may be purified by the simple effect of the natural selection through centuries of inbreeding) or variations in the reproductive management of the breed.

In this context, it is noteworthy that in a breed such as the PRE, characterized by a closed breeding system since the foundation of its studbook (1912), the absence of crossbreeding with horses from other breeds has led the loss of variability due to inbreeding, being a concern present in the breed from its inception. In fact, until about 40–50 years ago, the tendency was to gradually increase the average level of inbreeding of the population, since the breeders made a great effort to increase the relationship

of their animals with the best stallions in their studs. However, in recent decades, with the breed's genetic improvement program, ANCCE has made great efforts to raise awareness among breeders about the detrimental effects of inbreeding (Perdomo-González et al., 2020). It should be noted that the number of animals with high levels of inbreeding is currently much lower than the number of animals with low or intermediate levels. In addition, those animals with high levels of inbreeding are generally animals with a high genetic value for traits of interest to breeders (morphology, or sporting aptitude especially), but also with acceptable reproductive aptitudes. Thus, nowadays, breeders are making real efforts to avoid consanguineous matings and, in any case, try to ensure that common ancestors, which are the main cause of inbreeding, are animals of great relevance to the breed. Those animals which have much higher levels of inbreeding, may have managed to purge the deleterious impacts of inbreeding through natural selection or, alternatively, through the breeder's sustained artificial selection. While animals of recent generations do not usually have high levels of inbreeding even though they may present much more recent inbreeding (in terms of generations in which the common ancestor occurs). Consequently, this has resulted in a less pronounced manifestation of the inbreeding depression effect across numerous generations. Currently, genetic assessments for inbreeding depression load have been performed (Perdomo-González et al., 2021; Poyato-Bonilla et al., 2020). These evaluations make it possible to detect animals that, if they act as ancestors causing inbreeding, this is not only capable to counteract inbreeding depression and by that also causes a phenotypic improvement in their inbred descendants when compared with the population average.

Finally, in order to explore in greater depth, the reasons which account for the behaviour of inbreeding depression throughout the range of the inbreeding coefficients ( $F_{ROH}$  and  $F_{ped}$ ) used, further study is needed. With a larger number of animals, it may be possible to differentiate groups of animals with the same level of inbreeding but with different recent and ancient inbreeding coefficients, and thereby compare the inbreeding depression based on recent and ancient inbreeding.

## 5 | CONCLUSION

We have demonstrated the existence of inbreeding depression in reproductive traits of a large cohort of PRE mares. All the predicted residual phenotypes analysed (AFF, AIF, NF, PL and RE) were adversely affected by increasing genomic and pedigree-based inbreeding. All models that included genomic data to estimate predicted

residual phenotypes showed an improvement in estimation accuracy compared to models that lacked genomic data. In addition, the genomic inbreeding coefficient ( $F_{ROH}$ ) was found to capture more inbreeding depression than pedigree-based inbreeding ( $F_{ped}$ ). The segmented regression analysis provided evidences that the increase in genomic inbreeding (including pedigree-based inbreeding) does not affect reproductive traits homogeneously. Finally, we concluded that reproductive traits are very sensitive to inbreeding in this breed since they are affected by low levels of homozygosity.

## AUTHOR CONTRIBUTIONS

The authors' contributions are as follows: AM and MV conceived and designed the study; NL, CZ, DP and SDP obtained the data and conducted the research; NL, CZ, DP, MV, SDP and AM analysed and interpreted the data; NL and AM drafted the manuscript. All the authors revised the manuscript and approved the final version.

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## CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interests.


## DATA AVAILABILITY STATEMENT

The dataset supporting the results of this study was supplied by the Real Asociación Nacional de Criadores de Caballos de Pura Raza Español (ANCCE). The datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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