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Authors

Navas, FJ
Jordana, J
McLean, AK
et al.

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Modelling for the inheritance of multiple births and fertility in endangered equids: Determining risk factors and genetic parameters in donkeys (*Equus asinus*)

F.J. Navas [a, f], J. Jordana [b, f], A.K. McLean [c, f], J.M. León [d, f], C.J. Barba [e, f], A. Arando [a, f], J.V. Delgado [a, f]

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Highlights

- Genetic parameters enable the inclusion of these traits in donkey breeding programs.
- Combined selection for maximum foal number and historical foal number is feasible.
- Selection for twinning in donkeys may have traditionally been carried out indirectly.
- Genetic relationships enable developing donkeys' multiple birth production lines.

Abstract

Multiple births or twinning in equids are dangerous, undesirable situations that compromise the life of the dam and resulting offspring. However, embryo vitrification and freezing techniques take advantage of individuals whose multiple ovulations allow flushing more fertilised embryos from the oviduct to be collected, increasing the productivity and profitability of reproductive techniques. Embryo preservation is especially important in highly endangered populations such as certain donkey *(Equus asinus)* breeds; for which conventional reproductive techniques have previously been deemed inefficient. For instance, becoming an effective alternative to artificial insemination with frozen semen to preserve the individuals' genetic material. The objective of this study was to examine the historical foaling records of Andalusian donkeys to estimate prevalence, risk factors, phenotypic and genetic parameters for multiple births, assessing the cumulative foal number born per animal, maximum foal number per birth and multiple birth number per animal. We designed a Bayesian General Animal Mixed Model with single records considering the 'fixed' effects of birth year, birth season, birth month, sex, farm, location, and husbandry system. Age was considered and included as a linear and quadratic covariate. Gibbs sampling reported heritability estimates ranging from 0.18±0.101 to 0.24±0.078. Genetic and phenotypic correlations ranged from 0.496±0.298 to 0.846±0.152 and 0.206±0.063 to 0.607±0.054, respectively. Predicted breeding values obtained enable the potential selection against/for these traits, offering a new perspective for donkey breeding and conservation.

Previous

Next

Keywords

Donkey; Twinning; Heritability; Gibbs sampling; Risk factors

1. Introduction

The occurrence of multiple births has been addressed as one of the main causes of fetal and neonatal loss in equids (Jeffcott and Whitwell, 1973). The majority of twin pregnancies in horses (72.6%) terminates in abortion or stillbirth of both twins from eight months to term. Out of these terminated pregnancies, 64.5% ends from 3months gestation to term. In the remaining cases, either one (21%) or both twins (14.5%.) are born alive or survive after birth complications. However, the foals are usually born stunted or emaciated, which does not allow them to survive further from 2weeks of age (Jeffcott and Whitwell, 1973).

In the case of the donkey species, (Quaresma et al. (2015)) addressed the overall neonatal mortality for the first month of life to be near 9% of all births. These authors would also report that the percentage of twin foaling at full term was only around 3%, with a neonatal foal mortality rate of 40%. Hence, the selection of individuals that may be less prone to present multiple ovulation could be a preventive alternative to decrease the risks attached.

Contrastingly, the donkey is a species for which the most of its breed populations have been classed as endangered (Kugler et al., 2008) and that has been reported to be highly reproductively compromised as it happens with many other endangered populations (Navas et al., 2016). These reproductive compromises may be attributed to the deleterious effects of inbreeding in such populations (Navas et al., 2017). The long gestation cycle (a norm of 12months to give birth in the 13th month) (Weaver, 2008), fertility that steadily decreases over generations (Quaresma et al., 2015) and the highly inbred status of donkey breed populations (Navas et al., 2017; Quaresma, 2015) only contribute to worsening the endangerment risk situation that donkey breeds face worldwide.

Furthermore, highly standardised reproduction techniques in horses and other equids (Hearn and Summers, 1986) such as artificial insemination with frozen semen still represent a challenge in donkeys. These challenges have been directly related to the anatomical, histological and physiological differences that exist between the reproductive tracts of both species. Particularly, the most likely hypothesis regarding the inefficiency of frozen semen artificial insemination addresses the high immune response in the endometrium of jennies when compared to mares as the main cause. Jennies are more likely to get acute endometritis 6h after artificial insemination (AI) with frozen semen than mares, what has been attributed to the elimination of the modulating effects of seminal plasma discarded while preparing frozen doses (Miró and Papas, 2017; Rota et al., 2017; Saragusty et al., 2017). This context lays the basis for embryo vitrification and freezing to arise as new possibilities that may enable the preservation of the genetic material of donkeys belonging to populations for which the numbers rarely exceed 1000 individuals. This is supported as the pregnancy rates of 50% and 36% after the transfer of fresh and vitrified embryos, respectively (Panzani et al., 2017), overcome the best currently reported results for pregnancy rate (28%) obtained for uterine horn insemination using frozen-thawed semen (de Oliveira et al., 2016). The efficiency and profitability of such reproductive techniques could be improved relying on the higher ability of certain animals to develop multiple ovulations, even more, when those animals may be genetically prone to develop them at a higher rate.

Studies of the genetic background of multiple pregnancies and fertility in equines have reported very low heritabilities, in general (Van Vleck and Gregory, 1996). However, these findings normally ascribe to the capacity of the methodology implemented to fit the evaluation of such traits given their statistical nature (non-normal distribution and high skewness) (Van Tassell et al., 1998). In this context, horse breeding schemes progressively adopt new research methods such as the use of genetic markers and genotyping beadchips as a way to integrate the most recent genetic advances to their relatively stable traditional idiosyncrasies (Sieme and Distl, 2012). By contrast, no study approaching the genetic background behind donkey fertility has been conducted until the date, hence, efforts towards increasing the knowledge on the field have rather focused on risk factor evaluation or populational frequency studies.

The present paper describes a retrospective study over a period of 38years, given the birth year of the oldest animal registered in the studbook was 1980. The first aim of this paper was the assessment of the prolificacy, fertility, and frequency of multiple pregnancies in the historical population of Andalusian donkeys. To do this, we analyzed cumulative foal number born per animal, maximum foal number per birth and multiple birth number per animal traits. Second, we isolated and quantified the influence that non-genetic factors (farm, husbandry system, location, year of birth, birth season, birth month or age) may have on such traits. Then, we isolated and quantified the genetic additive component for the same traits through the estimation of the genetic parameters using Gibbs sampling. Afterward, we predicted breeding values as a way to assess the potential implementation of a bidirectional breeding strategy for/against multiple pregnancies. This strategy may simultaneously consist of animals selected against multiple births because of the gestation complications that they involve, while other individuals may promote the occurrence of multiple births, seeking higher conservation profitability based upon an increased number of embryos to collect while implementing assisted reproduction plans.

2. Materials and methods

2.1. Sample size and background

We studied the foaling recordings of 765 individuals registered in the historical pedigree record of the Andalusian donkey breed (181 jacks and 584 jennies). As age range was not normally distributed (*P*≤0.01 Shapiro-Francia W′ test for normality), we used minimum, Q1, median, Q3 and maximum to describe the age range in our sample. The minimum age in the range was six months, Q1 age was six years, the median age was ten years, Q3 age was 14years, and the maximum age was 29years. Such a wide age range was considered, given the fact that we assess reproductive traits in an endangered breed with therefore a limited number of individuals able to provide data. That is, we need to build a model that may suit the inclusion of cases like already dead animals from which we know their whole birth record, those animals for whom their reproductive life is still active and likely to continue or those for whom their reproductive life has not started yet. Hence, we included the age of birth in our model to correct for such cases to adjust the data for each animal to their reproductive moment. The youngest age at which both jacks and jennies gave birth for the first time was three and four years old, respectively (Navas et al., 2017). Moreover, it is often a decision of owners in particular not to breed the animals until they have been recognised as apt for reproduction and included in the main section of studbook of the breed what takes place when the animals turn 3years old.

The donkeys in the sample were the progeny of 93 jackstocks and 253 jennies. All the donkeys were registered in the breed's Spanish studbook. The relationships among all the individuals comprising the pedigree of the breed are routinely genetically tested using microsatellite genotyping and parentage tests performed on each mating and its resulting offspring (Table 1). The application of microsatellite molecular marker genotyping and parentage testing improves and ensures the reliability of the information in the pedigree as a way to counteract the small size of the sample tested. The 24 molecular markers used were recommended by the International Society of Animal Genetics for donkeys (Table 1). The DNA used for parentage tests was obtained from hair samples that are routinely taken when the inscription of each new animals takes place and from the historical bank of samples of the breed kept at the laboratory of applied molecular genetics of the University of Córdoba. All tests were carried out using a pedigree file provided by the Union of

Andalusian Donkey Breeders (UGRA). The pedigree file included 1017 animals (272 males and 745 females) born between January 1980 and July 2015 from which only 914 donkeys, 246 males, and 668 females, were alive during the development of the study.

Table 1. 24 specifical microsatellite primers (nuclear DNA) used for genotyping and parentage tests in donkeys.

Locus	Primers (5′→3′)	Sequence length/range (bp)
AHT4	F: AACCGCCTGAGCAAGGAAGT R: GCTCCCAGAGAGTTTACCCT	128–160
AHT05	F: ACGGACACATCCCTGCCTGC R: GCAGGCTAAGGAGGCTCAGC	124–154
ASB2	F:*CACTAAGTGTCGTTTCAGAAGG R: CACAACTGAGTTCTCTGATAGG	222–256
ASB23	F: GCAAGGATGAAGAGGGCAGC R: CTGGTGGGTTAGATGAGAAGTC	134–148
UCDEQ (CA)425	F: AGCTGCCTCGTTAATTCA R: CTCATGTCCGCTTGTCTC	222–242
HMS2	F: CTTGCAGTCGAATGTGTATTAAATG R: ACGGTGGCAACTGCCAAGGAAG	225–245
HMS3	F: CCAACTCTTTGTCACATAACAAGA R: CCATCCTCACTTTTTCACTTTGTT	152–170
HMS5	F: TAGTGTATCCGTCAGAGTTCAAAG R: GCAAGGAAGTCAGACTCCTGGA	97–111
HMS6	F: GAAGCTGCCAGTATTCAACCATTG R: CTCCATCTTGTGAAGTGTAACTCA	149–167
HSM7	F: CAGGAAACTCATGTTGATACCATC R: TGTTGTTGAAACATACCTTGACTGT	167–177
HTG6	F: CCTGCTTGGAGGCTGTGATAAGAT	78–84

	R: GTTCACTGAATGTCAAATTCTGCT	
HTG10	F: CAATTCCCGCCCCACCCCCGGCA	83–103
	R: TTTTTATTCTGATCTGTCACATTT	
HTG15	F: TCCTGATGGCAGAGCCAGGATTTG	116–134
	R: AATGTCACCATGCGGCACATGACT	
LEX3	F:ACATCTAACCAGTGCTGAGACT	194–220
	R:AAGAACTAGAACCTACAACTAGG	
VHL20	F: CAAGTCCTCTTACTTGAAGACTAG	75–105
	R: AACTCAGGGAGAATCTTCCTCAG	
TKY287	F:ATCAGAGAACACCAAGAAGG	215–245
	R:TCTCTGCTATAGGTAAGGTC	
TKY294	F:GATCTATGTGCTAGCAAACAC	210–235
	R:CTAGTGTTTCAGATAGCCTC	
TKY297	F:GTCTTTTTGTGCCTCGGTG	215–250
	R:TCAGGGGACAGTGGCAGCAG	
TKY301	F:AATGGTGGCTAATCAATGGG	140–170
	R:GTGTATGATGCCCTCATCTC	
TKY312	F:AACCTGGGTTTCTGTTGTTG	90–130
	R:GATCCTTCTTTTTATGGCTG	
TKY321	F:TTGTTGGGTTTAGGTATGAAGG	175–210
	R:GTGTCAATGTGACTTCAAGAAC	
TKY341	F:TATCCAGTCACCCATTTTAC	135–160
	R:TTGTGTCAGTACACTCTATG	
TKY343	F:TAGTCCCTATTTCTCCTGAG	135–170
	R:AAACCCACAGATACTCTAGA	
TKY344	F:GTGTCCATCAATGGATGAAG	75–115
	R:CTTAAGGCTAAATAATATCCC	

F: Forward primer; R: Reverse primer.

2.2. Birth-related traits

We studied prolificacy, fertility and multiple birth occurrence in the historical Andalusian donkey breed population through the assessment of cumulative foal number born per animal, maximum foal number per birth and multiple birth number per animal for each jack or jenny. To obtain this information, we contrasted the registries of the historical pedigree file with interviews with the 145 owners whose animals participated in the study. Owners were interviewed due to the fact that it is very likely for the early abortion of multiple gestations not to be registered if it is not in the veterinarian or owners' personal records. Then, from this initial sample of owners, we only considered the ones who affirmatively responded to the question in block 2 for the estimation of genetic parameters (90 out of 145 owners interviewed) as a veterinarian or theriogenologist had issued an official gestation diagnosis (simple or multiple). This excluding criterion was applied as a way to consider those cases when abortions had occurred. Many twin (and triplet) pregnancies in equids are already lost at very early stages and the aborted material stays mostly undetected by the owners, which could have distorted the true number of pregnancies with multiple conceptuses.

First, we summarised the cumulative foal number born per animal. That is for the total of 765 individuals, the number of offspring foaled (resulting from natural mating or artificial insemination) by each of 584 jennies or born to each of 181 jacks, either over their reproductive lifetime or up to July 2015 (absolute scale 0 to 40). Second, the maximum foal number per birth, or the maximum number resulting at any of all the deliveries through the life of each jenny, considering which jack was used to breed. That is, for the same 765 animals, the maximum number of offspring born in a single foaling event in which the individual (male or female) was part of either over its reproductive lifetime or up to July 2015 (absolute scale 0 to 3). Third, multiple birth number per animal, that is for the same 765 animals, the sum of all mating events resulting in multiple gestations either over the reproductive life of the individual (male or female) or up to July 2015 (absolute scale 0 to 5).

The units of study considered for descriptive statistics and populational data were each of the births occurring in the 91% of Andalusian donkey population and the characteristics of such births (number, moment, among others). The records for the remaining 9% of the population were not readily available or did not fulfil the requirements set for them to be included in the analyses (Supplementary Table S1). For genetic analyses, the unit of analysis that we considered was the lifetime parentship record of each animal separately to avoid the possibly occurring unmodelled covariance between sire and dam due to their mating and successful conception differences. That is to say; we summed every molecularly confirmed jack and jenny's birth registries separately so that for the data considered reliable. Given the BLUP methodology was applied (Parnell, 2004), data obtained can either belong directly from field observations and registries or indirectly, because of individuals being directly genealogically linked to common ancestors.

2.3. Interview description

A telephone survey was carried out to 145 different owners whose farms were located in Andalusia (southern Spain). The survey took place in June 2017. We interviewed owners regarding the specific foaling registry of all the animals historically present at their farms since the 1980s until 2017 and registered in the studbook of the breed at the moment that the survey took place. The oldest donkey from which there was information available had been born in 1984. All the interviews comprised a battery of 18 questions that were asked by the same interlocutor and each interview lasted for a mean time of 10min. Despite the lack of multiple births or gestation in their farms stated by the owners, all the questions were asked indistinctly. A description of the questions and options asked the owners is shown in Supplementary Table S1. Supplementary Table S2 defines the unordered categories or levels (extensive, semi-extensive, semi-intensive and intensive) of the husbandry system factor. There were open questions (regarding the location of the farms, the age of the animals or the number of animals present in the farms at the moment that the interviews took place) and closed questions (regarding the sex, the husbandry system under which the animals were handled, and the prevalence of multiple gestations from the past up to the date when the interview was performed). All the information provided by the owners was contrasted with the information provided by UGRA and the information present in the official stud-book of the breed.

2.4. Records description and scales

We organised the questions into three blocks (Supplementary Table S1). The first block aimed at describing the farms of the owners' interviewed to statistically assess the possible effects that may condition the prevalence of multiple gestations or births. We included the questions asked to the owners to classify or define the husbandry system under which their farms were managed in Supplementary Table S2. These questions based on the extension of territory to which the donkeys had access, whether the donkeys were reproductively handled and whether the owner held daily contact with them or they were handled just for minimum punctual health inspection and studbook inclusion. The second block comprised a single question related to whether the diagnosis by a veterinarian or theriogenologist had been requested. The second block comprised the excluding question of whether a theriogenologist or veterinarian had been requested for diagnosis and an official diagnose had been issued, as only the owners affirmatively responding to it were included in the statistical and genetic analyses. The third block consisted of questions regarding the assertive diagnosis of the multiple births, and the care and preventive measures taken in each case. When the animals had never given birth, had suffered from an undetected early embryonic loss nor had carried any embryo, we gave them a score of 0.

2.5. Population frequency statistical analysis (pedigree retrospective screening)

To achieve the first aim of the paper, that is to provide a picture of the prolificacy, fertility and multiple gestation status during the whole history of the Andalusian donkey breed, descriptive parameters were computed. Among these parameters, we calculated the average number of foals born per year and the highest number of births registered during the whole story of the breed for a certain year. Furthermore, the mean prevalence of multiple births per hundred births and the percentage of the population that had not given birth to any foal when the registries were also studied in the historical Andalusian donkey population. Then, to assess the occurrence of multiple gestations the proportion of single, twin and triplet pregnancies detected was also studied for the whole population. All triple pregnancies were interrupted.

2.6. Isolation and quantification of non-genetic risk factors

A Shapiro-Francia W′ test revealed that the data significantly deviated from a normal distribution ($P<0.001$) (Fig. 1) which was supported by Kurtosis values (Supplementary Table S3). Thus, we carried out a cross-sectional study employing Chi-square analysis to determine whether the categorical independent effects of birth year, birth season, birth month, sex, location, farm/owner, and husbandry system and the covariate of the age may randomly influence the dependent variables of cumulative foal number born per animal, maximum foal number per birth and multiple birth number per animal. We performed a Kruskal-Wallis H test to study the potentially existing differences between levels of the same factor except for age, as it is measured on a continuous scale (Table 2). We present Kruskal Wallis H Ranks for all the levels of the factors affecting historical foal number born per animal, maximum foal number per birth and multiple birth number per animal in Supplementary Table S4.

Afterward, we studied the pairwise comparisons between the levels of any dependent variables for which the Kruskal-Wallis test was significant, aiming at assessing whether there were differences between groups (levels) of the same factor. We used the Mann-Whitney U test for sex, as it only has two levels, jack and jenny, and Dunn's test for the rest of the factors. Bonferroni corrections for multiple comparisons were used to prevent the occurrence of Type I errors. Additionally, an independent-sample median test was carried out to assess the differences in the median between levels within the same factor.

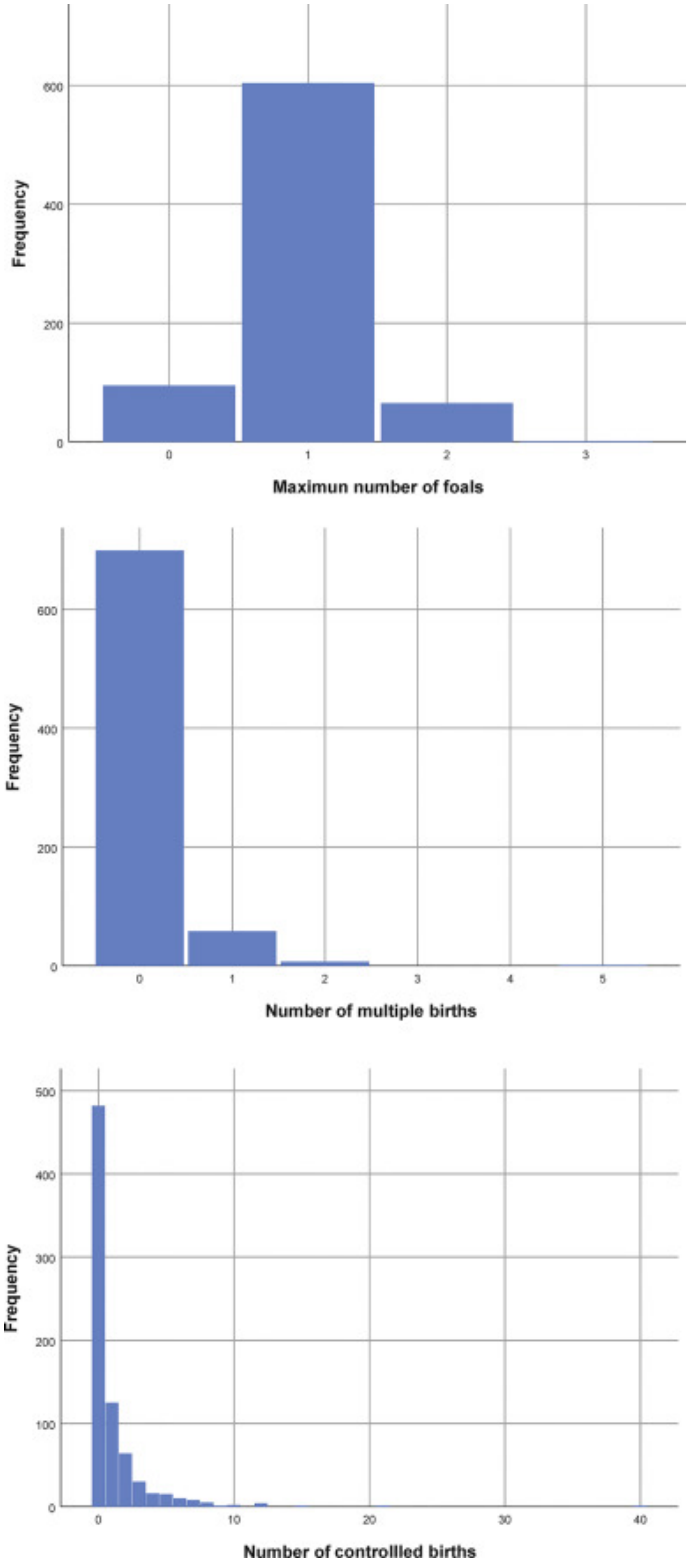
Frequency
600
400
200
0
0
1
2
3
Maximun number of foals
Frequency
600
400
200
0
0
1
2
3
4
5
Number of multiple births
Frequency
500
400
300
200
100
0
0
10
20
30
40
Number of controllled births

Fig. 1. Frequency distribution histograms for maximum foal number per birth, multiple birth number per animal for a given donkey and cumulative foal number born per donkey.

Table 2. Summary of the results for the Kruskal-Wallis H test and their partial eta-squared coefficients (ηp^2) for fixed effects and the covariate included in the model to test for birth-related traits in Andalusian donkeys.

Factor	Item	Cumulative foal number born per animal				Maximum foal number per birth			
Year of birth	χ^2	41.548				30.787			
	df	31				31			
	p-value	0.098				0.477			
	Levels	1984–2017				1984–2017			
	Mean rank	345.15–404.00				345.15–404.00			
	ηp^2	0.234				0.184			
Month of birth	χ^2	16.085				15.128			
	df	11				11			
	p-value	0.138				0.177			
	Levels	January, February, March, April, May, June, July, August, September, October, November, December				January, February, March, April, May, June, July, August, September, October, November, December			
	Mean rank	345.22–424.28				350.00–405.01			
	ηp^2	0.146				0.134			
Season of birth	χ^2	7.750				7.201			
	df	3				3			
	p-value	0.050				0.066			
	Levels	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn
	Mean rank	368.70	402.81	373.02	370.13	369.64	395.08	380.22	379.90
	ηp^2	0.099				0.093			

Sex	χ^2	12.348				3.676			
	df	1				1			
	p-value	0.001				0.050			
	Levels	Jack	Jenny			Jack		Jenny	
	Mean rank	418.85	371.89			396.39		378.85	
	ηp^2	0.124				0.074			
Farm/Owner	χ^2	302.220				321.748			
	df	91				91			
	p-value	<0.001				<0.001			
	Levels	1–92				1–92			
	Mean rank	162.00–732.00				350.00–744.75			
	ηp^2	0.626				0.558			
Husbandry system	χ^2	24.169				5.027			
	df	3				3			
	p-value	<0.001				0.170			
	Levels	Intensive	Semi intensive	Semi extensive	Extensive	Intensive	Semi intensive	Semi extensive	Extensive
	Mean rank	370.390	385.340	397.550	317.960	406.390	388.060	385.490	363.450
	ηp^2	0.176				0.076			
Location	χ^2	67.358				42.013			
	df	10				10			
	p-value	<0.001				<0.001			
	Levels	1–11				1–11			
	Mean rank	222.75–620.50				350.00–613.17			
	ηp^2	0.291				0.229			
Age (in years)	Spearman's rho	–0.137				0.085			
	p-value	<0.001				0.019			

Then, we quantified the strength effect of the factors reported above on cumulative foal number born per animal, maximum foal number per birth and multiple birth number per animal for each jack or jenny to understand the proportion of variability existing in these variables that may be isolatedly explained by each of the factors studied. To this aim, F values were computed from the Kruskal-Wallis H tests using the modified method of Murphy et al. (2014). Then, from F(dfn,dfd), we calculated partial eta squared (Lakens, 2013) following the methodology for non-standard evaluations in the research described and reported by Li et al., 2019.

Partial eta-squared (ηp^2), was computed to measure the strength of association between each categorical independent factor from the first set with the ordinal dependent variables of cumulative foal number born per animal (considered ordinal as described by (Ibarra et al., 2005), maximum foal number per birth and multiple birth number per animal using the Crosstabs procedure from SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016) (Table 2). Values labeled eta squared on some printouts from SPSS are actually partial eta^2. Similarly for age, Spearman's rho was computed to measure the strength of association between it and the ordinal dependent variables of cumulative foal number born per animal, maximum foal number per birth and multiple birth number per animal using the Bivariate procedure from SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016) (Table 2). All non-parametrical tests were carried out using the independent samples package from the non-parametrical task of SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016).

Once the separate effect of each factor had been computed, a categorical regression (CATREG) was used to describe how the variables in our study linearly depended on combinations of the factors considered (Table 2, Table 3). Then, the resulting regression equations could be used to predict cumulative foal number born per animal, maximum foal number per birth and multiple birth number per animal for any combination of the independent factors included in the model. Categorical Regression was carried out using the Optimal Scaling procedure from the Regression task from SPSS Statistics for Windows, Version 24.0, IBM Corp. (2016).

Table 3. Model summary of CATREG optimal linear regression with transformed variables.

Variable	**Multiple R**	**R Square**	**Adjusted R square**	**Apparent prediction error**	**Estimate**	**Std. error**	**Significance**
Cumulative foal number born per animal	0.687	0.472	0.267	0.528	1.497	0.780	0.001
Maximum foal number per birth	0.677	0.458	0.358	0.050	0.072	0.010	0.001
Multiple birth number per animal	0.959	0.919	0.671	0.026	0.156	0.068	0.001

2.7. Isolating and quantifying the additive genetic component: genetic model, phenotypic and genetic parameters

As we only considered one measure per animal, the model used was a Bayesian general linear mixed model with single records. All effects are random in a Bayesian analysis. However, we will follow the nomenclature methodology explained by Van Tassell and Van Vleck (1995) regarding 'fixed' effects and random effects as common in animal modelling. The factors submitted to the above described statistical procedures and which comprised the general animal mixed model consisted of the 'fixed' effects of birth season (summer, spring, autumn, and winter); sex (jack or jenny); the farm (92 farms/owners), the location (11 locations, clustering farms placed at the same municipality) and husbandry system (intensive, semi-intensive, semi-extensive and extensive).

At a previous stage of the study, we computed the double interaction between herd and year of birth (Herd*Birthyear) and the triple interaction between the herd, the year of birth and season of birth (Herd*Birthyear*Birth season) as these were the most regularly included in literature for the same kind of studies in other species such as goats or sheep. Then we tested for the repercussion of the inclusion of such interactions in the model used in the present paper (Eq. (1)). As results for adjusted R-squared for non-normal data may be misleading, Akaike's Information Criterion (AIC) and Bayesian Information Criterion (BIC) were computed both including and without including the interactions reported above. A summary of the results in Supplementary Table S5. Adjusted R-squared is used mainly to correct for overfitting, the phenomenon by which the residual sum of squares (RSS) of the model typically keep on decreasing by adding additional variables. We computed the expected prediction error of regression with 0.632 Bootstrap ("leave-one-out bootstrap") from 200 bootstrap samples (Efron, 1983; Kooij, 2007). In regression contexts (Yong, 2005), to choose the best predictive model we select the one that provides the minimum AIC or BIC (excluding the interaction in our case), denoted by AIC* or BIC*. Candidate models are represented by AICm or BICm (in our case the models including the interaction). We can compute delta AIC=AICm–AIC* or delta BIC=BICm–BIC*. Given M models, the magnitude of the delta AIC and BIC can be interpreted as evidence against a candidate model being the best model. The rules of thumb are <2, it is not worth more than a bare mention (for both AIC and BIC); between 2 and 6 and 4 and 7 for BIC and AIC, respectively, the evidence against the candidate model is positive; between 6 and 10 for BIC, the evidence against the candidate model is strong and >10, the evidence is very strong that is there is essentially the candidate model it is unlikely to be the best model (Fabozzi et al., 2014).

The multi-trait animal threshold models used for the analyses can be described as follows:

$$Y_{\mathrm{ijklmop}} = \mu + a_{\mathrm{ij}} + \mathrm{Sea}_k + \mathrm{Sex}_l + \mathrm{Far}_m + \mathrm{Sys}_n + \mathrm{Loc}_o + \mathrm{b1A}_q + \mathrm{b2A}^2{}_q + e_{\mathrm{ijklmnop}} \quad (1)$$

where $Y_{ijklmnop}$ is the separate record of *i*th trait for *j*th donkey (cumulative foal number born per animal (1 in matrix below), maximum foal number per birth (2 in matrix below) and multiple birth number per animal for a given donkey (3 in matrix below); μ is the overall mean for the trait; aij is the additive genetic effect of the *j*th donkey for *i*th trait, Sea_k is the fixed effect of the *k*th birth season

(k=summer, spring, autumn, winter); Sex_l is the fixed effect of the lth sex (l=jack, jenny); Far_m is the fixed effect of the mth farm/owner (m=1–92); Sys_n is the fixed effect of the nth husbandry system (n=intensive, semi-intensive, semi-extensive, extensive); Loc_o is the fixed effect of the oth Location (o=1–11); b1 and b2 are the linear and quadratic regression coefficients on age when the tests took place (A_p and $A^2{}_p$) and $e_{ijklmno}$ is the random residual effect associated with each record. No maternal effect was computed because of the low completeness level found in the pedigree, as 53.36% of the dams in the study were unknown (Navas et al., 2017). Such a lack of information could have represented a problem when performing genetic analyses. However, as our sample provides direct or indirect information from 91% of the animals included in the pedigree, we could save the possible drawback meant by the missing information. Then, the quality of the predicted genetic values estimated was quantified by reporting their reliability.

We included the age of the animals expressed in years as a linear and quadratic covariate to correct the variables measured according to the lifetime of each animal and specifically the cases in which the animals were too young to have given birth to their first foal/s. We included the effect of sex on our model to save the imbalance between sexes, even more, when we consider the vast differences between the offspring of males and females given the long duration of the gestation of the species.

In matrix notation, the multi-trait model used was:

$$\begin{bmatrix} y_1 \\ y_2 \\ y_3 \end{bmatrix} = \begin{bmatrix} X_1 & \cdot\ \cdot & \cdot\ \cdot \\ \cdot\ \cdot & X_2 & \cdot\ \cdot \\ \cdot\ \cdot & \cdot\ \cdot & X_3 \end{bmatrix} \begin{bmatrix} \beta_1 \\ \beta_2 \\ \beta_3 \end{bmatrix} + \begin{bmatrix} Z_1 & \cdot\ \cdot & \cdot\ \cdot \\ \cdot\ \cdot & Z_2 & \cdot\ \cdot \\ \cdot\ \cdot & \cdot\ \cdot & Z_3 \end{bmatrix} \begin{bmatrix} \alpha_1 \\ \alpha_2 \\ \alpha_3 \end{bmatrix} + \begin{bmatrix} \varepsilon_1 \\ \varepsilon_2 \\ \varepsilon_3 \end{bmatrix} \tag{2}$$

where y_1 to y_3 represent the phenotypical observation for each trait and animal. The vectors of ‘fixed’ effect for the three different traits considered (β_1 to $\beta_{3,}$) include all the effect related in the model described above and the vectors α_1 to α_2 and ε_1 to $\varepsilon_{2,}$ are random additive genetic and residual effects for each trait, respectively. The incidence matrices X_1 to X_3 and Z_1 to Z_3 associate elements of β_1 to β_3 and α_1 to α_2 with the records in y_1 to y_2.

If A is the matrix of additive genetic relationships among individuals, the mixed model equations (MME) used is as follows:

$$[\begin{matrix} X'X & X'Z \\ Z'XZ' & Z+A^{-1}k \end{matrix}][\begin{matrix} \beta \\ \alpha \end{matrix}] = [\begin{matrix} X'y \\ Z'y \end{matrix}] \tag{3}$$

Proxies of prolificacy (i.e. number of offspring produced in a single parturition) are calculated as sums over random time periods eventually censored by nature, and/or the will of the owner, and/or the timeframe of the study (each donkeys' lifetime, especially in animals that are too young to have given birth). Hence the importance of including, assessing and controlling factors such as owner and age of birth as reported above.

2.8. Institutional animal care and use committee statement

All farms included in the study followed specific codes of good practices for equids and particularly donkeys and therefore, the animals received humane care in compliance with the national guidelines for the care and use of laboratory and farm animals in research. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki. The Spanish Ministry of Economy and Competitivity through the Royal Decree-Law 53/2013 and its credited entity the Ethics Committee of Animal Experimentation from the University of Córdoba permitted the application of the protocols present in this study as cited in the 5th section of its 2nd article, as the animals assessed were used for credited zootechnical use. This national Decree follows the European Union Directive 2010/63/UE, from the 22nd of September of 2010.

3. Results

3.1. Interview results

Out of the 145 owners interviewed, we considered the information from 92 farms/owners. These owners had affirmatively responded to the question in the second block as they were the only who had requested information concerning diagnosis by their veterinarians or theriogenologists and therefore, were the only ones providing reliable information. Due to the particularities of the species and the breeding routines carried by the owners, the artificial insemination with fresh semen of the animals registered in the studbook was infrequent, and almost all the matings were performed naturally. No productive artificial insemination using frozen semen was registered. The matings of only 66 animals out of the 765 donkeys from which there was information (8.63% of the total sample) had resulted in multiple gestations. Out of this percentage, 1.04% of the animals developed multiple gestations in more than one occasion through their lives and only one of the animals was responsible for 0.13% of multiple gestations in the population (five multiple births out of 40 births through his life).

3.2. Population frequency statistical analysis (pedigree retrospective screening)

The average number of foals born per year reported a value of 28.19, reaching the highest number (71) in 2003. The mean prevalence of multiple births per hundred births in the Andalusian donkey population was 9.85%. The 11.18% of the population had not given birth to any foal when the registries were studied. The proportion of single, twins and triplets' pregnancies detected (all triple pregnancies were interrupted) was 90.15%, 9.70%, and 0.15%, respectively implying 604 single births records, 65 twin records, and 1 triplet birth record.

The pedigree of the donkeys in our sample was traced back six generations providing indirect information from 930 connected ancestors (91% of the historical population registered) and reporting an average inbreeding of 0.7% for the historical population. Although this average inbreeding coefficient could seem not to be alarming enough, it is only due to this value presumably being underestimated, as it happens in other endangered equid populations, given the low level of completeness reported for the Andalusian donkey breed population (Navas et al., 2017). The same authors reported the same parameter increased up to 1.51% when only those animals whose first-generation genealogy was known were considered. The percentage of females with progeny selected for breeding was 10.76% and 25% for males in the historical population. Historically breeding jacks were 2.98years older than breeding jennies on average. The average age of parents when their offspring was born was 8.08years (8.03 for jennies and 8.16 for jacks). The average generation interval was 7.40years (Navas et al., 2017).

3.3. Isolation and quantification of non-genetic risk factors

Shapiro-Francia W′ Test ($P<0.001$) and higher or lower kurtosis values than three on all the 'fixed' effects, the covariate and interaction showed that they highly significantly did not fit a normal distribution. The variability observed for the two traits analyzed was from moderate to high, with a coefficient of variation of 21.3% for the husbandry system effect and 82.2% for the effect of the farm/owner.

The results of Chi-Square, Partial eta (for each independent categorical-dependent ordinal pair of variables) and Spearman's rho correlation coefficient (for the effect of age on the ordinal dependent variables studied), testing for the existence of linear correlation are shown in Table 2. Partial eta effectively and statistically significantly measured the strength of collinearity that the sex and farm factors have on continuous variables of cumulative foal number born per animal, maximum foal number per birth and multiple birth number per animal for a given donkey.

Husbandry system reported highly statistically significant ($P<0.001$) collinearity with the cumulative foal number born per animal (Table 2). Kruskal-Wallis H test and Chi-square reported the effects birth year and birth month to be statistically nonsignificant ($P>0.05$) for the three dependent variables considered. The same test reported the rest of independent variables (sex, owner/farm and husbandry system) to be statistically significant ($P<0.05$) for all dependent variables except for husbandry system on maximum foal number per birth and multiple birth number per animal for a given donkey and birth season on maximum foal number per birth and multiple birth number per animal for a given donkey ($P>0.05$) (Table 2).

From the results of the Mann-Whitney *U* Test (Supplementary Table S6), we can conclude that cumulative foal number born per animal and maximum foal number per birth in jacks was statistically significantly higher than in jennies (U=46,363.500, $P<0.001$ and U=50,364.000, $P<005$). However, the opposite trend was described by multiple birth number per animal for a given donkey, which was statistically significantly higher in jennies than in jacks (U=47,730.000, $P<0.05$).

The results of the Dunn test in our study reported the fact that there were highly statistically significant differences for 44.69% of pairwise comparisons of farms/owners for maximum foal number per birth and from 5.45% to 12.73% of pairwise comparisons of location for multiple birth number per animal for a given donkey and of location for maximum foal number per birth, respectively (mostly involving differences between location 3 and others). The same test reported statistically significant differences between extensive, semi-extensive and semi-intensive husbandry systems ($P<0.05$) for maximum foal number per birth (Supplementary Table S7).

CATREG was performed on the 5 qualitative independent variables (birth season, sex, location, farm/owner, husbandry system) and age as a covariable with the three birth-related continuous variables (cumulative foal number born per animal, maximum foal number per birth and multiple birth number per animal for a given donkey) as dependent variables. Categorical regression quantifies categorical data by assigning numerical values to the categories, which results in an optimal linear regression equation for the transformed variables. CATREG is also the name of the program in SPSS that uses the Categorical Regression Analysis algorithm (Van der Kooij and Meulman, 2007). In this analysis, categorical variables are quantified by using optimal scaling, in order to reach the optimal regression model coefficients. “Optimal Scaling” is the quantification method of the variant variables in Gifi (1990). With the results from CATREG, it is still required to verify the statistical significance of the predictors. Consequently, CATREG is equivalent to a standard linear regression when the qualitative predictors are substituted by the linear and nonlinear transformed (quantified) values (Çilan and Can, 2014).

Optimal scaling transformations were carried out as described by (Van der Kooij and Meulman, 2007). According to these authors, at the same time that CATREG algorithm provides a very simple and efficient way to compute the regression coefficients in the constrained models for Ridge regression, the Lasso, and the Elastic Net it also prevents the inflation of R-squared and bias (towards zero) of the estimates of standard errors and thus, F-tests and *P*-values that is likely to occur. The estimated coefficients reflect how changes in the predictors affect the response. We present the summary results with the significant variables in Table 3, Table 4. The standardised coefficients (β) are listed in Table 4. CATREG reported all of the independent variables except for the birth year and sex to be significant for cumulative foal number born per animal. Sex was nonsignificant for the maximum foal number per birth and multiple birth number per animal. The birth season was nonsignificant for Multiple birth number per animal and husbandry system for cumulative foal number born per animal and multiple birth number per animal.

Table 4. Standardised coefficients and significance of CATREG model.

Variable	**Cumulative foal number born per animal**		**Maximum foal number per birth**		**Multiple birth number per animal**	
	Parameter					
Factor	**Standardised Coefficients (β)**	**Significance**	**Standardised Coefficients (β)**	**Significance**	**Standardised Coefficients (β)**	**Significance**
Birth season	0.098	0.013	0.086	0.000	0.031	0.993
Sex	0.435	0.000	0.020	0.391	0.006	0.902

Owner/Farm	0.478	0.000	0.592	0.000	0.921	0.000
Location	0.159	0.000	0.246	0.000	0.307	0.033
Husbandry system	0.032	0.439	0.045	0.139	0.096	0.636
Age (in years)	0.207	0.002	0.163	0.000	–0.059	0.620

There was a small to moderate monotonic (whether linear or not) significant (P<0.05) correlation between age and the three variables tested (Table 2). This correlation was inverse (–0.137) in cumulative foal number born per animal, that is if age increases the cumulative number of foals per donkey decreases, while it was direct, for maximum foal number per birth (0.085) and multiple birth number per animal for a given donkey (0.339), which parallelly increased with age. The number of standard deviations that a dependent variable will change per unit of standard deviation increase in the age (or age CATREG (β) standardised coefficients) is shown in Table 4. CATREG (β) standardised coefficients for age ranged from –0.059 to 0.207 for multiple birth number per animal and cumulative foal number born per birth, respectively.

Month and year of birth, Chi-square values were non-significant (P>0.05), thus they were not included in the CATREG analysis. Partial eta values ranged from 0.117 to 0.146 reporting a moderate association between the month of birth and the dependent variables of multiple birth number per animal for a given donkey and cumulative foal number born per animal. For the birth year, partial eta cumulative values ranged from 0.177 to 0.234 addressing a moderately high association between birth year and the dependent variables of multiple birth number per animal for a given donkey and cumulative foal number born per animal.

For the birth season, Chi-square values were only significant for cumulative foal number born per animal (P<0.05). Partial eta values ranged from 0.093 to 0.102 suggesting a low association between birth season and the dependent variables of cumulative foal number born per animal, maximum foal number per birth and multiple birth number per animal for a given donkey. CATREG standardised coefficient for the birth season and multiple birth number per animal was non-significant. However, CATREG standardised coefficients for maximum foal number per birth (0.098) and cumulative foal number born per animal (0.086) reported a low increase of the standard deviation of the birth year was needed to increase a unit of standard deviation in both dependent variables.

For sex, Chi-square values were all significant (P<0.05), thus there were statistical differences between jacks and jennies. Partial eta values ranged from 0.074 to 0.227 what reported a low to a moderately high association between sex and the dependent variables of maximum foal number per birth and cumulative foal number born per animal. CATREG standardised coefficient for sex and multiple birth number per animal for a given donkey cumulative and maximum foal number per birth were non-significant. However, CATREG standardised coefficients for cumulative foal number born per animal (0.435) reported a high increase of the standard deviation of sex was needed to increase a unit of standard deviation in cumulative foal number born per animal.

Owner/Farm, Chi-square values, were all significant (*P*<0.001), thus there were highly significant statistical differences between the values of the dependent variables for each of the 92 levels of the farm/owner factor. Partial eta values ranged from 0.330 to 0.626 what reported a high association between owner/farm and the dependent variables of cumulative multiple birth number per animal for a given donkey and cumulative foal number born per animal, respectively. CATREG standardised coefficient for owner/farm were all highly statistically significant (*P*<0.001). CATREG standardised coefficients ranging from 0.478 to 0.921 reported a high increase of the standard deviation of owner/farm was needed to increase a unit of standard deviation in all three dependent variables measured.

Location Chi-square values, were all significant (*P*<0.001), addressing differences between locations. Partial eta values ranged from 0.113 to 0.291 what reported a moderate to the moderately high association between location and the dependent variables of cumulative foal number born per animal, maximum foal number per birth, and multiple birth number per animal for a given donkey. CATREG standardised coefficient for the location was highly statistically significant (*P*<0.001 for Cumulative foal number born per animal and Maximum foal number per birth) and statistically significant (*P*<0.05) for Multiple birth number per animal. CATREG standardised coefficients ranging from 0.159 to 0.307 reported a moderate increase of the standard deviation of location was needed to increase a unit of standard deviation in all three dependent variables measured.

Husbandry system Chi-square value was only significant (*P*<0.001) for cumulative foal number born per animal, addressing differences among extensive, semi-extensive, semi-intensive and intensive husbandry systems. Partial eta value for this dependent variable was 0.176 what reported a moderately low association between husbandry system and the independent variables of cumulative foal number born per animal and multiple birth number per animal for a given donkey. CATREG standardised coefficient for owner/farm was not statistically significant (*P*>0.05) for any cumulative of the three dependent variables.

We show the factors affecting the three birth-related variables in order of importance according to the CATREG standardised coefficients (β) in Table 5. Since we used the stepwise method, there was no multicollinearity problem. The standardised solution for the regression equations can be found in Table 5 as well.

Table 5. Regression equations for maximum foal number per birth, multiple birth number per animal for a given donkey and cumulative foal number born per donkey.

General model regression equation	Legend

$Z'Y_{maxmulhis}=\beta farm*Zfarm+\beta location*Zlocation+\beta birthseason*Zbirthseason+\beta sex*Zsex+\beta age*Zage$	$Z'y_{maxmulhis}=$ score for eac variable (maximum fo number per birth, multip birth numbe per animal fo a given donke and cumulative foal number born per donkey). β=standardis coefficient fo each of the factors appearing in the subindex. Z=Z score for each of the factors appearing in the subindex.

Specific regression equations		Legend
Maximum foal number per birth	$Z'y_{max}=0.592(ZFarm)+0.086(ZBirthseason)+0.246(ZLocation)+0.163(ZAge)$	$Z'y_{max}$=Z score fo number per birt $\beta_{Farm}Z_{Farm}=0.59$ $\beta_{Birthseason}Z_{Births}$ $\beta_{Location}Z_{Location}$ $\beta_{Age}Z_{Age}=0.163(Z$
Multiple birth number per animal for a given donkey	$Z'y_{mul}=0.921(ZFarm)+0.307(ZLocation)$	$Z'y_{mul}$=Z score fo number per anin $\beta_{Farm}Z_{Farm}=0.921$ $\beta_{Location}Z_{Location}$

Cumulative foal number born per donkey	$Z'y_{his}=0.478(ZFarm)+0.098(ZBirthseason)+0.435(ZSex)+0.159(ZLocation)+0.207(ZAge)$	$Z'y_{his}$=Z score for number born pe $\beta_{Farm}Z_{Farm}=0.47$ $\beta_{Sex}Z_{Sex}=0.435(Z$ $\beta_{Birthseason}Z_{Births}$ $\beta_{Location}Z_{Location}$ $\beta_{Age}Z_{Age}=0.207(Z$

Non-significant effects for each variable were not included (P>0.05).

3.4. Interaction exclusion and general mixed model predictive power

The triple interaction was statistically nonsignificant (P>0.05) so that it was not included in the model. Although, the Herd*Birth year double interaction was statistically significant P<0.01, its inclusion within the model distorted the results in the following way so that we decided not to include such interaction. The model for cumulative foal number born per animal explained a higher percentage of the variance in the sample when we included the interaction. However, the estimation of the genetic parameters reported almost twice the standard error of the same model without including the interaction as stated below, that may have its basis on the high amount of possible levels of the interaction matched to a proportionally small sample. For maximum foal number per birth, there was a reduction in Adjusted R squared from 0.421 to 0.406 and the expected prediction error increased from 0.113 to 0.198 when we included the Herd*Birth year interaction. For multiple birth number per animal, one or more levels for the interaction did not occur in the sample. Furthermore, according to AIC and BIC (Akaike's Information Criterion and Bayesian Information Criterion, respectively) the model that excluded the interaction had higher predictive power as suggested in Supplementary Table S5 by its lowest values presented when compared to those reported for the model including the interaction. These results suggested that the inclusion of this interaction in the model may result in potentially distorting effects which were highlighted at the statistical level as expected prediction error could not be computed. The results of the genetic and phenotypic parameters estimated by a preliminary model including Herd*Birth year iteration supported such distorting effects, as there was an increase in the standard errors from the general animal mixed model used in our study (without including the interaction) 0.081 to 0.128 to 0.154 to 0.643 (including the interaction). As the previous statistical analysis had reported, the basis for such distorting effects may be the fact that the number of categories considered for herd*year interaction was 441, while the whole sample size was 765. This data may generate a statistical imbalance that may result in an overestimation of the effect of the interaction as it has been reported by literature (Schmidt et al., 2014), making it impossible to test for its effects properly, due to the lack of enough animals in the pedigree between whom to compare.

CATREG R squared coefficient obtained ranged from 0.458 to 0.919 for the maximum foal number per birth and multiple birth number per animal, respectively (Table 3).

Table 6. Estimated components of variance, heritability (h^2) and standard error (SE) for maximum foal number per birth, multiple birth number per animal for a given donkey and cumulative foal number born per donkey obtained from multivariate analyses for Mixed Animal Model using Gibbs sampling in Andalusian donkeys.

Trait	σ_a^2	σ_p^2	σ_e^2	**h^2±SE**
Maximum foal number per birth	0.0287	0.1456	0.1169	0.2000±0.1050
Multiple birth number per animal for a given donkey	0.0198	0.1076	0.0877	0.1800±0.1010
Cumulative foal number born per donkey	1.1252	4.6190	3.4888	0.2400±0.0780

Table 7. Estimated phenotypic (r_P) (above diagonal) and genetic (r_G) (below diagonal) correlations for maximum foal number per birth, multiple birth number per animal for a given donkey and cumulative foal number born per donkey obtained in bivariate analyses using Bayesian methods in Andalusian donkeys.

Traits	**Maximum foal number per birth**	**Multiple birth number per animal for a given donkey**	**Cumulative foal number born per donkey**
Maximum foal number per birth	–	0.607±0.054	0.206±0.063
Multiple birth number per animal for a given donkey	0.846±0.152	–	0.530±0.045
Cumulative foal number born per donkey	0.496±0.298	0.605±0.222	–

Table 8. Descriptive statistics of predicted breeding values (PBVs) for maximum foal number per birth, multiple birth number per animal for a given donkey and cumulative foal number born per donkey for all the donkeys included in the pedigree sorted by model and sex.

Sex	**Trait**	**Mean**	**SEM**	**95% confidence**	**Std. deviation**	**Median**	**Minimum**	**Maximum**	**Skewness**	**Kurtosis**

				interval for mean						
Jacks (*n*=272)	Maximum foal number per birth	0.009	0.002	0.005–0.014	0.037	0.003	−0.108	0.164	0.925	2.499
	Multiple birth number per animal for a given donkey	0.005	0.001	0.003–0.007	0.013	0.003	−0.037	0.054	0.763	1.790
	Cumulative foal number born per donkey	0.092	0.007	0.078–0.106	0.116	0.059	−0.159	0.645	1.125	1.751
Jennies (*n*=745)	Maximum foal number per birth	0.004	0.002	0.001–0.007	0.043	0.000	−0.157	0.190	0.035	2.086
	Multiple birth number per animal for a given donkey	0.002	0.001	0.001–0.003	0.014	0.000	−0.053	0.064	0.204	1.960
	Cumulative foal number born per donkey	0.038	0.003	0.031–0.044	0.091	0.005	−0.109	0.520	2.004	4.613

3.5. Isolating and quantifying the additive genetic component: phenotypic and genetic parameters, predicted breeding values and prediction accuracy (distribution and correlation)

The additive genetic component was isolated and quantified through the estimates for heritability, genetic and phenotypic variance estimated with Gibbs sampling shown in Table 6. Table 7 shows the genetic and phenotypic correlation chart. The results for the estimates of predicted breeding values (PBV) for both models (Bayesian general mixed animal model) separated in jacks and jennies are shown in Table 8. Regarding phenotypic parameters, we also present the results for the best linear unbiased estimators (BLUEs) obtained from the Gibbs sampling quantitative genetic analysis through posterior mean, including age as a linear and quadratic covariate, the 'fixed' effects of birth season, sex, farm/owner, location and husbandry system in Supplementary Table S8.

4. Discussion

According to literature, donkeys have a 13% higher fertility than horses (Debra and Hagstrom, 2004), reaching an incidence for multiple ovulations of 61% in Mammoth jennies and standard jennies. This higher incidence of multiple ovulation in donkeys translates in twinning occurring more frequently. Although the incidence of twins has been reported to be as high as 40% via ultrasound at day 21 in standard donkeys, for endangered donkey breeds such as Asinina de Miranda, the percentage of twin foaling at full term reduces to 2.85% (Quaresma et al., 2015). The rate of multiple ovulations in the donkey species varies with the reports from the literature, ranging from 5.3% to almost 70% (Quaresma, 2015) so that our results fall within the range reported for other donkey breeds.

In donkey breeding plans, selection has more frequently been applied to jackstocks, as historically, owners have only paid attention to them for the selection of mating couples, erroneously considering jennies a secondary item (Navas et al., 2017). This context can be compared to horses' and sets the complex environmental and genetic background behind the economically important trait of fertility.

Furthermore, the reproductive trends of this polygynous species have been reported to highly depend on the owner tastes for certain morphological or coat characteristics and local availability of the animals. Navas et al. (2017) suggested the typical excessive contribution of few ancestors to the gene pool of small critically endangered donkey populations may lead to narrow bottlenecks shortly whose hidden effects can only be controlled by tracking the populations. Among such hidden effects, the compromises exerted on the reproductive and immune system of the animals have been addressed to be some of the determinants of the difficulties experimented to conceive by individuals (Ober et al., 1999).

Such reproductive compromises have been suggested to be a direct cause of inbreeding depression in donkeys. However, the lack of completeness of the pedigree of endangered donkey populations and the irregular distribution through great extensions of territory makes the estimation of this parameter little reliable (Navas et al., 2017). Quaresma et al. (2015) reported the numbers obtained in 40 populations indicated an average value of 3.14 of lethal equivalents with 50% due to recessive lethal alleles.

Taberner et al. (2008) stated that multiple ovulations tend to repeat in several estrous cycles, which may support the existence of animals that present a certain cyclical predisposition towards multiple births. The relative frequencies for multiple pregnancies of certain donkeys were higher than for others, which suggested a genetic background behind multiple births, as it had previously been reported by Ginther (1992). Similarly, Quaresma et al. (2015) suggested an indirect selection of certain family lines may have been carried out in the Mammoth donkey, what may have resulted in the higher incidence of multiple ovulations reported by Blanchard et al. (1999).

Specific studies have assessed the possible repercussion of certain environmental factors on the fertility of donkeys. For example, in our study, the Chi-square values for the birth season were non-significant ($P>0.05$). Thus, there was not any statistical difference between the values of the dependent variables for each of the four levels of birth season. The findings by Contri et al. (2014) support our results. These authors reported estrous cycle can be detected during the whole year in jennies, with no differences in the estrous cycle length among seasons. Parallelly, the pattern of the plasma concentration of certain hormones such as E2 and P4 during the estrous cycle did not report any difference among seasons, although a larger diameter of the ovulating follicle was reported for spring and summer.

Breeding season and month significantly affected gestation and estrous cycle length in donkeys (Galisteo and Perez-Marin, 2010). However, these authors did not study whether the effect of the month may condition the occurrence of multiple births and fertility. Quaresma and Payan-Carreira (2015) reported the incidence of single, double, and triple ovulations to be 57.58%, 36.36%, and 6.06%, respectively. The same authors stated, multiple ovulations affected neither the length of the interovulatory interval nor the individual cycle stages ($P>0.05$) but lengthened the interval from the beginning of estrus to the last ovulation ($P=0.01$), which may support the results found by our study and those found by Galisteo and Perez-Marin (2010) as well.

No paper has reported the higher prevalence of multiple births or a higher likelihood of presenting a higher maximum number of foals depending on the husbandry techniques carried in the farms. The results found in our study for Dunn's and independent samples median tests suggested donkeys located at semi-extensive farms presented a higher likelihood of presenting higher maximum foal numbers per birth, followed by semi-intensive farms and extensive farms, respectively (Supplementary Table S7). The criteria used to classify the husbandry systems of the farms in the study (Table 3) may suggest that the access to more extensive territories, when owners provide regular reproductive care to the animals and the daily contact with the owners may have an increasing importance in the occurrence of a higher number of foals per birth. The higher strength effect of the farm factor on all the variables tested ranging from 0.598 to 0.873, for multiple birth number per animal and cumulative foal number born per animal, respectively supported the finding.

A higher relevance was attributed to jennies in having a cumulatively higher number of foals, a higher number of multiple offspring and a higher maximum number per birth. These values balanced (providing an equal relevance to jacks and jennies) as the number of foals and multiple births increased, as we can observe in the charts in Supplementary Table S5. Still there seems to be a very slight effect of specific jacks on promoting the obtention of a higher cumulative number of foals. This could be attributed to the reproductive characteristics of the jenny and breeding strategies of donkey owners, as it has already been suggested by Bresińska et al. (2004) and is addressed by the results of the Mann-Whitney *U* test of our study (Supplementary Table S5). According to our results, the fact that foal number born per animal and maximum foal number per birth in jacks was statistically significantly higher than in jennies could be attributed to the fact that jacks can act as the sire for several jennies at the same time, while jennies are going to be reproductively blocked for a whole year when they have become pregnant. The same test suggested that although jacks were likely to significantly reach a higher number of foals on a certain gestation through their lives when compared to jennies, jennies were statistically significantly more prone to develop multiple gestations through theirs. This could be supported by the greater chance of jacks to mate and the fact that multiple ovulations are a female trait, usually associated with endocrine changes that originate a sort of independence from the falling FSH values, that allow two (or more) dominant follicles to ovulate.

The potential imbalance between the partial genetic implication of equine stallions (jacks or horses) and dams (jennies or mares) has often been overlooked in literature, as only a few studies report a comparison between sires and dams (Lin et al., 2016; Mucha et al., 2012). This way, these studies rather focus on examining their genetic implications separately (Giesecke et al., 2010; Mahon and Cunningham, 1982) than make a combined effort to compare the effects of both factors.

Using Gibbs sampling methods, as we consider the relationship among the individuals present in the pedigree, regardless their sex, we can estimate genetic information for the animals from which we have direct observations, and predict such information for animals assessing the additive indirect observations obtained from their ancestors. Hence, we can get the information for a particular trait of an individual when it is naturally impossible or potentially difficult to obtain it. For instance, prolificacy in foals that are too young to give birth, milk production from a male or when fertility rates are unbalanced between sexes (i.e., the number of offspring that a male can produce compared to the number of offspring a female can give birth to) (Parnell, 2004).

Estimates of additive genetic variance for maximum foal number per birth and multiple birth number per animal for a given donkey were around the lowest margin of the values reported for twinning and fertility in horses. By contrast, the estimate of additive genetic variance for cumulative foal number born per donkey was around the highest margin reported for fertility in horses (Table 4), what resulted in higher heritabilities (Mucha et al., 2012). Sairanen et al. (2009) values for the heritability of foaling rate ranged between 3.4% and 3.7% in Standardbreds and between 5.5% and 9.8% in Finnhorses when the outcome of the foaling was considered to be a trait of the expected foal. However, the models used in such circumstances differed from ours. Interestingly, the low genetic component of variance did not affect heritability estimates which were moderate and ranged from 0.18 to 0.24 for the general linear model for multiple birth number per animal for a given donkey and cumulative foal number born per donkey, respectively. Furthermore, these heritability values were from moderately to highly accurate as suggested by the estimation error found ranging from 0.078 to 0.105 for cumulative foal number born per donkey and multiple birth number per animal for a given donkey for the generalised animal model, respectively (Table 6).

Our results resemble those at the upper limit for the heritabilities of fertility in stallions as reported in literature. Hamann et al. (2005) reported heritability estimates for stallion fertility to range from 0.03 to 0.15 for foaling rate per breeding season. In the same way, the same authors reported a marked maternal component for some traits such as pregnancy rate per oestrus (PRO) associated with breeding year and season, breeding centre, age of mares, breeding history of mares, type of covering (natural or artificial insemination), breeding management (number of coverings and time intervals between them), and type of semen (fresh within 24h, fresh and shipped within 48h or frozen/thawed) (Hamann et al., 2005).

Similarly, the use of genetic markers implemented in the study of mare multiple pregnancies, fecundity (defined as ratio of progeny number to number of pregnancies) and length of reproduction cycle has reported heritability estimates of mare fecundity and number of pregnancies that range from 0.03 to 0.07, respectively, and hence, proportionally present the same positive correlation that the traits considered in our study.

Genetic, phenotypic and environmental correlations widely vary from low to moderate and from negative to positive from one year to another. In general, genetic, phenotypic and environmental trends for fecundity and the number of pregnancies have been reported to be non-negative (Mahon and Cunningham, 1982) as it can be seen in Table 6, Table 7. However, these annual effects highly fluctuate, which may be attributed to the influence of some sires incorporated into population, as supported by Bresińska et al. (2004). These authors reported very high frequencies of twinning in some families in this population, hence the possibility to classify stallions into two opposite groups according to twinning level of their daughters.

Moioli et al. (2017) found similar SE for the same parameters and traits in the Maremmana local cattle breed whose sample size was similar to the one in our study. Among the common factors to the two studies, microsatellite genotyping of the pedigree relationships may have played an essential role in the estimation of such reliable genetic parameters. A mixed inheritance model of twinning has also been hypothesised for horses such as (Zöldág et al., 2001) and cattle. Studies on cattle showed similar conclusions. For instance, some bulls had several hundred daughters with no multiple births, and the top bull had daughters with a twinning rate of 12.9% (Karlsen et al., 2000).

From a theoretical perspective, low heritability estimates indicate a larger environmental influence, that may occur after an indirect selection process for a certain trait through time. Reproductive traits are well known to be lowlily heritable because of this. Such selection process derives then in fixation of such trait in the population. For example, (Van Vleck and Gregory, 1996) reported an increase in the average twinning rate of cattle of >25% in fifteen years. In general, these traits are troublesome in statistical modelling. One of the main assumptions of the classical methods is, among others, normality of residuals. Unfortunately, these traits do not hold the assumption as it happens in our study. Both the number of progenies per pregnancy and the number of pregnancies are typically discrete traits determined by many loci. But the twinning rate may also be expressed as a continuous trait (when it is summarised as fecundity, fertility or cumulated number of foals, as it happens in our study). Heritabilities of these traits can be underestimated since, in case of a deviation from the model assumption, the error variance is overestimated. Van Tassell et al. (1998) reported higher heritability estimates from the threshold than linear model for twinning cattle data, whereas Kadarmideen et al. (2016), obtained very close estimates via both types of models.

Several authors have suggested Bayesian inference Threshold models to be more suitable to analyze non-normally distributed functional traits in small samples (Johanson et al., 2001; Skotarczak et al., 2007; Van Tassell et al., 1998; Wolc et al., 2006). Furthermore, REML estimates tend to be included within the credible interval of the estimates obtained using Gibbs sampling methods, thus reporting similar results (Mucha et al., 2012).

Mostly all authors agree that the analysis should be based on the threshold model, what was also proved at a preliminary stage in our study when a linear model was tested and discarded. Still some papers using REML models appear (Sairanen et al., 2009). These results obtained clearly indicate that foaling rates, fertility or twinning are influenced by reproductive ability of dams, inbreeding, and other environmental factors. In this way, heritability estimates for both fecundity and number of pregnancies are low, and seem to be affected by some deviation from the assumption of the model employed (for instance, a skewed distribution) but also may benefit from the improvement of environmental factors deserves special attention.

Our estimates for phenotypic and residual variance are almost 4 to 6 times higher than genetic variance estimates. As it has been reported in horses (Mucha et al., 2012), the current analysis assumes that fertility and multiple births are determined by an infinite number of loci that contribute each with a minimal effect in what is called infinitesimal mode of inheritance. Hence, we can suppose, fertility may complexly depend on many physiological processes each of which is controlled by specific biochemical pathways.

The high value for genetic, phenotypic correlations between maximum foal number per birth and multiple birth number per animal for a given donkey could have been expected as the fact that an animal is more prone to have multiple births may make it more prone to have a higher maximum number of foals per birth. We found moderate genetic and low phenotypic correlations between maximum foal number per birth and cumulative foal number born per donkey. This finding may mean a weak relationship between animals that have a high cumulative number of offspring through their lives and the same animals having a high maximum number per birth, which may suggest a lower reproductive life for those animals producing multiple offspring. Genetic and phenotypic correlations between the number of multiple per animal and cumulative foal number born per donkey were moderately high, which suggests the higher the number of total offspring through the life of a given donkey is (that is the more fertile), the more likely these animals are to produce multiple births.

These correlations have been described as well in humans (Colletto et al., 2001; Rickard et al., 2012). For instance, all the findings by Mbarek et al. (2016) point to spontaneous twinning being a heritable trait and suggest the potential for polygenic inheritance as supported by the genetic correlations found by our analyses. The same authors reported that consistent with its effects on higher circulating FSH levels; the rs11031006-G allele also associates with a higher total lifetime number of children. Moreover, Boomsma et al. (1992), reported an increased frequency of the S allele in fathers of dizygotic twins. However, this may be a secondary effect of assortative mating for family size. The Andalusian donkey is a highly standardised breed for which assortative mating may have played an indirect role when seeking for obtaining specific phenotypical characteristics what may account for the low genetic variance for maximum foal number per birth and multiple birth number per animal for a given donkey.

Despite its demographic bottlenecks, the Andalusian donkey still maintains considerable levels of genetic variability for fertility and multiple birth traits (Navas et al., 2017). Given the favorable existing genetic relationships between the traits involved, these traits can play an essential role in a selection program aimed at improving the breeding efficiency of the animals. The potential opportunities arising from the incorporation of genomic information in the selection program should be investigated and implemented carefully in the future. Their contribution to reducing generation intervals and enhancing selection accuracy could result in extraordinary benefits for genetic progress, avoiding to detrimentally increase the inbreeding problems and endangerment risk from which the species suffers (Haberland et al., 2012). PBVs for multiple births and fertility show considerable variability, indicating a possibly effective selection based on genetic merit objective estimates. The moderate heritability values balance the high existing phenotypic variability, resulting in a moderately wide PBV distribution (Table 8). Implementing a systematic genetic evaluation procedure through the genetic information available, allowing the early selection of breeding animals becomes then one of the main aims of the study. However, the reduction of generation intervals, enhancing selection accuracy through multivariate animal models for functional traits, and thus, the reduction in the number of breeding jackstocks to compatible levels with an increased selection response, must consider the detrimental problems that are likely to appear because of an increase in inbreeding in breeds with such a low effective population number. In these breeds, the protection of genetic variability and minimizing inbreeding are primary concerns as they may prevent population bottlenecks from occurring. The incorporation of genetic markers in the functional selection against or for donkeys for multiple births or fertility is a still a developing possibility. Hence, the exceptional importance of the implementation of these validated assessment tools and new methods and the perspective to develop routinely studies assessing the same animals over several years. Genotyping Beadchip and expression microarrays could greatly enhance studies aimed at understanding equine reproductive physiology and pathology (Sieme and Distl, 2012). Still donkey fertility studies are far from the recent developments in equine genomics. The application of new genetic advances provides novel possibilities and tools for mapping fertility traits in the horse and for unraveling the causes of intersexuality and reproductive inborn defects.

Utilizing a large number of genome-wide equidistantly distributed markers offers the possibility for interrogation of the whole equine genome for associations and linkage at high resolution and this should greatly facilitate identification of causal genes for fertility and other complex traits, even those with low heritability.

5. Conclusions

The values found for genetic parameters enable the potential inclusion of these traits within breeding programs seeking the genetic progress of donkey breeds. Positive and moderate genetic correlations enable the combined selection for maximum foal number per birth and cumulative foal number born per donkey, with low detrimental effect for either one. Selection for multiple births or fertility in donkeys may have traditionally been carried out indirectly. Thus, the routine application of the assessment including a higher number of animals is required to standardize the valuation methodology implemented. However, this is a difficult task to achieve, considering the current extinction risk of donkey breed endangered populations. Functional traits related to fertility and prolificacy can play an essential role in a selection program aimed at improving the suitability of donkeys for their inclusion in embryo vitrification, or freezing assisted reproduction programs. The present results lay the basis for a bidirectional selection strategy. On one hand, the specific nature and the magnitude of the existing genetic relationships may make interesting to consider the possibility of developing and maintaining specialised lines relying on the ability of particular donkeys to develop multiple births within the Andalusian donkey breeding program, hence, increasing the productivity of assisted reproduction techniques. On the other hand, when embryo collection is not the purpose aimed at, selection could focus on the obtention of those individuals that may be less prone to develop multiple births, thus, avoiding the risks of multiple gestations, which in the end translates in the improvement of the reproductive welfare of the individuals.

The following are the supplementary data related to this article.Supplementary Table S1. Questions asked to the owner interviewed during the survey carried out regarding the fertility and prevalence of multiple births in donkeys.

Supplementary Table S2. Description of the levels included in the husbandry system fixed effect.

Supplementary Table S3. Descriptive statistics for fixed effects (yellow), interaction (green), covariates (red) and birth related variables (blue) in Andalusian donkeys (N=765).

Supplementary Table S4. Kruskal Wallis H Ranks for all the levels of the factors affecting historical foal number born per animal, maximum foal number per birth and multiple birth number per animal (the redder the lower value, the greener, the higher value).

Supplementary Table S4. Comparison of the model summary of stepwise linear Categorical regression with transformed variables including and without included the interaction of herd*birthyear.

Supplementary Table S6. Summary of the results of the Independent-Samples Mann-Whitney *U* Test and Dunn's test and Bonferroni's significance correction for the effects for whose levels there were statistically significant differences on the historical foal number born per animal, maximum foal number per birth and multiple birth number per animal (the redder the more significant, $P<0.05$, the greener the less significant, $P>0.05$).

Supplementary Table S7. Summary of the results of the independent sample median test for the effects for whose levels there were statistically significant differences on the historical foal number born per animal, maximum foal number per birth and multiple birth number per animal compared to the significances for the results of the Dunn's test for the same parameters (for *p*-value column, the redder the more significant, $P<0.05$, the greener the less significant, $P>0.05$, for the rest of the columns the redder the lower value, the greener, the higher value).

Supplementary Table S8. Estimates of non-genetic effects obtained from the Gibbs sampling quantitative genetic analysis, including age as a linear and quadratic covariate, the fixed effects of birth season, sex, farm/owner, location and husbandry system.

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Declaration of Competing Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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